Sudan University of Science and Technology

College of Post Graduate Studies

Phytochemical Studies on the Stems of *Pulcria crispa* and Seeds of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* and Biological Activity of Constituents

دراسة فيتوكيميائية لسيقان نبات الربل وبذور نبات الرجلة والكسبرة والكتان والفعالية البيولوجية للمكونات

A Thesis Submitted in Fulfillment of the Requirements of the Ph.D. Degree in Chemistry

By

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September, 2019

الإســـــــتـــهـــلال

﴿ بسم الله الرحمن الرحيم ﴾

فلينظر الإنسان إلى طعامه ﴿٢٤﴾ أنا
صببنا الماء صبا ﴿٢٥﴾ ثم شققنا
الأرض شقا ﴿٢٦﴾ فأنبتنا فيها حبا
﴿٢٧﴾ وعنبا وقضبا ﴿٢٨﴾ وزيتونا
ونخلا ﴿٢٩﴾ وحدائق غلبا ﴿٣٠﴾
وفاكهة وأبا ﴿٣١﴾ متاعا لكم
ولأنعامكم ﴿٣٢﴾ سورة عبس

Dedication

To,

My parents,

My sons,

And my sisters and brother

Acknowledgement

First and foremost, I thank **Almighty Allah, the Ever-Magnificent, the Ever-Thankful**, for his help and blessing.

I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor Dr. Mohamed Abdel karim, who has been always generous during all phases of the research, and I highly appreciate the efforts expended by my co-supervisor, Dr. Kamal Mohamed Saeed.

I owe a deep debt of gratitude to the Sudan University of Science and Technology for giving us an opportunity to accomplish this work. Thanks are extended to the Aromatic and Medicinal Plants Institute, Khartoum, Sudan for all facilities. Thanks for the National Research Center, Cairo for the spectral measurements.

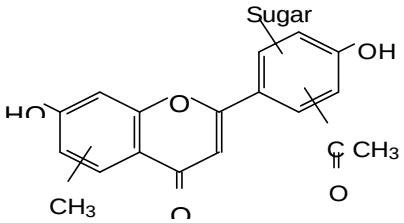
I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis.

I also would like to express my whole hearted thanks to my parents, for their generous support. They supported me throughout my entire life and particularly through the process of accomplishing the PhD degree.

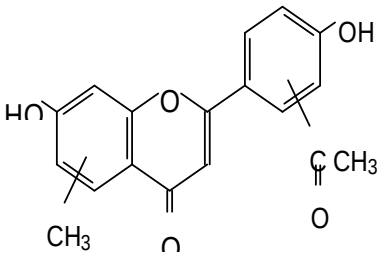
Abstract

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

The stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature. Qualitative tests on the alcoholic extract were positive for flavonoids. Conventional chromatographic techniques were used for isolation and purification of flavonoids. Two flavonoids (compounds I and II) were isolated from this plant. The partial structures of both compounds were deduced on the basis of their: IR, UV and NMR data and the following structures were suggested:



Compound I



Compound II

Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried. The results exhibited variable susceptibilities of microorganism for crude extract and different fractions.

In this study, the antioxidant capacity of the alcoholic extract of *Pulcriacrispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The GC-MS analysis of the *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* oils revealed the presence of 15, 14, 15 components respectively.

In the agar diffusion bioassay oils of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* failed to exhibit any antimicrobial activity. However, *Coriandrum sativam* oil showed significant antioxidant activity.

المستخلص

يشتمل البحث على دراسة كيميائية مفصلة للمحتوى الفلافونيدي لسيقان نبات الربل وكذلك التركيب الكيميائي لزيوت بذور كل من البقلة، الكزبرة وبذور الكتان. ايضا يتضمن البحث دراسة فعالية مستخلصات نبات الربل والزيوت المستخلصة كمضادات ميكروبات وكمضادات أكسدة.

لقد أستخلصت سيقان نبات الربل بواسطة الكحول الإيثيلي عند درجة الحرارة العادية و افاد المسح الفيتوكيميائى بوجود الفلافونيدات.

أنجز فصل المركبات بإستخدام كروماتوغرافيا العمود و كروماتوغرافيا الورق ، كما وتم التعرف على التركيب الكيميائي للمركبات المفصولة بإستخدام وسائل التحاليل الطيفية المختلفة : مطيافية الأشعة تحت الحمراء، الأشعة فوق البنفسجية ، الرنين النووي المغناطيسي للهيدروجين . وقد تم فصل مركبين ذوا طبيعة فلافونيدية من نبات الربل وهما المركب **II ,I.** تناولت الدراسة فعالية مستخلصات سيقان نبات الربل كمضادات ميكروبات وكذلك كمضادات أكسدة.

أثبت البحث ان مستخلص خلات اللإيثيل ذو فعالية كمضاد للبكتيريا. أما المستخلص الكحولي وبقية المستخلصات فقد اظهروا فعالية منخفضة كمضادات بكتيريا وفطرية. أوضحت الدراسة ان المستخلص الكحولي لنبات الريل ذو تاثير فعال كمضادات اكسدة.

اخضع زيت بذور البقلة، الكزبرة والكتان للكروماتوغرافيا الغازية - السائلة الملحقة بمطياف الكتلة . اوضحت هذه الدراسات وجود خمسة عشر ، أربعة عشر، وخمسة عشر مكونا بالزيت على التوالي.

تناولت الدراسة أيضا فعالية الزيوت المستخلصة من بذور البقلة ، الكزبرة والكتان كمضادات ميكروبات ولم تظهر النتائج أي تأثيريذكر. وأثبت البحث أن زيت بذور الكزبرة ذو فعالية عالية كمضاد أكسدة .

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INTRODUCTION

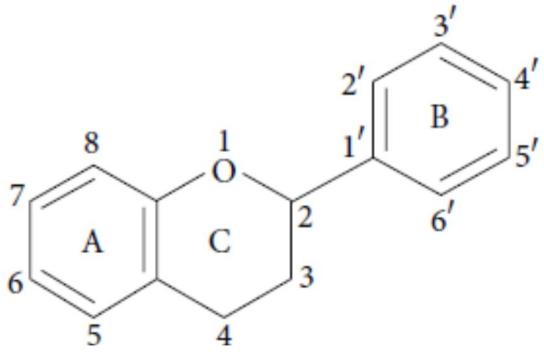
1.1 General overview

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities [1, 2]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [3]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [4]. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [5, 6]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [7, 8].

As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems [9, 10]. Flavonoids have ability to induce human protective enzyme systems.

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases [2, 8-

[10].Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin [11]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [12].The basic nucleus of flavonoids is displayed in Fig. 1.



(1)

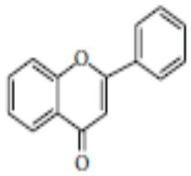
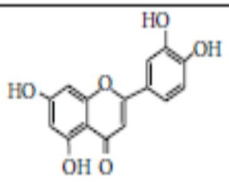
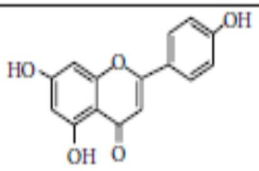
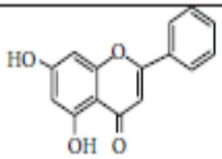
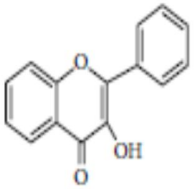
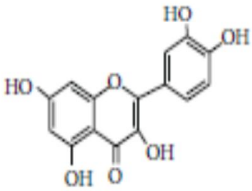
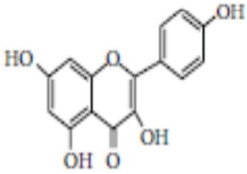
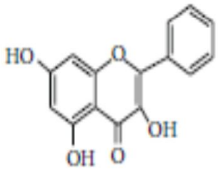
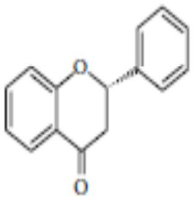
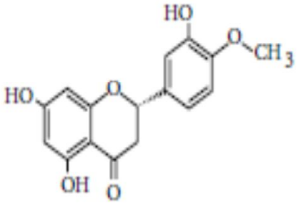
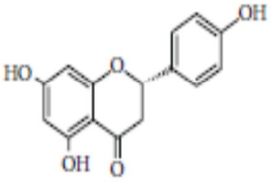
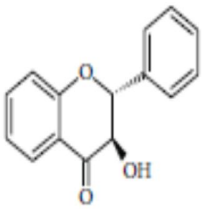
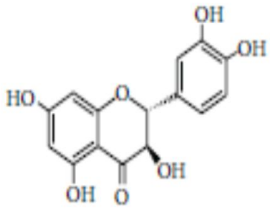
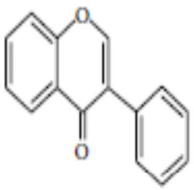
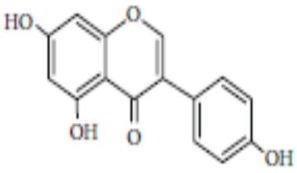
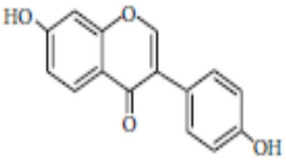
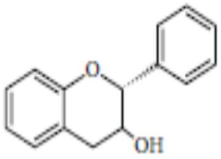
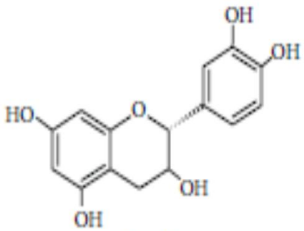
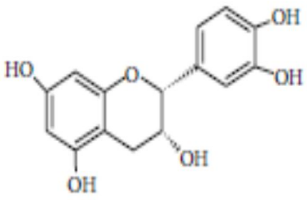
1.2 Chemistry of Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from orange. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified [13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (e.g. apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.

Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings [13].

Table (1): Structure of flavonoids

Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1.1). Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

Flavonols differ from flavanones by hydroxyl group at the 3-position and a C_2–C_3 double bond[14]. Flavonoids are often hydroxylation positions 3, 5, 7, 2, 3', 4', and 5'.Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[15].

1.2.1 Spectral Characteristics of Flavonoids

Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (235–285 nm) corresponds to the A ring absorption.

Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367 nm in kaempferol (3,5,7,4-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'- hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'- hydroxyl groups) [16]. The absence of a 3-hydroxyl group in flavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics [17]. Flavanones exhibit a very strong (Band II) absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only a shoulder for (Band I) at 326 and 327 nm. Band II appears as one peak (270

nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present.

As anthocyanins show distinctive (Band I) peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring and (Band II) peaks in the 240–280nmregion due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups [18].

1.3 Flavonoid- Rich Food and Medicinal Plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photo-synthesizing plant cells.

They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals [19].Thus flavonoids found in animals are of plant origin rather than being biosynthesized *in situ*. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [20].

Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg [21]. Preparation and processing of food may decrease flavonoid levels depending on the methods used.

For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644mg/L which suggest that the flavanones are concentrated in the cloud

during processing and storage [22]. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans [23].

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids [24, 25]. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases.

Table (2): Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry

Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavone – glabridin- a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals [26]. Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants [27, 28]. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people.

Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats [29].

Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia [30]. Table 3 summarizes some of the medicinal plants rich in flavonoid contents.

Table (3): Medicinal plants rich in flavonoids contents

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein

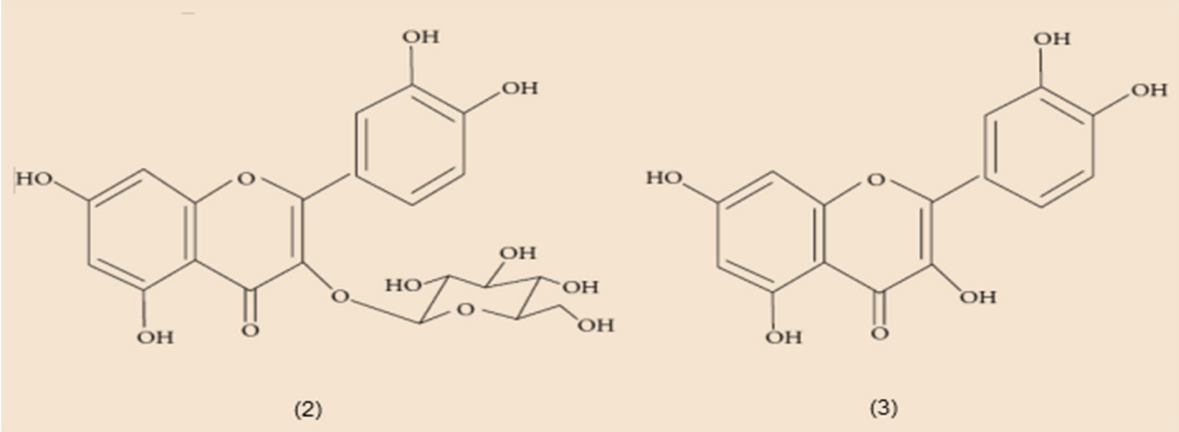
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for

example, hydroxy ethyl rutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and micro bleeding [31].

1.4 Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and p^{Ka}. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as glycosides (Figures 2 and 3). Aglycones can be easily absorbed by the small intestine, while flavonoid glycosides have to be converted into aglycone form before absorption [32].



The hydrophilic flavonoid glucoside such as quercetin are transported across the small intestine by the intestinal Na^+-dependent glucose cotransporter (SGLT1) [32].

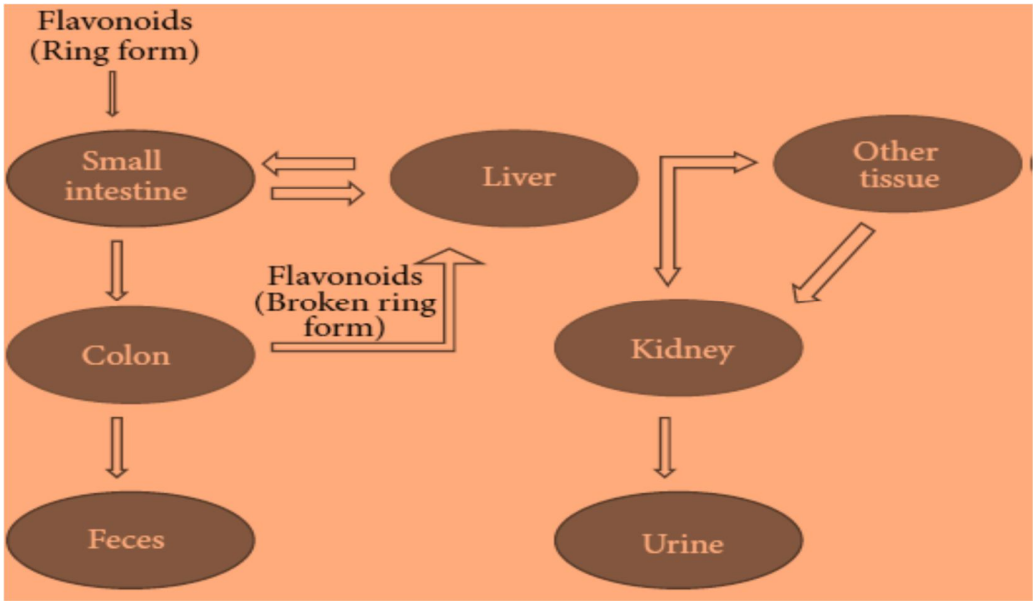
An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [33]. The substrate

specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [34].

The glycosides which are not substrates for these enzymes are transported toward the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [35]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected.

After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds [36]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [37]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [38]. The flavonoids secreted with bile in intestine and those that cannot be absorbed from the small intestine are degraded in the colon by intestinal micro flora which also break down the flavonoid ring structure (Sceme 1). Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [39]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine.

They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [40].



Sheme 1: Compartments involved in the metabolism of flavonoid

1.5 Biological Activities of Flavonoids

1.5.1 Antioxidant Activity

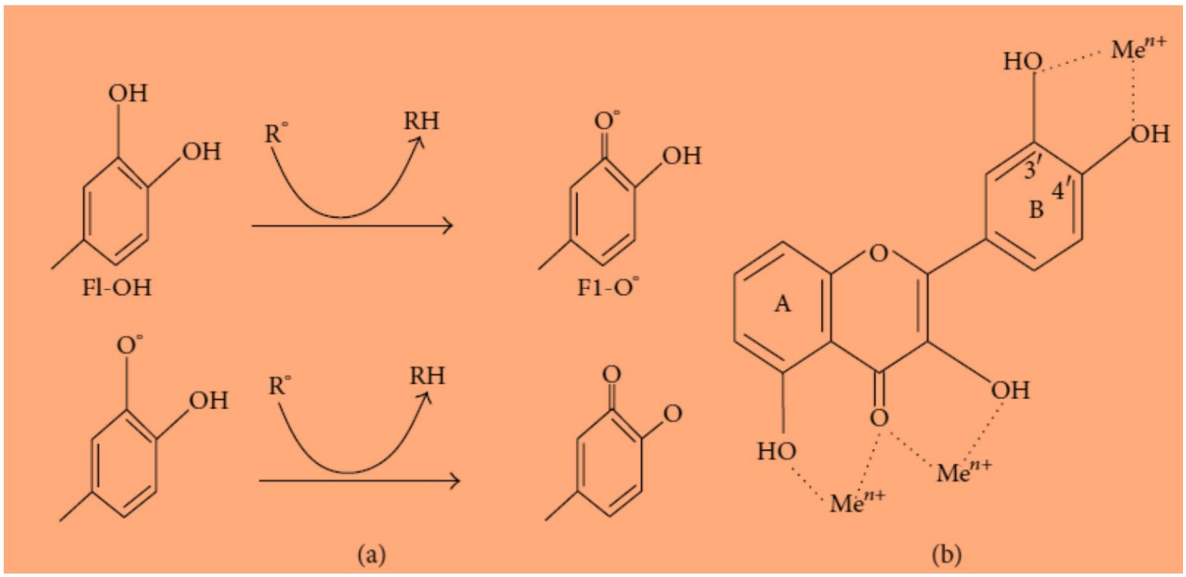
Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants.

The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure.

The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [4, 41]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and

peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [42].

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation;(2) Scavenging ROS; and (3) upregulation or protection of antioxidant defenses [43, 44]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [45]{see scheme 2}.



Scheme (2): (a) Scavenging of ROS (R∘) by flavonoids (Fl-OH) and

(b) binding sites for trace metals where Men+ indicates metal ions

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms [5, 25]. Free

metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation (Scheme 2(a)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [44, 46]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures [47]. The binding sites are shown in Schme 2(b). A 3', 4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [4]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro* [48]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable *ortho* semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential [49]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features [50].

The flavonoid heterocyclic contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH is crucial. Removal of a 3-OH annuls coplanarity and conjugation which

compromises scavenging ability [51]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity [50].

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides.

There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased [52]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position [53]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [54].

1.5.2 Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [55].

Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Glutamate-cysteine ligase catalytic subunit

(Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases [56]. It has been demonstrated that anthocyanin cyanidin-3-O-β- glucoside increases Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and pro-apoptotic signaling. Furthermore, this flavonoid lowers hepatic lipid peroxidation, inhibits the release of pro-inflammatory cytokines, and protects against the development of hepatic steatosis [56]. Silymarin is a flavonoid having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle *Silybum marianum* (Compositae). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers [57-59].

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl4-) induced injury. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl4 [60]. Similarly in an *in vivo* experiment flavonoids at of 50, 100, and 200mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver.

Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid [60].

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive

complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. *Equisetum arvense* flavonoids as well as hirustrin and avicularin isolated from some other sources is reported to provide protection against chemically induced hepatotoxicity in HepG2 cells [61, 62].

1.5.3 Antibacterial Activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid- rich plant extracts from different species have been reported to possess antibacterial activity [44, 46, 63, 64]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [65].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation.

Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [66, 67].

Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria [68, 69].

The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities [68, 70]. Robinetin, myricetin, and (−)-epigallocatechin

are known to inhibit DNA synthesis in *Proteus vulgaris.* Mori et al. [71] suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase [72].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes [73]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells.

The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity [74]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [75].

Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus* [76].

Haraguchi and colleagues [74] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A

inhibited incorporation of radioactive precursors intomacromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain [74].

There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents [77–79].

1.5.4 Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and pro-inflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [80].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells [81]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein

kinases [82, 83]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules [84]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful pro-inflammatory signaling molecules[85]. Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotopes *in vitro* [86].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration [87]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide [88]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.5.5 Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents [46, 89]. Consumption of onions and/or apples, two

major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon [90].The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods [91].

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [92]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [93]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in cogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor

agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial [94]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer [93].

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death [90, 95]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors [96].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P- 388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, andMKN-28), colon cancer cells (COLON320DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells [97] . It has been proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent [98].

Barnes [99] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones

genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats [100]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats [101]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al. [102]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [103]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects [104]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin [105].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [106]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor [44, 46, 107].Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals [108].

1.5.6 Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral

activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available.

Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso [109] demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication. Baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase [110].

Table (4): Antiviral activity of various flavonoids

Flavonoid	**Virus**
Quercetin	Rabies virus, herpes virus, parainfluenza virus, polio virus, mengo virus, and pseudorabies virus
Rutin	Parainfluenza virus, influenza virus, and potato virus
Apigenin	Immunodeficiency virus infection, Herpes simplex virus type, and Auzesky virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

Another study revealed inhibition of HIV-1 entry into cells expressing CD4 and chemokine coreceptors and antagonism of HIV-1 reverse transcriptase by the flavone *O*-glycoside [111]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase [110]. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a novel mechanism that probably involves inhibition of viral transcription [112].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV).

Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [110]. Studies have displayed that flavonols are more active than flavones against herpes simplex virus type 1 and the activity order was found to be galangin, kaempferol, and quercetin [110].

Zandi et al. [113] studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus [109]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [113]. List of some flavonoids and their efficacy against viruses is given in Table 4.

1. 6 Role of Flavonoids in Plants

1.6.1 Combating Oxidative Stress

Flavonoids have long been reported as serving multiple functions in plants [114].Various abiotic and biotic factors helps in the generation of ROS in plants leading to oxidative stress. Flavonoid biosynthesis in plants is almost exclusively enhanced due to oxidative stress. They have capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and quench ROS once they are formed [115]. Flavonoids discharged primary UV-B screening functions when early plants moved from water to colonize the land. Extent of antioxidant capacity and ability to absorb UV-wavelengths depends upon the nature of substitution on different rings of flavonoids. Dihydroxy B ring substituted flavonoids have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts have greater ability to absorb UV wavelengths [115].

The most reactive hydroxyl groups (7-OH in flavones or the 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in the aqueous cellular environment, protects the reactive hydroxyl groups from autooxidation [116], and allows the transport of flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and the cell wall [117]. Recent evidence shows that antioxidant flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation, that is, the chloroplast. Here they can easily quench H_2O_2, hydroxyl radical, and singlet oxygen [115, 118].

Oxidative stress due to an excess of excitation energy in the chloroplast may be aggravated under conditions that limit the diffusion of CO_2 to the carboxylation sites and the efficiency of carboxylation [115, 119]. The environmental limitations to CO_2 assimilation rate include drought/salinity,

low/high temperature, and nutrient scarcity. Under these conditions the activity of ROS detoxifying enzymes may significantly reduce in the chloroplast [120, 121], which in turn up regulates the biosynthesis of ROS scavenging flavonoids.

The reducing functions of flavonoids are of key significance in plants under severe stress conditions. These functional roles are concomitant with the very high concentration of dihydroxy B ring substituted flavonoids [122]. Flavonoids have been suggested as representing a secondary antioxidant defense system in plant tissues exposed to different stresses [115]. Lipid peroxidation is the common consequence of oxidative stress which disrupts the cell membrane integrity.

Quercetin 3-*O*-rutinoside (rutin) may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage [123].

1.6.2 Growth Regulator

Flavonoids carry out functional roles of amazing significance in plant-environment interactions.

Flavonoids (in the nanomolar range) may regulate auxin movement and catabolism. The ability of flavonoids to create auxin gradients translates into phenotypes with different morphoanatomical features [124]. The control of flavonoids on auxin movement may have immense value in the stress-induced morphogenic responses of plants such as the flight strategy of sessile organisms exposed to unfavorable environments [125]. Species rich in dihydroxy flavonoids exhibit phenotypes with strikingly different morphological traits as compared with those rich in monohydroxy flavonoids [126]. Dwarf bushy phenotypes with few, small, and thick leaves to direct sunlight irradiance are usually present in sunny environments, thus

protecting leaves located deep in the canopy from light-induced severe cellular homeostasis perturbations. On the contrary, shaded plants, which are rich in kaempferol and/or apigenin derivatives (having negligible concentrations of quercetin derivatives), have long internodes, and large leaf lamina coupled with reduced leaf thickness [125].

Flavonoids at the plasma membrane are effective inhibitors of PIN (pin formed) and MDR (multidrug resistance) glycoproteins that are involved in the cell to cell movement of auxin. The ability of flavonoids to inhibit the activity of the efflux facilitator PIN and MDR proteins depends on the presence of the catechol group in the B ring of the flavonoid skeleton. In addition flavonoids regulate the activity of IAA-oxidase with markedly different effects based on their chemical structure [127]. Recent evidence of a nuclear location of flavonoids (as well as of enzymes of flavonoid biosynthesis) supports that flavonoids are capable of modulating the activity of proteins involved in cell growth. Flavonoids may therefore act as transcriptional regulators [128, 129].

1.7 Microbial Production of Flavonoids

In response to the low production efficiency from plants and chemical synthesis, research groups have directed their attention to the production of flavonoids in microorganisms using metabolic engineering and synthetic biology [130].

Chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals [131]. Because of the rapid development in molecular biology tools and the flooding of genome information from a variety of organisms, combinatorial biosynthesis offers an advantage for production of rare and expensive natural products. It can be used in both simple and complex transformations without the tiresome blocking and deblocking steps that are

common in organic synthesis [132]. Several prokaryotes and eukaryotes such as *E. coli*, *Saccharomyces cerevisiae*, *Streptomyces venezuelae*, and *Phellinus igniarius,* a medicinal mushroom, have been used for production of flavonoids [12].

1.7.1 Phenylpropanoid Pathway

In plants naringenin chalcone is the precursor for a large number of flavonoids produced by the phenylpropanoid synthetic pathway. Fermentative production by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine, and tyrosine [133]. As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonialyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid, which is then activated to *p*-coumaroyl-CoA by the action of 4-coumarate:
CoA ligase. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or nonenzymatically *in vitro* [134].

1.7.2 Enhancement of Flavonoid Production

Combination of promoter and target genes; knockout of related genes; overexpression of malonyl-CoA; and construction of artificial P450 enzymes are the key molecular biology technology procedures used in the heterologous production of flavonoids.

Every gene from the phenylpropanoid pathway is cloned in the host under the control of the promoter which often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used to enhance production of flavonoids according to the need of specific host such as T7, ermE∗, and GAL1 promoter [12]. The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids. Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens* intracellular malonyl-CoA pool was amplified leading to increased production of flavonoids [135].

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It was proved in an experiment in which researchers knocked out the udg gene encoding for UDP-glucose dehydrogenase. This resulted in elimination of endogenous UDP-glucose consumption pathway leading to increase in intracellular concentration of UDP-glucose and as a consequence increment in the production of flavanones and anthocyanins was observed [136].

One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound cinnamate-4-hydroxylase [137].This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host. An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis

pathway [138]. Combining bacterial cells and eukaryotic cells in a pot enabled researchers to generate a wider library of natural and unnatural products than any of the previously reported systems. *De novo* production of the key flavonoid intermediate naringenin has been demonstrated for the first time from glucose using an engineered *S. cerevisiae* strain which led to fourfold higher concentrations than reported in earlier studies on de novo biosynthesis [139, 140].

1.8 The studied plant species

1.8.1 *Pulcaria crispa*

Pulicaria crispa is a genus of Asteraceae family. The Asteraceae family is one of largest families of flowering plant, with about 1,100 currently accepted genera and 25,000 species. It is characterized by the flowers being arranged in dense heads of many small florets, such as the daisy and dandelion [141].

Pulcaria crispa

The family is of worldwide distribution ,being absent only from the Antarctic mainland, and is particularly well represented in semiarid region of the tropics and subtropics, such as the Mediterranean region, Mexico, the cape province of South Africa grassland and bush land formation of Africa and montane floras throughout the world. Only in the tropical rain forests are they poorly represented [141]. The Asteraceae are of incalculably great indirect economic importance to man as major contributors to the diversity, and therefore, the stability and sustainable productivity, of the drier vegetation types throughout the world, especially in tropical and sub-tropical areas. In proportion to its size, however, the direct economic importance of the family is comparatively small. It includes food plants, sources of raw materials, medicinal and drug plants, ornamentals and succulents, and, on the debit side, weeds and poisonous plants [141].

1.8.1.1 Phytochemical, antibacterial screening and antioxidant activity of *Pulcaria crispa*

Many secondary metabolites have been detected - coumarins, tannins and alkaloids - on *P. crispa* [142]. Investigation of the flavonoids constituents of *Pulicaria crispa*, result in the isolation and identification of four flavonoids, quercein, quercetin-3-o-glucoside, quercetin7-o glycoside and quercetin-3-methyl ether [143]. The presence of these metabolites explains the various uses of this plant in traditional medicine. Alkaloids, comprising a large group of nitrogenous compounds, are widely used as cancer chemotherapeutic agents [144, 145] alkaloids also interfere with cell division The presence of alkaloids in *Pulicaria crispa* may be responsible for antibacterial activity. *Pulicaria crispa* is used in folk medicine for the treatment of colds, coughs, colic, excessive sweating and as carminative [146].

1.8.2 *Portulaca oleracea*

Portulaca oleracea is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution belonging to the Portulacaceae family. It is commonly known as *purslane* (USA and Australia), *rigla* (Sudan and Egypt), *pigweed* (England), *pourpier* (France), and *Ma-Chi-Xian* (China) [147]. It is distributed widely in the tropical and subtropical areas of the world including many parts of the United States and is eaten extensively as a potherb and is added to soups and salads around the Mediterranean and tropical Asian countries [148].

Portulaca oleracea

Americans and aborigines of Australia grind the seeds of this plant into flour for use in mush and bread [149]. *Portulaca oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties [150]. *Portulaca oleracea* has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic and so forth [151]. It exhibits a wide range of pharmacological effects, including antibacterial [152], antiulcerogenic [153], anti-inflammatory [154], antioxidant [155], and wound-healing [156] properties. It is listed by the World Health Organization as one of the most used medicinal plants, and it has been given the term “Global Panacea” [157]. The

Chinese folklore described it as “vegetable for long life” and it has been used for thousands of years in traditional Chinese Medicine [158, 159]. It is cold in nature and sour in taste and is used to cool the blood, stanch bleeding, clear heat, and resolve toxins. The dried aerial part of this plant is indicated for the treatment of fever, dysentery, diarrhea, carbuncle, eczema and hematochezia, with a recommended dose of 9–15 g, *Portulaca oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine [160–162].

1.8.2.2 Phytochemistry

Many constituents of *Portulaca oleracea* have been isolated, including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals.

One of the most effective constituents present in Chinese Herbal Medicines are flavonoids which are biologically active and possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. In the *Portulaca oleracea* plant, the flavonoids levels vary according to the part of the plant; the highest levels are present in the root followed by stem and the leaf; and seven different flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin [163]. However, only kaempferol and apigenin have been found in ethanolic extracts of leaves and stems, with the levels in the former being higher [157]. Portulacanones B–D, three homoisoflavonoids compounds, display selectively cytotoxic activities against three human cancer cell lines (SF-268,NCI-H460, and SGC-7901) [164]. Flavonoids are also widely present in foods such as fruits and vegetables [165].

In addition to flavonoids, another important chemical found in this plant is alkaloids including dopa, dopamine, and noradrenalin. The content of dopamine and noradrenalin is higher in leaves compared to stem and seeds. The amount of dopamine and noradrenalin obtained from leaves varies according to the solvents used in the extraction process, suggesting that the levels of these compounds are dependent on the solvents used during the extraction process [166].

Oleraceins A, B, C, D, and E are cyclodopa alkaloids isolated from this plant [167] and several analytes such as (3R)-3,5-bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone and 1,5-dimethyl-6-phenyl-1,2-dihydro-1,2,4-triazin-3(2H)-one display cytotoxic activities against human cancer cells [168].

Portulaca oleracea is also an excellent source of omega-3 fatty acids, which is usually present in oil and fat of fishes but not normally found in plants. Omega-3 fatty acids play an important role in the enhancement of immune function [169] and prevention and treatment of hypertension, coronary artery disease, cancer, and other inflammatory and autoimmune disorders [170]. It includes α-linolenic acid and linoleic acid, which are essential for normal growth, health promotion, and disease prevention in humans. Polysaccharides found in *Portulaca oleracea* are potential therapeutic agents for the treatment of diabetes mellitus owing to their modulation of blood lipids, metabolism, and decrease of blood glucose. *Portulaca oleracea* contains monoterpenes such as portulosides A and B, diterpenes such as portulene, and β-amyrin type triterpenoids [147, 171]; in addition, vitamins have also been isolated from the leaves of this plant. It contains the highest content of vitamin A which is a natural antioxidant playing an important role in vision, maintaining healthy mucus membranes and protecting against lung and oral cavity cancers among green leafy vegetables. This plant also

contains ascorbic acid, α-tocopherol, and B-complex vitamins, for example, niacin, pyridoxine, and riboflavin [171]. Furthermore it is rich in minerals like phosphorus, manganese, icon, calcium selenium [149], and the amino acids isoleucine, proline, leucine, lysine, phenylalanine, methionine, cystine, valine, threonine, and tyrosine [148]. Many other constituents have also been isolated from this plant, such as β-carotene, glutathione, melatonin, portulacerebroside A, catechol, and bergapten.

1.8.3 *Coriandrum sativam*

Coriander (*Coriandrum sativum*) being an annual herb is most commonly used for seasoning purpose. Seeds, leaves and roots are edible, although they have very distinct flavors and uses [172]. The herb has a light and fresh flavor. Coriander can be used as whole plant and can be processed because of its perishable nature of leaves and to increase the palatability of ripe fruits (seeds) before using it as flavoring agent in different food preparations. Whole plant of coriander mainly fresh leaves and ripe fruits are used for culinary purposes [172].

Coriandrum sativum

Coriander leaves have different taste than its seeds, with citrus overtones. Coriander plant is a rich reservoir of micronutrients and nutritional elements which leads us to focus our study on this herb [172]. Coriander is very low in saturated fat however,it contains good amount of linoleic acid which is a good source of α-tocopherol and vitamin K. Leaves of plant are rich source of vitamins while seeds are rich in polyphenols and essential oils. Coriander taste is devoted to its essential oil comprising a significant content of linoleic and furanocoumarins (coriandrine and dihydrocoriandrine). Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activity along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits and its protective effect to preserve the food for longer period [172].

Coriander contains high amount of essential oils that are very important for growth and for proper functioning of brain. The main essential fatty acids present in coriander include linoleic and linolenic acids. Linoleic acids belong to PUFA (polyunsaturated fatty acid) group. Dietary supplementation of coriander seed greatly affects the lipid composition of carcass by decreasing saturated fatty acid (SFA) contents (palmitic and stearic acids) and by increasing monounsaturated and polyunsaturated fatty acid (MUFA and PUFA) [173]. This plant is a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) isolated from the seeds and the aerial parts [174]. The high content of fats and protein in the fruits make distillation residues suitable for animal feed. Coriander fruits yield 5 to 7% of ash, 13% resin, astringent principle, malic acid and alkaloids. Coriander oil contains coriandrol, jireniol and vebriniol [175].

1.8.4 *Linum usitatissimum*

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* Figure 1.8 was given by Linnaeus in his book "Species Plantarum" [176]. It is an annual herbaceous plant with shallow root system.

The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are specialized development of this species [177].

The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber purpose are tall growing with straight culms and have fewer secondary branches.

Linum usitatissimum

The Mediterranean and Southwest Asia have both been proposed as the center of origin [203]; however the exact location is uncertain [178]. The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the stearoyl-ACP desaturase II

(*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop [179].

Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [180] making it ideal for paints, varnishes, and inks due to its fast polymerization properties.

Increasing demand for edible oil sources with significant percentages of omega-3 fatty acids is resulting in consumption of flaxseed as a functional food. Flaxseed is also added to animal feed to improve animal reproductive performance and health [181, 182].

Research objectives

1. Extraction of flavonoids and oils from some plants
2. GC-MS studies on the extracted oils.
3. Elucidation of structures of flavonoids.
4. Biological activity of isolates.

MATERIAL AND METHOD

2.1. Materials

2.1.1 Plant material

Stems of *pulcaria crispa* were collected from White Nile state (Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum – Sudan. The seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were collected from Jeddah-Saudi Arabia.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m length; 0.25mm diameter; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.1.3-Test organisms

Test organisms used in the antimicrobial assay are: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

2.2- Methods

2.2.1- Preparations of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminum chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. distilled water.

- **Potassium iodide solution:** 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

100 g) of powdered air- dried stems of *pulcaria crispa* were extracted with 95% aqueous ethanol. The solvent was removed under reduced pressure giving the prepared extract. This prepared extract (PE) was used for phytochemical screening.

2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) were evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5

ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.2.2- Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

2.2.2.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Mayer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.2.4- Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and

the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.2.5 -Test for saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml)of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand for one hour and then checked for foam formation.

2.2.2.6 - Test for coumarins

(0.2 g) of the prepared extract were dissolved in 10 ml distilled water in test tube and a filter paper was attached to the test tube to be saturated with the vapor after being spotted o with 0.5N KOH. Then the filter paper was inspected under UV light. The presence of coumarins is indicated by the ability of the spot to absorb the UV light.

2.2.2.7- Test for anthraquinone glycosides

(0.2 g) of the prepared extract was boiled with 10 ml of 0.5 N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. (5ml) of the benzene solution was shacked with (3ml) of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraxquinones is indicated by pink or red color in the alkaline layer.

2.2.3- Extraction of flavonoids

The air - dried powdered stem of a *pulcria crispa* (3 kg) were extracted by maceration with ethanol (95%). The extract was concentrated under reduced pressure to give a crude product.

2.2.4- Column chromatography

Open column (70× 4 cm) was used for fractionation of the ethanolic extracts of *pulcaria crispa*. Silica gel with particle size 60-120 mesh ,from LOBA chemicals, was used as stationary phase. The composition of the mobile phase (ethyl acetate: methanol) was determined by TLC experiments.

The ethanolic extract of *pulcaria crispa* (5 g) was mixed with 10 g of silica gel and then applied on the top of the column. Elution commenced by EthOAC - MeOH ;(3:7, fraction I), (1:1, fraction II) and (0:1, fraction III).

2.2.5- paper chromatography

Fraction II – being rich in phenolics- was subjected to paper chromatography where pure flavonoids - compounds I and II -were isolated using the solvent system: butanol: acetic acid: water (4: 1: 12, upper layer).

2.2.6-Extraction of oils

Powdered seeds of *Portulaca oleracea* (*Coriandrum sativam* and *Linum usitatissimum*) - (500g) for each - were macerated with n-hexane . The solvent was removed under reduced pressure to yield the oil.

2.2.7-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added,then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes. The hexane layer was then separated.(5µl) of the hexane extract

were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.8- GC-MS analysis

The oils from *Portulaca oleracea, Coriandrum sativam* and *Linum usitatissimum* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 µm, thickness) was used. Helium (purity; 99.99 %) was used as carrier gas.

2.2.9- Biological activity

2.2.9.1- Preparations of chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa*

Chloroform fraction of *pulcaria crispa* was prepared by suspending the ethanolic extract of the leaves in the least amount of distilled water and then shaking with chloroform in a seperatory funnel. Evaporation of the chloroform layer under reduced pressure gave the required fraction. Other fractions were prepared in a similar way.

2.2.9.2- Antimicrobial assay

The ethanolic, chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa* as well as the essential oils extracted from the seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were evaluated for their antimicrobial activity against five standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*) using the cup plate agar method with some minor modifications.

2.2.9.2.1- Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 108- 104 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o}C for 24 hours.

2.2.9.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.9.3- Testing for antibacterial activity

The cup plate agar diffusion method was adopted with, some minor modification, to assess the antibacterial activity (2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a single test sample.

The agar discs were removed and cups were filled with 0.1 ml of each test sample using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^{o} C for one hours.

The above procedure was repeated for different concentrations of extracts and standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.9.4- Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but this time Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above

2.2.9.5-Antioxidant activity

The DPPH radical scavenging capacity was determined. In 96-wells plate, the test samples were allowed to react with 2.2 Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37° C. the concentration of DPPH was kept as (300μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests were run in triplicate. Propyl gallate was used as a positive standard at concentrations 0.5 mM.

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RESULT AND DISSCUSSION

Powdered air-dried stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature for 48hr. The crude product obtained after evaporation of the solvent under reduced pressure. The crude extract was fractionating by separator funnel and the three fractions were collected (ethyl acetate, chloroform and butanol fractions) and screened for its antimicrobial activity. The crude was subjected to silica gel column chromatography and eluted with ethyl acetate - methanol mixture (3: 7, fraction I), (1: 1, fraction II) and pure methanol (fraction III). Three fractions were collected. The fraction II was subjected to paper chromatography where pure components: **compound I** and **II** have been isolated. The structures of isolated flavonoids have been elucidated via a combination of spectra techniques.

GC-MS analysis on seed oil of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and the identification of the constituents. The oils was screened for antimicrobial and antioxidant activity against standard organisms.

3.1- *Pulcaria crispa*

3.1.1- Phytochemical screening

Pulcaria crispa was screened for major secondary metabolites and the results are displayed in table 3.1.

Table 3.1: Phytochemical screening of *pulcaria crispa*

Test	**Result**
Saponin	++
Cumarin	+
Alkaloids	+
Flavonoids	+
Tanin	+++
Steroids	-
Triterpens	-
Anthraquinone	-

+: Trace

++: Moderate

++: High

- : Negative

3.1.2- Antimicrobial assay

The crude extract, ethyl acetate, chloroform and butanol fractions have been screened for antimicrobial activity against five human pathogens. The results are depicted in table 3.2 Tables 3.3 and 3.4 display the antimicrobial activity of standard drugs.

Table 3.2: Antimicrobial activity of crude and three fractions

	Ec	Ps	Sa	Bs	Ca
Crude extract	_	_	_	_	10
Ethyle acetate fraction	12	10	13	_	12
Choroform fraction	_	_	_	_	11
Butanol fraction	_	_	10	_	10

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

The results were interpreted in a commonly used term :< 9 mm: in active; 9-12 mm: partially active; 13-18 mm: active; >18 very active.

The crude extract showed partial antifungal activity against *Candida albicans* (see table 3.2 and the appendix).

Ethyl acetate fraction showed activity against *Staphylococcus aureus.* It also exhibited partial Antibacterial activity against *Escherichia coli,*

Pseudomonas aeroginosa and *Candida albicans*.The choroform fraction showed partial anticandidal activity.

Butanol fraction showed partial Antibacterial activity against *Staphylococcus aureus* and *Candida albicans*.

Table 3.3: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.4: Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

3.1.3- Antioxidant assay

The antioxidant capacity of crude extract has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test extract against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.5) crude extract exhibited significant anti- oxidant activity.

Table 3.5: Radical scavenging activity of crude extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Crude extract	82.00%

3.1.4-Characterization of compound I

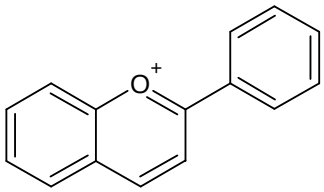
Compound I was isolated from *Pulcaria crispa* stems as yellow powder.

Table 3.6: IR data of compound I

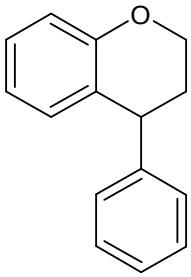
Functional group	**Wave number /cm^{-1}**
(C-H, Ar)	630.51
(C-O, ether)	1273.70
(C=C, Ar)	1378.30
(C=O)	1727.92
(OH)	3368.88
(C-H) Alkane	2921.62

The IR spectrum of compound I **(**Fig.3.1) showed all the anticipated bands of flavonids. Since

the infrared showed carbonyl absorption then the possibility that compound I being an anthocyanin or catechin is dismissed since these classes are characterized by absence of a carbonyl function.



Anthocyanin



Catechin

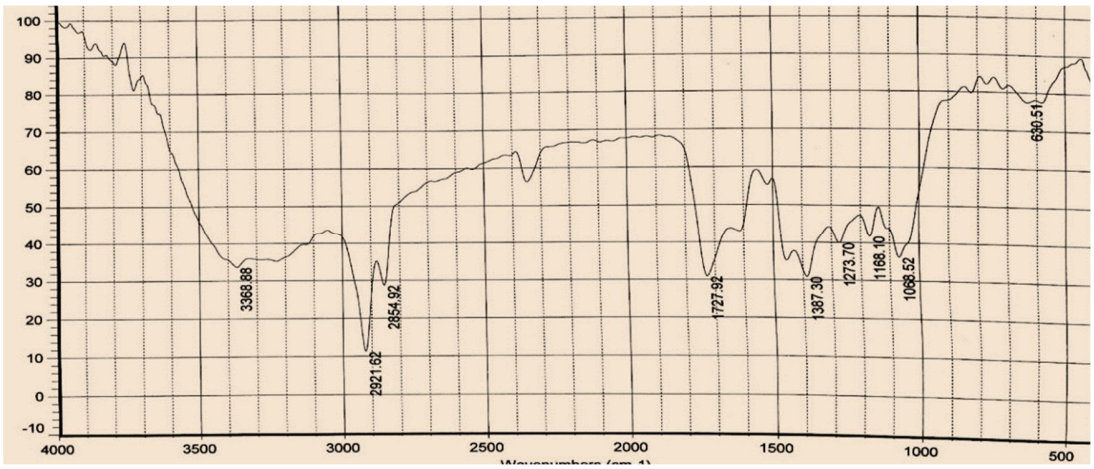
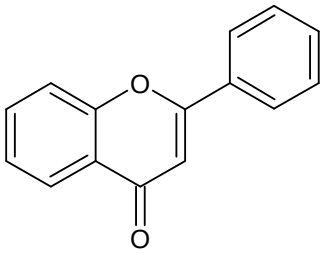
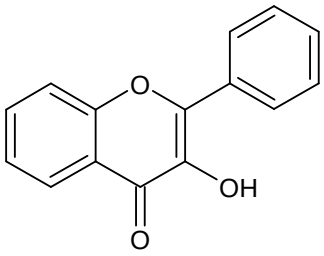


Fig 3.1: IR spectrum of compound I

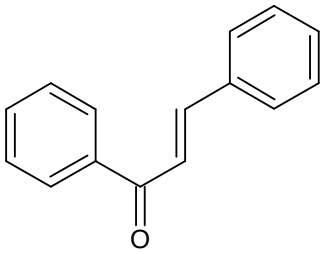
The UV spectrum of compound I revealed two major peaks at 262 and 345 nm (Fig.3.2). The presence of two peaks is a characteristic feature of flavones, flavonols, chalcones, and aurones.



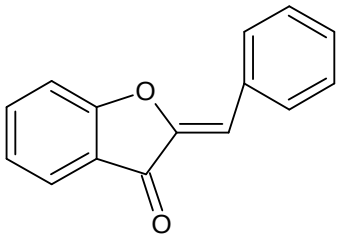
Flavone



Flavonol



Chalcone



Aurone

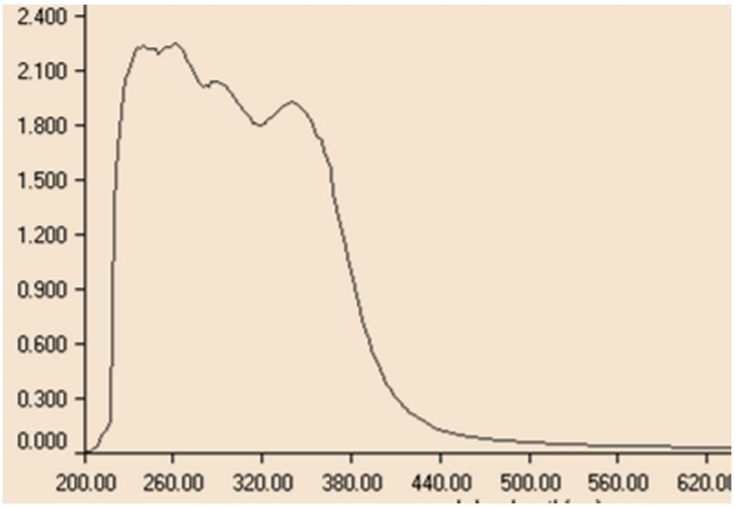
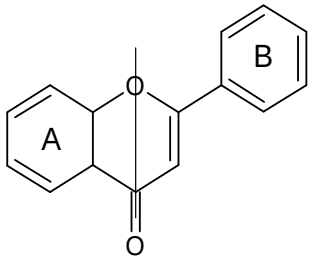


Fig 3.2: UV spectrum of compound I

The UV spectra of most flavonoids consist of two major absorption maxima, one of which occur in the range 220 – 290 nm (band II) and other in the range 300 – 400 nm (band I). The appearance of both band I and II demonstrates conjugation between the benzoyl and cinnamoyl systems.



Benzoyl Cinnamoyl

Band I is considerable to be associated with the absorption of cinnamoyl system, while band II originates from the benzoyl system.

Compound I showed both band II and I (fig.3.2) at 262 and 345nm respectively. Such absorption indicates that this compound is either a flavone, flavonol, chalcone or aurone. But aurones and chalcones absorb in

the range 365-430nm (band I). Flavones absorb in the range 300-350 nm, while flavonols absorb in the range 356-385. Thus compound I is a flavone.
Considerable structural feature have also been obtained using UV shift reagents such as sodium methoxide, sodium acetate, aluminum trichloride. These reagents produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl groups in the flavonoid molecule.
Use has been made of the effect of sodium methoxide on the UV spectra of flavonoids for detection of free 3 – and/or 4` –hydroxyl groups.
On addition of NaOMe to a methanolic solution of compound I a 42 nm bathochromic shift in band I without decrease in intensity (fig.3.3) was observed indicating the presence of a 4`-OH function.

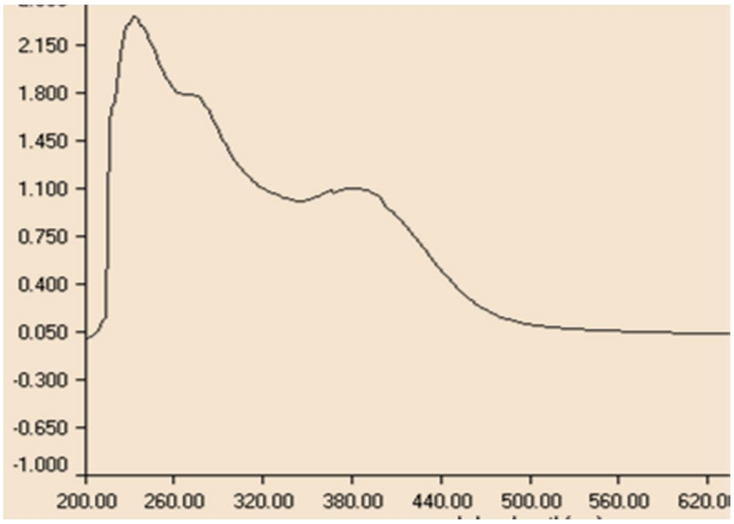


Fig 3.3: The UV spectrum of compound I in sodium methoxide

Sodium acetate is particularly useful diagnostic reagent for the specific detection of 7 –hydroxy group. When a methanolic solution of compound I **was** treated with excess powdered sodium acetate, and 16nm bathochromic shift in band II (fig.3.4) was observed suggesting a 7 –OH group.

When the previous solution of compound I was treated with excess powdered boric acid, no bathochromic shift was observed (fig.3.5) this indicate absence of *ortho*- dihydroxyl groups.

The shift reagent aluminum trichloride is diagnostic of 3 –hydroxyl group, 5 –hydroxyl group and catechol moieties[216].When a methanolic solution of compound I was treated with a few drops of the shift reagent $AlCl_3$ no bathochromic shift (fig.3.6) was observed and this indicates absence of 3 –OH, 5 –OH and catechol moieties.

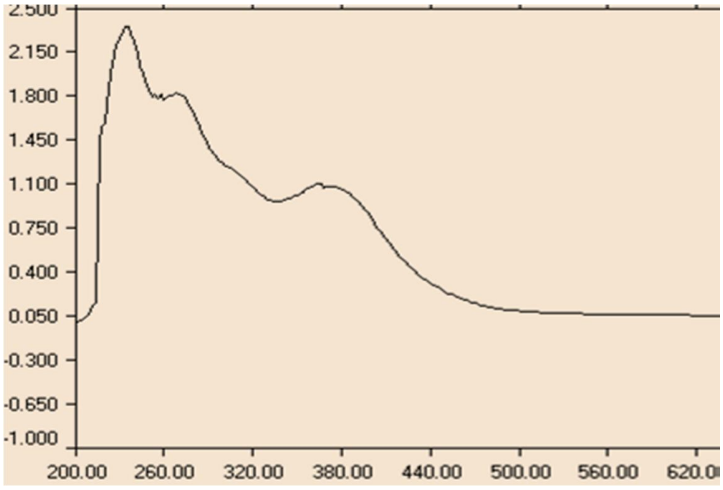


Fig 3.4: UV spectrum of compound I in sodium acetate

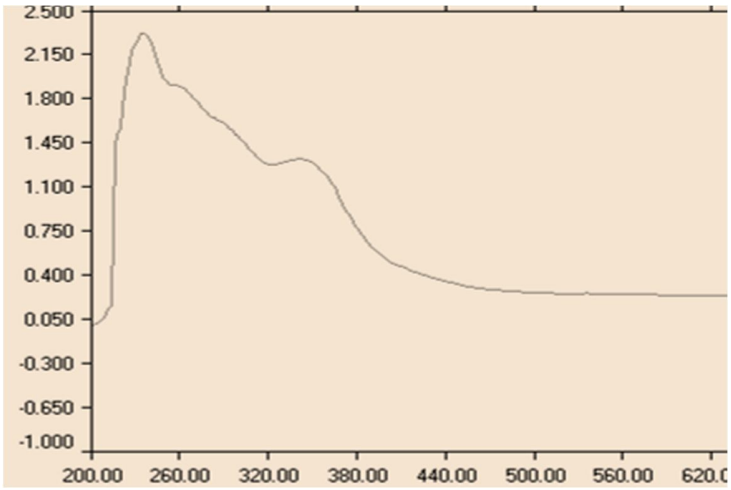


Fig 3.5: UV spectrum of compound I in sodium acetate/boric acid

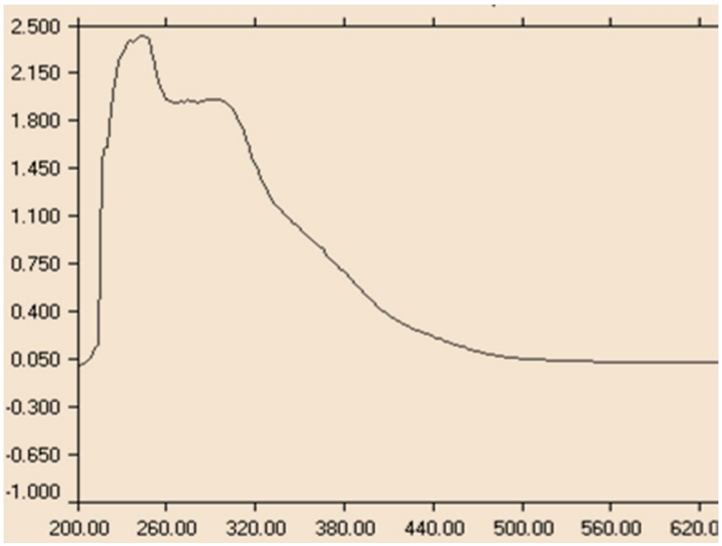


Fig 3.6: UV spectrum of compound I in aluminum trichloride

The ^{1}HNMR spectrum (Fig.3.7) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.1 ppm accounts for an acetyl function. The multiplet at δ 4.23-5.45 ppm is due to a sugar moiety (not identified in this study). The aromatic protons appeared as a multiplet (δ 6.68-7.65 ppm).

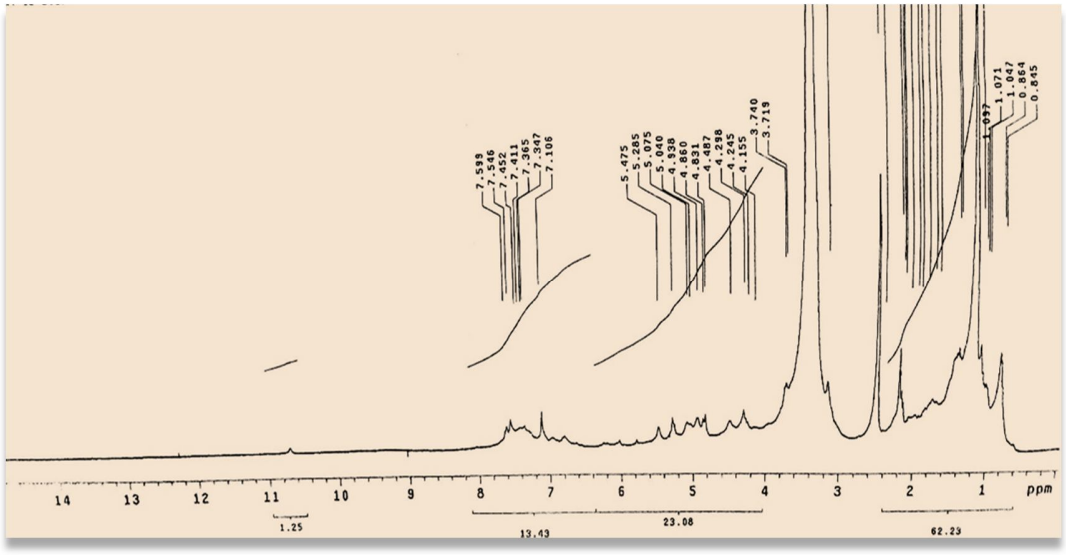
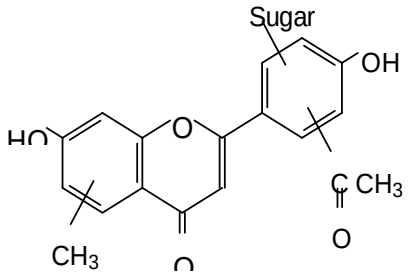


Fig 3.7: ^{1}HNMR spectrum of compound I

On the basis of the above spectral data, the following partial structure was proposed for compound I:



3.1.5- Characterization of compound II

Compound II was isolated as white-yellowish powder from *Pulcaria crispa* stem. The IR data of compound II is presented in the following table (see also Fig.3.8).

Table 3.7: IR data of compound II

Functional group	N(cm^{-1})
(C-H, Ar)	2085.7
(C-O, ether)	1017.15
(C=C, Ar)	1386.55
(C=O)	1643.49
(OH)	3419.34
(C-H) Alkane	2832.88

Compound II – which showed a carbonyl absorption-cannot be an anthocyanin or a catechin since IR spectrum gave carbonyl stretching at 1643.49 cm^{-1}. It could belong to one of the other classes of flavonoids. Anthocyanin and catechins are devoid of carbonyl.

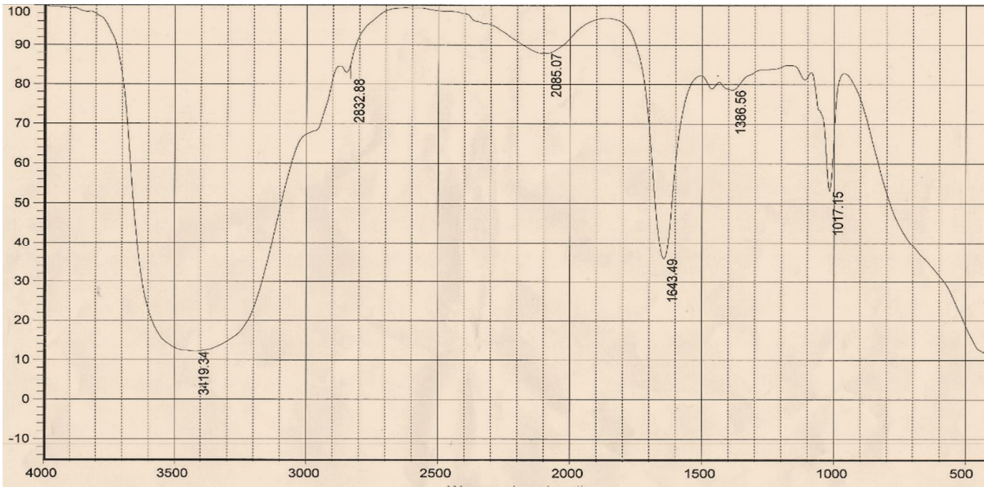
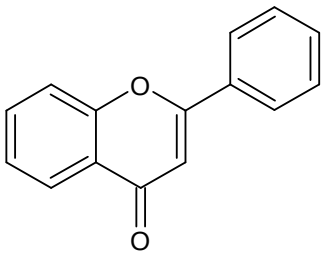


Fig 3.8: IR spectrum of compound II

Table 3.8: UV spectral data of compound II

λ_{max} (nm)	
Band I	Band II
330	240

The UV spectrum of compound II is depicted in (fig.3.9). The spectrum revealed two major peaks at 240 and 330 nm. Such absorption is a characteristic feature of flavones.



Flavone

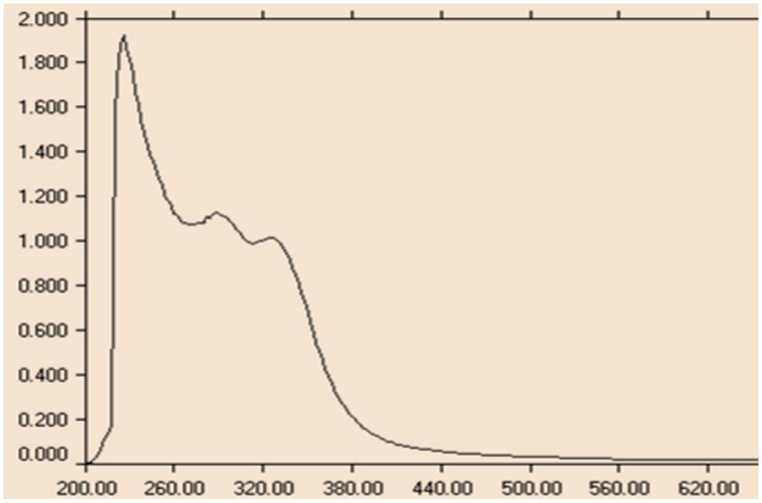


Fig 3.9: UV spectrum of compound II

Considerable structural feature have also been obtained using UV shift reagents: sodium methoxide, sodium acetate, aluminum trichloride.

On addition of NaOMe to a methanolic solution of compound II a 45nm bathochromic shift in band I indicative the presence of a 4`-OH function was observed (Fig.3.10).

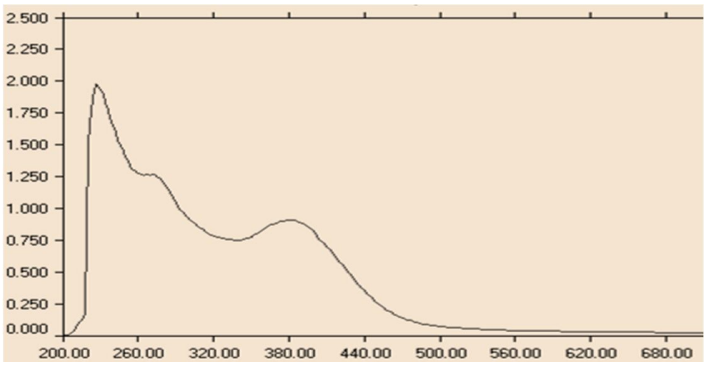


Fig 3.10: The UV spectrum of compound II in sodium methoxide

When a methanolic solution of compound II was treated with excess powdered of the shift reagent NaOAC, a 30 bathochromic shift in band II (fig.3.11) was observed and this indicates presence of a 7 –OH group.

When the previous solution of compound II was treated with excess powdered of boric acid, no bathochromic shift in both band II and I was observed (fig.3.12) this indicate absence of *ortho*- dihydroxyl groups.

The aluminium chloride spectrum showed no bathochromic shift (fig.3.13) indicating absence of 3 –OH, 5 –OH and catechol moieties.

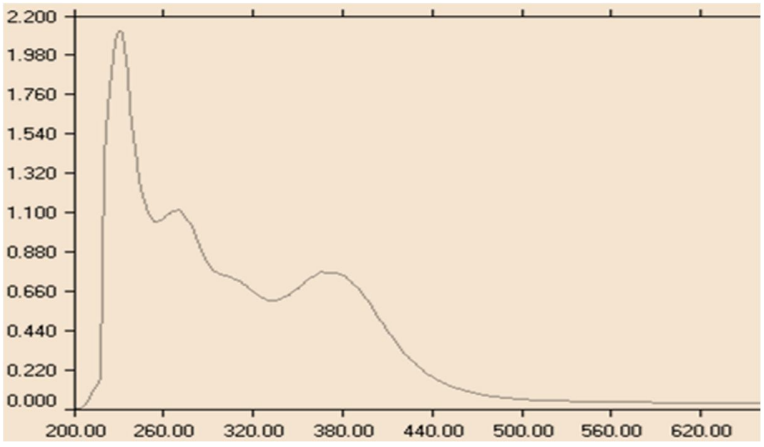


Fig 3.11: UV spectrum of compound II in sodium acetate

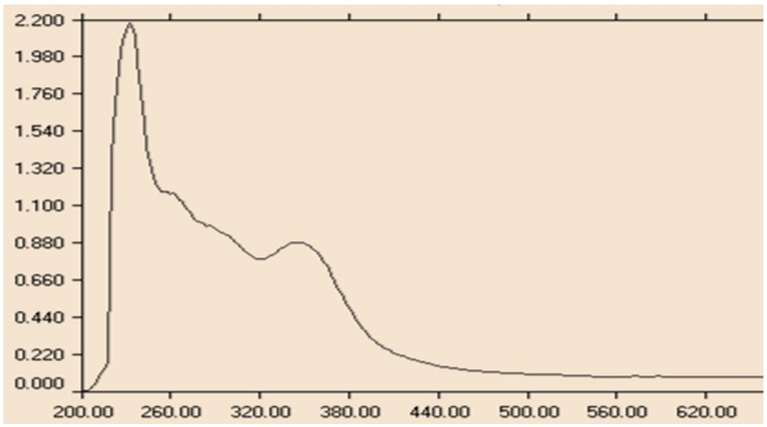


Fig 3.12: UV spectrum of compound II in sodium acetate/boric acid

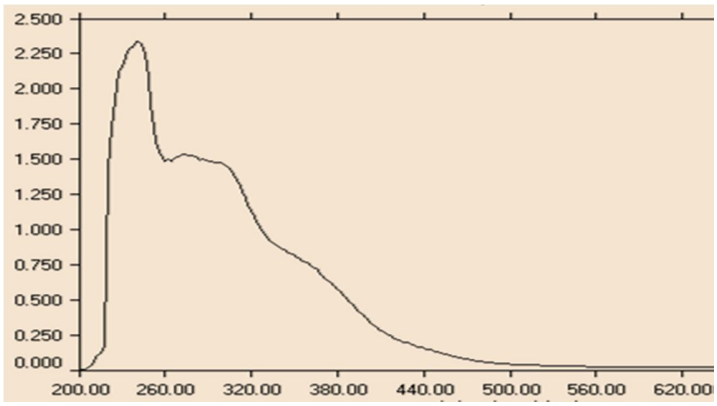


Fig 3.13: Aluminum trichloride spectrum of compound II

The ^{1}HNMR spectrum (Fig.3.14) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.4 ppm accounts for an acetyl function. The aromatic protons appeared as a multiplet (δ 6.68-7.59 ppm).

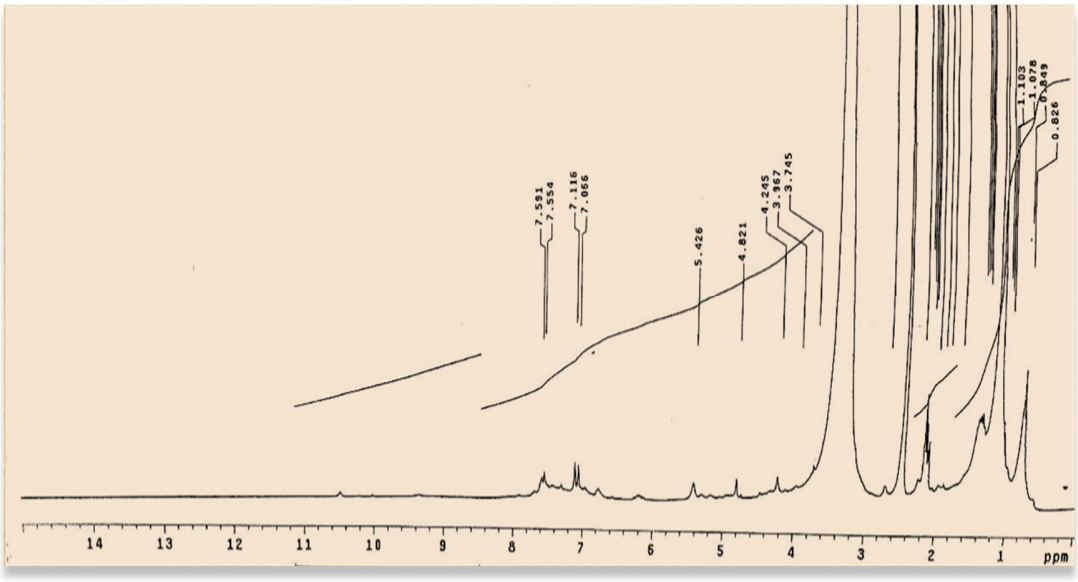
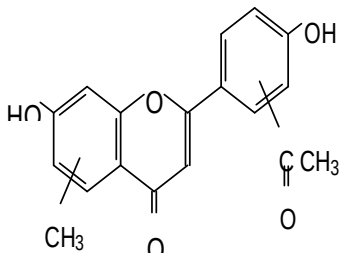


Fig 3.14: [1]HNMR spectrum of compound II

On the basis of the above spectral data, the following partial structure was proposed for compound II:



Compound II

3.2- *Portulaca oleracea*

3.2.1- GC-MS analysis of *Portulaca oleracea* oil

GC-MS analysis of *Portulaca oleracea* oil was conducted and the identification of the constituents was done by comparison of retentions times and MS data with the GC-MS library. Structures of constituents of the oil were further confirmed by interpreting the observed fragmentation pattern

.Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.2- constituents of oil

The GC-MS analysis of the studied oil revealed the presence of 15 components (Table: 3.9).The typical total ion chromatograms (TIC) of n-hexane extract are shown in (fig.3.15).

Table: 3.9: Constituents of *Portulaca oleracea* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.316	237128	0.08
2.	Pentadecanoic acid, methyl ester	14.373	170885	0.05
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.152	161664	0.05
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.195	705542	0.23
5.	Hexadecanoic acid, methyl ester	15.404	75687986	24.19
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	251818	0.08
7.	Heptadecanoic acid, methyl ester	16.367	775909	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.068	116899705	37.37
9.	9-Octadecenoic acid (Z)-, methyl ester	17.130	23526885	7.52
10.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.153	59248358	18.94
11.	Methyl stearate	17.307	22933709	7.33
12.	cis-11-Eicosenoic acid, methyl ester	18.883	1307281	0.42
13.	Eicosanoic acid, methyl ester	19.084	6202098	1.98
14.	Docosanoic acid, methyl ester	20.716	3045550	0.97
15.	Tetracosanoic acid, methyl ester	22.226	1688258	0.54

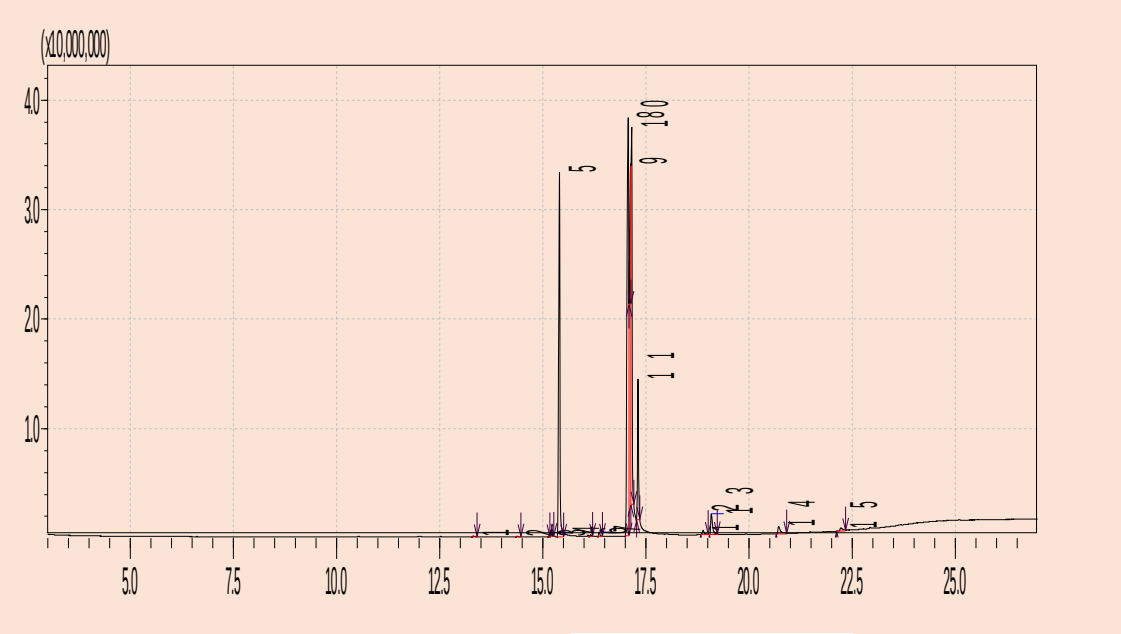


Fig 3.15: Chromatograms of *Portulaca oleracea* seed oil

The following esters were the major components:

9, 12-Octadecadienoic acid, methyl ester (37.37%)

The EI mass spectrum of 9,12-octadecadienoic acid, methyl ester shown in (fig. 3.16).The peak at m/z 294, which appeared at R.T. 17.068 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z 263 corresponds to loss of a methoxyl function.

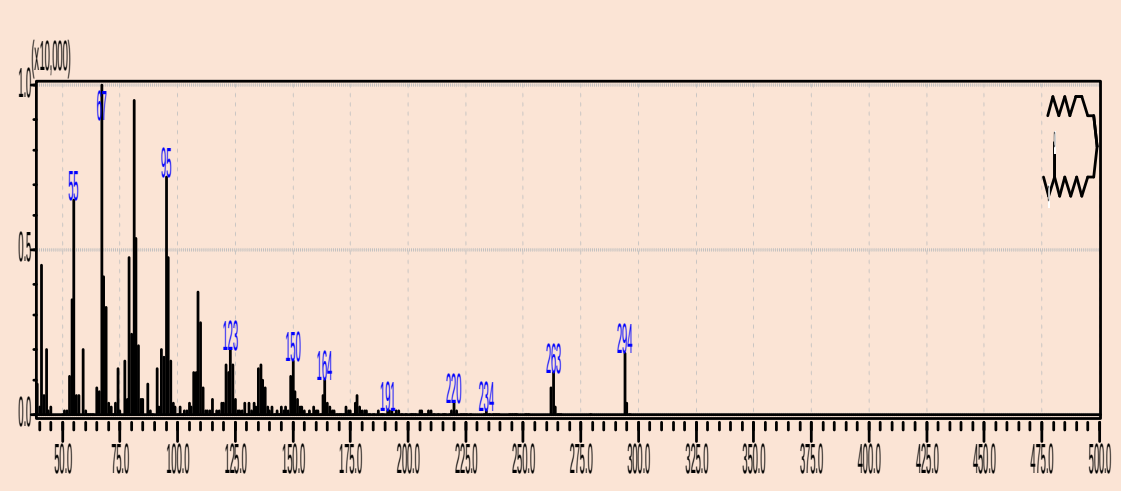


Fig 3.16: Mass spectrum of 9, 12-Octadecadienoic acid, methyl ester

Hexadecanoic acid, methyl ester (24.19%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.17).The peak at m/z 270 (at R.T. 15.404) corresponds $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is due to loss of a methoxyl .

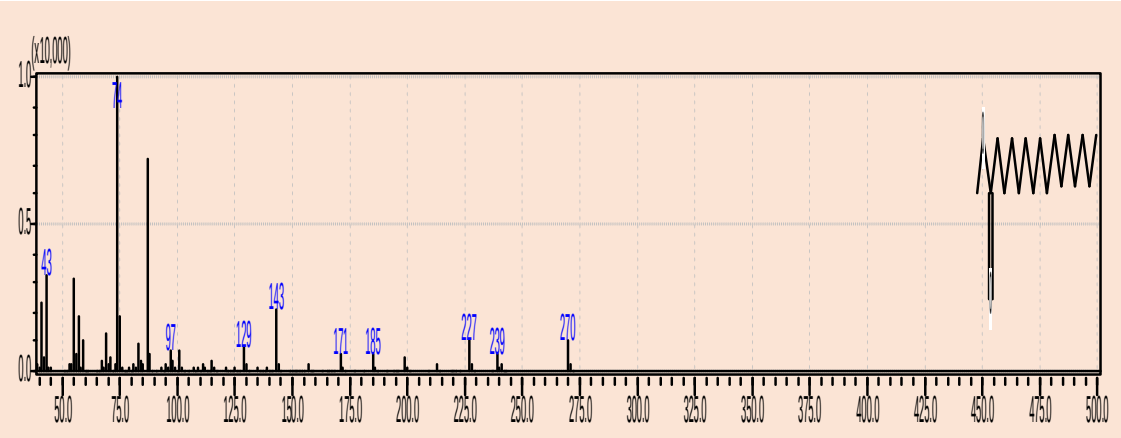


Fig 3.17: Mass spectrum of hexadecanoic acid, methyl ester

9,12,15-Octadecatrienoic acid, methyl ester (18.94%)

Fig. 3.18 demonstrates the mass spectrum of 9, 12, 15-octadecanoic acid, methyl ester. The signal at m/z 292(R.T. 17.153 in total ion chromatogram) corresponds to the molecular ion: $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z261 accounts for loss of a methoxyl .

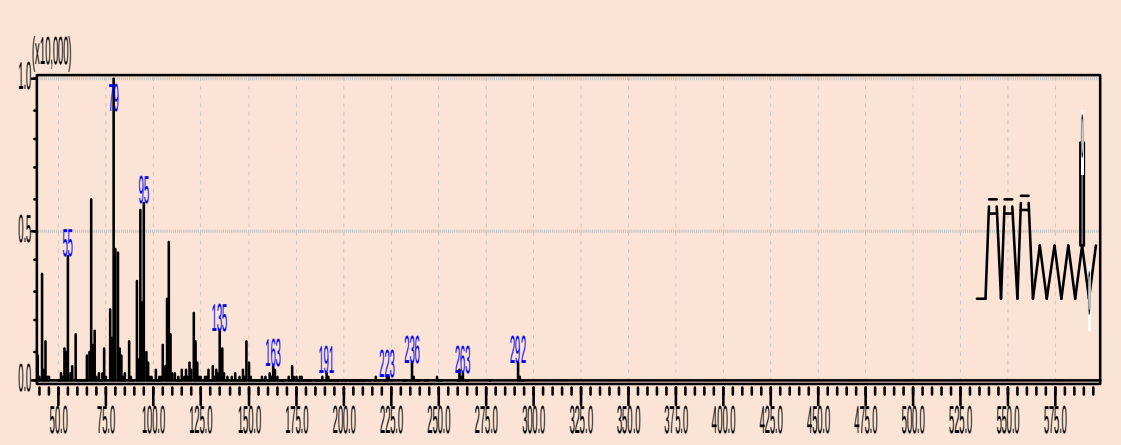


Fig 3.18: Mass spectrum of 9,12,15-Octadecatrienoic acid, methyl ester

3.2.3- Antimicrobial activity

The oil was screened for antimicrobial activity against five standard organisms. The oil not showed any activity against all test organisms

3.2.4- Antioxidant activity

The antioxidant capacity of *Portulaca oleracea* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.10) *Portulaca oleracea* seed oil exhibited weak antioxidant activity.

Table 3.10: Radical scavenging activity of *Portulaca oleracea* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Portulaca oleracea oil	29.00%

3.3- *Coriandrum sativam*

3.3.1- GC-MS analysis of *Coriandrum sativam* oil

GC-MS analysis of *Coriandrum sativam* was carried out . The GC-MS analysis revealed the presence of 14 components (table: 3.11).The typical total ion chromatograms (TIC) of n-hexane extract is presented in (fig.3.19).

Table: 3.11: Constituents of *Coriandrum sativam* oil

ID#	Name	Ret. Time	Area	Area%
1.	Methyl tetradecanoate	13.312	215647	0.03
2.	Pentadecanoic acid, methyl ester	14.372	231442	0.04
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.169	1910240	0.29
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.192	3233931	0.50
5.	Hexadecanoic acid, methyl ester	15.392	41125489	6.32
6.	Heptadecanoic acid, methyl ester	16.367	287117	0.04
7.	7,10-Octadecadienoic acid, methyl ester	16.913	1741544	0.27
8.	6,9-Octadecadienoic acid, methyl ester	16.962	543578	0.08
9.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.062	100831481	15.50
10.	9-Octadecenoic acid (Z)-, methyl ester	17.189	485955106	74.69
11.	Methyl stearate	17.313	9449169	1.45
12.	cis-11-Eicosenoic acid, methyl ester	18.865	3707972	0.57
13.	Eicosanoic acid, methyl ester	19.084	1068295	0.16
14.	Docosanoic acid, methyl ester	20.719	362910	0.06

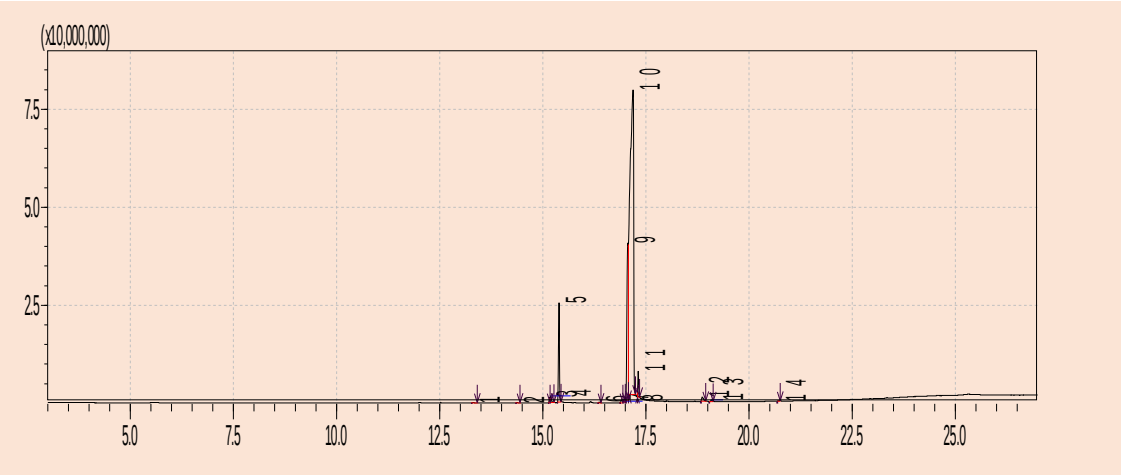


Fig 3.19: Chromatograms of *Coriandrum sativam* seed oil

The following esters were detected as major components:

9-Octadecenoic acid, methyl ester (74.69%)

The EI mass spectrum of 9-octadecenoic acid, methyl ester is shown in (fig. 3.20).The signal which appeared at m/z 264, with R.T. 17.198 corresponds $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z 235 is due to loss of a methoxyl group.

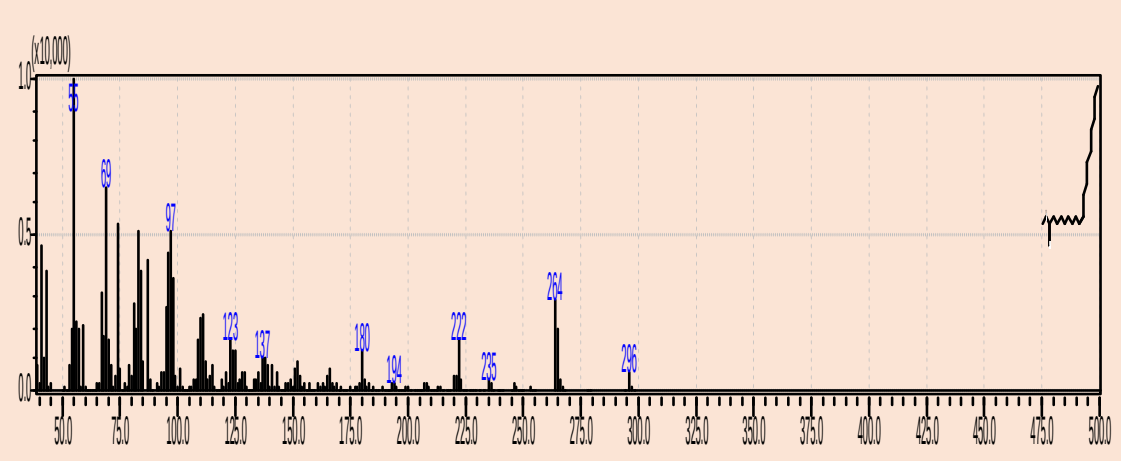


Fig 3.20: Mass spectrum of 9-Octadecenoic acid, methyl ester

9, 12-Octadecadienoic acid, methyl ester (15.50%)

Fig. 3.21 presents the mass spectrum of 9, 12-octadecadienoic acid, methyl ester .The peak at m/z 294, with R.T. 17.062 in total ion chromatogram, corresponds the molecular ion : $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 accounts for loss of a methoxyl function.

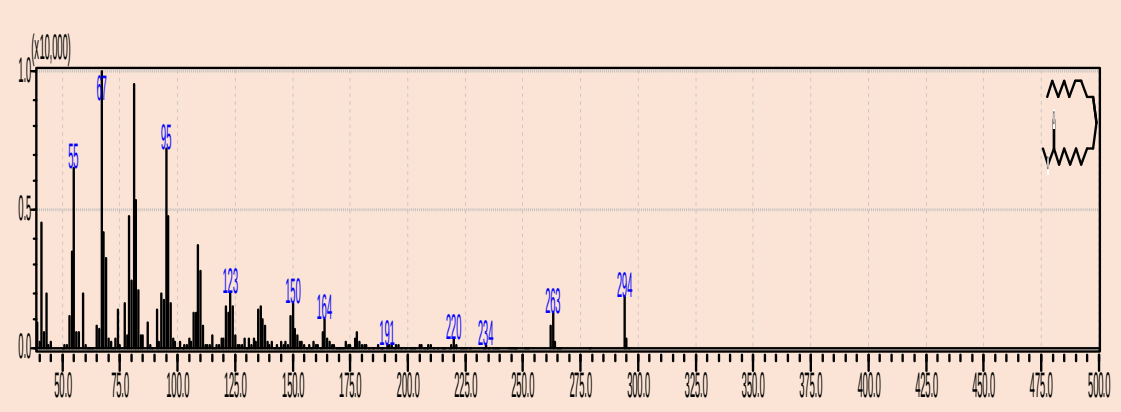


Fig 3.21: Mass spectrum 9, 12-Octadecadienoic acid, methyl ester

3.3.3- Antimicrobial activity

The studied oil was evaluated for antimicrobial activity against five standard human pathogens. The oil not showed any activity against all test organisms

3.3.4- Antioxidant activity

The antioxidant capacity of *Coriandrum sativam* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.12) *Coriandrum sativam* seed oil exhibited moderate antioxidant activity.

Table 3.12: Radical scavenging activity of *Coriandrum sativam* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Coriandrum sativam oil	52.00%

3.4- *Linum usitatissimum*

3.4.1- GC-MS analysis of *Linum usitatissimum* oil

GC-MS analysis of *Linum usitatissimum* was conducted. The analysis revealed the presence of 15 components (table: 3.13).The typical total ion chromatograms (TIC) of n-hexane extract is shown in (fig.3.22)

Table: 3.13: Constituents of *Linum usitatissimum* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.317	189936	0.07
2.	Pentadecanoic acid, methyl ester	14.373	70754	0.03
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.155	104077	0.04
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.196	476941	0.19
5.	Hexadecanoic acid, methyl ester	15.392	35779007	14.12
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	270452	0.11
7.	Heptadecanoic acid, methyl ester	16.365	508829	0.20
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.053	64486368	25.44
9.	9-Octadecenoic acid (Z)-, hexadecyl ester	17.155	69889815	27.58
10	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.164	50002070	19.73
11	Methyl stearate	17.307	28380442	11.20
12	cis-11-Eicosenoic acid, methyl ester	18.887	1343975	0.53
13	Eicosanoic acid, methyl ester	19.092	684609	0.27
14	Docosanoic acid, methyl ester	20.722	818972	0.32
15	Tetracosanoic acid, methyl ester	22.233	436726	0.17

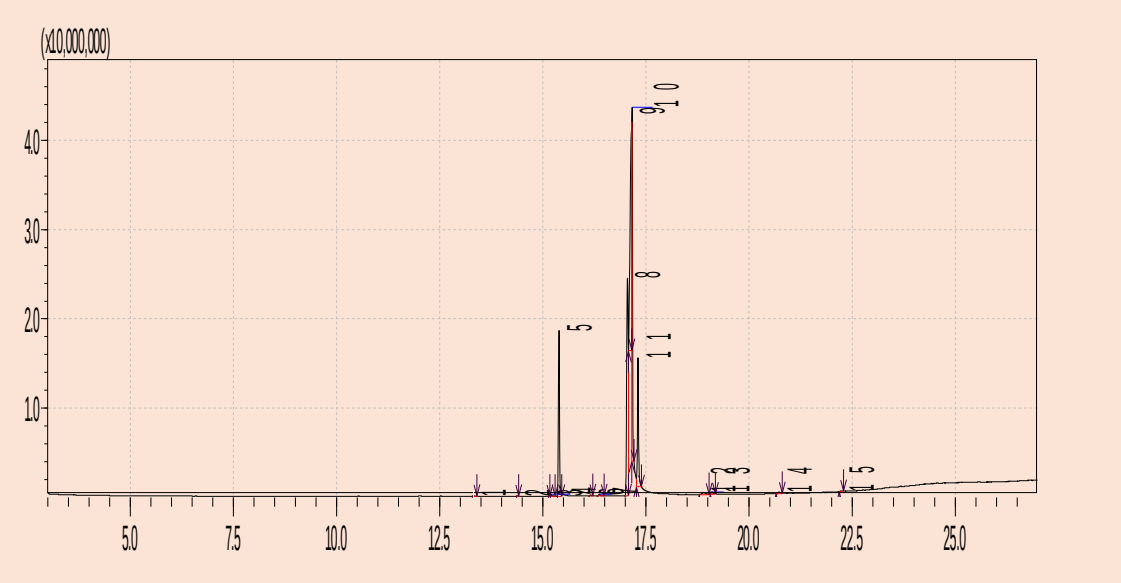


Fig 3.22: Chromatograms of *Linum usitatissimum* seed oil

The following esters were found as major components:

9-Octadecenoic acid, hexadecyl ester (27.58%)

The EI mass spectrum of 9-Octadecenoic acid, hexadecyl ester is shown in (fig. 3.23).The peak at m/z 295, which appeared at R.T. 17.155 in total ion chromatogram, corresponds to $M^{+}[C_{34}H_{66}O_2]^{+}$ .The peak at m/z 264 corresponds to loss of a methoxyl function.

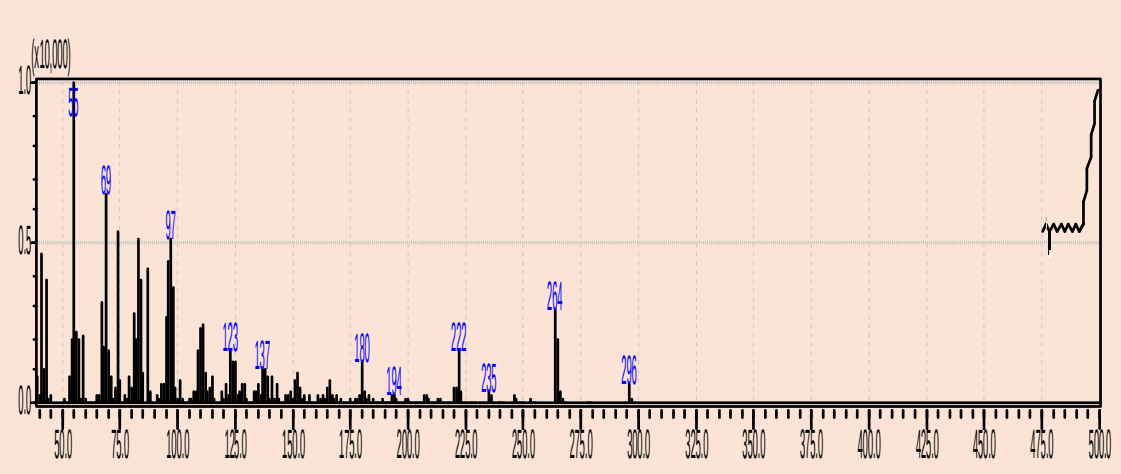


Fig 3.23: Mass spectrum of 9-Octadecenoic acid, hexadecyl ester

9, 12-Octadecadienoic acid, methyl ester (25.44%)

The EI mass spectrum of 9, 12-octadecadienoic acid, methyl ester is shown in (fig.3.24). The peak at m/z 294, which appeared at R.T. 17.053 corresponds $M^{+}[C_{19}H_{34}O_2]^{+}$ while the sighal at m/z 263 is due to loss of a methoxyl group.

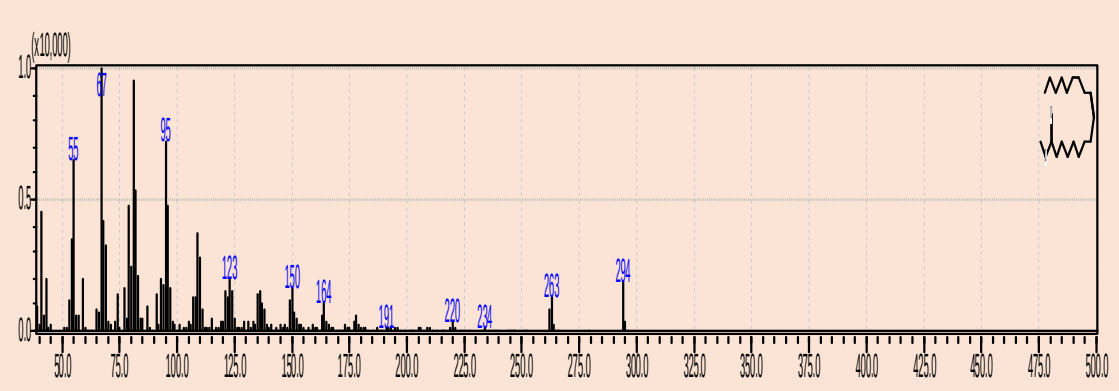


Fig 3.24: Mass spectrum of 9, 12- Octadecadienoic acid, methyl ester

9, 12, 15-Octadecatrienoic acid, methyl ester (19.73%)

Figure 3.25 shows the mass spectrum of 9, 12, 15-octadecatrienoic acid, methyl ester. The peak at m/z 292 - R.T. 17.164- in total ion chromatogram, corresponds the molecular ion : $M^{+}[C_{19}H_{32}O_2]^{+}$,while the sighal at m/z 261 accouts for loss of a methoxyl .

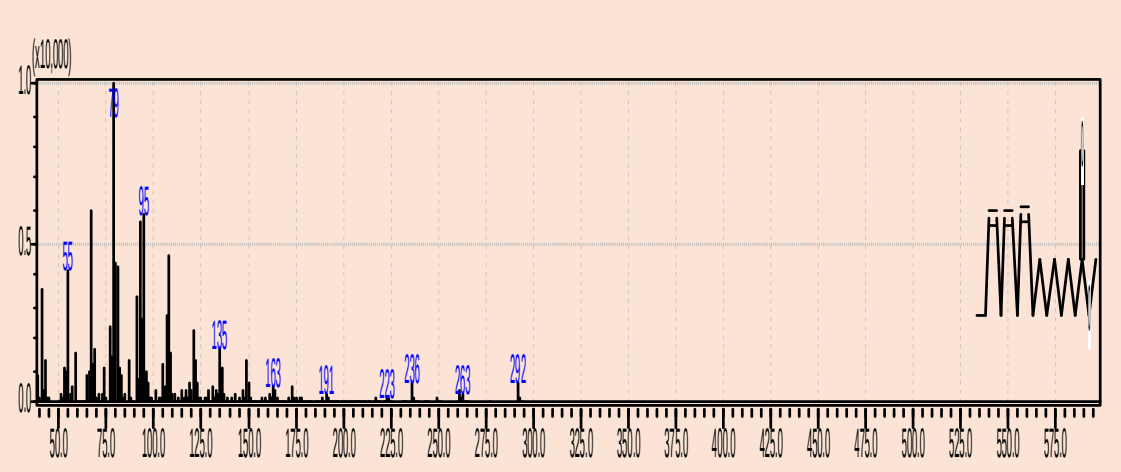


Fig 3.25: Mass spectrum of 9, 12, 15-Octadecatrienoic acid, methyl ester

Hexadecanoic acid, methyl ester (14.12%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.26). The moleculat ion : $M^{+}[C_{17}H_{34}O_2]^{+}$ appeared as expected at m/z 270(R.T. 15.392).The peak at m/z 239 is attributed to loss of a methoxyl .

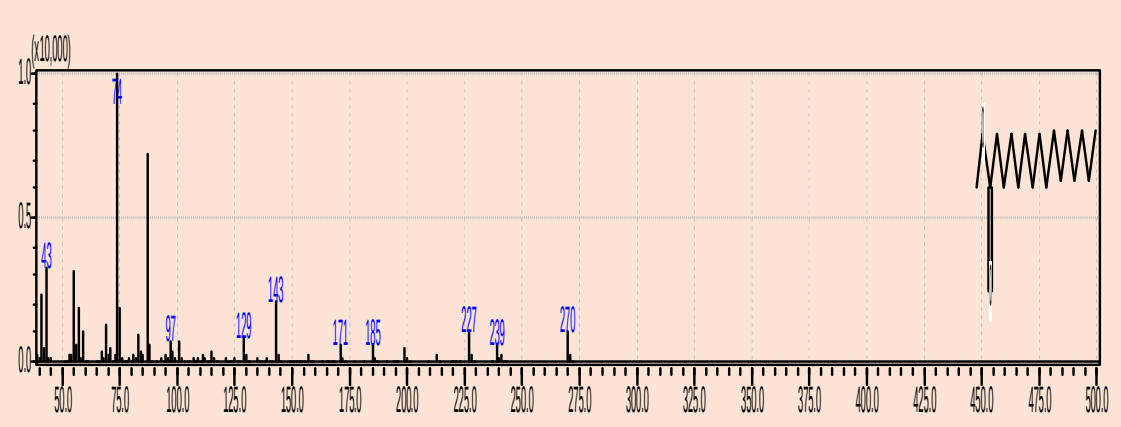


Fig 3.26: Mass spectrum of Hexadecanoic acid, methyl ester

Methyl stearate (11.20%)

The mass spectrum of methyl stearate is presented in (fig.3.27). The signal which appeared at m/z 298 -with R.T. 17.307 in total ion chromatogram - corresponds $M^{+}[C_{19}H_{38}O_2]^{+}$ .The peak at m/z 267 is attributed to loss of a methoxyl .

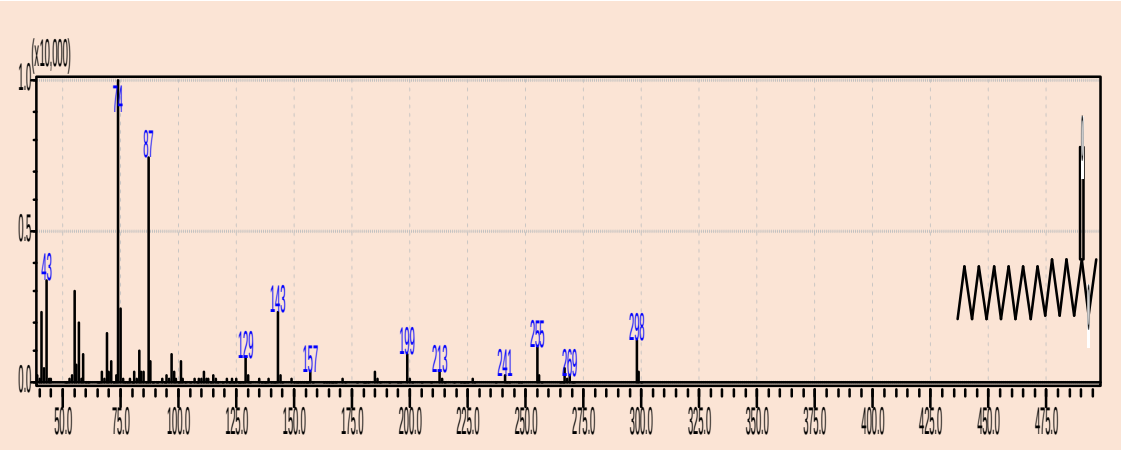


Fig 3.27: Mass spectrum of methyl stearate

3.4.2- Antioxidant activity

The antioxidant capacity of *Linum usitatissimum* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.14) *Linum usitatissimum* seed oil exhibited moderate antioxidant activity.

Table 3.14: Radical scavenging activity of *Linum usitatissimum* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Linum usitatissimum oil	48.00%

Conclusion

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

Two flavonoids (compounds I and II) were isolated from the stems of *Pulcaria crispa* .The partial structures of both compounds were deduced on the basis of their : IR, UV and NMR data and partial structures were suggested. Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried out . The results exhibited variable susceptibilities of microorganism for crude extract and different fractions. The antioxidant capacity of the alcoholic extract of *Pulcria crispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The oils were also assessed for their antimicrobial activity and promising results were obtained.

Recommendations

The following is highly recommended:

1. Other phytochemicals of the studied plant may be isolated and their structures may be elucidated and the biological activity could be screened.
2. The isolated falvonoids may be subjected to 2D NMR experiments for full characterization.
3. The isolated falvonoids and oil may be screened for other biological effects such as antiinflammatory, antidiabetic , antiviral, antilashmenial ..etc.

Sudan University of Science and Technology

College of Post Graduate Studies

Phytochemical Studies on the Stems of *Pulcria crispa* and Seeds of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* and Biological Activity of Constituents

دراسة فيتوكيميائية لسيقان نبات الربل وبذور نبات الرجلة والكسبرة والكتان والفعالية البيولوجية للمكونات

A Thesis Submitted in Fulfillment of the Requirements of the Ph.D. Degree in Chemistry

By

Lubna Akasha Abdelmalik Mohamed

(B.Sc. (Hons.) Chemical Laboratories, M.Sc. in Chemistry)

Supervisor

Prof: Mohamed Abdel Karim Mohamed

September, 2019

الإســـــــتـــهـــلال

﴿ بسم الله الرحمن الرحيم ﴾

فلينظر الإنسان إلى طعامه ﴿٢٤﴾ أنا
صببنا الماء صبا ﴿٢٥﴾ ثم شققنا
الأرض شقا ﴿٢٦﴾ فأنبتنا فيها حبا
﴿٢٧﴾ وعنبا وقضبا ﴿٢٨﴾ وزيتونا
ونخلا ﴿٢٩﴾ وحدائق غلبا ﴿٣٠﴾
وفاكهة وأبا ﴿٣١﴾ متاعا لكم
ولأنعامكم ﴿٣٢﴾ سورة عبس

Dedication

To,

My parents,

My sons,

And my sisters and brother

Acknowledgement

First and foremost, I thank **Almighty Allah, the Ever-Magnificent, the Ever-Thankful**, for his help and blessing.

I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor Dr. Mohamed Abdel karim, who has been always generous during all phases of the research, and I highly appreciate the efforts expended by my co-supervisor, Dr. Kamal Mohamed Saeed.

I owe a deep debt of gratitude to the Sudan University of Science and Technology for giving us an opportunity to accomplish this work. Thanks are extended to the Aromatic and Medicinal Plants Institute, Khartoum, Sudan for all facilities. Thanks for the National Research Center, Cairo for the spectral measurements.

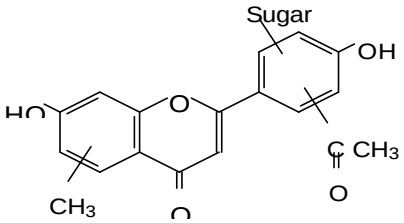
I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis.

I also would like to express my whole hearted thanks to my parents, for their generous support. They supported me throughout my entire life and particularly through the process of accomplishing the PhD degree.

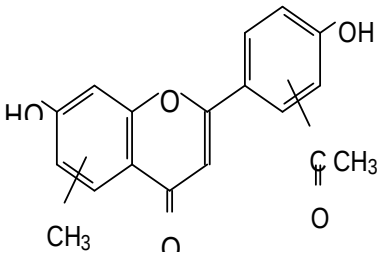
Abstract

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

The stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature. Qualitative tests on the alcoholic extract were positive for flavonoids. Conventional chromatographic techniques were used for isolation and purification of flavonoids. Two flavonoids (compounds I and II) were isolated from this plant. The partial structures of both compounds were deduced on the basis of their: IR, UV and NMR data and the following structures were suggested:



Compound I



Compound II

Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried. The results exhibited variable susceptibilities of microorganism for crude extract and different fractions.

In this study, the antioxidant capacity of the alcoholic extract of *Pulcriacrispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The GC-MS analysis of the *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* oils revealed the presence of 15, 14, 15 components respectively.

In the agar diffusion bioassay oils of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* failed to exhibit any antimicrobial activity. However, *Coriandrum sativam* oil showed significant antioxidant activity.

المستخلص

يشتمل البحث على دراسة كيميائية مفصلة للمحتوى الفلافونيدي لسيقان نبات الربل وكذلك التركيب الكيميائي لزيوت بذور كل من البقلة، الكزبرة وبذور الكتان. ايضا يتضمن البحث دراسة فعالية مستخلصات نبات الربل والزيوت المستخلصة كمضادات ميكروبات وكمضادات أكسدة.

لقد أستخلصت سيقان نبات الربل بواسطة الكحول الإيثيلي عند درجة الحرارة العادية و افاد المسح الفيتوكيميائى بوجود الفلافونيدات.

أنجز فصل المركبات بإستخدام كروماتوغرافيا العمود و كروماتوغرافيا الورق ، كما وتم التعرف على التركيب الكيميائي للمركبات المفصولة بإستخدام وسائل التحاليل الطيفية المختلفة : مطيافية الأشعة تحت الحمراء، الأشعة فوق البنفسجية ، الرنين النووي المغناطيسي للهيدروجين . وقد تم فصل مركبين ذوا طبيعة فلافونيدية من نبات الربل وهما المركب **II ,I.** تناولت الدراسة فعالية مستخلصات سيقان نبات الربل كمضادات ميكروبات وكذلك كمضادات أكسدة.

أثبت البحث ان مستخلص خلات اللإيثيل ذو فعالية كمضاد للبكتيريا. أما المستخلص الكحولي وبقية المستخلصات فقد اظهروا فعالية منخفضة كمضادات بكتيريا وفطرية. أوضحت الدراسة ان المستخلص الكحولي لنبات الريل ذو تاثير فعال كمضادات اكسدة.

اخضع زيت بذور البقلة، الكزبرة والكتان للكروماتوغرافيا الغازية - السائلة الملحقة بمطياف الكتلة . اوضحت هذه الدراسات وجود خمسة عشر ، أربعة عشر، وخمسة عشر مكونا بالزيت على التوالي.

تناولت الدراسة أيضا فعالية الزيوت المستخلصة من بذور البقلة ، الكزبرة والكتان كمضادات ميكروبات ولم تظهر النتائج أي تأثيريذكر. وأثبت البحث أن زيت بذور الكزبرة ذو فعالية عالية كمضاد أكسدة .

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INTRODUCTION

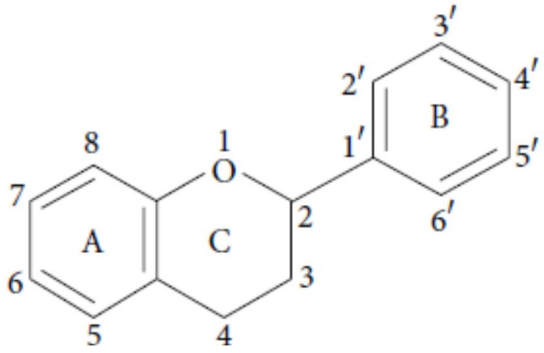
1.1 General overview

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities [1, 2]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [3]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [4]. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [5, 6]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [7, 8].

As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems [9, 10]. Flavonoids have ability to induce human protective enzyme systems.

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases [2, 8-

[10].Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin [11]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [12].The basic nucleus of flavonoids is displayed in Fig. 1.



(1)

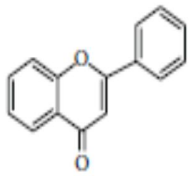
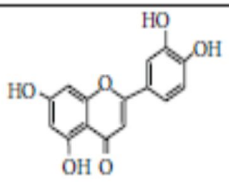
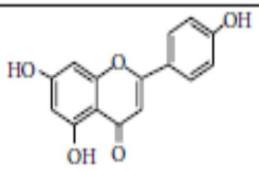
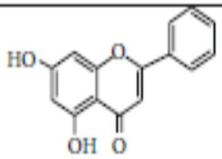
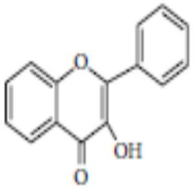
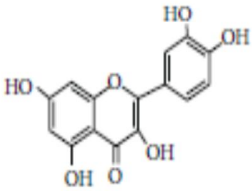
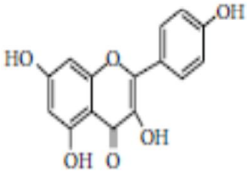
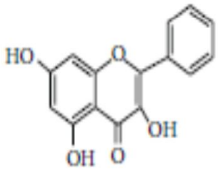
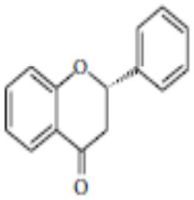
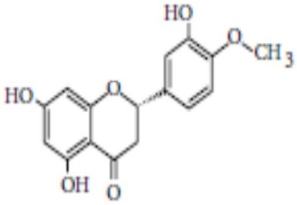
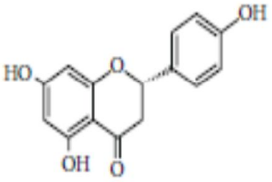
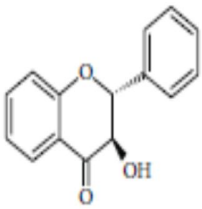
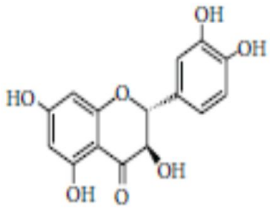
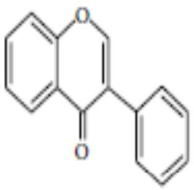
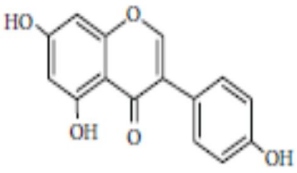
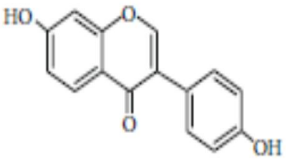
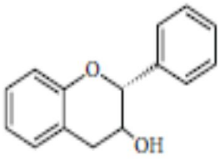
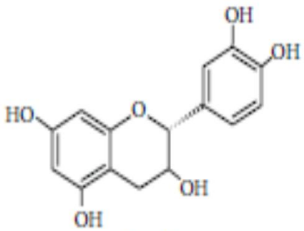
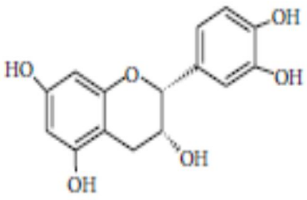
1.2 Chemistry of Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from orange. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified [13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (e.g. apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.

Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings [13].

Table (1): Structure of flavonoids

Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1.1). Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

Flavonols differ from flavanones by hydroxyl group at the 3-position and a C_2–C_3 double bond[14]. Flavonoids are often hydroxylation positions 3, 5, 7, 2, 3', 4', and 5'.Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[15].

1.2.1 Spectral Characteristics of Flavonoids

Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (235–285 nm) corresponds to the A ring absorption.

Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367 nm in kaempferol (3,5,7,4-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'- hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'- hydroxyl groups) [16]. The absence of a 3-hydroxyl group in flavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics [17]. Flavanones exhibit a very strong (Band II) absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only a shoulder for (Band I) at 326 and 327 nm. Band II appears as one peak (270

nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present.

As anthocyanins show distinctive (Band I) peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring and (Band II) peaks in the 240–280nmregion due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups [18].

1.3 Flavonoid- Rich Food and Medicinal Plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photo-synthesizing plant cells.

They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals [19].Thus flavonoids found in animals are of plant origin rather than being biosynthesized *in situ*. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [20].

Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg [21]. Preparation and processing of food may decrease flavonoid levels depending on the methods used.

For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644mg/L which suggest that the flavanones are concentrated in the cloud

during processing and storage [22]. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans [23].

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids [24, 25]. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases.

Table (2): Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry

Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavone – glabridin- a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals [26]. Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants [27, 28]. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people.

Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats [29].

Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia [30]. Table 3 summarizes some of the medicinal plants rich in flavonoid contents.

Table (3): Medicinal plants rich in flavonoids contents

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein

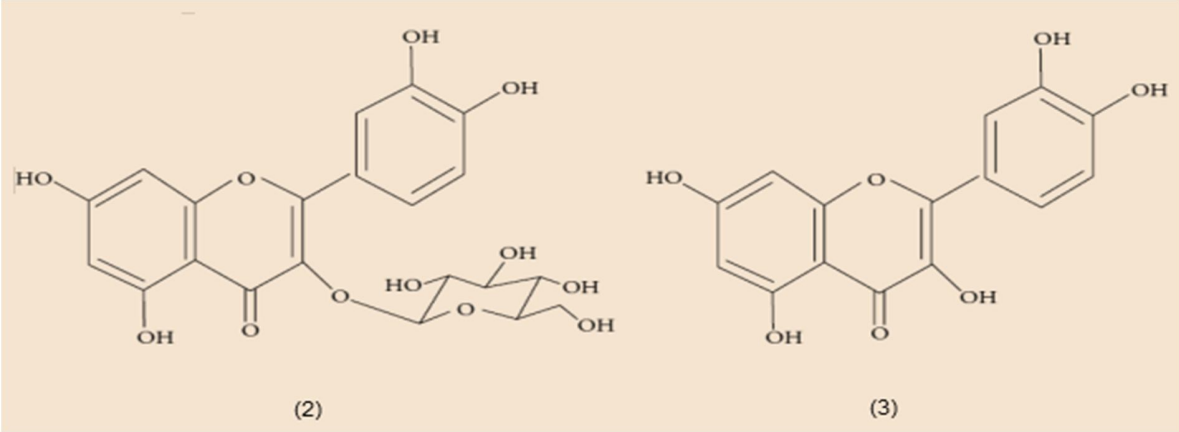
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for

example, hydroxy ethyl rutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and micro bleeding [31].

1.4 Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and p^{Ka}. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as glycosides (Figures 2 and 3). Aglycones can be easily absorbed by the small intestine, while flavonoid glycosides have to be converted into aglycone form before absorption [32].



The hydrophilic flavonoid glucoside such as quercetin are transported across the small intestine by the intestinal Na^+-dependent glucose cotransporter (SGLT1) [32].

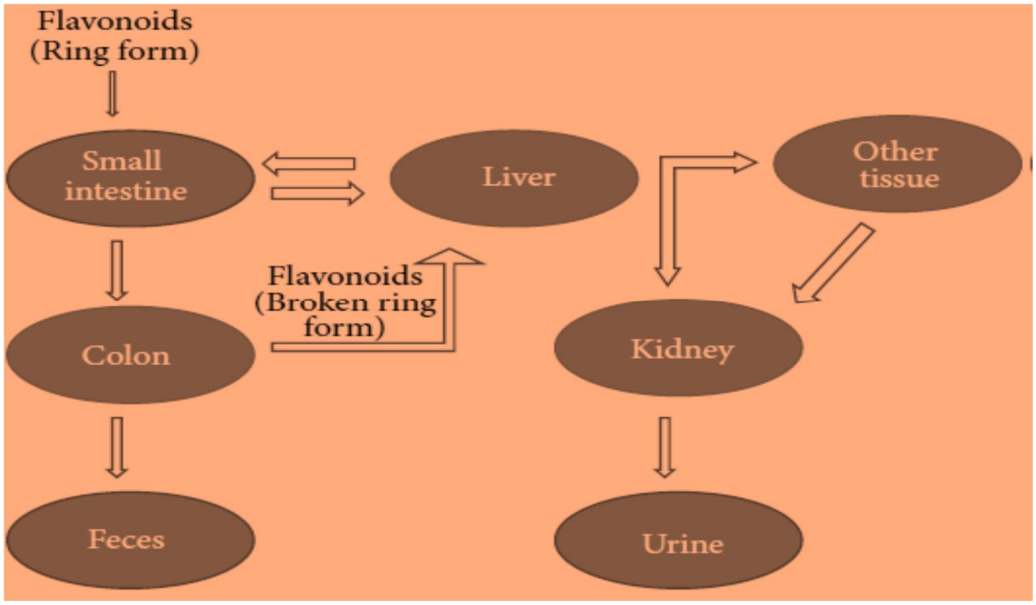
An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [33]. The substrate

specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [34].

The glycosides which are not substrates for these enzymes are transported toward the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [35]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected.

After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds [36]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [37]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [38]. The flavonoids secreted with bile in intestine and those that cannot be absorbed from the small intestine are degraded in the colon by intestinal micro flora which also break down the flavonoid ring structure (Sceme 1). Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [39]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine.

They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [40].



Sheme 1: Compartments involved in the metabolism of flavonoid

1.5 Biological Activities of Flavonoids

1.5.1 Antioxidant Activity

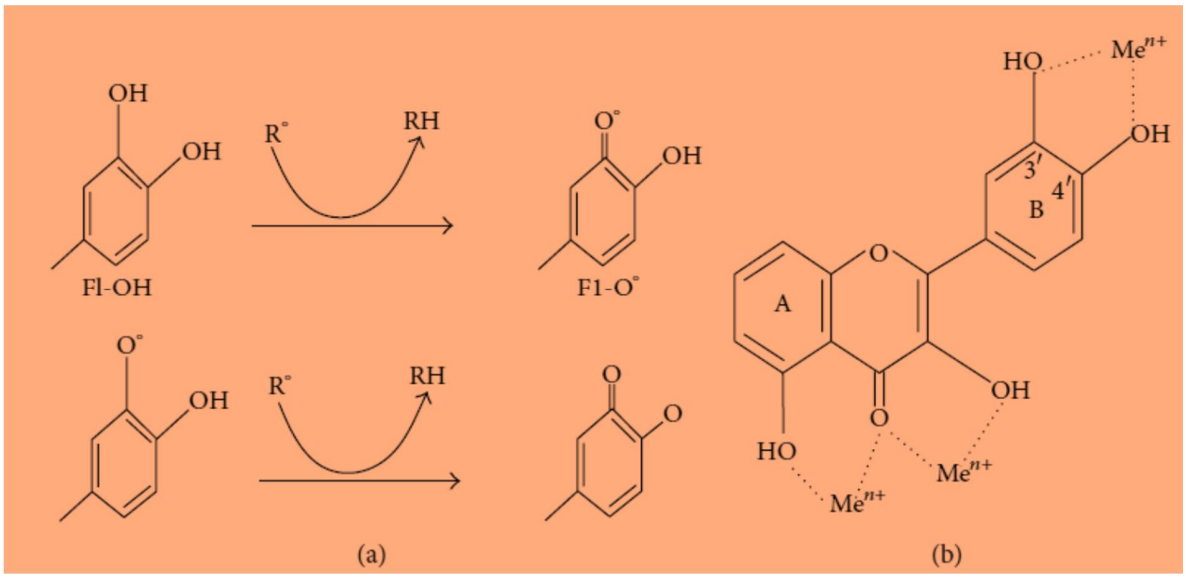
Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants.

The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure.

The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [4, 41]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and

peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [42].

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation;(2) Scavenging ROS; and (3) upregulation or protection of antioxidant defenses [43, 44]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [45]{see scheme 2}.



Scheme (2): (a) Scavenging of ROS (R∘) by flavonoids (Fl-OH) and

(b) binding sites for trace metals where Men+ indicates metal ions

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms [5, 25]. Free

metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation (Scheme 2(a)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [44, 46]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures [47]. The binding sites are shown in Schme 2(b). A 3', 4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [4]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro* [48]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable *ortho* semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential [49]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features [50].

The flavonoid heterocyclic contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH is crucial. Removal of a 3-OH annuls coplanarity and conjugation which

compromises scavenging ability [51]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity [50].

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides.

There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased [52]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position [53]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [54].

1.5.2 Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [55].

Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Glutamate-cysteine ligase catalytic subunit

(Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases [56]. It has been demonstrated that anthocyanin cyanidin-3-O-β- glucoside increases Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and pro-apoptotic signaling. Furthermore, this flavonoid lowers hepatic lipid peroxidation, inhibits the release of pro-inflammatory cytokines, and protects against the development of hepatic steatosis [56]. Silymarin is a flavonoid having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle *Silybum marianum* (Compositae). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers [57-59].

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl4-) induced injury. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl4 [60]. Similarly in an *in vivo* experiment flavonoids at of 50, 100, and 200mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver.

Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid [60].

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive

complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. *Equisetum arvense* flavonoids as well as hirustrin and avicularin isolated from some other sources is reported to provide protection against chemically induced hepatotoxicity in HepG2 cells [61, 62].

1.5.3 Antibacterial Activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid- rich plant extracts from different species have been reported to possess antibacterial activity [44, 46, 63, 64]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [65].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation.

Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [66, 67].

Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria [68, 69].

The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities [68, 70]. Robinetin, myricetin, and (−)-epigallocatechin

are known to inhibit DNA synthesis in *Proteus vulgaris.* Mori et al. [71] suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase [72].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes [73]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells.

The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity [74]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [75].

Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus* [76].

Haraguchi and colleagues [74] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A

inhibited incorporation of radioactive precursors intomacromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain [74].

There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents [77–79].

1.5.4 Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and pro-inflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [80].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells [81]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein

kinases [82, 83]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules [84]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful pro-inflammatory signaling molecules[85]. Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotopes *in vitro* [86].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration [87]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide [88]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.5.5 Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents [46, 89]. Consumption of onions and/or apples, two

major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon [90].The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods [91].

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [92]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [93]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in cogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor

agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial [94]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer [93].

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death [90, 95]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors [96].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P- 388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, andMKN-28), colon cancer cells (COLON320DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells [97] . It has been proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent [98].

Barnes [99] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones

genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats [100]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats [101]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al. [102]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [103]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects [104]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin [105].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [106]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor [44, 46, 107].Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals [108].

1.5.6 Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral

activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available.

Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso [109] demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication. Baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase [110].

Table (4): Antiviral activity of various flavonoids

Flavonoid	**Virus**
Quercetin	Rabies virus, herpes virus, parainfluenza virus, polio virus, mengo virus, and pseudorabies virus
Rutin	Parainfluenza virus, influenza virus, and potato virus
Apigenin	Immunodeficiency virus infection, Herpes simplex virus type, and Auzesky virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

Another study revealed inhibition of HIV-1 entry into cells expressing CD4 and chemokine coreceptors and antagonism of HIV-1 reverse transcriptase by the flavone *O*-glycoside [111]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase [110]. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a novel mechanism that probably involves inhibition of viral transcription [112].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV).

Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [110]. Studies have displayed that flavonols are more active than flavones against herpes simplex virus type 1 and the activity order was found to be galangin, kaempferol, and quercetin [110].

Zandi et al. [113] studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus [109]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [113]. List of some flavonoids and their efficacy against viruses is given in Table 4.

1. 6 Role of Flavonoids in Plants

1.6.1 Combating Oxidative Stress

Flavonoids have long been reported as serving multiple functions in plants [114].Various abiotic and biotic factors helps in the generation of ROS in plants leading to oxidative stress. Flavonoid biosynthesis in plants is almost exclusively enhanced due to oxidative stress. They have capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and quench ROS once they are formed [115]. Flavonoids discharged primary UV-B screening functions when early plants moved from water to colonize the land. Extent of antioxidant capacity and ability to absorb UV-wavelengths depends upon the nature of substitution on different rings of flavonoids. Dihydroxy B ring substituted flavonoids have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts have greater ability to absorb UV wavelengths [115].

The most reactive hydroxyl groups (7-OH in flavones or the 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in the aqueous cellular environment, protects the reactive hydroxyl groups from autooxidation [116], and allows the transport of flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and the cell wall [117]. Recent evidence shows that antioxidant flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation, that is, the chloroplast. Here they can easily quench H_2O_2, hydroxyl radical, and singlet oxygen [115, 118].

Oxidative stress due to an excess of excitation energy in the chloroplast may be aggravated under conditions that limit the diffusion of CO_2 to the carboxylation sites and the efficiency of carboxylation [115, 119]. The environmental limitations to CO_2 assimilation rate include drought/salinity,

low/high temperature, and nutrient scarcity. Under these conditions the activity of ROS detoxifying enzymes may significantly reduce in the chloroplast [120, 121], which in turn up regulates the biosynthesis of ROS scavenging flavonoids.

The reducing functions of flavonoids are of key significance in plants under severe stress conditions. These functional roles are concomitant with the very high concentration of dihydroxy B ring substituted flavonoids [122]. Flavonoids have been suggested as representing a secondary antioxidant defense system in plant tissues exposed to different stresses [115]. Lipid peroxidation is the common consequence of oxidative stress which disrupts the cell membrane integrity.

Quercetin 3-*O*-rutinoside (rutin) may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage [123].

1.6.2 Growth Regulator

Flavonoids carry out functional roles of amazing significance in plant-environment interactions.

Flavonoids (in the nanomolar range) may regulate auxin movement and catabolism. The ability of flavonoids to create auxin gradients translates into phenotypes with different morphoanatomical features [124]. The control of flavonoids on auxin movement may have immense value in the stress-induced morphogenic responses of plants such as the flight strategy of sessile organisms exposed to unfavorable environments [125]. Species rich in dihydroxy flavonoids exhibit phenotypes with strikingly different morphological traits as compared with those rich in monohydroxy flavonoids [126]. Dwarf bushy phenotypes with few, small, and thick leaves to direct sunlight irradiance are usually present in sunny environments, thus

protecting leaves located deep in the canopy from light-induced severe cellular homeostasis perturbations. On the contrary, shaded plants, which are rich in kaempferol and/or apigenin derivatives (having negligible concentrations of quercetin derivatives), have long internodes, and large leaf lamina coupled with reduced leaf thickness [125].

Flavonoids at the plasma membrane are effective inhibitors of PIN (pin formed) and MDR (multidrug resistance) glycoproteins that are involved in the cell to cell movement of auxin. The ability of flavonoids to inhibit the activity of the efflux facilitator PIN and MDR proteins depends on the presence of the catechol group in the B ring of the flavonoid skeleton. In addition flavonoids regulate the activity of IAA-oxidase with markedly different effects based on their chemical structure [127]. Recent evidence of a nuclear location of flavonoids (as well as of enzymes of flavonoid biosynthesis) supports that flavonoids are capable of modulating the activity of proteins involved in cell growth. Flavonoids may therefore act as transcriptional regulators [128, 129].

1.7 Microbial Production of Flavonoids

In response to the low production efficiency from plants and chemical synthesis, research groups have directed their attention to the production of flavonoids in microorganisms using metabolic engineering and synthetic biology [130].

Chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals [131]. Because of the rapid development in molecular biology tools and the flooding of genome information from a variety of organisms, combinatorial biosynthesis offers an advantage for production of rare and expensive natural products. It can be used in both simple and complex transformations without the tiresome blocking and deblocking steps that are

common in organic synthesis [132]. Several prokaryotes and eukaryotes such as *E. coli*, *Saccharomyces cerevisiae*, *Streptomyces venezuelae*, and *Phellinus igniarius,* a medicinal mushroom, have been used for production of flavonoids [12].

1.7.1 Phenylpropanoid Pathway

In plants naringenin chalcone is the precursor for a large number of flavonoids produced by the phenylpropanoid synthetic pathway. Fermentative production by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine, and tyrosine [133]. As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonialyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid, which is then activated to *p*-coumaroyl-CoA by the action of 4-coumarate:
CoA ligase. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or nonenzymatically *in vitro* [134].

1.7.2 Enhancement of Flavonoid Production

Combination of promoter and target genes; knockout of related genes; overexpression of malonyl-CoA; and construction of artificial P450 enzymes are the key molecular biology technology procedures used in the heterologous production of flavonoids.

Every gene from the phenylpropanoid pathway is cloned in the host under the control of the promoter which often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used to enhance production of flavonoids according to the need of specific host such as T7, ermE∗, and GAL1 promoter [12]. The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids. Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens* intracellular malonyl-CoA pool was amplified leading to increased production of flavonoids [135].

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It was proved in an experiment in which researchers knocked out the udg gene encoding for UDP-glucose dehydrogenase. This resulted in elimination of endogenous UDP-glucose consumption pathway leading to increase in intracellular concentration of UDP-glucose and as a consequence increment in the production of flavanones and anthocyanins was observed [136].

One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound cinnamate-4-hydroxylase [137].This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host. An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis

pathway [138]. Combining bacterial cells and eukaryotic cells in a pot enabled researchers to generate a wider library of natural and unnatural products than any of the previously reported systems. *De novo* production of the key flavonoid intermediate naringenin has been demonstrated for the first time from glucose using an engineered *S. cerevisiae* strain which led to fourfold higher concentrations than reported in earlier studies on de novo biosynthesis [139, 140].

1.8 The studied plant species

1.8.1 *Pulcaria crispa*

Pulicaria crispa is a genus of Asteraceae family. The Asteraceae family is one of largest families of flowering plant, with about 1,100 currently accepted genera and 25,000 species. It is characterized by the flowers being arranged in dense heads of many small florets, such as the daisy and dandelion [141].

Pulcaria crispa

The family is of worldwide distribution ,being absent only from the Antarctic mainland, and is particularly well represented in semiarid region of the tropics and subtropics, such as the Mediterranean region, Mexico, the cape province of South Africa grassland and bush land formation of Africa and montane floras throughout the world. Only in the tropical rain forests are they poorly represented [141]. The Asteraceae are of incalculably great indirect economic importance to man as major contributors to the diversity, and therefore, the stability and sustainable productivity, of the drier vegetation types throughout the world, especially in tropical and sub-tropical areas. In proportion to its size, however, the direct economic importance of the family is comparatively small. It includes food plants, sources of raw materials, medicinal and drug plants, ornamentals and succulents, and, on the debit side, weeds and poisonous plants [141].

1.8.1.1 Phytochemical, antibacterial screening and antioxidant activity of *Pulcaria crispa*

Many secondary metabolites have been detected - coumarins, tannins and alkaloids - on *P. crispa* [142]. Investigation of the flavonoids constituents of *Pulicaria crispa*, result in the isolation and identification of four flavonoids, quercein, quercetin-3-o-glucoside, quercetin7-o glycoside and quercetin-3-methyl ether [143]. The presence of these metabolites explains the various uses of this plant in traditional medicine. Alkaloids, comprising a large group of nitrogenous compounds, are widely used as cancer chemotherapeutic agents [144, 145] alkaloids also interfere with cell division The presence of alkaloids in *Pulicaria crispa* may be responsible for antibacterial activity. *Pulicaria crispa* is used in folk medicine for the treatment of colds, coughs, colic, excessive sweating and as carminative [146].

1.8.2 *Portulaca oleracea*

Portulaca oleracea is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution belonging to the Portulacaceae family. It is commonly known as *purslane* (USA and Australia), *rigla* (Sudan and Egypt), *pigweed* (England), *pourpier* (France), and *Ma-Chi-Xian* (China) [147]. It is distributed widely in the tropical and subtropical areas of the world including many parts of the United States and is eaten extensively as a potherb and is added to soups and salads around the Mediterranean and tropical Asian countries [148].

Portulaca oleracea

Americans and aborigines of Australia grind the seeds of this plant into flour for use in mush and bread [149]. *Portulaca oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties [150]. *Portulaca oleracea* has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic and so forth [151]. It exhibits a wide range of pharmacological effects, including antibacterial [152], antiulcerogenic [153], anti-inflammatory [154], antioxidant [155], and wound-healing [156] properties. It is listed by the World Health Organization as one of the most used medicinal plants, and it has been given the term “Global Panacea” [157]. The

Chinese folklore described it as “vegetable for long life” and it has been used for thousands of years in traditional Chinese Medicine [158, 159]. It is cold in nature and sour in taste and is used to cool the blood, stanch bleeding, clear heat, and resolve toxins. The dried aerial part of this plant is indicated for the treatment of fever, dysentery, diarrhea, carbuncle, eczema and hematochezia, with a recommended dose of 9–15 g, *Portulaca oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine [160–162].

1.8.2.2 Phytochemistry

Many constituents of *Portulaca oleracea* have been isolated, including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals.

One of the most effective constituents present in Chinese Herbal Medicines are flavonoids which are biologically active and possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. In the *Portulaca oleracea* plant, the flavonoids levels vary according to the part of the plant; the highest levels are present in the root followed by stem and the leaf; and seven different flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin [163]. However, only kaempferol and apigenin have been found in ethanolic extracts of leaves and stems, with the levels in the former being higher [157]. Portulacanones B–D, three homoisoflavonoids compounds, display selectively cytotoxic activities against three human cancer cell lines (SF-268,NCI-H460, and SGC-7901) [164]. Flavonoids are also widely present in foods such as fruits and vegetables [165].

In addition to flavonoids, another important chemical found in this plant is alkaloids including dopa, dopamine, and noradrenalin. The content of dopamine and noradrenalin is higher in leaves compared to stem and seeds. The amount of dopamine and noradrenalin obtained from leaves varies according to the solvents used in the extraction process, suggesting that the levels of these compounds are dependent on the solvents used during the extraction process [166].

Oleraceins A, B, C, D, and E are cyclodopa alkaloids isolated from this plant [167] and several analytes such as (3R)-3,5-bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone and 1,5-dimethyl-6-phenyl-1,2-dihydro-1,2,4-triazin-3(2H)-one display cytotoxic activities against human cancer cells [168].

Portulaca oleracea is also an excellent source of omega-3 fatty acids, which is usually present in oil and fat of fishes but not normally found in plants. Omega-3 fatty acids play an important role in the enhancement of immune function [169] and prevention and treatment of hypertension, coronary artery disease, cancer, and other inflammatory and autoimmune disorders [170]. It includes α-linolenic acid and linoleic acid, which are essential for normal growth, health promotion, and disease prevention in humans. Polysaccharides found in *Portulaca oleracea* are potential therapeutic agents for the treatment of diabetes mellitus owing to their modulation of blood lipids, metabolism, and decrease of blood glucose. *Portulaca oleracea* contains monoterpenes such as portulosides A and B, diterpenes such as portulene, and β-amyrin type triterpenoids [147, 171]; in addition, vitamins have also been isolated from the leaves of this plant. It contains the highest content of vitamin A which is a natural antioxidant playing an important role in vision, maintaining healthy mucus membranes and protecting against lung and oral cavity cancers among green leafy vegetables. This plant also

contains ascorbic acid, α-tocopherol, and B-complex vitamins, for example, niacin, pyridoxine, and riboflavin [171]. Furthermore it is rich in minerals like phosphorus, manganese, icon, calcium selenium [149], and the amino acids isoleucine, proline, leucine, lysine, phenylalanine, methionine, cystine, valine, threonine, and tyrosine [148]. Many other constituents have also been isolated from this plant, such as β-carotene, glutathione, melatonin, portulacerebroside A, catechol, and bergapten.

1.8.3 *Coriandrum sativam*

Coriander (*Coriandrum sativum*) being an annual herb is most commonly used for seasoning purpose. Seeds, leaves and roots are edible, although they have very distinct flavors and uses [172]. The herb has a light and fresh flavor. Coriander can be used as whole plant and can be processed because of its perishable nature of leaves and to increase the palatability of ripe fruits (seeds) before using it as flavoring agent in different food preparations. Whole plant of coriander mainly fresh leaves and ripe fruits are used for culinary purposes [172].

Coriandrum sativum

Coriander leaves have different taste than its seeds, with citrus overtones. Coriander plant is a rich reservoir of micronutrients and nutritional elements which leads us to focus our study on this herb [172]. Coriander is very low in saturated fat however,it contains good amount of linoleic acid which is a good source of α-tocopherol and vitamin K. Leaves of plant are rich source of vitamins while seeds are rich in polyphenols and essential oils. Coriander taste is devoted to its essential oil comprising a significant content of linoleic and furanocoumarins (coriandrine and dihydrocoriandrine). Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activity along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits and its protective effect to preserve the food for longer period [172].

Coriander contains high amount of essential oils that are very important for growth and for proper functioning of brain. The main essential fatty acids present in coriander include linoleic and linolenic acids. Linoleic acids belong to PUFA (polyunsaturated fatty acid) group. Dietary supplementation of coriander seed greatly affects the lipid composition of carcass by decreasing saturated fatty acid (SFA) contents (palmitic and stearic acids) and by increasing monounsaturated and polyunsaturated fatty acid (MUFA and PUFA) [173]. This plant is a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) isolated from the seeds and the aerial parts [174]. The high content of fats and protein in the fruits make distillation residues suitable for animal feed. Coriander fruits yield 5 to 7% of ash, 13% resin, astringent principle, malic acid and alkaloids. Coriander oil contains coriandrol, jireniol and vebriniol [175].

1.8.4 *Linum usitatissimum*

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* Figure 1.8 was given by Linnaeus in his book "Species Plantarum" [176]. It is an annual herbaceous plant with shallow root system.

The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are specialized development of this species [177].

The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber purpose are tall growing with straight culms and have fewer secondary branches.

Linum usitatissimum

The Mediterranean and Southwest Asia have both been proposed as the center of origin [203]; however the exact location is uncertain [178]. The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the stearoyl-ACP desaturase II

(*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop [179].

Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [180] making it ideal for paints, varnishes, and inks due to its fast polymerization properties.

Increasing demand for edible oil sources with significant percentages of omega-3 fatty acids is resulting in consumption of flaxseed as a functional food. Flaxseed is also added to animal feed to improve animal reproductive performance and health [181, 182].

Research objectives

1. Extraction of flavonoids and oils from some plants
2. GC-MS studies on the extracted oils.
3. Elucidation of structures of flavonoids.
4. Biological activity of isolates.

MATERIAL AND METHOD

2.1. Materials

2.1.1 Plant material

Stems of *pulcaria crispa* were collected from White Nile state (Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum – Sudan. The seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were collected from Jeddah-Saudi Arabia.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m length; 0.25mm diameter; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.1.3-Test organisms

Test organisms used in the antimicrobial assay are: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

2.2- Methods

2.2.1- Preparations of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminum chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. distilled water.

- **Potassium iodide solution:** 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

100 g) of powdered air- dried stems of *pulcaria crispa* were extracted with 95% aqueous ethanol. The solvent was removed under reduced pressure giving the prepared extract. This prepared extract (PE) was used for phytochemical screening.

2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) were evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5

ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.2.2- Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

2.2.2.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Mayer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.2.4- Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and

the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.2.5 -Test for saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml)of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand for one hour and then checked for foam formation.

2.2.2.6 - Test for coumarins

(0.2 g) of the prepared extract were dissolved in 10 ml distilled water in test tube and a filter paper was attached to the test tube to be saturated with the vapor after being spotted o with 0.5N KOH. Then the filter paper was inspected under UV light. The presence of coumarins is indicated by the ability of the spot to absorb the UV light.

2.2.2.7- Test for anthraquinone glycosides

(0.2 g) of the prepared extract was boiled with 10 ml of 0.5 N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. (5ml) of the benzene solution was shacked with (3ml) of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraxquinones is indicated by pink or red color in the alkaline layer.

2.2.3- Extraction of flavonoids

The air - dried powdered stem of a *pulcria crispa* (3 kg) were extracted by maceration with ethanol (95%). The extract was concentrated under reduced pressure to give a crude product.

2.2.4- Column chromatography

Open column (70× 4 cm) was used for fractionation of the ethanolic extracts of *pulcaria crispa*. Silica gel with particle size 60-120 mesh ,from LOBA chemicals, was used as stationary phase. The composition of the mobile phase (ethyl acetate: methanol) was determined by TLC experiments.

The ethanolic extract of *pulcaria crispa* (5 g) was mixed with 10 g of silica gel and then applied on the top of the column. Elution commenced by EthOAC - MeOH ;(3:7, fraction I), (1:1, fraction II) and (0:1, fraction III).

2.2.5- paper chromatography

Fraction II – being rich in phenolics- was subjected to paper chromatography where pure flavonoids - compounds I and II -were isolated using the solvent system: butanol: acetic acid: water (4: 1: 12, upper layer).

2.2.6-Extraction of oils

Powdered seeds of *Portulaca oleracea* (*Coriandrum sativam* and *Linum usitatissimum*) - (500g) for each - were macerated with n-hexane . The solvent was removed under reduced pressure to yield the oil.

2.2.7-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added,then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes. The hexane layer was then separated.(5µl) of the hexane extract

were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.8- GC-MS analysis

The oils from *Portulaca oleracea, Coriandrum sativam* and *Linum usitatissimum* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 µm, thickness) was used. Helium (purity; 99.99 %) was used as carrier gas.

2.2.9- Biological activity

2.2.9.1- Preparations of chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa*

Chloroform fraction of *pulcaria crispa* was prepared by suspending the ethanolic extract of the leaves in the least amount of distilled water and then shaking with chloroform in a seperatory funnel. Evaporation of the chloroform layer under reduced pressure gave the required fraction. Other fractions were prepared in a similar way.

2.2.9.2- Antimicrobial assay

The ethanolic, chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa* as well as the essential oils extracted from the seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were evaluated for their antimicrobial activity against five standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*) using the cup plate agar method with some minor modifications.

2.2.9.2.1- Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 108- 104 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o}C for 24 hours.

2.2.9.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.9.3- Testing for antibacterial activity

The cup plate agar diffusion method was adopted with, some minor modification, to assess the antibacterial activity (2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a single test sample.

The agar discs were removed and cups were filled with 0.1 ml of each test sample using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^{o} C for one hours.

The above procedure was repeated for different concentrations of extracts and standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.9.4- Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but this time Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above

2.2.9.5-Antioxidant activity

The DPPH radical scavenging capacity was determined. In 96-wells plate, the test samples were allowed to react with 2.2 Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37° C. the concentration of DPPH was kept as (300μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests were run in triplicate. Propyl gallate was used as a positive standard at concentrations 0.5 mM.

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RESULT AND DISSCUSSION

Powdered air-dried stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature for 48hr. The crude product obtained after evaporation of the solvent under reduced pressure. The crude extract was fractionating by separator funnel and the three fractions were collected (ethyl acetate, chloroform and butanol fractions) and screened for its antimicrobial activity. The crude was subjected to silica gel column chromatography and eluted with ethyl acetate - methanol mixture (3: 7, fraction I), (1: 1, fraction II) and pure methanol (fraction III). Three fractions were collected. The fraction II was subjected to paper chromatography where pure components: **compound I** and **II** have been isolated. The structures of isolated flavonoids have been elucidated via a combination of spectra techniques.

GC-MS analysis on seed oil of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and the identification of the constituents. The oils was screened for antimicrobial and antioxidant activity against standard organisms.

3.1- *Pulcaria crispa*

3.1.1- Phytochemical screening

Pulcaria crispa was screened for major secondary metabolites and the results are displayed in table 3.1.

Table 3.1: Phytochemical screening of *pulcaria crispa*

Test	**Result**
Saponin	++
Cumarin	+
Alkaloids	+
Flavonoids	+
Tanin	+++
Steroids	-
Triterpens	-
Anthraquinone	-

+: Trace

++: Moderate

++: High

- : Negative

3.1.2- Antimicrobial assay

The crude extract, ethyl acetate, chloroform and butanol fractions have been screened for antimicrobial activity against five human pathogens. The results are depicted in table 3.2 Tables 3.3 and 3.4 display the antimicrobial activity of standard drugs.

Table 3.2: Antimicrobial activity of crude and three fractions

	Ec	Ps	Sa	Bs	Ca
Crude extract	_	_	_	_	10
Ethyle acetate fraction	12	10	13	_	12
Choroform fraction	_	_	_	_	11
Butanol fraction	_	_	10	_	10

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

The results were interpreted in a commonly used term :< 9 mm: in active; 9-12 mm: partially active; 13-18 mm: active; >18 very active.

The crude extract showed partial antifungal activity against *Candida albicans* (see table 3.2 and the appendix).

Ethyl acetate fraction showed activity against *Staphylococcus aureus.* It also exhibited partial Antibacterial activity against *Escherichia coli,*

Pseudomonas aeroginosa and *Candida albicans*.The choroform fraction showed partial anticandidal activity.

Butanol fraction showed partial Antibacterial activity against *Staphylococcus aureus* and *Candida albicans*.

Table 3.3: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.4: Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

3.1.3- Antioxidant assay

The antioxidant capacity of crude extract has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test extract against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.5) crude extract exhibited significant anti- oxidant activity.

Table 3.5: Radical scavenging activity of crude extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Crude extract	82.00%

3.1.4-Characterization of compound I

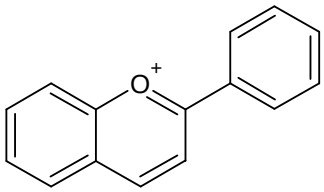
Compound I was isolated from *Pulcaria crispa* stems as yellow powder.

Table 3.6: IR data of compound I

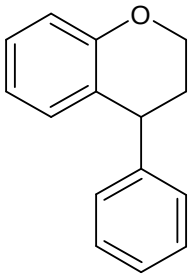
Functional group	**Wave number /cm^{-1}**
(C-H, Ar)	630.51
(C-O, ether)	1273.70
(C=C, Ar)	1378.30
(C=O)	1727.92
(OH)	3368.88
(C-H) Alkane	2921.62

The IR spectrum of compound I **(**Fig.3.1) showed all the anticipated bands of flavonids. Since

the infrared showed carbonyl absorption then the possibility that compound I being an anthocyanin or catechin is dismissed since these classes are characterized by absence of a carbonyl function.



Anthocyanin



Catechin

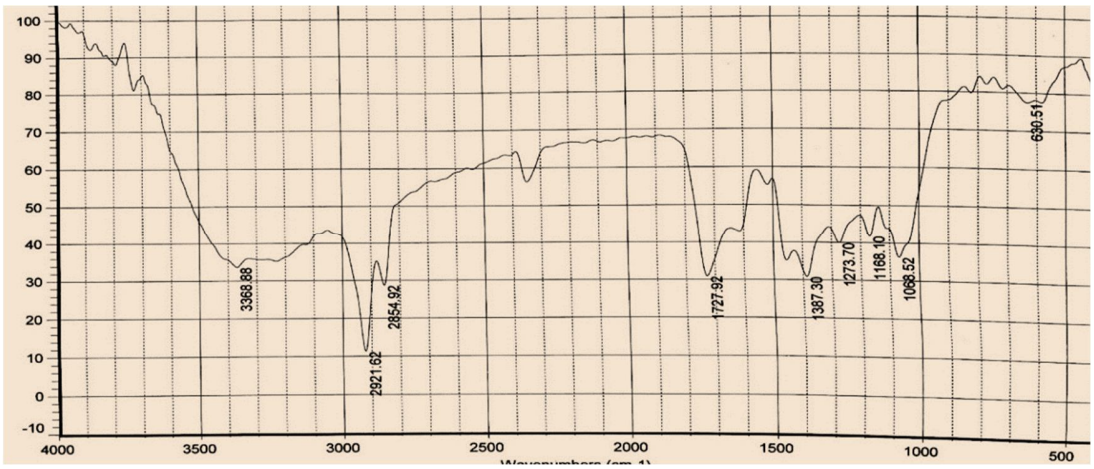
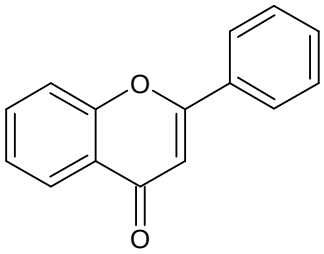
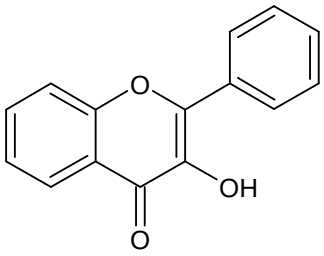


Fig 3.1: IR spectrum of compound I

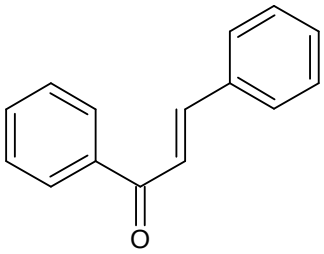
The UV spectrum of compound I revealed two major peaks at 262 and 345 nm (Fig.3.2). The presence of two peaks is a characteristic feature of flavones, flavonols, chalcones, and aurones.



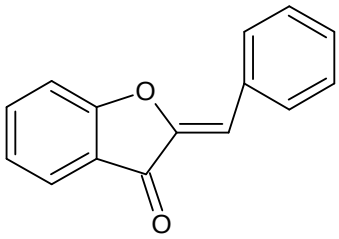
Flavone



Flavonol



Chalcone



Aurone

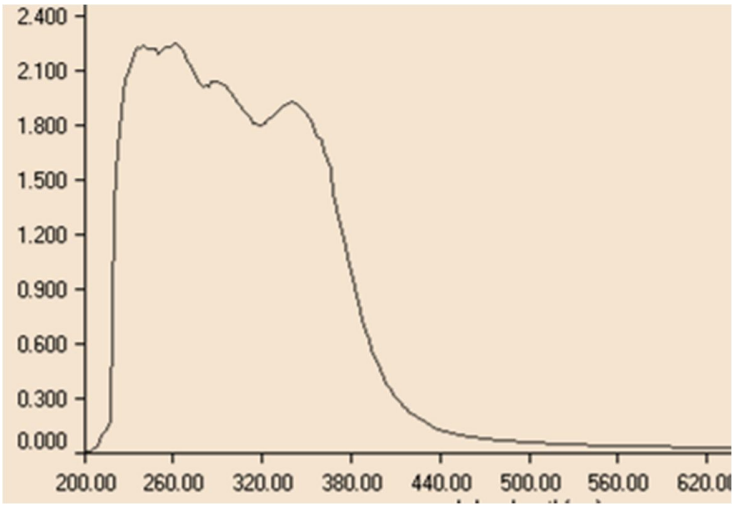
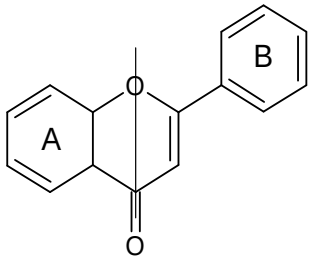


Fig 3.2: UV spectrum of compound I

The UV spectra of most flavonoids consist of two major absorption maxima, one of which occur in the range 220 – 290 nm (band II) and other in the range 300 – 400 nm (band I). The appearance of both band I and II demonstrates conjugation between the benzoyl and cinnamoyl systems.



Benzoyl Cinnamoyl

Band I is considerable to be associated with the absorption of cinnamoyl system, while band II originates from the benzoyl system.

Compound I showed both band II and I (fig.3.2) at 262 and 345nm respectively. Such absorption indicates that this compound is either a flavone, flavonol, chalcone or aurone. But aurones and chalcones absorb in

the range 365-430nm (band I). Flavones absorb in the range 300-350 nm, while flavonols absorb in the range 356-385. Thus compound I is a flavone.
Considerable structural feature have also been obtained using UV shift reagents such as sodium methoxide, sodium acetate, aluminum trichloride. These reagents produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl groups in the flavonoid molecule.
Use has been made of the effect of sodium methoxide on the UV spectra of flavonoids for detection of free 3 – and/or 4` –hydroxyl groups.
On addition of NaOMe to a methanolic solution of compound I a 42 nm bathochromic shift in band I without decrease in intensity (fig.3.3) was observed indicating the presence of a 4`-OH function.

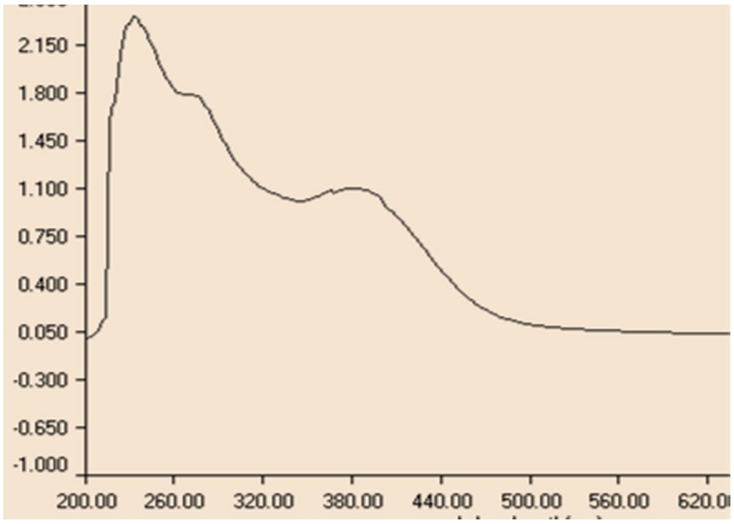


Fig 3.3: The UV spectrum of compound I in sodium methoxide

Sodium acetate is particularly useful diagnostic reagent for the specific detection of 7 –hydroxy group. When a methanolic solution of compound I **was** treated with excess powdered sodium acetate, and 16nm bathochromic shift in band II (fig.3.4) was observed suggesting a 7 –OH group.

When the previous solution of compound I was treated with excess powdered boric acid, no bathochromic shift was observed (fig.3.5) this indicate absence of *ortho*- dihydroxyl groups.

The shift reagent aluminum trichloride is diagnostic of 3 –hydroxyl group, 5 –hydroxyl group and catechol moieties[216].When a methanolic solution of compound I was treated with a few drops of the shift reagent $AlCl_3$ no bathochromic shift (fig.3.6) was observed and this indicates absence of 3 –OH, 5 –OH and catechol moieties.

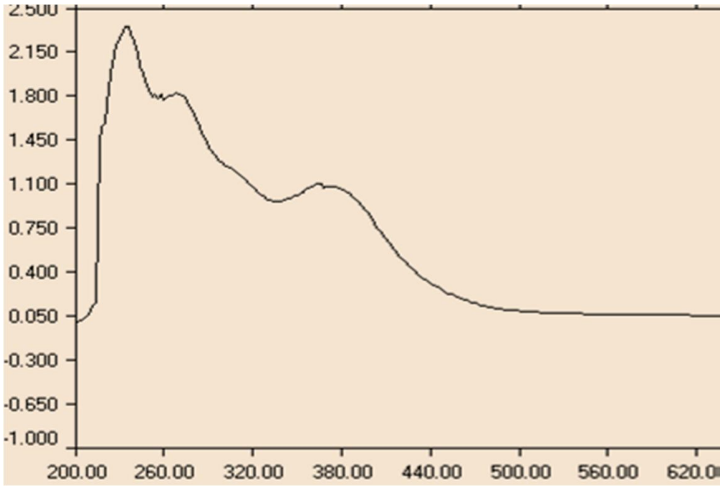


Fig 3.4: UV spectrum of compound I in sodium acetate

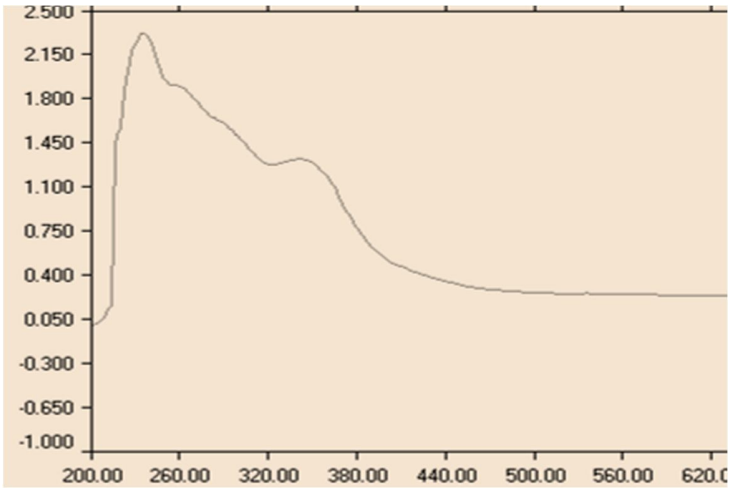


Fig 3.5: UV spectrum of compound I in sodium acetate/boric acid

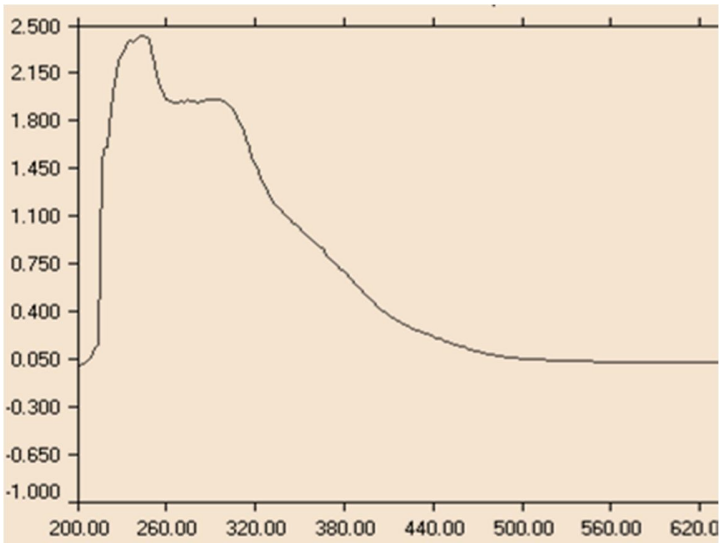


Fig 3.6: UV spectrum of compound I in aluminum trichloride

The ^{1}HNMR spectrum (Fig.3.7) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.1 ppm accounts for an acetyl function. The multiplet at δ 4.23-5.45 ppm is due to a sugar moiety (not identified in this study). The aromatic protons appeared as a multiplet (δ 6.68-7.65 ppm).

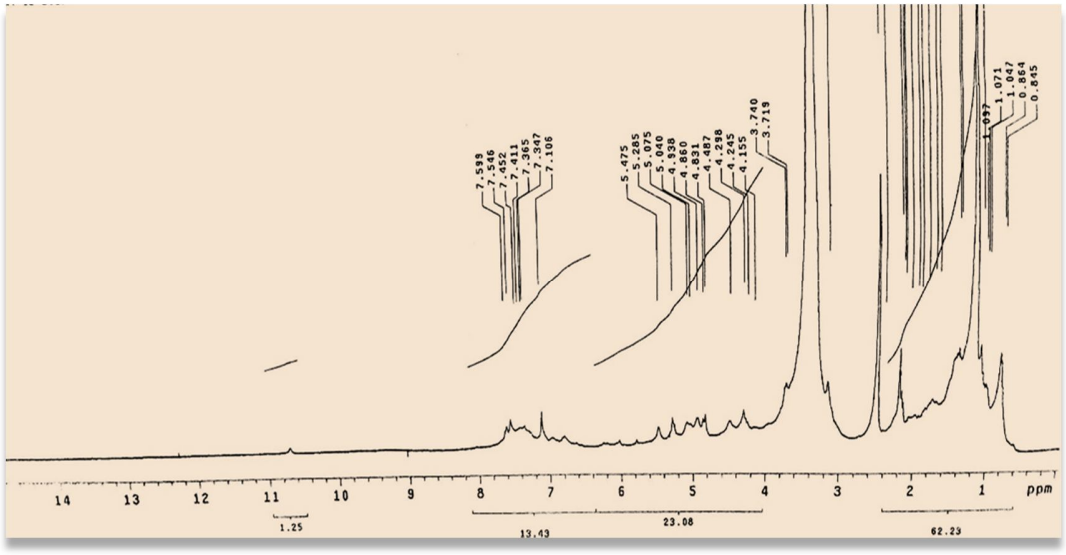
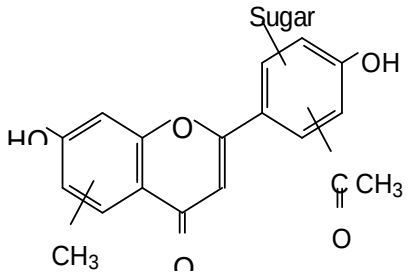


Fig 3.7: ^{1}HNMR spectrum of compound I

On the basis of the above spectral data, the following partial structure was proposed for compound I:



3.1.5- Characterization of compound II

Compound II was isolated as white-yellowish powder from *Pulcaria crispa* stem. The IR data of compound II is presented in the following table (see also Fig.3.8).

Table 3.7: IR data of compound II

Functional group	N(cm^{-1})
(C-H, Ar)	2085.7
(C-O, ether)	1017.15
(C=C, Ar)	1386.55
(C=O)	1643.49
(OH)	3419.34
(C-H) Alkane	2832.88

Compound II – which showed a carbonyl absorption-cannot be an anthocyanin or a catechin since IR spectrum gave carbonyl stretching at 1643.49 cm^{-1}. It could belong to one of the other classes of flavonoids. Anthocyanin and catechins are devoid of carbonyl.

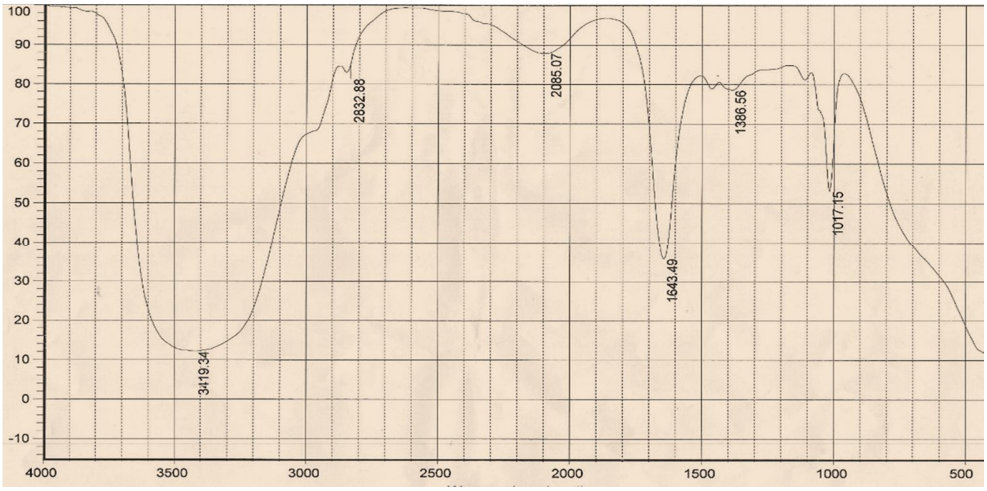
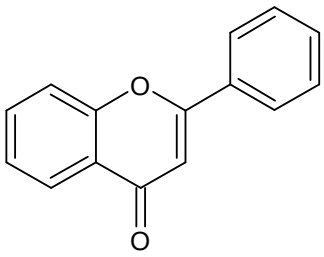


Fig 3.8: IR spectrum of compound II

Table 3.8: UV spectral data of compound II

λ_{max} (nm)	
Band I	Band II
330	240

The UV spectrum of compound II is depicted in (fig.3.9). The spectrum revealed two major peaks at 240 and 330 nm. Such absorption is a characteristic feature of flavones.



Flavone

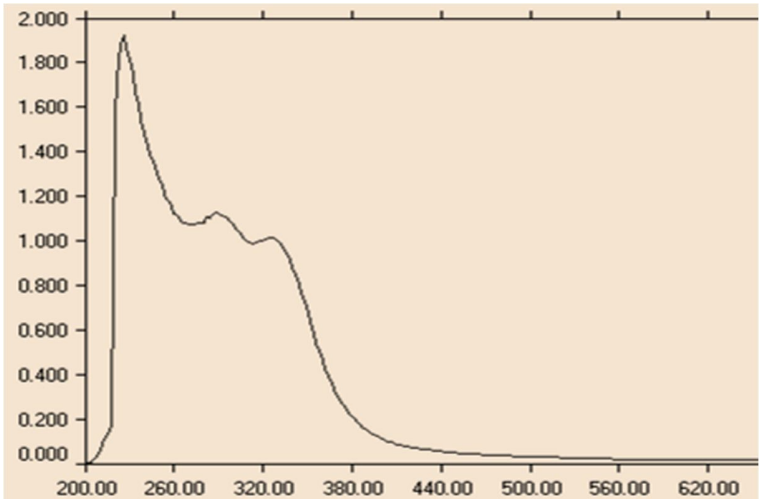


Fig 3.9: UV spectrum of compound II

Considerable structural feature have also been obtained using UV shift reagents: sodium methoxide, sodium acetate, aluminum trichloride.

On addition of NaOMe to a methanolic solution of compound II a 45nm bathochromic shift in band I indicative the presence of a 4`-OH function was observed (Fig.3.10).

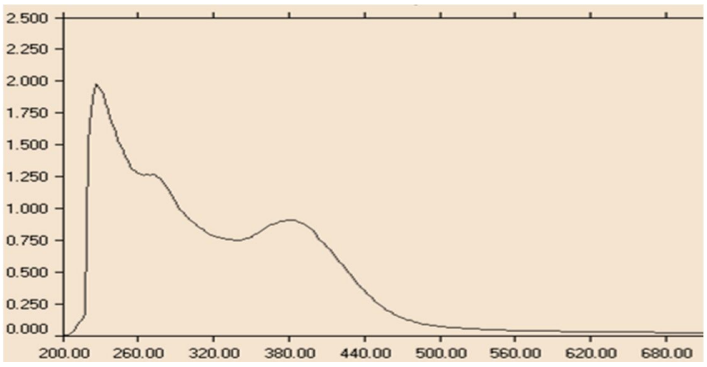


Fig 3.10: The UV spectrum of compound II in sodium methoxide

When a methanolic solution of compound II was treated with excess powdered of the shift reagent NaOAC, a 30 bathochromic shift in band II (fig.3.11) was observed and this indicates presence of a 7 –OH group.

When the previous solution of compound II was treated with excess powdered of boric acid, no bathochromic shift in both band II and I was observed (fig.3.12) this indicate absence of *ortho*- dihydroxyl groups.

The aluminium chloride spectrum showed no bathochromic shift (fig.3.13) indicating absence of 3 –OH, 5 –OH and catechol moieties.

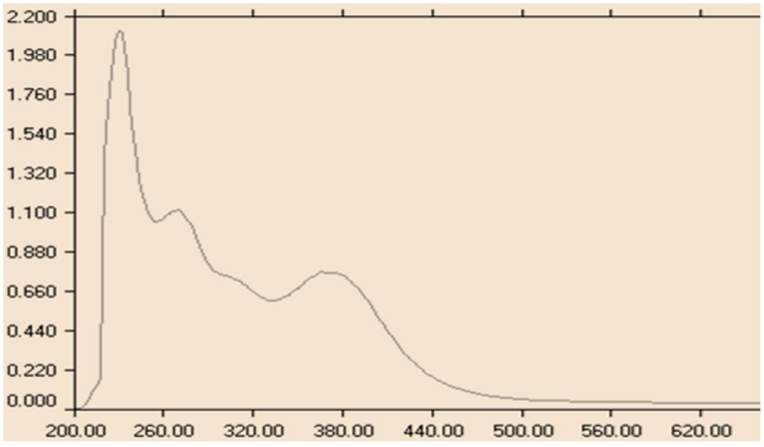


Fig 3.11: UV spectrum of compound II in sodium acetate

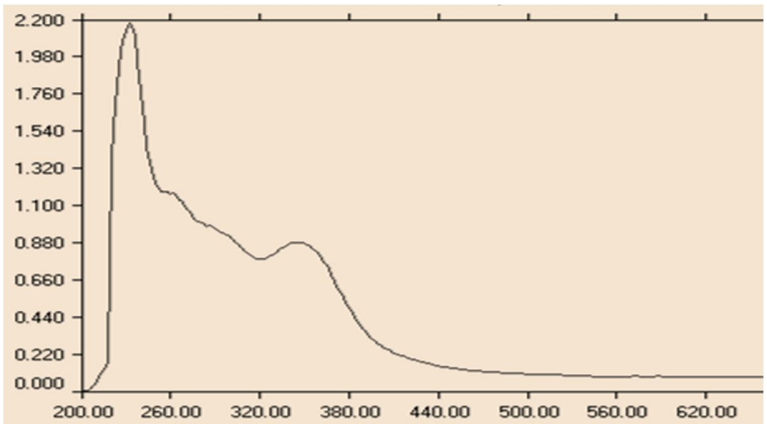


Fig 3.12: UV spectrum of compound II in sodium acetate/boric acid

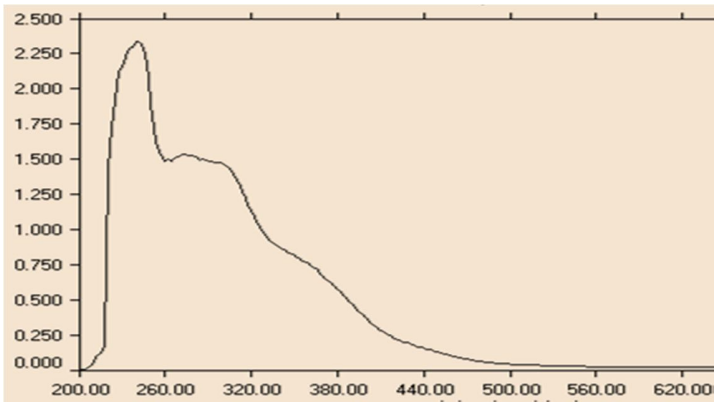


Fig 3.13: Aluminum trichloride spectrum of compound II

The ^{1}HNMR spectrum (Fig.3.14) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.4 ppm accounts for an acetyl function. The aromatic protons appeared as a multiplet (δ 6.68-7.59 ppm).

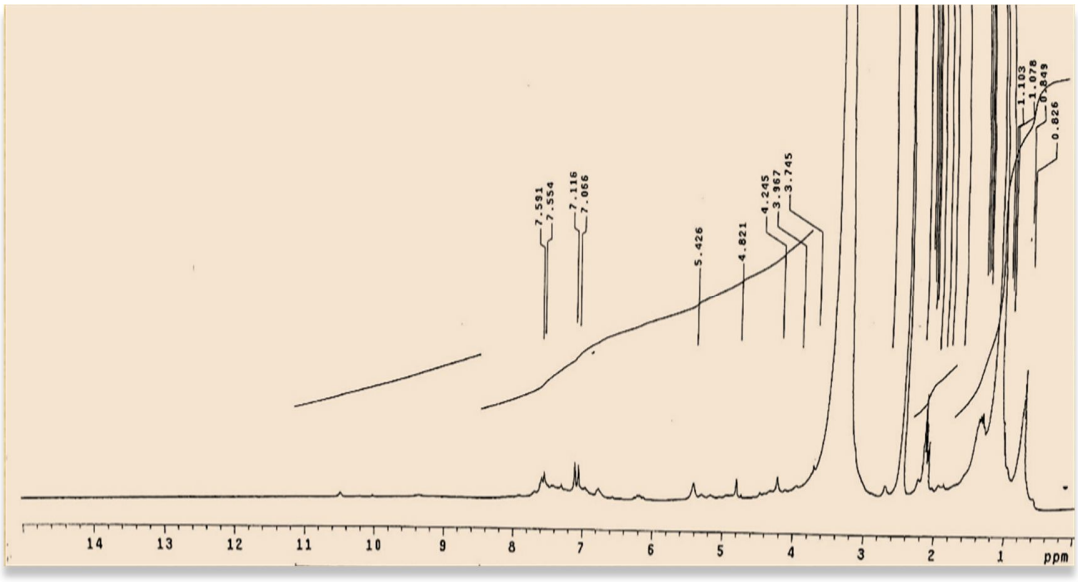
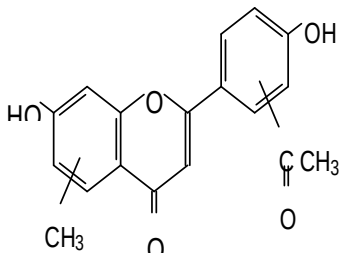


Fig 3.14: [1]HNMR spectrum of compound II

On the basis of the above spectral data, the following partial structure was proposed for compound II:



Compound II

3.2- *Portulaca oleracea*

3.2.1- GC-MS analysis of *Portulaca oleracea* oil

GC-MS analysis of *Portulaca oleracea* oil was conducted and the identification of the constituents was done by comparison of retentions times and MS data with the GC-MS library. Structures of constituents of the oil were further confirmed by interpreting the observed fragmentation pattern

.Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.2- constituents of oil

The GC-MS analysis of the studied oil revealed the presence of 15 components (Table: 3.9).The typical total ion chromatograms (TIC) of n-hexane extract are shown in (fig.3.15).

Table: 3.9: Constituents of *Portulaca oleracea* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.316	237128	0.08
2.	Pentadecanoic acid, methyl ester	14.373	170885	0.05
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.152	161664	0.05
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.195	705542	0.23
5.	Hexadecanoic acid, methyl ester	15.404	75687986	24.19
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	251818	0.08
7.	Heptadecanoic acid, methyl ester	16.367	775909	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.068	116899705	37.37
9.	9-Octadecenoic acid (Z)-, methyl ester	17.130	23526885	7.52
10.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.153	59248358	18.94
11.	Methyl stearate	17.307	22933709	7.33
12.	cis-11-Eicosenoic acid, methyl ester	18.883	1307281	0.42
13.	Eicosanoic acid, methyl ester	19.084	6202098	1.98
14.	Docosanoic acid, methyl ester	20.716	3045550	0.97
15.	Tetracosanoic acid, methyl ester	22.226	1688258	0.54

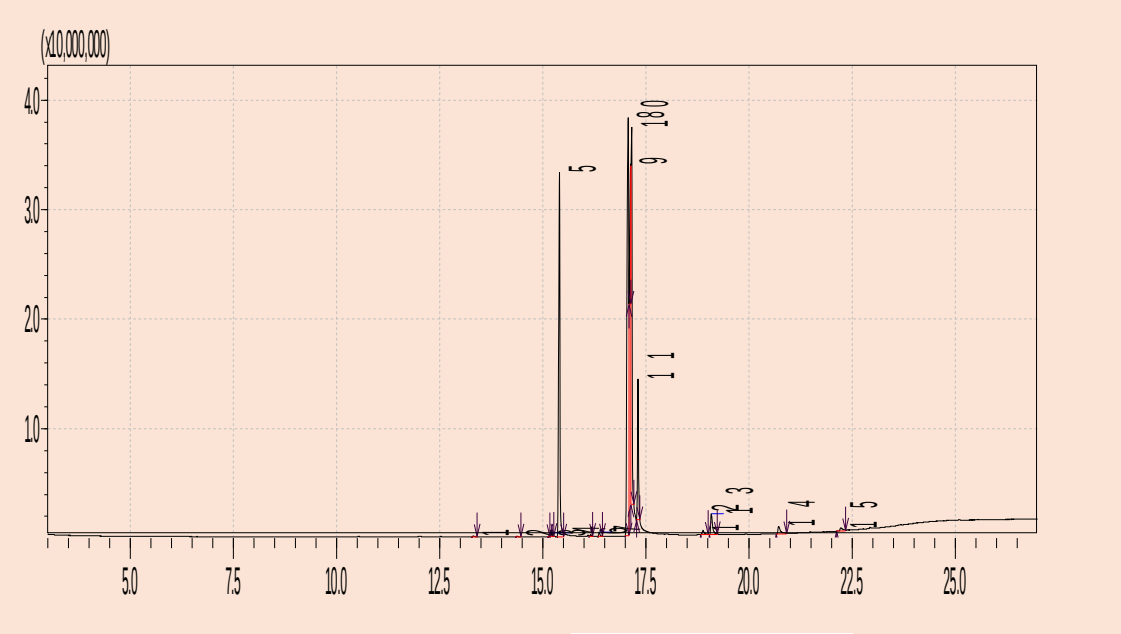


Fig 3.15: Chromatograms of *Portulaca oleracea* seed oil

The following esters were the major components:

9, 12-Octadecadienoic acid, methyl ester (37.37%)

The EI mass spectrum of 9,12-octadecadienoic acid, methyl ester shown in (fig. 3.16).The peak at m/z 294, which appeared at R.T. 17.068 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z 263 corresponds to loss of a methoxyl function.

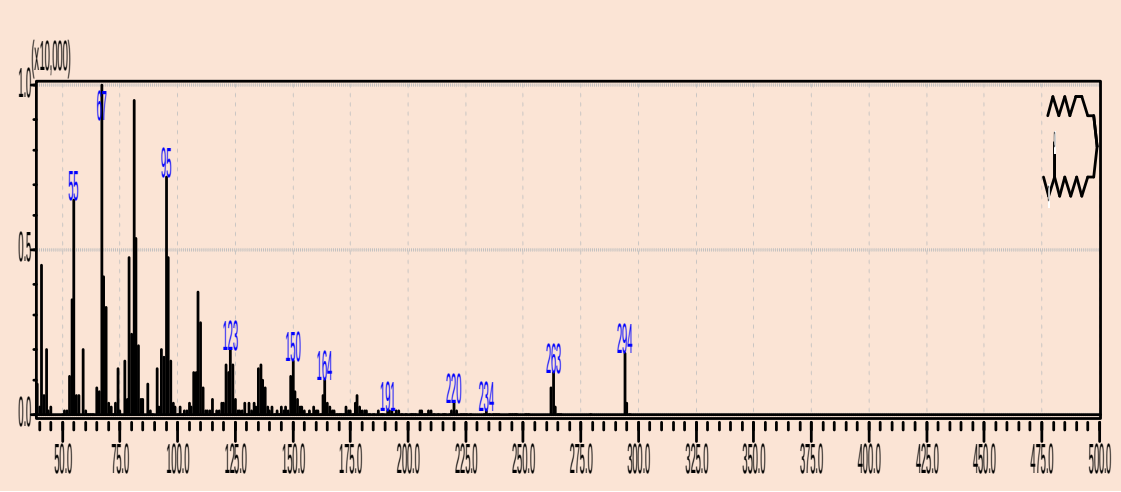


Fig 3.16: Mass spectrum of 9, 12-Octadecadienoic acid, methyl ester

Hexadecanoic acid, methyl ester (24.19%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.17).The peak at m/z 270 (at R.T. 15.404) corresponds $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is due to loss of a methoxyl .

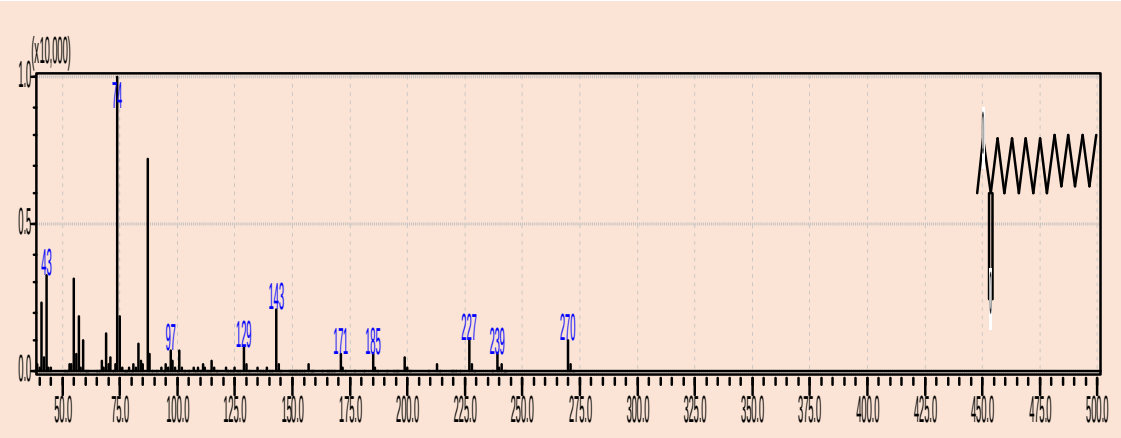


Fig 3.17: Mass spectrum of hexadecanoic acid, methyl ester

9,12,15-Octadecatrienoic acid, methyl ester (18.94%)

Fig. 3.18 demonstrates the mass spectrum of 9, 12, 15-octadecanoic acid, methyl ester. The signal at m/z 292(R.T. 17.153 in total ion chromatogram) corresponds to the molecular ion: $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z261 accounts for loss of a methoxyl .

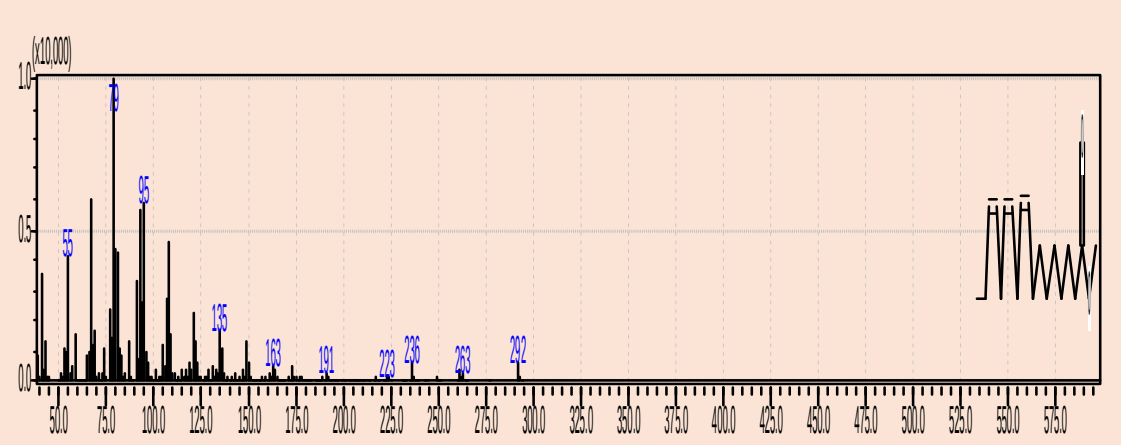


Fig 3.18: Mass spectrum of 9,12,15-Octadecatrienoic acid, methyl ester

3.2.3- Antimicrobial activity

The oil was screened for antimicrobial activity against five standard organisms. The oil not showed any activity against all test organisms

3.2.4- Antioxidant activity

The antioxidant capacity of *Portulaca oleracea* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.10) *Portulaca oleracea* seed oil exhibited weak antioxidant activity.

Table 3.10: Radical scavenging activity of *Portulaca oleracea* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Portulaca oleracea oil	29.00%

3.3- *Coriandrum sativam*

3.3.1- GC-MS analysis of *Coriandrum sativam* oil

GC-MS analysis of *Coriandrum sativam* was carried out . The GC-MS analysis revealed the presence of 14 components (table: 3.11).The typical total ion chromatograms (TIC) of n-hexane extract is presented in (fig.3.19).

Table: 3.11: Constituents of *Coriandrum sativam* oil

ID#	Name	Ret. Time	Area	Area%
1.	Methyl tetradecanoate	13.312	215647	0.03
2.	Pentadecanoic acid, methyl ester	14.372	231442	0.04
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.169	1910240	0.29
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.192	3233931	0.50
5.	Hexadecanoic acid, methyl ester	15.392	41125489	6.32
6.	Heptadecanoic acid, methyl ester	16.367	287117	0.04
7.	7,10-Octadecadienoic acid, methyl ester	16.913	1741544	0.27
8.	6,9-Octadecadienoic acid, methyl ester	16.962	543578	0.08
9.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.062	100831481	15.50
10.	9-Octadecenoic acid (Z)-, methyl ester	17.189	485955106	74.69
11.	Methyl stearate	17.313	9449169	1.45
12.	cis-11-Eicosenoic acid, methyl ester	18.865	3707972	0.57
13.	Eicosanoic acid, methyl ester	19.084	1068295	0.16
14.	Docosanoic acid, methyl ester	20.719	362910	0.06

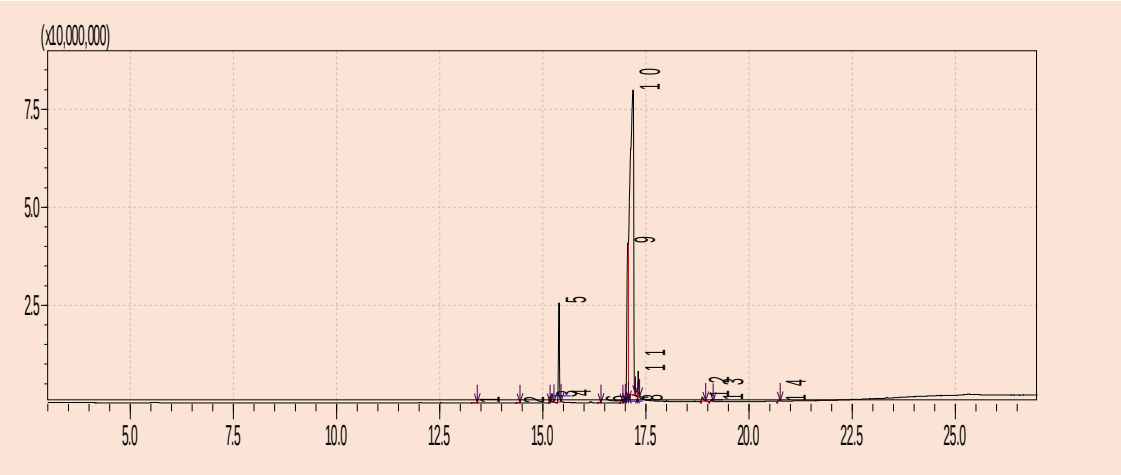


Fig 3.19: Chromatograms of *Coriandrum sativam* seed oil

The following esters were detected as major components:

9-Octadecenoic acid, methyl ester (74.69%)

The EI mass spectrum of 9-octadecenoic acid, methyl ester is shown in (fig. 3.20).The signal which appeared at m/z 264, with R.T. 17.198 corresponds $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z 235 is due to loss of a methoxyl group.

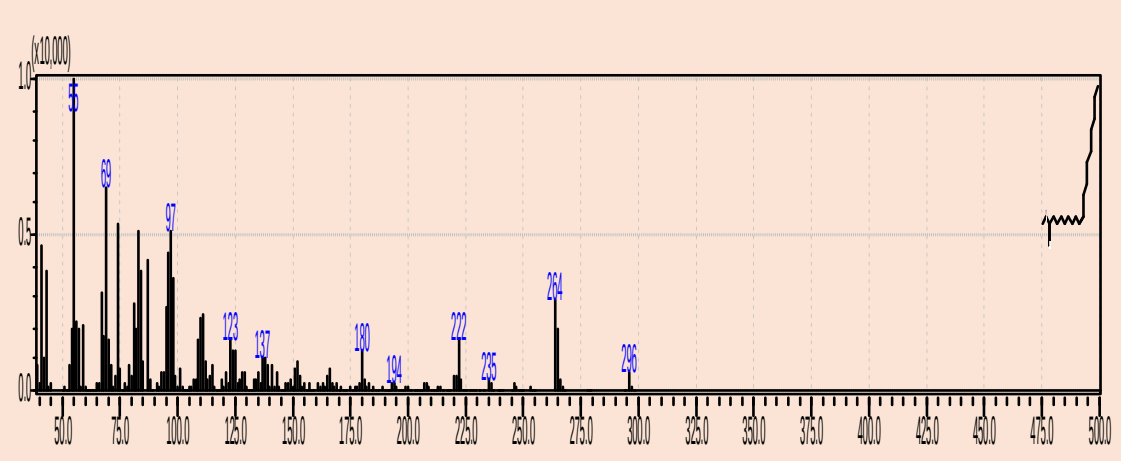


Fig 3.20: Mass spectrum of 9-Octadecenoic acid, methyl ester

9, 12-Octadecadienoic acid, methyl ester (15.50%)

Fig. 3.21 presents the mass spectrum of 9, 12-octadecadienoic acid, methyl ester .The peak at m/z 294, with R.T. 17.062 in total ion chromatogram, corresponds the molecular ion : $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 accounts for loss of a methoxyl function.

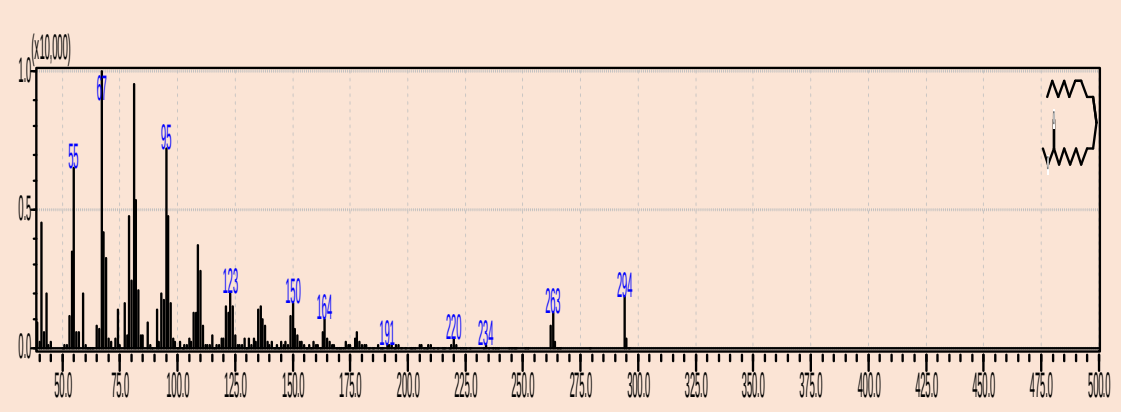


Fig 3.21: Mass spectrum 9, 12-Octadecadienoic acid, methyl ester

3.3.3- Antimicrobial activity

The studied oil was evaluated for antimicrobial activity against five standard human pathogens. The oil not showed any activity against all test organisms

3.3.4- Antioxidant activity

The antioxidant capacity of *Coriandrum sativam* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.12) *Coriandrum sativam* seed oil exhibited moderate antioxidant activity.

Table 3.12: Radical scavenging activity of *Coriandrum sativam* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Coriandrum sativam oil	52.00%

3.4- *Linum usitatissimum*

3.4.1- GC-MS analysis of *Linum usitatissimum* oil

GC-MS analysis of *Linum usitatissimum* was conducted. The analysis revealed the presence of 15 components (table: 3.13).The typical total ion chromatograms (TIC) of n-hexane extract is shown in (fig.3.22)

Table: 3.13: Constituents of *Linum usitatissimum* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.317	189936	0.07
2.	Pentadecanoic acid, methyl ester	14.373	70754	0.03
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.155	104077	0.04
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.196	476941	0.19
5.	Hexadecanoic acid, methyl ester	15.392	35779007	14.12
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	270452	0.11
7.	Heptadecanoic acid, methyl ester	16.365	508829	0.20
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.053	64486368	25.44
9.	9-Octadecenoic acid (Z)-, hexadecyl ester	17.155	69889815	27.58
10	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.164	50002070	19.73
11	Methyl stearate	17.307	28380442	11.20
12	cis-11-Eicosenoic acid, methyl ester	18.887	1343975	0.53
13	Eicosanoic acid, methyl ester	19.092	684609	0.27
14	Docosanoic acid, methyl ester	20.722	818972	0.32
15	Tetracosanoic acid, methyl ester	22.233	436726	0.17

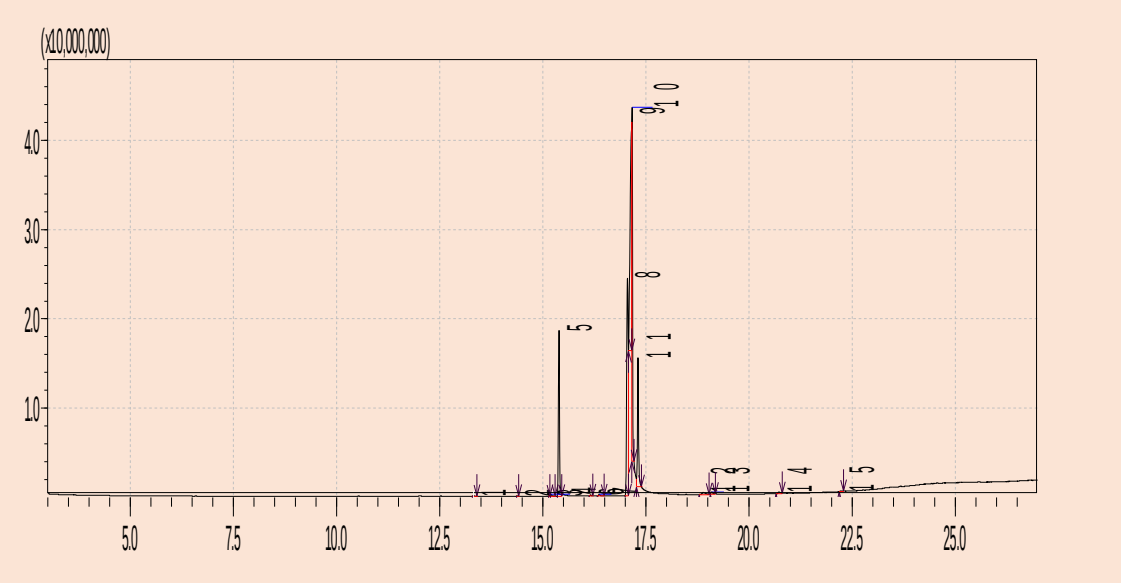


Fig 3.22: Chromatograms of *Linum usitatissimum* seed oil

The following esters were found as major components:

9-Octadecenoic acid, hexadecyl ester (27.58%)

The EI mass spectrum of 9-Octadecenoic acid, hexadecyl ester is shown in (fig. 3.23).The peak at m/z 295, which appeared at R.T. 17.155 in total ion chromatogram, corresponds to $M^{+}[C_{34}H_{66}O_2]^{+}$ .The peak at m/z 264 corresponds to loss of a methoxyl function.

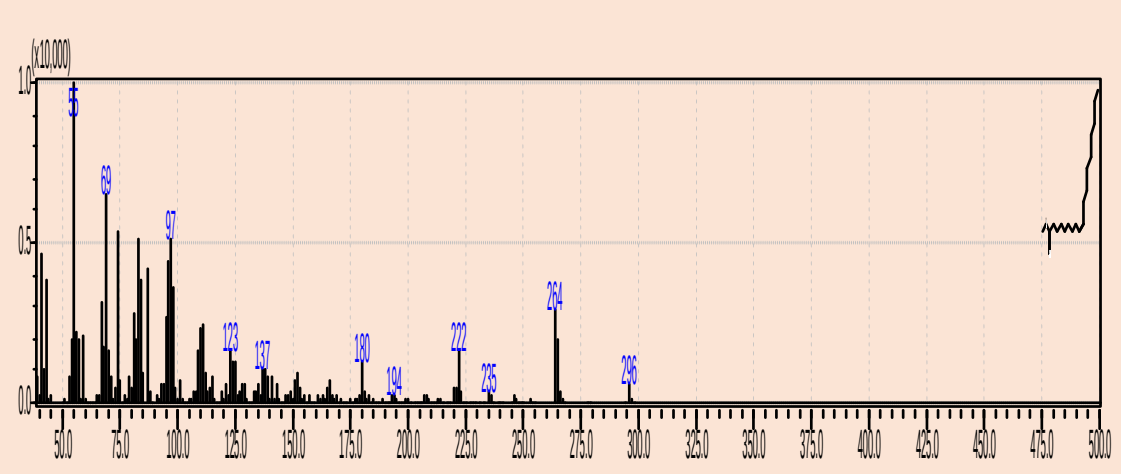


Fig 3.23: Mass spectrum of 9-Octadecenoic acid, hexadecyl ester

9, 12-Octadecadienoic acid, methyl ester (25.44%)

The EI mass spectrum of 9, 12-octadecadienoic acid, methyl ester is shown in (fig.3.24). The peak at m/z 294, which appeared at R.T. 17.053 corresponds $M^{+}[C_{19}H_{34}O_2]^{+}$ while the sighal at m/z 263 is due to loss of a methoxyl group.

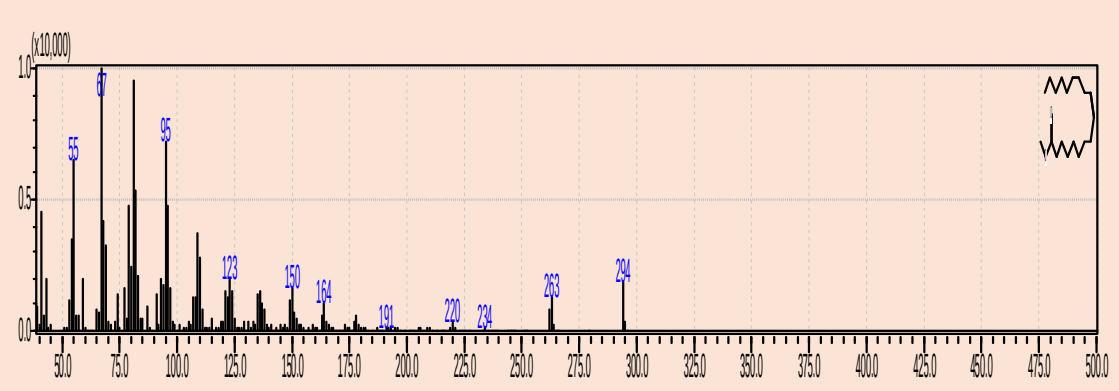


Fig 3.24: Mass spectrum of 9, 12- Octadecadienoic acid, methyl ester

9, 12, 15-Octadecatrienoic acid, methyl ester (19.73%)

Figure 3.25 shows the mass spectrum of 9, 12, 15-octadecatrienoic acid, methyl ester. The peak at m/z 292 - R.T. 17.164- in total ion chromatogram, corresponds the molecular ion : $M^{+}[C_{19}H_{32}O_2]^{+}$,while the sighal at m/z 261 accouts for loss of a methoxyl .

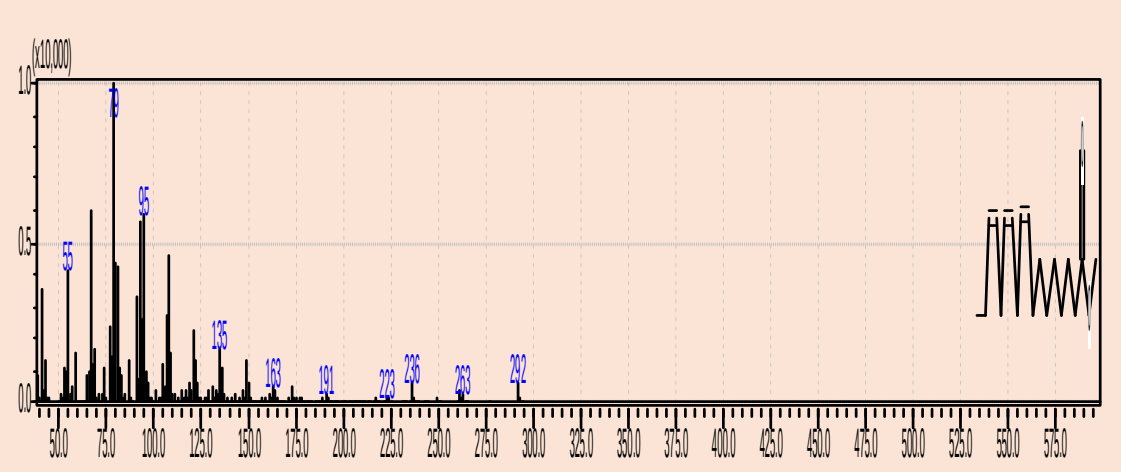


Fig 3.25: Mass spectrum of 9, 12, 15-Octadecatrienoic acid, methyl ester

Hexadecanoic acid, methyl ester (14.12%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.26). The moleculat ion : $M^{+}[C_{17}H_{34}O_2]^{+}$ appeared as expected at m/z 270(R.T. 15.392).The peak at m/z 239 is attributed to loss of a methoxyl .

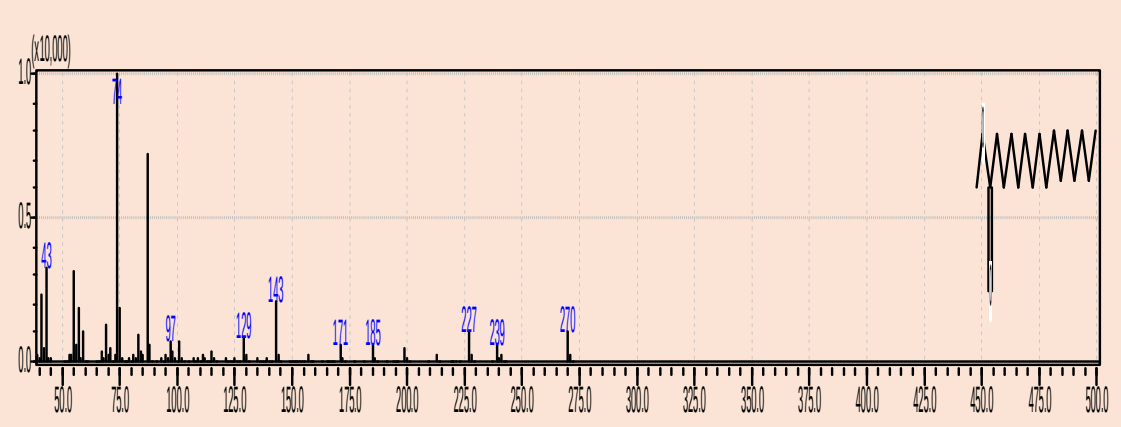


Fig 3.26: Mass spectrum of Hexadecanoic acid, methyl ester

Methyl stearate (11.20%)

The mass spectrum of methyl stearate is presented in (fig.3.27). The signal which appeared at m/z 298 -with R.T. 17.307 in total ion chromatogram - corresponds $M^{+}[C_{19}H_{38}O_2]^{+}$ .The peak at m/z 267 is attributed to loss of a methoxyl .

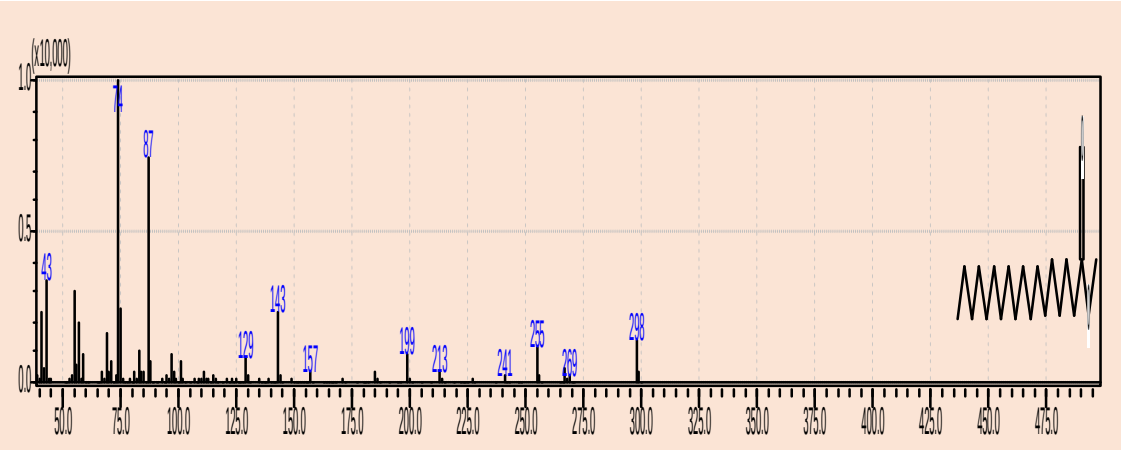


Fig 3.27: Mass spectrum of methyl stearate

3.4.2- Antioxidant activity

The antioxidant capacity of *Linum usitatissimum* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.14) *Linum usitatissimum* seed oil exhibited moderate antioxidant activity.

Table 3.14: Radical scavenging activity of *Linum usitatissimum* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Linum usitatissimum oil	48.00%

Conclusion

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

Two flavonoids (compounds I and II) were isolated from the stems of *Pulcaria crispa* .The partial structures of both compounds were deduced on the basis of their : IR, UV and NMR data and partial structures were suggested. Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried out . The results exhibited variable susceptibilities of microorganism for crude extract and different fractions. The antioxidant capacity of the alcoholic extract of *Pulcria crispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The oils were also assessed for their antimicrobial activity and promising results were obtained.

Recommendations

The following is highly recommended:

1. Other phytochemicals of the studied plant may be isolated and their structures may be elucidated and the biological activity could be screened.
2. The isolated falvonoids may be subjected to 2D NMR experiments for full characterization.
3. The isolated falvonoids and oil may be screened for other biological effects such as antiinflammatory, antidiabetic , antiviral, antilashmenial ..etc.

Sudan University of Science and Technology

College of Post Graduate Studies

Phytochemical Studies on the Stems of *Pulcria crispa* and Seeds of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* and Biological Activity of Constituents

دراسة فيتوكيميائية لسيقان نبات الربل وبذور نبات الرجلة والكسبرة والكتان والفعالية البيولوجية للمكونات

A Thesis Submitted in Fulfillment of the Requirements of the Ph.D. Degree in Chemistry

By

Lubna Akasha Abdelmalik Mohamed

(B.Sc. (Hons.) Chemical Laboratories, M.Sc. in Chemistry)

Supervisor

Prof: Mohamed Abdel Karim Mohamed

September, 2019

الإســـــــتـــهـــلال

﴿ بسم الله الرحمن الرحيم ﴾

فلينظر الإنسان إلى طعامه ﴿٢٤﴾ أنا
صببنا الماء صبا ﴿٢٥﴾ ثم شققنا
الأرض شقا ﴿٢٦﴾ فأنبتنا فيها حبا
﴿٢٧﴾ وعنبا وقضبا ﴿٢٨﴾ وزيتونا
ونخلا ﴿٢٩﴾ وحدائق غلبا ﴿٣٠﴾
وفاكهة وأبا ﴿٣١﴾ متاعا لكم
ولأنعامكم ﴿٣٢﴾ سورة عبس

Dedication

To,

My parents,

My sons,

And my sisters and brother

Acknowledgement

First and foremost, I thank **Almighty Allah, the Ever-Magnificent, the Ever-Thankful**, for his help and blessing.

I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor Dr. Mohamed Abdel karim, who has been always generous during all phases of the research, and I highly appreciate the efforts expended by my co-supervisor, Dr. Kamal Mohamed Saeed.

I owe a deep debt of gratitude to the Sudan University of Science and Technology for giving us an opportunity to accomplish this work. Thanks are extended to the Aromatic and Medicinal Plants Institute, Khartoum, Sudan for all facilities. Thanks for the National Research Center, Cairo for the spectral measurements.

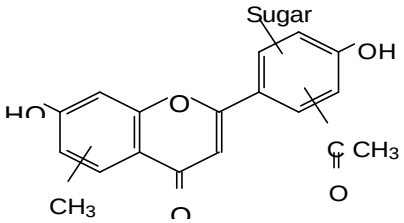
I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis.

I also would like to express my whole hearted thanks to my parents, for their generous support. They supported me throughout my entire life and particularly through the process of accomplishing the PhD degree.

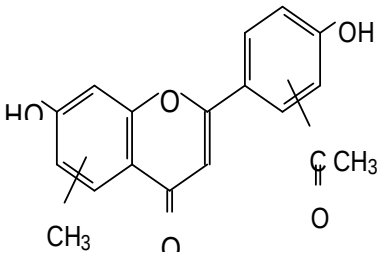
Abstract

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

The stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature. Qualitative tests on the alcoholic extract were positive for flavonoids. Conventional chromatographic techniques were used for isolation and purification of flavonoids. Two flavonoids (compounds I and II) were isolated from this plant. The partial structures of both compounds were deduced on the basis of their: IR, UV and NMR data and the following structures were suggested:



Compound I



Compound II

Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried. The results exhibited variable susceptibilities of microorganism for crude extract and different fractions.

In this study, the antioxidant capacity of the alcoholic extract of *Pulcriacrispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The GC-MS analysis of the *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* oils revealed the presence of 15, 14, 15 components respectively.

In the agar diffusion bioassay oils of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* failed to exhibit any antimicrobial activity. However, *Coriandrum sativam* oil showed significant antioxidant activity.

المستخلص

يشتمل البحث على دراسة كيميائية مفصلة للمحتوى الفلافونيدي لسيقان نبات الربل وكذلك التركيب الكيميائي لزيوت بذور كل من البقلة، الكزبرة وبذور الكتان. ايضا يتضمن البحث دراسة فعالية مستخلصات نبات الربل والزيوت المستخلصة كمضادات ميكروبات وكمضادات أكسدة.

لقد أستخلصت سيقان نبات الربل بواسطة الكحول الإيثيلي عند درجة الحرارة العادية و افاد المسح الفيتوكيميائى بوجود الفلافونيدات.

أنجز فصل المركبات بإستخدام كروماتوغرافيا العمود و كروماتوغرافيا الورق ، كما وتم التعرف على التركيب الكيميائي للمركبات المفصولة بإستخدام وسائل التحاليل الطيفية المختلفة : مطيافية الأشعة تحت الحمراء، الأشعة فوق البنفسجية ، الرنين النووي المغناطيسي للهيدروجين . وقد تم فصل مركبين ذوا طبيعة فلافونيدية من نبات الربل وهما المركب **II ,I.** تناولت الدراسة فعالية مستخلصات سيقان نبات الربل كمضادات ميكروبات وكذلك كمضادات أكسدة.

أثبت البحث ان مستخلص خلات اللإيثيل ذو فعالية كمضاد للبكتيريا. أما المستخلص الكحولي وبقية المستخلصات فقد اظهروا فعالية منخفضة كمضادات بكتيريا وفطرية. أوضحت الدراسة ان المستخلص الكحولي لنبات الريل ذو تاثير فعال كمضادات اكسدة.

اخضع زيت بذور البقلة، الكزبرة والكتان للكروماتوغرافيا الغازية - السائلة الملحقة بمطياف الكتلة . اوضحت هذه الدراسات وجود خمسة عشر ، أربعة عشر، وخمسة عشر مكونا بالزيت على التوالي.

تناولت الدراسة أيضا فعالية الزيوت المستخلصة من بذور البقلة ، الكزبرة والكتان كمضادات ميكروبات ولم تظهر النتائج أي تأثيريذكر. وأثبت البحث أن زيت بذور الكزبرة ذو فعالية عالية كمضاد أكسدة .

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INTRODUCTION

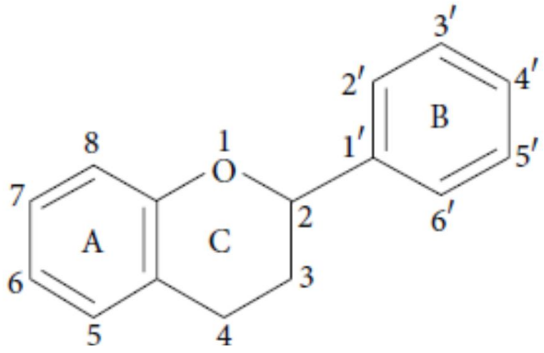
1.1 General overview

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities [1, 2]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [3]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [4]. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [5, 6]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [7, 8].

As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems [9, 10]. Flavonoids have ability to induce human protective enzyme systems.

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases [2, 8-

[10].Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin [11]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [12].The basic nucleus of flavonoids is displayed in Fig. 1.



(1)

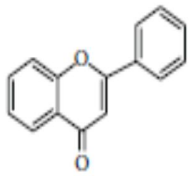
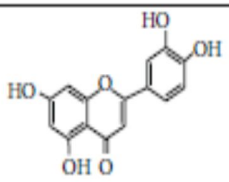
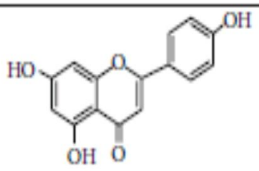
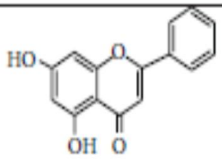
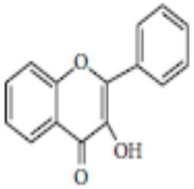
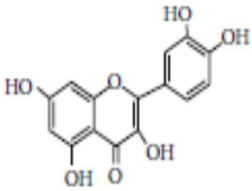
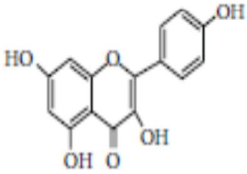
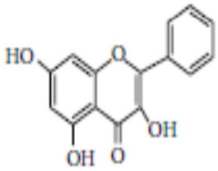
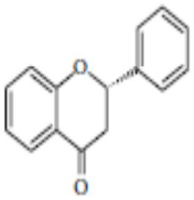
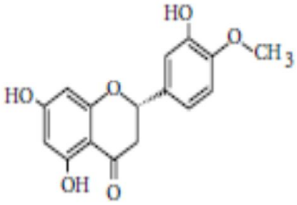
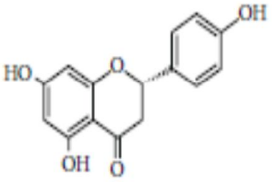
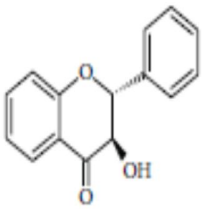
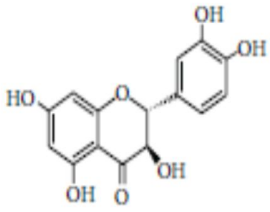
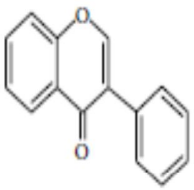
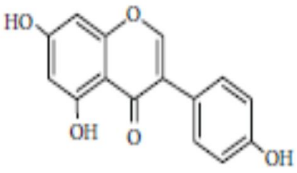
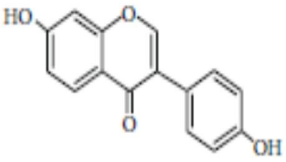
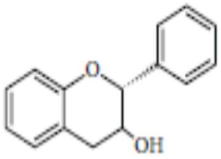
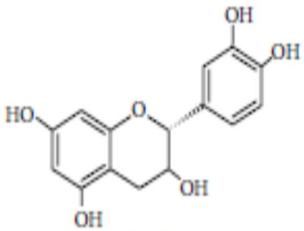
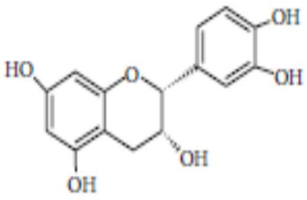
1.2 Chemistry of Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from orange. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified [13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (e.g. apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.

Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings [13].

Table (1): Structure of flavonoids

Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1.1). Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

Flavonols differ from flavanones by hydroxyl group at the 3-position and a C_2–C_3 double bond[14]. Flavonoids are often hydroxylation positions 3, 5, 7, 2, 3', 4', and 5'.Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[15].

1.2.1 Spectral Characteristics of Flavonoids

Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (235–285 nm) corresponds to the A ring absorption.

Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367 nm in kaempferol (3,5,7,4-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'- hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'- hydroxyl groups) [16]. The absence of a 3-hydroxyl group in flavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics [17]. Flavanones exhibit a very strong (Band II) absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only a shoulder for (Band I) at 326 and 327 nm. Band II appears as one peak (270

nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present.

As anthocyanins show distinctive (Band I) peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring and (Band II) peaks in the 240–280nmregion due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups [18].

1.3 Flavonoid- Rich Food and Medicinal Plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photo-synthesizing plant cells.

They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals [19].Thus flavonoids found in animals are of plant origin rather than being biosynthesized *in situ*. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [20].

Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg [21]. Preparation and processing of food may decrease flavonoid levels depending on the methods used.

For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644mg/L which suggest that the flavanones are concentrated in the cloud

during processing and storage [22]. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans [23].

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids [24, 25]. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases.

Table (2): Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry

Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavone – glabridin- a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals [26]. Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants [27, 28]. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people.

Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats [29].

Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia [30]. Table 3 summarizes some of the medicinal plants rich in flavonoid contents.

Table (3): Medicinal plants rich in flavonoids contents

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein

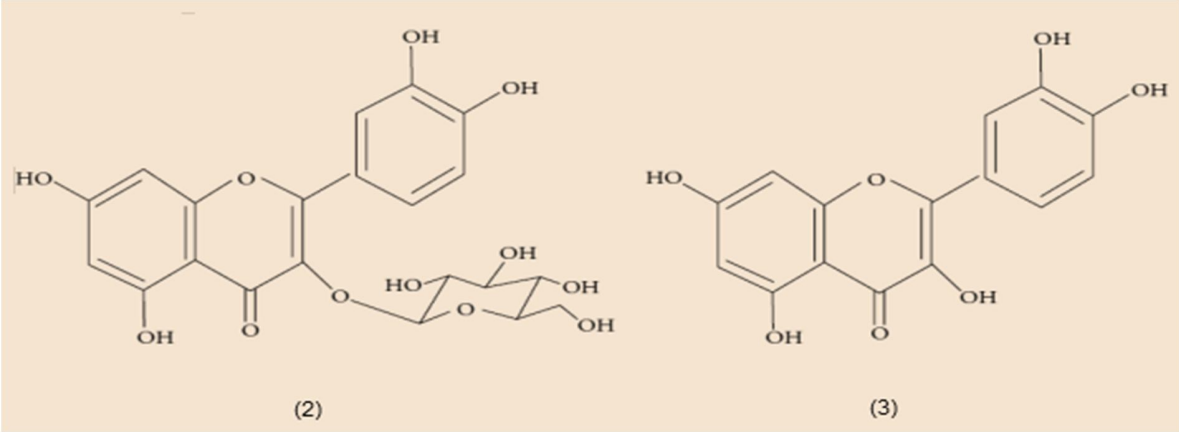
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for

example, hydroxy ethyl rutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and micro bleeding [31].

1.4 Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and p^{Ka}. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as glycosides (Figures 2 and 3). Aglycones can be easily absorbed by the small intestine, while flavonoid glycosides have to be converted into aglycone form before absorption [32].



The hydrophilic flavonoid glucoside such as quercetin are transported across the small intestine by the intestinal Na^+-dependent glucose cotransporter (SGLT1) [32].

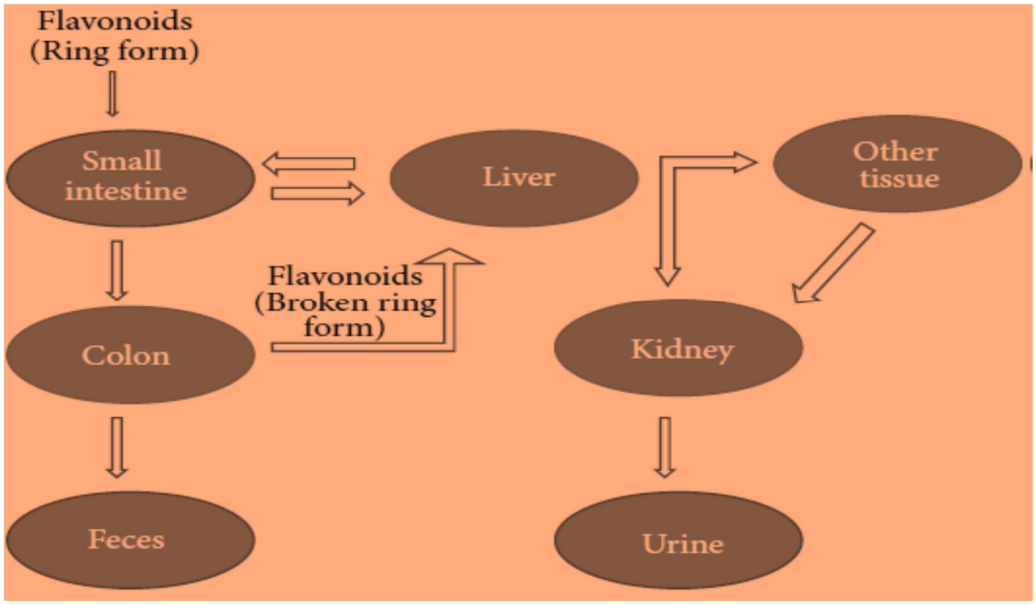
An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [33]. The substrate

specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [34].

The glycosides which are not substrates for these enzymes are transported toward the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [35]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected.

After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds [36]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [37]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [38]. The flavonoids secreted with bile in intestine and those that cannot be absorbed from the small intestine are degraded in the colon by intestinal micro flora which also break down the flavonoid ring structure (Sceme 1). Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [39]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine.

They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [40].



Sheme 1: Compartments involved in the metabolism of flavonoid

1.5 Biological Activities of Flavonoids

1.5.1 Antioxidant Activity

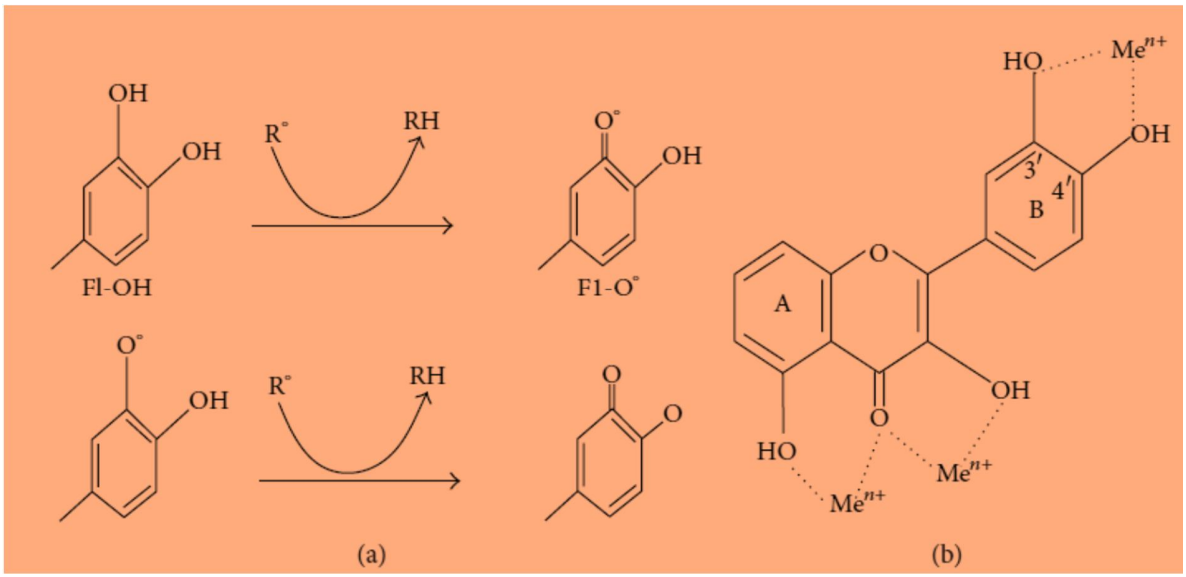
Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants.

The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure.

The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [4, 41]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and

peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [42].

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation;(2) Scavenging ROS; and (3) upregulation or protection of antioxidant defenses [43, 44]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [45]{see scheme 2}.



Scheme (2): (a) Scavenging of ROS (R∘) by flavonoids (Fl-OH) and

(b) binding sites for trace metals where Men+ indicates metal ions

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms [5, 25]. Free

metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation (Scheme 2(a)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [44, 46]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures [47]. The binding sites are shown in Schme 2(b). A 3', 4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [4]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro* [48]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable *ortho* semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential [49]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features [50].

The flavonoid heterocyclic contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH is crucial. Removal of a 3-OH annuls coplanarity and conjugation which

compromises scavenging ability [51]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity [50].

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides.

There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased [52]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position [53]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [54].

1.5.2 Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [55].

Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Glutamate-cysteine ligase catalytic subunit

(Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases [56]. It has been demonstrated that anthocyanin cyanidin-3-O-β- glucoside increases Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and pro-apoptotic signaling. Furthermore, this flavonoid lowers hepatic lipid peroxidation, inhibits the release of pro-inflammatory cytokines, and protects against the development of hepatic steatosis [56]. Silymarin is a flavonoid having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle *Silybum marianum* (Compositae). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers [57-59].

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl4-) induced injury. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl4 [60]. Similarly in an *in vivo* experiment flavonoids at of 50, 100, and 200mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver.

Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid [60].

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive

complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. *Equisetum arvense* flavonoids as well as hirustrin and avicularin isolated from some other sources is reported to provide protection against chemically induced hepatotoxicity in HepG2 cells [61, 62].

1.5.3 Antibacterial Activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid- rich plant extracts from different species have been reported to possess antibacterial activity [44, 46, 63, 64]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [65].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation.

Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [66, 67].

Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria [68, 69].

The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities [68, 70]. Robinetin, myricetin, and (−)-epigallocatechin

are known to inhibit DNA synthesis in *Proteus vulgaris.* Mori et al. [71] suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase [72].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes [73]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells.

The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity [74]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [75].

Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus* [76].

Haraguchi and colleagues [74] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A

inhibited incorporation of radioactive precursors intomacromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain [74].

There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents [77–79].

1.5.4 Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and pro-inflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [80].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells [81]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein

kinases [82, 83]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules [84]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful pro-inflammatory signaling molecules[85]. Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotopes *in vitro* [86].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration [87]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide [88]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.5.5 Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents [46, 89]. Consumption of onions and/or apples, two

major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon [90].The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods [91].

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [92]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [93]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in cogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor

agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial [94]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer [93].

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death [90, 95]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors [96].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P- 388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, andMKN-28), colon cancer cells (COLON320DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells [97] . It has been proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent [98].

Barnes [99] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones

genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats [100]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats [101]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al. [102]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [103]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects [104]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin [105].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [106]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor [44, 46, 107].Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals [108].

1.5.6 Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral

activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available.

Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso [109] demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication. Baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase [110].

Table (4): Antiviral activity of various flavonoids

Flavonoid	**Virus**
Quercetin	Rabies virus, herpes virus, parainfluenza virus, polio virus, mengo virus, and pseudorabies virus
Rutin	Parainfluenza virus, influenza virus, and potato virus
Apigenin	Immunodeficiency virus infection, Herpes simplex virus type, and Auzesky virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

Another study revealed inhibition of HIV-1 entry into cells expressing CD4 and chemokine coreceptors and antagonism of HIV-1 reverse transcriptase by the flavone *O*-glycoside [111]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase [110]. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a novel mechanism that probably involves inhibition of viral transcription [112].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV).

Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [110]. Studies have displayed that flavonols are more active than flavones against herpes simplex virus type 1 and the activity order was found to be galangin, kaempferol, and quercetin [110].

Zandi et al. [113] studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus [109]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [113]. List of some flavonoids and their efficacy against viruses is given in Table 4.

1. 6 Role of Flavonoids in Plants

1.6.1 Combating Oxidative Stress

Flavonoids have long been reported as serving multiple functions in plants [114].Various abiotic and biotic factors helps in the generation of ROS in plants leading to oxidative stress. Flavonoid biosynthesis in plants is almost exclusively enhanced due to oxidative stress. They have capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and quench ROS once they are formed [115]. Flavonoids discharged primary UV-B screening functions when early plants moved from water to colonize the land. Extent of antioxidant capacity and ability to absorb UV-wavelengths depends upon the nature of substitution on different rings of flavonoids. Dihydroxy B ring substituted flavonoids have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts have greater ability to absorb UV wavelengths [115].

The most reactive hydroxyl groups (7-OH in flavones or the 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in the aqueous cellular environment, protects the reactive hydroxyl groups from autooxidation [116], and allows the transport of flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and the cell wall [117]. Recent evidence shows that antioxidant flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation, that is, the chloroplast. Here they can easily quench H_2O_2, hydroxyl radical, and singlet oxygen [115, 118].

Oxidative stress due to an excess of excitation energy in the chloroplast may be aggravated under conditions that limit the diffusion of CO_2 to the carboxylation sites and the efficiency of carboxylation [115, 119]. The environmental limitations to CO_2 assimilation rate include drought/salinity,

low/high temperature, and nutrient scarcity. Under these conditions the activity of ROS detoxifying enzymes may significantly reduce in the chloroplast [120, 121], which in turn up regulates the biosynthesis of ROS scavenging flavonoids.

The reducing functions of flavonoids are of key significance in plants under severe stress conditions. These functional roles are concomitant with the very high concentration of dihydroxy B ring substituted flavonoids [122]. Flavonoids have been suggested as representing a secondary antioxidant defense system in plant tissues exposed to different stresses [115]. Lipid peroxidation is the common consequence of oxidative stress which disrupts the cell membrane integrity.

Quercetin 3-*O*-rutinoside (rutin) may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage [123].

1.6.2 Growth Regulator

Flavonoids carry out functional roles of amazing significance in plant-environment interactions.

Flavonoids (in the nanomolar range) may regulate auxin movement and catabolism. The ability of flavonoids to create auxin gradients translates into phenotypes with different morphoanatomical features [124]. The control of flavonoids on auxin movement may have immense value in the stress-induced morphogenic responses of plants such as the flight strategy of sessile organisms exposed to unfavorable environments [125]. Species rich in dihydroxy flavonoids exhibit phenotypes with strikingly different morphological traits as compared with those rich in monohydroxy flavonoids [126]. Dwarf bushy phenotypes with few, small, and thick leaves to direct sunlight irradiance are usually present in sunny environments, thus

protecting leaves located deep in the canopy from light-induced severe cellular homeostasis perturbations. On the contrary, shaded plants, which are rich in kaempferol and/or apigenin derivatives (having negligible concentrations of quercetin derivatives), have long internodes, and large leaf lamina coupled with reduced leaf thickness [125].

Flavonoids at the plasma membrane are effective inhibitors of PIN (pin formed) and MDR (multidrug resistance) glycoproteins that are involved in the cell to cell movement of auxin. The ability of flavonoids to inhibit the activity of the efflux facilitator PIN and MDR proteins depends on the presence of the catechol group in the B ring of the flavonoid skeleton. In addition flavonoids regulate the activity of IAA-oxidase with markedly different effects based on their chemical structure [127]. Recent evidence of a nuclear location of flavonoids (as well as of enzymes of flavonoid biosynthesis) supports that flavonoids are capable of modulating the activity of proteins involved in cell growth. Flavonoids may therefore act as transcriptional regulators [128, 129].

1.7 Microbial Production of Flavonoids

In response to the low production efficiency from plants and chemical synthesis, research groups have directed their attention to the production of flavonoids in microorganisms using metabolic engineering and synthetic biology [130].

Chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals [131]. Because of the rapid development in molecular biology tools and the flooding of genome information from a variety of organisms, combinatorial biosynthesis offers an advantage for production of rare and expensive natural products. It can be used in both simple and complex transformations without the tiresome blocking and deblocking steps that are

common in organic synthesis [132]. Several prokaryotes and eukaryotes such as *E. coli*, *Saccharomyces cerevisiae*, *Streptomyces venezuelae*, and *Phellinus igniarius,* a medicinal mushroom, have been used for production of flavonoids [12].

1.7.1 Phenylpropanoid Pathway

In plants naringenin chalcone is the precursor for a large number of flavonoids produced by the phenylpropanoid synthetic pathway. Fermentative production by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine, and tyrosine [133]. As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonialyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid, which is then activated to *p*-coumaroyl-CoA by the action of 4-coumarate:
CoA ligase. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or nonenzymatically *in vitro* [134].

1.7.2 Enhancement of Flavonoid Production

Combination of promoter and target genes; knockout of related genes; overexpression of malonyl-CoA; and construction of artificial P450 enzymes are the key molecular biology technology procedures used in the heterologous production of flavonoids.

Every gene from the phenylpropanoid pathway is cloned in the host under the control of the promoter which often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used to enhance production of flavonoids according to the need of specific host such as T7, ermE∗, and GAL1 promoter [12]. The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids. Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens* intracellular malonyl-CoA pool was amplified leading to increased production of flavonoids [135].

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It was proved in an experiment in which researchers knocked out the udg gene encoding for UDP-glucose dehydrogenase. This resulted in elimination of endogenous UDP-glucose consumption pathway leading to increase in intracellular concentration of UDP-glucose and as a consequence increment in the production of flavanones and anthocyanins was observed [136].

One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound cinnamate-4-hydroxylase [137].This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host. An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis

pathway [138]. Combining bacterial cells and eukaryotic cells in a pot enabled researchers to generate a wider library of natural and unnatural products than any of the previously reported systems. *De novo* production of the key flavonoid intermediate naringenin has been demonstrated for the first time from glucose using an engineered *S. cerevisiae* strain which led to fourfold higher concentrations than reported in earlier studies on de novo biosynthesis [139, 140].

1.8 The studied plant species

1.8.1 *Pulcaria crispa*

Pulicaria crispa is a genus of Asteraceae family. The Asteraceae family is one of largest families of flowering plant, with about 1,100 currently accepted genera and 25,000 species. It is characterized by the flowers being arranged in dense heads of many small florets, such as the daisy and dandelion [141].

Pulcaria crispa

The family is of worldwide distribution ,being absent only from the Antarctic mainland, and is particularly well represented in semiarid region of the tropics and subtropics, such as the Mediterranean region, Mexico, the cape province of South Africa grassland and bush land formation of Africa and montane floras throughout the world. Only in the tropical rain forests are they poorly represented [141]. The Asteraceae are of incalculably great indirect economic importance to man as major contributors to the diversity, and therefore, the stability and sustainable productivity, of the drier vegetation types throughout the world, especially in tropical and sub-tropical areas. In proportion to its size, however, the direct economic importance of the family is comparatively small. It includes food plants, sources of raw materials, medicinal and drug plants, ornamentals and succulents, and, on the debit side, weeds and poisonous plants [141].

1.8.1.1 Phytochemical, antibacterial screening and antioxidant activity of *Pulcaria crispa*

Many secondary metabolites have been detected - coumarins, tannins and alkaloids - on *P. crispa* [142]. Investigation of the flavonoids constituents of *Pulicaria crispa*, result in the isolation and identification of four flavonoids, quercein, quercetin-3-o-glucoside, quercetin7-o glycoside and quercetin-3-methyl ether [143]. The presence of these metabolites explains the various uses of this plant in traditional medicine. Alkaloids, comprising a large group of nitrogenous compounds, are widely used as cancer chemotherapeutic agents [144, 145] alkaloids also interfere with cell division The presence of alkaloids in *Pulicaria crispa* may be responsible for antibacterial activity. *Pulicaria crispa* is used in folk medicine for the treatment of colds, coughs, colic, excessive sweating and as carminative [146].

1.8.2 *Portulaca oleracea*

Portulaca oleracea is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution belonging to the Portulacaceae family. It is commonly known as *purslane* (USA and Australia), *rigla* (Sudan and Egypt), *pigweed* (England), *pourpier* (France), and *Ma-Chi-Xian* (China) [147]. It is distributed widely in the tropical and subtropical areas of the world including many parts of the United States and is eaten extensively as a potherb and is added to soups and salads around the Mediterranean and tropical Asian countries [148].

Portulaca oleracea

Americans and aborigines of Australia grind the seeds of this plant into flour for use in mush and bread [149]. *Portulaca oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties [150]. *Portulaca oleracea* has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic and so forth [151]. It exhibits a wide range of pharmacological effects, including antibacterial [152], antiulcerogenic [153], anti-inflammatory [154], antioxidant [155], and wound-healing [156] properties. It is listed by the World Health Organization as one of the most used medicinal plants, and it has been given the term “Global Panacea” [157]. The

Chinese folklore described it as “vegetable for long life” and it has been used for thousands of years in traditional Chinese Medicine [158, 159]. It is cold in nature and sour in taste and is used to cool the blood, stanch bleeding, clear heat, and resolve toxins. The dried aerial part of this plant is indicated for the treatment of fever, dysentery, diarrhea, carbuncle, eczema and hematochezia, with a recommended dose of 9–15 g, *Portulaca oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine [160–162].

1.8.2.2 Phytochemistry

Many constituents of *Portulaca oleracea* have been isolated, including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals.

One of the most effective constituents present in Chinese Herbal Medicines are flavonoids which are biologically active and possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. In the *Portulaca oleracea* plant, the flavonoids levels vary according to the part of the plant; the highest levels are present in the root followed by stem and the leaf; and seven different flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin [163]. However, only kaempferol and apigenin have been found in ethanolic extracts of leaves and stems, with the levels in the former being higher [157]. Portulacanones B–D, three homoisoflavonoids compounds, display selectively cytotoxic activities against three human cancer cell lines (SF-268,NCI-H460, and SGC-7901) [164]. Flavonoids are also widely present in foods such as fruits and vegetables [165].

In addition to flavonoids, another important chemical found in this plant is alkaloids including dopa, dopamine, and noradrenalin. The content of dopamine and noradrenalin is higher in leaves compared to stem and seeds. The amount of dopamine and noradrenalin obtained from leaves varies according to the solvents used in the extraction process, suggesting that the levels of these compounds are dependent on the solvents used during the extraction process [166].

Oleraceins A, B, C, D, and E are cyclodopa alkaloids isolated from this plant [167] and several analytes such as (3R)-3,5-bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone and 1,5-dimethyl-6-phenyl-1,2-dihydro-1,2,4-triazin-3(2H)-one display cytotoxic activities against human cancer cells [168].

Portulaca oleracea is also an excellent source of omega-3 fatty acids, which is usually present in oil and fat of fishes but not normally found in plants. Omega-3 fatty acids play an important role in the enhancement of immune function [169] and prevention and treatment of hypertension, coronary artery disease, cancer, and other inflammatory and autoimmune disorders [170]. It includes α-linolenic acid and linoleic acid, which are essential for normal growth, health promotion, and disease prevention in humans. Polysaccharides found in *Portulaca oleracea* are potential therapeutic agents for the treatment of diabetes mellitus owing to their modulation of blood lipids, metabolism, and decrease of blood glucose. *Portulaca oleracea* contains monoterpenes such as portulosides A and B, diterpenes such as portulene, and β-amyrin type triterpenoids [147, 171]; in addition, vitamins have also been isolated from the leaves of this plant. It contains the highest content of vitamin A which is a natural antioxidant playing an important role in vision, maintaining healthy mucus membranes and protecting against lung and oral cavity cancers among green leafy vegetables. This plant also

contains ascorbic acid, α-tocopherol, and B-complex vitamins, for example, niacin, pyridoxine, and riboflavin [171]. Furthermore it is rich in minerals like phosphorus, manganese, icon, calcium selenium [149], and the amino acids isoleucine, proline, leucine, lysine, phenylalanine, methionine, cystine, valine, threonine, and tyrosine [148]. Many other constituents have also been isolated from this plant, such as β-carotene, glutathione, melatonin, portulacerebroside A, catechol, and bergapten.

1.8.3 *Coriandrum sativam*

Coriander (*Coriandrum sativum*) being an annual herb is most commonly used for seasoning purpose. Seeds, leaves and roots are edible, although they have very distinct flavors and uses [172]. The herb has a light and fresh flavor. Coriander can be used as whole plant and can be processed because of its perishable nature of leaves and to increase the palatability of ripe fruits (seeds) before using it as flavoring agent in different food preparations. Whole plant of coriander mainly fresh leaves and ripe fruits are used for culinary purposes [172].

Coriandrum sativum

Coriander leaves have different taste than its seeds, with citrus overtones. Coriander plant is a rich reservoir of micronutrients and nutritional elements which leads us to focus our study on this herb [172]. Coriander is very low in saturated fat however,it contains good amount of linoleic acid which is a good source of α-tocopherol and vitamin K. Leaves of plant are rich source of vitamins while seeds are rich in polyphenols and essential oils. Coriander taste is devoted to its essential oil comprising a significant content of linoleic and furanocoumarins (coriandrine and dihydrocoriandrine). Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activity along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits and its protective effect to preserve the food for longer period [172].

Coriander contains high amount of essential oils that are very important for growth and for proper functioning of brain. The main essential fatty acids present in coriander include linoleic and linolenic acids. Linoleic acids belong to PUFA (polyunsaturated fatty acid) group. Dietary supplementation of coriander seed greatly affects the lipid composition of carcass by decreasing saturated fatty acid (SFA) contents (palmitic and stearic acids) and by increasing monounsaturated and polyunsaturated fatty acid (MUFA and PUFA) [173]. This plant is a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) isolated from the seeds and the aerial parts [174]. The high content of fats and protein in the fruits make distillation residues suitable for animal feed. Coriander fruits yield 5 to 7% of ash, 13% resin, astringent principle, malic acid and alkaloids. Coriander oil contains coriandrol, jireniol and vebriniol [175].

1.8.4 *Linum usitatissimum*

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* Figure 1.8 was given by Linnaeus in his book "Species Plantarum" [176]. It is an annual herbaceous plant with shallow root system.

The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are specialized development of this species [177].

The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber purpose are tall growing with straight culms and have fewer secondary branches.

Linum usitatissimum

The Mediterranean and Southwest Asia have both been proposed as the center of origin [203]; however the exact location is uncertain [178]. The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the stearoyl-ACP desaturase II

(*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop [179].

Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [180] making it ideal for paints, varnishes, and inks due to its fast polymerization properties.

Increasing demand for edible oil sources with significant percentages of omega-3 fatty acids is resulting in consumption of flaxseed as a functional food. Flaxseed is also added to animal feed to improve animal reproductive performance and health [181, 182].

Research objectives

1. Extraction of flavonoids and oils from some plants
2. GC-MS studies on the extracted oils.
3. Elucidation of structures of flavonoids.
4. Biological activity of isolates.

MATERIAL AND METHOD

2.1. Materials

2.1.1 Plant material

Stems of *pulcaria crispa* were collected from White Nile state (Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum – Sudan. The seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were collected from Jeddah-Saudi Arabia.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m length; 0.25mm diameter; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.1.3-Test organisms

Test organisms used in the antimicrobial assay are: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

2.2- Methods

2.2.1- Preparations of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminum chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. distilled water.

- **Potassium iodide solution:** 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

100 g) of powdered air- dried stems of *pulcaria crispa* were extracted with 95% aqueous ethanol. The solvent was removed under reduced pressure giving the prepared extract. This prepared extract (PE) was used for phytochemical screening.

2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) were evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5

ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.2.2- Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

2.2.2.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Mayer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.2.4- Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and

the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.2.5 -Test for saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml)of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand for one hour and then checked for foam formation.

2.2.2.6 - Test for coumarins

(0.2 g) of the prepared extract were dissolved in 10 ml distilled water in test tube and a filter paper was attached to the test tube to be saturated with the vapor after being spotted o with 0.5N KOH. Then the filter paper was inspected under UV light. The presence of coumarins is indicated by the ability of the spot to absorb the UV light.

2.2.2.7- Test for anthraquinone glycosides

(0.2 g) of the prepared extract was boiled with 10 ml of 0.5 N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. (5ml) of the benzene solution was shacked with (3ml) of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraxquinones is indicated by pink or red color in the alkaline layer.

2.2.3- Extraction of flavonoids

The air - dried powdered stem of a *pulcria crispa* (3 kg) were extracted by maceration with ethanol (95%). The extract was concentrated under reduced pressure to give a crude product.

2.2.4- Column chromatography

Open column (70× 4 cm) was used for fractionation of the ethanolic extracts of *pulcaria crispa*. Silica gel with particle size 60-120 mesh ,from LOBA chemicals, was used as stationary phase. The composition of the mobile phase (ethyl acetate: methanol) was determined by TLC experiments.

The ethanolic extract of *pulcaria crispa* (5 g) was mixed with 10 g of silica gel and then applied on the top of the column. Elution commenced by EthOAC - MeOH ;(3:7, fraction I), (1:1, fraction II) and (0:1, fraction III).

2.2.5- paper chromatography

Fraction II – being rich in phenolics- was subjected to paper chromatography where pure flavonoids - compounds I and II -were isolated using the solvent system: butanol: acetic acid: water (4: 1: 12, upper layer).

2.2.6-Extraction of oils

Powdered seeds of *Portulaca oleracea* (*Coriandrum sativam* and *Linum usitatissimum*) - (500g) for each - were macerated with n-hexane . The solvent was removed under reduced pressure to yield the oil.

2.2.7-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added,then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes. The hexane layer was then separated.(5µl) of the hexane extract

were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.8- GC-MS analysis

The oils from *Portulaca oleracea, Coriandrum sativam* and *Linum usitatissimum* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 µm, thickness) was used. Helium (purity; 99.99 %) was used as carrier gas.

2.2.9- Biological activity

2.2.9.1- Preparations of chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa*

Chloroform fraction of *pulcaria crispa* was prepared by suspending the ethanolic extract of the leaves in the least amount of distilled water and then shaking with chloroform in a seperatory funnel. Evaporation of the chloroform layer under reduced pressure gave the required fraction. Other fractions were prepared in a similar way.

2.2.9.2- Antimicrobial assay

The ethanolic, chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa* as well as the essential oils extracted from the seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were evaluated for their antimicrobial activity against five standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*) using the cup plate agar method with some minor modifications.

2.2.9.2.1- Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 108- 104 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o}C for 24 hours.

2.2.9.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.9.3- Testing for antibacterial activity

The cup plate agar diffusion method was adopted with, some minor modification, to assess the antibacterial activity (2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a single test sample.

The agar discs were removed and cups were filled with 0.1 ml of each test sample using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^{o} C for one hours.

The above procedure was repeated for different concentrations of extracts and standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.9.4- Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but this time Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above

2.2.9.5-Antioxidant activity

The DPPH radical scavenging capacity was determined. In 96-wells plate, the test samples were allowed to react with 2.2 Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37° C. the concentration of DPPH was kept as (300μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests were run in triplicate. Propyl gallate was used as a positive standard at concentrations 0.5 mM.

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RESULT AND DISSCUSSION

Powdered air-dried stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature for 48hr. The crude product obtained after evaporation of the solvent under reduced pressure. The crude extract was fractionating by separator funnel and the three fractions were collected (ethyl acetate, chloroform and butanol fractions) and screened for its antimicrobial activity. The crude was subjected to silica gel column chromatography and eluted with ethyl acetate - methanol mixture (3: 7, fraction I), (1: 1, fraction II) and pure methanol (fraction III). Three fractions were collected. The fraction II was subjected to paper chromatography where pure components: **compound I** and **II** have been isolated. The structures of isolated flavonoids have been elucidated via a combination of spectra techniques.

GC-MS analysis on seed oil of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and the identification of the constituents. The oils was screened for antimicrobial and antioxidant activity against standard organisms.

3.1- *Pulcaria crispa*

3.1.1- Phytochemical screening

Pulcaria crispa was screened for major secondary metabolites and the results are displayed in table 3.1.

Table 3.1: Phytochemical screening of *pulcaria crispa*

Test	**Result**
Saponin	++
Cumarin	+
Alkaloids	+
Flavonoids	+
Tanin	+++
Steroids	-
Triterpens	-
Anthraquinone	-

+: Trace

++: Moderate

++: High

- : Negative

3.1.2- Antimicrobial assay

The crude extract, ethyl acetate, chloroform and butanol fractions have been screened for antimicrobial activity against five human pathogens. The results are depicted in table 3.2 Tables 3.3 and 3.4 display the antimicrobial activity of standard drugs.

Table 3.2: Antimicrobial activity of crude and three fractions

	Ec	Ps	Sa	Bs	Ca
Crude extract	_	_	_	_	10
Ethyle acetate fraction	12	10	13	_	12
Choroform fraction	_	_	_	_	11
Butanol fraction	_	_	10	_	10

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

The results were interpreted in a commonly used term :< 9 mm: in active; 9-12 mm: partially active; 13-18 mm: active; >18 very active.

The crude extract showed partial antifungal activity against *Candida albicans* (see table 3.2 and the appendix).

Ethyl acetate fraction showed activity against *Staphylococcus aureus.* It also exhibited partial Antibacterial activity against *Escherichia coli,*

Pseudomonas aeroginosa and *Candida albicans*.The choroform fraction showed partial anticandidal activity.

Butanol fraction showed partial Antibacterial activity against *Staphylococcus aureus* and *Candida albicans*.

Table 3.3: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.4: Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

3.1.3- Antioxidant assay

The antioxidant capacity of crude extract has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test extract against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.5) crude extract exhibited significant anti- oxidant activity.

Table 3.5: Radical scavenging activity of crude extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Crude extract	82.00%

3.1.4-Characterization of compound I

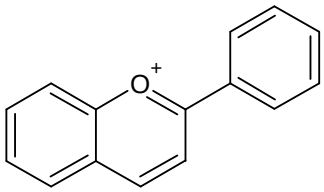
Compound I was isolated from *Pulcaria crispa* stems as yellow powder.

Table 3.6: IR data of compound I

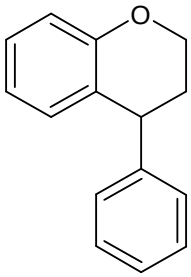
Functional group	**Wave number /cm^{-1}**
(C-H, Ar)	630.51
(C-O, ether)	1273.70
(C=C, Ar)	1378.30
(C=O)	1727.92
(OH)	3368.88
(C-H) Alkane	2921.62

The IR spectrum of compound I **(**Fig.3.1) showed all the anticipated bands of flavonids. Since

the infrared showed carbonyl absorption then the possibility that compound I being an anthocyanin or catechin is dismissed since these classes are characterized by absence of a carbonyl function.



Anthocyanin



Catechin

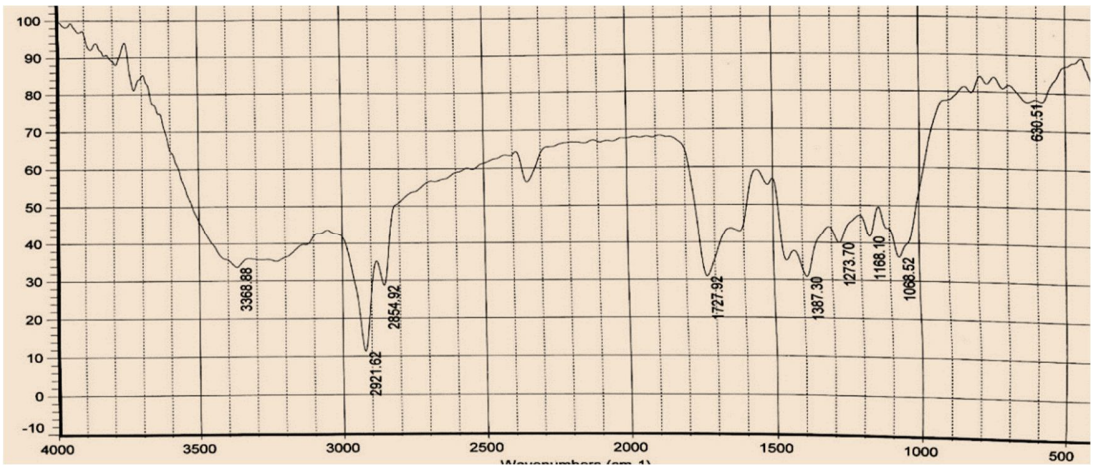
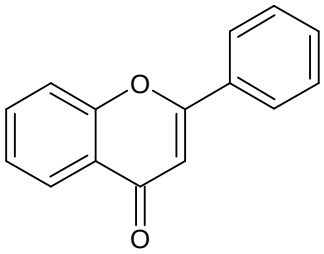
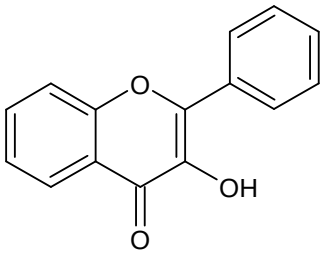


Fig 3.1: IR spectrum of compound I

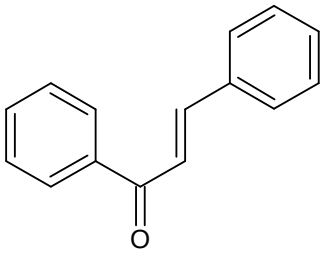
The UV spectrum of compound I revealed two major peaks at 262 and 345 nm (Fig.3.2). The presence of two peaks is a characteristic feature of flavones, flavonols, chalcones, and aurones.



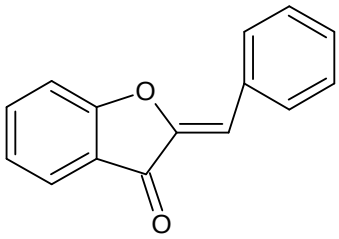
Flavone



Flavonol



Chalcone



Aurone

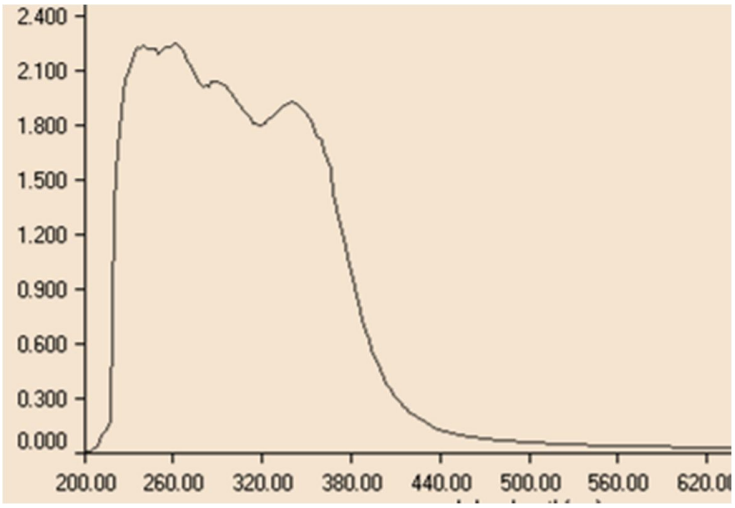
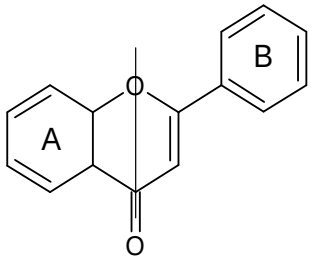


Fig 3.2: UV spectrum of compound I

The UV spectra of most flavonoids consist of two major absorption maxima, one of which occur in the range 220 – 290 nm (band II) and other in the range 300 – 400 nm (band I). The appearance of both band I and II demonstrates conjugation between the benzoyl and cinnamoyl systems.



Benzoyl Cinnamoyl

Band I is considerable to be associated with the absorption of cinnamoyl system, while band II originates from the benzoyl system.

Compound I showed both band II and I (fig.3.2) at 262 and 345nm respectively. Such absorption indicates that this compound is either a flavone, flavonol, chalcone or aurone. But aurones and chalcones absorb in

the range 365-430nm (band I). Flavones absorb in the range 300-350 nm, while flavonols absorb in the range 356-385. Thus compound I is a flavone.
Considerable structural feature have also been obtained using UV shift reagents such as sodium methoxide, sodium acetate, aluminum trichloride. These reagents produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl groups in the flavonoid molecule.
Use has been made of the effect of sodium methoxide on the UV spectra of flavonoids for detection of free 3 – and/or 4` –hydroxyl groups.
On addition of NaOMe to a methanolic solution of compound I a 42 nm bathochromic shift in band I without decrease in intensity (fig.3.3) was observed indicating the presence of a 4`-OH function.

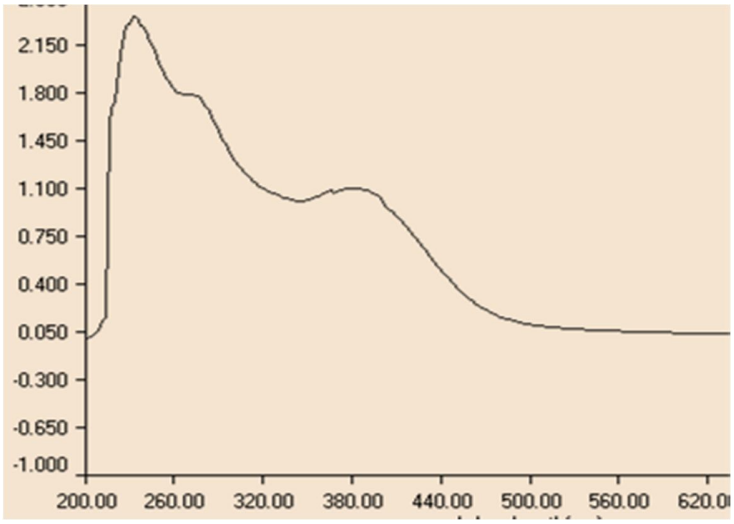


Fig 3.3: The UV spectrum of compound I in sodium methoxide

Sodium acetate is particularly useful diagnostic reagent for the specific detection of 7 –hydroxy group. When a methanolic solution of compound I **was** treated with excess powdered sodium acetate, and 16nm bathochromic shift in band II (fig.3.4) was observed suggesting a 7 –OH group.

When the previous solution of compound I was treated with excess powdered boric acid, no bathochromic shift was observed (fig.3.5) this indicate absence of *ortho*- dihydroxyl groups.

The shift reagent aluminum trichloride is diagnostic of 3 –hydroxyl group, 5 –hydroxyl group and catechol moieties[216].When a methanolic solution of compound I was treated with a few drops of the shift reagent $AlCl_3$ no bathochromic shift (fig.3.6) was observed and this indicates absence of 3 –OH, 5 –OH and catechol moieties.

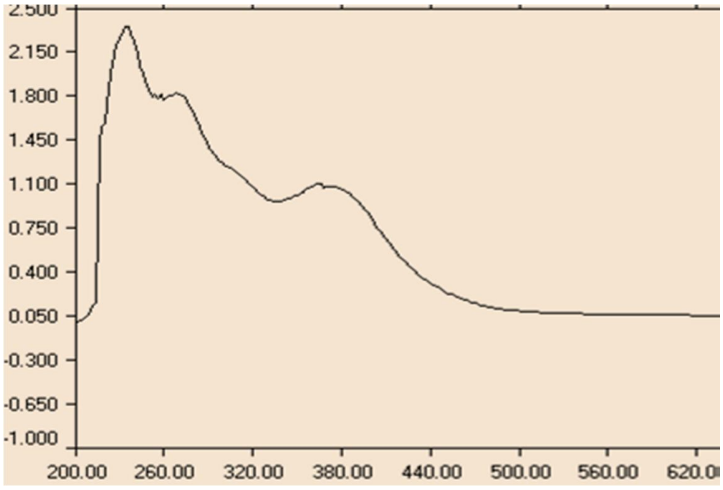


Fig 3.4: UV spectrum of compound I in sodium acetate

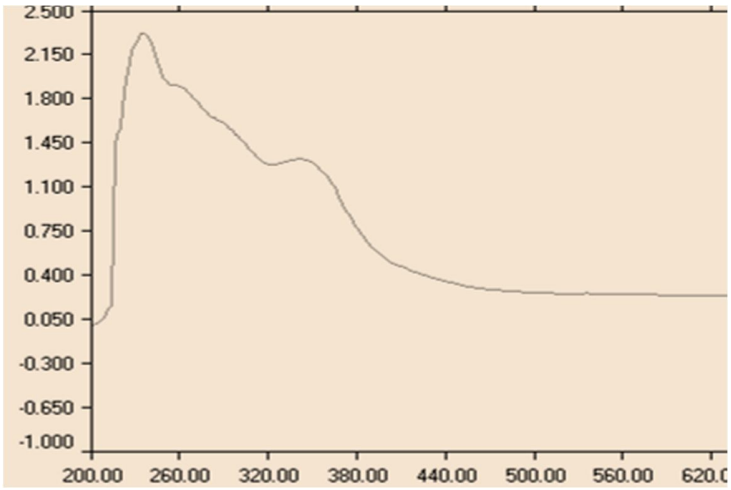


Fig 3.5: UV spectrum of compound I in sodium acetate/boric acid

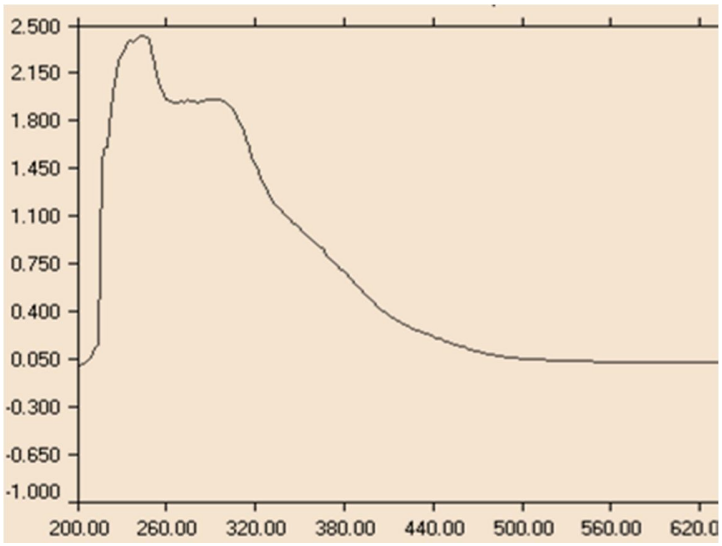


Fig 3.6: UV spectrum of compound I in aluminum trichloride

The ^{1}HNMR spectrum (Fig.3.7) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.1 ppm accounts for an acetyl function. The multiplet at δ 4.23-5.45 ppm is due to a sugar moiety (not identified in this study). The aromatic protons appeared as a multiplet (δ 6.68-7.65 ppm).

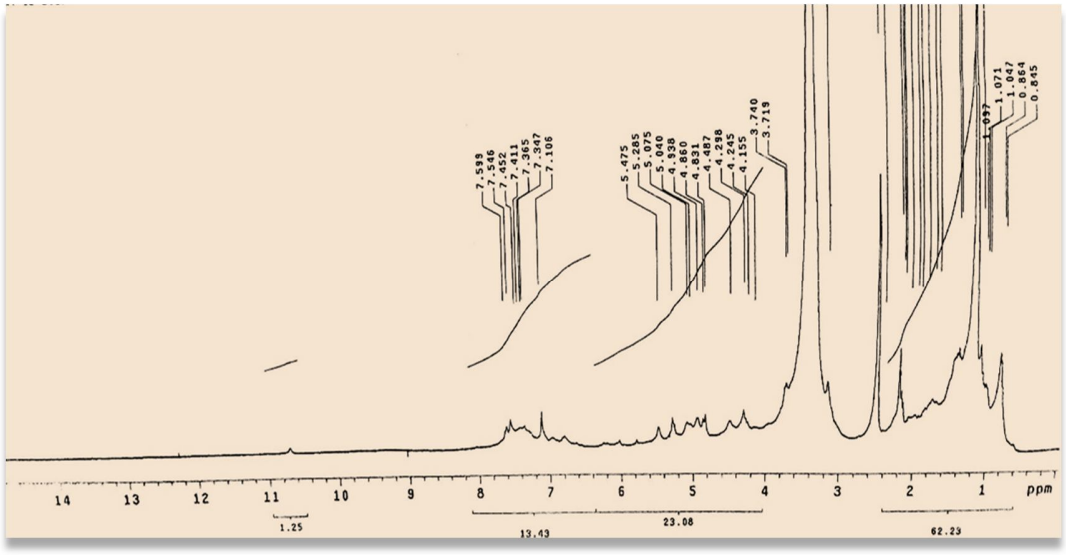
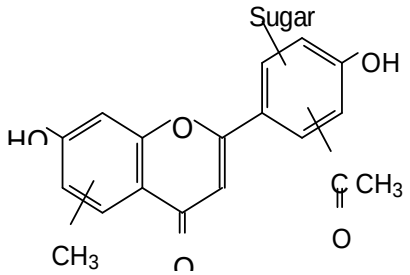


Fig 3.7: ^{1}HNMR spectrum of compound I

On the basis of the above spectral data, the following partial structure was proposed for compound I:



3.1.5- Characterization of compound II

Compound II was isolated as white-yellowish powder from *Pulcaria crispa* stem. The IR data of compound II is presented in the following table (see also Fig.3.8).

Table 3.7: IR data of compound II

Functional group	N(cm^{-1})
(C-H, Ar)	2085.7
(C-O, ether)	1017.15
(C=C, Ar)	1386.55
(C=O)	1643.49
(OH)	3419.34
(C-H) Alkane	2832.88

Compound II – which showed a carbonyl absorption-cannot be an anthocyanin or a catechin since IR spectrum gave carbonyl stretching at 1643.49 cm^{-1}. It could belong to one of the other classes of flavonoids. Anthocyanin and catechins are devoid of carbonyl.

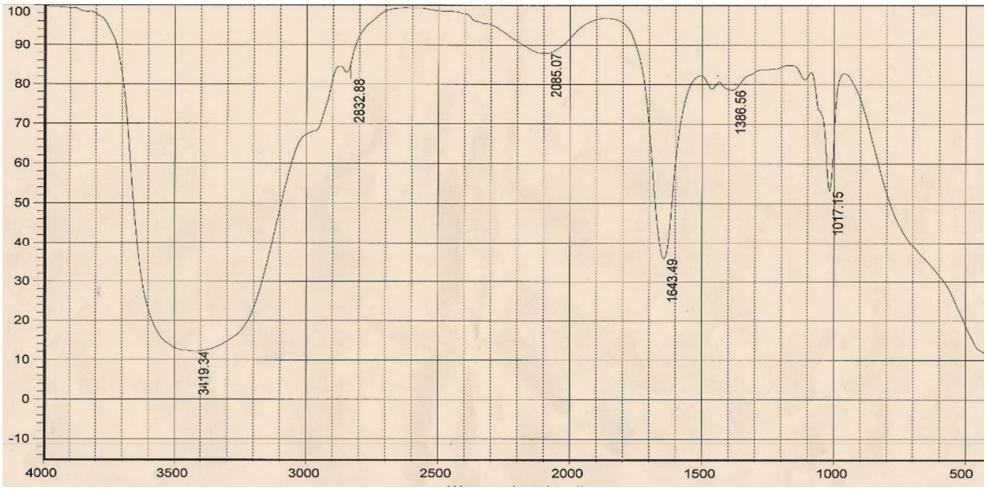
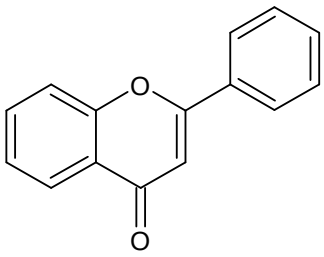


Fig 3.8: IR spectrum of compound II

Table 3.8: UV spectral data of compound II

λ_{max} (nm)	
Band I	Band II
330	240

The UV spectrum of compound II is depicted in (fig.3.9). The spectrum revealed two major peaks at 240 and 330 nm. Such absorption is a characteristic feature of flavones.



Flavone

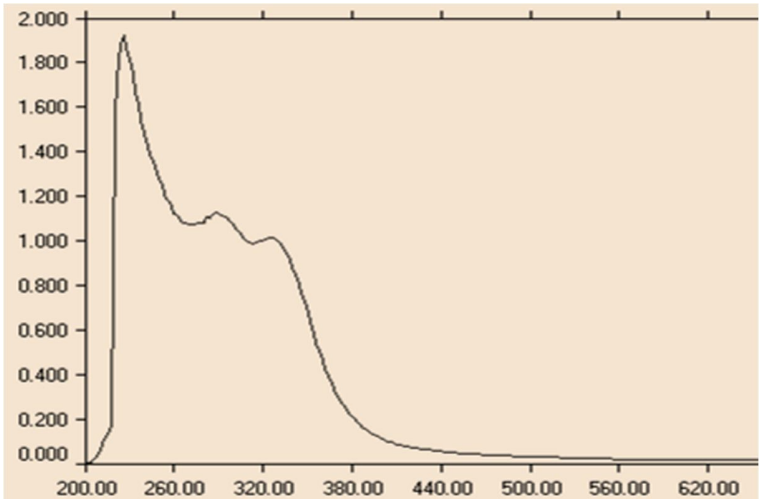


Fig 3.9: UV spectrum of compound II

Considerable structural feature have also been obtained using UV shift reagents: sodium methoxide, sodium acetate, aluminum trichloride.

On addition of NaOMe to a methanolic solution of compound II a 45nm bathochromic shift in band I indicative the presence of a 4`-OH function was observed (Fig.3.10).

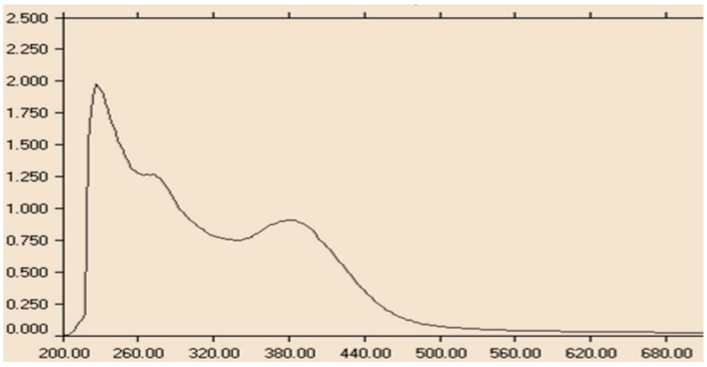


Fig 3.10: The UV spectrum of compound II in sodium methoxide

When a methanolic solution of compound II was treated with excess powdered of the shift reagent NaOAC, a 30 bathochromic shift in band II (fig.3.11) was observed and this indicates presence of a 7 –OH group.

When the previous solution of compound II was treated with excess powdered of boric acid, no bathochromic shift in both band II and I was observed (fig.3.12) this indicate absence of *ortho*- dihydroxyl groups.

The aluminium chloride spectrum showed no bathochromic shift (fig.3.13) indicating absence of 3 –OH, 5 –OH and catechol moieties.

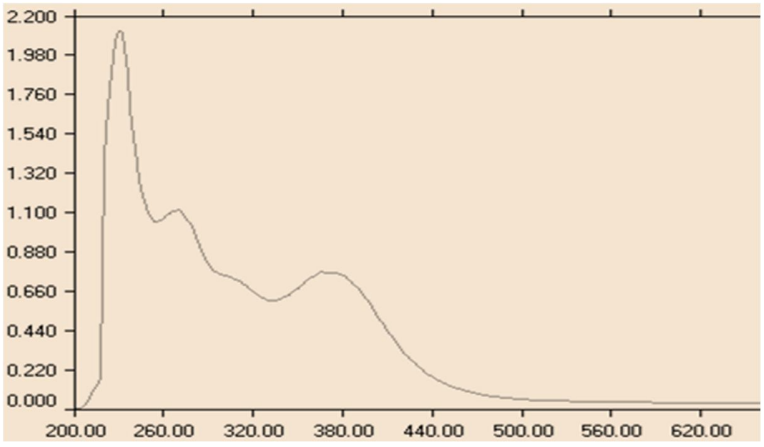


Fig 3.11: UV spectrum of compound II in sodium acetate

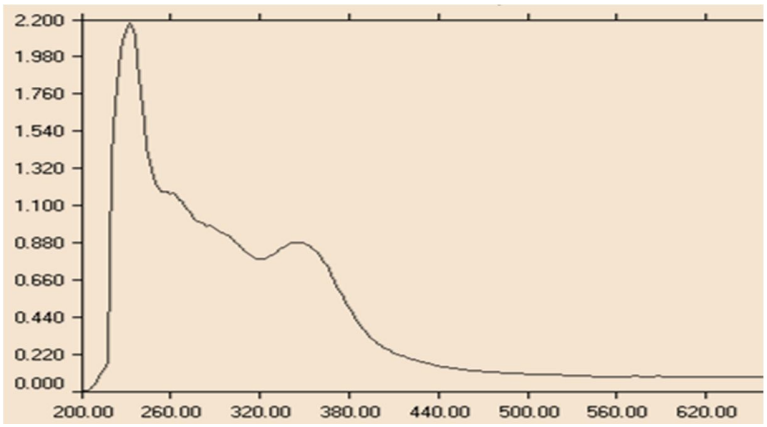


Fig 3.12: UV spectrum of compound II in sodium acetate/boric acid

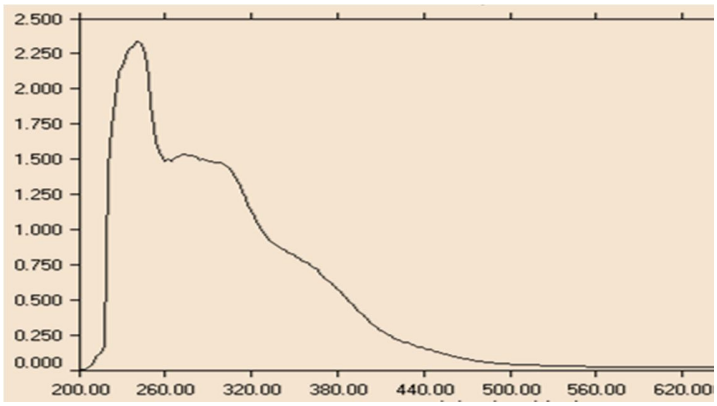


Fig 3.13: Aluminum trichloride spectrum of compound II

The ^{1}HNMR spectrum (Fig.3.14) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.4 ppm accounts for an acetyl function. The aromatic protons appeared as a multiplet (δ 6.68-7.59 ppm).

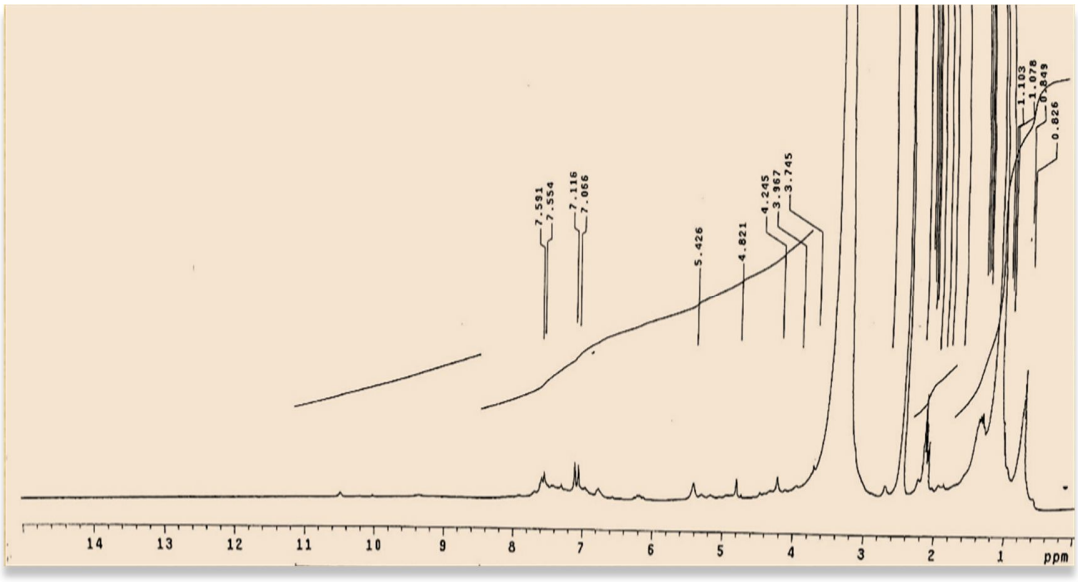
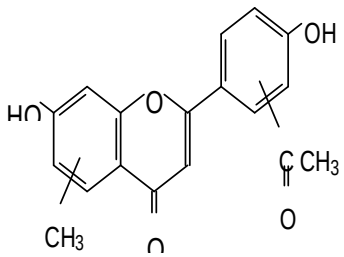


Fig 3.14: [1]HNMR spectrum of compound II

On the basis of the above spectral data, the following partial structure was proposed for compound II:



Compound II

3.2- *Portulaca oleracea*

3.2.1- GC-MS analysis of *Portulaca oleracea* oil

GC-MS analysis of *Portulaca oleracea* oil was conducted and the identification of the constituents was done by comparison of retentions times and MS data with the GC-MS library. Structures of constituents of the oil were further confirmed by interpreting the observed fragmentation pattern

.Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.2- constituents of oil

The GC-MS analysis of the studied oil revealed the presence of 15 components (Table: 3.9).The typical total ion chromatograms (TIC) of n-hexane extract are shown in (fig.3.15).

Table: 3.9: Constituents of *Portulaca oleracea* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.316	237128	0.08
2.	Pentadecanoic acid, methyl ester	14.373	170885	0.05
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.152	161664	0.05
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.195	705542	0.23
5.	Hexadecanoic acid, methyl ester	15.404	75687986	24.19
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	251818	0.08
7.	Heptadecanoic acid, methyl ester	16.367	775909	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.068	116899705	37.37
9.	9-Octadecenoic acid (Z)-, methyl ester	17.130	23526885	7.52
10.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.153	59248358	18.94
11.	Methyl stearate	17.307	22933709	7.33
12.	cis-11-Eicosenoic acid, methyl ester	18.883	1307281	0.42
13.	Eicosanoic acid, methyl ester	19.084	6202098	1.98
14.	Docosanoic acid, methyl ester	20.716	3045550	0.97
15.	Tetracosanoic acid, methyl ester	22.226	1688258	0.54

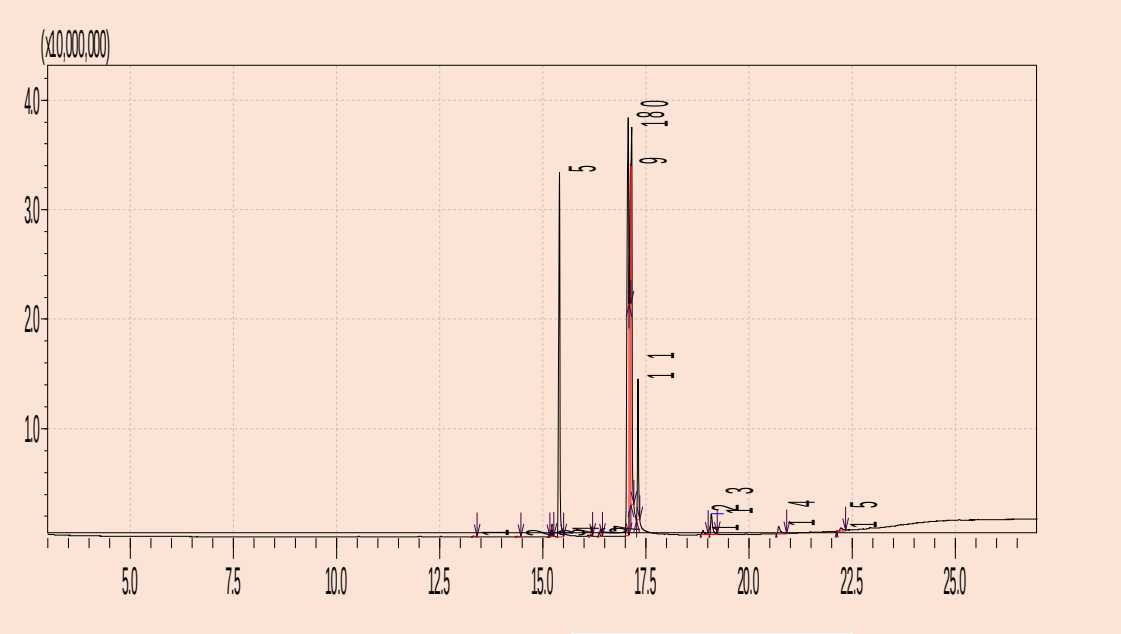


Fig 3.15: Chromatograms of *Portulaca oleracea* seed oil

The following esters were the major components:

9, 12-Octadecadienoic acid, methyl ester (37.37%)

The EI mass spectrum of 9,12-octadecadienoic acid, methyl ester shown in (fig. 3.16).The peak at m/z 294, which appeared at R.T. 17.068 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z 263 corresponds to loss of a methoxyl function.

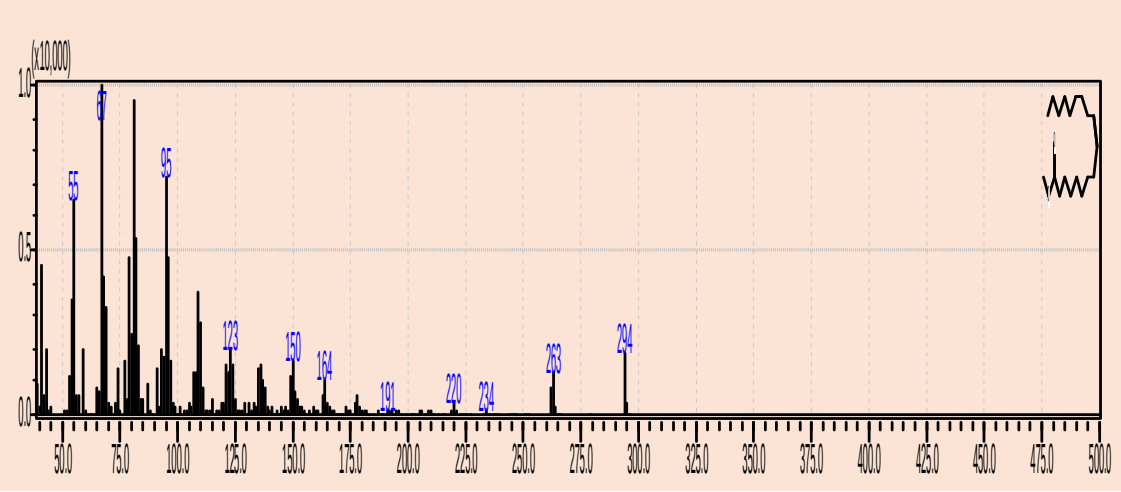


Fig 3.16: Mass spectrum of 9, 12-Octadecadienoic acid, methyl ester

Hexadecanoic acid, methyl ester (24.19%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.17).The peak at m/z 270 (at R.T. 15.404) corresponds $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is due to loss of a methoxyl .

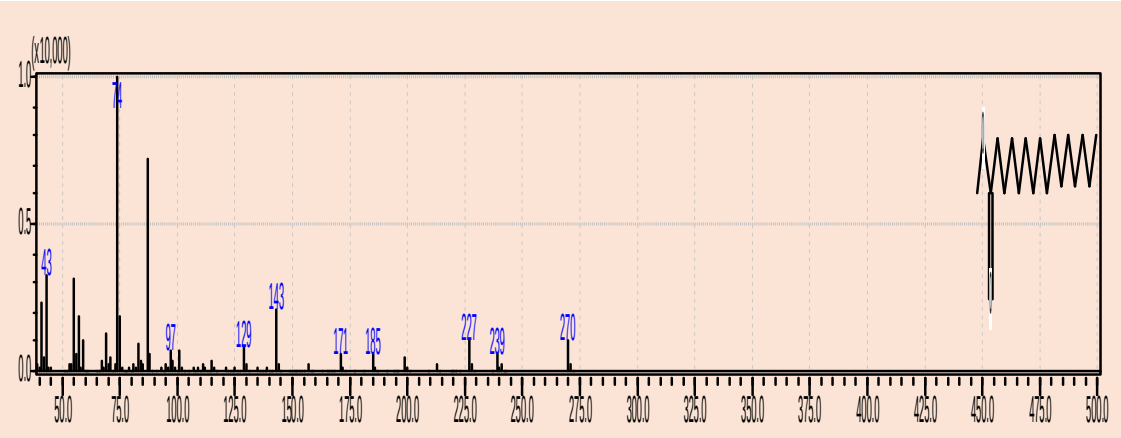


Fig 3.17: Mass spectrum of hexadecanoic acid, methyl ester

9,12,15-Octadecatrienoic acid, methyl ester (18.94%)

Fig. 3.18 demonstrates the mass spectrum of 9, 12, 15-octadecanoic acid, methyl ester. The signal at m/z 292(R.T. 17.153 in total ion chromatogram) corresponds to the molecular ion: $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z261 accounts for loss of a methoxyl .

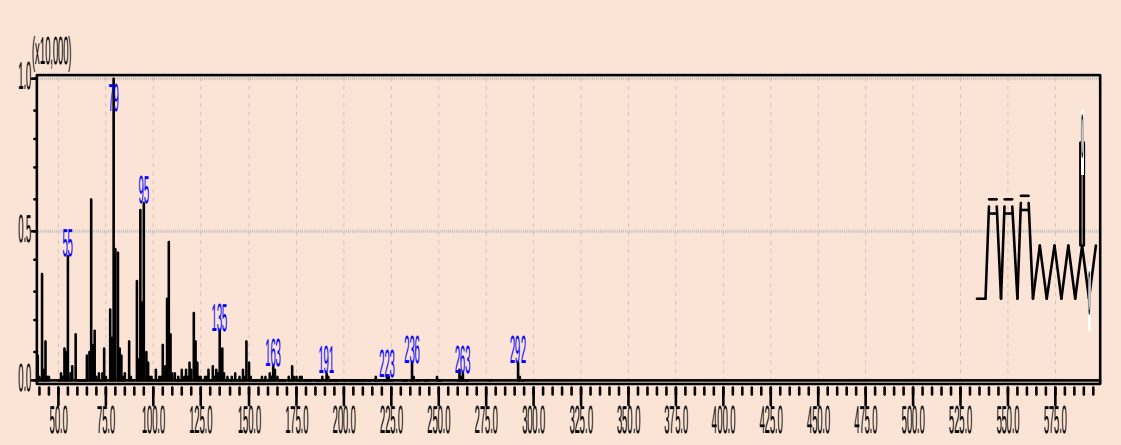


Fig 3.18: Mass spectrum of 9,12,15-Octadecatrienoic acid, methyl ester

3.2.3- Antimicrobial activity

The oil was screened for antimicrobial activity against five standard organisms. The oil not showed any activity against all test organisms

3.2.4- Antioxidant activity

The antioxidant capacity of *Portulaca oleracea* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.10) *Portulaca oleracea* seed oil exhibited weak antioxidant activity.

Table 3.10: Radical scavenging activity of *Portulaca oleracea* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Portulaca oleracea oil	29.00%

3.3- *Coriandrum sativam*

3.3.1- GC-MS analysis of *Coriandrum sativam* oil

GC-MS analysis of *Coriandrum sativam* was carried out . The GC-MS analysis revealed the presence of 14 components (table: 3.11).The typical total ion chromatograms (TIC) of n-hexane extract is presented in (fig.3.19).

Table: 3.11: Constituents of *Coriandrum sativam* oil

ID#	Name	Ret. Time	Area	Area%
1.	Methyl tetradecanoate	13.312	215647	0.03
2.	Pentadecanoic acid, methyl ester	14.372	231442	0.04
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.169	1910240	0.29
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.192	3233931	0.50
5.	Hexadecanoic acid, methyl ester	15.392	41125489	6.32
6.	Heptadecanoic acid, methyl ester	16.367	287117	0.04
7.	7,10-Octadecadienoic acid, methyl ester	16.913	1741544	0.27
8.	6,9-Octadecadienoic acid, methyl ester	16.962	543578	0.08
9.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.062	100831481	15.50
10.	9-Octadecenoic acid (Z)-, methyl ester	17.189	485955106	74.69
11.	Methyl stearate	17.313	9449169	1.45
12.	cis-11-Eicosenoic acid, methyl ester	18.865	3707972	0.57
13.	Eicosanoic acid, methyl ester	19.084	1068295	0.16
14.	Docosanoic acid, methyl ester	20.719	362910	0.06

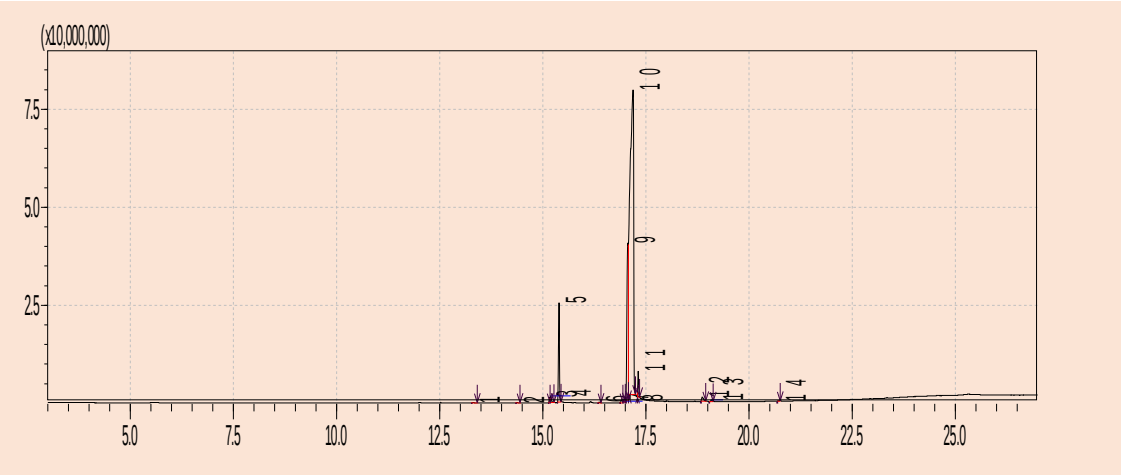


Fig 3.19: Chromatograms of *Coriandrum sativam* seed oil

The following esters were detected as major components:

9-Octadecenoic acid, methyl ester (74.69%)

The EI mass spectrum of 9-octadecenoic acid, methyl ester is shown in (fig. 3.20).The signal which appeared at m/z 264, with R.T. 17.198 corresponds $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z 235 is due to loss of a methoxyl group.

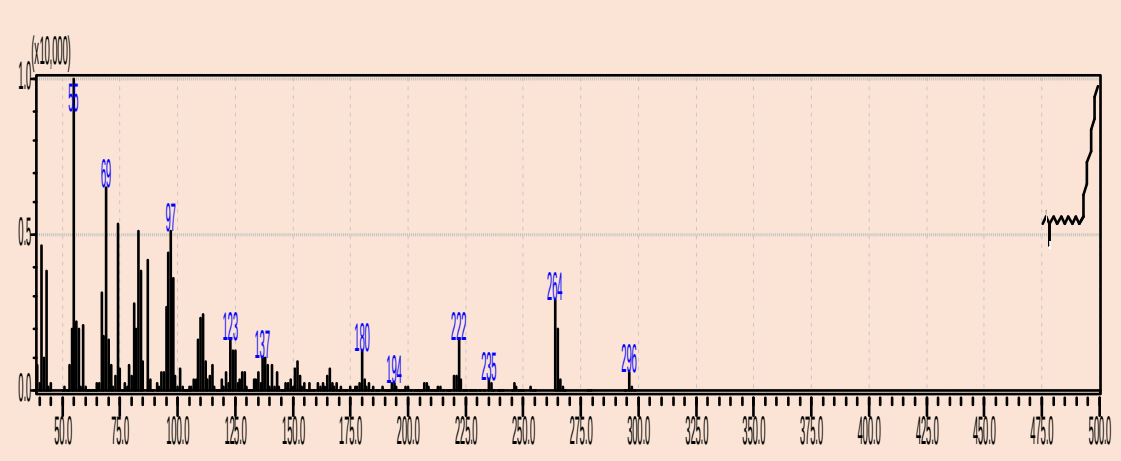


Fig 3.20: Mass spectrum of 9-Octadecenoic acid, methyl ester

9, 12-Octadecadienoic acid, methyl ester (15.50%)

Fig. 3.21 presents the mass spectrum of 9, 12-octadecadienoic acid, methyl ester .The peak at m/z 294, with R.T. 17.062 in total ion chromatogram, corresponds the molecular ion : $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 accounts for loss of a methoxyl function.

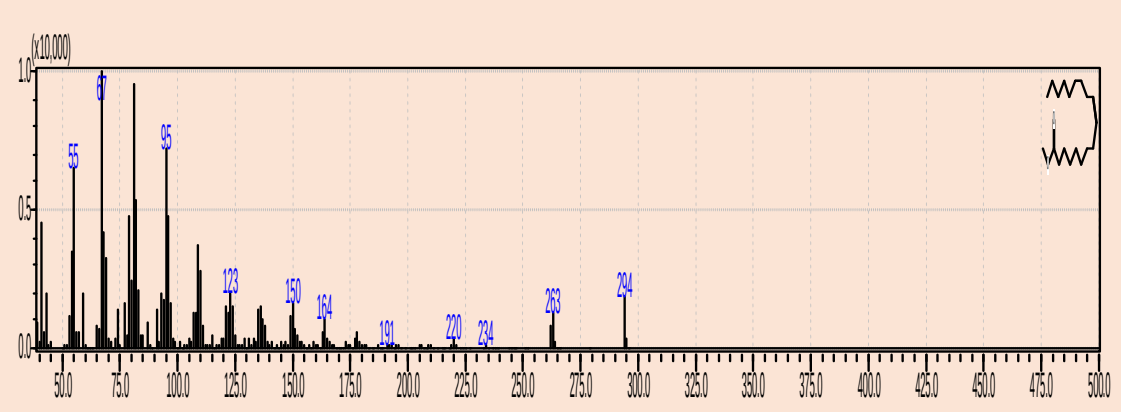


Fig 3.21: Mass spectrum 9, 12-Octadecadienoic acid, methyl ester

3.3.3- Antimicrobial activity

The studied oil was evaluated for antimicrobial activity against five standard human pathogens. The oil not showed any activity against all test organisms

3.3.4- Antioxidant activity

The antioxidant capacity of *Coriandrum sativam* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.12) *Coriandrum sativam* seed oil exhibited moderate antioxidant activity.

Table 3.12: Radical scavenging activity of *Coriandrum sativam* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Coriandrum sativam oil	52.00%

3.4- *Linum usitatissimum*

3.4.1- GC-MS analysis of *Linum usitatissimum* oil

GC-MS analysis of *Linum usitatissimum* was conducted. The analysis revealed the presence of 15 components (table: 3.13).The typical total ion chromatograms (TIC) of n-hexane extract is shown in (fig.3.22)

Table: 3.13: Constituents of *Linum usitatissimum* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.317	189936	0.07
2.	Pentadecanoic acid, methyl ester	14.373	70754	0.03
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.155	104077	0.04
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.196	476941	0.19
5.	Hexadecanoic acid, methyl ester	15.392	35779007	14.12
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	270452	0.11
7.	Heptadecanoic acid, methyl ester	16.365	508829	0.20
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.053	64486368	25.44
9.	9-Octadecenoic acid (Z)-, hexadecyl ester	17.155	69889815	27.58
10	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.164	50002070	19.73
11	Methyl stearate	17.307	28380442	11.20
12	cis-11-Eicosenoic acid, methyl ester	18.887	1343975	0.53
13	Eicosanoic acid, methyl ester	19.092	684609	0.27
14	Docosanoic acid, methyl ester	20.722	818972	0.32
15	Tetracosanoic acid, methyl ester	22.233	436726	0.17

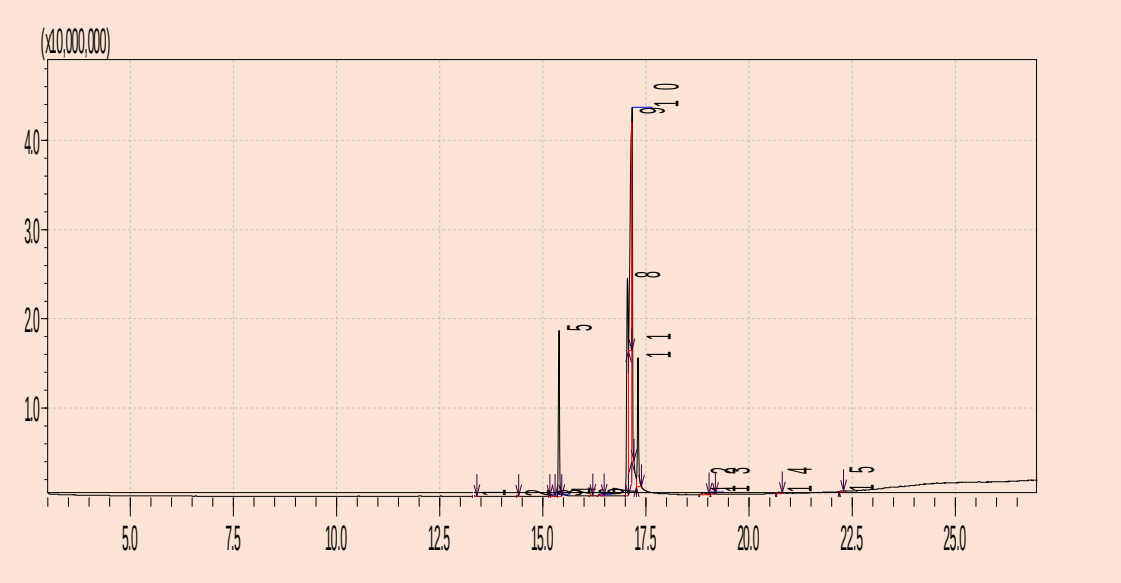


Fig 3.22: Chromatograms of *Linum usitatissimum* seed oil

The following esters were found as major components:

9-Octadecenoic acid, hexadecyl ester (27.58%)

The EI mass spectrum of 9-Octadecenoic acid, hexadecyl ester is shown in (fig. 3.23).The peak at m/z 295, which appeared at R.T. 17.155 in total ion chromatogram, corresponds to $M^{+}[C_{34}H_{66}O_2]^{+}$ .The peak at m/z 264 corresponds to loss of a methoxyl function.

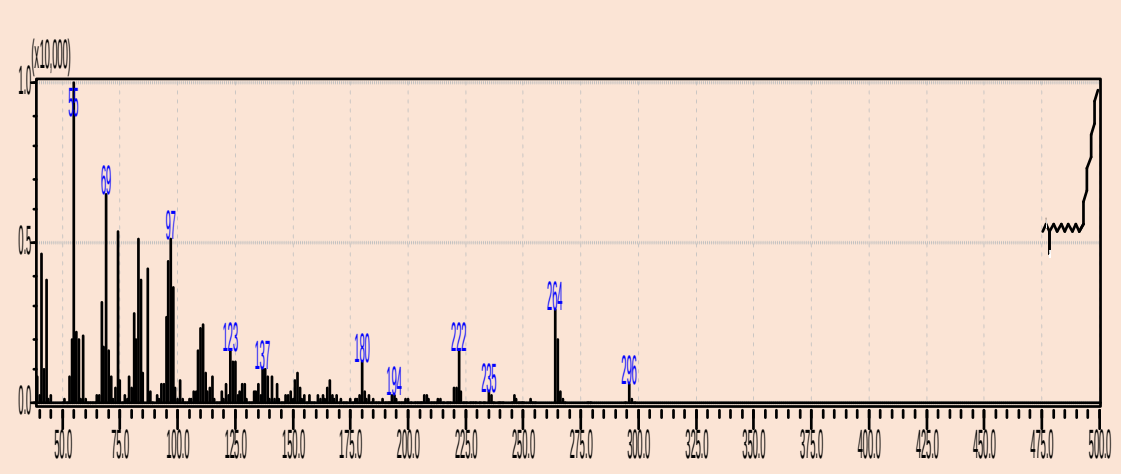


Fig 3.23: Mass spectrum of 9-Octadecenoic acid, hexadecyl ester

9, 12-Octadecadienoic acid, methyl ester (25.44%)

The EI mass spectrum of 9, 12-octadecadienoic acid, methyl ester is shown in (fig.3.24). The peak at m/z 294, which appeared at R.T. 17.053 corresponds $M^{+}[C_{19}H_{34}O_2]^{+}$ while the sighal at m/z 263 is due to loss of a methoxyl group.

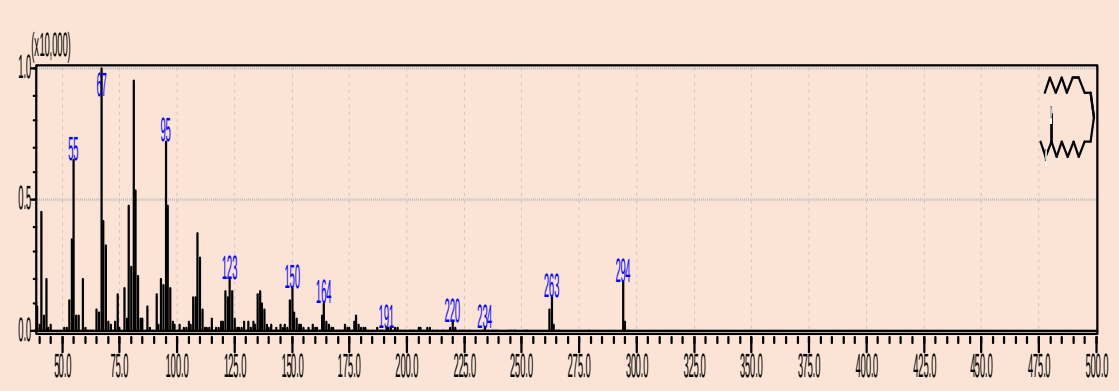


Fig 3.24: Mass spectrum of 9, 12- Octadecadienoic acid, methyl ester

9, 12, 15-Octadecatrienoic acid, methyl ester (19.73%)

Figure 3.25 shows the mass spectrum of 9, 12, 15-octadecatrienoic acid, methyl ester. The peak at m/z 292 - R.T. 17.164- in total ion chromatogram, corresponds the molecular ion : $M^{+}[C_{19}H_{32}O_2]^{+}$,while the sighal at m/z 261 accouts for loss of a methoxyl .

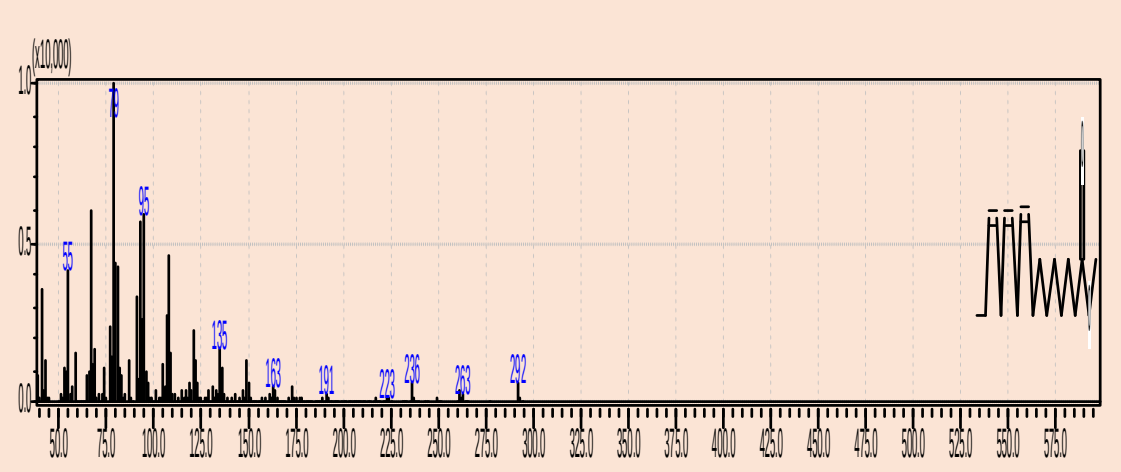


Fig 3.25: Mass spectrum of 9, 12, 15-Octadecatrienoic acid, methyl ester

Hexadecanoic acid, methyl ester (14.12%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.26). The moleculat ion : $M^{+}[C_{17}H_{34}O_2]^{+}$ appeared as expected at m/z 270(R.T. 15.392).The peak at m/z 239 is attributed to loss of a methoxyl .

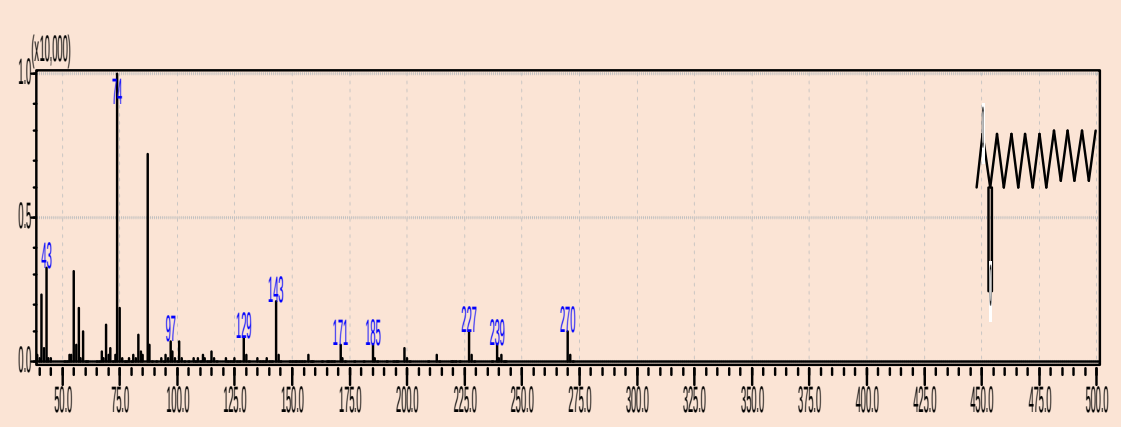


Fig 3.26: Mass spectrum of Hexadecanoic acid, methyl ester

Methyl stearate (11.20%)

The mass spectrum of methyl stearate is presented in (fig.3.27). The signal which appeared at m/z 298 -with R.T. 17.307 in total ion chromatogram - corresponds $M^{+}[C_{19}H_{38}O_2]^{+}$ .The peak at m/z 267 is attributed to loss of a methoxyl .

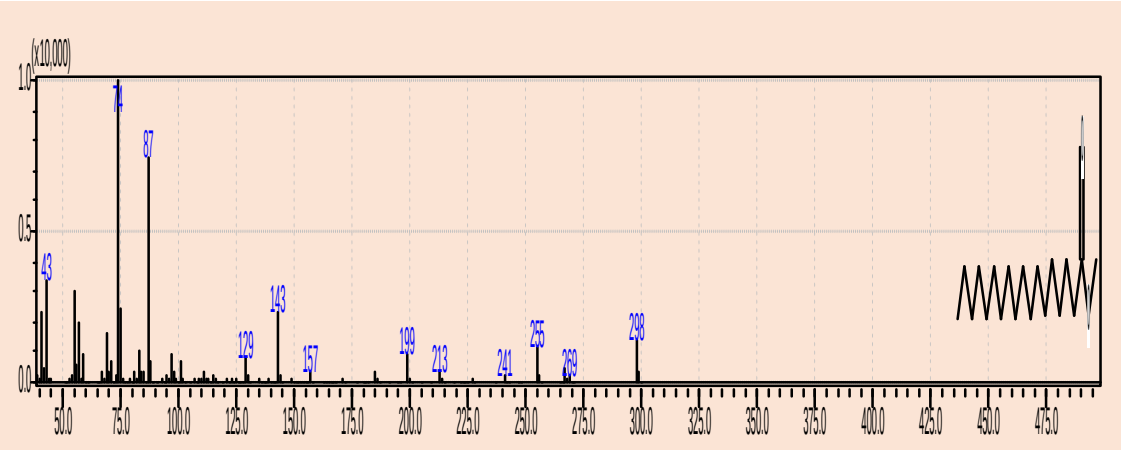


Fig 3.27: Mass spectrum of methyl stearate

3.4.2- Antioxidant activity

The antioxidant capacity of *Linum usitatissimum* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.14) *Linum usitatissimum* seed oil exhibited moderate antioxidant activity.

Table 3.14: Radical scavenging activity of *Linum usitatissimum* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Linum usitatissimum oil	48.00%

Conclusion

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

Two flavonoids (compounds I and II) were isolated from the stems of *Pulcaria crispa* .The partial structures of both compounds were deduced on the basis of their : IR, UV and NMR data and partial structures were suggested. Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried out . The results exhibited variable susceptibilities of microorganism for crude extract and different fractions. The antioxidant capacity of the alcoholic extract of *Pulcria crispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The oils were also assessed for their antimicrobial activity and promising results were obtained.

Recommendations

The following is highly recommended:

1. Other phytochemicals of the studied plant may be isolated and their structures may be elucidated and the biological activity could be screened.
2. The isolated falvonoids may be subjected to 2D NMR experiments for full characterization.
3. The isolated falvonoids and oil may be screened for other biological effects such as antiinflammatory, antidiabetic , antiviral, antilashmenial ..etc.

Sudan University of Science and Technology

College of Post Graduate Studies

Phytochemical Studies on the Stems of *Pulcria crispa* and Seeds of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* and Biological Activity of Constituents

دراسة فيتوكيميائية لسيقان نبات الربل وبذور نبات الرجلة والكسبرة والكتان والفعالية البيولوجية للمكونات

A Thesis Submitted in Fulfillment of the Requirements of the Ph.D. Degree in Chemistry

By

Lubna Akasha Abdelmalik Mohamed

(B.Sc. (Hons.) Chemical Laboratories, M.Sc. in Chemistry)

Supervisor

Prof: Mohamed Abdel Karim Mohamed

September, 2019

الإســـــــتـــهـــلال

﴿ بسم الله الرحمن الرحيم ﴾

فلينظر الإنسان إلى طعامه ﴿٢٤﴾ أنا
صببنا الماء صبا ﴿٢٥﴾ ثم شققنا
الأرض شقا ﴿٢٦﴾ فأنبتنا فيها حبا
﴿٢٧﴾ وعنبا وقضبا ﴿٢٨﴾ وزيتونا
ونخلا ﴿٢٩﴾ وحدائق غلبا ﴿٣٠﴾
وفاكهة وأبا ﴿٣١﴾ متاعا لكم
ولأنعامكم ﴿٣٢﴾ سورة عبس

Dedication

To,

My parents,

My sons,

And my sisters and brother

Acknowledgement

First and foremost, I thank **Almighty Allah, the Ever-Magnificent, the Ever-Thankful**, for his help and blessing.

I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor Dr. Mohamed Abdel karim, who has been always generous during all phases of the research, and I highly appreciate the efforts expended by my co-supervisor, Dr. Kamal Mohamed Saeed.

I owe a deep debt of gratitude to the Sudan University of Science and Technology for giving us an opportunity to accomplish this work. Thanks are extended to the Aromatic and Medicinal Plants Institute, Khartoum, Sudan for all facilities. Thanks for the National Research Center, Cairo for the spectral measurements.

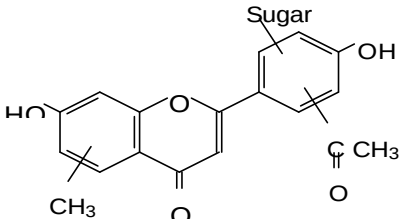
I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis.

I also would like to express my whole hearted thanks to my parents, for their generous support. They supported me throughout my entire life and particularly through the process of accomplishing the PhD degree.

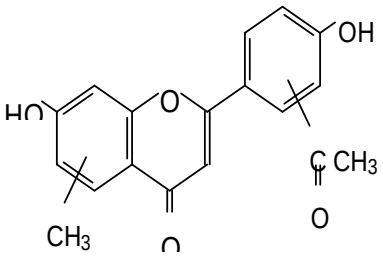
Abstract

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

The stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature. Qualitative tests on the alcoholic extract were positive for flavonoids. Conventional chromatographic techniques were used for isolation and purification of flavonoids. Two flavonoids (compounds I and II) were isolated from this plant. The partial structures of both compounds were deduced on the basis of their: IR, UV and NMR data and the following structures were suggested:



Compound I



Compound II

Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried. The results exhibited variable susceptibilities of microorganism for crude extract and different fractions.

In this study, the antioxidant capacity of the alcoholic extract of *Pulcriacrispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The GC-MS analysis of the *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* oils revealed the presence of 15, 14, 15 components respectively.

In the agar diffusion bioassay oils of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* failed to exhibit any antimicrobial activity. However, *Coriandrum sativam* oil showed significant antioxidant activity.

المستخلص

يشتمل البحث على دراسة كيميائية مفصلة للمحتوى الفلافونيدي لسيقان نبات الربل وكذلك التركيب الكيميائي لزيوت بذور كل من البقلة، الكزبرة وبذور الكتان. ايضا يتضمن البحث دراسة فعالية مستخلصات نبات الربل والزيوت المستخلصة كمضادات ميكروبات وكمضادات أكسدة.

لقد أستخلصت سيقان نبات الربل بواسطة الكحول الإيثيلي عند درجة الحرارة العادية و افاد المسح الفيتوكيميائى بوجود الفلافونيدات.

أنجز فصل المركبات بإستخدام كروماتوغرافيا العمود و كروماتوغرافيا الورق ، كما وتم التعرف على التركيب الكيميائي للمركبات المفصولة بإستخدام وسائل التحاليل الطيفية المختلفة : مطيافية الأشعة تحت الحمراء، الأشعة فوق البنفسجية ، الرنين النووي المغناطيسي للهيدروجين . وقد تم فصل مركبين ذوا طبيعة فلافونيدية من نبات الربل وهما المركب **II ,I.** تناولت الدراسة فعالية مستخلصات سيقان نبات الربل كمضادات ميكروبات وكذلك كمضادات أكسدة.

أثبت البحث ان مستخلص خلات اللإيثيل ذو فعالية كمضاد للبكتيريا. أما المستخلص الكحولي وبقية المستخلصات فقد اظهروا فعالية منخفضة كمضادات بكتيريا وفطرية. أوضحت الدراسة ان المستخلص الكحولي لنبات الريل ذو تاثير فعال كمضادات اكسدة.

اخضع زيت بذور البقلة، الكزبرة والكتان للكروماتوغرافيا الغازية - السائلة الملحقة بمطياف الكتلة . اوضحت هذه الدراسات وجود خمسة عشر ، أربعة عشر، وخمسة عشر مكونا بالزيت على التوالي.

تناولت الدراسة أيضا فعالية الزيوت المستخلصة من بذور البقلة ، الكزبرة والكتان كمضادات ميكروبات ولم تظهر النتائج أي تأثيريذكر. وأثبت البحث أن زيت بذور الكزبرة ذو فعالية عالية كمضاد أكسدة .

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INTRODUCTION

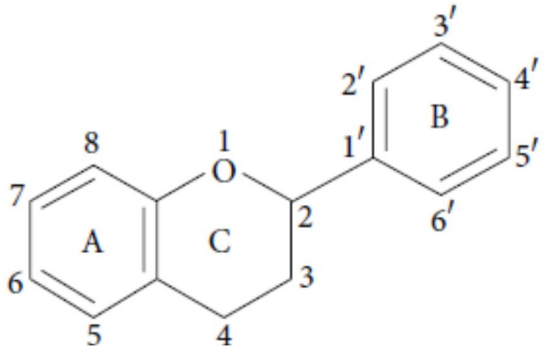
1.1 General overview

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities [1, 2]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [3]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [4]. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [5, 6]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [7, 8].

As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems [9, 10]. Flavonoids have ability to induce human protective enzyme systems.

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases [2, 8-

[10].Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin [11]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [12].The basic nucleus of flavonoids is displayed in Fig. 1.



(1)

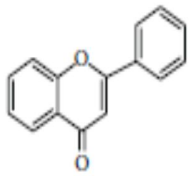
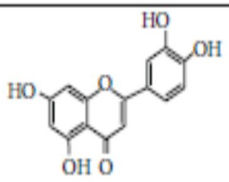
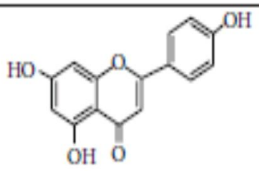
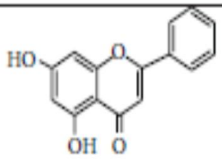
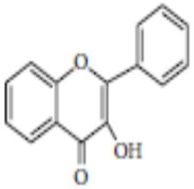
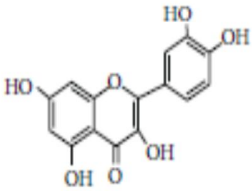
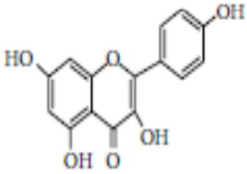
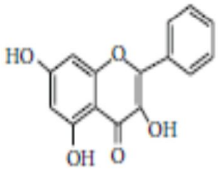
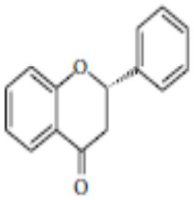
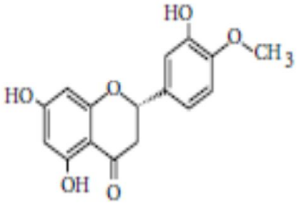
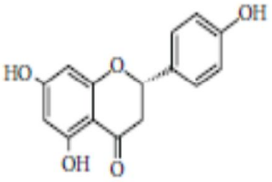
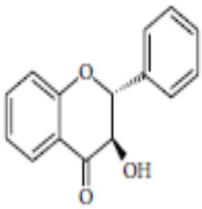
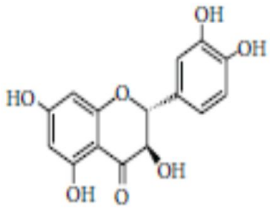
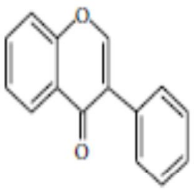
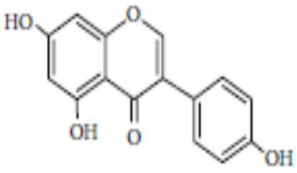
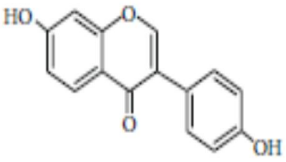
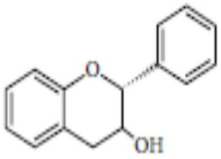
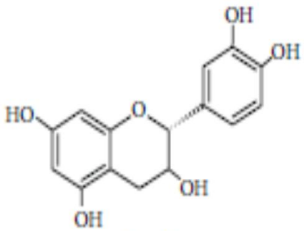
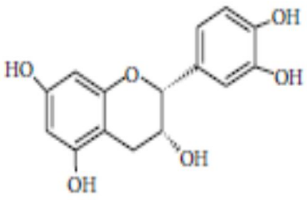
1.2 Chemistry of Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from orange. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified [13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (e.g. apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.

Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings [13].

Table (1): Structure of flavonoids

Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1.1). Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

Flavonols differ from flavanones by hydroxyl group at the 3-position and a C_2–C_3 double bond[14]. Flavonoids are often hydroxylation positions 3, 5, 7, 2, 3', 4', and 5'.Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[15].

1.2.1 Spectral Characteristics of Flavonoids

Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (235–285 nm) corresponds to the A ring absorption.

Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367 nm in kaempferol (3,5,7,4-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'- hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'- hydroxyl groups) [16]. The absence of a 3-hydroxyl group in flavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics [17]. Flavanones exhibit a very strong (Band II) absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only a shoulder for (Band I) at 326 and 327 nm. Band II appears as one peak (270

nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present.

As anthocyanins show distinctive (Band I) peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring and (Band II) peaks in the 240–280nmregion due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups [18].

1.3 Flavonoid- Rich Food and Medicinal Plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photo-synthesizing plant cells.

They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals [19].Thus flavonoids found in animals are of plant origin rather than being biosynthesized *in situ*. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [20].

Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg [21]. Preparation and processing of food may decrease flavonoid levels depending on the methods used.

For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644mg/L which suggest that the flavanones are concentrated in the cloud

during processing and storage [22]. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans [23].

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids [24, 25]. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases.

Table (2): Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry

Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavone – glabridin- a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals [26]. Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants [27, 28]. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people.

Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats [29].

Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia [30]. Table 3 summarizes some of the medicinal plants rich in flavonoid contents.

Table (3): Medicinal plants rich in flavonoids contents

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein

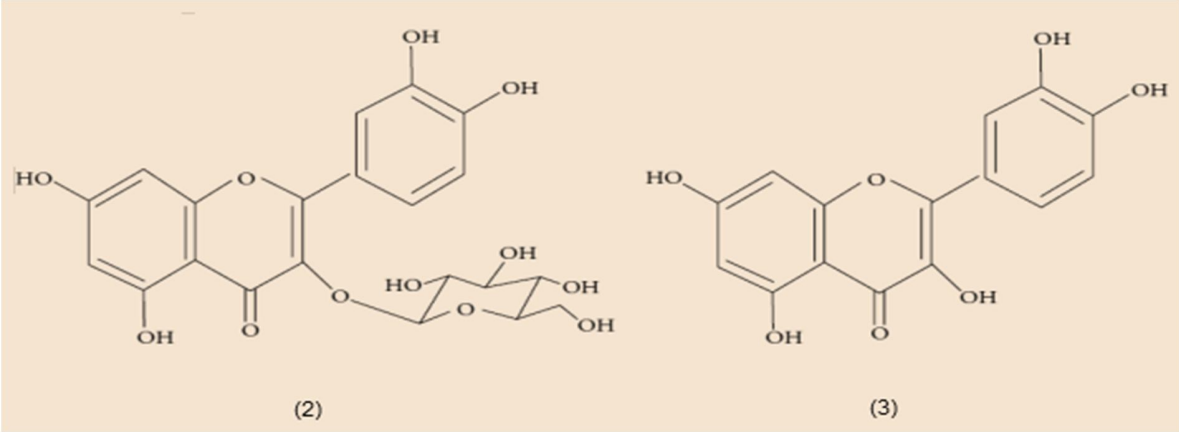
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for

example, hydroxy ethyl rutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and micro bleeding [31].

1.4 Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and p^{Ka}. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as glycosides (Figures 2 and 3). Aglycones can be easily absorbed by the small intestine, while flavonoid glycosides have to be converted into aglycone form before absorption [32].



The hydrophilic flavonoid glucoside such as quercetin are transported across the small intestine by the intestinal Na^+-dependent glucose cotransporter (SGLT1) [32].

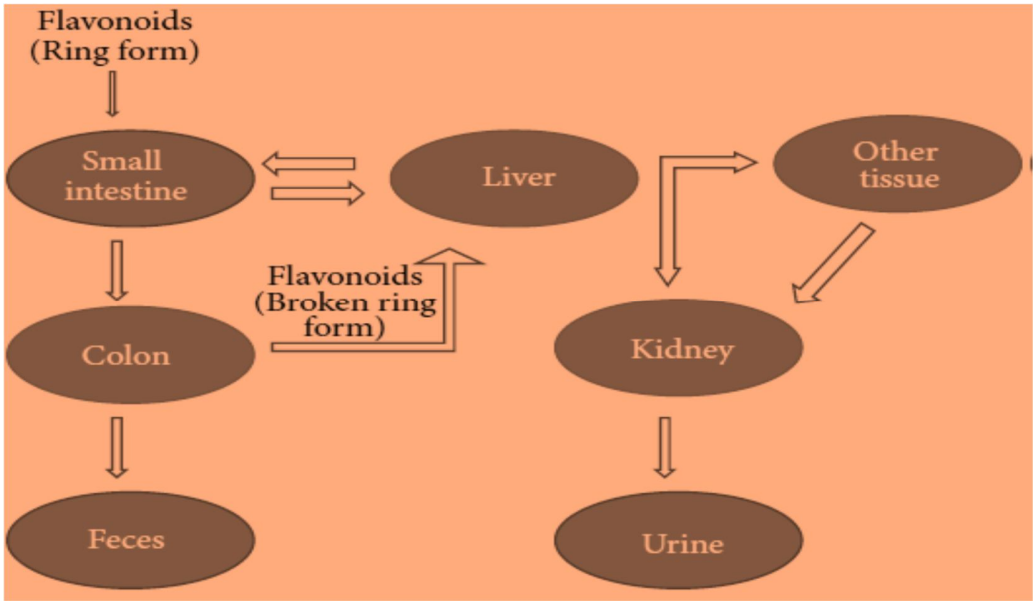
An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [33]. The substrate

specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [34].

The glycosides which are not substrates for these enzymes are transported toward the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [35]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected.

After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds [36]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [37]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [38]. The flavonoids secreted with bile in intestine and those that cannot be absorbed from the small intestine are degraded in the colon by intestinal micro flora which also break down the flavonoid ring structure (Sceme 1). Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [39]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine.

They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [40].



Sheme 1: Compartments involved in the metabolism of flavonoid

1.5 Biological Activities of Flavonoids

1.5.1 Antioxidant Activity

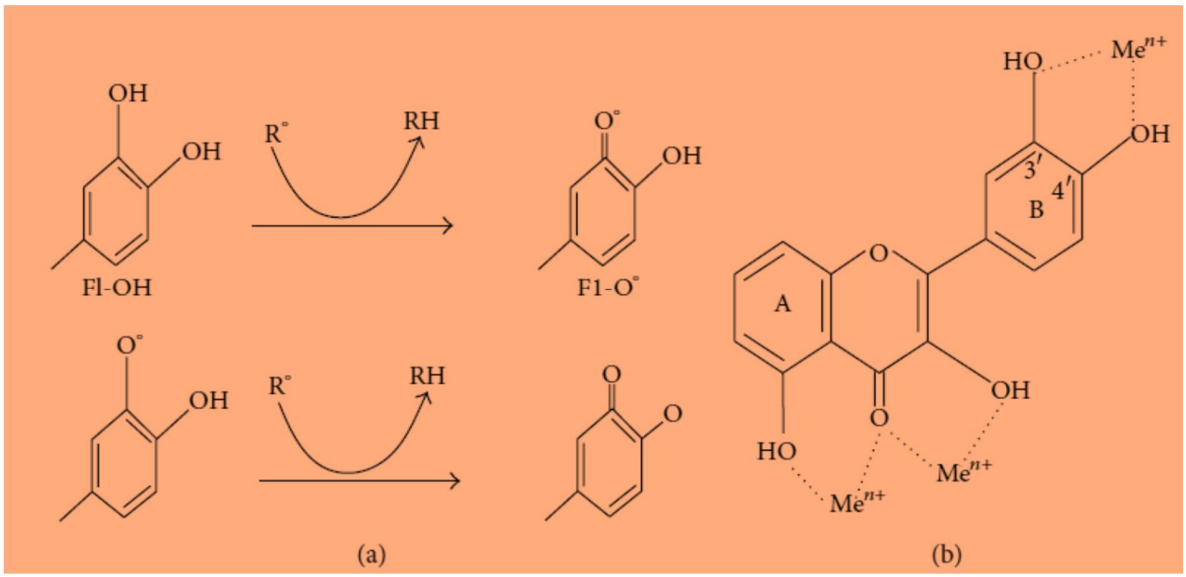
Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants.

The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure.

The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [4, 41]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and

peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [42].

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation;(2) Scavenging ROS; and (3) upregulation or protection of antioxidant defenses [43, 44]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [45]{see scheme 2}.



Scheme (2): (a) Scavenging of ROS (R∘) by flavonoids (Fl-OH) and

(b) binding sites for trace metals where Men+ indicates metal ions

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms [5, 25]. Free

metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation (Scheme 2(a)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [44, 46]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures [47]. The binding sites are shown in Schme 2(b). A 3', 4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [4]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro* [48]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable *ortho* semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential [49]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features [50].

The flavonoid heterocyclic contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH is crucial. Removal of a 3-OH annuls coplanarity and conjugation which

compromises scavenging ability [51]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity [50].

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides.

There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased [52]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position [53]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [54].

1.5.2 Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [55].

Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Glutamate-cysteine ligase catalytic subunit

(Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases [56]. It has been demonstrated that anthocyanin cyanidin-3-O-β- glucoside increases Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and pro-apoptotic signaling. Furthermore, this flavonoid lowers hepatic lipid peroxidation, inhibits the release of pro-inflammatory cytokines, and protects against the development of hepatic steatosis [56]. Silymarin is a flavonoid having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle *Silybum marianum* (Compositae). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers [57-59].

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl4-) induced injury. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl4 [60]. Similarly in an *in vivo* experiment flavonoids at of 50, 100, and 200mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver.

Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid [60].

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive

complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. *Equisetum arvense* flavonoids as well as hirustrin and avicularin isolated from some other sources is reported to provide protection against chemically induced hepatotoxicity in HepG2 cells [61, 62].

1.5.3 Antibacterial Activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid- rich plant extracts from different species have been reported to possess antibacterial activity [44, 46, 63, 64]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [65].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation.

Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [66, 67].

Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria [68, 69].

The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities [68, 70]. Robinetin, myricetin, and (−)-epigallocatechin

are known to inhibit DNA synthesis in *Proteus vulgaris.* Mori et al. [71] suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase [72].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes [73]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells.

The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity [74]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [75].

Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus* [76].

Haraguchi and colleagues [74] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A

inhibited incorporation of radioactive precursors intomacromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain [74].

There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents [77–79].

1.5.4 Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and pro-inflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [80].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells [81]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein

kinases [82, 83]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules [84]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful pro-inflammatory signaling molecules[85]. Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotopes *in vitro* [86].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration [87]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide [88]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.5.5 Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents [46, 89]. Consumption of onions and/or apples, two

major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon [90].The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods [91].

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [92]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [93]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in cogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor

agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial [94]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer [93].

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death [90, 95]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors [96].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P- 388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, andMKN-28), colon cancer cells (COLON320DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells [97] . It has been proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent [98].

Barnes [99] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones

genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats [100]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats [101]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al. [102]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [103]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects [104]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin [105].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [106]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor [44, 46, 107].Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals [108].

1.5.6 Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral

activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available.

Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso [109] demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication. Baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase [110].

Table (4): Antiviral activity of various flavonoids

Flavonoid	**Virus**
Quercetin	Rabies virus, herpes virus, parainfluenza virus, polio virus, mengo virus, and pseudorabies virus
Rutin	Parainfluenza virus, influenza virus, and potato virus
Apigenin	Immunodeficiency virus infection, Herpes simplex virus type, and Auzesky virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

Another study revealed inhibition of HIV-1 entry into cells expressing CD4 and chemokine coreceptors and antagonism of HIV-1 reverse transcriptase by the flavone *O*-glycoside [111]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase [110]. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a novel mechanism that probably involves inhibition of viral transcription [112].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV).

Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [110]. Studies have displayed that flavonols are more active than flavones against herpes simplex virus type 1 and the activity order was found to be galangin, kaempferol, and quercetin [110].

Zandi et al. [113] studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus [109]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [113]. List of some flavonoids and their efficacy against viruses is given in Table 4.

1. 6 Role of Flavonoids in Plants

1.6.1 Combating Oxidative Stress

Flavonoids have long been reported as serving multiple functions in plants [114].Various abiotic and biotic factors helps in the generation of ROS in plants leading to oxidative stress. Flavonoid biosynthesis in plants is almost exclusively enhanced due to oxidative stress. They have capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and quench ROS once they are formed [115]. Flavonoids discharged primary UV-B screening functions when early plants moved from water to colonize the land. Extent of antioxidant capacity and ability to absorb UV-wavelengths depends upon the nature of substitution on different rings of flavonoids. Dihydroxy B ring substituted flavonoids have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts have greater ability to absorb UV wavelengths [115].

The most reactive hydroxyl groups (7-OH in flavones or the 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in the aqueous cellular environment, protects the reactive hydroxyl groups from autooxidation [116], and allows the transport of flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and the cell wall [117]. Recent evidence shows that antioxidant flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation, that is, the chloroplast. Here they can easily quench H_2O_2, hydroxyl radical, and singlet oxygen [115, 118].

Oxidative stress due to an excess of excitation energy in the chloroplast may be aggravated under conditions that limit the diffusion of CO_2 to the carboxylation sites and the efficiency of carboxylation [115, 119]. The environmental limitations to CO_2 assimilation rate include drought/salinity,

low/high temperature, and nutrient scarcity. Under these conditions the activity of ROS detoxifying enzymes may significantly reduce in the chloroplast [120, 121], which in turn up regulates the biosynthesis of ROS scavenging flavonoids.

The reducing functions of flavonoids are of key significance in plants under severe stress conditions. These functional roles are concomitant with the very high concentration of dihydroxy B ring substituted flavonoids [122]. Flavonoids have been suggested as representing a secondary antioxidant defense system in plant tissues exposed to different stresses [115]. Lipid peroxidation is the common consequence of oxidative stress which disrupts the cell membrane integrity.

Quercetin 3-*O*-rutinoside (rutin) may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage [123].

1.6.2 Growth Regulator

Flavonoids carry out functional roles of amazing significance in plant-environment interactions.

Flavonoids (in the nanomolar range) may regulate auxin movement and catabolism. The ability of flavonoids to create auxin gradients translates into phenotypes with different morphoanatomical features [124]. The control of flavonoids on auxin movement may have immense value in the stress-induced morphogenic responses of plants such as the flight strategy of sessile organisms exposed to unfavorable environments [125]. Species rich in dihydroxy flavonoids exhibit phenotypes with strikingly different morphological traits as compared with those rich in monohydroxy flavonoids [126]. Dwarf bushy phenotypes with few, small, and thick leaves to direct sunlight irradiance are usually present in sunny environments, thus

protecting leaves located deep in the canopy from light-induced severe cellular homeostasis perturbations. On the contrary, shaded plants, which are rich in kaempferol and/or apigenin derivatives (having negligible concentrations of quercetin derivatives), have long internodes, and large leaf lamina coupled with reduced leaf thickness [125].

Flavonoids at the plasma membrane are effective inhibitors of PIN (pin formed) and MDR (multidrug resistance) glycoproteins that are involved in the cell to cell movement of auxin. The ability of flavonoids to inhibit the activity of the efflux facilitator PIN and MDR proteins depends on the presence of the catechol group in the B ring of the flavonoid skeleton. In addition flavonoids regulate the activity of IAA-oxidase with markedly different effects based on their chemical structure [127]. Recent evidence of a nuclear location of flavonoids (as well as of enzymes of flavonoid biosynthesis) supports that flavonoids are capable of modulating the activity of proteins involved in cell growth. Flavonoids may therefore act as transcriptional regulators [128, 129].

1.7 Microbial Production of Flavonoids

In response to the low production efficiency from plants and chemical synthesis, research groups have directed their attention to the production of flavonoids in microorganisms using metabolic engineering and synthetic biology [130].

Chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals [131]. Because of the rapid development in molecular biology tools and the flooding of genome information from a variety of organisms, combinatorial biosynthesis offers an advantage for production of rare and expensive natural products. It can be used in both simple and complex transformations without the tiresome blocking and deblocking steps that are

common in organic synthesis [132]. Several prokaryotes and eukaryotes such as *E. coli*, *Saccharomyces cerevisiae*, *Streptomyces venezuelae*, and *Phellinus igniarius,* a medicinal mushroom, have been used for production of flavonoids [12].

1.7.1 Phenylpropanoid Pathway

In plants naringenin chalcone is the precursor for a large number of flavonoids produced by the phenylpropanoid synthetic pathway. Fermentative production by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine, and tyrosine [133]. As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonialyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid, which is then activated to *p*-coumaroyl-CoA by the action of 4-coumarate:
CoA ligase. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or nonenzymatically *in vitro* [134].

1.7.2 Enhancement of Flavonoid Production

Combination of promoter and target genes; knockout of related genes; overexpression of malonyl-CoA; and construction of artificial P450 enzymes are the key molecular biology technology procedures used in the heterologous production of flavonoids.

Every gene from the phenylpropanoid pathway is cloned in the host under the control of the promoter which often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used to enhance production of flavonoids according to the need of specific host such as T7, ermE∗, and GAL1 promoter [12]. The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids. Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens* intracellular malonyl-CoA pool was amplified leading to increased production of flavonoids [135].

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It was proved in an experiment in which researchers knocked out the udg gene encoding for UDP-glucose dehydrogenase. This resulted in elimination of endogenous UDP-glucose consumption pathway leading to increase in intracellular concentration of UDP-glucose and as a consequence increment in the production of flavanones and anthocyanins was observed [136].

One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound cinnamate-4-hydroxylase [137].This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host. An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis

pathway [138]. Combining bacterial cells and eukaryotic cells in a pot enabled researchers to generate a wider library of natural and unnatural products than any of the previously reported systems. *De novo* production of the key flavonoid intermediate naringenin has been demonstrated for the first time from glucose using an engineered *S. cerevisiae* strain which led to fourfold higher concentrations than reported in earlier studies on de novo biosynthesis [139, 140].

1.8 The studied plant species

1.8.1 *Pulcaria crispa*

Pulicaria crispa is a genus of Asteraceae family. The Asteraceae family is one of largest families of flowering plant, with about 1,100 currently accepted genera and 25,000 species. It is characterized by the flowers being arranged in dense heads of many small florets, such as the daisy and dandelion [141].

Pulcaria crispa

The family is of worldwide distribution ,being absent only from the Antarctic mainland, and is particularly well represented in semiarid region of the tropics and subtropics, such as the Mediterranean region, Mexico, the cape province of South Africa grassland and bush land formation of Africa and montane floras throughout the world. Only in the tropical rain forests are they poorly represented [141]. The Asteraceae are of incalculably great indirect economic importance to man as major contributors to the diversity, and therefore, the stability and sustainable productivity, of the drier vegetation types throughout the world, especially in tropical and sub-tropical areas. In proportion to its size, however, the direct economic importance of the family is comparatively small. It includes food plants, sources of raw materials, medicinal and drug plants, ornamentals and succulents, and, on the debit side, weeds and poisonous plants [141].

1.8.1.1 Phytochemical, antibacterial screening and antioxidant activity of *Pulcaria crispa*

Many secondary metabolites have been detected - coumarins, tannins and alkaloids - on *P. crispa* [142]. Investigation of the flavonoids constituents of *Pulicaria crispa*, result in the isolation and identification of four flavonoids, quercein, quercetin-3-o-glucoside, quercetin7-o glycoside and quercetin-3-methyl ether [143]. The presence of these metabolites explains the various uses of this plant in traditional medicine. Alkaloids, comprising a large group of nitrogenous compounds, are widely used as cancer chemotherapeutic agents [144, 145] alkaloids also interfere with cell division The presence of alkaloids in *Pulicaria crispa* may be responsible for antibacterial activity. *Pulicaria crispa* is used in folk medicine for the treatment of colds, coughs, colic, excessive sweating and as carminative [146].

1.8.2 *Portulaca oleracea*

Portulaca oleracea is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution belonging to the Portulacaceae family. It is commonly known as *purslane* (USA and Australia), *rigla* (Sudan and Egypt), *pigweed* (England), *pourpier* (France), and *Ma-Chi-Xian* (China) [147]. It is distributed widely in the tropical and subtropical areas of the world including many parts of the United States and is eaten extensively as a potherb and is added to soups and salads around the Mediterranean and tropical Asian countries [148].

Portulaca oleracea

Americans and aborigines of Australia grind the seeds of this plant into flour for use in mush and bread [149]. *Portulaca oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties [150]. *Portulaca oleracea* has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic and so forth [151]. It exhibits a wide range of pharmacological effects, including antibacterial [152], antiulcerogenic [153], anti-inflammatory [154], antioxidant [155], and wound-healing [156] properties. It is listed by the World Health Organization as one of the most used medicinal plants, and it has been given the term “Global Panacea” [157]. The

Chinese folklore described it as “vegetable for long life” and it has been used for thousands of years in traditional Chinese Medicine [158, 159]. It is cold in nature and sour in taste and is used to cool the blood, stanch bleeding, clear heat, and resolve toxins. The dried aerial part of this plant is indicated for the treatment of fever, dysentery, diarrhea, carbuncle, eczema and hematochezia, with a recommended dose of 9–15 g, *Portulaca oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine [160–162].

1.8.2.2 Phytochemistry

Many constituents of *Portulaca oleracea* have been isolated, including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals.

One of the most effective constituents present in Chinese Herbal Medicines are flavonoids which are biologically active and possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. In the *Portulaca oleracea* plant, the flavonoids levels vary according to the part of the plant; the highest levels are present in the root followed by stem and the leaf; and seven different flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin [163]. However, only kaempferol and apigenin have been found in ethanolic extracts of leaves and stems, with the levels in the former being higher [157]. Portulacanones B–D, three homoisoflavonoids compounds, display selectively cytotoxic activities against three human cancer cell lines (SF-268,NCI-H460, and SGC-7901) [164]. Flavonoids are also widely present in foods such as fruits and vegetables [165].

In addition to flavonoids, another important chemical found in this plant is alkaloids including dopa, dopamine, and noradrenalin. The content of dopamine and noradrenalin is higher in leaves compared to stem and seeds. The amount of dopamine and noradrenalin obtained from leaves varies according to the solvents used in the extraction process, suggesting that the levels of these compounds are dependent on the solvents used during the extraction process [166].

Oleraceins A, B, C, D, and E are cyclodopa alkaloids isolated from this plant [167] and several analytes such as (3R)-3,5-bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone and 1,5-dimethyl-6-phenyl-1,2-dihydro-1,2,4-triazin-3(2H)-one display cytotoxic activities against human cancer cells [168].

Portulaca oleracea is also an excellent source of omega-3 fatty acids, which is usually present in oil and fat of fishes but not normally found in plants. Omega-3 fatty acids play an important role in the enhancement of immune function [169] and prevention and treatment of hypertension, coronary artery disease, cancer, and other inflammatory and autoimmune disorders [170]. It includes α-linolenic acid and linoleic acid, which are essential for normal growth, health promotion, and disease prevention in humans. Polysaccharides found in *Portulaca oleracea* are potential therapeutic agents for the treatment of diabetes mellitus owing to their modulation of blood lipids, metabolism, and decrease of blood glucose. *Portulaca oleracea* contains monoterpenes such as portulosides A and B, diterpenes such as portulene, and β-amyrin type triterpenoids [147, 171]; in addition, vitamins have also been isolated from the leaves of this plant. It contains the highest content of vitamin A which is a natural antioxidant playing an important role in vision, maintaining healthy mucus membranes and protecting against lung and oral cavity cancers among green leafy vegetables. This plant also

contains ascorbic acid, α-tocopherol, and B-complex vitamins, for example, niacin, pyridoxine, and riboflavin [171]. Furthermore it is rich in minerals like phosphorus, manganese, icon, calcium selenium [149], and the amino acids isoleucine, proline, leucine, lysine, phenylalanine, methionine, cystine, valine, threonine, and tyrosine [148]. Many other constituents have also been isolated from this plant, such as β-carotene, glutathione, melatonin, portulacerebroside A, catechol, and bergapten.

1.8.3 *Coriandrum sativam*

Coriander (*Coriandrum sativum*) being an annual herb is most commonly used for seasoning purpose. Seeds, leaves and roots are edible, although they have very distinct flavors and uses [172]. The herb has a light and fresh flavor. Coriander can be used as whole plant and can be processed because of its perishable nature of leaves and to increase the palatability of ripe fruits (seeds) before using it as flavoring agent in different food preparations. Whole plant of coriander mainly fresh leaves and ripe fruits are used for culinary purposes [172].

Coriandrum sativum

Coriander leaves have different taste than its seeds, with citrus overtones. Coriander plant is a rich reservoir of micronutrients and nutritional elements which leads us to focus our study on this herb [172]. Coriander is very low in saturated fat however,it contains good amount of linoleic acid which is a good source of α-tocopherol and vitamin K. Leaves of plant are rich source of vitamins while seeds are rich in polyphenols and essential oils. Coriander taste is devoted to its essential oil comprising a significant content of linoleic and furanocoumarins (coriandrine and dihydrocoriandrine). Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activity along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits and its protective effect to preserve the food for longer period [172].

Coriander contains high amount of essential oils that are very important for growth and for proper functioning of brain. The main essential fatty acids present in coriander include linoleic and linolenic acids. Linoleic acids belong to PUFA (polyunsaturated fatty acid) group. Dietary supplementation of coriander seed greatly affects the lipid composition of carcass by decreasing saturated fatty acid (SFA) contents (palmitic and stearic acids) and by increasing monounsaturated and polyunsaturated fatty acid (MUFA and PUFA) [173]. This plant is a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) isolated from the seeds and the aerial parts [174]. The high content of fats and protein in the fruits make distillation residues suitable for animal feed. Coriander fruits yield 5 to 7% of ash, 13% resin, astringent principle, malic acid and alkaloids. Coriander oil contains coriandrol, jireniol and vebriniol [175].

1.8.4 *Linum usitatissimum*

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* Figure 1.8 was given by Linnaeus in his book "Species Plantarum" [176]. It is an annual herbaceous plant with shallow root system.

The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are specialized development of this species [177].

The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber purpose are tall growing with straight culms and have fewer secondary branches.

Linum usitatissimum

The Mediterranean and Southwest Asia have both been proposed as the center of origin [203]; however the exact location is uncertain [178]. The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the stearoyl-ACP desaturase II

(*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop [179].

Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [180] making it ideal for paints, varnishes, and inks due to its fast polymerization properties.

Increasing demand for edible oil sources with significant percentages of omega-3 fatty acids is resulting in consumption of flaxseed as a functional food. Flaxseed is also added to animal feed to improve animal reproductive performance and health [181, 182].

Research objectives

1. Extraction of flavonoids and oils from some plants
2. GC-MS studies on the extracted oils.
3. Elucidation of structures of flavonoids.
4. Biological activity of isolates.

MATERIAL AND METHOD

2.1. Materials

2.1.1 Plant material

Stems of *pulcaria crispa* were collected from White Nile state (Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum – Sudan. The seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were collected from Jeddah-Saudi Arabia.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m length; 0.25mm diameter; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.1.3-Test organisms

Test organisms used in the antimicrobial assay are: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

2.2- Methods

2.2.1- Preparations of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminum chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. distilled water.

- **Potassium iodide solution:** 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

100 g) of powdered air- dried stems of *pulcaria crispa* were extracted with 95% aqueous ethanol. The solvent was removed under reduced pressure giving the prepared extract. This prepared extract (PE) was used for phytochemical screening.

2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) were evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5

ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.2.2- Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

2.2.2.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Mayer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.2.4- Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and

the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.2.5 -Test for saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml)of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand for one hour and then checked for foam formation.

2.2.2.6 - Test for coumarins

(0.2 g) of the prepared extract were dissolved in 10 ml distilled water in test tube and a filter paper was attached to the test tube to be saturated with the vapor after being spotted o with 0.5N KOH. Then the filter paper was inspected under UV light. The presence of coumarins is indicated by the ability of the spot to absorb the UV light.

2.2.2.7- Test for anthraquinone glycosides

(0.2 g) of the prepared extract was boiled with 10 ml of 0.5 N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. (5ml) of the benzene solution was shacked with (3ml) of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraxquinones is indicated by pink or red color in the alkaline layer.

2.2.3- Extraction of flavonoids

The air - dried powdered stem of a *pulcria crispa* (3 kg) were extracted by maceration with ethanol (95%). The extract was concentrated under reduced pressure to give a crude product.

2.2.4- Column chromatography

Open column (70× 4 cm) was used for fractionation of the ethanolic extracts of *pulcaria crispa*. Silica gel with particle size 60-120 mesh ,from LOBA chemicals, was used as stationary phase. The composition of the mobile phase (ethyl acetate: methanol) was determined by TLC experiments.

The ethanolic extract of *pulcaria crispa* (5 g) was mixed with 10 g of silica gel and then applied on the top of the column. Elution commenced by EthOAC - MeOH ;(3:7, fraction I), (1:1, fraction II) and (0:1, fraction III).

2.2.5- paper chromatography

Fraction II – being rich in phenolics- was subjected to paper chromatography where pure flavonoids - compounds I and II -were isolated using the solvent system: butanol: acetic acid: water (4: 1: 12, upper layer).

2.2.6-Extraction of oils

Powdered seeds of *Portulaca oleracea* (*Coriandrum sativam* and *Linum usitatissimum*) - (500g) for each - were macerated with n-hexane . The solvent was removed under reduced pressure to yield the oil.

2.2.7-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added,then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes. The hexane layer was then separated.(5µl) of the hexane extract

were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.8- GC-MS analysis

The oils from *Portulaca oleracea, Coriandrum sativam* and *Linum usitatissimum* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 µm, thickness) was used. Helium (purity; 99.99 %) was used as carrier gas.

2.2.9- Biological activity

2.2.9.1- Preparations of chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa*

Chloroform fraction of *pulcaria crispa* was prepared by suspending the ethanolic extract of the leaves in the least amount of distilled water and then shaking with chloroform in a seperatory funnel. Evaporation of the chloroform layer under reduced pressure gave the required fraction. Other fractions were prepared in a similar way.

2.2.9.2- Antimicrobial assay

The ethanolic, chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa* as well as the essential oils extracted from the seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were evaluated for their antimicrobial activity against five standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*) using the cup plate agar method with some minor modifications.

2.2.9.2.1- Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 108- 104 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o}C for 24 hours.

2.2.9.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.9.3- Testing for antibacterial activity

The cup plate agar diffusion method was adopted with, some minor modification, to assess the antibacterial activity (2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a single test sample.

The agar discs were removed and cups were filled with 0.1 ml of each test sample using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^{o} C for one hours.

The above procedure was repeated for different concentrations of extracts and standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.9.4- Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but this time Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above

2.2.9.5-Antioxidant activity

The DPPH radical scavenging capacity was determined. In 96-wells plate, the test samples were allowed to react with 2.2 Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37° C. the concentration of DPPH was kept as (300μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests were run in triplicate. Propyl gallate was used as a positive standard at concentrations 0.5 mM.

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RESULT AND DISSCUSSION

Powdered air-dried stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature for 48hr. The crude product obtained after evaporation of the solvent under reduced pressure. The crude extract was fractionating by separator funnel and the three fractions were collected (ethyl acetate, chloroform and butanol fractions) and screened for its antimicrobial activity. The crude was subjected to silica gel column chromatography and eluted with ethyl acetate - methanol mixture (3: 7, fraction I), (1: 1, fraction II) and pure methanol (fraction III). Three fractions were collected. The fraction II was subjected to paper chromatography where pure components: **compound I** and **II** have been isolated. The structures of isolated flavonoids have been elucidated via a combination of spectra techniques.

GC-MS analysis on seed oil of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and the identification of the constituents. The oils was screened for antimicrobial and antioxidant activity against standard organisms.

3.1- *Pulcaria crispa*

3.1.1- Phytochemical screening

Pulcaria crispa was screened for major secondary metabolites and the results are displayed in table 3.1.

Table 3.1: Phytochemical screening of *pulcaria crispa*

Test	**Result**
Saponin	++
Cumarin	+
Alkaloids	+
Flavonoids	+
Tanin	+++
Steroids	-
Triterpens	-
Anthraquinone	-

+: Trace

++: Moderate

++: High

- : Negative

3.1.2- Antimicrobial assay

The crude extract, ethyl acetate, chloroform and butanol fractions have been screened for antimicrobial activity against five human pathogens. The results are depicted in table 3.2 Tables 3.3 and 3.4 display the antimicrobial activity of standard drugs.

Table 3.2: Antimicrobial activity of crude and three fractions

	Ec	Ps	Sa	Bs	Ca
Crude extract	_	_	_	_	10
Ethyle acetate fraction	12	10	13	_	12
Choroform fraction	_	_	_	_	11
Butanol fraction	_	_	10	_	10

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

The results were interpreted in a commonly used term :< 9 mm: in active; 9-12 mm: partially active; 13-18 mm: active; >18 very active.

The crude extract showed partial antifungal activity against *Candida albicans* (see table 3.2 and the appendix).

Ethyl acetate fraction showed activity against *Staphylococcus aureus.* It also exhibited partial Antibacterial activity against *Escherichia coli,*

Pseudomonas aeroginosa and *Candida albicans*.The choroform fraction showed partial anticandidal activity.

Butanol fraction showed partial Antibacterial activity against *Staphylococcus aureus* and *Candida albicans*.

Table 3.3: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.4: Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

3.1.3- Antioxidant assay

The antioxidant capacity of crude extract has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test extract against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.5) crude extract exhibited significant anti- oxidant activity.

Table 3.5: Radical scavenging activity of crude extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Crude extract	82.00%

3.1.4-Characterization of compound I

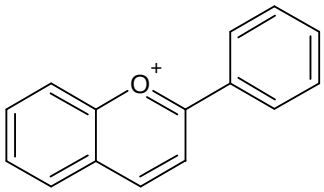
Compound I was isolated from *Pulcaria crispa* stems as yellow powder.

Table 3.6: IR data of compound I

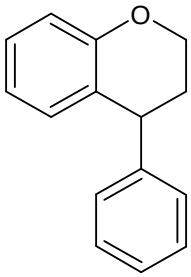
Functional group	**Wave number /cm^{-1}**
(C-H, Ar)	630.51
(C-O, ether)	1273.70
(C=C, Ar)	1378.30
(C=O)	1727.92
(OH)	3368.88
(C-H) Alkane	2921.62

The IR spectrum of compound I **(**Fig.3.1) showed all the anticipated bands of flavonids. Since

the infrared showed carbonyl absorption then the possibility that compound I being an anthocyanin or catechin is dismissed since these classes are characterized by absence of a carbonyl function.



Anthocyanin



Catechin

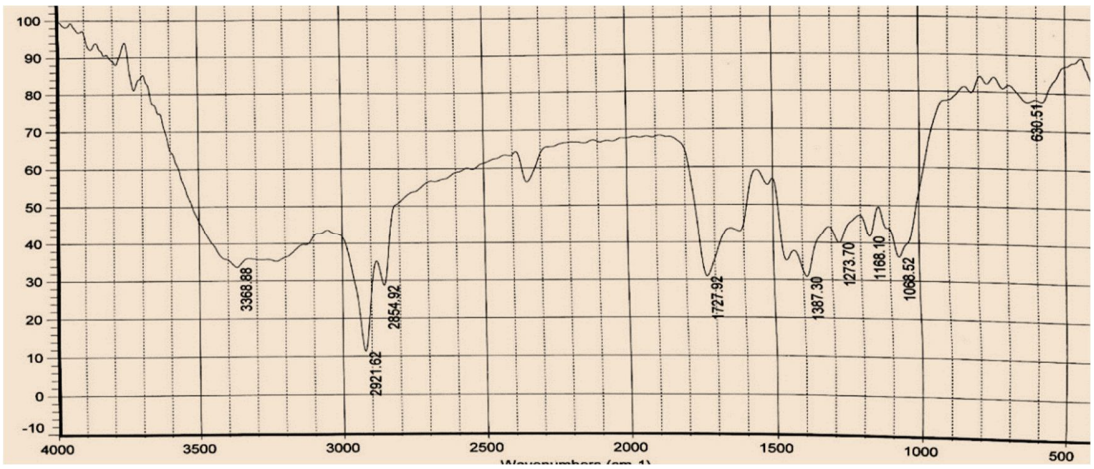
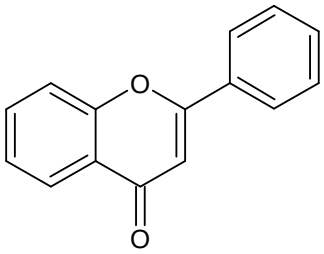
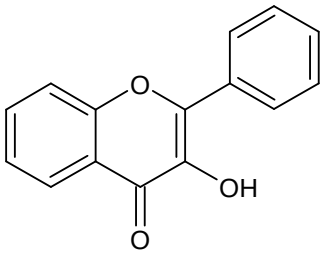


Fig 3.1: IR spectrum of compound I

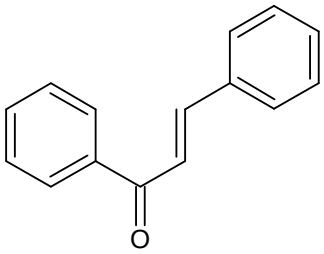
The UV spectrum of compound I revealed two major peaks at 262 and 345 nm (Fig.3.2). The presence of two peaks is a characteristic feature of flavones, flavonols, chalcones, and aurones.



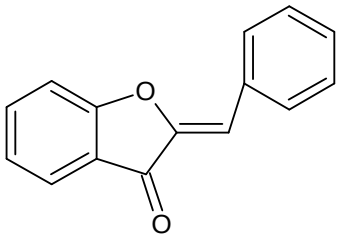
Flavone



Flavonol



Chalcone



Aurone

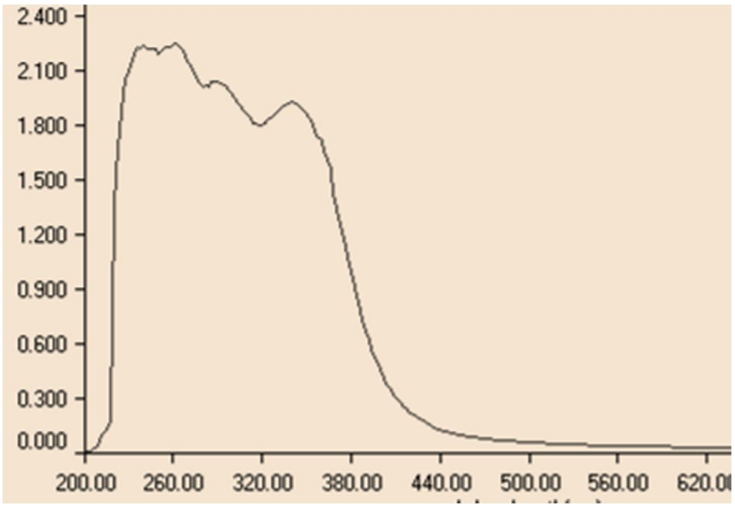
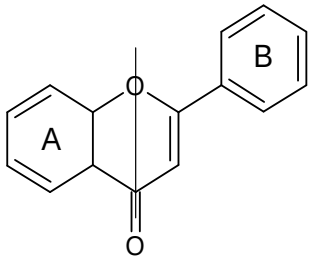


Fig 3.2: UV spectrum of compound I

The UV spectra of most flavonoids consist of two major absorption maxima, one of which occur in the range 220 – 290 nm (band II) and other in the range 300 – 400 nm (band I). The appearance of both band I and II demonstrates conjugation between the benzoyl and cinnamoyl systems.



Benzoyl Cinnamoyl

Band I is considerable to be associated with the absorption of cinnamoyl system, while band II originates from the benzoyl system.

Compound I showed both band II and I (fig.3.2) at 262 and 345nm respectively. Such absorption indicates that this compound is either a flavone, flavonol, chalcone or aurone. But aurones and chalcones absorb in

the range 365-430nm (band I). Flavones absorb in the range 300-350 nm, while flavonols absorb in the range 356-385. Thus compound I is a flavone.
Considerable structural feature have also been obtained using UV shift reagents such as sodium methoxide, sodium acetate, aluminum trichloride. These reagents produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl groups in the flavonoid molecule.
Use has been made of the effect of sodium methoxide on the UV spectra of flavonoids for detection of free 3 – and/or 4` –hydroxyl groups.
On addition of NaOMe to a methanolic solution of compound I a 42 nm bathochromic shift in band I without decrease in intensity (fig.3.3) was observed indicating the presence of a 4`-OH function.

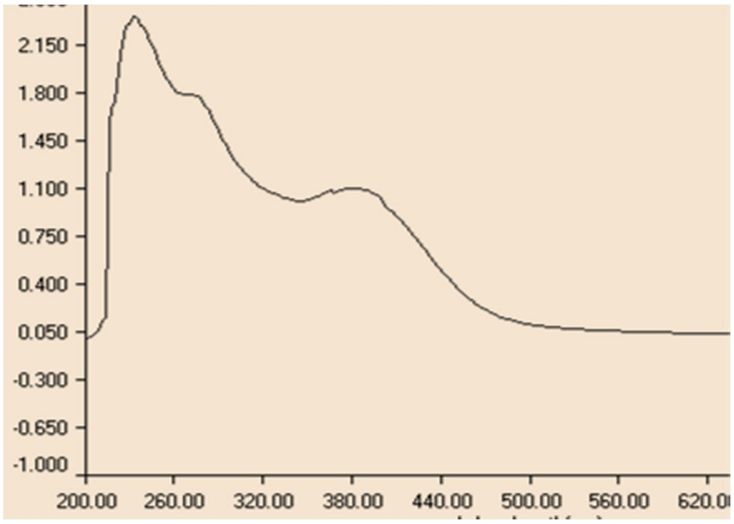


Fig 3.3: The UV spectrum of compound I in sodium methoxide

Sodium acetate is particularly useful diagnostic reagent for the specific detection of 7 –hydroxy group. When a methanolic solution of compound I **was** treated with excess powdered sodium acetate, and 16nm bathochromic shift in band II (fig.3.4) was observed suggesting a 7 –OH group.

When the previous solution of compound I was treated with excess powdered boric acid, no bathochromic shift was observed (fig.3.5) this indicate absence of *ortho*- dihydroxyl groups.

The shift reagent aluminum trichloride is diagnostic of 3 –hydroxyl group, 5 –hydroxyl group and catechol moieties[216].When a methanolic solution of compound I was treated with a few drops of the shift reagent $AlCl_3$ no bathochromic shift (fig.3.6) was observed and this indicates absence of 3 –OH, 5 –OH and catechol moieties.

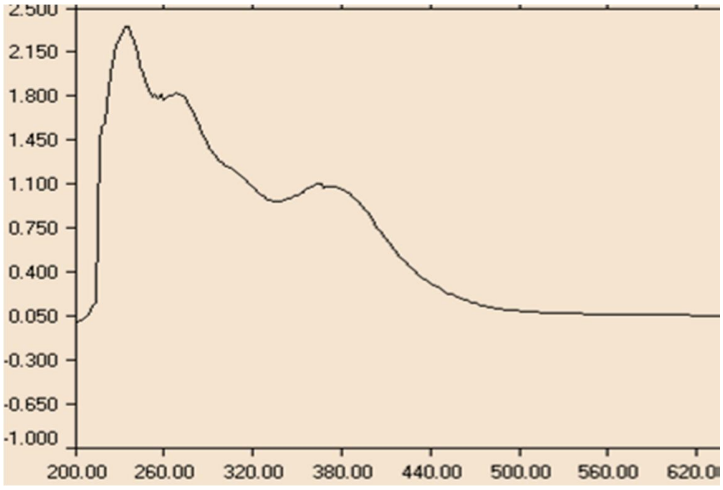


Fig 3.4: UV spectrum of compound I in sodium acetate

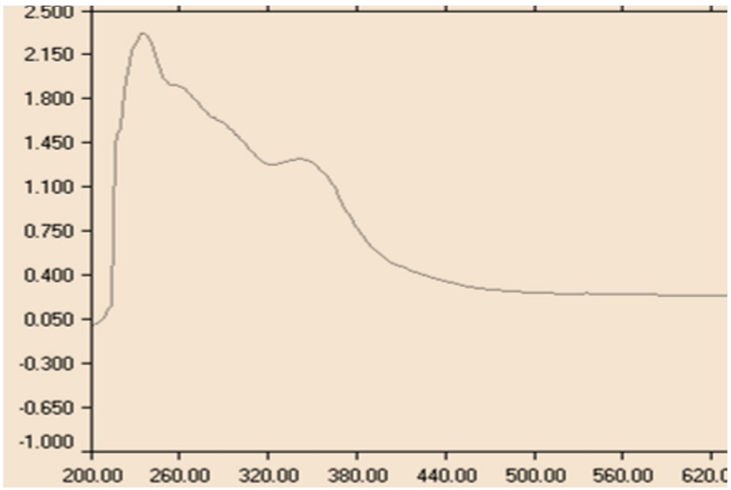


Fig 3.5: UV spectrum of compound I in sodium acetate/boric acid

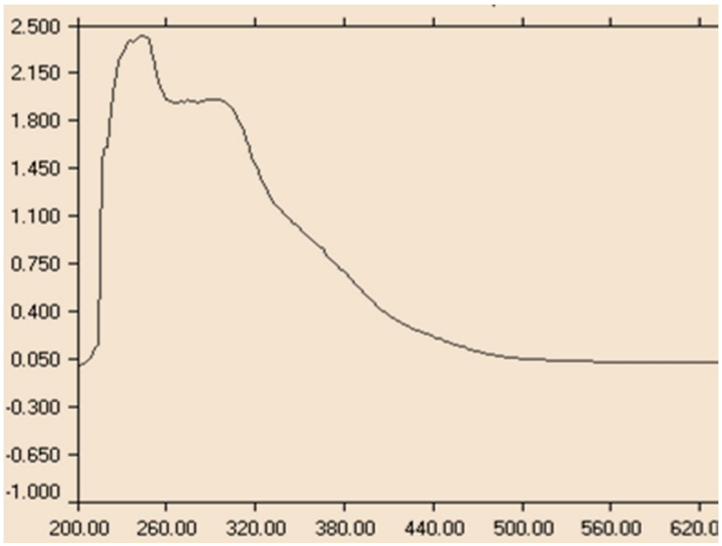


Fig 3.6: UV spectrum of compound I in aluminum trichloride

The ^{1}HNMR spectrum (Fig.3.7) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.1 ppm accounts for an acetyl function. The multiplet at δ 4.23-5.45 ppm is due to a sugar moiety (not identified in this study). The aromatic protons appeared as a multiplet (δ 6.68-7.65 ppm).

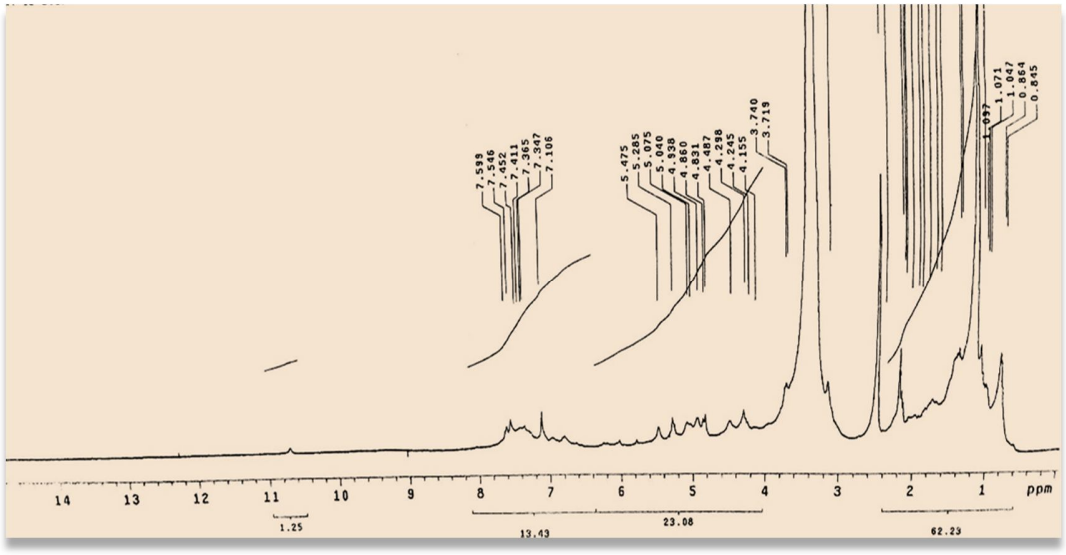
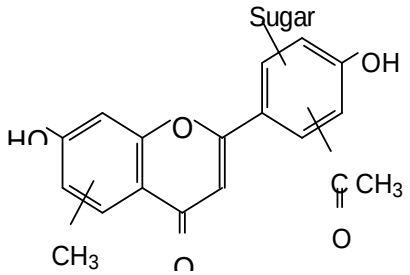


Fig 3.7: ^{1}HNMR spectrum of compound I

On the basis of the above spectral data, the following partial structure was proposed for compound I:



3.1.5- Characterization of compound II

Compound II was isolated as white-yellowish powder from *Pulcaria crispa* stem. The IR data of compound II is presented in the following table (see also Fig.3.8).

Table 3.7: IR data of compound II

Functional group	N(cm^{-1})
(C-H, Ar)	2085.7
(C-O, ether)	1017.15
(C=C, Ar)	1386.55
(C=O)	1643.49
(OH)	3419.34
(C-H) Alkane	2832.88

Compound II – which showed a carbonyl absorption-cannot be an anthocyanin or a catechin since IR spectrum gave carbonyl stretching at 1643.49 cm^{-1}. It could belong to one of the other classes of flavonoids. Anthocyanin and catechins are devoid of carbonyl.

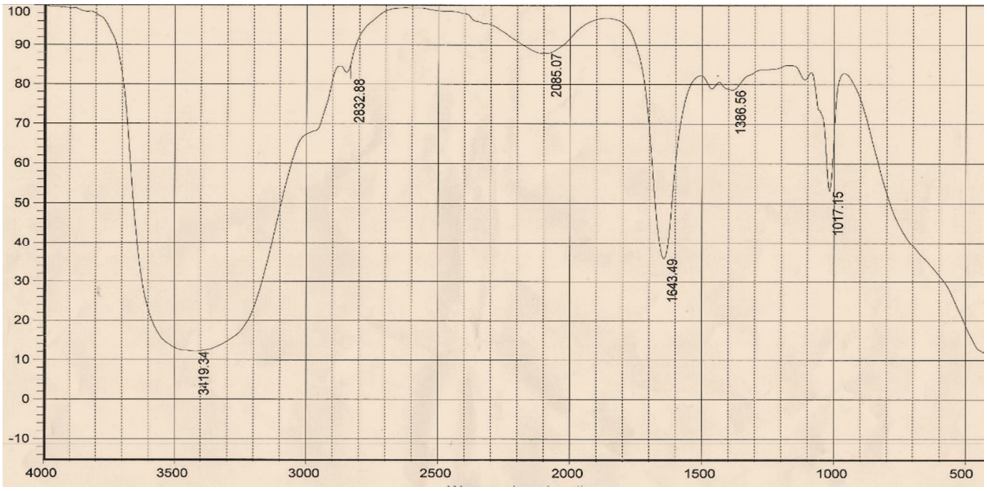
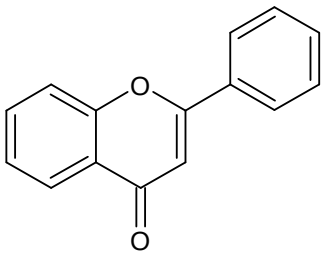


Fig 3.8: IR spectrum of compound II

Table 3.8: UV spectral data of compound II

λ_{max} (nm)	
Band I	Band II
330	240

The UV spectrum of compound II is depicted in (fig.3.9). The spectrum revealed two major peaks at 240 and 330 nm. Such absorption is a characteristic feature of flavones.



Flavone

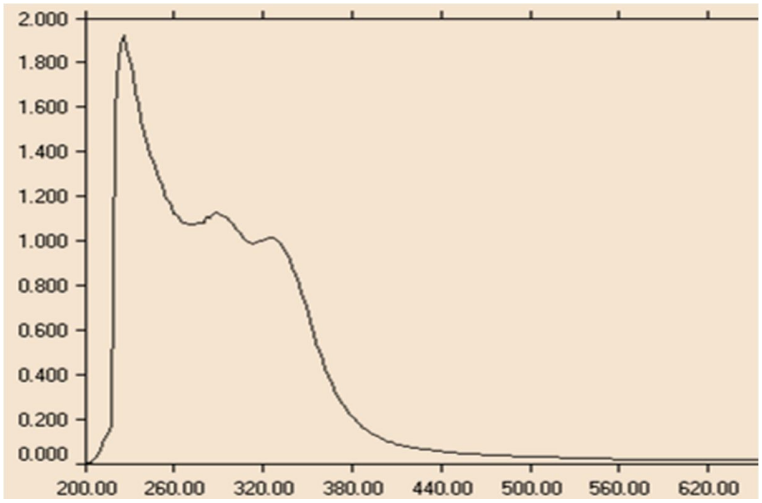


Fig 3.9: UV spectrum of compound II

Considerable structural feature have also been obtained using UV shift reagents: sodium methoxide, sodium acetate, aluminum trichloride.

On addition of NaOMe to a methanolic solution of compound II a 45nm bathochromic shift in band I indicative the presence of a 4`-OH function was observed (Fig.3.10).

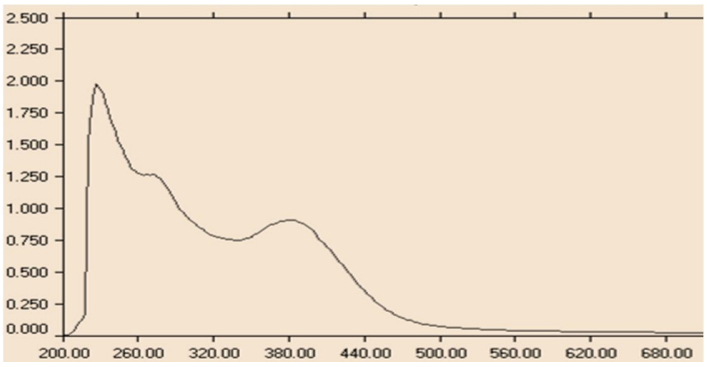


Fig 3.10: The UV spectrum of compound II in sodium methoxide

When a methanolic solution of compound II was treated with excess powdered of the shift reagent NaOAC, a 30 bathochromic shift in band II (fig.3.11) was observed and this indicates presence of a 7 –OH group.

When the previous solution of compound II was treated with excess powdered of boric acid, no bathochromic shift in both band II and I was observed (fig.3.12) this indicate absence of *ortho*- dihydroxyl groups.

The aluminium chloride spectrum showed no bathochromic shift (fig.3.13) indicating absence of 3 –OH, 5 –OH and catechol moieties.

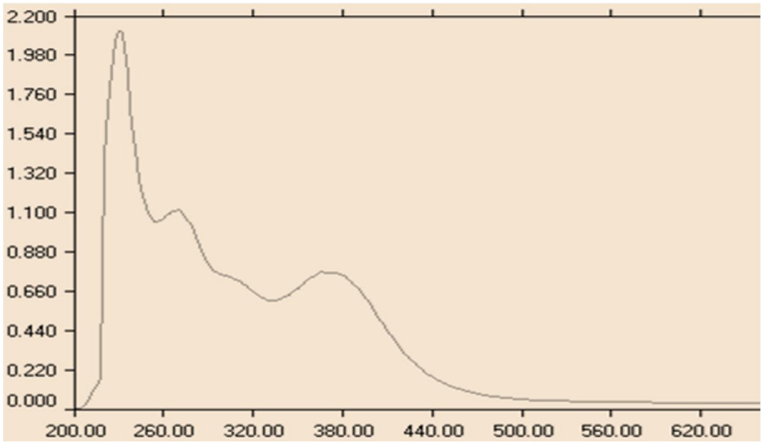


Fig 3.11: UV spectrum of compound II in sodium acetate

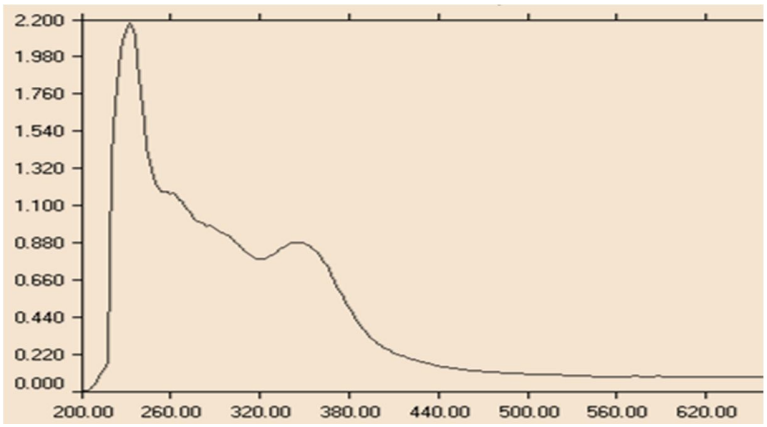


Fig 3.12: UV spectrum of compound II in sodium acetate/boric acid

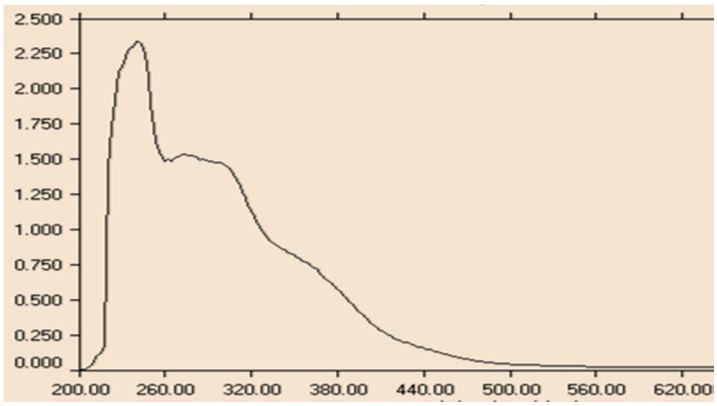


Fig 3.13: Aluminum trichloride spectrum of compound II

The ^{1}HNMR spectrum (Fig.3.14) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.4 ppm accounts for an acetyl function. The aromatic protons appeared as a multiplet (δ 6.68-7.59 ppm).

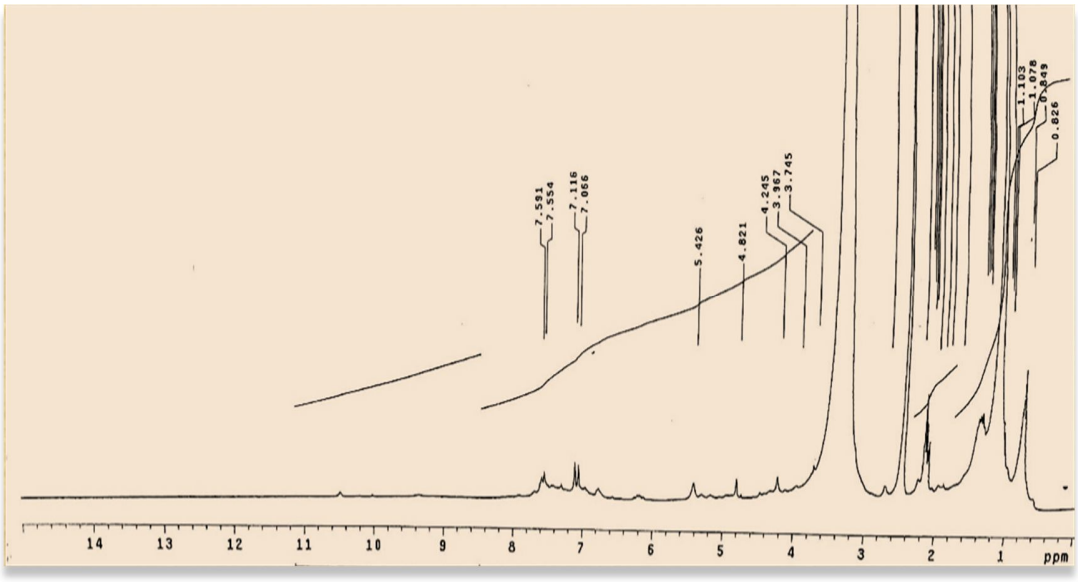
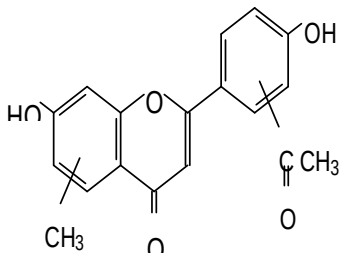


Fig 3.14: [1]HNMR spectrum of compound II

On the basis of the above spectral data, the following partial structure was proposed for compound II:



Compound II

3.2- *Portulaca oleracea*

3.2.1- GC-MS analysis of *Portulaca oleracea* oil

GC-MS analysis of *Portulaca oleracea* oil was conducted and the identification of the constituents was done by comparison of retentions times and MS data with the GC-MS library. Structures of constituents of the oil were further confirmed by interpreting the observed fragmentation pattern

.Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.2- constituents of oil

The GC-MS analysis of the studied oil revealed the presence of 15 components (Table: 3.9).The typical total ion chromatograms (TIC) of n-hexane extract are shown in (fig.3.15).

Table: 3.9: Constituents of *Portulaca oleracea* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.316	237128	0.08
2.	Pentadecanoic acid, methyl ester	14.373	170885	0.05
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.152	161664	0.05
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.195	705542	0.23
5.	Hexadecanoic acid, methyl ester	15.404	75687986	24.19
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	251818	0.08
7.	Heptadecanoic acid, methyl ester	16.367	775909	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.068	116899705	37.37
9.	9-Octadecenoic acid (Z)-, methyl ester	17.130	23526885	7.52
10.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.153	59248358	18.94
11.	Methyl stearate	17.307	22933709	7.33
12.	cis-11-Eicosenoic acid, methyl ester	18.883	1307281	0.42
13.	Eicosanoic acid, methyl ester	19.084	6202098	1.98
14.	Docosanoic acid, methyl ester	20.716	3045550	0.97
15.	Tetracosanoic acid, methyl ester	22.226	1688258	0.54

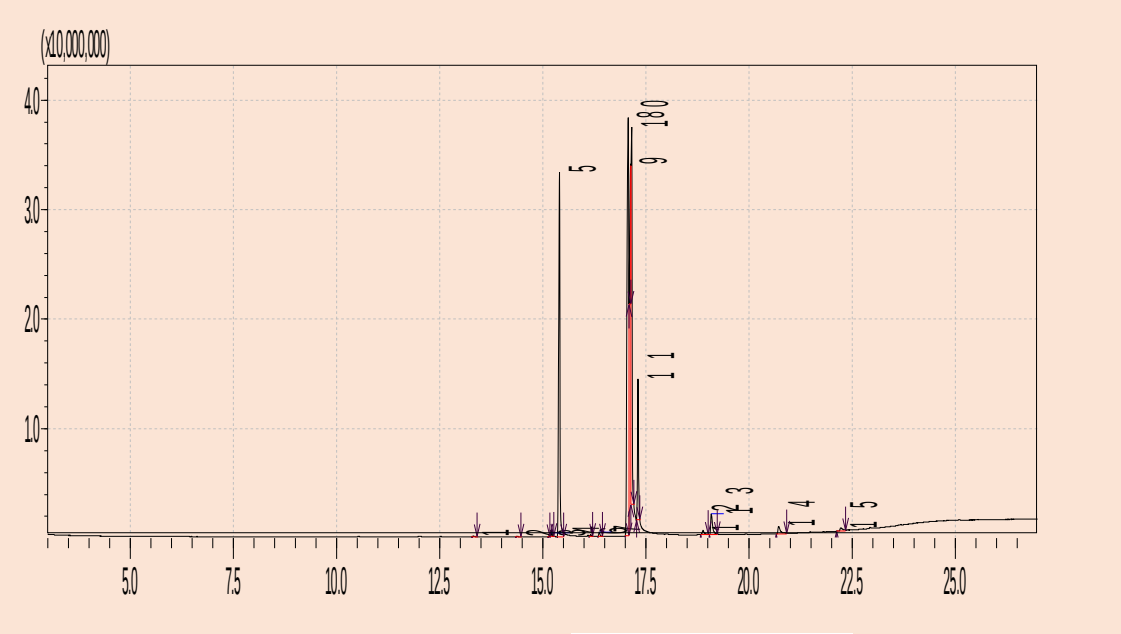


Fig 3.15: Chromatograms of *Portulaca oleracea* seed oil

The following esters were the major components:

9, 12-Octadecadienoic acid, methyl ester (37.37%)

The EI mass spectrum of 9,12-octadecadienoic acid, methyl ester shown in (fig. 3.16).The peak at m/z 294, which appeared at R.T. 17.068 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z 263 corresponds to loss of a methoxyl function.

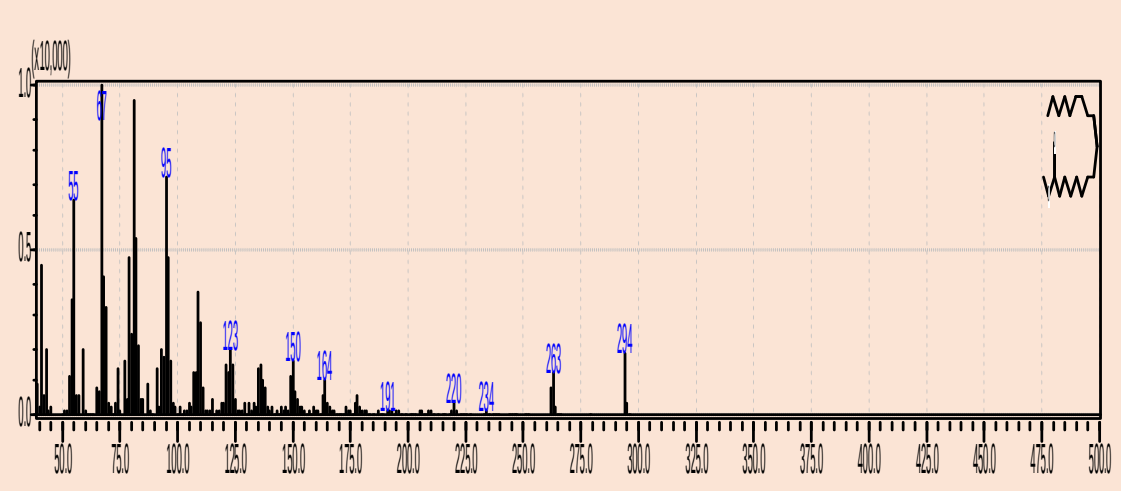


Fig 3.16: Mass spectrum of 9, 12-Octadecadienoic acid, methyl ester

Hexadecanoic acid, methyl ester (24.19%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.17).The peak at m/z 270 (at R.T. 15.404) corresponds $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is due to loss of a methoxyl .

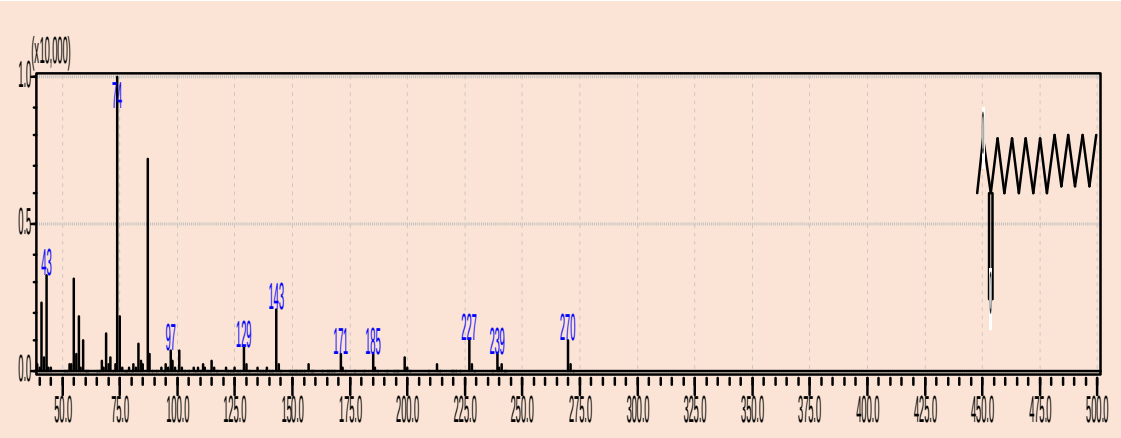


Fig 3.17: Mass spectrum of hexadecanoic acid, methyl ester

9,12,15-Octadecatrienoic acid, methyl ester (18.94%)

Fig. 3.18 demonstrates the mass spectrum of 9, 12, 15-octadecanoic acid, methyl ester. The signal at m/z 292(R.T. 17.153 in total ion chromatogram) corresponds to the molecular ion: $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z261 accounts for loss of a methoxyl .

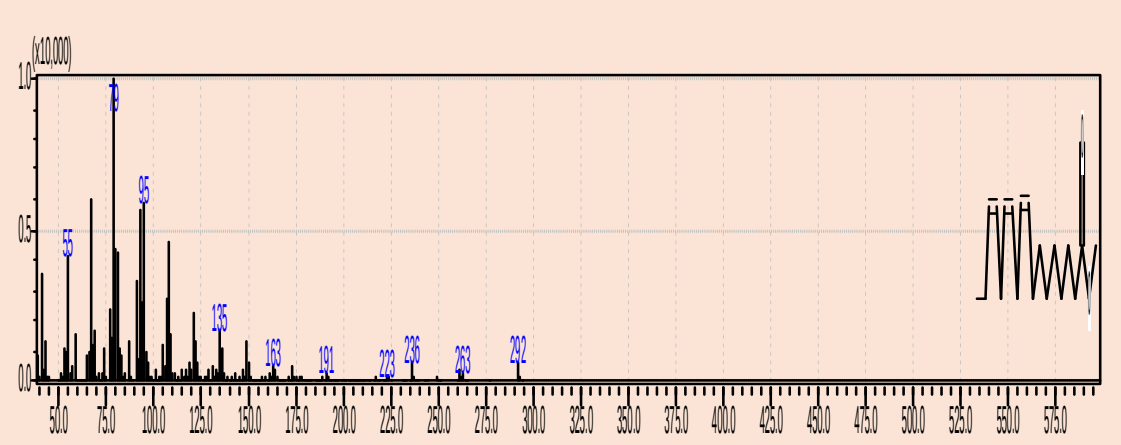


Fig 3.18: Mass spectrum of 9,12,15-Octadecatrienoic acid, methyl ester

3.2.3- Antimicrobial activity

The oil was screened for antimicrobial activity against five standard organisms. The oil not showed any activity against all test organisms

3.2.4- Antioxidant activity

The antioxidant capacity of *Portulaca oleracea* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.10) *Portulaca oleracea* seed oil exhibited weak antioxidant activity.

Table 3.10: Radical scavenging activity of *Portulaca oleracea* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Portulaca oleracea oil	29.00%

3.3- *Coriandrum sativam*

3.3.1- GC-MS analysis of *Coriandrum sativam* oil

GC-MS analysis of *Coriandrum sativam* was carried out . The GC-MS analysis revealed the presence of 14 components (table: 3.11).The typical total ion chromatograms (TIC) of n-hexane extract is presented in (fig.3.19).

Table: 3.11: Constituents of *Coriandrum sativam* oil

ID#	Name	Ret. Time	Area	Area%
1.	Methyl tetradecanoate	13.312	215647	0.03
2.	Pentadecanoic acid, methyl ester	14.372	231442	0.04
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.169	1910240	0.29
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.192	3233931	0.50
5.	Hexadecanoic acid, methyl ester	15.392	41125489	6.32
6.	Heptadecanoic acid, methyl ester	16.367	287117	0.04
7.	7,10-Octadecadienoic acid, methyl ester	16.913	1741544	0.27
8.	6,9-Octadecadienoic acid, methyl ester	16.962	543578	0.08
9.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.062	100831481	15.50
10.	9-Octadecenoic acid (Z)-, methyl ester	17.189	485955106	74.69
11.	Methyl stearate	17.313	9449169	1.45
12.	cis-11-Eicosenoic acid, methyl ester	18.865	3707972	0.57
13.	Eicosanoic acid, methyl ester	19.084	1068295	0.16
14.	Docosanoic acid, methyl ester	20.719	362910	0.06

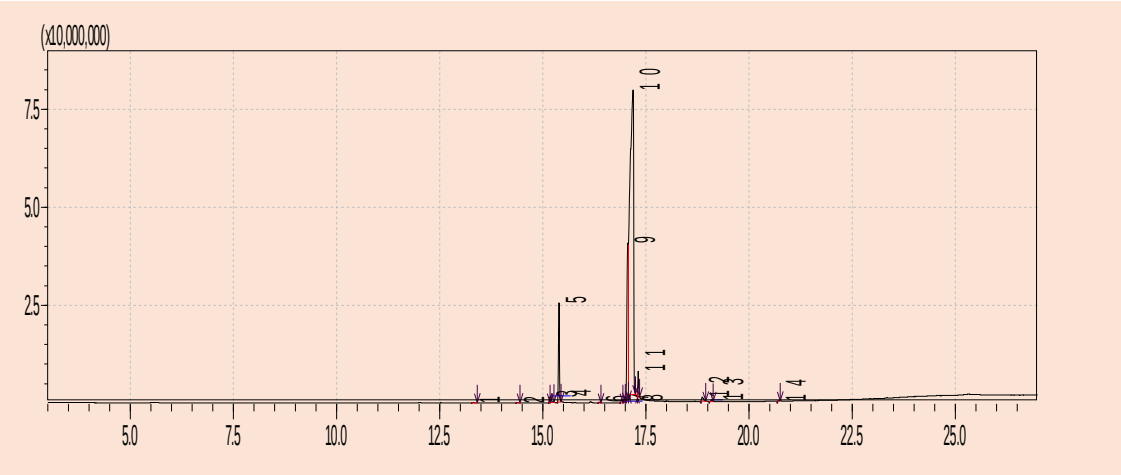


Fig 3.19: Chromatograms of *Coriandrum sativam* seed oil

The following esters were detected as major components:

9-Octadecenoic acid, methyl ester (74.69%)

The EI mass spectrum of 9-octadecenoic acid, methyl ester is shown in (fig. 3.20).The signal which appeared at m/z 264, with R.T. 17.198 corresponds $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z 235 is due to loss of a methoxyl group.

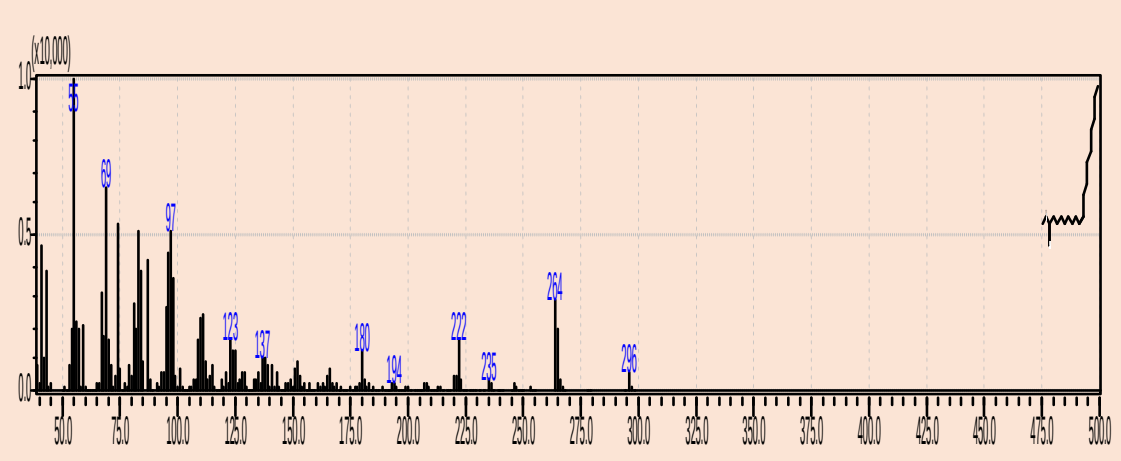


Fig 3.20: Mass spectrum of 9-Octadecenoic acid, methyl ester

9, 12-Octadecadienoic acid, methyl ester (15.50%)

Fig. 3.21 presents the mass spectrum of 9, 12-octadecadienoic acid, methyl ester .The peak at m/z 294, with R.T. 17.062 in total ion chromatogram, corresponds the molecular ion : $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 accounts for loss of a methoxyl function.

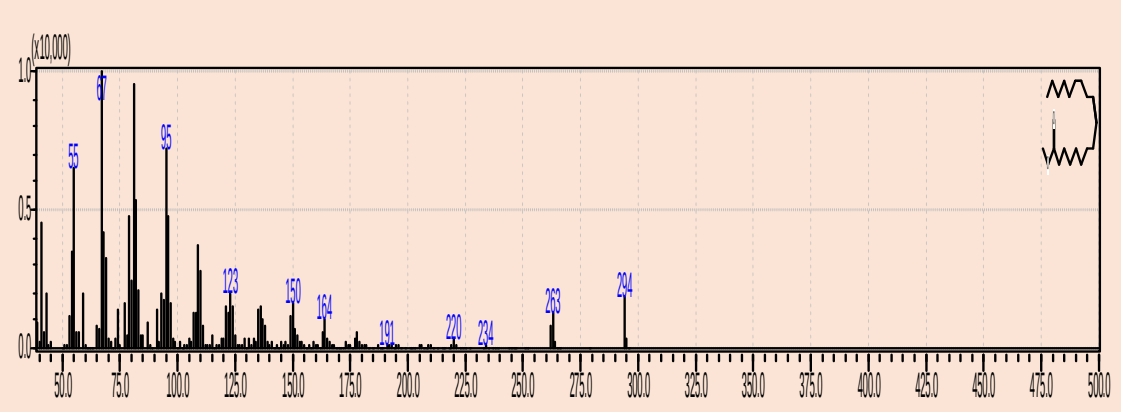


Fig 3.21: Mass spectrum 9, 12-Octadecadienoic acid, methyl ester

3.3.3- Antimicrobial activity

The studied oil was evaluated for antimicrobial activity against five standard human pathogens. The oil not showed any activity against all test organisms

3.3.4- Antioxidant activity

The antioxidant capacity of *Coriandrum sativam* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.12) *Coriandrum sativam* seed oil exhibited moderate antioxidant activity.

Table 3.12: Radical scavenging activity of *Coriandrum sativam* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Coriandrum sativam oil	52.00%

3.4- *Linum usitatissimum*

3.4.1- GC-MS analysis of *Linum usitatissimum* oil

GC-MS analysis of *Linum usitatissimum* was conducted. The analysis revealed the presence of 15 components (table: 3.13).The typical total ion chromatograms (TIC) of n-hexane extract is shown in (fig.3.22)

Table: 3.13: Constituents of *Linum usitatissimum* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.317	189936	0.07
2.	Pentadecanoic acid, methyl ester	14.373	70754	0.03
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.155	104077	0.04
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.196	476941	0.19
5.	Hexadecanoic acid, methyl ester	15.392	35779007	14.12
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	270452	0.11
7.	Heptadecanoic acid, methyl ester	16.365	508829	0.20
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.053	64486368	25.44
9.	9-Octadecenoic acid (Z)-, hexadecyl ester	17.155	69889815	27.58
10	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.164	50002070	19.73
11	Methyl stearate	17.307	28380442	11.20
12	cis-11-Eicosenoic acid, methyl ester	18.887	1343975	0.53
13	Eicosanoic acid, methyl ester	19.092	684609	0.27
14	Docosanoic acid, methyl ester	20.722	818972	0.32
15	Tetracosanoic acid, methyl ester	22.233	436726	0.17

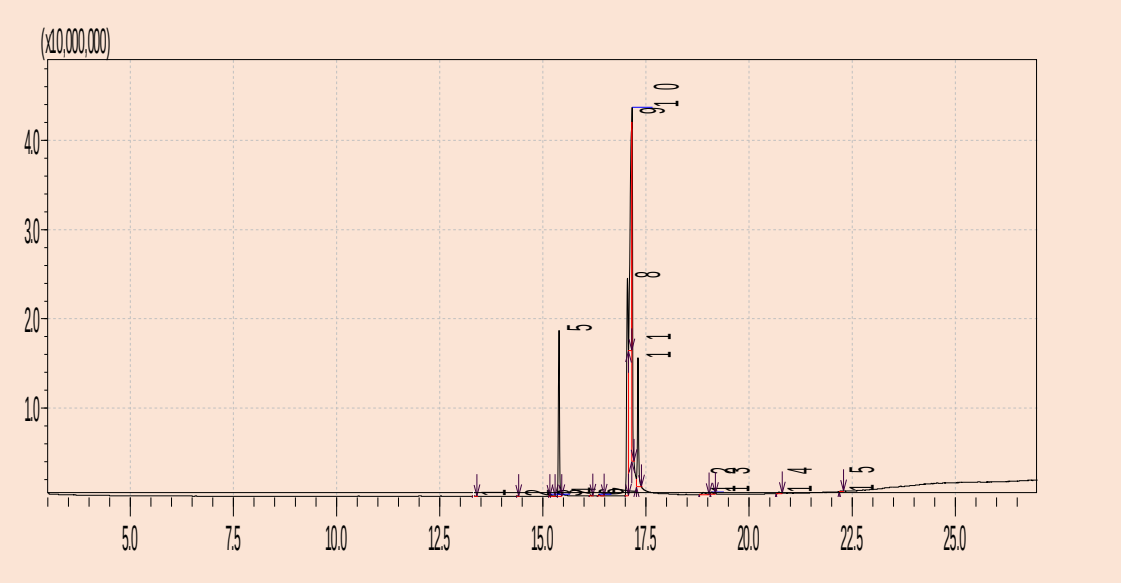


Fig 3.22: Chromatograms of *Linum usitatissimum* seed oil

The following esters were found as major components:

9-Octadecenoic acid, hexadecyl ester (27.58%)

The EI mass spectrum of 9-Octadecenoic acid, hexadecyl ester is shown in (fig. 3.23).The peak at m/z 295, which appeared at R.T. 17.155 in total ion chromatogram, corresponds to $M^{+}[C_{34}H_{66}O_2]^{+}$ .The peak at m/z 264 corresponds to loss of a methoxyl function.

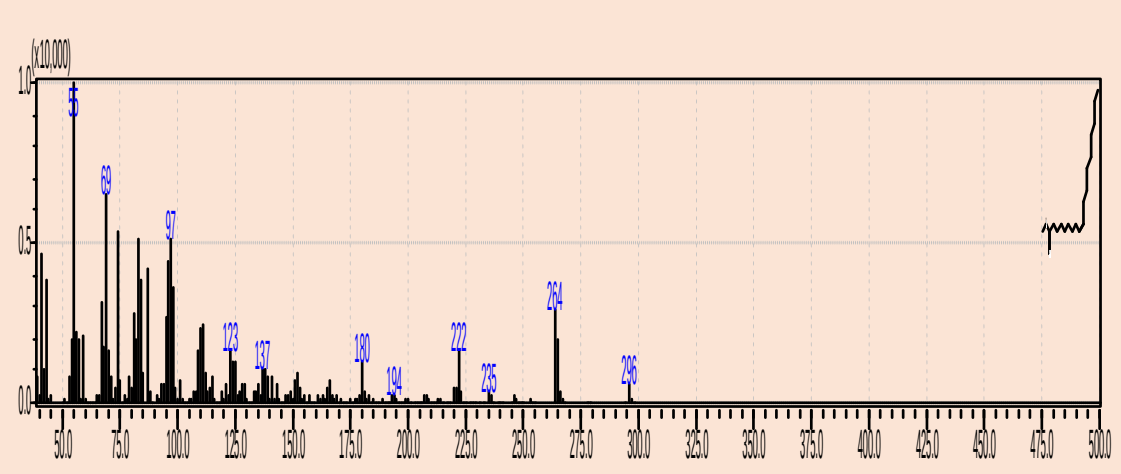


Fig 3.23: Mass spectrum of 9-Octadecenoic acid, hexadecyl ester

9, 12-Octadecadienoic acid, methyl ester (25.44%)

The EI mass spectrum of 9, 12-octadecadienoic acid, methyl ester is shown in (fig.3.24). The peak at m/z 294, which appeared at R.T. 17.053 corresponds $M^{+}[C_{19}H_{34}O_2]^{+}$ while the sighal at m/z 263 is due to loss of a methoxyl group.

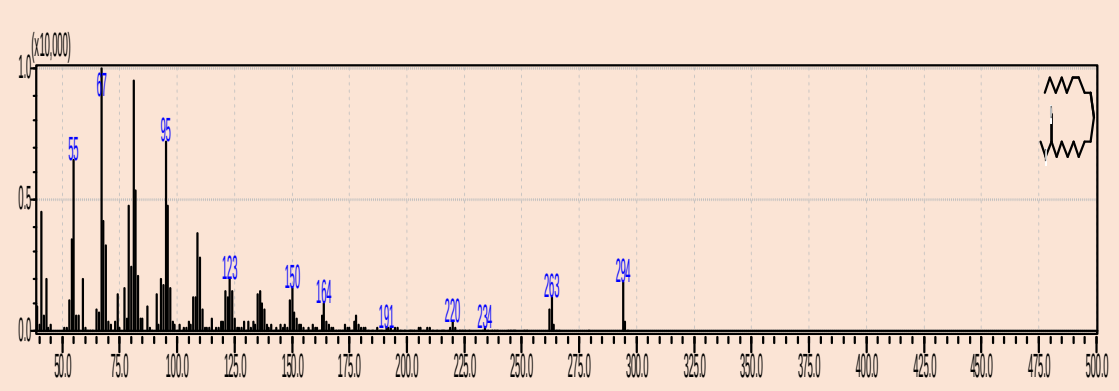


Fig 3.24: Mass spectrum of 9, 12- Octadecadienoic acid, methyl ester

9, 12, 15-Octadecatrienoic acid, methyl ester (19.73%)

Figure 3.25 shows the mass spectrum of 9, 12, 15-octadecatrienoic acid, methyl ester. The peak at m/z 292 - R.T. 17.164- in total ion chromatogram, corresponds the molecular ion : $M^{+}[C_{19}H_{32}O_2]^{+}$,while the sighal at m/z 261 accouts for loss of a methoxyl .

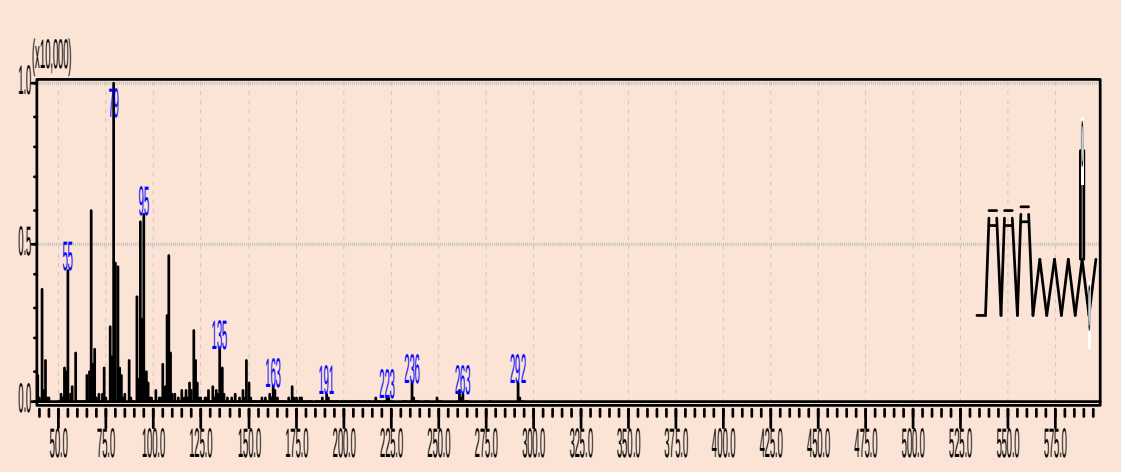


Fig 3.25: Mass spectrum of 9, 12, 15-Octadecatrienoic acid, methyl ester

Hexadecanoic acid, methyl ester (14.12%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.26). The moleculat ion : $M^{+}[C_{17}H_{34}O_2]^{+}$ appeared as expected at m/z 270(R.T. 15.392).The peak at m/z 239 is attributed to loss of a methoxyl .

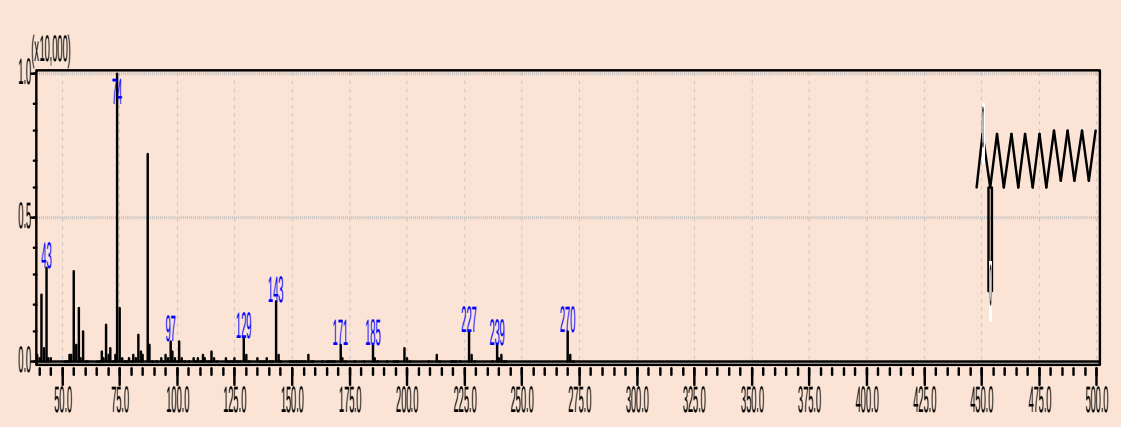


Fig 3.26: Mass spectrum of Hexadecanoic acid, methyl ester

Methyl stearate (11.20%)

The mass spectrum of methyl stearate is presented in (fig.3.27). The signal which appeared at m/z 298 -with R.T. 17.307 in total ion chromatogram - corresponds $M^{+}[C_{19}H_{38}O_2]^{+}$ .The peak at m/z 267 is attributed to loss of a methoxyl .

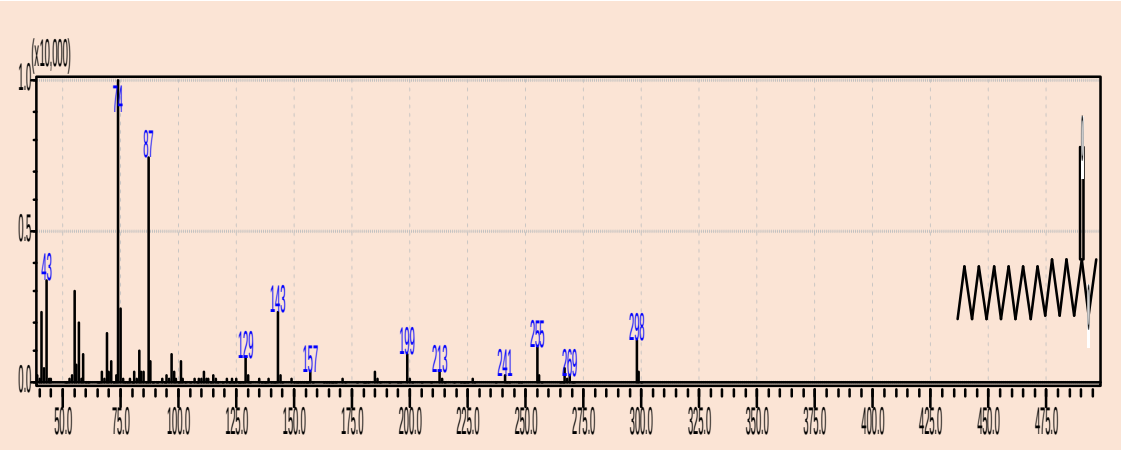


Fig 3.27: Mass spectrum of methyl stearate

3.4.2- Antioxidant activity

The antioxidant capacity of *Linum usitatissimum* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.14) *Linum usitatissimum* seed oil exhibited moderate antioxidant activity.

Table 3.14: Radical scavenging activity of *Linum usitatissimum* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Linum usitatissimum oil	48.00%

Conclusion

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

Two flavonoids (compounds I and II) were isolated from the stems of *Pulcaria crispa* .The partial structures of both compounds were deduced on the basis of their : IR, UV and NMR data and partial structures were suggested. Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried out . The results exhibited variable susceptibilities of microorganism for crude extract and different fractions. The antioxidant capacity of the alcoholic extract of *Pulcria crispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The oils were also assessed for their antimicrobial activity and promising results were obtained.

Recommendations

The following is highly recommended:

1. Other phytochemicals of the studied plant may be isolated and their structures may be elucidated and the biological activity could be screened.
2. The isolated falvonoids may be subjected to 2D NMR experiments for full characterization.
3. The isolated falvonoids and oil may be screened for other biological effects such as antiinflammatory, antidiabetic , antiviral, antilashmenial ..etc.

Sudan University of Science and Technology

College of Post Graduate Studies

Phytochemical Studies on the Stems of *Pulcria crispa* and Seeds of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* and Biological Activity of Constituents

دراسة فيتوكيميائية لسيقان نبات الربل وبذور نبات الرجلة والكسبرة والكتان والفعالية البيولوجية للمكونات

A Thesis Submitted in Fulfillment of the Requirements of the Ph.D. Degree in Chemistry

By

Lubna Akasha Abdelmalik Mohamed

(B.Sc. (Hons.) Chemical Laboratories, M.Sc. in Chemistry)

Supervisor

Prof: Mohamed Abdel Karim Mohamed

September, 2019

الإســـــــتـــهـــلال

﴿ بسم الله الرحمن الرحيم ﴾

فلينظر الإنسان إلى طعامه ﴿٢٤﴾ أنا
صببنا الماء صبا ﴿٢٥﴾ ثم شققنا
الأرض شقا ﴿٢٦﴾ فأنبتنا فيها حبا
﴿٢٧﴾ وعنبا وقضبا ﴿٢٨﴾ وزيتونا
ونخلا ﴿٢٩﴾ وحدائق غلبا ﴿٣٠﴾
وفاكهة وأبا ﴿٣١﴾ متاعا لكم
ولأنعامكم ﴿٣٢﴾ سورة عبس

Dedication

To,

My parents,

My sons,

And my sisters and brother

Acknowledgement

First and foremost, I thank **Almighty Allah, the Ever-Magnificent, the Ever-Thankful**, for his help and blessing.

I am grateful to some people, who worked hard with me from the beginning till the completion of the present research particularly my supervisor Dr. Mohamed Abdel karim, who has been always generous during all phases of the research, and I highly appreciate the efforts expended by my co-supervisor, Dr. Kamal Mohamed Saeed.

I owe a deep debt of gratitude to the Sudan University of Science and Technology for giving us an opportunity to accomplish this work. Thanks are extended to the Aromatic and Medicinal Plants Institute, Khartoum, Sudan for all facilities. Thanks for the National Research Center, Cairo for the spectral measurements.

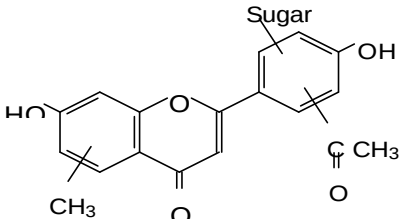
I would like to take this opportunity to say warm thanks to all my beloved friends, who have been so supportive along the way of doing my thesis.

I also would like to express my whole hearted thanks to my parents, for their generous support. They supported me throughout my entire life and particularly through the process of accomplishing the PhD degree.

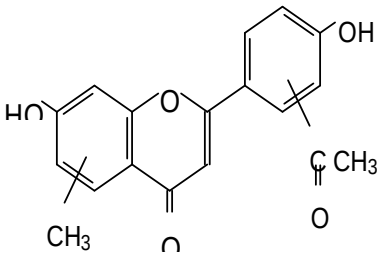
Abstract

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

The stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature. Qualitative tests on the alcoholic extract were positive for flavonoids. Conventional chromatographic techniques were used for isolation and purification of flavonoids. Two flavonoids (compounds I and II) were isolated from this plant. The partial structures of both compounds were deduced on the basis of their: IR, UV and NMR data and the following structures were suggested:



Compound I



Compound II

Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried. The results exhibited variable susceptibilities of microorganism for crude extract and different fractions.

In this study, the antioxidant capacity of the alcoholic extract of *Pulcriacrispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The GC-MS analysis of the *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* oils revealed the presence of 15, 14, 15 components respectively.

In the agar diffusion bioassay oils of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* failed to exhibit any antimicrobial activity. However, *Coriandrum sativam* oil showed significant antioxidant activity.

المستخلص

يشتمل البحث على دراسة كيميائية مفصلة للمحتوى الفلافونيدي لسيقان نبات الربل وكذلك التركيب الكيميائي لزيوت بذور كل من البقلة، الكزبرة وبذور الكتان. ايضا يتضمن البحث دراسة فعالية مستخلصات نبات الربل والزيوت المستخلصة كمضادات ميكروبات وكمضادات أكسدة.

لقد أستخلصت سيقان نبات الربل بواسطة الكحول الإيثيلي عند درجة الحرارة العادية و افاد المسح الفيتوكيميائى بوجود الفلافونيدات.

أنجز فصل المركبات بإستخدام كروماتوغرافيا العمود و كروماتوغرافيا الورق ، كما وتم التعرف على التركيب الكيميائي للمركبات المفصولة بإستخدام وسائل التحاليل الطيفية المختلفة : مطيافية الأشعة تحت الحمراء، الأشعة فوق البنفسجية ، الرنين النووي المغناطيسي للهيدروجين . وقد تم فصل مركبين ذوا طبيعة فلافونيدية من نبات الربل وهما المركب **II ,I.** تناولت الدراسة فعالية مستخلصات سيقان نبات الربل كمضادات ميكروبات وكذلك كمضادات أكسدة.

أثبت البحث ان مستخلص خلات اللإيثيل ذو فعالية كمضاد للبكتيريا. أما المستخلص الكحولي وبقية المستخلصات فقد اظهروا فعالية منخفضة كمضادات بكتيريا وفطرية. أوضحت الدراسة ان المستخلص الكحولي لنبات الريل ذو تاثير فعال كمضادات اكسدة.

اخضع زيت بذور البقلة، الكزبرة والكتان للكروماتوغرافيا الغازية - السائلة الملحقة بمطياف الكتلة . اوضحت هذه الدراسات وجود خمسة عشر ، أربعة عشر، وخمسة عشر مكونا بالزيت على التوالي.

تناولت الدراسة أيضا فعالية الزيوت المستخلصة من بذور البقلة ، الكزبرة والكتان كمضادات ميكروبات ولم تظهر النتائج أي تأثيريذكر. وأثبت البحث أن زيت بذور الكزبرة ذو فعالية عالية كمضاد أكسدة .

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INTRODUCTION

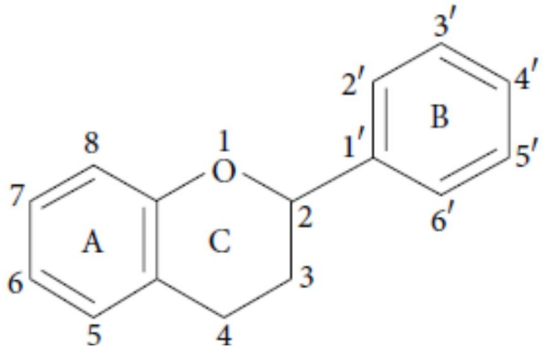
1.1 General overview

Flavonoids consist of a large group of polyphenolic compounds having a benzo-γ-pyrone structure and are ubiquitously present in plants. They are synthesized by phenylpropanoid pathway. Available reports tend to show that secondary metabolites of phenolic nature including flavonoids are responsible for a variety of pharmacological activities [1, 2]. Flavonoids are hydroxylated phenolic substances and are known to be synthesized by plants in response to microbial infection [3]. Their activities are structure dependent. The chemical nature of flavonoids depends on their structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization [4]. Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions [5, 6]. The chelation of metals could be crucial in the prevention of radical generation which damage target biomolecules [7, 8].

As a dietary component, flavonoids are thought to have health-promoting properties due to their high antioxidant capacity both *in vivo* and *in vitro* systems [9, 10]. Flavonoids have ability to induce human protective enzyme systems.

The number of studies has suggested protective effects of flavonoids against many infectious (bacterial and viral diseases) and degenerative diseases such as cardiovascular diseases, cancers, and other age-related diseases [2, 8-

[10].Flavonoids also act as a secondary antioxidant defense system in plant tissues exposed to different abiotic and biotic stresses. Flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation. They also regulate growth factors in plants such as auxin [11]. Biosynthetic genes have been assembled in several bacteria and fungi for enhanced production of flavonoids [12].The basic nucleus of flavonoids is displayed in Fig. 1.



(1)

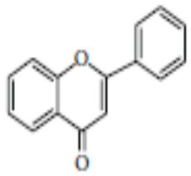
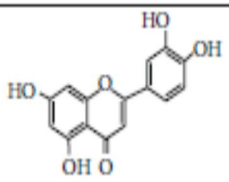
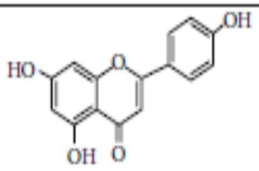
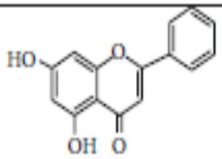
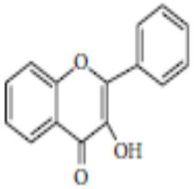
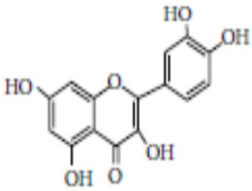
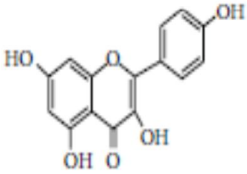
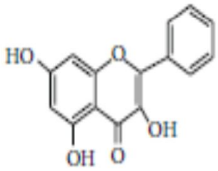
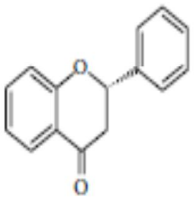
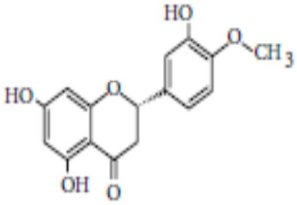
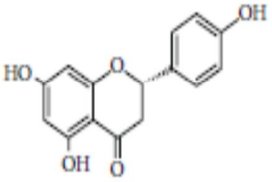
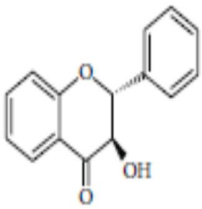
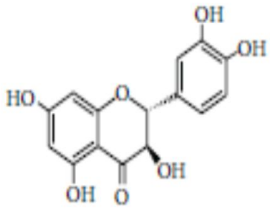
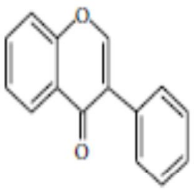
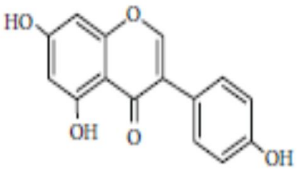
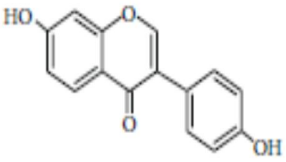
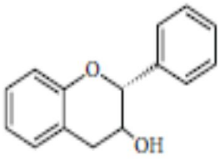
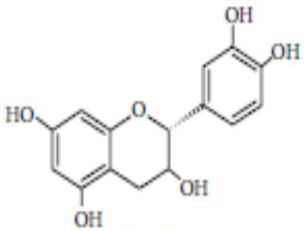
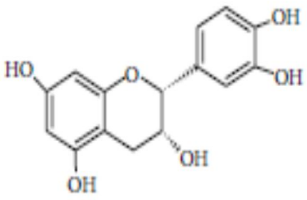
1.2 Chemistry of Flavonoids

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. In 1930 a new substance was isolated from orange. At that time it was believed to be a member of a new class of vitamins and was designated as vitamin P. Later on it became clear that this substance was a flavonoid (rutin) and till now more than 4000 varieties of flavonoids have been identified [13].

Chemically flavonoids are based upon a fifteen-carbon skeleton consisting of two benzene rings (A and B as shown in Figure 1) linked via a heterocyclic pyrane ring (C). They can be divided into a variety of classes such as flavones (e.g. apigenin, and luteolin), flavonols (e.g., quercetin, kaempferol, myricetin, and fisetin), flavanones (e.g., flavanone, hesperetin, and naringenin), and others.

Their general structures are shown in Table 1. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within a class differ in the pattern of substitution of the A and B rings [13].

Table (1): Structure of flavonoids

Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Flavonoids occur as aglycones, glycosides, and methylated derivatives. The basic flavonoid structure is aglycone (Figure 1.1). Six-member ring condensed with the benzene ring is either a α-pyrone (flavonols and flavanones) or its dihydro derivative (flavonols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

Flavonols differ from flavanones by hydroxyl group at the 3-position and a C_2–C_3 double bond[14]. Flavonoids are often hydroxylation positions 3, 5, 7, 2, 3', 4', and 5'.Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose[15].

1.2.1 Spectral Characteristics of Flavonoids

Studies on flavonoids by spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320–385nm) represents the B ring absorption, while Band II (235–285 nm) corresponds to the A ring absorption.

Functional groups attached to the flavonoid skeleton may cause a shift in absorption such as from 367 nm in kaempferol (3,5,7,4-hydroxyl groups) to 371 nm in quercetin (3,5,7,3',4'- hydroxyl groups) and to 374 nm in myricetin (3,5,7,3',4',5'- hydroxyl groups) [16]. The absence of a 3-hydroxyl group in flavones distinguishes them from flavonols. Flavanones have a saturated heterocyclic C ring, with no conjugation between the A and B rings, as determined by their UV spectral characteristics [17]. Flavanones exhibit a very strong (Band II) absorption maximum between 270 and 295 nm, namely, 288 nm (naringenin) and 285 nm (taxifolin), and only a shoulder for (Band I) at 326 and 327 nm. Band II appears as one peak (270

nm) in compounds with a mono substituted B ring, but as two peaks or one peak (258 nm) with a shoulder (272 nm) when a di-, tri-, or *o*-substituted B ring is present.

As anthocyanins show distinctive (Band I) peak in the 450–560 nm region due to hydroxyl cinnamoyl system of the B ring and (Band II) peaks in the 240–280nmregion due to the benzoyl system of the A ring, the colour of the anthocyanins varies with the number and position of the hydroxyl groups [18].

1.3 Flavonoid- Rich Food and Medicinal Plants

Flavonoids are the most common and widely distributed group of plant phenolic compounds, occurring virtually in all plant parts, particularly the photo-synthesizing plant cells.

They are a major coloring component of flowering plants. Flavonoids are an integral part of human and animal diet. Some food sources containing different classes of flavonoids are given in Table 2. Being phytochemicals, flavonoids cannot be synthesized by humans and animals [19].Thus flavonoids found in animals are of plant origin rather than being biosynthesized *in situ*. Flavonols are the most abundant flavonoids in foods. Flavonoids in food are generally responsible for colour, taste, prevention of fat oxidation, and protection of vitamins and enzymes [20].

Flavonoids found in the highest amounts in the human diet include the soy isoflavones, flavonols, and the flavones. Although most fruits and some legumes contain catechins, the levels vary from 4.5 to 610mg/kg [21]. Preparation and processing of food may decrease flavonoid levels depending on the methods used.

For example, in a recent study, orange juices were found to contain 81–200mg/L soluble flavanones, while the content in the cloud was 206–644mg/L which suggest that the flavanones are concentrated in the cloud

during processing and storage [22]. Accurate estimation of the average dietary intake of flavonoids is difficult, because of the wide varieties of available flavonoids and the extensive distribution in various plants and also the diverse consumption in humans [23].

Recently there has been an upsurge of interest in the therapeutic potential of medicinal plants which might be due to their phenolic compounds, specifically to flavonoids [24, 25]. Flavonoids have been consumed by humans since the advent of human life on earth, that is, for about 4 million years. They have extensive biological properties that promote human health and help reduce the risk of diseases.

Table (2): Classification, structure, and food sources of some dietary flavonoids

Class	Flavonoid	Dietary source
Flavanol	(+)-Catechin (−)-Epicatechin Epigallocatechin	Tea
Flavone	Chrysin, apigenin Rutin, luteolin, and luteolin glucosides	Fruit skins, red wine, buckwheat, red pepper, and tomato skin
Flavonol	Kaempferol, quercetin, myricetin, and tamarixetin	Onion, red wine, olive oil, berries, and grapefruit.
Flavanone	Naringin, naringenin, taxifolin, and hesperidin	Citrus fruits, grapefruits, lemons, and oranges
Isoflavone	Genistin, daidzin	Soyabean
Anthocyanidin	Apigenidin, cyanidin	Cherry, easberry, and strawberry

Oxidative modification of LDL cholesterol is thought to play a key role during atherosclerosis. The isoflavone – glabridin- a major polyphenolic compound found in *Glycyrrhiza glabra* (Fabaceae), inhibits LDL oxidation via a mechanism involving scavenging of free radicals [26]. Several epidemiologic studies have suggested that drinking either green or black tea may lower blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease. Flavonoids are also known to influence the quality and stability of foods by acting as flavorants, colorants, and antioxidants [27, 28]. Flavonoids contained in berries may have a positive effect against Parkinson's disease and may help to improve memory in elderly people.

Antihypertensive effect has been observed in total flavonoid fraction of *Astragalus complanatus* in hypertensive rats [29].

Intake of antioxidant flavonoids has been inversely related to the risk of incidence of dementia [30]. Table 3 summarizes some of the medicinal plants rich in flavonoid contents.

Table (3): Medicinal plants rich in flavonoids contents

Plant	Family	Flavonoid
Aloe vera	Asphodelaceae	Luteolin
Acalypha indica	Euphorbiaceae	Kaempferol glycosides
Azadirachta indica	Meliaceae	Quercetin
Andrographis paniculata	Acanthaceae	5-hydroxy-7,8-dimethoxyflavone
Bacopa moneirra	Scrophulariaceae	Luteolin
Betula pendula	Betulaceae	Quercetrin
Butea monospermea	Fabaceae	Genistein

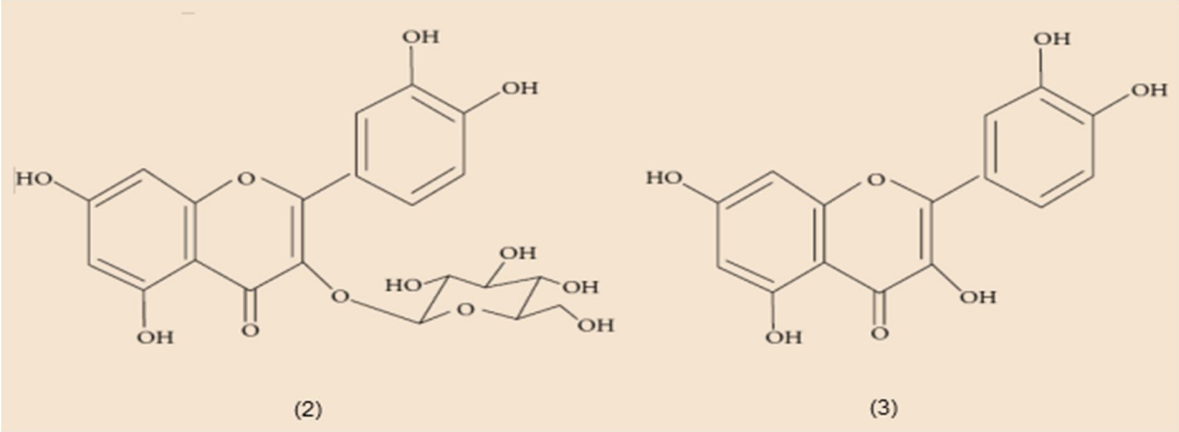
Bauhinia monandra	Fabaceae	Quercetin-3-O-rutinoside
Brysonima crassa	Malphigaceae	(+)-catechin
Calendula officinalis	Compositae	isorhamnetin
Cannabis sativa	Compositae	Quercetin
Citrus medica	Rutaceae	hesperidin
Clerodendrum phlomidis	Verbenaceae	Pectolinarigenin
Clitoria ternatea	Fabaceae	kaempferol-3-neohesperidoside
Glyccheriza glabra	Leguminosae	Liquiritin
Mimosa pudica	Mimosoideae	Isoquercetin
Limnophila indica	Scrophulariaceae	3,4-methlenedioxyflavone
Mentha longifolia	Lamiaceae	Luteolin-7-O-glycoside
Momordica charantia	Curcurbitaceae	Luteolin
Oroxylum indicum	Bignoniaceaea	Chrysin
Passiflora incarnate	Passifloraceae	Vitexin
Pongamia pinnata	Fabaceae	Pongaflavonol
Tephrosia purpurea	Fabaceae	Purpurin
Tilia cordata	Tiliaceae	hyperoside

Solubility may play major role in the therapeutic efficacy of flavonoids. Low solubility of flavonoid aglycones in water coupled with its short residence time in the intestine as well as its lower absorption does not allow humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy. The low solubility of the flavonoids in water often presents a problem for its medicinal applications. Hence, the development of semisynthetic, water-soluble flavonoids, for

example, hydroxy ethyl rutosides and inositol-2-phosphatequercetin, has been implicated for the treatment of hypertension and micro bleeding [31].

1.4 Metabolism of Flavonoids in Humans

The absorption of the dietary flavonoids liberated from the food by chewing will depend on its physicochemical properties such as molecular size, configuration, lipophilicity, solubility, and p^{Ka}. The flavonoid can be absorbed from the small intestine or has to go to the colon before absorption. It may depend upon structure of flavonoid, that is, whether it is glycoside or aglycone. Most flavonoids, except for the subclass of catechins, are present in plants bound to sugars as glycosides (Figures 2 and 3). Aglycones can be easily absorbed by the small intestine, while flavonoid glycosides have to be converted into aglycone form before absorption [32].



The hydrophilic flavonoid glucoside such as quercetin are transported across the small intestine by the intestinal Na^+-dependent glucose cotransporter (SGLT1) [32].

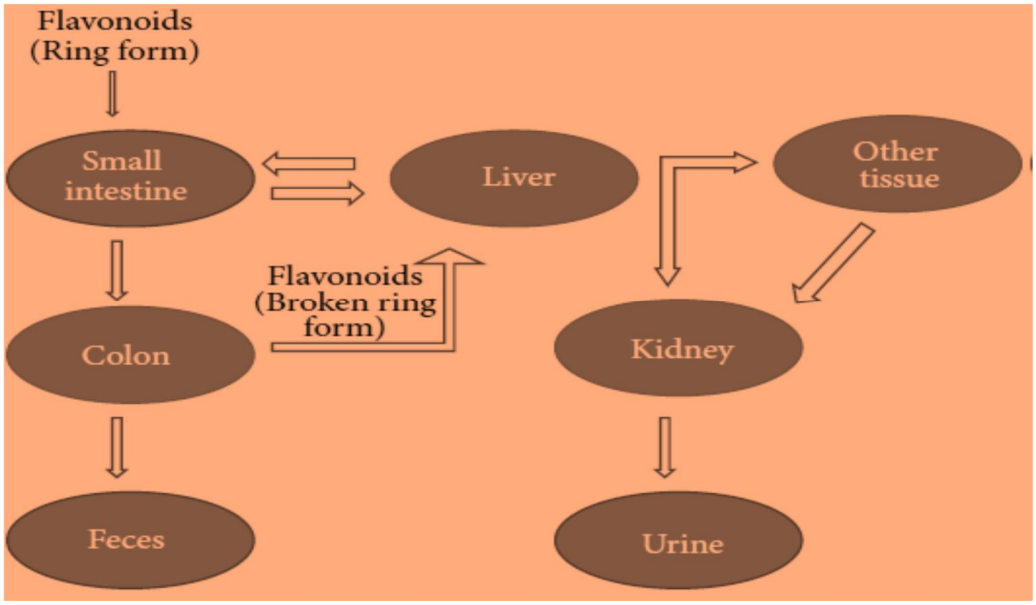
An alternative mechanism suggests that flavonoid glucosides are hydrolyzed by lactase phloridzin hydrolase (LPH), a β-glucosidase on the outside of the brush border membrane of the small intestine. Subsequently, the liberated aglycone can be absorbed across the small intestine [33]. The substrate

specificity of this LPH enzyme varies significantly in a broad range of glycosides (glucosides, galactosides, arabinosides, xylosides, and rhamnosides) of flavonoids [34].

The glycosides which are not substrates for these enzymes are transported toward the colon where bacteria have ability to hydrolyze flavonoid glycosides, but simultaneously they will also degrade the liberated flavonoid aglycones [35]. Since absorption capacity of the colon is far less than that of the small intestine, only trivial absorption of these glycosides is to be expected.

After absorption, the flavonoids are conjugated in the liver by glucuronidation, sulfation, or methylation or metabolized to smaller phenolic compounds [36]. Due to these conjugation reactions, no free flavonoid aglycones can be found in plasma or urine, except for catechins [37]. Depending on the food source bioavailability of certain flavonoids differs markedly; for example, the absorption of quercetin from onions is fourfold greater than that from apple or tea [38]. The flavonoids secreted with bile in intestine and those that cannot be absorbed from the small intestine are degraded in the colon by intestinal micro flora which also break down the flavonoid ring structure (Sceme 1). Oligomeric flavonoids may be hydrolyzed to monomers and dimers under influence of acidic conditions in the stomach. Larger molecules reach the colon where they are degraded by bacteria. The sugar moiety of flavonoid glycosides is an important determinant of their bioavailability. Dimerization has been shown to reduce bioavailability. Among all the subclasses of flavonoids, isoflavones exhibit the highest bioavailability [39]. After ingestion of green tea, flavonoid content is absorbed rapidly as shown by their elevated levels in plasma and urine.

They enter the systemic circulation soon after ingestion and cause a significant increase in plasma antioxidant status [40].



Sheme 1: Compartments involved in the metabolism of flavonoid

1.5 Biological Activities of Flavonoids

1.5.1 Antioxidant Activity

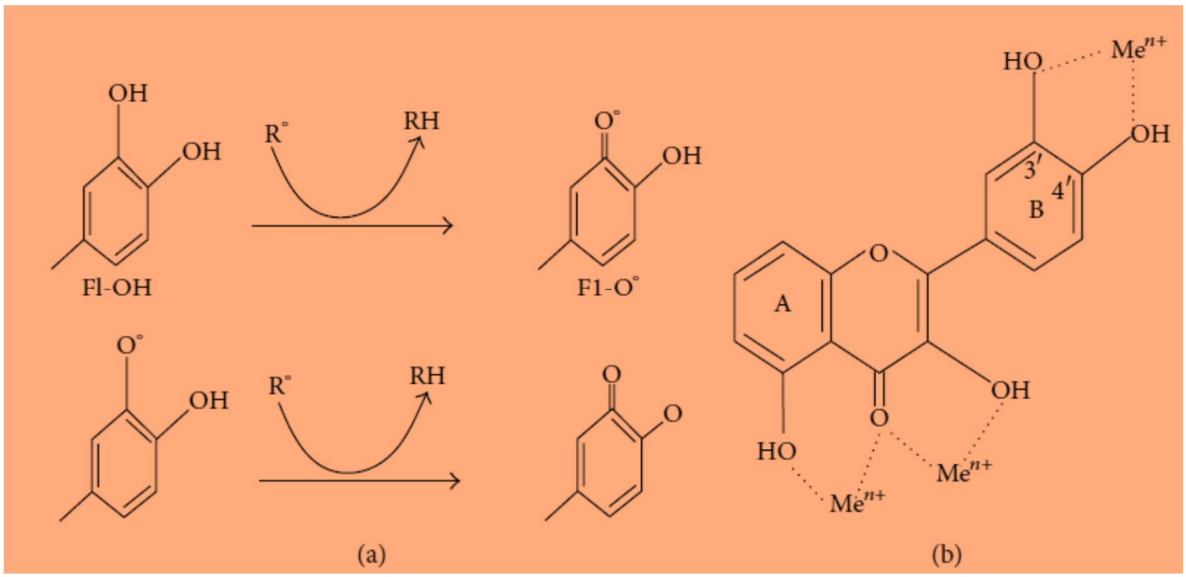
Flavonoids possess many biochemical properties, but the best described property of almost every group of flavonoids is their capacity to act as antioxidants.

The antioxidant activity of flavonoids depends upon the arrangement of functional groups about the nuclear structure.

The configuration, substitution, and total number of hydroxyl groups substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability [4, 41]. The B ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxyl, and

peroxynitrite radicals, stabilizing them and giving rise to a relatively stable flavonoids radical [42].

Mechanisms of antioxidant action can include (1) suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation;(2) Scavenging ROS; and (3) upregulation or protection of antioxidant defenses [43, 44]. Flavonoid action involves most of the mechanisms mentioned above. Some of the effects mediated by them may be the combined result of radical scavenging activity and the interaction with enzyme functions. Flavonoids inhibit the enzymes involved in ROS generation, that is, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase, NADH oxidase, and so forth [45]{see scheme 2}.



Scheme (2): (a) Scavenging of ROS (R∘) by flavonoids (Fl-OH) and

(b) binding sites for trace metals where Men+ indicates metal ions

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanisms [5, 25]. Free

metal ions enhance ROS formation by the reduction of hydrogen peroxide with generation of the highly reactive hydroxyl radical. Due to their lower redox potentials flavonoids (OH) are thermodynamically able to reduce highly oxidizing free radicals (redox potentials in the range 2.13–1.0V) such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation (Scheme 2(a)). Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation [44, 46]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties. Trace metals bind at specific positions of different rings of flavonoid structures [47]. The binding sites are shown in Schme 2(b). A 3', 4'-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trait of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [4]. Epicatechin and rutin are strong radical scavengers and inhibitors of lipid peroxidation *in vitro* [48]. Because of oxidation on the B ring of flavonoids having catechol group a fairly stable *ortho* semiquinone radical is formed which is strong scavengers. Flavones lacking catechol system on oxidation lead to formation of unstable radicals exhibit weak scavenging potential [49]. The literature shows that flavonoids having an unsaturated 2-3 bond in conjugation with a 4-oxo function are more potent antioxidants than the flavonoids lacking one or both features. Conjugation between the A and B rings allows a resonance effect of the aromatic nucleus that provides stability to the flavonoid radical. Free radical scavenging by flavonoids is potentiated by the presence of both the elements besides other structural features [50].

The flavonoid heterocyclic contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH is crucial. Removal of a 3-OH annuls coplanarity and conjugation which

compromises scavenging ability [51]. It is proposed that B ring OH groups form hydrogen bonds with the 3-OH, aligning the B ring with the heterocycle and A ring. Due to this intramolecular hydrogen bonding, the influence of a 3-OH is enhanced by the presence of a 3',4'-catechol, elucidating the potent antioxidant activity of flavan-3-ols and flavon-3-ols that possess the latter feature. Generally O-methylation of hydroxyl groups of flavonoids decreases their radical scavenging capacity [50].

Occurrence, position, structure, and total number of sugar moieties in flavonoid (flavonoids glycosides) play an important role in antioxidant activity. Aglycones are more potent antioxidants than their corresponding glycosides.

There are reports that the antioxidant properties of flavonol glycosides from tea declined as the number of glycosidic moieties increased [52]. Though glycosides are usually weaker antioxidants than aglycones, bioavailability is sometimes enhanced by a glucose moiety. In the diet, flavonoid glycosidic moieties occur most frequently at the 3- or 7-position [53]. Increasing degree of polymerization enhances the effectiveness of procyanidins against a variety of radical species. Procyanidin dimers and trimers are more effective than monomeric flavonoids against superoxide anion. Tetramers exhibit greater activity against peroxynitrite and superoxide mediated oxidation than trimers, while heptamers and hexamers demonstrate significantly greater superoxide scavenging properties than trimers and tetramers [54].

1.5.2 Hepatoprotective Activity

Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [55].

Different chronic diseases such as diabetes may lead to development of hepatic clinical manifestations. Glutamate-cysteine ligase catalytic subunit

(Gclc) expression, glutathione, and ROS levels are reported to be decreased in liver of diabetic mice. Anthocyanins have drawn increasing attention because of their preventive effect against various diseases [56]. It has been demonstrated that anthocyanin cyanidin-3-O-β- glucoside increases Gclc transcription. Increased Gclc expression results in a decrease in hepatic ROS levels and pro-apoptotic signaling. Furthermore, this flavonoid lowers hepatic lipid peroxidation, inhibits the release of pro-inflammatory cytokines, and protects against the development of hepatic steatosis [56]. Silymarin is a flavonoid having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle *Silybum marianum* (Compositae). Silymarin has been reported to stimulate enzymatic activity of DNA-dependent RNA polymerase 1 and subsequent biosynthesis of RNA and protein, resulting in DNA biosynthesis and cell proliferation leading to liver regeneration only in damaged livers [57-59].

Hepatoprotective activities were observed in flavonoids isolated from *Laggera alata* against carbon-tetrachloride (CCl4-) induced injury. Flavonoids at a concentration range of 1–100 μg/mL improved cell viability and inhibited cellular leakage of hepatocyte aspartate aminotransferase (AST) and alanine aminotransferase (ALT) caused by CCl4 [60]. Similarly in an *in vivo* experiment flavonoids at of 50, 100, and 200mg/kg oral doses significantly reduced the levels of AST, ALT, total protein, and albumin in serum and the hydroxyproline and sialic acid levels in liver.

Histopathological examinations also revealed the improvement in damaged liver with the treatment of flavonoid [60].

Several clinical investigations have shown the efficacy and safety of flavonoids in the treatment of hepatobiliary dysfunction and digestive

complaints, such as sensation of fullness, loss of appetite, nausea, and abdominal pain. *Equisetum arvense* flavonoids as well as hirustrin and avicularin isolated from some other sources is reported to provide protection against chemically induced hepatotoxicity in HepG2 cells [61, 62].

1.5.3 Antibacterial Activity

Flavonoids are known to be synthesized by plants in response to microbial infection; thus it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Flavonoid- rich plant extracts from different species have been reported to possess antibacterial activity [44, 46, 63, 64]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [65].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation.

Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes [66, 67].

Catechins, the most reduced form of the C3 unit in flavonoid compounds, have been extensively researched due to their antimicrobial activity. These compounds are reported for their *in vitro* antibacterial activity against *Vibrio cholerae*, *Streptococcus mutans*, *Shigella*, and other bacteria [68, 69].

The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S. mutans*, probably due to complexing activities [68, 70]. Robinetin, myricetin, and (−)-epigallocatechin

are known to inhibit DNA synthesis in *Proteus vulgaris.* Mori et al. [71] suggested that the B ring of the flavonoids may interchelate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3',4'-pentahydroxyflavone against *Escherichia coli* DNA gyrase [72].

Naringenin and sophoraflavanone G have intensive antibacterial activity against methicillin resistant *Staphylococcus aureus* (MRSA) and streptococci. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner layers of membranes [73]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells.

The 5,7-dihydroxylation of the A ring and 2',4'-or 2',6'- dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity [74]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C8 and C10 chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [75].

Osawa et al. have shown that 5-hydroxyflavanones and 5-hydroxyisoflavanones with one, two, or three additional hydroxyl groups at the 7, 2' and 4' positions inhibited the growth of *S. mutans* and *Streptococcus sobrinus* [76].

Haraguchi and colleagues [74] studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus.* They observed that licochalcone A

inhibited incorporation of radioactive precursors intomacromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome *c* in the bacterial respiratory electron transport chain [74].

There are many examples that lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents [77–79].

1.5.4 Anti-Inflammatory Activity

Inflammation is a normal biological process in response to tissue injury, microbial pathogen infection, and chemical irritation. Inflammation is initiated by migration of immune cells from blood vessels and release of mediators at the site of damage. This process is followed by recruitment of inflammatory cells, release of ROS, RNS, and pro-inflammatory cytokines to eliminate foreign pathogens, and repairing injured tissues. In general, normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders [80].

The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals. Certain members of flavonoids significantly affect the function of the immune system and inflammatory cells [81]. A number of flavonoids such as hesperidin, apigenin, luteolin, and quercetin are reported to possess anti-inflammatory and analgesic effects. Flavonoids may affect specifically the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein

kinases [82, 83]. The inhibition of kinases is due to the competitive binding of flavonoids with ATP at catalytic sites on the enzymes. These enzymes are involved in signal transduction and cell activation processes involving cells of the immune system. It has been reported that flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes, and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules [84]. Flavonoids also inhibit phosphodiesterases involved in cell activation. Much of the anti-inflammatory effect of flavonoid is on the biosynthesis of protein cytokines that mediate adhesion of circulating leukocytes to sites of injury. Certain flavonoids are potent inhibitors of the production of prostaglandins, a group of powerful pro-inflammatory signaling molecules[85]. Reversal of the carrageenan induced inflammatory changes has been observed with silymarin treatment. It has been found that quercetin inhibit mitogen stimulated immunoglobulin secretion of IgG, IgM, and IgA isotopes *in vitro* [86].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10mM concentration [87]. The effect of flavonoid on platelets has been related to the inhibition of arachidonic acid metabolism by carbon monoxide [88]. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function.

1.5.5 Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemopreventive agents [46, 89]. Consumption of onions and/or apples, two

major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon [90].The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods [91].

Several mechanisms have been proposed for the effect of flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [92]. Major molecular mechanisms of action of flavonoids are given as follows:

(1) down regulation of mutant p53 protein,

(2) cell cycle arrest,

(3) tyrosine kinase inhibition,

(4) inhibition of heat shock proteins,

(5) estrogen receptor binding capacity,

(6) inhibition of expression of Ras proteins.

Mutations of p53 are among the most common genetic abnormalities in human cancers. The inhibition of expression of p53 may lead to arrest the cancer cells in the G2-M phase of the cell cycle. Flavonoids are found to down regulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [93]. Tyrosine kinases are a family of proteins located in or near the cell membrane involved in the transduction of growth factor signals to the nucleus. Their expression is thought to be involved in cogenesis via an ability to override normal regulatory growth control. Drugs inhibiting tyrosine kinase activity are thought to be possible antitumor

agents without the cytotoxic side effects seen with conventional chemotherapy. Quercetin was the first tyrosine kinase inhibiting compound tested in a human phase I trial [94]. Heat shock proteins form a complex with mutant p53, which allows tumor cells to bypass normal mechanisms of cell cycle arrest. Heat shock proteins also allow for improved cancer cell survival under different bodily stresses. Flavonoids are known to inhibit production of heat shock proteins in several malignant cell lines, including breast cancer, leukemia, and colon cancer [93].

Recently it has been shown that the flavanol epigallocatechin-3-gallate inhibited fatty acid synthase (FAS) activity and lipogenesis in prostate cancer cells, an effect that is strongly associated with growth arrest and cell death [90, 95]. In contrast to most normal tissues expression of FAS is markedly increased in various human cancers. Up regulation of FAS occurs early in tumor development and is further enhanced in more advanced tumors [96].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included P- 388 leukemia cells, gastric cancer cells (HGC-27, NUGC-2, NKN-7, andMKN-28), colon cancer cells (COLON320DM), human breast cancer cells, human squamous and gliosarcoma cells, and ovarian cancer cells [97] . It has been proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent [98].

Barnes [99] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In a study to determine effects of isoflavones

genistein, daidzein, and biochanin A on mammary carcinogenesis, genistein was found to suppress the development of chemically induced mammary cancer without reproductive or endocrinological toxicities. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats [100]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats [101]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al. [102]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity [103]. A carbonyl function at C-4 of the flavone nucleus was found to be essential for their activity. Flavone-8-acetic acid has also been shown to have antitumor effects [104]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10- epoxide-2 on mouse skin [105].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk [106]. It is well known that due to oxidative stress cancer initiation may take place and thus potent antioxidants show potential to combat progression of carcinogenesis. Potential of antioxidant as an anticancer agent depends on its competence as an oxygen radical inactivator and inhibitor [44, 46, 107].Therefore diets rich in radical scavengers would diminish the cancer-promoting action of some radicals [108].

1.5.6 Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral

activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available.

Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso [109] demonstrated that flavan-3-o1 was more effective than flavones and flavonones in selective inhibition of HIV-1, HIV-2, and similar immunodeficiency virus infections. Baicalin, a flavonoid isolated from *Scutellaria baicalensis* (Lamieaceae), inhibits HIV-1 infection and replication. Baicalein and other flavonoids such as robustaflavone and hinokiflavone have also been shown to inhibit HIV-1 reverse transcriptase [110].

Table (4): Antiviral activity of various flavonoids

Flavonoid	**Virus**
Quercetin	Rabies virus, herpes virus, parainfluenza virus, polio virus, mengo virus, and pseudorabies virus
Rutin	Parainfluenza virus, influenza virus, and potato virus
Apigenin	Immunodeficiency virus infection, Herpes simplex virus type, and Auzesky virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

Another study revealed inhibition of HIV-1 entry into cells expressing CD4 and chemokine coreceptors and antagonism of HIV-1 reverse transcriptase by the flavone *O*-glycoside [111]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase [110]. It has also been reported that the flavonoids chrysin, acacetin, and apigenin prevent HIV-1 activation via a novel mechanism that probably involves inhibition of viral transcription [112].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV).

Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [110]. Studies have displayed that flavonols are more active than flavones against herpes simplex virus type 1 and the activity order was found to be galangin, kaempferol, and quercetin [110].

Zandi et al. [113] studied the antidengue virus properties of quercetin, hesperetin, naringin, and daidzein at different stages of DENV-2 (dengue virus type-2) infection and replication cycle. Quercetin was found to be most effective against DENV-2 in Vero cells. Many flavonoids, namely, dihydroquercetin, dihydrofisetin, leucocyanidin, pelargonidin chloride, and catechin, show activity against several types of virus including HSV, respiratory syncytial virus, polio virus and Sindbis virus [109]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [113]. List of some flavonoids and their efficacy against viruses is given in Table 4.

1. 6 Role of Flavonoids in Plants

1.6.1 Combating Oxidative Stress

Flavonoids have long been reported as serving multiple functions in plants [114].Various abiotic and biotic factors helps in the generation of ROS in plants leading to oxidative stress. Flavonoid biosynthesis in plants is almost exclusively enhanced due to oxidative stress. They have capacity to absorb the most energetic solar wavelengths (i.e., UV-B and UV-A), inhibit the generation of ROS, and quench ROS once they are formed [115]. Flavonoids discharged primary UV-B screening functions when early plants moved from water to colonize the land. Extent of antioxidant capacity and ability to absorb UV-wavelengths depends upon the nature of substitution on different rings of flavonoids. Dihydroxy B ring substituted flavonoids have a greater antioxidant capacity, while their monohydroxy B ring substituted counterparts have greater ability to absorb UV wavelengths [115].

The most reactive hydroxyl groups (7-OH in flavones or the 3-OH in flavonols) in flavonoids are generally glycosylated. Glycosylation increases solubility in the aqueous cellular environment, protects the reactive hydroxyl groups from autooxidation [116], and allows the transport of flavonoids from the endoplasmic reticulum to various cellular compartments and their secretion to the plasma membrane and the cell wall [117]. Recent evidence shows that antioxidant flavonoids are located in the nucleus of mesophyll cells and within centers of ROS generation, that is, the chloroplast. Here they can easily quench H_2O_2, hydroxyl radical, and singlet oxygen [115, 118].

Oxidative stress due to an excess of excitation energy in the chloroplast may be aggravated under conditions that limit the diffusion of CO_2 to the carboxylation sites and the efficiency of carboxylation [115, 119]. The environmental limitations to CO_2 assimilation rate include drought/salinity,

low/high temperature, and nutrient scarcity. Under these conditions the activity of ROS detoxifying enzymes may significantly reduce in the chloroplast [120, 121], which in turn up regulates the biosynthesis of ROS scavenging flavonoids.

The reducing functions of flavonoids are of key significance in plants under severe stress conditions. These functional roles are concomitant with the very high concentration of dihydroxy B ring substituted flavonoids [122]. Flavonoids have been suggested as representing a secondary antioxidant defense system in plant tissues exposed to different stresses [115]. Lipid peroxidation is the common consequence of oxidative stress which disrupts the cell membrane integrity.

Quercetin 3-*O*-rutinoside (rutin) may interact with the polar head of phospholipids at water lipid interface, enhancing membrane rigidity and consequently protecting membranes from oxidative damage [123].

1.6.2 Growth Regulator

Flavonoids carry out functional roles of amazing significance in plant-environment interactions.

Flavonoids (in the nanomolar range) may regulate auxin movement and catabolism. The ability of flavonoids to create auxin gradients translates into phenotypes with different morphoanatomical features [124]. The control of flavonoids on auxin movement may have immense value in the stress-induced morphogenic responses of plants such as the flight strategy of sessile organisms exposed to unfavorable environments [125]. Species rich in dihydroxy flavonoids exhibit phenotypes with strikingly different morphological traits as compared with those rich in monohydroxy flavonoids [126]. Dwarf bushy phenotypes with few, small, and thick leaves to direct sunlight irradiance are usually present in sunny environments, thus

protecting leaves located deep in the canopy from light-induced severe cellular homeostasis perturbations. On the contrary, shaded plants, which are rich in kaempferol and/or apigenin derivatives (having negligible concentrations of quercetin derivatives), have long internodes, and large leaf lamina coupled with reduced leaf thickness [125].

Flavonoids at the plasma membrane are effective inhibitors of PIN (pin formed) and MDR (multidrug resistance) glycoproteins that are involved in the cell to cell movement of auxin. The ability of flavonoids to inhibit the activity of the efflux facilitator PIN and MDR proteins depends on the presence of the catechol group in the B ring of the flavonoid skeleton. In addition flavonoids regulate the activity of IAA-oxidase with markedly different effects based on their chemical structure [127]. Recent evidence of a nuclear location of flavonoids (as well as of enzymes of flavonoid biosynthesis) supports that flavonoids are capable of modulating the activity of proteins involved in cell growth. Flavonoids may therefore act as transcriptional regulators [128, 129].

1.7 Microbial Production of Flavonoids

In response to the low production efficiency from plants and chemical synthesis, research groups have directed their attention to the production of flavonoids in microorganisms using metabolic engineering and synthetic biology [130].

Chemical synthesis of flavonoids requires extreme reaction conditions and toxic chemicals [131]. Because of the rapid development in molecular biology tools and the flooding of genome information from a variety of organisms, combinatorial biosynthesis offers an advantage for production of rare and expensive natural products. It can be used in both simple and complex transformations without the tiresome blocking and deblocking steps that are

common in organic synthesis [132]. Several prokaryotes and eukaryotes such as *E. coli*, *Saccharomyces cerevisiae*, *Streptomyces venezuelae*, and *Phellinus igniarius,* a medicinal mushroom, have been used for production of flavonoids [12].

1.7.1 Phenylpropanoid Pathway

In plants naringenin chalcone is the precursor for a large number of flavonoids produced by the phenylpropanoid synthetic pathway. Fermentative production by *E. coli* carrying an artificially assembled phenylpropanoid pathway was the first example to show that a nearly complete biosynthetic pathway in plants was established in a heterologous microorganism for production of flavanones from the amino acid precursors, phenylalanine, and tyrosine [133]. As the first step in the phenylpropanoid pathway in plants, phenylalanine is deaminated to yield cinnamic acid by the action of phenylalanine ammonialyase (PAL). Cinnamic acid is hydroxylated by cinnamate-4-hydroxylase (C4H) to *p*-coumaric acid, which is then activated to *p*-coumaroyl-CoA by the action of 4-coumarate:
CoA ligase. Chalcone synthase (CHS) catalyzes the stepwise condensation of three acetate units from malonyl-CoA with *p*-coumaroyl-CoA to yield naringenin chalcone. Naringenin chalcone is converted to naringenin by chalcone isomerase (CHI) or nonenzymatically *in vitro* [134].

1.7.2 Enhancement of Flavonoid Production

Combination of promoter and target genes; knockout of related genes; overexpression of malonyl-CoA; and construction of artificial P450 enzymes are the key molecular biology technology procedures used in the heterologous production of flavonoids.

Every gene from the phenylpropanoid pathway is cloned in the host under the control of the promoter which often plays an important role in the heterologous expression of secondary metabolites. Several promoters have been used to enhance production of flavonoids according to the need of specific host such as T7, ermE∗, and GAL1 promoter [12]. The extremely low concentration of malonyl-CoA in the microbial cell was one of the drawbacks in the microbiological production of flavonoids. Through the coordinated overexpression of acetyl-CoA carboxylase genes from *Photorhabdus luminescens* intracellular malonyl-CoA pool was amplified leading to increased production of flavonoids [135].

Supplication of UDP-glucose is also a key effector in the biosynthesis of flavonoids. It was proved in an experiment in which researchers knocked out the udg gene encoding for UDP-glucose dehydrogenase. This resulted in elimination of endogenous UDP-glucose consumption pathway leading to increase in intracellular concentration of UDP-glucose and as a consequence increment in the production of flavanones and anthocyanins was observed [136].

One of the barriers to the production of flavonoids and their related compounds in microorganisms by means of assembling biosynthetic genes to form an artificial pathway is the difficulty in expression of active, membrane-bound cinnamate-4-hydroxylase [137].This enzyme is not expressed efficiently in bacteria due to its instability and the lack of its cognate cytochrome P450 reductase in the host. An advantage of flavonoid production in yeasts or fungi is their ability to express functionally active microsomal cytochrome P450 enzymes, which are usually difficult to be expressed in an active form in bacterial cells. There are various microsomal cytochrome P450 enzymes that are involved in the flavonoid biosynthesis

pathway [138]. Combining bacterial cells and eukaryotic cells in a pot enabled researchers to generate a wider library of natural and unnatural products than any of the previously reported systems. *De novo* production of the key flavonoid intermediate naringenin has been demonstrated for the first time from glucose using an engineered *S. cerevisiae* strain which led to fourfold higher concentrations than reported in earlier studies on de novo biosynthesis [139, 140].

1.8 The studied plant species

1.8.1 *Pulcaria crispa*

Pulicaria crispa is a genus of Asteraceae family. The Asteraceae family is one of largest families of flowering plant, with about 1,100 currently accepted genera and 25,000 species. It is characterized by the flowers being arranged in dense heads of many small florets, such as the daisy and dandelion [141].

Pulcaria crispa

The family is of worldwide distribution ,being absent only from the Antarctic mainland, and is particularly well represented in semiarid region of the tropics and subtropics, such as the Mediterranean region, Mexico, the cape province of South Africa grassland and bush land formation of Africa and montane floras throughout the world. Only in the tropical rain forests are they poorly represented [141]. The Asteraceae are of incalculably great indirect economic importance to man as major contributors to the diversity, and therefore, the stability and sustainable productivity, of the drier vegetation types throughout the world, especially in tropical and sub-tropical areas. In proportion to its size, however, the direct economic importance of the family is comparatively small. It includes food plants, sources of raw materials, medicinal and drug plants, ornamentals and succulents, and, on the debit side, weeds and poisonous plants [141].

1.8.1.1 Phytochemical, antibacterial screening and antioxidant activity of *Pulcaria crispa*

Many secondary metabolites have been detected - coumarins, tannins and alkaloids - on *P. crispa* [142]. Investigation of the flavonoids constituents of *Pulicaria crispa*, result in the isolation and identification of four flavonoids, quercein, quercetin-3-o-glucoside, quercetin7-o glycoside and quercetin-3-methyl ether [143]. The presence of these metabolites explains the various uses of this plant in traditional medicine. Alkaloids, comprising a large group of nitrogenous compounds, are widely used as cancer chemotherapeutic agents [144, 145] alkaloids also interfere with cell division The presence of alkaloids in *Pulicaria crispa* may be responsible for antibacterial activity. *Pulicaria crispa* is used in folk medicine for the treatment of colds, coughs, colic, excessive sweating and as carminative [146].

1.8.2 *Portulaca oleracea*

Portulaca oleracea is a warm-climate, herbaceous succulent annual plant with a cosmopolitan distribution belonging to the Portulacaceae family. It is commonly known as *purslane* (USA and Australia), *rigla* (Sudan and Egypt), *pigweed* (England), *pourpier* (France), and *Ma-Chi-Xian* (China) [147]. It is distributed widely in the tropical and subtropical areas of the world including many parts of the United States and is eaten extensively as a potherb and is added to soups and salads around the Mediterranean and tropical Asian countries [148].

Portulaca oleracea

Americans and aborigines of Australia grind the seeds of this plant into flour for use in mush and bread [149]. *Portulaca oleracea* also provides a source of nutritional benefits owing to its rich omega-3 fatty acids and antioxidant properties [150]. *Portulaca oleracea* has been used as a folk medicine in many countries, acting as a febrifuge, antiseptic and so forth [151]. It exhibits a wide range of pharmacological effects, including antibacterial [152], antiulcerogenic [153], anti-inflammatory [154], antioxidant [155], and wound-healing [156] properties. It is listed by the World Health Organization as one of the most used medicinal plants, and it has been given the term “Global Panacea” [157]. The

Chinese folklore described it as “vegetable for long life” and it has been used for thousands of years in traditional Chinese Medicine [158, 159]. It is cold in nature and sour in taste and is used to cool the blood, stanch bleeding, clear heat, and resolve toxins. The dried aerial part of this plant is indicated for the treatment of fever, dysentery, diarrhea, carbuncle, eczema and hematochezia, with a recommended dose of 9–15 g, *Portulaca oleracea* has a high potential to be used as human and animal food and to be utilized as a pharmacological agent in medicine [160–162].

1.8.2.2 Phytochemistry

Many constituents of *Portulaca oleracea* have been isolated, including flavonoids, alkaloids, fatty acids, terpenoids, polysaccharides, vitamins, sterols, proteins, and minerals.

One of the most effective constituents present in Chinese Herbal Medicines are flavonoids which are biologically active and possess a wide range of pharmacological properties such as antibacterial, antivirus, anti-inflammation, and antioxidation properties. In the *Portulaca oleracea* plant, the flavonoids levels vary according to the part of the plant; the highest levels are present in the root followed by stem and the leaf; and seven different flavonoids are present in this plant, including kaempferol, myricetin, luteolin, apigenin, quercetin, genistein, and genistin [163]. However, only kaempferol and apigenin have been found in ethanolic extracts of leaves and stems, with the levels in the former being higher [157]. Portulacanones B–D, three homoisoflavonoids compounds, display selectively cytotoxic activities against three human cancer cell lines (SF-268,NCI-H460, and SGC-7901) [164]. Flavonoids are also widely present in foods such as fruits and vegetables [165].

In addition to flavonoids, another important chemical found in this plant is alkaloids including dopa, dopamine, and noradrenalin. The content of dopamine and noradrenalin is higher in leaves compared to stem and seeds. The amount of dopamine and noradrenalin obtained from leaves varies according to the solvents used in the extraction process, suggesting that the levels of these compounds are dependent on the solvents used during the extraction process [166].

Oleraceins A, B, C, D, and E are cyclodopa alkaloids isolated from this plant [167] and several analytes such as (3R)-3,5-bis(3-methoxy-4-hydroxyphenyl)-2,3-dihydro-2(1H)-pyridinone and 1,5-dimethyl-6-phenyl-1,2-dihydro-1,2,4-triazin-3(2H)-one display cytotoxic activities against human cancer cells [168].

Portulaca oleracea is also an excellent source of omega-3 fatty acids, which is usually present in oil and fat of fishes but not normally found in plants. Omega-3 fatty acids play an important role in the enhancement of immune function [169] and prevention and treatment of hypertension, coronary artery disease, cancer, and other inflammatory and autoimmune disorders [170]. It includes α-linolenic acid and linoleic acid, which are essential for normal growth, health promotion, and disease prevention in humans. Polysaccharides found in *Portulaca oleracea* are potential therapeutic agents for the treatment of diabetes mellitus owing to their modulation of blood lipids, metabolism, and decrease of blood glucose. *Portulaca oleracea* contains monoterpenes such as portulosides A and B, diterpenes such as portulene, and β-amyrin type triterpenoids [147, 171]; in addition, vitamins have also been isolated from the leaves of this plant. It contains the highest content of vitamin A which is a natural antioxidant playing an important role in vision, maintaining healthy mucus membranes and protecting against lung and oral cavity cancers among green leafy vegetables. This plant also

contains ascorbic acid, α-tocopherol, and B-complex vitamins, for example, niacin, pyridoxine, and riboflavin [171]. Furthermore it is rich in minerals like phosphorus, manganese, icon, calcium selenium [149], and the amino acids isoleucine, proline, leucine, lysine, phenylalanine, methionine, cystine, valine, threonine, and tyrosine [148]. Many other constituents have also been isolated from this plant, such as β-carotene, glutathione, melatonin, portulacerebroside A, catechol, and bergapten.

1.8.3 *Coriandrum sativam*

Coriander (*Coriandrum sativum*) being an annual herb is most commonly used for seasoning purpose. Seeds, leaves and roots are edible, although they have very distinct flavors and uses [172]. The herb has a light and fresh flavor. Coriander can be used as whole plant and can be processed because of its perishable nature of leaves and to increase the palatability of ripe fruits (seeds) before using it as flavoring agent in different food preparations. Whole plant of coriander mainly fresh leaves and ripe fruits are used for culinary purposes [172].

Coriandrum sativum

Coriander leaves have different taste than its seeds, with citrus overtones. Coriander plant is a rich reservoir of micronutrients and nutritional elements which leads us to focus our study on this herb [172]. Coriander is very low in saturated fat however,it contains good amount of linoleic acid which is a good source of α-tocopherol and vitamin K. Leaves of plant are rich source of vitamins while seeds are rich in polyphenols and essential oils. Coriander taste is devoted to its essential oil comprising a significant content of linoleic and furanocoumarins (coriandrine and dihydrocoriandrine). Coriander is also well known for its antioxidant, anti-diabetic, anti-mutagenic, antianxiety and antimicrobial activity along with analgesic and hormone balancing effect that promotes its use in foods due to numerous health benefits and its protective effect to preserve the food for longer period [172].

Coriander contains high amount of essential oils that are very important for growth and for proper functioning of brain. The main essential fatty acids present in coriander include linoleic and linolenic acids. Linoleic acids belong to PUFA (polyunsaturated fatty acid) group. Dietary supplementation of coriander seed greatly affects the lipid composition of carcass by decreasing saturated fatty acid (SFA) contents (palmitic and stearic acids) and by increasing monounsaturated and polyunsaturated fatty acid (MUFA and PUFA) [173]. This plant is a potential source of lipids (rich in petroselinic acid) and an essential oil (high in linalool) isolated from the seeds and the aerial parts [174]. The high content of fats and protein in the fruits make distillation residues suitable for animal feed. Coriander fruits yield 5 to 7% of ash, 13% resin, astringent principle, malic acid and alkaloids. Coriander oil contains coriandrol, jireniol and vebriniol [175].

1.8.4 *Linum usitatissimum*

Flax or linseed is among the oldest crop plants cultivated for the purpose of oil and fiber. It belongs to the genus *Linum* and family Linaceae. The botanical name, *Linum usitatissimum* Figure 1.8 was given by Linnaeus in his book "Species Plantarum" [176]. It is an annual herbaceous plant with shallow root system.

The common names flax and linseed are used in North America and Asia, respectively, for *L. usitatissimum*. Oilseed varieties and fiber varieties are specialized development of this species [177].

The cultivars grown primarily for seed/oil purpose are relatively short in height and possess more secondary branches and seed bolls (seed capsule). The cultivars grown for fiber purpose are tall growing with straight culms and have fewer secondary branches.

Linum usitatissimum

The Mediterranean and Southwest Asia have both been proposed as the center of origin [203]; however the exact location is uncertain [178]. The initial use of flax has also been debated. Based on archeological evidence, it was proposed that flax was used first for fiber. However, a more recent comparative study of genetic diversity of the stearoyl-ACP desaturase II

(*sad2*) locus from flax and pale flax (*L. angustifolium*) showed reduced diversity in the cultivated species. This suggests that flax may have been initially selected as an oilseed crop [179].

Flaxseed oil is an excellent source of the omega-3 fatty acid linolenic acid with typical levels of 55% in the oil [180] making it ideal for paints, varnishes, and inks due to its fast polymerization properties.

Increasing demand for edible oil sources with significant percentages of omega-3 fatty acids is resulting in consumption of flaxseed as a functional food. Flaxseed is also added to animal feed to improve animal reproductive performance and health [181, 182].

Research objectives

1. Extraction of flavonoids and oils from some plants
2. GC-MS studies on the extracted oils.
3. Elucidation of structures of flavonoids.
4. Biological activity of isolates.

MATERIAL AND METHOD

2.1. Materials

2.1.1 Plant material

Stems of *pulcaria crispa* were collected from White Nile state (Sudan). The plant was identified and authenticated by the Medicinal and Aromatic Plants Research Institute, Khartoum – Sudan. The seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were collected from Jeddah-Saudi Arabia.

2.1.2- Instruments

UV spectra were run on a Shimadzu UV – 2401PC UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer. NMR spectra were measured on a Joel ECA 500 NMR Spectrophotometer. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m length; 0.25mm diameter; 0.25 μm, thickness) was used for GC-MS analysis**.**

2.1.3-Test organisms

Test organisms used in the antimicrobial assay are: *Bacillus subtilis* (Gram +ve), *Staphylococcus aureus* (Gram +ve), *Pseudomonas aeroginosa* (Gram –ve), *Escherichia coli* (Gram –ve) and the fungal species *Candida albicans.*

2.2- Methods

2.2.1- Preparations of reagents for phytochemical screening.

2.2.1.1-Flavonoid test reagents

- Aluminum chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml distilled water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

2.1.2- Alkaloid test reagents

Maeyer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. distilled water.

- **Potassium iodide solution:** 5 g in 10 ml. distilled water

The two solutions were combined and then diluted with distilled water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and (2 g) of potassium iodide in (100 ml) distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

100 g) of powdered air- dried stems of *pulcaria crispa* were extracted with 95% aqueous ethanol. The solvent was removed under reduced pressure giving the prepared extract. This prepared extract (PE) was used for phytochemical screening.

2.2.2.1 Test for unsaturated sterols and for triterpenes

(10 ml) of the (PE) were evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chloroform solution was dehydrated over anhydrous sodium sulphite. (5

ml) portion of the solution was mixed with (0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

2.2.2.2- Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on a water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

2.2.2.3- Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated.

Filtrate was divided into two portions:

To one portion a few drops of Mayer reagent were added to the other portion few drops of Wagner reagent were added.

2.2.2.4- Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled, filtrated and

the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

2.2.2.5 -Test for saponins

(1 g) of dried powdered plant material was placed in a clean test tube. (10 ml)of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds, and allowed to stand for one hour and then checked for foam formation.

2.2.2.6 - Test for coumarins

(0.2 g) of the prepared extract were dissolved in 10 ml distilled water in test tube and a filter paper was attached to the test tube to be saturated with the vapor after being spotted o with 0.5N KOH. Then the filter paper was inspected under UV light. The presence of coumarins is indicated by the ability of the spot to absorb the UV light.

2.2.2.7- Test for anthraquinone glycosides

(0.2 g) of the prepared extract was boiled with 10 ml of 0.5 N KOH containing 1ml of 3% hydrogen peroxide solution. The mixture was extracted by shaking with 10 ml of benzene. (5ml) of the benzene solution was shacked with (3ml) of 10% ammonium hydroxide solution and the two layers were allowed to separate. The presence of anthraxquinones is indicated by pink or red color in the alkaline layer.

2.2.3- Extraction of flavonoids

The air - dried powdered stem of a *pulcria crispa* (3 kg) were extracted by maceration with ethanol (95%). The extract was concentrated under reduced pressure to give a crude product.

2.2.4- Column chromatography

Open column (70× 4 cm) was used for fractionation of the ethanolic extracts of *pulcaria crispa*. Silica gel with particle size 60-120 mesh ,from LOBA chemicals, was used as stationary phase. The composition of the mobile phase (ethyl acetate: methanol) was determined by TLC experiments.

The ethanolic extract of *pulcaria crispa* (5 g) was mixed with 10 g of silica gel and then applied on the top of the column. Elution commenced by EthOAC - MeOH ;(3:7, fraction I), (1:1, fraction II) and (0:1, fraction III).

2.2.5- paper chromatography

Fraction II – being rich in phenolics- was subjected to paper chromatography where pure flavonoids - compounds I and II -were isolated using the solvent system: butanol: acetic acid: water (4: 1: 12, upper layer).

2.2.6-Extraction of oils

Powdered seeds of *Portulaca oleracea* (*Coriandrum sativam* and *Linum usitatissimum*) - (500g) for each - were macerated with n-hexane . The solvent was removed under reduced pressure to yield the oil.

2.2.7-Esterification of oil

A Methanolic solution of sodium hydroxide was prepared by dissolving (2g) of sodium hydroxide in 100ml methanol.A stock solution of methanolic sulphuric acid was prepared by mixing (1ml)of concentrated sulphuric acid with (99ml) methanol.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added,then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes. The hexane layer was then separated.(5µl) of the hexane extract

were mixed with 5ml diethyl ether . The solution was filtered and the filtrate (1µl) was injected in the GC-MS vial.

2.2.8- GC-MS analysis

The oils from *Portulaca oleracea, Coriandrum sativam* and *Linum usitatissimum* were analyzed by gas chromatography – mass spectrometry. A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m, length; 0.25mm diameter; 0.25 µm, thickness) was used. Helium (purity; 99.99 %) was used as carrier gas.

2.2.9- Biological activity

2.2.9.1- Preparations of chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa*

Chloroform fraction of *pulcaria crispa* was prepared by suspending the ethanolic extract of the leaves in the least amount of distilled water and then shaking with chloroform in a seperatory funnel. Evaporation of the chloroform layer under reduced pressure gave the required fraction. Other fractions were prepared in a similar way.

2.2.9.2- Antimicrobial assay

The ethanolic, chloroform, ethyl acetate and n-butanol fractions of *pulcaria crispa* as well as the essential oils extracted from the seeds of *Portulaca oleracea*, *Coriandrum sativam* and *Linum usitatissimum* were evaluated for their antimicrobial activity against five standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Candida albicans*) using the cup plate agar method with some minor modifications.

2.2.9.2.1- Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were distributed onto agar slopes and incubated at 37^{o} C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce suspension containing about 108- 104 colony forming units per ml. The suspension was stored in refrigerator at 4^{o}C until used. The average number of viable organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volume (0-02 ml) of the appropriate dilution was transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37^{o}C for 24 hours.

2.2.9.2.2- Preparation of fungal suspensions

Fungal cultures were maintained on Sabouraud dextrose agar incubated at 25^{o}C for four days. The fungal growth was harvested, washed with sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.9.3- Testing for antibacterial activity

The cup plate agar diffusion method was adopted with, some minor modification, to assess the antibacterial activity (2 ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45^{o} C in water bath.

(20 ml) aliquots of the incubated nutrient agar were distributed into sterile Petri dishes. The agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10 mm in diameter) were cut using sterile cork borer (No. 4). Each of the halves was designed for a single test sample.

The agar discs were removed and cups were filled with 0.1 ml of each test sample using adjustable volume microliter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37^{o} C for one hours.

The above procedure was repeated for different concentrations of extracts and standard antimicrobial chemotherapeutics. After incubation the diameters of the resultant growth inhibition zones were measures.

2.2.9.4- Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but this time Sabouraud dextrose agar was used. Samples were used here by the same concentrations used above

2.2.9.5-Antioxidant activity

The DPPH radical scavenging capacity was determined. In 96-wells plate, the test samples were allowed to react with 2.2 Di (4-tert-octylphenyl)-1-picryl-stable free radical (DPPH) for half an hour at 37° C. the concentration of DPPH was kept as (300μM). The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517nm using multiple reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests were run in triplicate. Propyl gallate was used as a positive standard at concentrations 0.5 mM.

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RESULT AND DISSCUSSION

Powdered air-dried stems of *Pulcaria crispa* were extracted with 95% ethanol at ambient temperature for 48hr. The crude product obtained after evaporation of the solvent under reduced pressure. The crude extract was fractionating by separator funnel and the three fractions were collected (ethyl acetate, chloroform and butanol fractions) and screened for its antimicrobial activity. The crude was subjected to silica gel column chromatography and eluted with ethyl acetate - methanol mixture (3: 7, fraction I), (1: 1, fraction II) and pure methanol (fraction III). Three fractions were collected. The fraction II was subjected to paper chromatography where pure components: **compound I** and **II** have been isolated. The structures of isolated flavonoids have been elucidated via a combination of spectra techniques.

GC-MS analysis on seed oil of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and the identification of the constituents. The oils was screened for antimicrobial and antioxidant activity against standard organisms.

3.1- *Pulcaria crispa*

3.1.1- Phytochemical screening

Pulcaria crispa was screened for major secondary metabolites and the results are displayed in table 3.1.

Table 3.1: Phytochemical screening of *pulcaria crispa*

Test	**Result**
Saponin	++
Cumarin	+
Alkaloids	+
Flavonoids	+
Tanin	+++
Steroids	-
Triterpens	-
Anthraquinone	-

+: Trace

++: Moderate

++: High

- : Negative

3.1.2- Antimicrobial assay

The crude extract, ethyl acetate, chloroform and butanol fractions have been screened for antimicrobial activity against five human pathogens. The results are depicted in table 3.2 Tables 3.3 and 3.4 display the antimicrobial activity of standard drugs.

Table 3.2: Antimicrobial activity of crude and three fractions

	Ec	Ps	Sa	Bs	Ca
Crude extract	_	_	_	_	10
Ethyle acetate fraction	12	10	13	_	12
Choroform fraction	_	_	_	_	11
Butanol fraction	_	_	10	_	10

Ec = *Escherichia coli*

Pa = *Pseudomonas aeroginosa*

Sa = *Staphylococcus aureus*

Bs = *Bacillus subtilles*

Ca = *Candida albicans*

The results were interpreted in a commonly used term :< 9 mm: in active; 9-12 mm: partially active; 13-18 mm: active; >18 very active.

The crude extract showed partial antifungal activity against *Candida albicans* (see table 3.2 and the appendix).

Ethyl acetate fraction showed activity against *Staphylococcus aureus.* It also exhibited partial Antibacterial activity against *Escherichia coli,*

Pseudomonas aeroginosa and *Candida albicans*.The choroform fraction showed partial anticandidal activity.

Butanol fraction showed partial Antibacterial activity against *Staphylococcus aureus* and *Candida albicans*.

Table 3.3: Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-
	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 3.4: Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	Ca
Clotrimazole	30	38
	15	31
	7.5	29

3.1.3- Antioxidant assay

The antioxidant capacity of crude extract has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test extract against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.5) crude extract exhibited significant anti- oxidant activity.

Table 3.5: Radical scavenging activity of crude extract

Sample	Antioxidant activity
Propyl gallate	92.00%
Crude extract	82.00%

3.1.4-Characterization of compound I

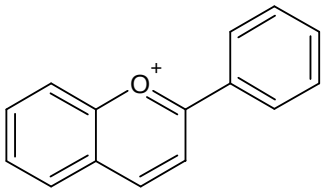
Compound I was isolated from *Pulcaria crispa* stems as yellow powder.

Table 3.6: IR data of compound I

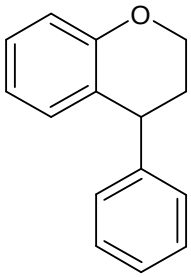
Functional group	**Wave number /cm^{-1}**
(C-H, Ar)	630.51
(C-O, ether)	1273.70
(C=C, Ar)	1378.30
(C=O)	1727.92
(OH)	3368.88
(C-H) Alkane	2921.62

The IR spectrum of compound I **(**Fig.3.1) showed all the anticipated bands of flavonids. Since

the infrared showed carbonyl absorption then the possibility that compound I being an anthocyanin or catechin is dismissed since these classes are characterized by absence of a carbonyl function.



Anthocyanin



Catechin

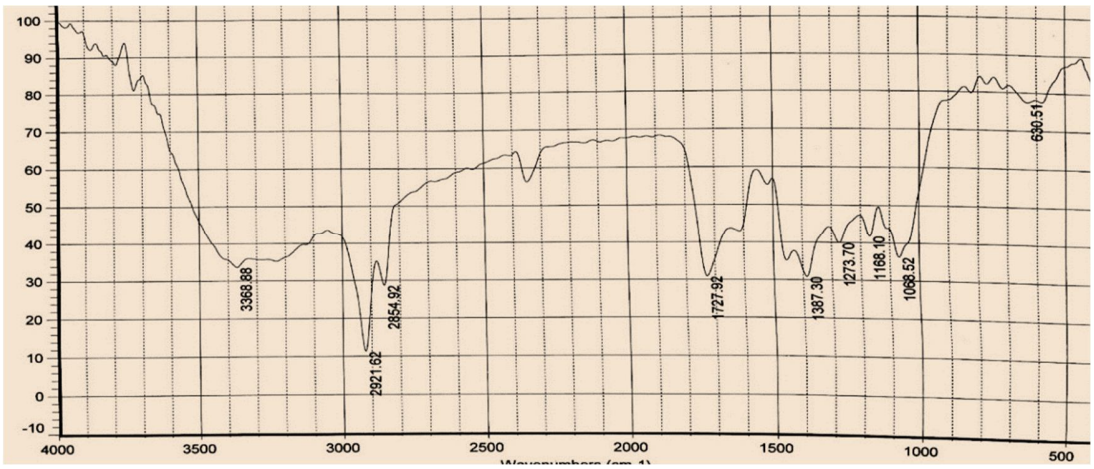
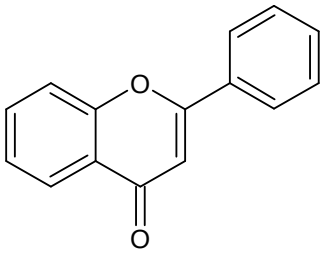
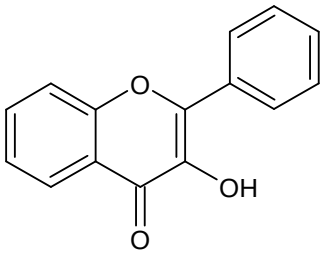


Fig 3.1: IR spectrum of compound I

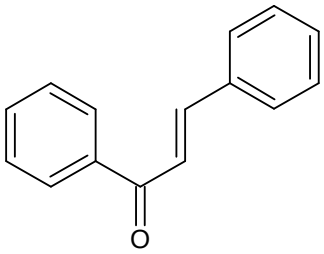
The UV spectrum of compound I revealed two major peaks at 262 and 345 nm (Fig.3.2). The presence of two peaks is a characteristic feature of flavones, flavonols, chalcones, and aurones.



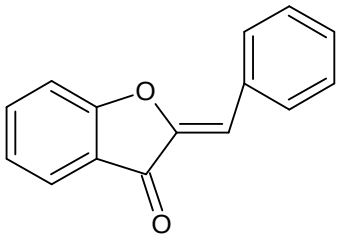
Flavone



Flavonol



Chalcone



Aurone

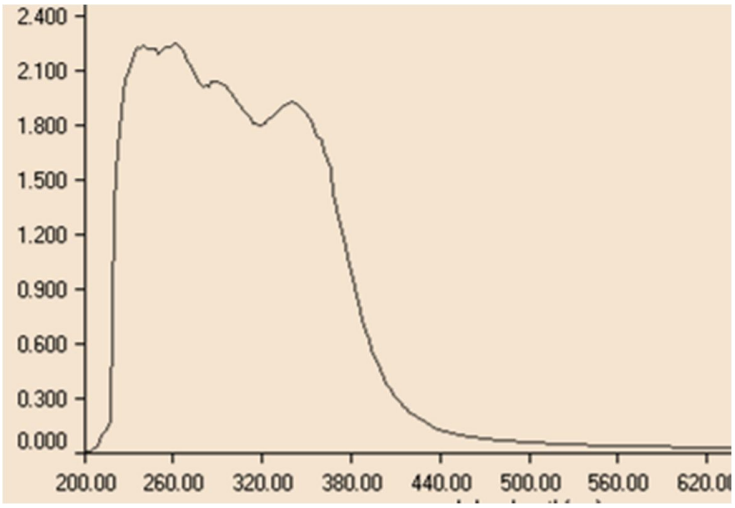
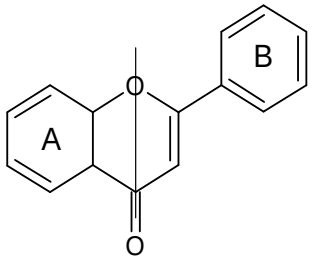


Fig 3.2: UV spectrum of compound I

The UV spectra of most flavonoids consist of two major absorption maxima, one of which occur in the range 220 – 290 nm (band II) and other in the range 300 – 400 nm (band I). The appearance of both band I and II demonstrates conjugation between the benzoyl and cinnamoyl systems.



Benzoyl Cinnamoyl

Band I is considerable to be associated with the absorption of cinnamoyl system, while band II originates from the benzoyl system.

Compound I showed both band II and I (fig.3.2) at 262 and 345nm respectively. Such absorption indicates that this compound is either a flavone, flavonol, chalcone or aurone. But aurones and chalcones absorb in

the range 365-430nm (band I). Flavones absorb in the range 300-350 nm, while flavonols absorb in the range 356-385. Thus compound I is a flavone.
Considerable structural feature have also been obtained using UV shift reagents such as sodium methoxide, sodium acetate, aluminum trichloride. These reagents produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl groups in the flavonoid molecule.
Use has been made of the effect of sodium methoxide on the UV spectra of flavonoids for detection of free 3 – and/or 4` –hydroxyl groups.
On addition of NaOMe to a methanolic solution of compound I a 42 nm bathochromic shift in band I without decrease in intensity (fig.3.3) was observed indicating the presence of a 4`-OH function.

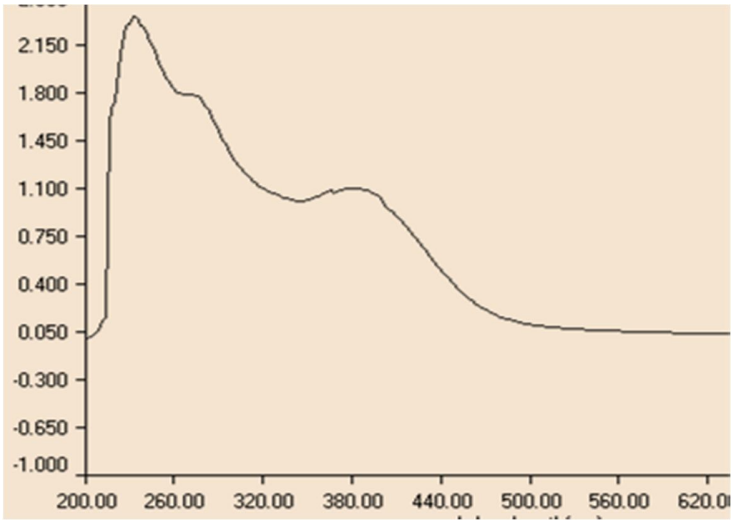


Fig 3.3: The UV spectrum of compound I in sodium methoxide

Sodium acetate is particularly useful diagnostic reagent for the specific detection of 7 –hydroxy group. When a methanolic solution of compound I **was** treated with excess powdered sodium acetate, and 16nm bathochromic shift in band II (fig.3.4) was observed suggesting a 7 –OH group.

When the previous solution of compound I was treated with excess powdered boric acid, no bathochromic shift was observed (fig.3.5) this indicate absence of *ortho*- dihydroxyl groups.

The shift reagent aluminum trichloride is diagnostic of 3 –hydroxyl group, 5 –hydroxyl group and catechol moieties[216].When a methanolic solution of compound I was treated with a few drops of the shift reagent $AlCl_3$ no bathochromic shift (fig.3.6) was observed and this indicates absence of 3 –OH, 5 –OH and catechol moieties.

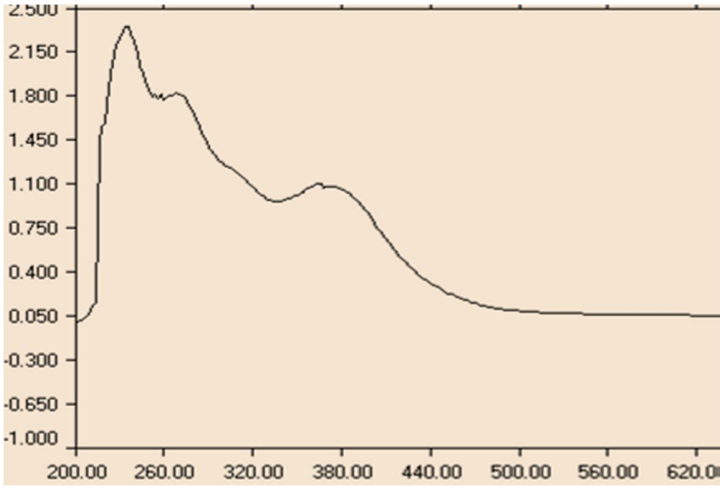


Fig 3.4: UV spectrum of compound I in sodium acetate

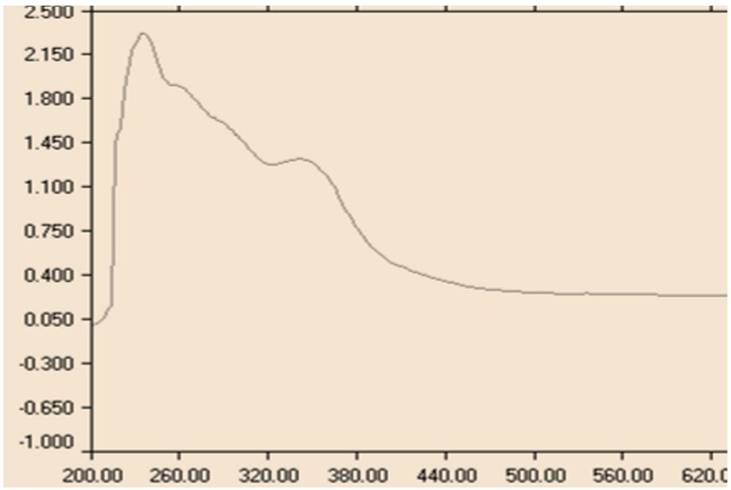


Fig 3.5: UV spectrum of compound I in sodium acetate/boric acid

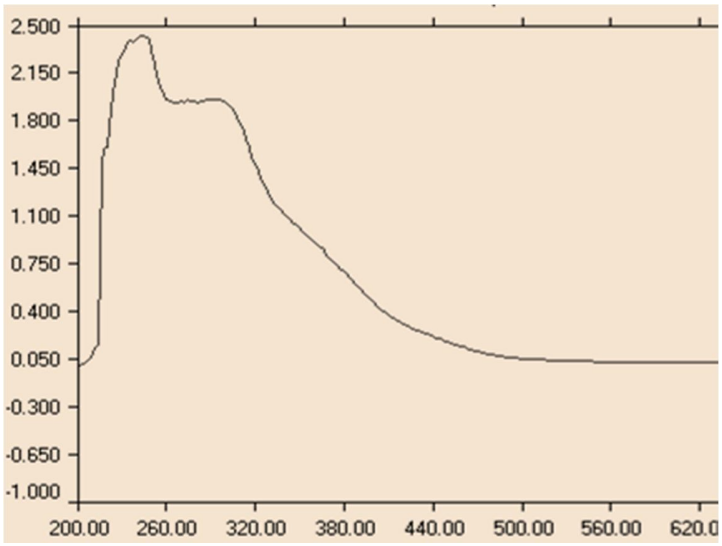


Fig 3.6: UV spectrum of compound I in aluminum trichloride

The ^{1}HNMR spectrum (Fig.3.7) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.1 ppm accounts for an acetyl function. The multiplet at δ 4.23-5.45 ppm is due to a sugar moiety (not identified in this study). The aromatic protons appeared as a multiplet (δ 6.68-7.65 ppm).

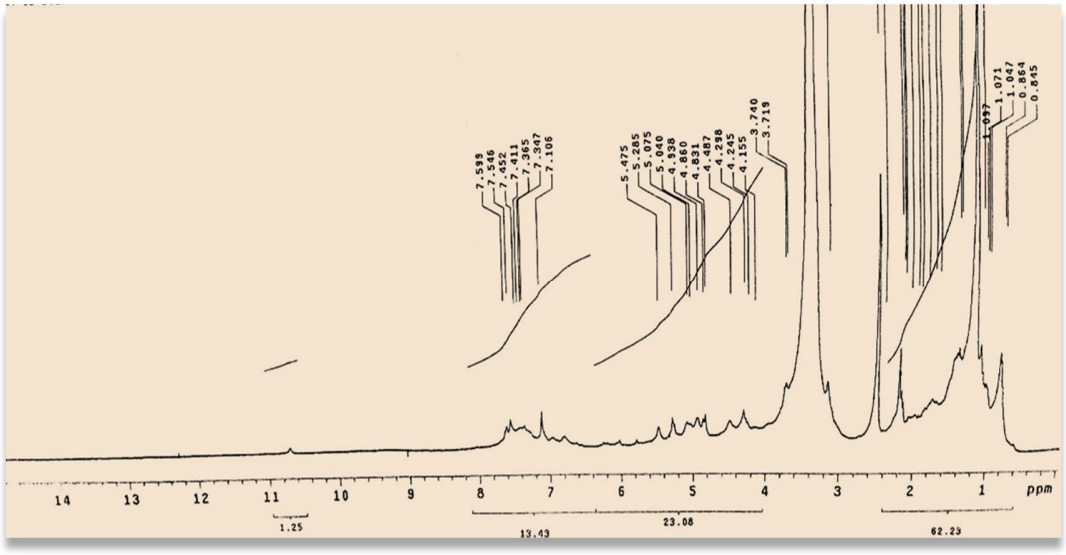
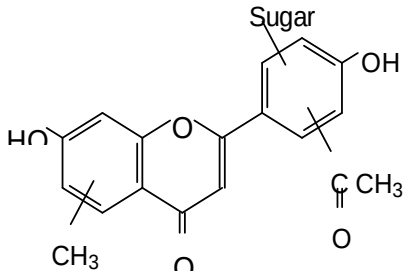


Fig 3.7: ^{1}HNMR spectrum of compound I

On the basis of the above spectral data, the following partial structure was proposed for compound I:



3.1.5- Characterization of compound II

Compound II was isolated as white-yellowish powder from *Pulcaria crispa* stem. The IR data of compound II is presented in the following table (see also Fig.3.8).

Table 3.7: IR data of compound II

Functional group	N(cm^{-1})
(C-H, Ar)	2085.7
(C-O, ether)	1017.15
(C=C, Ar)	1386.55
(C=O)	1643.49
(OH)	3419.34
(C-H) Alkane	2832.88

Compound II – which showed a carbonyl absorption-cannot be an anthocyanin or a catechin since IR spectrum gave carbonyl stretching at 1643.49 cm^{-1}. It could belong to one of the other classes of flavonoids. Anthocyanin and catechins are devoid of carbonyl.

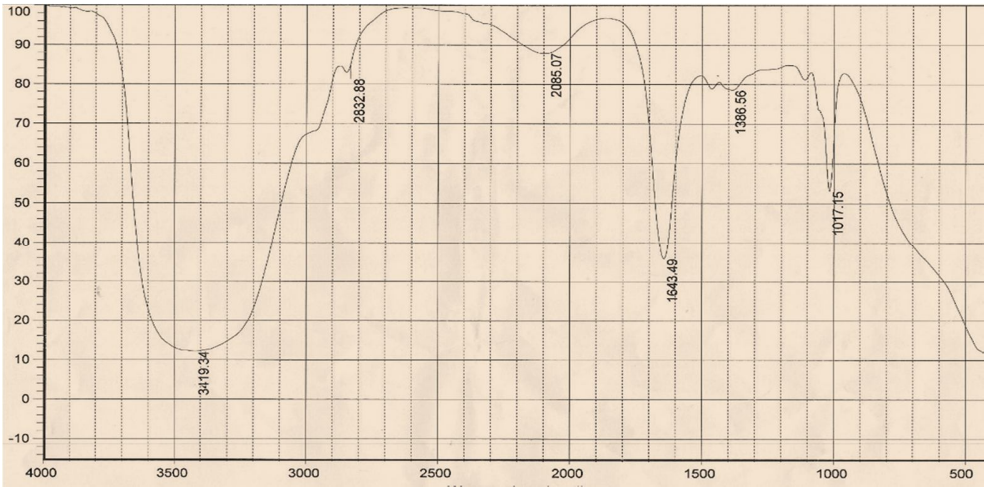
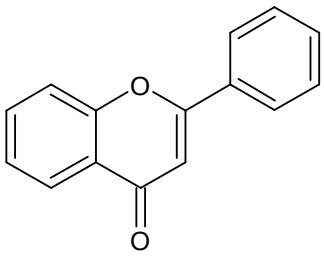


Fig 3.8: IR spectrum of compound II

Table 3.8: UV spectral data of compound II

λ_{max} (nm)	
Band I	Band II
330	240

The UV spectrum of compound II is depicted in (fig.3.9). The spectrum revealed two major peaks at 240 and 330 nm. Such absorption is a characteristic feature of flavones.



Flavone

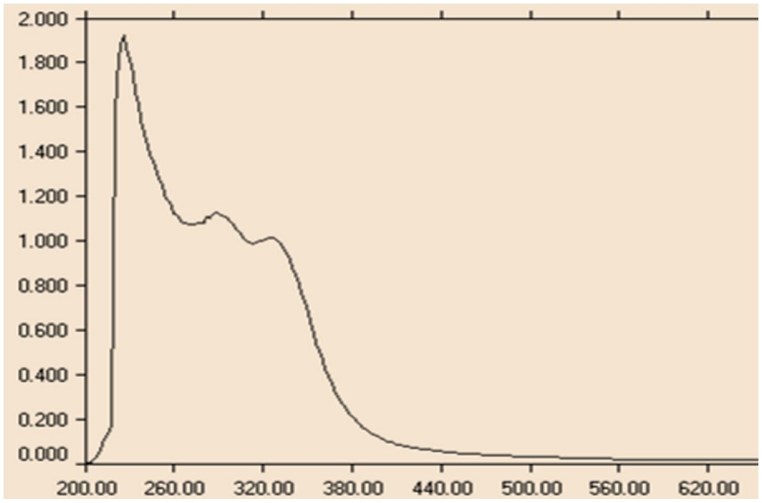


Fig 3.9: UV spectrum of compound II

Considerable structural feature have also been obtained using UV shift reagents: sodium methoxide, sodium acetate, aluminum trichloride.

On addition of NaOMe to a methanolic solution of compound II a 45nm bathochromic shift in band I indicative the presence of a 4`-OH function was observed (Fig.3.10).

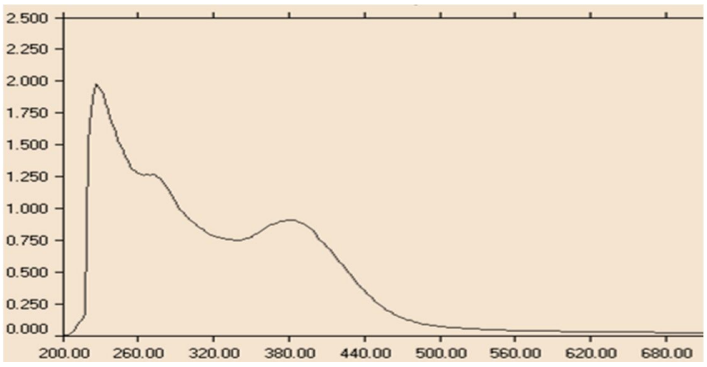


Fig 3.10: The UV spectrum of compound II in sodium methoxide

When a methanolic solution of compound II was treated with excess powdered of the shift reagent NaOAC, a 30 bathochromic shift in band II (fig.3.11) was observed and this indicates presence of a 7 –OH group.

When the previous solution of compound II was treated with excess powdered of boric acid, no bathochromic shift in both band II and I was observed (fig.3.12) this indicate absence of *ortho*- dihydroxyl groups.

The aluminium chloride spectrum showed no bathochromic shift (fig.3.13) indicating absence of 3 –OH, 5 –OH and catechol moieties.

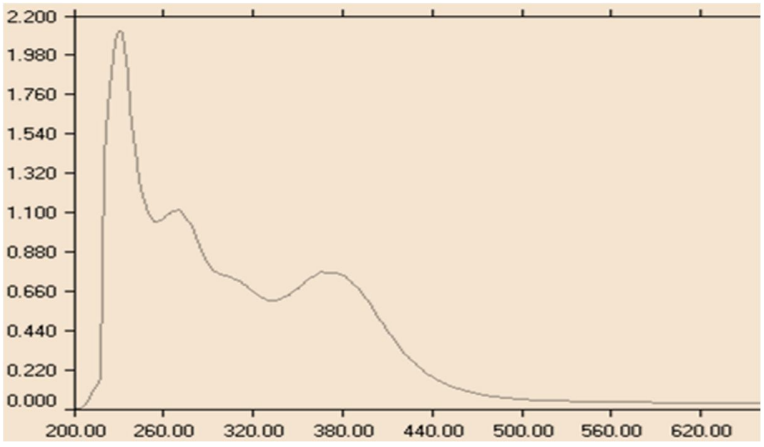


Fig 3.11: UV spectrum of compound II in sodium acetate

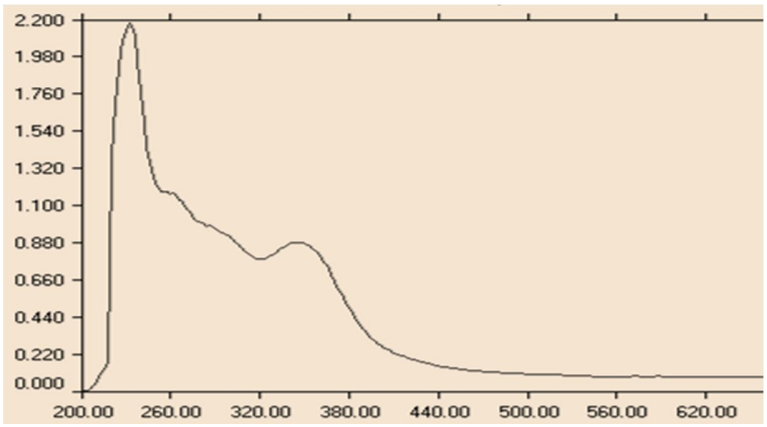


Fig 3.12: UV spectrum of compound II in sodium acetate/boric acid

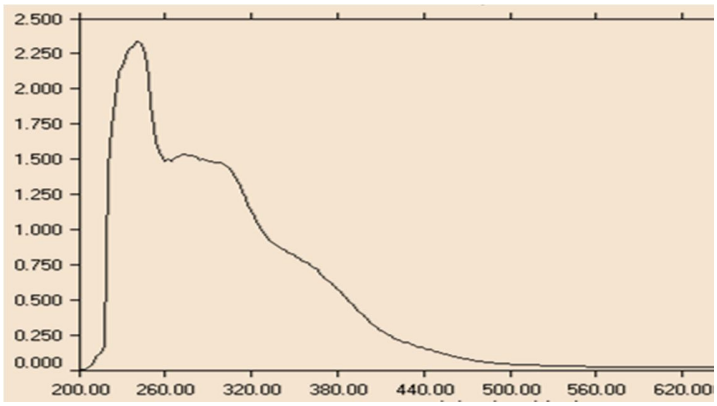


Fig 3.13: Aluminum trichloride spectrum of compound II

The ^{1}HNMR spectrum (Fig.3.14) showed a signal at δ 1.23 ppm assigned for a methyl group. The signal at δ 2.4 ppm accounts for an acetyl function. The aromatic protons appeared as a multiplet (δ 6.68-7.59 ppm).

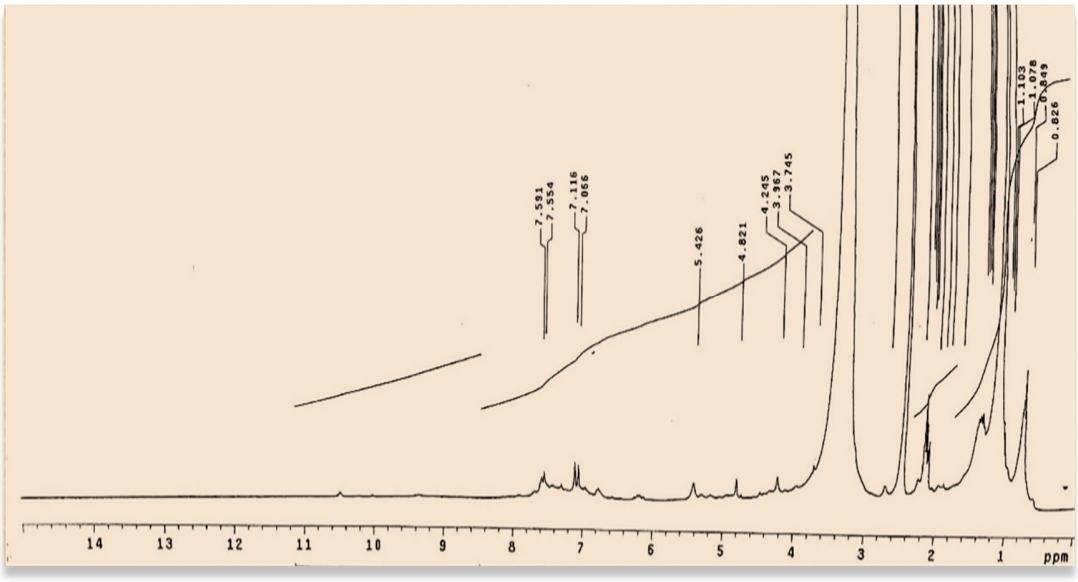
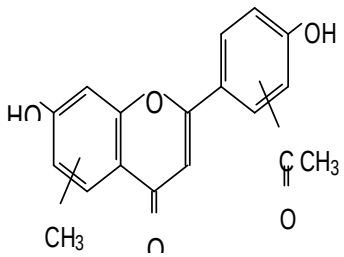


Fig 3.14: [1]HNMR spectrum of compound II

On the basis of the above spectral data, the following partial structure was proposed for compound II:



Compound II

3.2- *Portulaca oleracea*

3.2.1- GC-MS analysis of *Portulaca oleracea* oil

GC-MS analysis of *Portulaca oleracea* oil was conducted and the identification of the constituents was done by comparison of retentions times and MS data with the GC-MS library. Structures of constituents of the oil were further confirmed by interpreting the observed fragmentation pattern

.Comparison of the mass spectra with the database on MS library revealed about 90-95% match.

3.2.2- constituents of oil

The GC-MS analysis of the studied oil revealed the presence of 15 components (Table: 3.9).The typical total ion chromatograms (TIC) of n-hexane extract are shown in (fig.3.15).

Table: 3.9: Constituents of *Portulaca oleracea* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.316	237128	0.08
2.	Pentadecanoic acid, methyl ester	14.373	170885	0.05
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.152	161664	0.05
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.195	705542	0.23
5.	Hexadecanoic acid, methyl ester	15.404	75687986	24.19
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	251818	0.08
7.	Heptadecanoic acid, methyl ester	16.367	775909	0.25
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.068	116899705	37.37
9.	9-Octadecenoic acid (Z)-, methyl ester	17.130	23526885	7.52
10.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.153	59248358	18.94
11.	Methyl stearate	17.307	22933709	7.33
12.	cis-11-Eicosenoic acid, methyl ester	18.883	1307281	0.42
13.	Eicosanoic acid, methyl ester	19.084	6202098	1.98
14.	Docosanoic acid, methyl ester	20.716	3045550	0.97
15.	Tetracosanoic acid, methyl ester	22.226	1688258	0.54

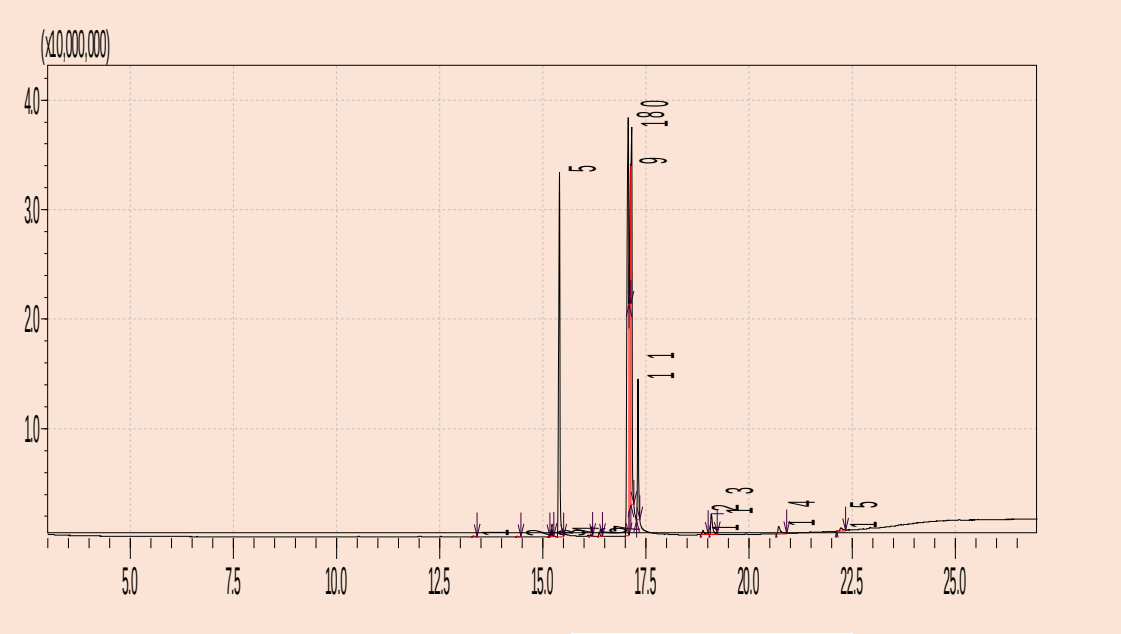


Fig 3.15: Chromatograms of *Portulaca oleracea* seed oil

The following esters were the major components:

9, 12-Octadecadienoic acid, methyl ester (37.37%)

The EI mass spectrum of 9,12-octadecadienoic acid, methyl ester shown in (fig. 3.16).The peak at m/z 294, which appeared at R.T. 17.068 in total ion chromatogram, corresponds to $M^{+}[C_{19}H_{34}O_2]^{+}$.The peak at m/z 263 corresponds to loss of a methoxyl function.

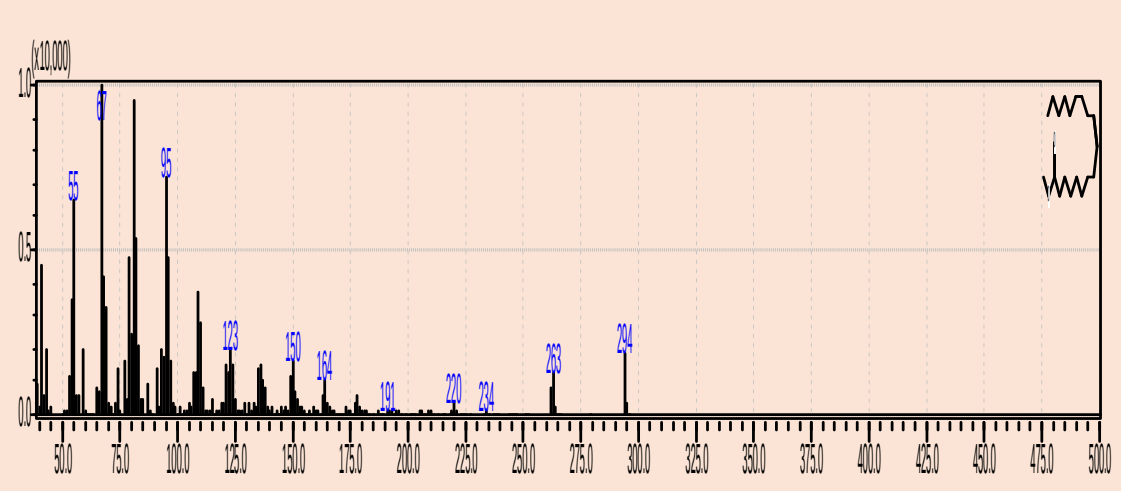


Fig 3.16: Mass spectrum of 9, 12-Octadecadienoic acid, methyl ester

Hexadecanoic acid, methyl ester (24.19%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.17).The peak at m/z 270 (at R.T. 15.404) corresponds $M^+[C_{17}H_{34}O_2]^+$ .The signal at m/z 239 is due to loss of a methoxyl .

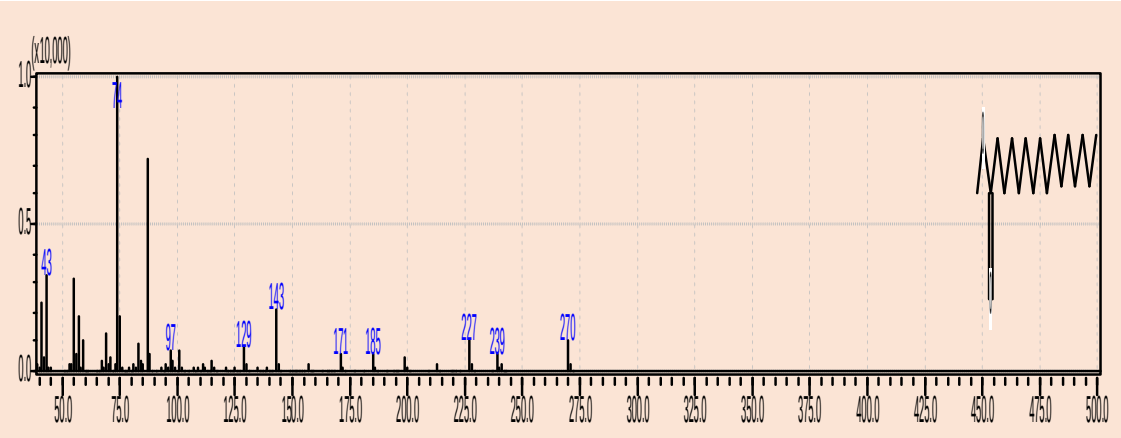


Fig 3.17: Mass spectrum of hexadecanoic acid, methyl ester

9,12,15-Octadecatrienoic acid, methyl ester (18.94%)

Fig. 3.18 demonstrates the mass spectrum of 9, 12, 15-octadecanoic acid, methyl ester. The signal at m/z 292(R.T. 17.153 in total ion chromatogram) corresponds to the molecular ion: $M^+[C_{19}H_{32}O_2]^+$ .The peak at m/z261 accounts for loss of a methoxyl .

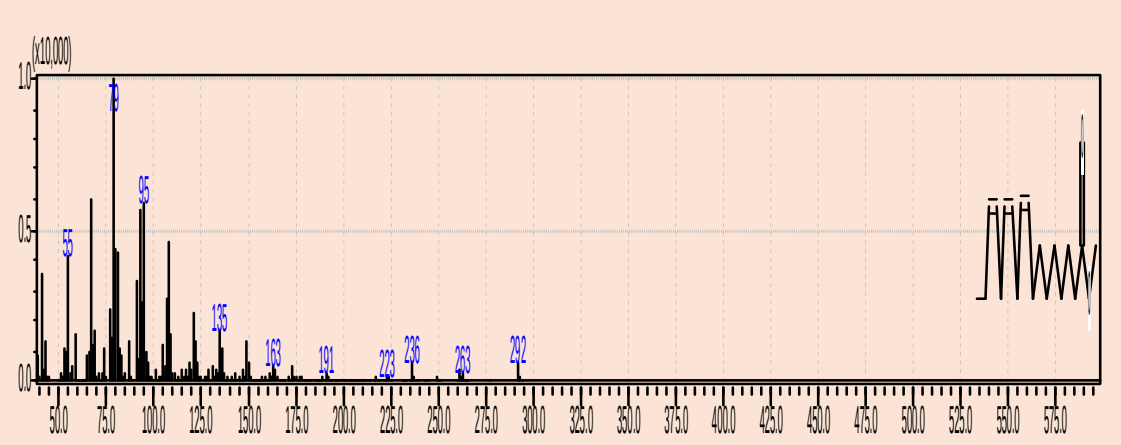


Fig 3.18: Mass spectrum of 9,12,15-Octadecatrienoic acid, methyl ester

3.2.3- Antimicrobial activity

The oil was screened for antimicrobial activity against five standard organisms. The oil not showed any activity against all test organisms

3.2.4- Antioxidant activity

The antioxidant capacity of *Portulaca oleracea* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.10) *Portulaca oleracea* seed oil exhibited weak antioxidant activity.

Table 3.10: Radical scavenging activity of *Portulaca oleracea* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Portulaca oleracea oil	29.00%

3.3- *Coriandrum sativam*

3.3.1- GC-MS analysis of *Coriandrum sativam* oil

GC-MS analysis of *Coriandrum sativam* was carried out . The GC-MS analysis revealed the presence of 14 components (table: 3.11).The typical total ion chromatograms (TIC) of n-hexane extract is presented in (fig.3.19).

Table: 3.11: Constituents of *Coriandrum sativam* oil

ID#	Name	Ret. Time	Area	Area%
1.	Methyl tetradecanoate	13.312	215647	0.03
2.	Pentadecanoic acid, methyl ester	14.372	231442	0.04
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.169	1910240	0.29
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.192	3233931	0.50
5.	Hexadecanoic acid, methyl ester	15.392	41125489	6.32
6.	Heptadecanoic acid, methyl ester	16.367	287117	0.04
7.	7,10-Octadecadienoic acid, methyl ester	16.913	1741544	0.27
8.	6,9-Octadecadienoic acid, methyl ester	16.962	543578	0.08
9.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.062	100831481	15.50
10.	9-Octadecenoic acid (Z)-, methyl ester	17.189	485955106	74.69
11.	Methyl stearate	17.313	9449169	1.45
12.	cis-11-Eicosenoic acid, methyl ester	18.865	3707972	0.57
13.	Eicosanoic acid, methyl ester	19.084	1068295	0.16
14.	Docosanoic acid, methyl ester	20.719	362910	0.06

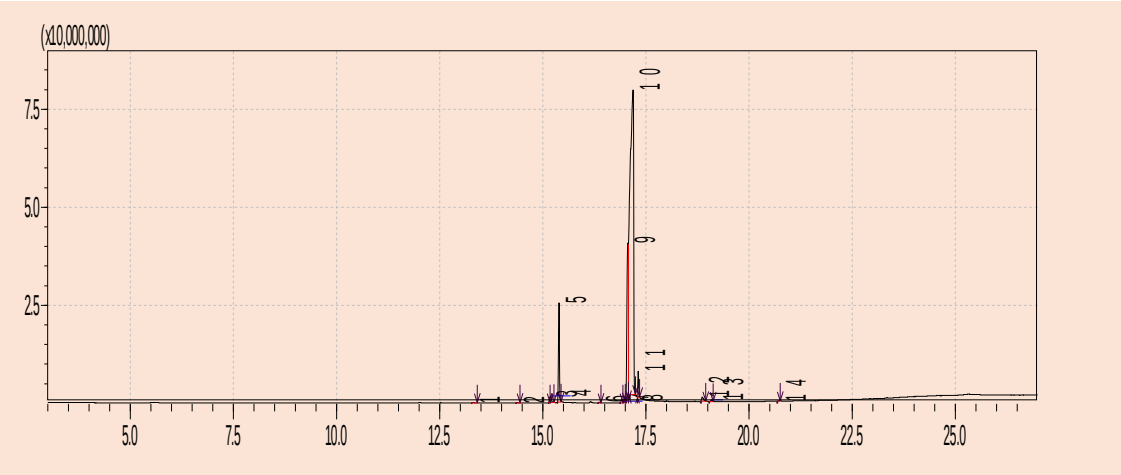


Fig 3.19: Chromatograms of *Coriandrum sativam* seed oil

The following esters were detected as major components:

9-Octadecenoic acid, methyl ester (74.69%)

The EI mass spectrum of 9-octadecenoic acid, methyl ester is shown in (fig. 3.20).The signal which appeared at m/z 264, with R.T. 17.198 corresponds $M^+[C_{19}H_{36}O_2]^+$ .The peak at m/z 235 is due to loss of a methoxyl group.

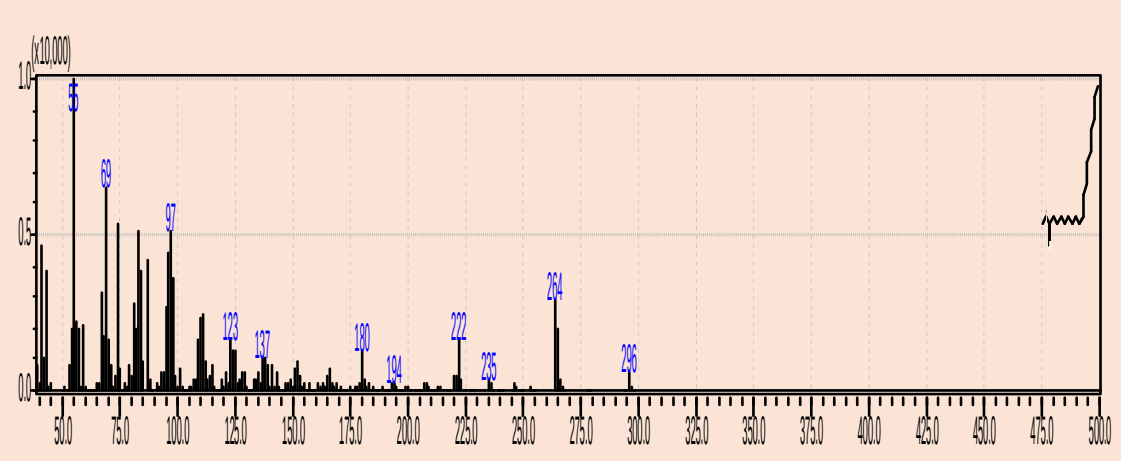


Fig 3.20: Mass spectrum of 9-Octadecenoic acid, methyl ester

9, 12-Octadecadienoic acid, methyl ester (15.50%)

Fig. 3.21 presents the mass spectrum of 9, 12-octadecadienoic acid, methyl ester .The peak at m/z 294, with R.T. 17.062 in total ion chromatogram, corresponds the molecular ion : $M^+[C_{19}H_{34}O_2]^+$ .The signal at m/z 263 accounts for loss of a methoxyl function.

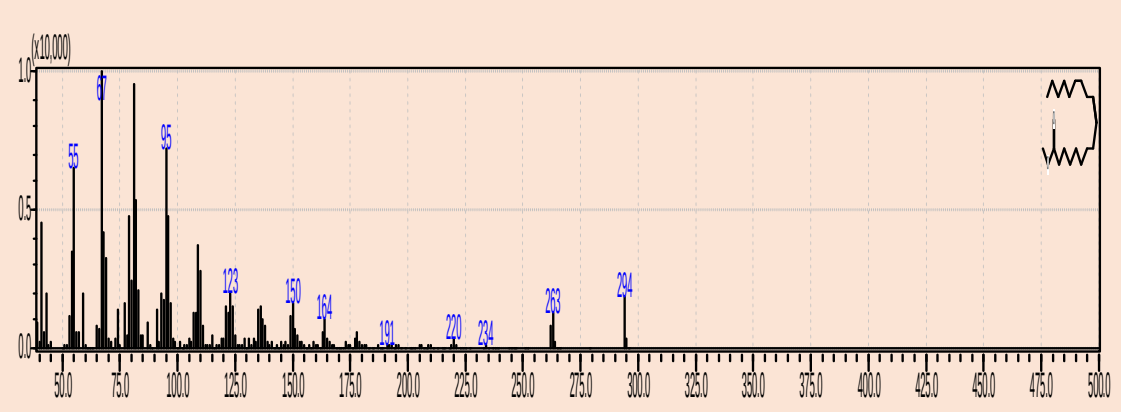


Fig 3.21: Mass spectrum 9, 12-Octadecadienoic acid, methyl ester

3.3.3- Antimicrobial activity

The studied oil was evaluated for antimicrobial activity against five standard human pathogens. The oil not showed any activity against all test organisms

3.3.4- Antioxidant activity

The antioxidant capacity of *Coriandrum sativam* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.12) *Coriandrum sativam* seed oil exhibited moderate antioxidant activity.

Table 3.12: Radical scavenging activity of *Coriandrum sativam* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Coriandrum sativam oil	52.00%

3.4- *Linum usitatissimum*

3.4.1- GC-MS analysis of *Linum usitatissimum* oil

GC-MS analysis of *Linum usitatissimum* was conducted. The analysis revealed the presence of 15 components (table: 3.13).The typical total ion chromatograms (TIC) of n-hexane extract is shown in (fig.3.22)

Table: 3.13: Constituents of *Linum usitatissimum* oil

ID#	Name	Ret.Time	Area	Area%
1.	Methyl tetradecanoate	13.317	189936	0.07
2.	Pentadecanoic acid, methyl ester	14.373	70754	0.03
3.	7-Hexadecenoic acid, methyl ester, (Z)-	15.155	104077	0.04
4.	9-Hexadecenoic acid, methyl ester, (Z)-	15.196	476941	0.19
5.	Hexadecanoic acid, methyl ester	15.392	35779007	14.12
6.	cis-10-Heptadecenoic acid, methyl ester	16.159	270452	0.11
7.	Heptadecanoic acid, methyl ester	16.365	508829	0.20
8.	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	17.053	64486368	25.44
9.	9-Octadecenoic acid (Z)-, hexadecyl ester	17.155	69889815	27.58
10	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	17.164	50002070	19.73
11	Methyl stearate	17.307	28380442	11.20
12	cis-11-Eicosenoic acid, methyl ester	18.887	1343975	0.53
13	Eicosanoic acid, methyl ester	19.092	684609	0.27
14	Docosanoic acid, methyl ester	20.722	818972	0.32
15	Tetracosanoic acid, methyl ester	22.233	436726	0.17

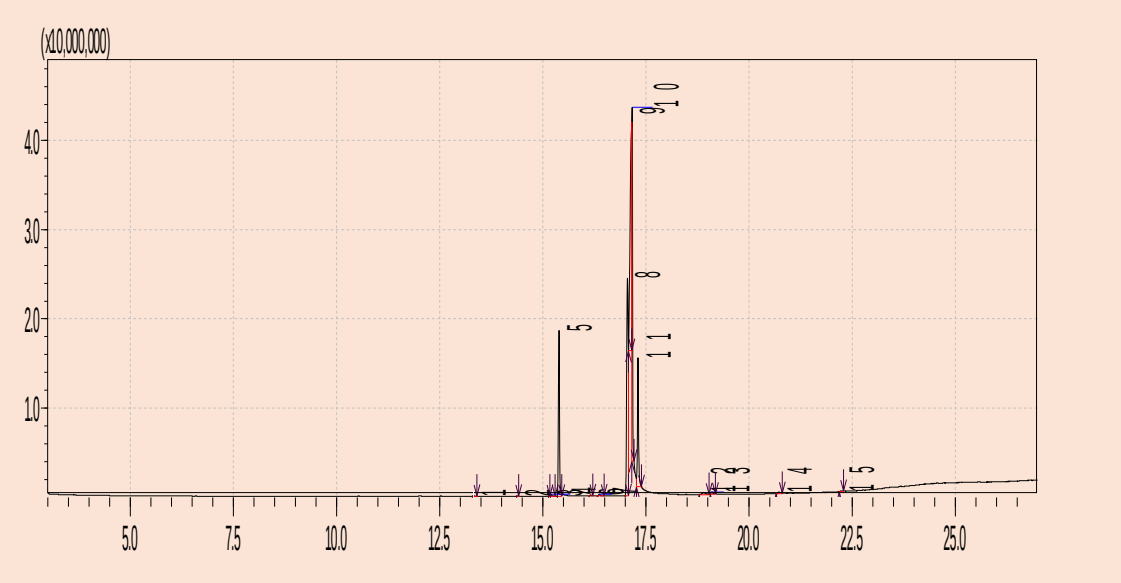


Fig 3.22: Chromatograms of *Linum usitatissimum* seed oil

The following esters were found as major components:

9-Octadecenoic acid, hexadecyl ester (27.58%)

The EI mass spectrum of 9-Octadecenoic acid, hexadecyl ester is shown in (fig. 3.23).The peak at m/z 295, which appeared at R.T. 17.155 in total ion chromatogram, corresponds to $M^{+}[C_{34}H_{66}O_2]^{+}$ .The peak at m/z 264 corresponds to loss of a methoxyl function.

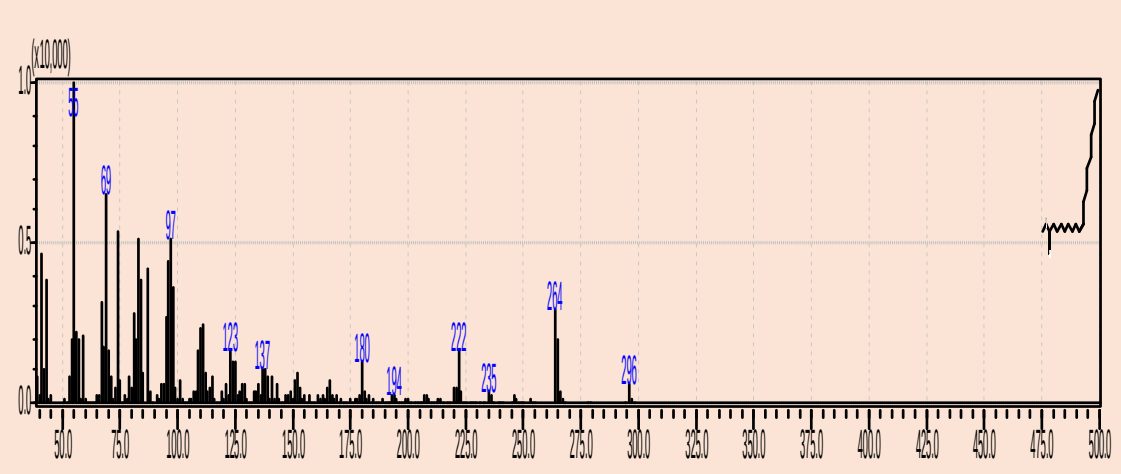


Fig 3.23: Mass spectrum of 9-Octadecenoic acid, hexadecyl ester

9, 12-Octadecadienoic acid, methyl ester (25.44%)

The EI mass spectrum of 9, 12-octadecadienoic acid, methyl ester is shown in (fig.3.24). The peak at m/z 294, which appeared at R.T. 17.053 corresponds $M^{+}[C_{19}H_{34}O_2]^{+}$ while the sighal at m/z 263 is due to loss of a methoxyl group.

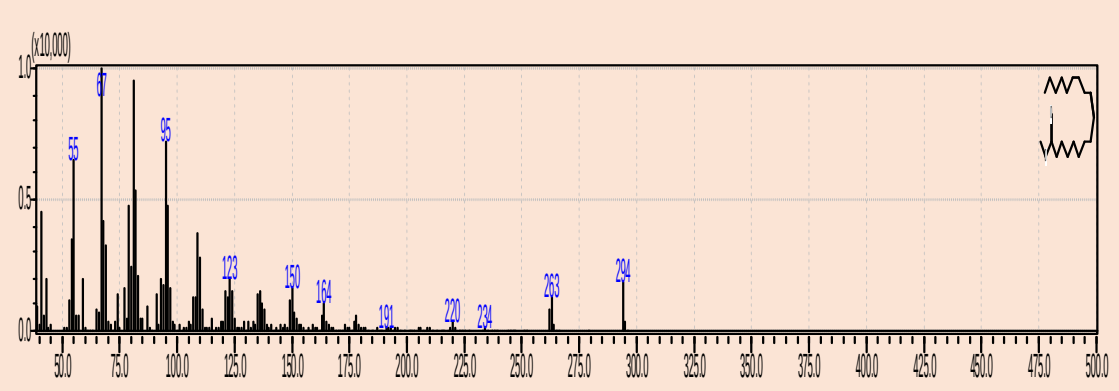


Fig 3.24: Mass spectrum of 9, 12- Octadecadienoic acid, methyl ester

9, 12, 15-Octadecatrienoic acid, methyl ester (19.73%)

Figure 3.25 shows the mass spectrum of 9, 12, 15-octadecatrienoic acid, methyl ester. The peak at m/z 292 - R.T. 17.164- in total ion chromatogram, corresponds the molecular ion : $M^{+}[C_{19}H_{32}O_2]^{+}$,while the sighal at m/z 261 accouts for loss of a methoxyl .

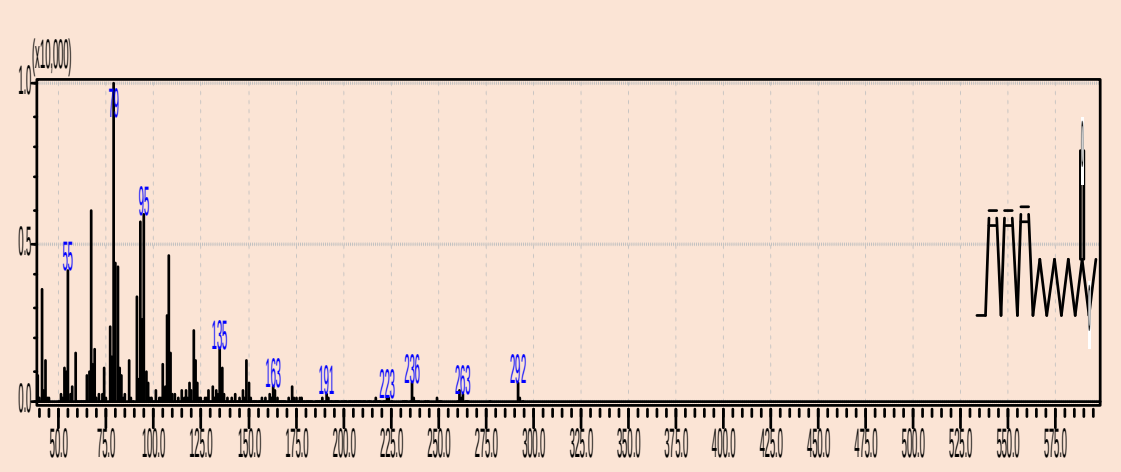


Fig 3.25: Mass spectrum of 9, 12, 15-Octadecatrienoic acid, methyl ester

Hexadecanoic acid, methyl ester (14.12%)

Mass spectrum of hexadecanoic acid, methyl ester is shown in (fig.3.26). The moleculat ion : $M^{+}[C_{17}H_{34}O_2]^{+}$ appeared as expected at m/z 270(R.T. 15.392).The peak at m/z 239 is attributed to loss of a methoxyl .

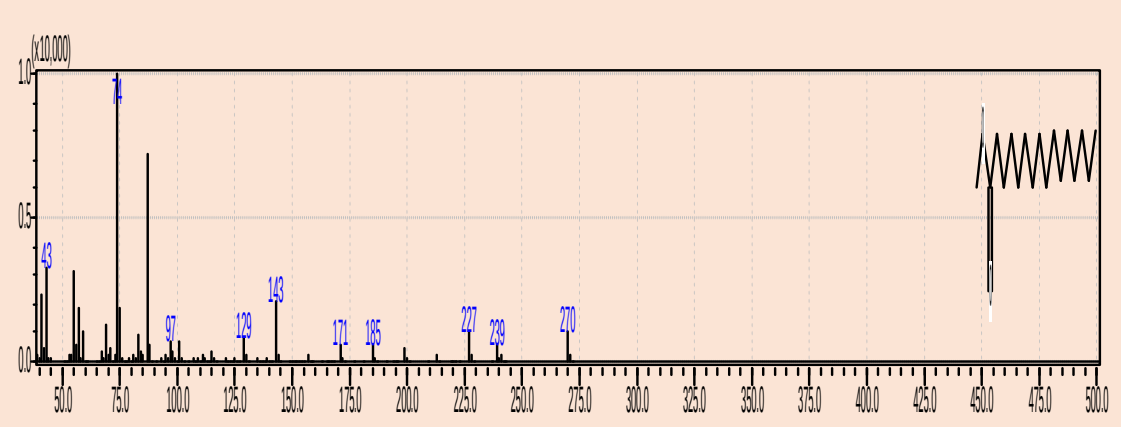


Fig 3.26: Mass spectrum of Hexadecanoic acid, methyl ester

Methyl stearate (11.20%)

The mass spectrum of methyl stearate is presented in (fig.3.27). The signal which appeared at m/z 298 -with R.T. 17.307 in total ion chromatogram - corresponds $M^{+}[C_{19}H_{38}O_2]^{+}$ .The peak at m/z 267 is attributed to loss of a methoxyl .

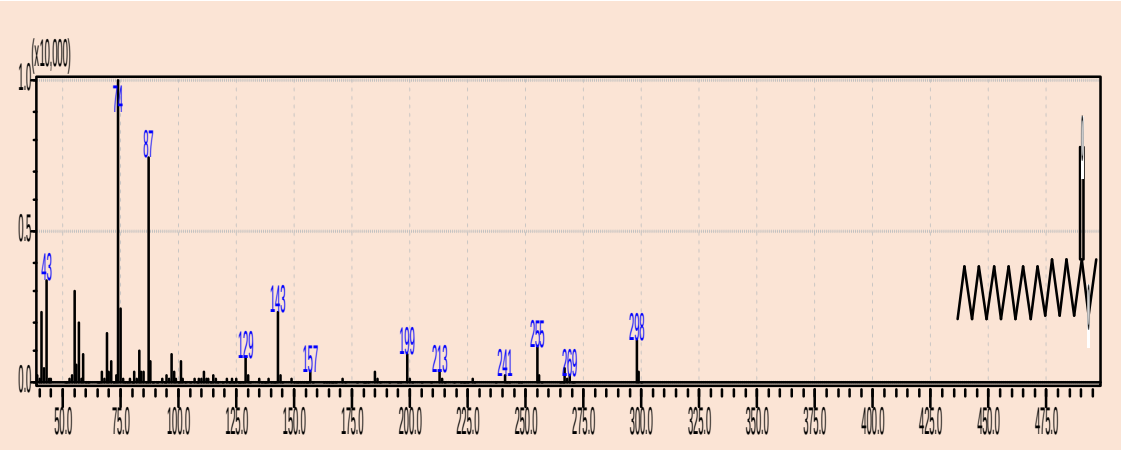


Fig 3.27: Mass spectrum of methyl stearate

3.4.2- Antioxidant activity

The antioxidant capacity of *Linum usitatissimum* seed oil has been measured. Evaluation of the antioxidant activity was carried out by measuring the capacity of the test compound against stable DPPH radical. The change in color is measured spectrophotometrically at 517 nm. As depicted in Table (3.14) *Linum usitatissimum* seed oil exhibited moderate antioxidant activity.

Table 3.14: Radical scavenging activity of *Linum usitatissimum* seed oil

Sample	Antioxidant activity
Propyl gallate	92.00%
Linum usitatissimum oil	48.00%

Conclusion

This research was carried out to study the flavonoids of *Pulcaria crispa* stem and oils from *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum.*

Two flavonoids (compounds I and II) were isolated from the stems of *Pulcaria crispa* .The partial structures of both compounds were deduced on the basis of their : IR, UV and NMR data and partial structures were suggested. Evaluation of the antimicrobial activity of the ethanol extract and different fractions of *Pulcaria crispa* was carried out . The results exhibited variable susceptibilities of microorganism for crude extract and different fractions. The antioxidant capacity of the alcoholic extract of *Pulcria crispa* was screened. The extract showed significant antioxidant activity using the DPPH bioassay.

GC-MS analysis of *Portulaca oleracea, Coriandrum sativam and Linum usitatissimum* was conducted and different constituents were identified. The oils were also assessed for their antimicrobial activity and promising results were obtained.

Recommendations

The following is highly recommended:

1. Other phytochemicals of the studied plant may be isolated and their structures may be elucidated and the biological activity could be screened.
2. The isolated falvonoids may be subjected to 2D NMR experiments for full characterization.
3. The isolated falvonoids and oil may be screened for other biological effects such as antiinflammatory, antidiabetic , antiviral, antilashmenial ..etc.