Sudan University of Science and Technology

The Graduate College

Physiochemical Characterization of the Flavonoids from *Combretum aculeatum*

توضيح تركيب فلافونيدات نبات الشحيط بالطرق الفيزيوكيميائية

A Thesis Submitted in Fulfillment of the Requirement`s of the Ph.D. in chemistry

By:

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March, 2015

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بسم الله الرحمن الرحيم

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صدق الله العظيم

سورة البقرة، الاية: (32)

Dedication

To my parents

My Husband

Sister and brothers

Acknowledgement

First of all, thanks to Allah, Almighty for helping me to finish this work, it would not has be possible without his help. I would like to thank my supervisor Prof. Mohamed Abdel Karim Mohamed for his tremendous help, andvice and support during the period of this study.

Thanks are due to for Dr. Wafa Tawfig and the staff of the National Reawearch Center-Cairo for all facilities.

Special thanks are also extended to the laboratory staff of the faculty of Science, Sudan University of Science and Technology for their kind help. Thanks are extended to Dongla University for funding this research.

My deepest thanks are due to my family and friends who encouraged me to complete this work.

Abstract

Photochemical screening of methanolic extract of *combretum aculeaum* leaves revealed the prsence of flavonoids, steroids, alkaloids, saponins, tannins, coumarins and anthraquinone glycosides. Cyanogenic glycosides were absent.

From ethyl acetate extract of *combretum aculeatem*, four flavonoids-compounds I-IV were isolated and purified by different chromatographic techniques.These compounds were identified via spectroscopic tools: UV, ^{1}HNMR and MS.All isolates were found to be substituted flavones.

When methanolic and ethyl acetate extract were screened for their antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, staphyloccus aureus, pseudomonas aeruginosa, Candida albicans* and *Aspergillus niger*), The methanolic extract exhibited significant antibacterial and moderate antifungal activity against the tested human pathogens, while the ethyl acetate fraction was compeletely inactive.

In vitro anticxidant activity for the methanolic and ethyl acetate extracts was conducted and significant results were obtained for both methanolic and ethyl acetate extracts.

الخلاصة

اجريت اختبارات فيتوكيميائية لمستخلص الكحول المثيلي لاوراق نبات الشحيط حيث اتضح انه يحتوي على الفلافونيدات، الاسترويدات، القلويدات، الصابونينات، التانينات، الكومرينات، الجلايكوسيدات الانثراكوينية، الا ان الجلايكوسيدات السياجينية لم تكن موجودة بالنبات.

من مستخلص االثيل استات لاوراق نبات الشحيط ثم فصل اربعة مركبات فلافونيدية وتمت التنقيه بالطرق الكروموتغرافيه المختلفة.حددت التراكيب لهذه المركبات بواسطة اطياف : الاشعة فوق البنفسجية، الرنين النووي المغنطيسي وطيف الكتلة. كل المركبات المفصولة كانت مركبات فلافون مستبدله.

اجريت التحاليل الميكربيولوجية لمستخلص الميثانول والايثايل استات ضد (الاشريكية القولونية، العصوية الرقيقة، المكورات العنقودية الذهبية، الزائفة الزنجارية، المبيضات البيض والرشاشيات النيجرا).حيث اثبتت التحاليل ان للمستخلص الميثانولى فعالية عاليه ضد البكتريا ومعتدله ضد الفطريات اما مستخلص الايثايل استات فقد كان غير فعال.

تمت دراسة التأثير المضاد للاكسدة لمستخلصي الكحول الميثيلي والايثايل استات واوضحت النتائج ان لكلا المستخلصين فعالية عاليه ضد الاكسدة.

Table Contents

Chapter one

Introduction

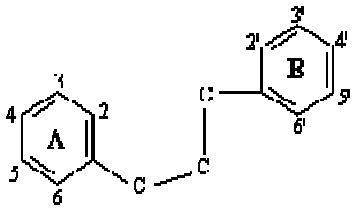
I- Introduction

1.1- General approach

Botanical products have been used for medicinal purposes by human civilizations over the course of thousands of years. Not surprisingly, many of the pharmaceuticals used today are derived in part from natural substances. Flavonoids are an excellent example of a safe and effective botanical compound that has natural biological activity in physiological processes [(1)].

Flavonoids are a group of natural compounds with variable phenolic structures and are found in plants. They are located in the cells or on the surface of various plant organs[(2)]. These compounds are usually found in our daily diet in vegetables, fruits, grains, seeds, herbs, barks , roots , stems , flowers, spices and beverages[(3-5)] .

The chemical structure of flavonoid is based on a C_{15} skeleton(C_6- C_3 - C_6), two aromatic rings A and B connected to three carbon chain(1)



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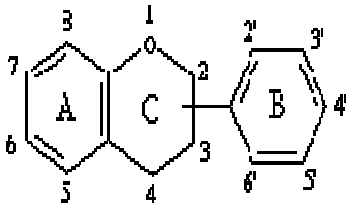
They contain a chroman ring (C-ring) bearing a second aromatic ring (B-ring) at the C-2, C-3, or C-4 position(2).



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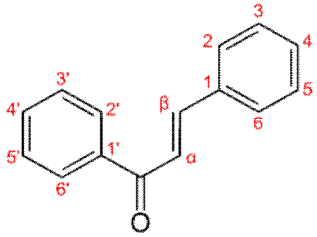
The heterocyclic six-membered C-ring is sometimes replaced by a five-membered ring(3) or the acyclic form(4) .



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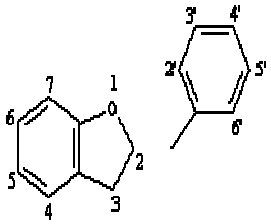


(4)

Flavanoids , the most important class of phenolic compounds , are secondary metabolites produced by plants and are found in a non glycosylated form (aglycone) or attached to a sugar molecule (glycoside). The glycoside residues can be attached to O or C atoms of the flavonoids, giving rise to *O*- glycosides, *C*- glycosides and *O*-*C*-glycosides.

Variation in C ring provides subclasses as: flavones , flavonols , flavanones , anthocyanins , isoflavones , chalcones, aurones , and

others. Biflavonoids are a type of flavonoids with general formula scheme $(C_6\text{-}C_3\text{-}C_6)_2$.

Flavonoids also occur as oligomeric (condensed tannins or proanthocyanidins). Flavonoid oligomers are obtained by oxidative coupling of at least two units of aryl-substituted benzopyran rings or its substituted derivatives[1] .

More than 8.000 flavonoids have been identified[6], and this number is constantly growing because of the great structural diversty arising from the various hydroxylation,methoxylation,glycosylation ,and acylation patterns.

Flavonoids function as antioxidants. They inhibit lipoxygenase and cyclo- oxygenase enzymes and lipids peroxidation[7,8].

Many flavonoids are endowed with biological activities, such as anti-inflammatory, antallergic, antischemic, antiplatelet, immunomodulatory, and antitumor activity[9,10].The number of studies has suggested protective effects of flavonoids against many infections (bacterial and viral diseases) and degenerative diseas such as cardiovascular diseases and age- related diseases [11,12] .

These polyphenols have a wide variety of physiological functions in plants ranging from affecting plant pigmentation, flavor, growth and reproduction. They provide an innate immunity and resistance against pathogens (bacterial, fungal and viral) and also against herbivores and insects [13]. Flavonoids are also involved in electron transport during

photosynthesis, acting as an antioxidant against the effects of ultraviolet light[14].

Flavonids in foods are generally responsible for color, taste, prevention of fat oxidation and protection of vitamins and enzymes [15] . They are also known to influence the quality and stability of food by acting as flavorant , colornate and antioxidant [16,17].

1.2- Classification of Flavonoids

Flavonoids can be subdivided into different subgroups or classes. The various classes of flavonoids differ in the level of oxidation and pattern of substitution of the C ring, while individual compounds within class differ in the pattern of substitution of the A and B rings[6].

For six memberd hetro cyclic C ring , the subgroups depend on the carbon of the C ring on which B ring is attached, and the degree of unsaturation and oxidation of the C ring.

Flavonoids in which B ring is linked in position 3 of the ring C are called isoflavones . Those in which B ring is linked in position 4, neoflavonoids, while those in which the B ring is linked in position 2 can be further subdivided into several subgroups on the basis of the structural features of the C ring. These subgroup are: flavones, flavonols, flavanones, flavanonols, flavanol (catechins), anthocyanins, leucoanthocyanidin and .oligomeric flavonoids (proanthocyanidins) ,which consist of monomeric unites of flavans linked through carbon-carbon and ether linkages.

Flavonoids with five- memberd hetrocyclic C ring are called aurones. Finally, flavonoids with open C ring are called chalcones . Biflavonoids consist of two flavanoids linked through either a carbon – carbon or oxygen bonds.

Flavonoids are often hydroxylated in 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 [(18)]. Monosaccharides or combinations (di ,tri , and so on)may also be found combined to hydroxyl groups of flavonoids . Most of flavonoids occur as O-glycosides but a view occur as C-glycoside ,which are stable to hydrolysis[(19)].

The subgroubs of flavonoids in which the B ring is linked in position 2 of the C ring are derived frorm 2-phenylbenzopyrone or 2-phenylchromen -4-one.

1.3-Flavones

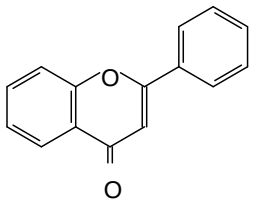
Flavones (flavus = yellow) is the root from which the word flavonoid is derived [(20)]. They have a double bond between positions 2 and 3 and a keto function in position 4 of the C ring . Flavones(5) are based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzo pyran -4-one).

Most flavones of vegetables and fruits has a hydroxyl group in position 5 of the A ring, while the hydroxylation in other positions, for the most part . occurs in position 7 of the A ring or 3′ and 4′ of the B ring and this

may vary according to the taxonomic classification of the particular vegetable or fruit. Flavones glycosylation occur primarily in position 5 ,while methylation and acylation on the hydroxyl groups of the B ring .

Flavones are not distributed widely. However, with significant occurrence been reported in only celery, parsley and some herbs, In addition polymethoxylated flavones have found in cetrus species [21] . Fruit skins , buck wheat , red pepper and tomato skin are dietary sources for flavones apigenin , rutin , luteolin , luteolin glucosides and chrystin[22].

In recent years, scientific and public interest in flavones has grown enormously due to their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus and certain cancers[23]. Flavones have effects on CYP (P450) activity [24,25]which are enzymes that metabolize most drugs in the body.



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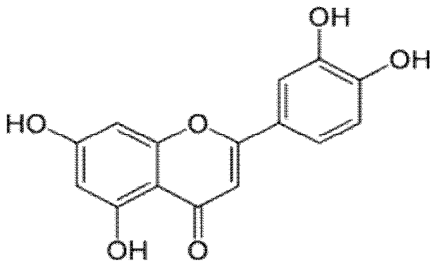
Examples of flavones are: apigenin(6) and luteolin (7)



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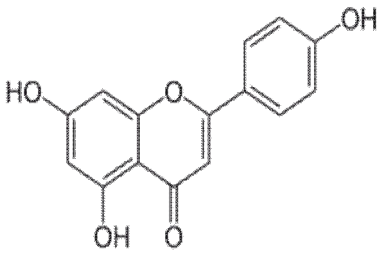

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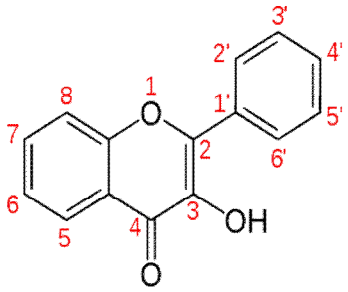
1.4 -flavonols

Flavonol (8) compared to flavones, have a hydroxyl group in position 3 of the C ring, which may also be glycosylated. Again, like flavones, flavonols are very diverse in methylation , hydroxylation and glycosylation patterns.

Flavonols are very widely distributed in plants and are the most abundant flavonoids in foods .They occur most frequently in glycosidic combination. They are found in fruits , vegetables [26, 27] onions , olive oil , berries , grapefruit , tea , broccoli and apples [22].

Flavonols are inhibitor of CYP2C9 and CYP3A4[24,25] which are enzymes that metabolize most drugs in the body.

Example of flavonols include: quercetin(9) , myricetin (10) and kaempferol (11)



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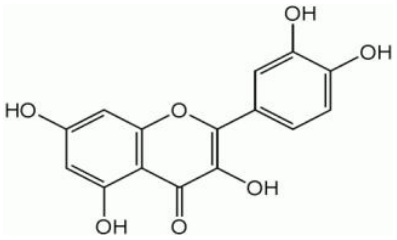

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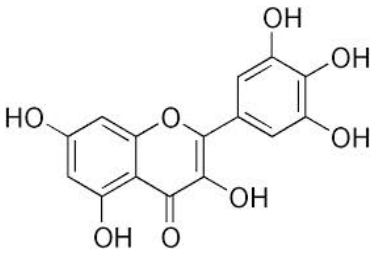

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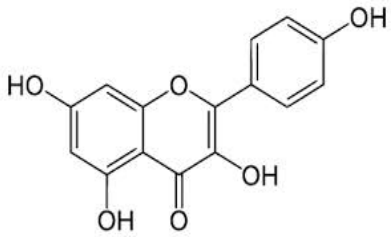

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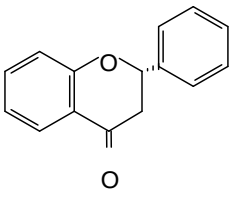
1.5- Flavanones

Flavanones (12) - also called dihydroflavones- have have a saturated C ring ; therefore, unlike flavones, the double bond between positions 2 and 3 is saturated and this is the only structural difference between these two subgroups of flavonoids. Thus, in flavanones , C-2 bears one hydrogen atom in addition to the pinolic B – ring, and C-3 two hydrogen atoms. Two stereoisomeric forms of each flavanone structure are possible , since C-2 is a center of asymmetry (epimeric center). Consequently, the B-ring can be either in the (2S)- or (2R)-

configuration.The great majority of the flavanones isolated from plants are laevorotatory flavanones, because the enzymatic reaction catalyzing the conversion of chalcones to flavanones is stereospecific .

The flavanones can be multi-hydroxylated, and several hydroxyl groups can be glycosylated and/or methylated. They are generally glycosylated at postion seven to give flavanone glycosides.

some flavanones have unique patterns of substitution, for example, furanoflavanones, prenylated flavanones, pyranoflavanon or benzylated flavanones,giving a great number of substituted derivatives [28] .Flavanone are present in high level in citrus fruits , grapefruits ,lemons and orange[22].Example of flavanones include : hesperidin (13), and naringenin (14) which is considered to have bioactivity effect on human health as antioxidant , anti-inflammatory and immune system modulator[30].



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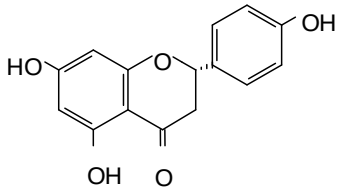

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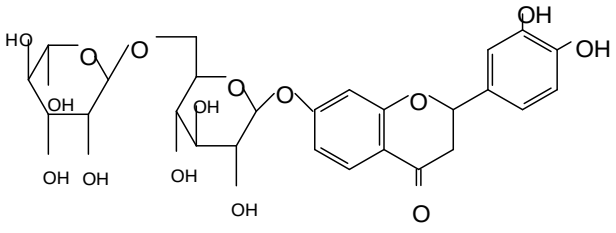

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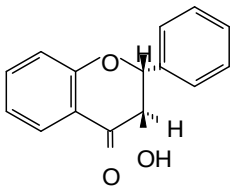
1.6- Dihydroflavonols

Dihydroflavonols, are the 3-hydroxy derivatives of flavanones.They are highly diversified and multisubstituted subgroup. The hydroxyl group at C-3 in dihydroflavonols can be methylated, glycosylated, or esterified. Dihydroflavonols have two asymmetric carbons at C-2 and C- 3 . The most common configuration is:(2R,3R)- configuration(15) [28] . The most significant biological property is their anti microbial activity [(20)]. Examples are: taxifolin (dihydroquericetin-16) and ampelopsin (dihydro myricetin -17) .



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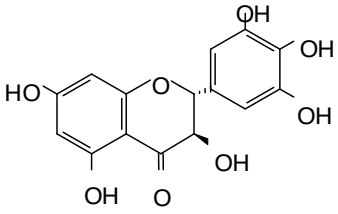

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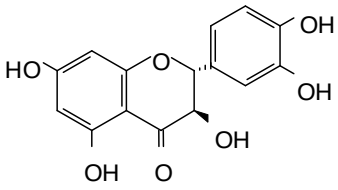

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1.7- Flavanols

Flavanols are also known as flavan-3-ol as the hydroxyl group is almost always bound to position 3 of C ring; they are called catechins as well. Unlike many flavonoids, there is no double bond between positions 2

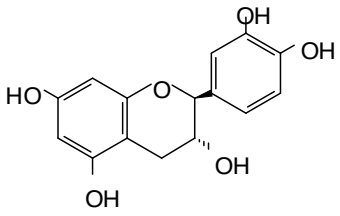
and 3; another distinctive features, e.g. compared to flavanonols, with which they share a hydroxyl group in position 3, is the lack of a carbonyl group, that is, a keto group, in position 4. This particular chemical structure allows flavanols to have two chiral centers in the molecule, on positions 2 and 3, then four possible diastereoisomers are formed. Epicatechin is the isomer with the *cis* configuration and catechin is the one with the *trans* configuration. Each of these configurations has two stereoisomers, namely, (+)-epicatechin and (-)-epicatechin, (+)-catechin and (-)-catechin.(+)-Catechin and (-)-epicatechin are the two isomers most often present in edible plants. Another important feature of flavanols and particulary of catechines (18) and epicatechines(19) is ability to form oligomeric and polymeric compounds called proanthocyanidins [30].

The catechines are abundant in tea,some cocoa and chocolates[31]. They are also present in human diet in fruits and vegetables [32].

Flavanols , usually from cocoa beans or tea are beleived to keep arteries flexible , increase small vessel circulation , reduce blood pressure and protect against sun burns[33]. Mice fed with catechines showed decreased level of aging , lowering of oxidation stress in proteins [34].

Epigallocatechin(20) and gallocatechin contain an additional phenolic hydroxyl group when compared to epicatechin and catechin, respectively. .

Catechin gallates are gallic acid esters of the catechins . Examples of this class include: epigallocatechin gallate(21), which is commonly the most abundant catechin in tea.



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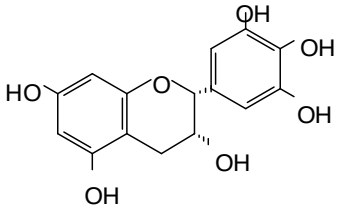

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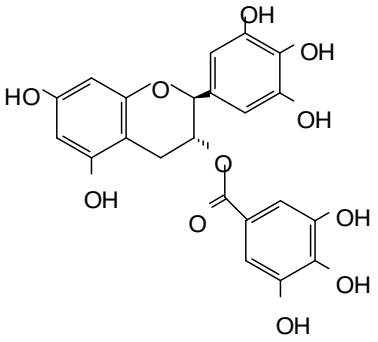

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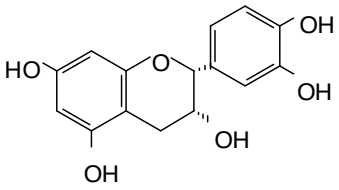

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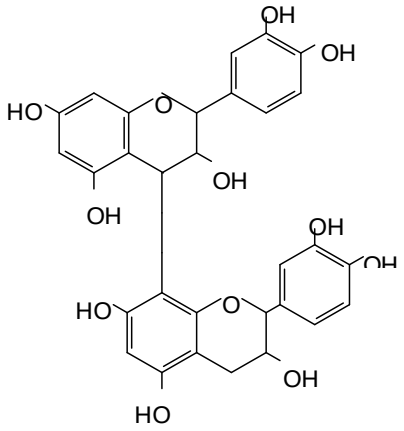
1.8-Proanthocyanidins

Proanthocyanidins are oligomeric flavonoids , mainly found in grapes. They are dimers or oligomers of catechin and epicatechin and their gallic acid esters. Proanthocyanidins are traditionally considered to be condensed tannins. Flavanols and oligomers (containing 2–7 monomeric units) are known as strong antioxidants .

Proathocyanidins can be found in many plants , most notably apples , grape seed , grape skin ,cinnamon , cocoa , pine bark . Also they are found in fruits from wild shrubs such as chock berry , howthorn , and sea buck thorn[35].Proanthocyanidins are converted into anthocyanidins under acidic conditions . Examples of proanthocyanidins include: B2 dimer (22).



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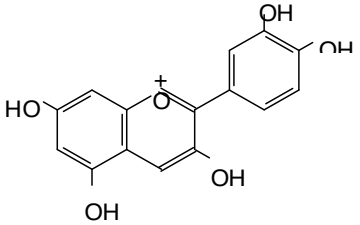
1.9- Anthocyanidins and anthocyanins

Anthocyanidins(23) are flavonoids lacking keto oxygen at position 4 . Chemically ,they are oxygenated derivetives of 2- phenylbenzobyrylium or flavylium salts and they are considered as common plant pigments.

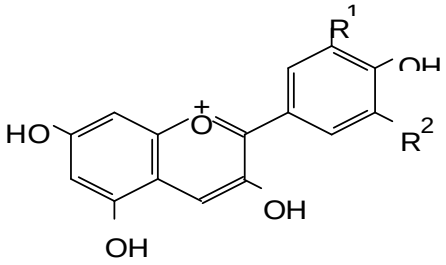


(23)

Anthocyanins (glycosylated anthocyanidins) are water -soluble vacular pigments. they occure in all tissues of higher plants including leaves,stems,roots,flowers and fruits [36]. Major source of anthocyanins are cherries , berries , purple grapes[37].They are responsible for the red , purle and blue colors of many fruits, vegetables, cerenal grains and flowers.They support pollination and seed dispersal, and protect photo-synthetic tissues against photoinhabitation [38].

Anthocyanins can be used as PH indicators because of their color change with P^H. They are pink in acidic solution, purple in neutral solution, greenish-yellow in alkaline solution and colourless in very alkaline solution. Generally degraded at higher PH, however some of them are resistant to the gradation at PH 8 and can be used as food color [39].

The most common anthocyanidins are: cyanidin, delphinidin, peonidin, petuniodin, malvidin, pelargonidin which only differ in hydroxylation and methoxylation on their B rings as in (24) .



(24)

Pelargonidin; $R^1 = R^2 = H$

Cyanidin ; R^1= OH , R^2 = H

Delphinidin; R^1= OH , R^2 = OH

Peonidin; R^1= OCH_3 , R^2 = H

Petuniodin; $R^1 = OCH_3$, $R^2 = OH$

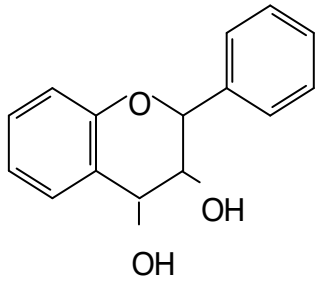
Malvidin ; $R^1 = OCH_3$, $R^2 = OCH_3$

The anthocyanins differ with respect to glycosylation of hydroxyl groups , nature of glycosylation , substitution and potential aliphatic and aromatic acylation [40].

Several studies have shown that anthocyanins display a wide range of biological activities including antioxidant, anti-inflammatory, antimicrobial and anti-carcinogenic activities. In addition they display a variety of effects on blood vessels, platelets and lipoproteins able to reduce the risk of coronary heart diseases [37].

1.10-Leucoanthocyanidin (flavan-3,4-diols)

Leucoanthocyanidin(25) are considerd as derivatives of anthocyanidins and hence are termed leucoanthocyanidin. These flavonoids have been identified in the heartwood of different Acacia species , grape seed extract , and the maritime pine tree which has potential for improving circulating function and for treating inflammatory disorders. Leucoanthocyanidins have been demonstrated to be intermediates in anthocyanidin biosynthesis in flowers of Matthiola incana . Example is leucopeonidin(26) which is found in bark of *Ficus bengalensis* , and shows anti- diabetic effect [41].



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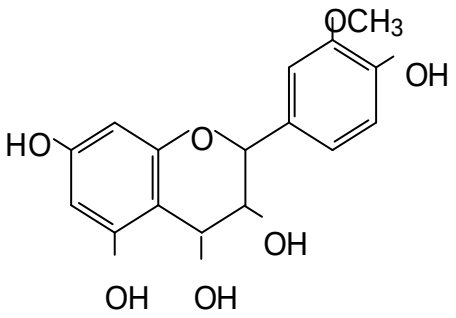

26

1.11- Isoflavonoids

Isoflavonoids are subgroup of flavonoids in which the B ring is attached to position 3 of the C ring. They have structural similarities to estrogens, such as estrodiol.They are also called phytoestrogens. Isoflavonoids are derived from 3-phenyl chromen-4 -one -3-phenyl -1,4 benzopyrone .

Isoflavonoids are divided into seven categories : isoflavones, isoflavanes, isoflavanones, coumestane, pterocarpanes, retinoids, and coumaronochromones [42-44] .As in isoflav-3-ene (27) and coumestane (28).

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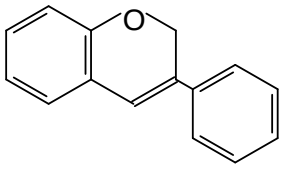

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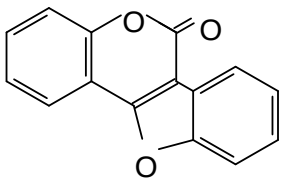
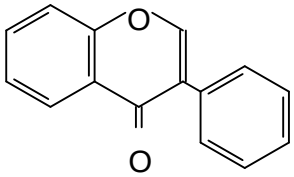


28

Isoflavones (29) constitute the largest and the most studied group of isoflavonoids. There are several natural source of isoflavones, but the most important are soybeans and red clover. Genistein(30) and daidzein(31) are the main isoflavonoids found in soy along with glycetein biochanin and fomononetin [45], they are also found in the red clovers [46]. All these isoflavone aglycones are mostly found as 7-O-glucosides and 6''-O-malonyl-7-O-glucosides. Many isoflavones act as phytoestrogens in mammals. some are termed antioxidants . More evidence [43] indicates that soybean isoflavones may often protect against a wide range of human diseases , including breast, bowel, prostate and other cancers, cardiovascular disease and osteoporosis.



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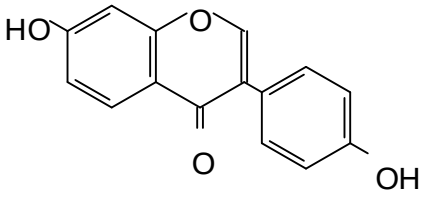

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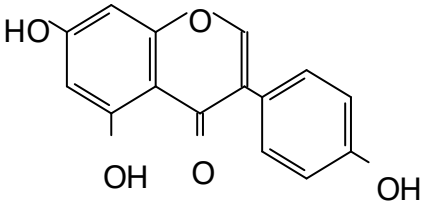
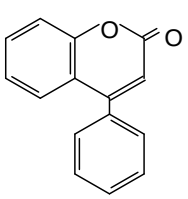


31

1.12- Neoflavonoids

Neoflavonoids are a class of polyphenolic compounds which belong to flavonoids (in the narrow sense) .They have the B ring attached at position 4 of the C ring . They have limited distribution . Neoflavonoids have three main subdivisions of structure [47] and they are derived from the 4 - phenylcoumarine (32) . Neoflavans - 3 , 4-dihydro -4- arylcoumarins(33) , neoflavens (34) possess the 4 phenyl chromen back bone .



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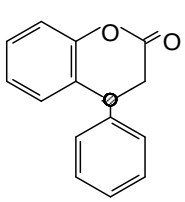

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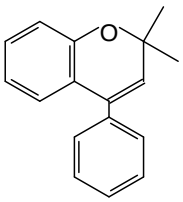
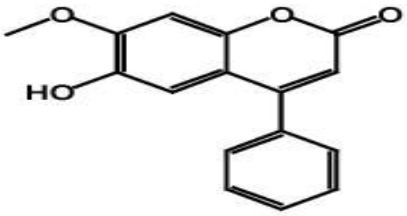


34

Neoflavonoids are not often found in food plants, but dalbergin (35) is the most common and relatively widely distributed neoflavone in the plant kingdom [48].



(35)

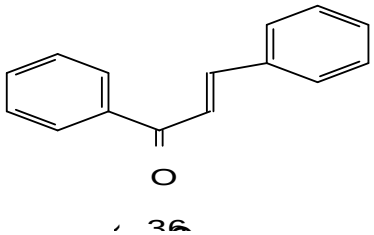
1.13- chalcones and dihydrochalcones

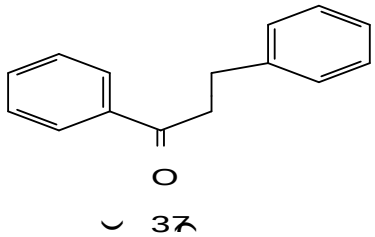
chalcones and dihydrochalcones are characterized by the presence of two aromatic rings linked by three carbon bridge that is unsaturated as in chalcones(36) and saturated in dihydrochalcones(37). The numbering convention of cbalcones and dihydrochalcones differ from that used for other flavonoids.

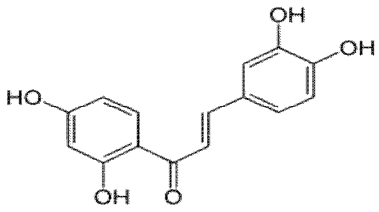
Chalcones are also known as benzalacetophenone or benzylidene acetophenone. They play an essential role [49] in flavonoid biosynthetic path and contributes signicantly to the total amounts of plant flavonoid. They are specially abundant in fruits (citrus) ,apples, vegetables and spices [50] .Chalcone may exist in *cis* and *trans* isomeric forms of which , the *trans* form is the stable isomer [51].

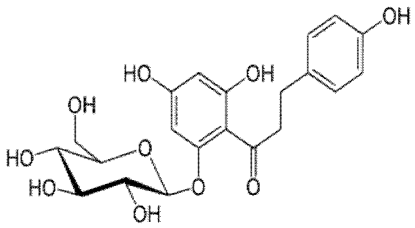
Most of the chalcones. Have radical quenching properties due to presence of phenolic group that have raised interest in using the chalcone-rich plant extracts as therapeutic compounds or food preservatives [52]. Naturally occurring chalcones as well as synthetic chalcones analogues have been revised for their wide biological acivities such as anti-inflammatory anti- tumor anti-malaria antioxidant . anti-bacterial [53]. Example of a chalcone is butein (38).

Dihydrochalcones are directly related to the chalcones and are derived from them by reduction of the α , β- double bond . Example is phloridzin(39) which is the most abundant phenolic compounds in seeds of apple.









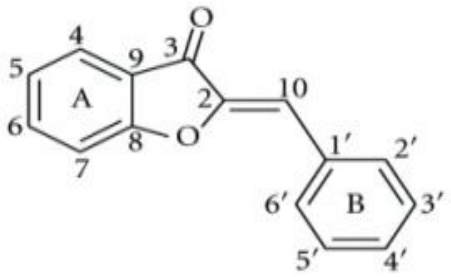
(38) (39)

1.14- Aurones

Aurones constitute a sub class of flavonoids with a five- membred ring C .There are two isomers of the aurone(40) molecule, with (E) and (Z) configuration. Most aurones are in (Z) configuration, which is the most stable form.

Aurones like (Z)-2-benzylidenebenzofuran-3-(2H)-ones, constitute a subclass of flavonoids which occur rarely in nature. They are responsible for the bright yellow color of some popular ornamental flowers and are biosynthesized from chalcones by the key enzyme aureusidin synthase. Although the studies of the biological activities of aurones are still limited, these natural products and their synthetic analogues have proved to be promising bioactive compounds with a broad spectrum of activity

including anticancer ,antimicrobial, antioxidant and antileishmanial properties , whereas they possess enzyme inhibitory, or enzyme-inducing activity [54]. Example of this class is leptosidin (41).



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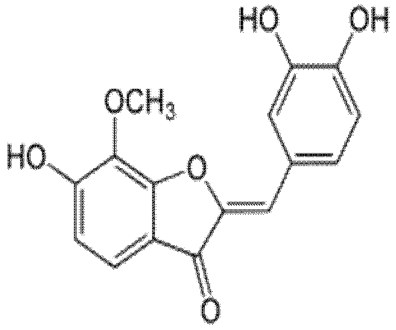


(41)

1.15- Biflavonoids

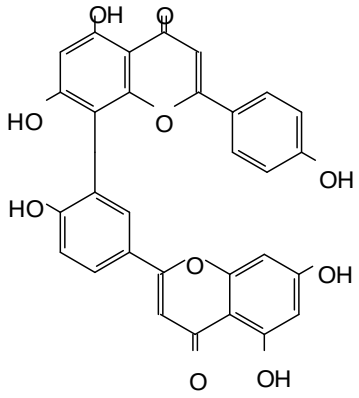
The term biflavonoids was proposed by Harbone to designate the dimer of apigenin , which is clearly distinct from other dimeric flavonoids such as proanthocyanidin . Biflavonoids consist of two flavonoids linked through either a C – C or C – O bond . They involve member of flavonoids and their distribution is limited to several species.

Biflavonoids are classified into seven major types: agathisflavones, cupressuflavones, amentoflavones, hinoflavones, robustaflavones, miscellaneous synthetic biflavonoids, and Garcinia biflavonoids (GBs) . The latter are generated from linkage variations of two monomers, generally leading to C-3/C-8" biflavanones, C-3/C-8"-linked biflavone, and C-3/C-8"-coupled flavanone-flavone analogs. All these dimers (except for biflavones) carry at least one stereogenic center, but also

show atropisomeric behavior due to restricted rotation about the central axis.

Biflavonoids are found in the rind of green citrus fruits , rose hips , and black currant .Many pharmacological effects of biflavonoids have been assessed, such as their ability to inhibit histamine release and platelet adhesion. Anti-inflammatory as well as antibacterial activity related to biflavones have also been reported [55] .

Amentoflavones (42) and hinokiflavones (43) are biflavonoids from dimers of apigenin , the difference between them is the linkage .



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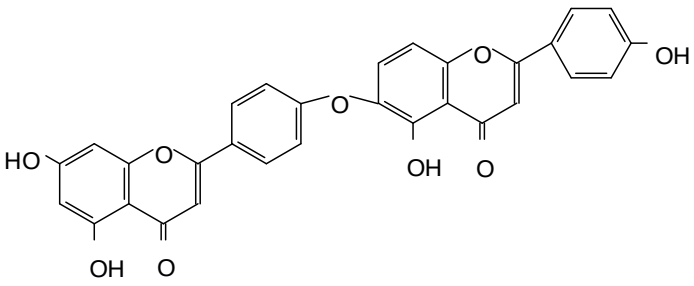
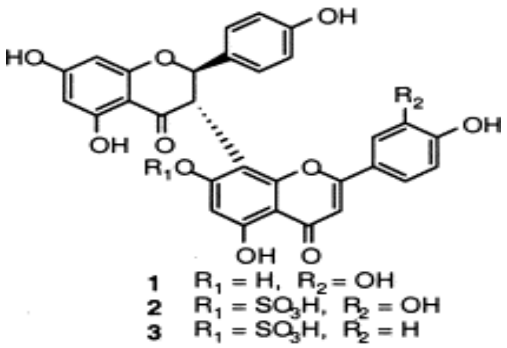


(43)

Mixed biflavonoids are exemplified by: flavone– flavanone dimers (44), flavanone-(3→8″)-flavone biflavonoids.



(44)

1.16- Synthesis of flavonoids

1.16.1 Biosynthesis of flavonoids

The biosynthesis of flavonoids involves a complex network of routes based principally on the shikimate and phenylpropanoid pathways.

The $C_6 - C_3 - C_6$ flavonoid structure is the producer of two separate biosynthesis pathways. The bridge and aromatic B-ring constitute a phenylpropanoid unit synthesized from *P*-coumaryl CoA . the six carbons of ring-A originate from the condensation of three acetate units via the malonic acid pathway . Both flavonoids precursers are derived from carbohydrates.

P-coumaroyl CoA is synthesized via phenyl alanine which is synthesized by enzymes of the shikimate/arogenet pathway in three enzymes steps. Malonyl CoA is synthesized from acetyl CoA by the enzymes of acetyl CoA carboxylase).

The fusion of these two parts involves the step- wise condensation of *P*-coumaroyl coA with three malonyl CoA residues, each of which donate

two carbon atoms , in a reaction catalyzed by chalcone synthase (CHS). The product of this reaction is naringenin (a chalcone). Slight modification in the pathway involves the production of isoflavones , that are derived from isoliquiritigenin which , unlike naringenin lacks two hydroxyl groups.

The next step in the flavonoid biosynthesis is the stereospecific conversion of a naringenin to a flavanone by chalcone isomerase (CHI).From these central intermediates , the pathway diverse into several biosynthesis branches , each resulting in a different class of flavonoids: isoflavones , flavones , flavonols , flavan-3-ols and anthocyanins. Additional structural elaboration , mainly through glycosylation but also via acylation and alkylation, gives the huge variety of flavonoids structures seen throughout plant kindom .

In legumes , CHI , also catalyses the conversion of isoliquiritigenin to liquiritigenin [56].

During the biosynthesis of isoflavonoids , the enzyme isoflavone synthase (IFS) catalyses the first step which involves conversion naringenin and isoliquiritigeni into the isoflavones genisteins and daidzein , respectively [54]. Further metapolism of the isoflavones, characterized by the fate of daidzein , result in a 4- or 7- methylation to produce isoforrmononelin , which andegoes a series of reations including , hydroxylation,reduction , and dehydration to form phytalexin medicarpin . Otherless , well-characterized steps result in a

range of isoflavonoids including coumestanes , rotenoids and pterocarpins.

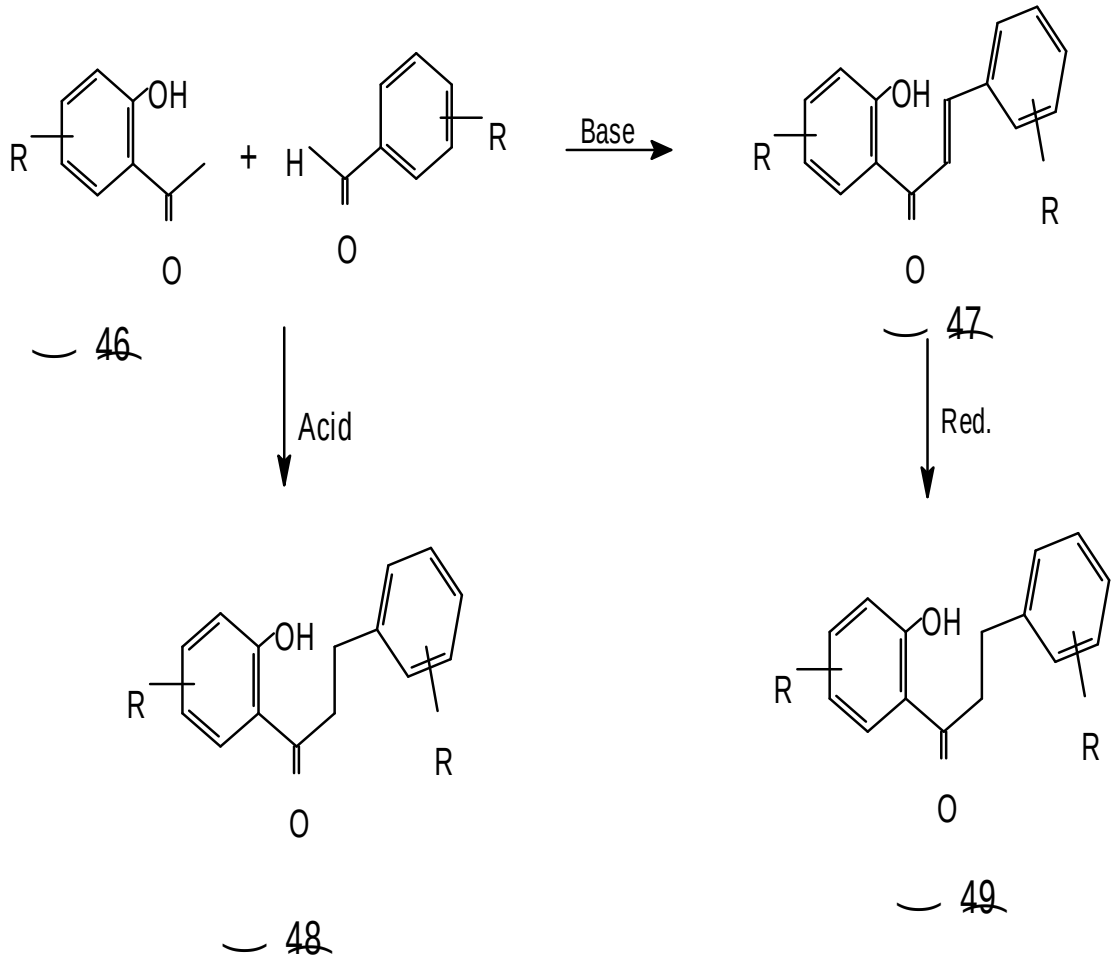
Two different enzyme systems : FNS I , FNS II have been reported [57] for the conversion of flavanone (naringenin) to flavone (apigenin). The conversion involves the introduction of a double bond .

Aurones are derived from chalcones, the biosynthetic mechanism has only recently been clarified, and some aspects of the enzymatic process still await *in vivo* proof . Polyphenol oxidase (PPO) can catalyze conversion of either 2',4',6', 4-etrahydroxychalcone (naringenin chalcone) or 2',4',6',3,4-pentahydroxychalcone to

4- etrahydroxychalcone (naringenin chalcone) or 2',4',6',3,4-pentahydroxychalcone toaureusidin (3',4'-hydroxylated) or bracteatin (3',4',5'-hydroxylated), respectively [57].

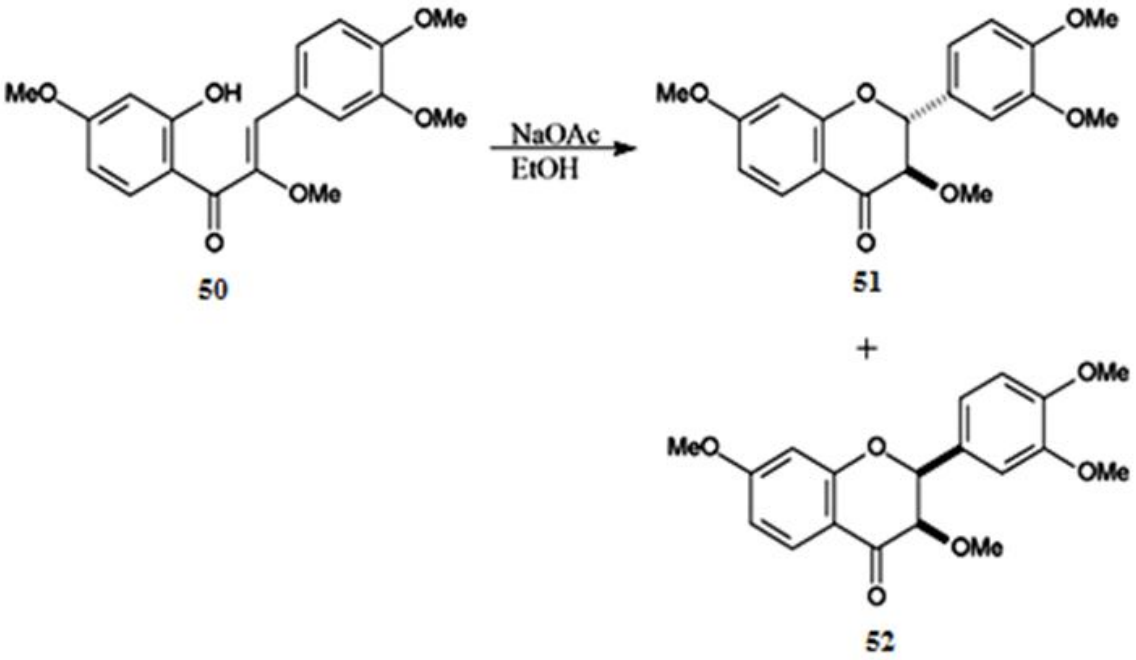
1.16.2-Chemical synthesis of flavonoids

Chalcones and dihydrochalcones are considered to be the primary C_6-C_3-C_6 precursors and constitute intermediates in the synthesis of flavonoids. Chalcones are readily accessible via two well-established routes comprising a base-catalyzed aldol condensation or acid-mediated aldolization of 2-hydroxyacetophenones(45) and benzaldehydes(46).The base-catalyzed aldol condensation is usually the preferred route towards chalcone(47) formation, since under acidic conditions cyclization of the ensuing chalcone leads to formation of corresponding racemic flavanones(48) . Dihydrochalcones (49) are generally obtained via reduction (H_2/Pd) of the preceding chalcones [58] . (Scheme2.2).



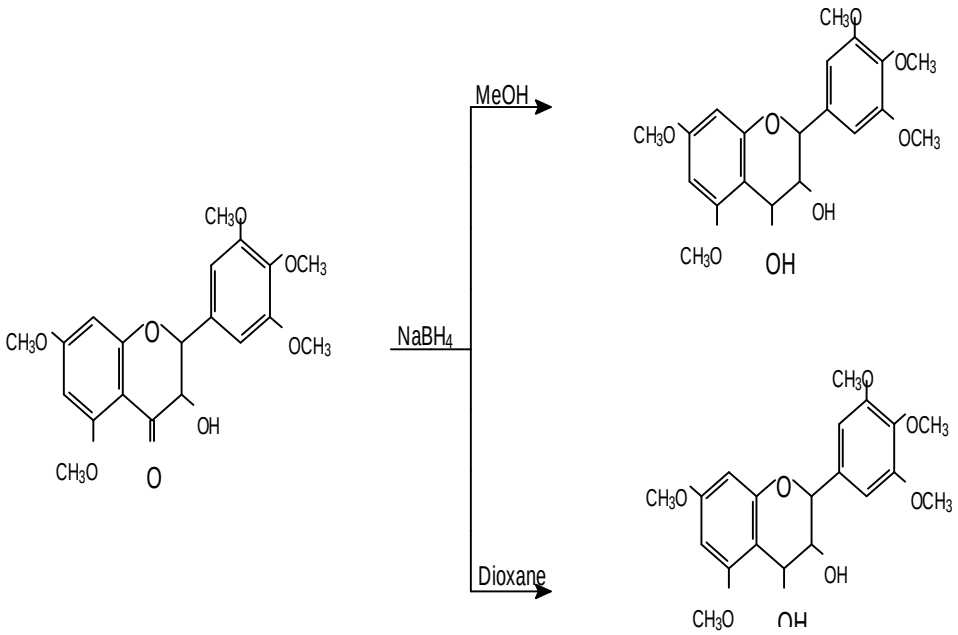
Scheme 2.2: Synthesis of chalcones

Although the Algar-Flynn-Oyamada protocol was demonstrated to form of racemic dihydroflavonols in moderate to good yields,cyclization of 2′-hydroxy-α,3,4,4′-tetramethoxychalcone(50) with sodium acetate in ethanol furnished both (51)and(52) in 22% and 11% yields, respectively. However, this method is not applicable to cyclization of α- OH-chalcones [58].

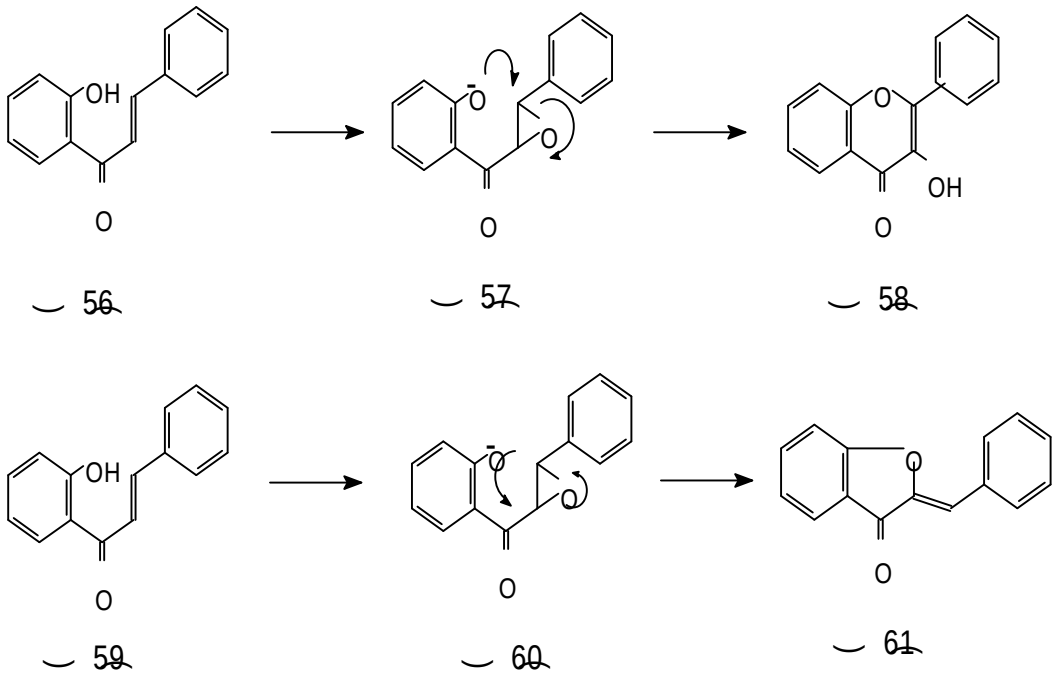


50 51 52

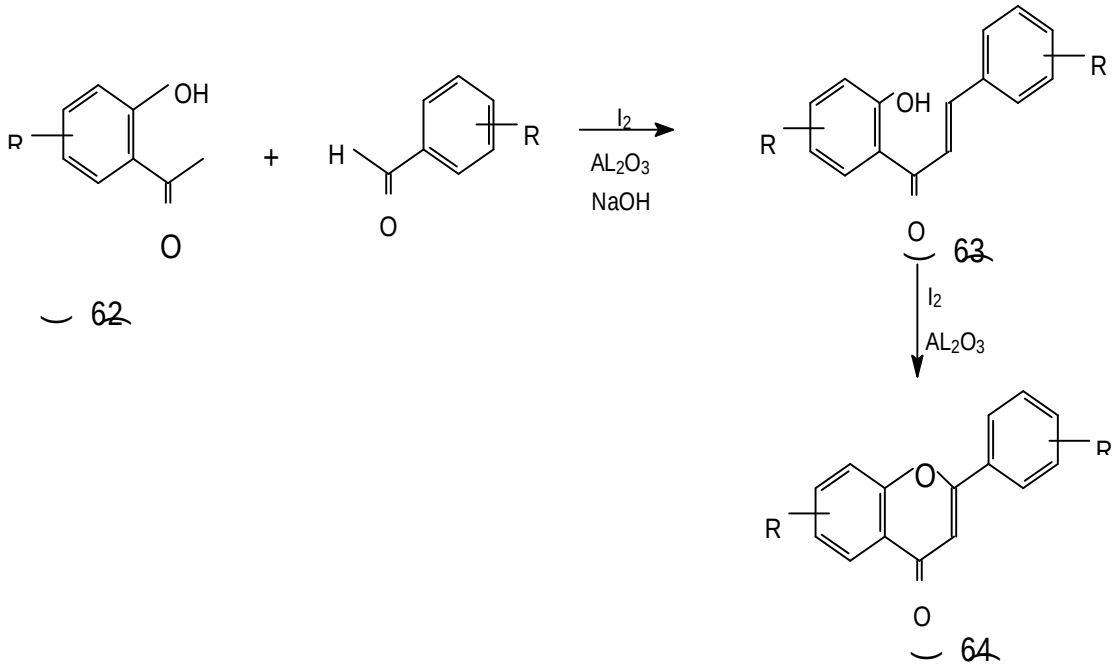
One of the most common ways for the synthesis of flavan-3-ols and the closely related flavan-3,4-diol analogues involves the reductive transformation of dihydroflavonols. Reduction of the dihydroflavonols(53) with sodium borohydride in methanol affords the 2,3-*trans*-3,4-*trans*-flavan-3,4-diols(54) while reduction in an aprotic solvent like dioxane yielded [58] the C4-epimers (55) exclusively.



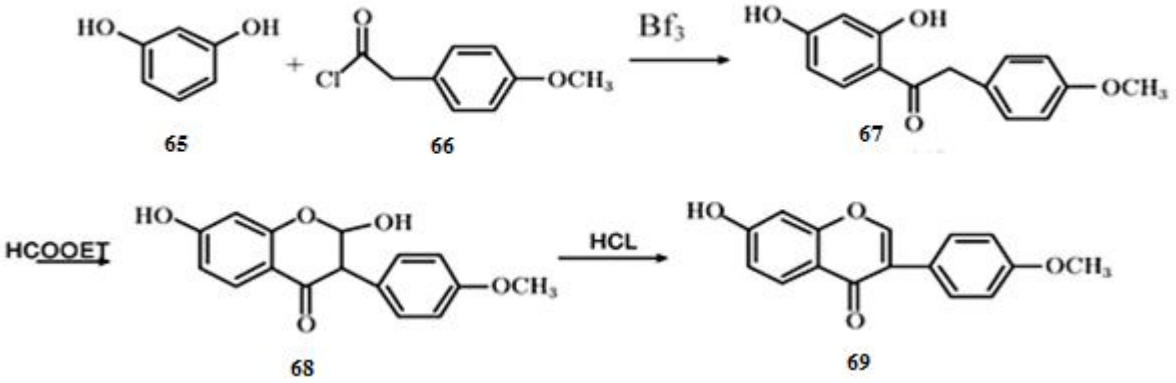
In Algar-Flynn- Oymada method , oxidation of 2-hydroxychalcone (56) with H_2O_2 in basic conditions gave corresponding flavonol (57) which is an epoxide derivatives. Then , an intermolecular nucleophlic substitution of flavonol at the benzylic carbon and a concomitant oxidation afforded flavonol (58) . In contrast, nucleophlic substitution on the other side of the epoxide , i.e., α- postion of ketone gave (61) as by product .Solvent and temperatue are the major factors for this reaction. High temperature is beneficial to the formation of flavonol.



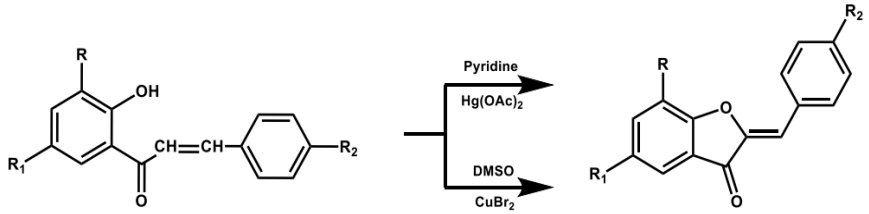
Traditionally, flavones have been prepared by Baker-Venkatraman rearrangement and Claisen-Schmidt condensation, which involves the conversion of 2-hydroxyacetophenones into benzoyl esters, followed by rearrangement in basic media to 1,3-diphenylpropane-1,3-diones which upon cyclization under acidic conditions furnishes flavones.On the other hand hydroxychalcone[63] synthesized from 2- Hydroxyacetophenone [62] and benzaldehyde under Claisen-Schmidt conditions can undergo oxidative cyclization to furnish flavone ring[64].



There is many approach to synthesize isoflavones . The classic one is cyclization of phenyl benzyl ketone derivatives[67] obtained from resorcinol[65] and the benzyl ketone [66] . Many agents , such as ethyl orthoformate , $Zn(CN)_2$\Hcl , DMF/$POCl_3$, and ethyl formate /Na , can be used in the cyclization step . However , these reagents have specific requirements for their structures , for example , ethyl orthoformate is only applied for the compounds which contain 2,3,6-trihydroxyl phenyl substrate and DMF/$POCl_3$ is only applied to oxidation derivatives of resorcinol. The synthesis of formononetion [69] is one example [59,60].



The following reaction illustrates the synthesis of aurones ,like [70] , where 2'-hydroxychalcone is used as a synthon [61].



(70)

R= H-, Br-; R1= H-, CH3-; R2= H-, OCH3-, Cl-

1.17- Biological activities of flavonoids

1.17.1 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 mehanisms of antioxidant activity such as radical scavenging and metal ion chelation ability[62].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 flavonoid radical[63]. Mechanisms of antioxidant action can include:

suppression of ROS formation either by inhibition of enzymes or by chelating trace elements involved in free radical generation; scavenging ROS; and upregulation or protection of antioxidant defenses [64]

Lipid peroxidation is a common consequence of oxidative stress. Flavonoid protect lipids against oxidative damage by various mechanism [65]. 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 are thermodynamically able to reduce highly oxidizing free radicals , such as superoxide, peroxyl, alkoxyl, and hydroxyl radicals by hydrogen atom donation . Because of their capacity to chelate metal ions (iron, copper, etc.), flavonoids also inhibit free radical generation[64]. Quercetin in particular is known for its iron-chelating and iron-stabilizing properties.

The presence of a 3′,4′-catechol structure in the B ring firmly enhances inhibition of lipid peroxidation. This trend of flavonoids makes them most effective scavengers of peroxyl, superoxide, and peroxynitrite radicals [65]. Epicatechin and rutin are strong radical scavengers and

inhibitors of lipid peroxidation *in vitro* [66]. Oxidation of the B rings of flavonoids having catechol group afford fairly stable orthosemiquinone radicals which are strong scavengers. Flavones lacking catechol systems afford, on oxidation, unstable radicals exhibiting weak scavenging potential [67].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 [68].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 elements besides other structural features .The flavonoid heterocyclic C ring contributes to antioxidant activity by permitting conjugation between the aromatic rings and the presence of a free 3-OH afford more potential. 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 radicals scavenging capacity [69].

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 increases [70].

1.17.2- Hepatoprotective activity

The hepatoprotective activity of flavonoids has been well studied. It plays an important role in healthy liver function .Several flavonoids such as catechin, apigenin, quercetin, naringenin, rutin, and venoruton are reported for their hapatoprotective activities [71].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. Zhu et. al.[72]demonstrated that anthocyanin: cyanidin-3-O-β-glucoside (Cy-G) increases hepatic Gclc expression.. Increased Gclc expression results in a decrease in hepatic ROS levels and proapoptotic signalling. Furthermore, Cy-G treatment lowers hepatic lipid peroxidation, inhibits the release of proinflammatory cytokines, and protects against the development of hepatic steatosis [72]. Silymarin is a flavonoids having three structural components silibinin, silydianine, and silychristine extracted from the seeds and fruit of milk thistle (*Silybum marianum* - Compositeae). 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 [73].

Silymarin has clinical applications in the treatment of cirrhosis, ischemic injury, and toxic hepatitis induced by various toxins such as acetaminophen, and toxic mushroom [74].

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 are reported to provide protection against chemically -induced hepatotoxicity in HepG2 cells [75].

1.17.3-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 [64,76].Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity [77].Antibacterial flavonoids might have 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.

Catechins, the most reduced form of the C_3 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 [78]. Naringenin and sophoraflavanone G have intensive antibacterial activity against *Staphylococcus aureus* (MRSA) and *Streptococci* [79]. A hydroxyl group at position 5 in flavanones and flavones is important for their activity against MRSA. Substitution with C_8 and C_{10} chains may also enhance the antistaphylococcal activity of flavonoids belonging to the flavan-3-ol class [80].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* [81].

1.17.4- Antifungal activity

Owing to the widespread ability of flavonoids to inhibit spore germination of plant pathogens, they have been proposed for use against fungal pathogens of man [82]. A prenylated flavanone isolated from the shrub *Eysenhardtia texana* has been identified as 5,7,4-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2S)-flavanone and shown to possess activity against the opportunistic pathogen *Candida albicans* [83]. The flavonoid:7-hydroxy-3,4-methylenedioxyflavan, isolated from

Terminalia bellerica fruit rind, has also been shown to possess activity against *C. albicans* [84]. Two flavones from *Artemisia giraldi,* identified

as: 6,7,4-trihydroxy-3,5-dimethoxyflavone and 5,5- dihydroxy-8,2,4-trimethoxyflavone, together with 5,7,4- trihydroxy-3,5-dimethoxyflavone have been reported to exhibit activity against *Aspergillus flavus* [85], species of fungi that causes invasive disease in immunosuppressed patients [86]. The activity of propolis against dermatophytes and Candida species has been attributed at least partially to its high flavonoid content . Galangin, [87] a flavonol commonly found in propolis , has been shown to have inhibitory activity against *Aspergillus tamarii, A.flavus, Cladosporium sphaerospermum, Penicillium digitatum* and *Penicillium italicum* [88].

1.17.5-Antiviral activlty

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 [89] ,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 [(90)]. 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 [(91)].Catechins are also known to inhibit DNA polymerases of HIV-1. 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 [(92)].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against *herpes simplex* virus (HSV) . Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection [(90)]. 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 [(90)].

Zandi et al.[(93)] 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 [89]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action [93]. List of some flavonoids and their efficacy against viruses is given in Table(1.1) below [22]:

Table 1.1: Antiviral activity of some 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	Parainfluenza virus, influenza virus, and potato virus
Naringin	Respiratory syncytial virus
Luteolin	Auzesky virus
Morin	Potato virus
Galangin	Herpes simplex virus type

1.17.6- Antiinflammatory 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 proinflammatory 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[94].

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 proteinkinase [95].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 transmisstion 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 [96]. 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 proinflammatory signaling molecules [97].

Several flavonoids are reported to inhibit platelet adhesion, aggregation, and secretion significantly at 1–10 mM concentration[98].The effect of flavonoid on platelets has been related to the inhibition of arachidonic

acid metabolism by carbon monoxide. Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function [99,100] .

1.17.7- 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 [101]. 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 [101].

Several mechanisms have been proposed for the effect flavonoids on the initiation and promotion stages of the carcinogenicity including influences on development and hormonal activities [102]. Flavonoids are found to downregulate expression of mutant p53 protein to nearly undetectable levels in human breast cancer cell lines [103].Mutations of p53 are among the most common genetic abnormalities in human cancers. 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 [100,104].In contrast to most normal tissues expression of FAS is markedly increased in various human cancers . 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*.

Barnes [105] has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models. In an 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. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol -induced colon and mammary cancers in rats [106].Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity[107] .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[108].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk[109].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 [64].

1.17.8- Cardiotonic activity of flavonoids

Flavonoids play a significant role in maintaining vascular health. This is because of their anti-oxidative properties. Studies have shown that intake of polyphenols including flavonoids is associated with reduced

risk of coronary heart diseases [110]. Numerous flavonoids of dietary significance have been shown to impart beneficial impact on parameters associated with atherosclerosis, including lipoprotein oxidation, blood platelet aggregation, and vascular reactivity [111,112].For example, in a study[113], 3',4'-dihydroxyflavonol has been shown to reduce injury after myocardial ischemia and reperfusion. It acts both as antioxidant and as vasorelaxant .Furthermore, epidemiological studies show an inverse correlation between dietary flavonoid intake and mortality from coronary heart disease ,which is explained in part by the inhibition of low-density lipoprotein oxidation and reduced platelet aggregability

1.17.9-Endocrine effect of flavonoids

Certain flavonoids exhibit hormone-like activities. They show resemblance to estrogen and other steroid hormones and are referred to as phytoestrogens. Estrogen, apart from its classical hormonal effect, possesses a neuroprotective effect on the brain. Studies have been carried out by various research groups to investigate the estrogenic activity of genistein, daidzein and equol at various levels that include the molecular, preclinical and clinical. The studies determined their potential for treatment of chronic diseases such as hormone-dependent cancer, cardiovascular disorders and osteoporosis[114].

One of the studies suggested that genistein is the most promising compound to prevent postmenopausal bone loss in women[115].Studies have shown that ingestion of dietary genistein resulted in concentration

changes of hormones, such as insulin, thyroid hormones, cortisone and corticosterone, as well as lipid metabolic changes [116].

1.17.10-Neuroprotective property of Flavonoids

Neurodegerative disease seems to result from the combined effect of oxidative stress, inflammation and transition metal accumulation. Alzheimers and related dementias are among some of the major disorder of neurodegeneration. This was attested to by one of the studies which suggested that higher consumption of dietary flavonoids, especially flavonols, is associated with lower population rates of dementias [117].Similarly, another study suggested that citrus flavanones such as hesperidin, hesperetin, and neohesperidin could traverse the blood-brain barrier and may play effective role in the intervention for neurodegenerative diseases[118].

1.17.11- Antidiabetic activity of flavonoids

Flavonoids have been studied for their antidiabetic activity, although there are only a few studies in relation to this. It may be thought that flavonoids may help to repair beta cell function by reducing free radical-induced tissue damage. They also reduce the hyperglycemic effects controlling the blood sugar levels. Studies have shown that intake of specific types of lavonoids, including quercetin and myricetin, is inversely associated with the risk of type 2 diabetes [119]. This is attested by another study which showed that quercetin may relieve diabetic symptoms .The study illustrated the mechanism for protective effects of quercetin on diabetes - induced hepatic injury. Quercetin is found to

inhibit enzyme aldose reductase. It is the first enzyme of the sorbitol-aldose reductase pathway. It plays an active role in converting glucose (sugar) to sorbitol (a sugar alcohol) in the body. Hyperglycemia leads to increased generation of sorbitol in the body. This result in development of secondary problems, such as neuropathy, retinopathy, diabetic cataracts, and nephropathy [120]. Quercetin may therefore, be beneficial in the nutritional management of diabetes and its complications.

1.17.12- Cosmetic application of flavonoids

Skin forms the first line of defense to the external environment. Upcoming research studies have confirmed the effect of flavonoids on skin health. Flavonoids work as anti-oxidizing agents and free radical scavengers. They penetrate deeper into the skin and protect it from UV radiation damage. Green tea (*Camellia sinensis*) and *Ginkgo biloba* extracts in cosmetic formulations have been suggested to protect the skin against UV-induced damage and skin aging. They evidenced good skin penetration and retention [121]. Myricetin is considered to be the flavonoid which has the capacity to neutralize the effects of free radicals which cause photoaging within the skin. The protective effects of myricetin on ultraviolet B - induced damage to keratinocytes has been studied [122] and it was proposed that myricetin is the compound which may be used for further development of anti-aging components for skin care applications. Similarly the ability of quercetin to guard the skin against UV radiation induced damage has been studied [123] and it was suggested that the protective effect of quercetin against UV damage may

in part be attributed to its capacity to scavenge free radicals generated by UV rays.

1.18- *Combretum aculeatum*

Classification*:*

Phylum Magnolioph

Class Magnoliopside

Order Matales

Family Combretaceae

Genus Combretum

Synonyms: *Combretum leuconili* Schweinf., *C. holstii*

Vernacular/common names: Bularal, laonadi, laongi, (Peulh), agersigil (Tamachek), kodentabga (Mooré), shihheit (Sudan), gedajedo, mardaf (Somali)

Distribution and habitat:

Combretum aculeatum is sub-Sahelian dry zone species with a distribution range stretching across Africa from Senegal and Mauritania, to Somalia and Tanzania. It is widespread in dry areas, in bushland, woodland, savannah, and wooded grassland. It is often found along rivers, riverine forest and ground water forests, as well as on rocky slopes. It grows in bushland on fixed dunes, on sandy alluvium or in rocky places. It has a wide edaphic adaptation growing on alluvial soils and sandy, stony or clay soils. It can grow at altitudes of up to 1800 miles. It is reported to withstand flooding; however in the seasonally flooded areas of Sudan, it is restricted to termite mounds, which are

generally above the flood level. Its distribution is irregular and is locally common. There is no recorded threat to *C. aculeatum*; however, the species is strongly browsed and regeneration suffers in heavily overgrazed areas[124].

Uses:

C . aculeatum is important source of nutrients for animals , which consume the leaves , flower and young shoots . its appreciated for its nutritive value and also for its palatability to stall fed and browsing sheep. The green leaves and young branches are much sought after as browse by both wild and domestic animals, and even the fallen leaves are eaten. Seeds of *C. aculeatum* are edible and in some places used for consumption, they are also eaten by wild and domestic animals[125].

Medicinal use:

The plant is used for its purgative and diuretic properties. It is used to treat blennorrhoea, colic, diarrhoea,intestinal worms, wounds, fever, gastritis,and loss of appetite. Some more speculative traditional uses include treatment against female sterility and mental disorders. Water in which the leaves have been boiled is drunk in northwest Senegal to promote micturition in cases when venereal disease obstructs the urethra. It is also used in Burkina Faso and Senegal for leprosy. In Senegal, the Soce tribe claims that a root decoction has a well-established reputation in the treatment of catarrha; the Serer tribe uses sap from the center of the stem for eye troubles. The boiled roots are

taken in Kenya for stomach upsets. Also the macerations of the roots of *C. aculeatum* are used to enhance wounds healing and the water extract of its roots is used as a purgative and as a poultice for skin tuberculosis in Sudan [124,126]

Botanical description:

Combretum aculeatum is a climbing shrub that subsists often on its annual shoots after been eaten. It can grow up to 4 m, even taller if support is available. The bark is fibrous grey-beige or dark red, with brown rhytidome, greenish or pale yellow slash. It often has long sarmentose branches. The leaves are alternate to sub-opposite. They can vary in size on the same branch. The blades can grow up to 7 cm long and 5 cm wide, but are usually smaller. They are elliptic, obovate or orbicular with acute to emarginated apex; both surfaces are pubescent . The nerves are pinnate, more or less prominent, with 4-6 pairs of mostly fused Lateral nerves. Petioles are 1-10 mm long, and persist after the rest of the leaf has fallen, forming a recurved spine that is up to 30 mm long. Its hairy branches with curved thorns allow the plant to hook onto surrounding trees and shrubs. The yellowish-white fragrant flowers are bisexual, with greenish to dark red sepals .The petals are 4-8 mm long by 1-2 mm wide, oblanceolate to obovate to spatulate, and pubescent on the back. The inflorescence is spike like, from the axils of the leaves. The amenfilaments are longer than the petals .

Flowering occurs at the end of the dry season and during rainly season . In Sudan flowering occurs from march to june and fruits from july to November.A tree can beer flowers and ripe fruit simultaneously .

The pale yellow or pale reddish fruit is an ovoid samara. It is 5-winged, 1-2 by 1-3 cm, and with a stalk 0.6-1 cm long. The papery, yellow-brown wings are0.4-0.6 cm wide[124].

Combretum aculeatum

Preliminary phytochemical screening of *Combretum aculeatum* leaves revealed that it is rich of tannins, unsaturated sterols and or triterpenes

and flavonoids .Also there is presence of coumarins in low concentration and trace of alkaloids [127] .

Extracts of leave and root show anti-cercarial activity against cercariae of *Schistosoma mansonia* [127]. Extracts of *C. aculeatum* showed anti-microbial activity against Gram-positive bacteria (*Bacillus subtilis* and *staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* , *Pseudomonas aeruginosa* and *Proteus vulgaris*)[128].

In Sudan, the genus *Combretum* represents a common constituent of forests on high drained rainfall savanna on wells or alluvial soils along streams, rivers and valleys in south Kassala, Kordofan and south Darfor[129].

Phytochemical studies carried out in the genus *Combretum* have demonstrated the occurrence of many classes of constituents, including triterpenes, flavonoids, lignans and amino acids . Also phenanthrene , dihydrophenanthrene , methoxylated flavonoids , stibenes were reported from the different species [130] .

About 24 different species of *Combretum* are well known in traditional medicine and used for treatment of an array of human disorders, such as abdominal pain, back-pain, cough, cold, conjunctivitis, diarrhea, dysmenorrhea, earache, fever, headache, fighting worms, infertility in women, fattening babies, leprosy, eumonia, swelling caused by mumps, scorpion stings and snake bites [131,132].

Since the 1970s, several unusual compounds have also been isolated from *Combretum* species, for example,9,10-dihydrophenanthrenes and a

substituted bibenzyl from *C. molle* [130] was isolated. Eleven triterpenes and theirglycosides were isolated from *C. laxum* among them, oleanane - , ursane- glycosides, arjunolic acid, arjunglucoside II, bellericoside, chebuloside II, quadranoside IV, asiatic acid and betulinic acid[133].Alkaloids (combretine and betonicine) were isolated from the leaves of *C. micranthum* [134]. Some flavonoids, rhamnoctrin, quercetin-5,3'-dimetylether , ramnazi and kaempferol were isolated from *C. erythrophyllum*[135],as well as quercetrin, kaempferol and pinocembrin (flavanone) from *C. apiculatum C rdamonin*. A Chalcone was a also isolated from *C.apiculatum* [136] and ellagic acid derivatives from *C. kraussii* [137].Combretastatins, a group of stilbenes, have been isolated from several species of *Combretum* [138].

Several phytochemical investigations on this genus focus mainly on pentacyclic triterpenoids, various polyphenols like flavonoids and stilbenoids [139]. GC/ MS showed presence of triterpenoids and stilbenoids in dichloromethane fractions of leaf and stem bark of *C. aculeatum, C. glutinosum and C. micranthum* . Ursolic acid was identified in the leaf extracts of all the three species whereas combretastatin A4 was found in small amounts only in the bark extract of *C glutinosum* species[140].

Oleanene-type of pentacyclic terpenoids containing 29-carboxyl-1α-hydroxyl groups were isolated from various species of *Combretum* e.g. *C.molle* and *C. imberbe* confirming chemotaxonomically significant bifurcation in triterpenoids synthesis in *Combretum* species[141].

1.19- Aim of this study

This study was aimed to:

Phytochemical screening of the medicinally important species *Combretum aculeatum*.

Extraction of flavonoids from the targeted species.

Isolation of plant phenolics via chromatographic techniques.

Elucidation of structures of isolates via sensitive spectroscopic techniques.

Evaluation of the antimicrobial potential of isolates against some standard human pathogens.

Chapter Two

Materials and Methods

2. Material and Methods

2.1. Materials

2.1.1 Plant material

The leaves of *Combretum aculeatum* were collected from Sunga, Gadaref State in October 2011. The plant was authenticated by the Department of Phytochemistry and taxonomy, National Research Center, *Khartoum*.

2.1.2 Instruments

Uv- visible spectrophotometer (Shimadzu UV – 2401PC) .

Joel ECA 500 NMR spectrophotometer.

Joel Mass Spectrometer (JMS- AX500).

2.2. Methods

2.2.1 Preperations of reagents for phytochemical screening.

2.2.1.2 Flavonoid and phenolic test reagents

- Alumnium 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.

2.2.1.3 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 leaves of *Combretum aculeatum* was extracted with 80% aqueous methanol (soxhelt) until exhaustion. This prepared extract(PE) was used for phytochemical screening.

2.2.3 Phytochemical screening

The prepared extract of *Combretum aculeatum* (PE) was used for following tests:

2.2.3.1- Test for unsaturated sterols and for triterpenes

10 ml of the (PE) was 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 chlorform solution was dehydrated over sodium sulphite anhydrous. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride, followed by two drops of concentrated sulphuric acid. Two separate layers (green, red) were observed.

2.2.3.2- Test for flavonoids

20 ml of the (PE) was evaporated to dryness on 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. Red colour was observed.
- To 3 ml. of the filtrate few drops of aluminium chloride solution were added. A dark yellow colour was formed.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.

2.2.3.3- Test for alkaloids

10 ml of the (PE) were evaporated to dryness on water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for minutes, then cooled and divided into two portions:

To one portion a few drops of Maeyer reagent were added. A white precipitated appeared, to the other portion few drops of Wagner reagent were added. A brown precipitate appeared.

2.2.3.4- Test for tannins

10 ml of (PE) was 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 was treated with few drops of ferric chloride solution. A dark blue colour was observed.

2.2.3.5- Test for Saponins

1 g of dried powdered *Combretum aculeatum* leaves 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. Honey comb was formed.

2.2.3.6- Test for cyanogenic glycosides

Three grams of powdered *Combretum aculeatum* leaves were placed in Erlenmeyer flask and sufficient water was added to moisten the leaves powder, followed by 1 ml. of chloroform. A piece of freshly prepared sodium picrate paper was carefully inserted between the split crock which was used to stopper the flask. The colour of sodium picrate paper changed from yellow to red**.**

2.2.3.7- Test for anthraquinone glycosides

Five grams of powdered leaves were boiled with 10 ml of 0.5 N potassium hydroxide containing 1 ml. or 3% hydrogen peroxide solution. The mixture was extracted with 10 ml. of benzene. 5 ml of benzene solution was shaken with 3 ml of 10% ammonium hydroxide solution and the lower layer was allowed to separate, there was no change in colour**.**

2.2.3.8 Test for coumarins

Three grams of powdered leaves of *Combretum aculeatum* were treated with 20 ml distilled water in a test tube . A filter paper was treated with a drop of 0.5 N potassium hydroxide solution. The filter paper was attached to the test tube to be saturated with the vapor and then it was inspected under UV light. The spot was able to absorb the UV light.

2.2.4 Extraction of flavonoids

(1 kg) Of powdered air-dried leaves of *Combretum aculeatum* was macerated with 80% aqueous methanol (4L) for 24hr. at room temperature with occasional shaking and then filtered off . The extraction process was repeated two more times with the same solvent. Combined filtrates were concentrated under reduced pressure using rotary evaporator at 40^{o} C until all methanol was removed yielding a crude product, which was suspended in 300 ml water and left overnight in a refrigerator and then filtrated. The aqueous filtrate (500 ml.) was partitioned successively with n-hexane, dichloromethane, ethyl acetate and butanol.

2.2.5- column chromatograpy (CC)

Open column (80 4 cm) was used for fractionation the ethyl acetate fraction. Silica gel with particle size 100-200 mesh from LOBA chemicals was used as stationary phase.

The composition of the mobile phase (dichloromethane CH_2Cl_2: methanol CH_4OH) was determined by TLC analysis.

The column was packed with slurry of silica gel with dichloromethane and then allowed to equilibrate for two hours before use.

The ethyl acetate fraction 5 g was mixed with 10 g of silica gel then applied on the top of the column. Elution commenced by CH_2Cl_2:MeOH

95:5 in increasing order of polarity stepwise until 100% methanol. Fractions of 10 ml were collected, and concentrated under reduced pressure then investigated by thin layer chromatography (TLC) analysis using different solvent systems.

The spots were visualized under UV lights using both short and long wave lengths with and without exposure toNH_3 and sprayed with NA reagent. Similar fractions were combined, concentrated to dryness .

2.2.6- Preparative paper chromatography

Preparative paper chromatography was carried out for each fraction using BAW (Butanol: Acetic: Water 4:1:5 upper layer) as solvent system. The developed chromatograms were dried, sprayed with NA reagent and the colours (bands) which developed was observed in UV light (λ = 366, 254 nm), then determined with smooth lines using a pencil.

The equivalent bands from each paper was then cut out into small strips and slurred with methanol. After several hours of contact with occasional shaking, the liquid was filtrated and evaporated to dryness.In this way compounds I-IV were isolated.

Thin layer chromatography (TLC) was used to determine the purity of isolated flavonoids using solvent system CH_2Cl_2 :;MeOH 70:30.

2.2.7- Biological activity

2.2.7.1- Preparations of crude methanolic and ethyl acetate extracts for biological study

- Methanol extract: of *Combretum aculeatum* was prepared by macerating 100g of the air dried powdered leaves in successive portions of methanol (100%) till exhaustion. The methanolic extract was evaporated under reduced pressure to obtain a semi – solid residue.

- Ethyl acetate fraction was prepared by suspending the semisolid residue obtained from the methanolic extract in the least amount of distilled water, then shaking with successive portions of ethyl acetate till exhaustion. The ethyl acetate extract was evaporated under reduced pressure to obtain the residue.

2.2.7.2 -Antimicrobial assay

The methanolic extract and ethyl acetate fraction were screened for their antimicrobial activity against six standard human pathogens (*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*) using the cup plate agar method with some minor modifications.

2.2.7.2.1- 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 10^8- 10^4 colony forming units per ml. The suspension was stored in refrigerator at 4^oC 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 dilutions were 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 to dry, and then incubated at 37^o C for 24 hours.

2.2.7.2.2- Preparation of fungal suspensions

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

2.2.7.2.3 -Testing for antibacterial activity

The cup plate agar diffusion method was adopted with some minor modification, to assess the antibacterial activity of the methanolic extract and ethyl acetate fraction of *Combretum aculeatum* .Two ml of the

standardized bacterial stock suspention 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 and 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 one of the extracts.

The agar discs were removed and cups were filled with(0.1) ml of each extract using adjustable volume microtiter 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 24 hours.

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

2.2.7.2.4 -Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar dextrose agar was used. Samples were used here by the same concentrations used above.

2.2.8- Antioxidant assay

Evaluation of the antioxidant activity of methanolic and ethyl acetate extracts was carried out by measuring the decolourizing capacity of each extract against stable DPPH radical. The change in colour is measured by UV Spectrophotometer at 516 nm.

Freshly prepared methanolic solution of DPPH (10^{-4}) (1,1 diphenyl- 2-picryl hydrazyl) was mixed with the tested samples. Also the fresh methanolic solution of DPPH was mixed with trolox (reference compound). They were shaken vigorously then kept in the dark at room temperature for 30 minutes.

The absorbance of the resulting solutions was measured spectrophtometrically at 516 nm. Also a blank sample containing the same amount of methanol and DPPH solutions was prepared and measure . All determinations were carried out in triplicates.

Any decrease in the intensity of the purple colour, indicates that the smaple exerts an oxidant activity.

The radical scavenging activity of the tested samples is expressed as "inhibition percent" it is calculated with the following formula:

$$\text{Antioxidant activity} = \frac{\text{Control absorbance - Sample absorbance}}{\text{Control absorbance}} \times 100$$

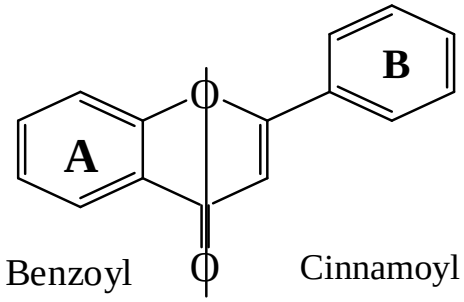
Chapter three

Result and Discussion

3- Results and Disscussion

Phytochemical screening of Combretum aculeatum leaves revealed the presence of flavonoids, steroids, tannins, alkaloids, saponins, coumarins and anthraquinone glycosides .Cyanogenic glycosides was absent .

Most flavonoids show two absorption bands; band I and II. Band I is considered to be associated with the absorption of the cinnamoyl system, while band II originates from the benzoyl system.Due to conjugation between benzoyl and cinnamoyl chromophores, flavones, flavonols, chalcones and aurones give both bands I, II while isoflavones,dihydroflavonols, dihydrochalcones and flavanones give only band II due to loss of conjugation between the carbonyl function and ring B.



The UV absorption of flavones, flavonols, chalcones and aurones is depicted in table (3-1).

Table (3.1) : The UV absorption of flavones, flavonols, chalcones and aurones[1]

Flavonoid class	Band I	Band II
Flavones	330-350	250-270
Flavonols	350-390	250-280
Chalcones	365-390	240-260
Aurones	390-430	240-270

Compound I

In its UV spectrum , compound I gave λ_{max} 271,346nm (Fig, 1).Such absorption is characteristic of flavones[12].

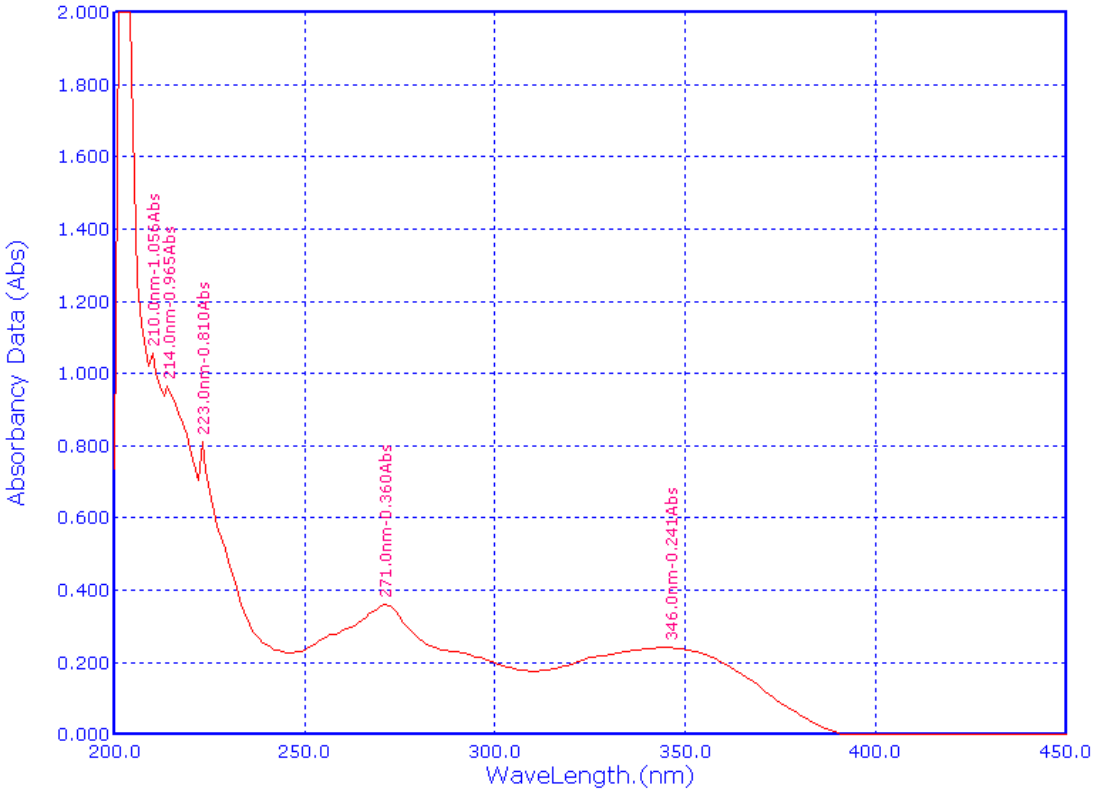


Fig (1): UV spectrum of compound 1

Some significant structural features are gained by using the so called UV shift reagents, these are: sodium methoxide, sodium acetate, aluminum chloride, hydrochloric acid and boric acid. The strong base sodium methoxide ionizes to some extent all hydroxyl groups on the flavonoid nucleus. However, use has been made of the effect of this reagent on the UV spectra of flavonoids for the detection of free 3-and/or 4`-hydroxyl groups. In presence of such functions, this reagent gives characteristic bathochromic shifts, but with decrease in intensity in case of a 3-OH function. When sodium methoxide was added to a methanolic solution of compound I (Fig.2) a bathochromic shift was observed.This clearly indicates the presence of a 4`-hydroxyl.

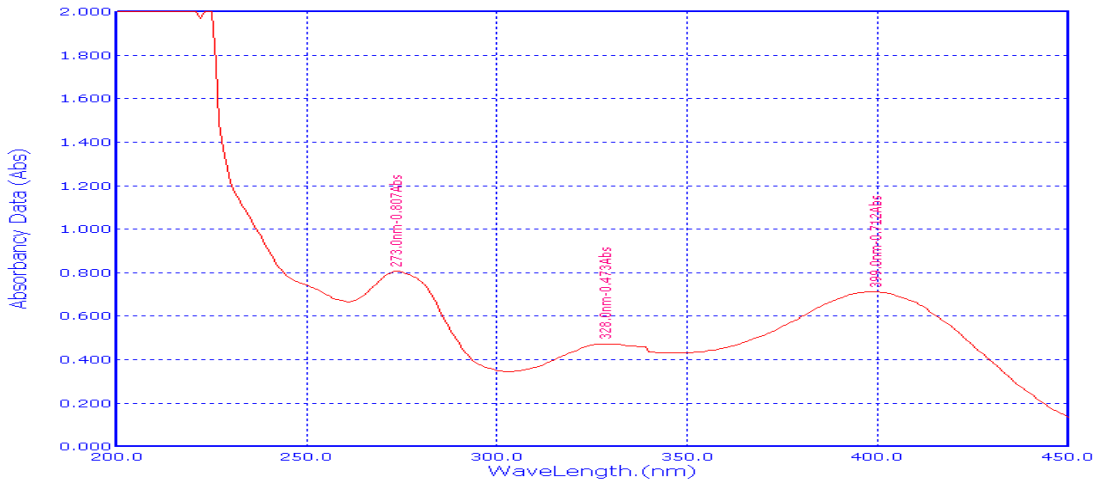


Fig.2 : Sodium methoxide spectrum of compound I

On the other hand the shift reagent , sodium acetate is a weaker base than sodium methoxide and as such ionizes the more acidic hydroxyl groups .Thus it is diagnostic of a 7-OH group. Ionization of the 7-hydroxyl group mainly affects band II (whereas ionization of the 3-

and/or 4`-hydroxl groups mainly affects band I) [12,13] . When sodium acetate was added to a methanolic solution of compound I, a bathochromic shift was observed(Fig.3)indicating a 7-OH function. Flavonoids containing free 7-hydroxy group, with few exception, exhibits a diagnostic 5-20 nm band II bathochromic shift in the presence of NaOAc [12,20].

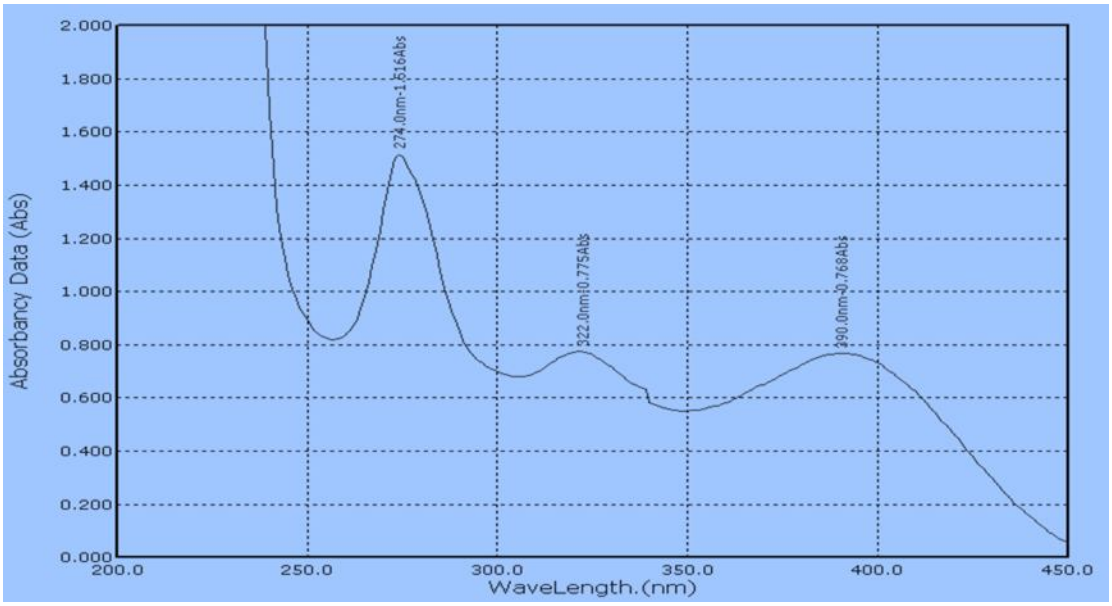


Fig.3: The UV spectrum of compound 1in sodium acetate

With aluminum chloride, flavonoids which contain hydroxyl groups at C-3 or C-5 form complexes. In addition, aluminum chloride forms complexes with flavonoids which contain catechol systems. The complexes formed between $AlCl_3$ and the A-and B-ring ortho-dihydroxyl groups, with few exceptions ,decompose in acidic media. In contrast the $AlCl_3$ complexes between the C-4 keto function and either the 3-or 5-hydroxyl group are stable in the presence of acid [20,24].

When $AlCl_3$ was added to a methanolic solution of compound 1, band II shifted bathochromically (Fig. 4) . However, the spectrum decomposed in HCl(Fig.5) indicating a B ring catechol moiety.The same bathochromic shift was observed in the boric acid spectrum (Fig.6).

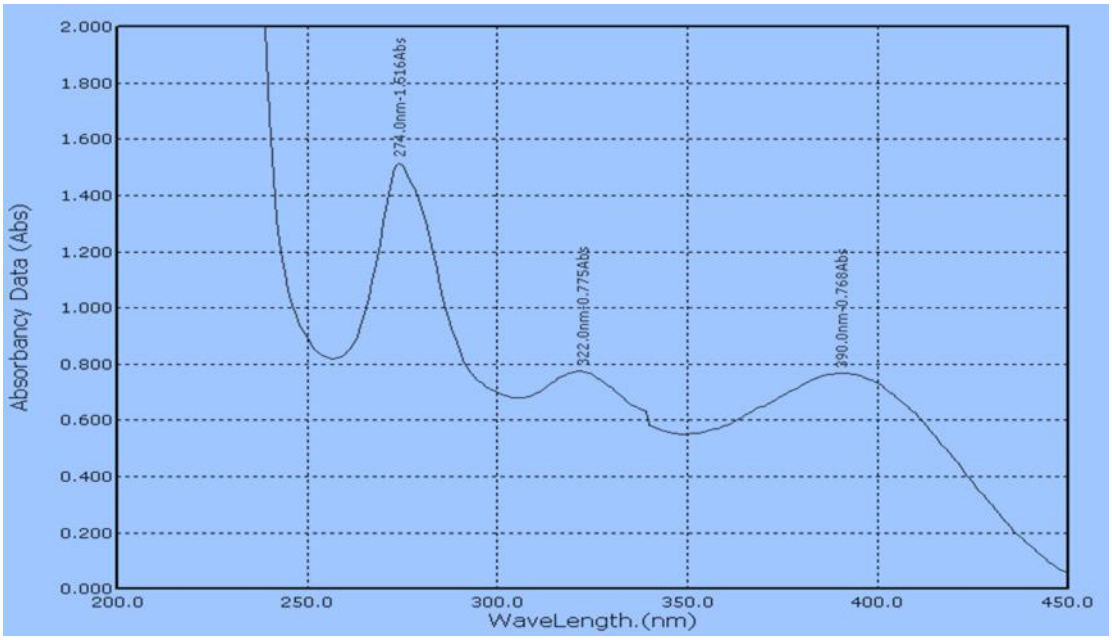


Fig.4 : Aluminium chloride spectrum of compound I

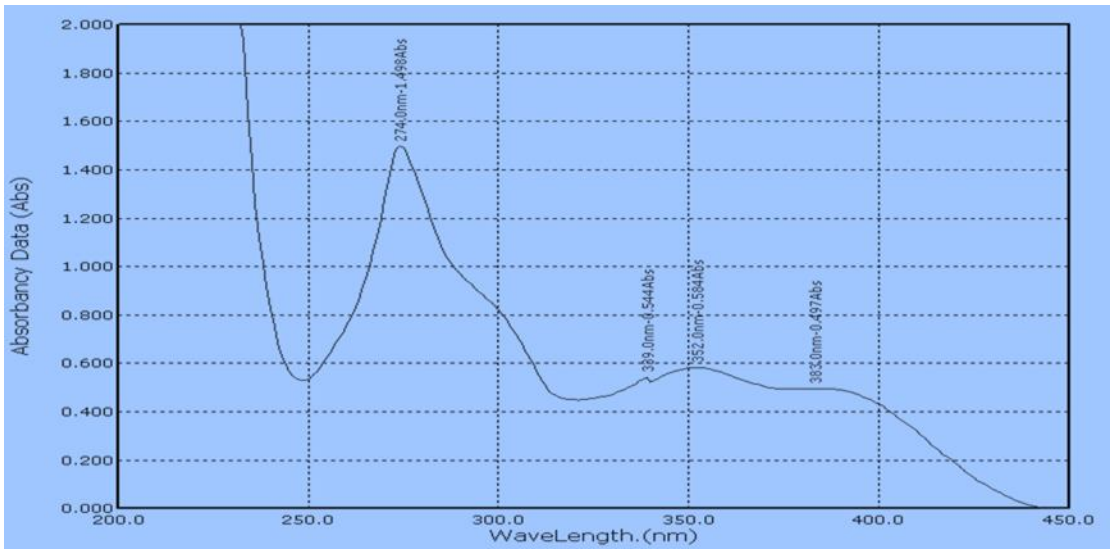


Fig. 5: $AlCl_3$/HCl spectrum of compound I

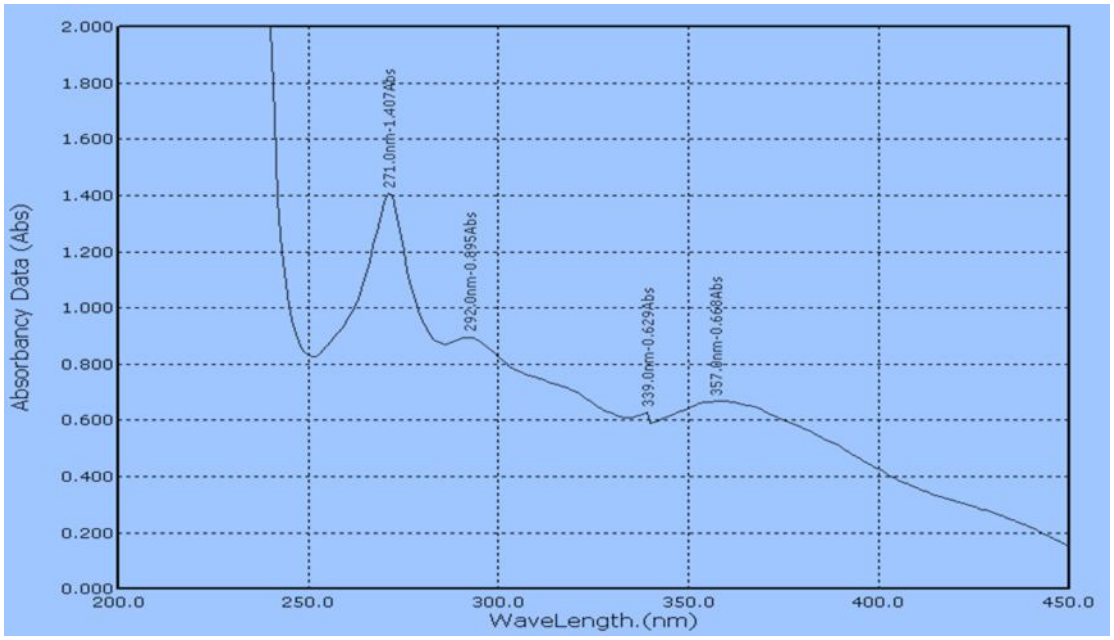


Fig.6: Boric acid spectrum of compound I

The ^{1}HNMR spectrum revealed(Fig.7) a pattern characteristic of flavones. The signals at δ6.2 and δ6.8ppm were assigned for C_6- and C_8- protons respectively. The latter proton resonates at higher field due to the deshielding effect of the 4- keto function[12]. The olefinic proton of the heterocyclic C ring resonated as singlet at δ6.2ppm due to the anisotropic effect of the π electrons and the electron-wthdrawal effect of the 4 keto function.The B ring protons resonate at δ7.4,7.6 ppm, while the C_5 –H resonates well downfield due to the deshielding influence of the neighbouring 4-keto function [12,24].The resonance at δ2,2ppm was assigned for an aromatic acetyl function, which was assigned for the B ring on the basis of the retro Diels-Alder fission (Scheme I).

The mass spectrum(Fig.8) gave m/z 312 for the molecular ion.The retro Diels-Alder cleavage gave(Scheme I) m/z 91 for intact A ring which cites evidence for the substitution pattern of compound I.

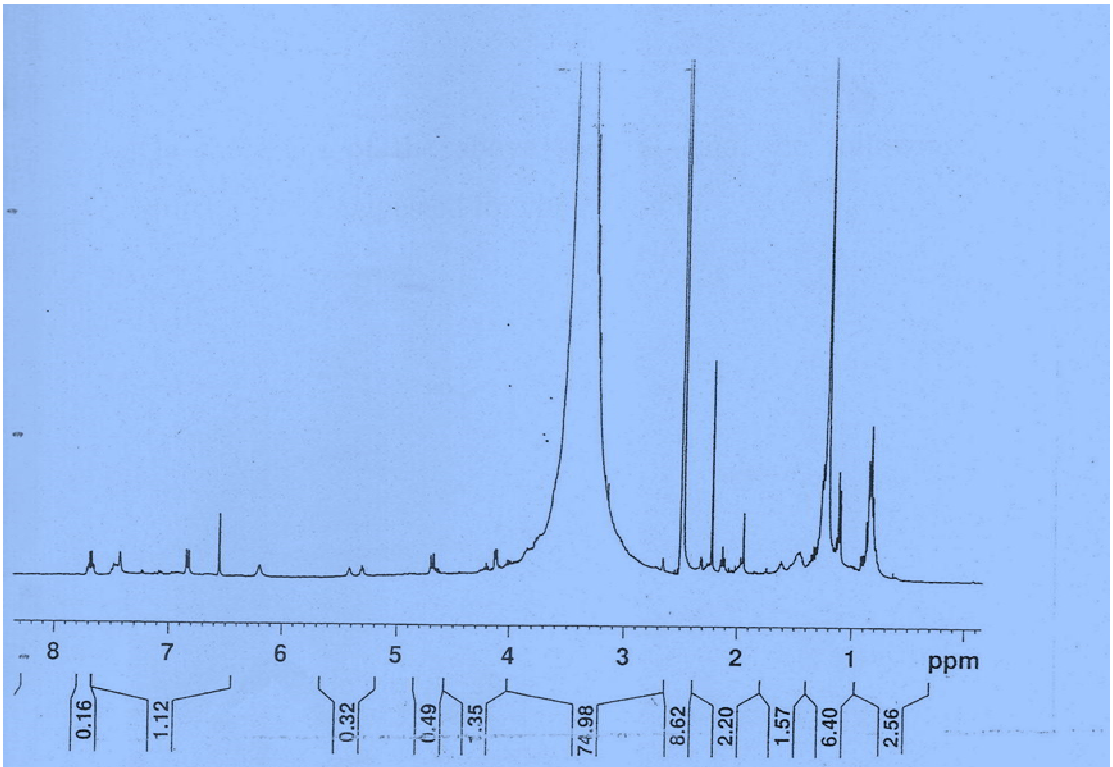


Fig. 7: ^{1}HNMR spectrum of compound I

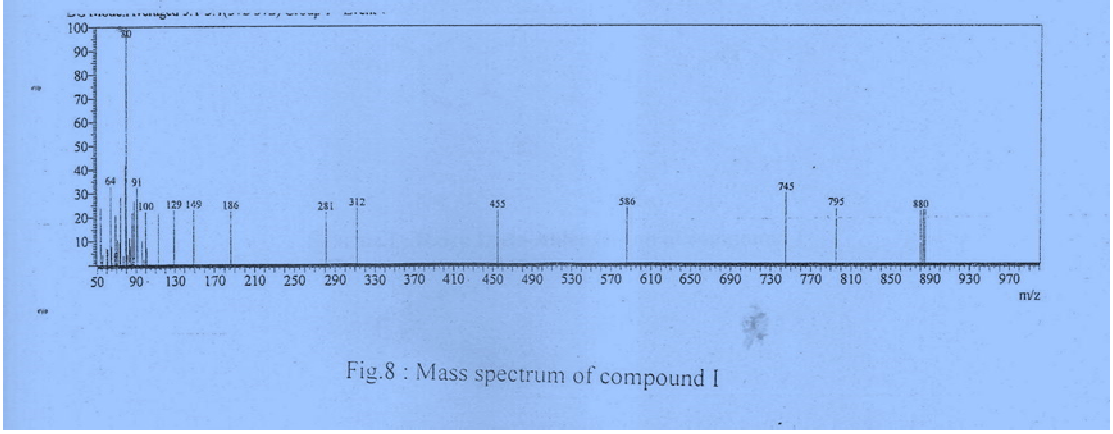
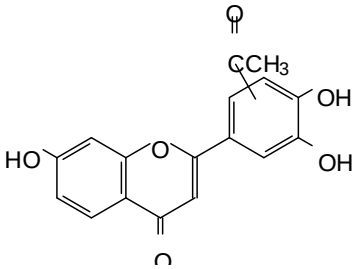
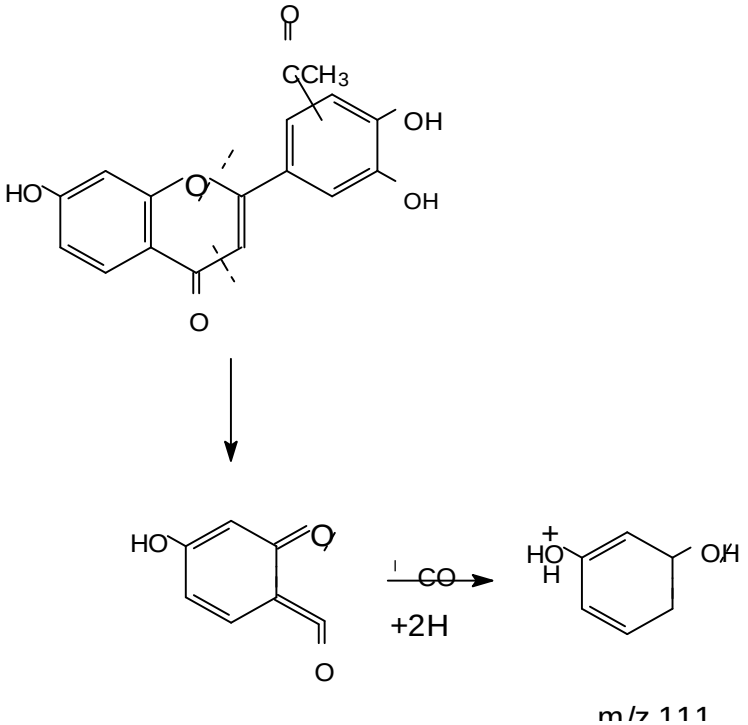


Fig.8 : Mass spectrum of compound I

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



Compound I



Scheme I : Retro Diels-Alder fission of compound I

Compound II

The UV spectrum(Fig.9) of compound II gave λ_{max} 271,330nm . Such absorption is characteristic of flavones [12,14].

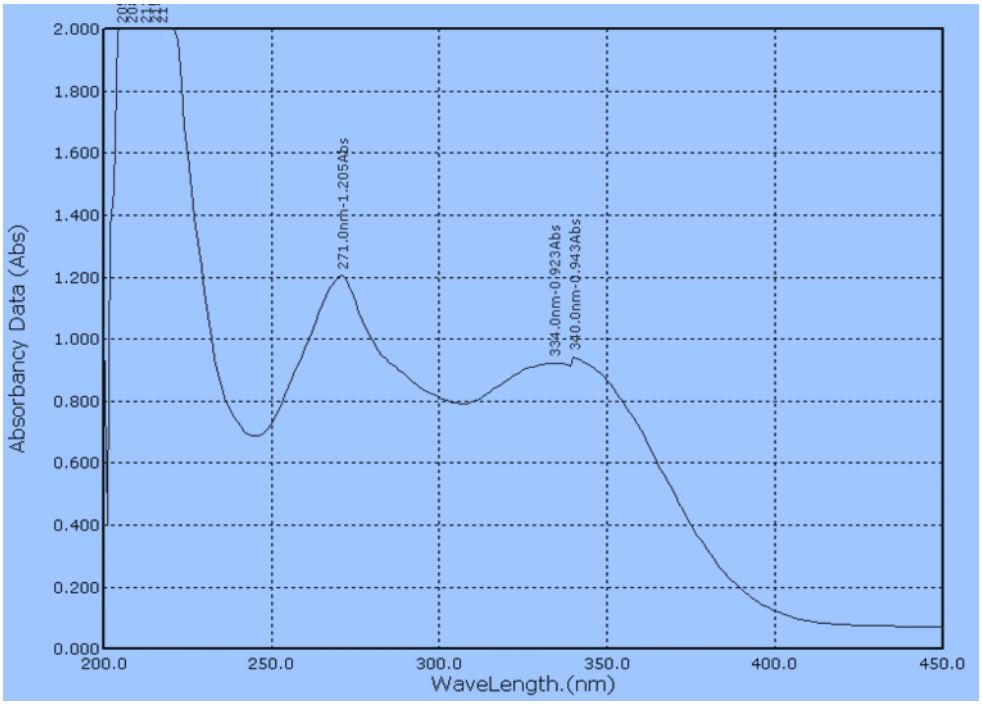


Fig.9: UV spectrum of compound II

When sodium methoxide was added to a methanolic solution of compound II, band I shifted bathochromically to 385nm with increase in intensity indicating(Fig.10) a 4`-OH function [24] .

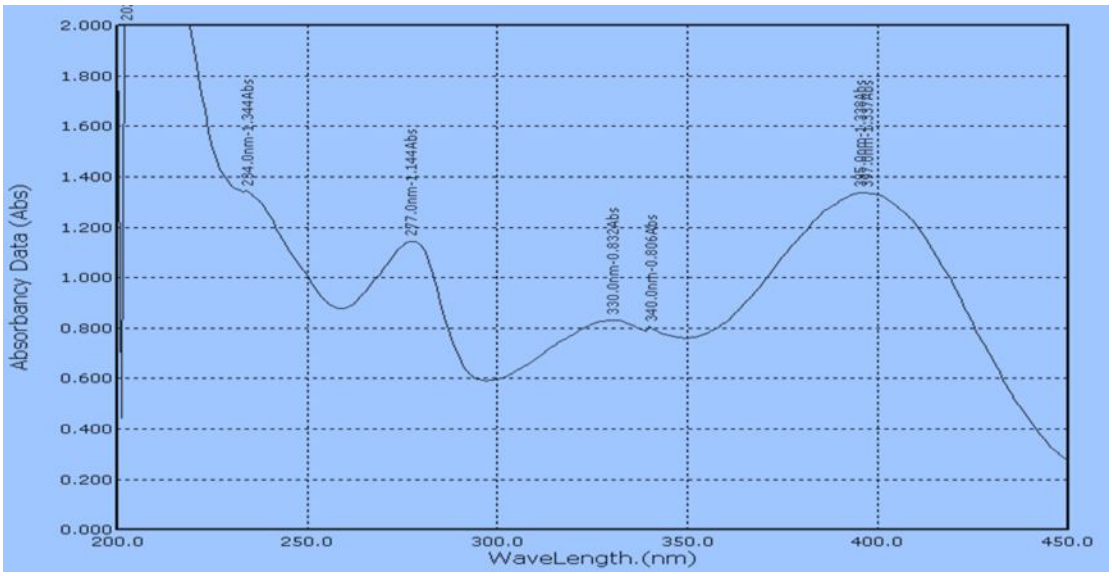


Fig.10: Sodium methoxide spectrum of compound II

A bathochromic shift was observed when compound II was treated with the shift reagent: sodim acetate and this suggests a 7-OH function(Fig.11).

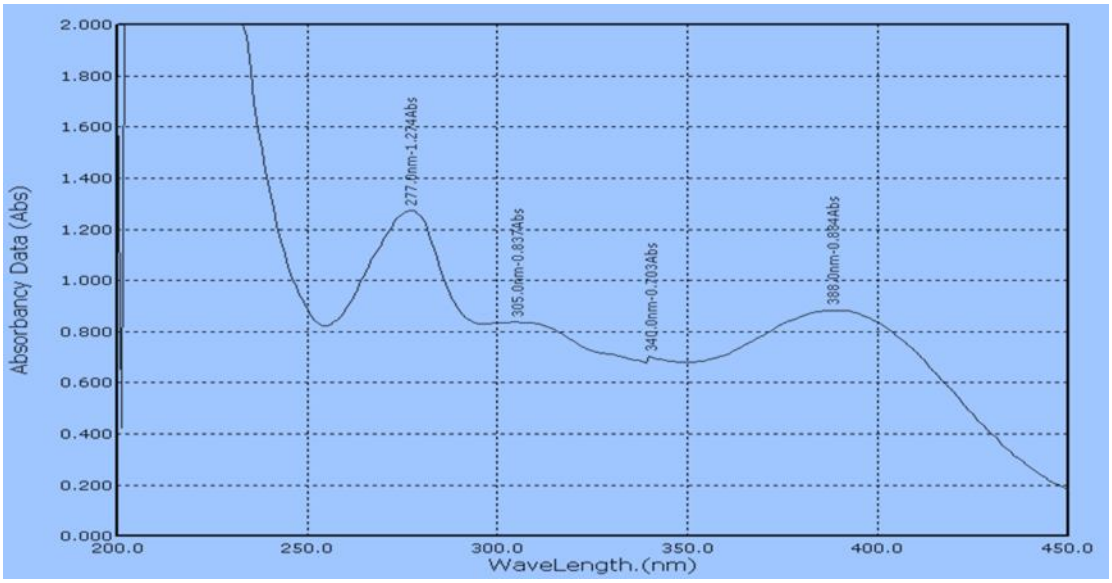


Fig.11: Sodium acetate spectrum of compound II

The aluminium chloride spectrum(Fig.12) revealed a bathochromic shift in band II. The spectrum did not degenerate(Fig.13) on addition of HCl indicating a 5-OH function.The boric acid spectrum (Fig.14)which did not reveal any bathochromic shift – cited additional evidence for absence of catechol systems.

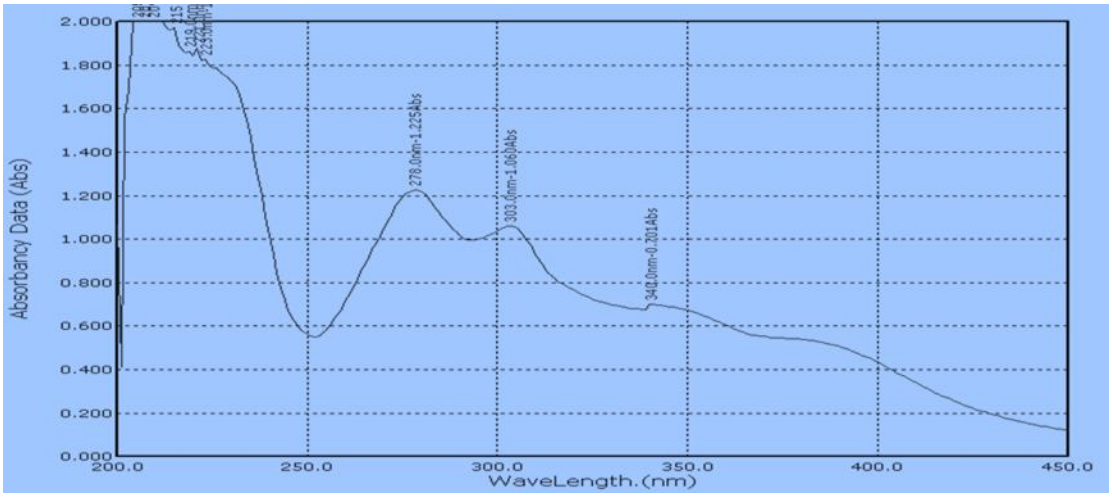


Fig.12: Aluminium chloride spectrum of compound II

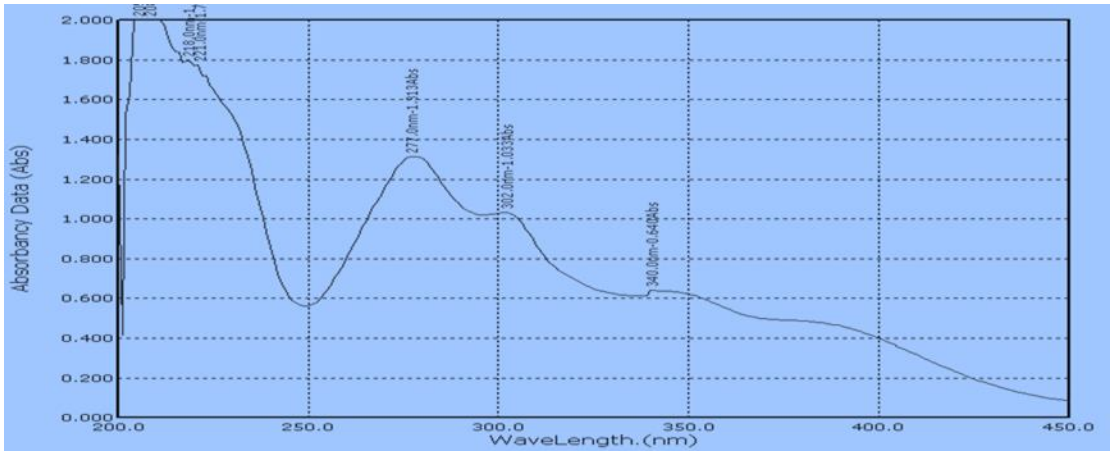


Fig.13: Aluminium chloride /HCl spectrum of compound II

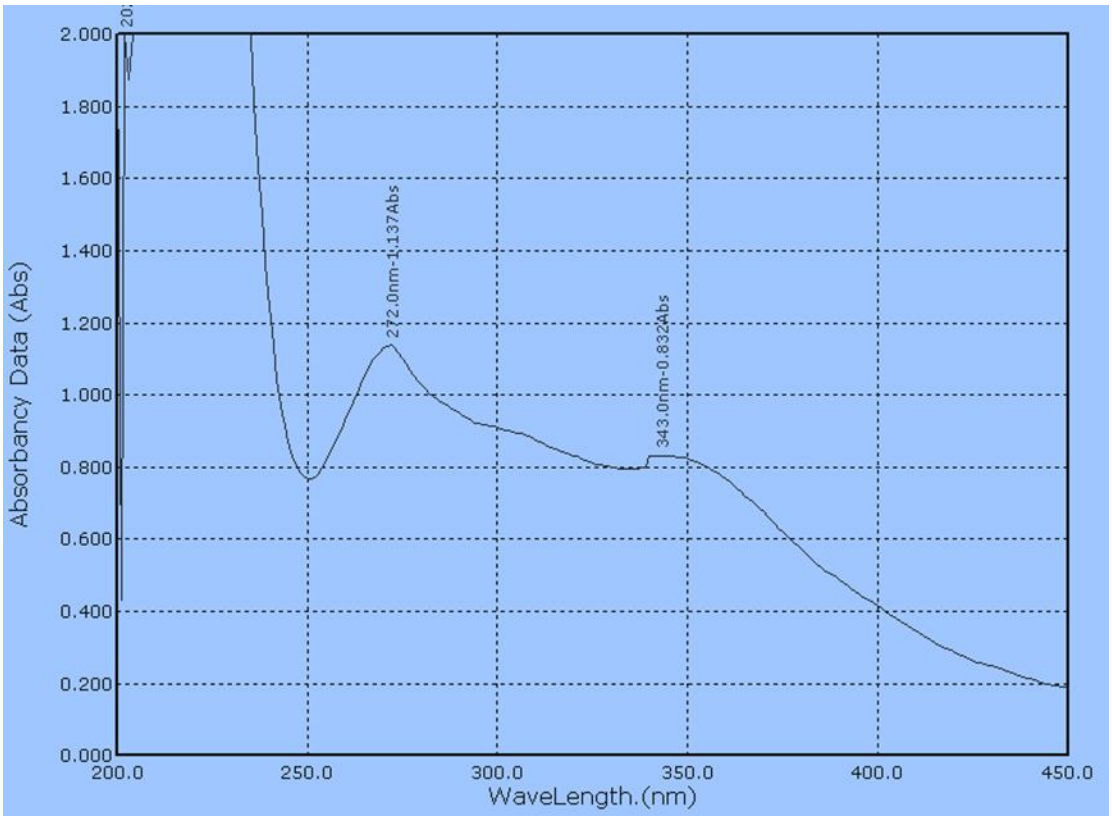


Fig.14: Boric acid spectrum of compound II

The ^{1}HNMR spectrum (Fig.15) gave a signal at δ6.11 ppm characteristic of C_6-H.The signal at δ6.71 was attributed to C_8 proton,while the resonance δ8.07 was assigned for B ring proton. The signals at δ1.3 and δ3.54 account for a methyl and methoxyl groups.The resonances at δ 1.98 and δ2.28 ppm were assigned for two acetyl functions.On the basis of the retro Diels-Alder fission (Scheme II) the methyl , methoxyl and acetyl functions were assigned for B ring of the flavones.

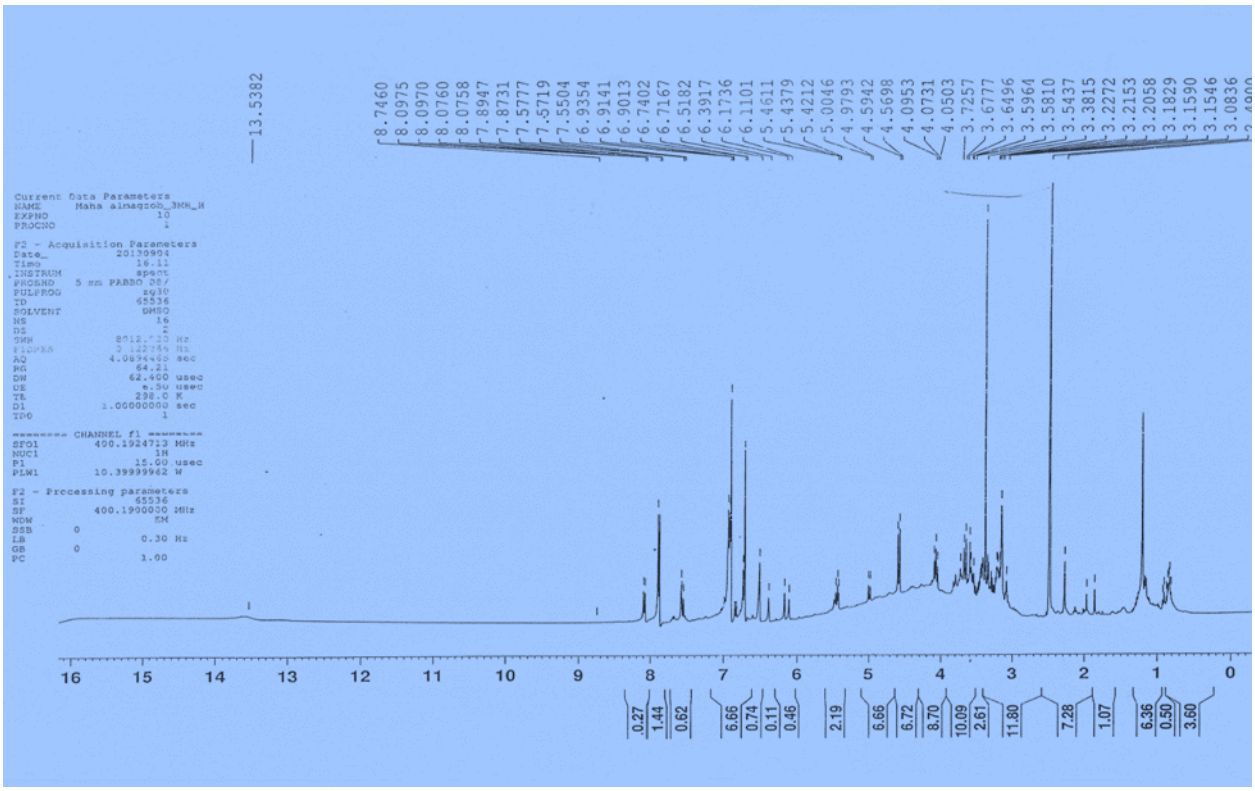


Fig.15: ^{1}HNMR spectrum of compound II

The mass spectrum (Fig. 16) gave m/z398 for M^{+} + 2H. The retro Diels-Alder fission (Scheme II) which gave intact A ring fragment - cited evience evidence for the substitution pattern of ring A.

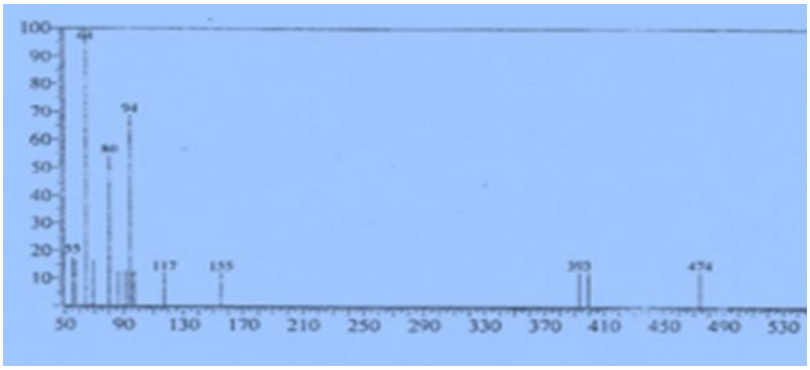
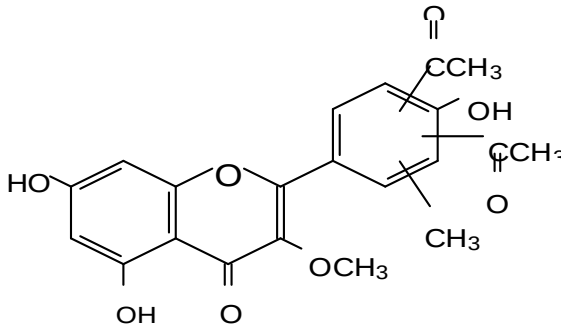
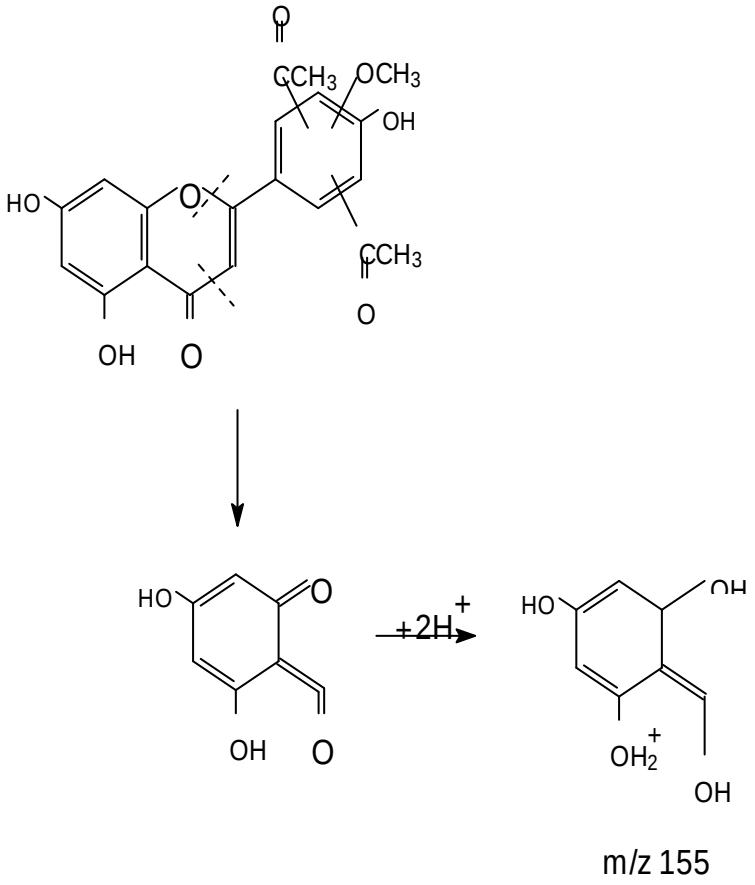


Fig.16: Mass spectrum of compound II

On the basis of the above argument the following tentative structure was proposed for compound II:



Compound II



m/z 155

Retro Diels-Alder fission of compound II

Compound III

The UV spectrum (Fig.17) gave λ_{max} 271,336 nm which is characteristic of flavones.

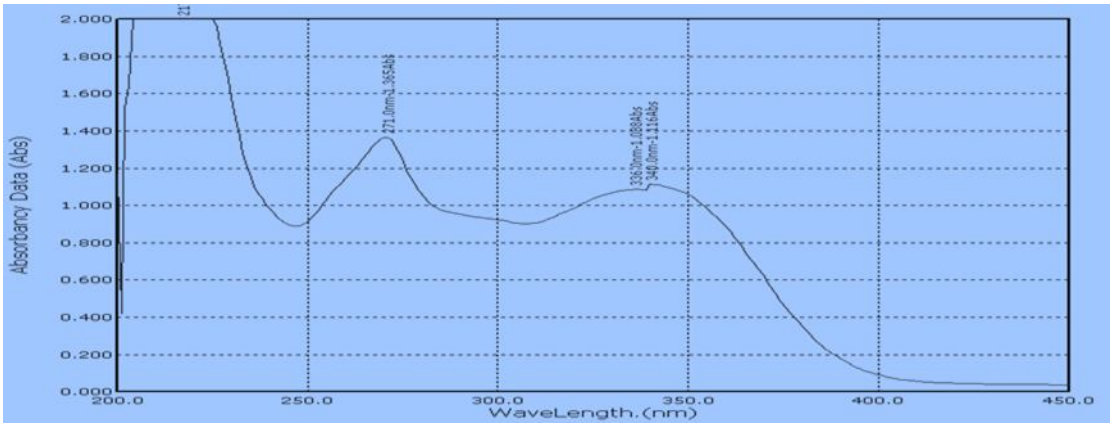


Fig.17: UV spectrum of compound III

When compound III was treated with the shift reagent sodium methoxide a bathochromic shift -with increase in intensity-was observed indicating a free OH group at C_4·(Fig.18) .

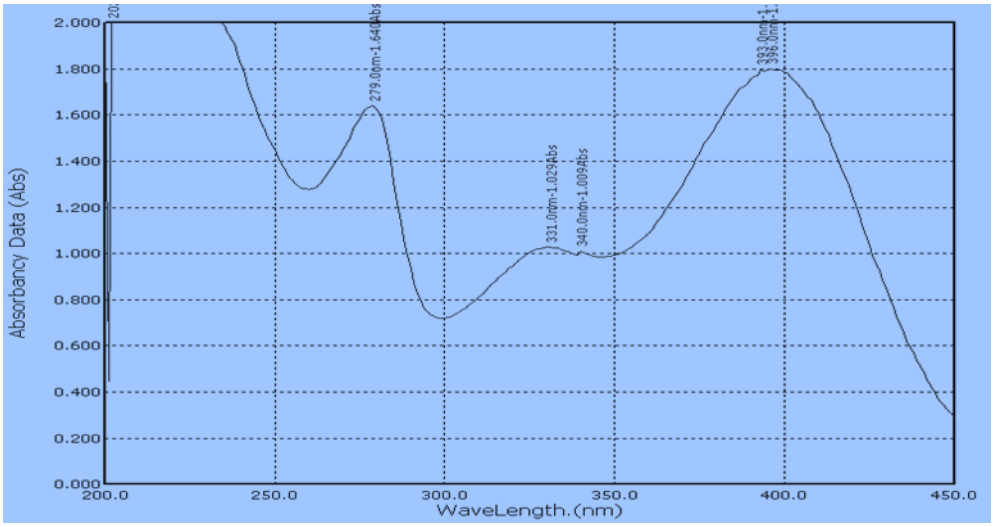
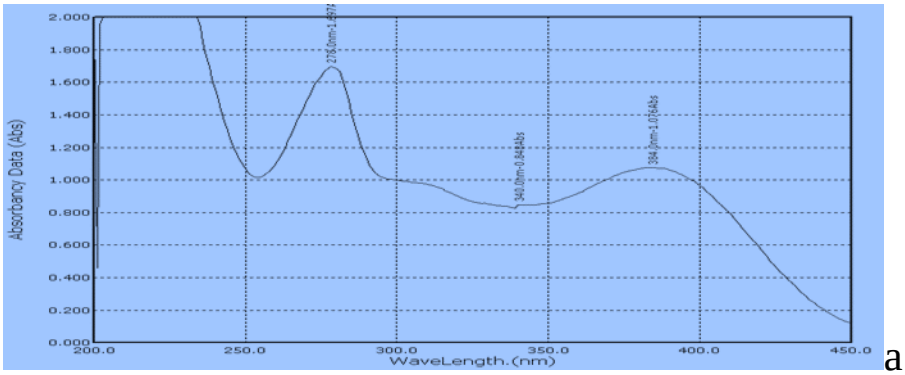


Fig.18: Sodium methoxide spectrum of compound III

The sodium acetate spectrum(Fig.19) revealed a bathochromic shift which is diagnostic of a free 7-OH group.The aluminium chloride spectrum (Fig.20) also showed bathochromic shift.



a

Fig.19: Sodium acetate spectrum of compound III

However , the spectrum degenerated(Fig.21) on addition of acid indicating a catechol system.The boric acid spectrum- which revealed a bathochromic shift cited additional evidence in favour of the catechol moiety(Fig.22).

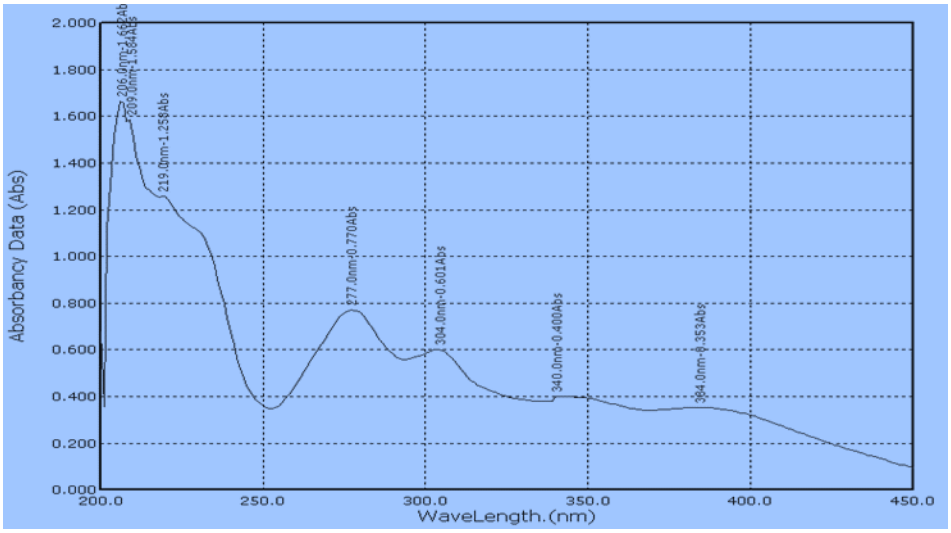


Fig.20: Aluminium chloride spectrum of compound III

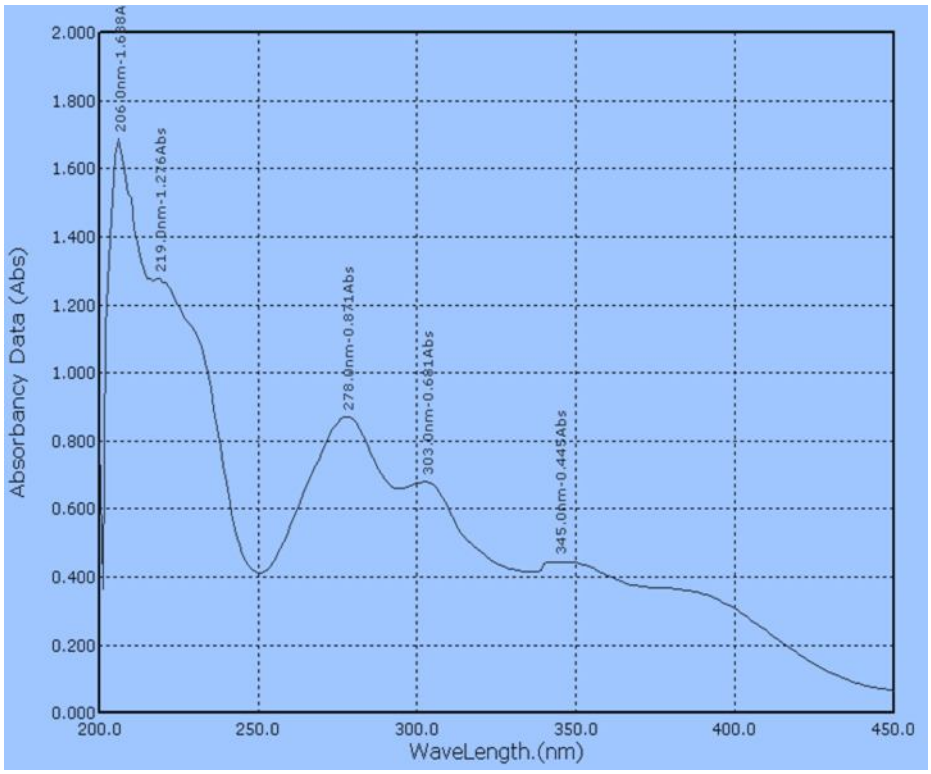


Fig.21: Aluminium chloride /HCl spectrum of compound III

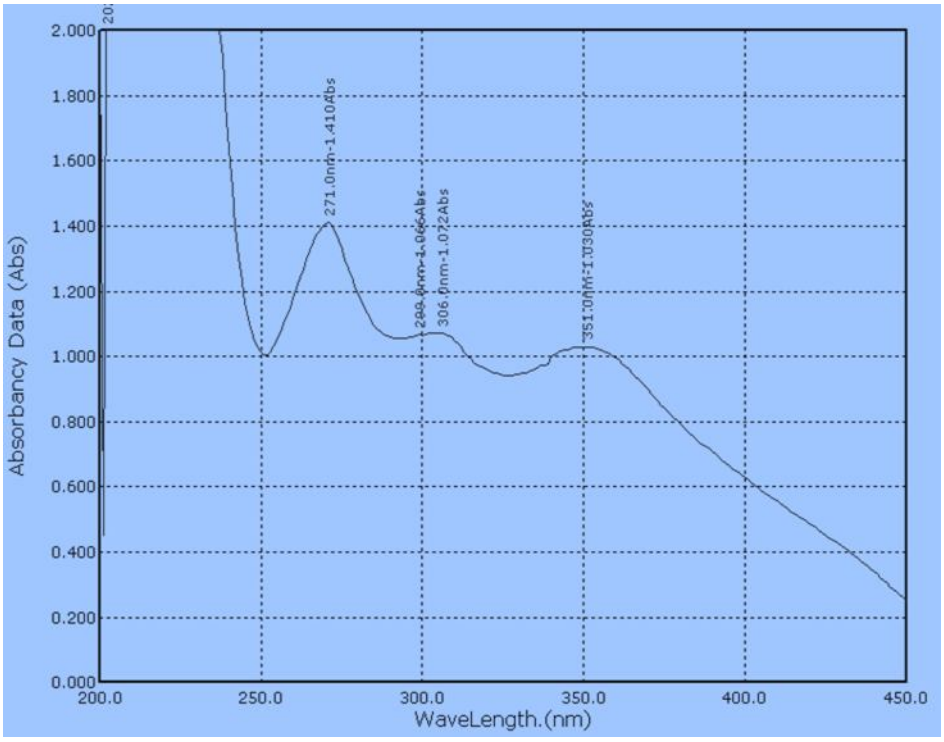


Fig.22: Boric acid spectrum of compound III

The ^{1}HNMR spectrum(Fig.23) revealed a pattern characteristic of flavones. The signals at δ6.21 and δ6.87 were assigned for C_6 – and C_8 – proton respectively[20].Usually C_6 – H resonates [24] at higher field relative to the C_8 – proton. The singlet at δ6.56 ppm accounts for the olefinic proton at the heterocyclic C ring, while the signals at δ7.36,7.67ppm accounts for B ring protons.Such protons resonate at lower field relative to A ring protons due to he deshielding influence of the the heterocyclic C ring [20]. The C_5- proton resonates well downfield at δ 7.98ppm due to the deshielding influence of the 4 keto function.

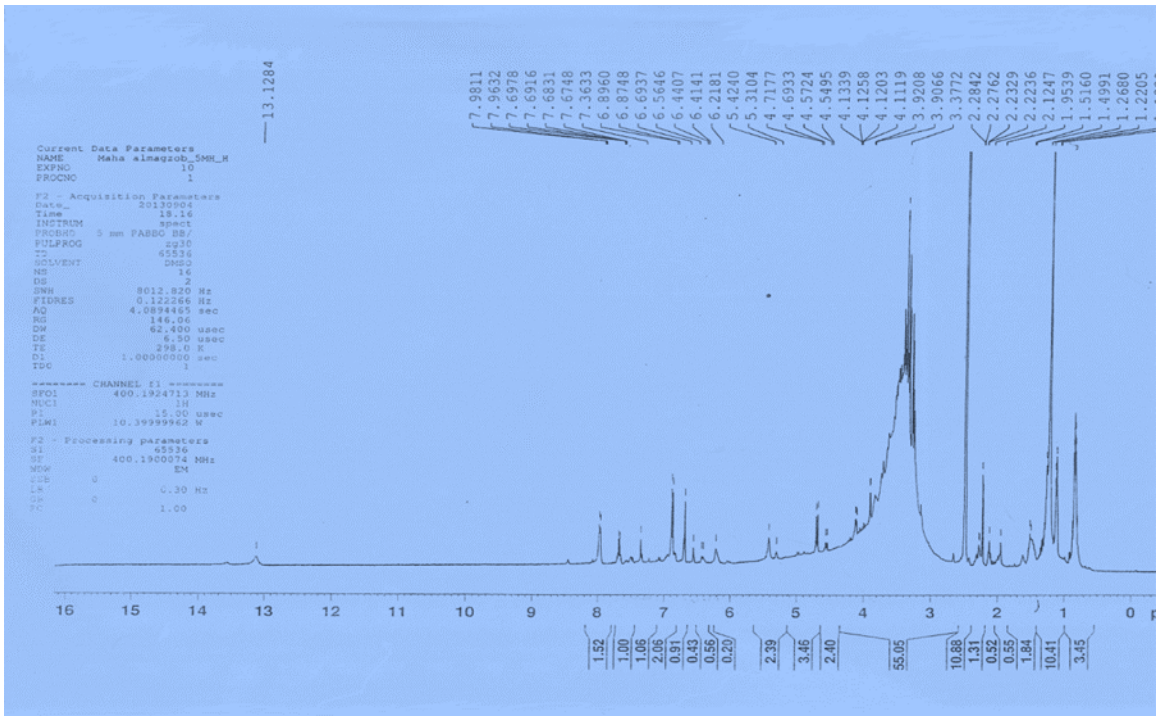


Fig.23: ^{1}HNMR spectrum of compound III

The mass spectrum (Fig.24) gave m/z288 for M^{+} +2H and the retro Diels – Alder fission(Scheme 3)- which revealed a fragment for intact B ring at m/z149 - cites evidence for the substitution pattern of ring A.

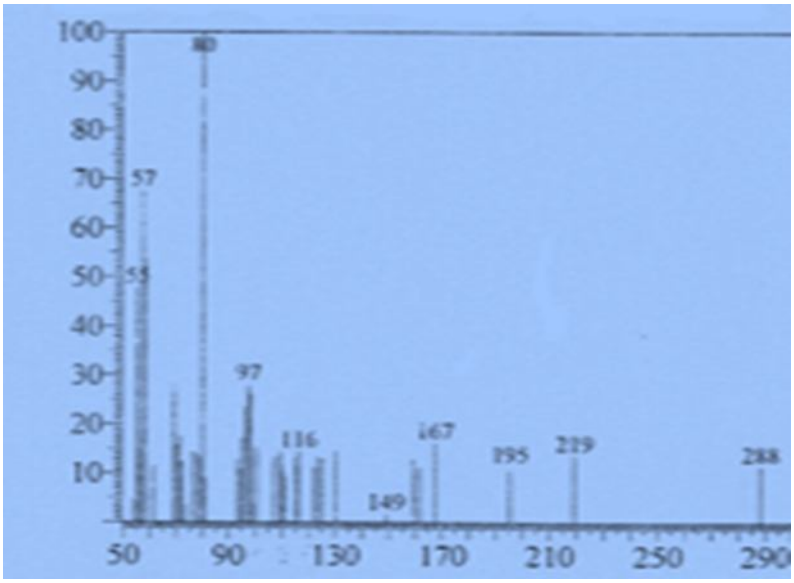
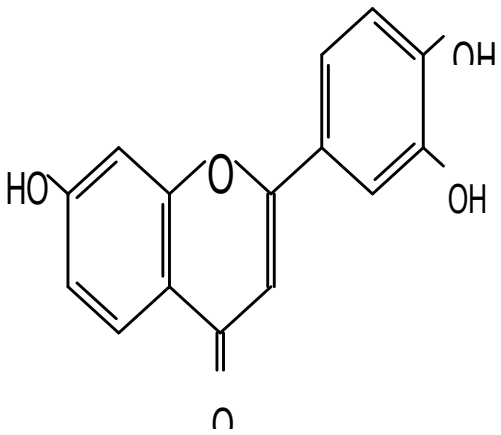
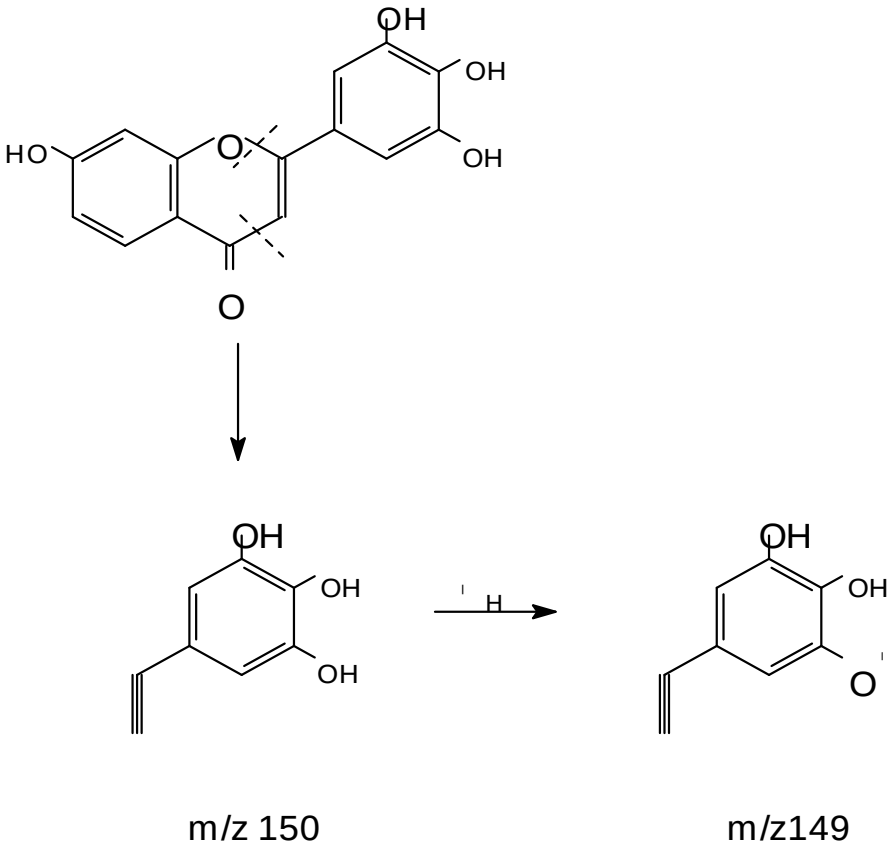


Fig.24: mass spectrum of compound III

On the basis of the above argument , the following structure was suggested for compound III.



Compound III



Retro Diels-Alder fission of compound III

Compound IV

In the UV ,compound IV absorbs at λ_{max} 271,328nm(Fig.25). This absorption is characteristic of flavones [20,24].When the shift reagent , sodium methoxide, was added to a methanolic solution of compound IV a bathochromic shift was observed indicating a 4`-OH function(Fig.26).

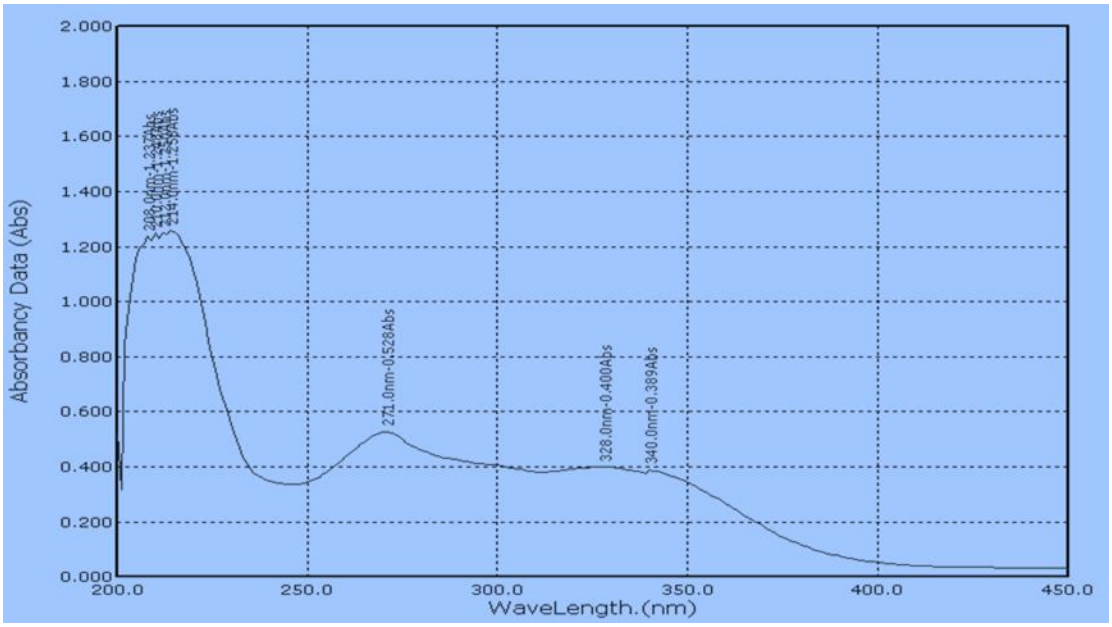


Fig.25: UV spectrum of compound IV

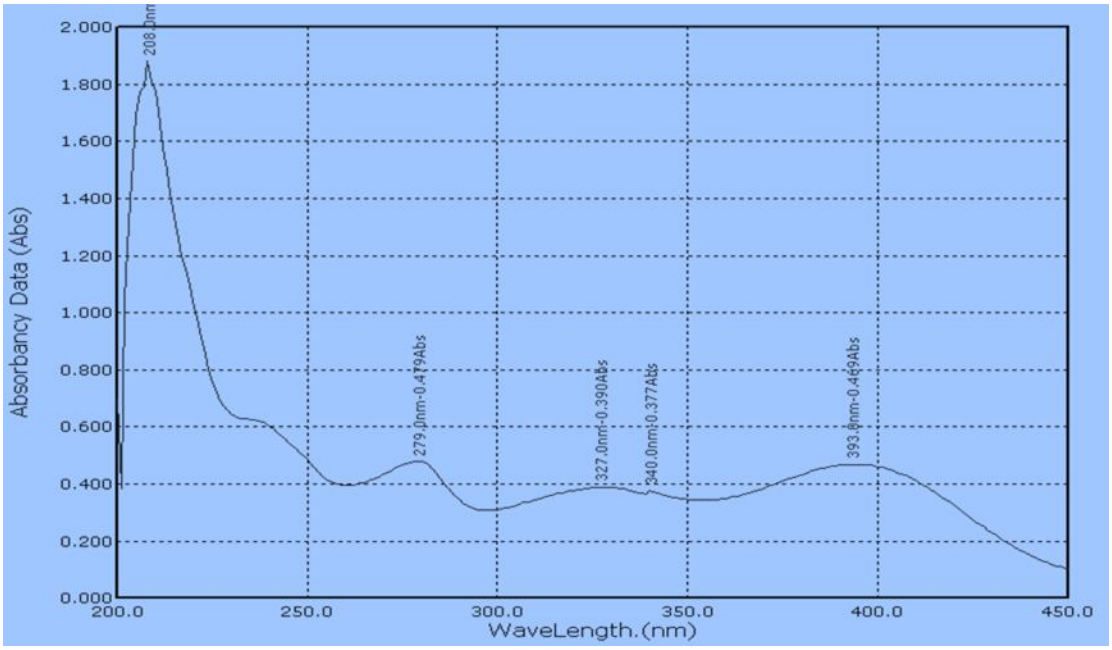


Fig:26: Sodium methoxide spectrum of compound IV

The sodium acetate spectrum (Fig.27) revealed a bathochromic shift which is diagnostic of a 7-OH.

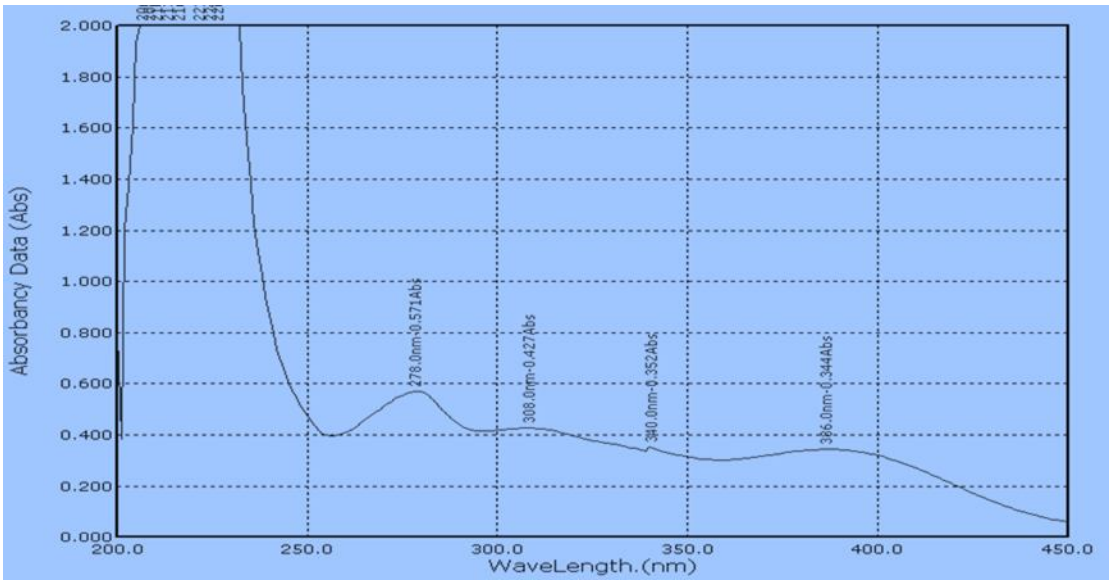


Fig.27: Sodium acetate spectrum of compound IV

Non bathochromic shift was observed in the aluminium chloride spectrum and this indicates absence of catechol systems as well as a 5-OH function(Fig.28).The same trend was observed in the boric acid spectrum which is diagnostic of catechol systems(Fig.29).

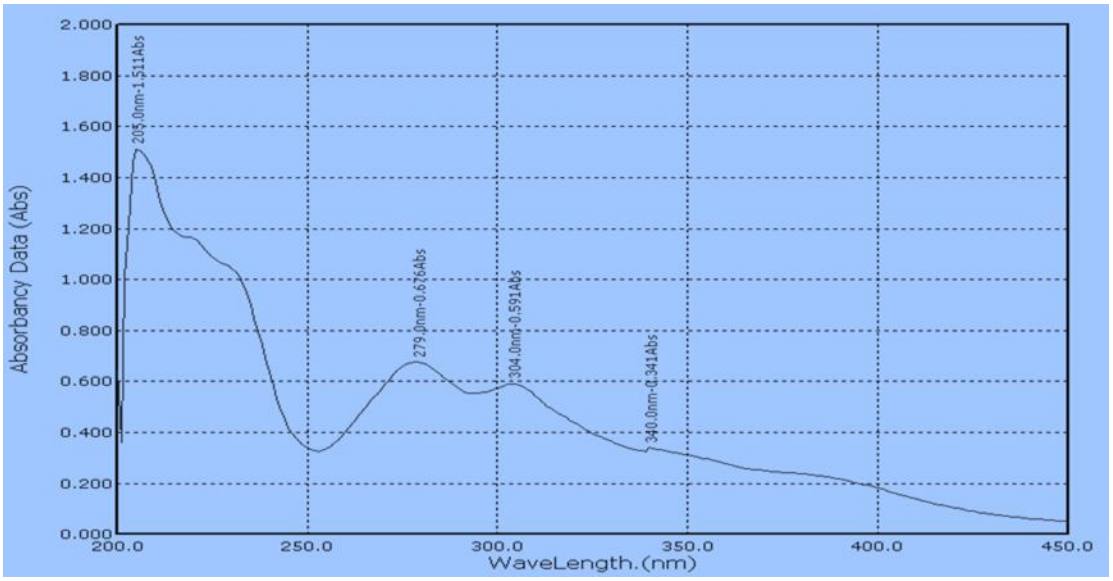


Fig.28: Aluminium chloride spectrum of compound IV

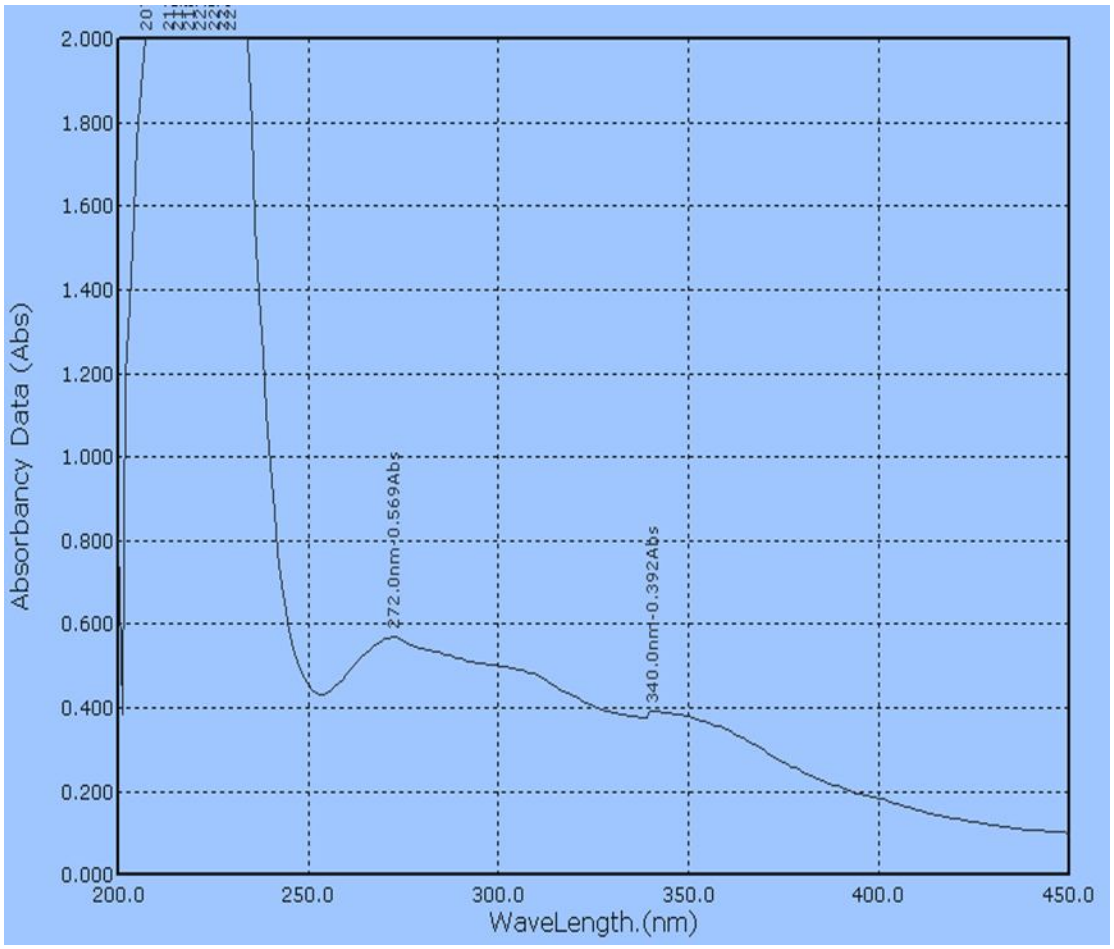


Fig.29: Boric acid spectrum of compound IV

The ^{1}HNMR spectrum (Fig.30) gave a signal at δ0.84 assigned for a methyl group. The resonance at δ3.91 accounts for a methoxyl function, while The double doublet at δ6.70 and δ6.93 is characteristic of C_6- and C_8- protons respectively.The lowfield signal at δ9.05 was attributed to C_5-H and the resonances at δ7.69 and δ 7.86 ppm account for the B ring protons.The mass spectrum (Fig.31) gave m/z300 for M^+ + 2H.

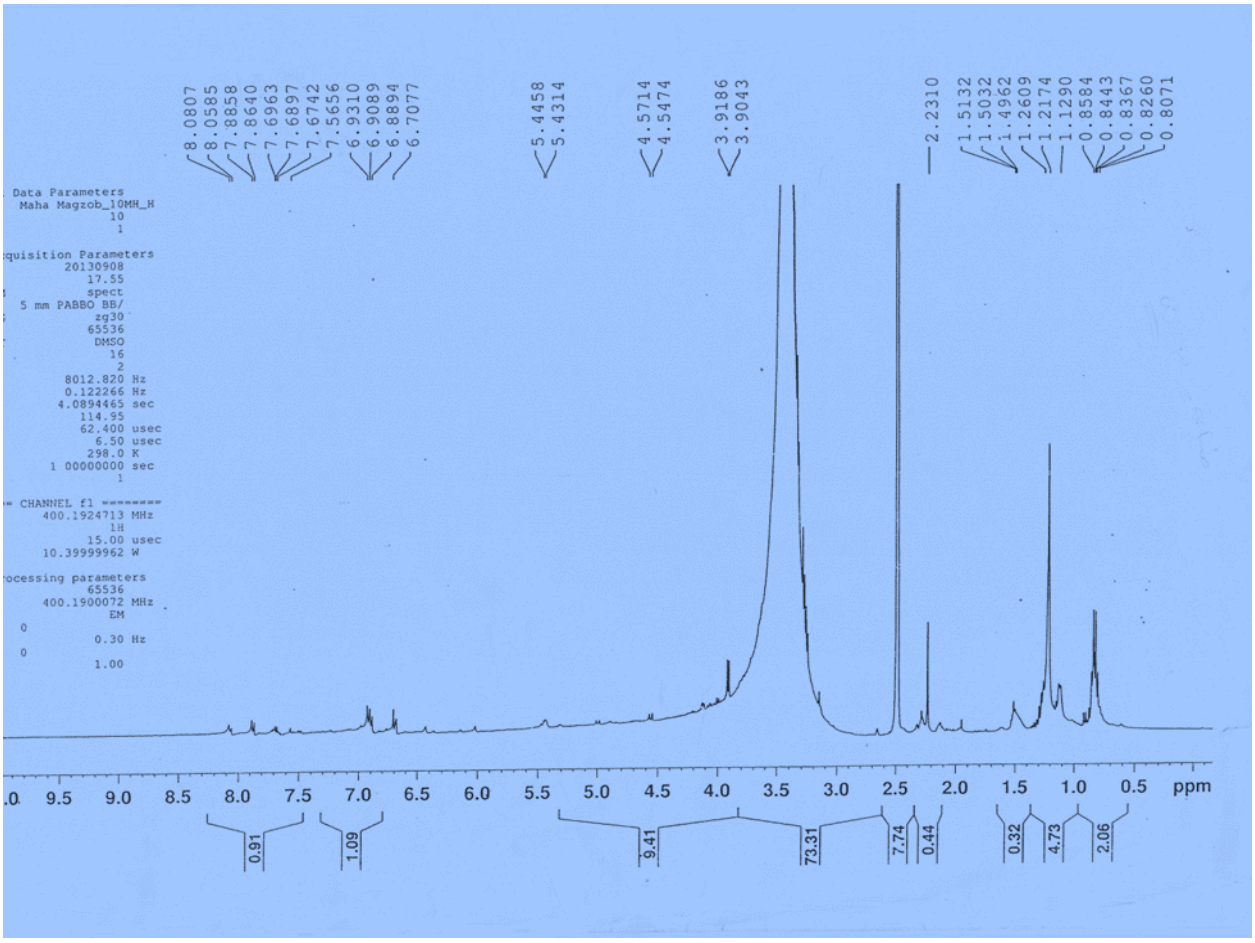


Fig.30: ^{1}HNMR spectrum of compound IV

The mass spectrum (Fig.31) gave m/z300 for M^{+} + 2H.

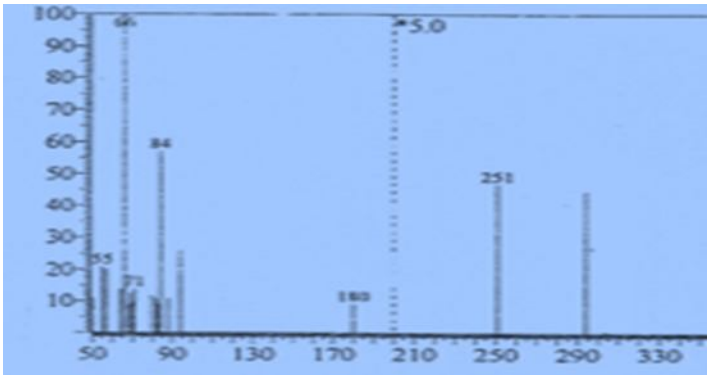
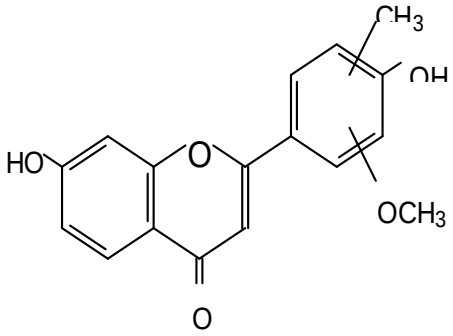


Fig.31: Mass spectrum of compound IV

Thus the following tentative structure was assigned for this flavones:



Compound IV

Antimicrobial assay

The methanolic and ethyl acetate extracts of *Combretum aculeatum* were evaluated for their antimicrobial potential against six standard human pathogens(*Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Candida albicans and Aspergillus niger*). The methanolic extracts showed significant activity against *Escherichia coli* and *Pseudomonas aeruginosa* . It also showed moderate activity against *Bacillus subtilis* and staphynococcus. This extract also exhibited significant antifungal activity against the fungi : *Candida albicans and Aspergillus niger*.However, the ethyl acetate extract was devoid of activity(Inhibition zones are depicted in Table 3.

Table 3.2: Inhibition zones for methanolic and ethyl acetate extracts

	Inhibition zone diameter(mm/mg sample)					
Extract	*Ec	Pa	Bs	Sa	Ca	An
Methanolic	22	30	17	17	19	22
Ethyl acetate	–	–	–	–	–	–

*Ec= *Escherichia coli*

Bs= *Bacillus subtilis*

Sa= *Staphylococcus aureus*

Pa= *Pseudomonas aeruginosa*

Ca= *Candida albicans*

An= *Aspergillus niger*

Antioxidant assay

In vitro antioxidant assay for the methanolic and ethyl acetate extracts of *Combretum aculeatum* was conducted . Evaluation of the antioxidant activity was carried out by measuring the capacity of each extract against stable DPPH radical. The change in colour is measured spectrophotometrically at 516nm. As depicted in Table (3.3) both methanolic and ethyl acetate extracts exhibited significant anti- oxidant activity.

Table 3.3: Radical scavenging activity of *combretum aculeatum* extracts

Samlpe	Mean absorbance	Antioxidant activity(%)
Trolox	0.0275	96.50
Methanolic extract	0.050	93.70
Ethyl acetate extract	0.0525	93.50

Conclusion and recommendations

Conclusion

The present thesis affords comprehensive studies on the chemical structure of flavonoids found in alocal plant, Combretum aculeatum which exhibits antimicrobial and anti oxidant activities.

The plant has been selected to be the subject of this thesis due to its used in folk medicine and the lack of literature concerning its flavonoidic constituents.

- Phytochemical screening of Combretum aculeatum leaves revealed the presence of flavonoids, steroids, tannins, alkaloids, saponins, coumarins and anthraquinone glycosides .Cyanogenic glycosides was absent .

- Four substituted flavones (I, II, III and Iv) were isolated by fractionation of the ethyl acetate extract of Combretum aculeatum over silica gel column followed by papper chromatography .

-Methanol extract of Combreatum aculeatum exhibited significant anti microbial activity ,how ever the ethyl acetate extract was devoid of activity.

The methanolic extracts showed significant activity against Escherichia coli and Pseudomonas aeruginosa . It also showed moderate activity against Bacillus subtilis and staphylococcus. This extract also exhibited significant antifungal activity against the fungi : Candida albicans and Aspergillus niger .

- Both methanolic and ethyl acetate extracts exhibited significant anti-oxidant activity.

Recommendations

-The structures of isolated flavonoids may be confirmed by further spectra scopic studies as used of 13CNMR and tow dimentional-NMR (COSY, HSQC and HMBC) .

- Other phyto chemicals (steroids, tannins, alkaloids, etc.) of the studied species may investigated .

- Clinical trails should be performed in order to support all the above invistigations and to facilitate their pharmaceutical formulations.

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