Sudan University of Science and Technology

College of Graduate Studies

Characterization of Flavonoids from *Albizia amara* Leaves and their Biological Activity on Microbes

توصيف فلافونيدات أوراق نبات العرد وتأثيرها الحيوي علي الميكروبات

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

***By*:**

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الإستفتاحية

بسم الله الرحمن الرحيم

((وَيَسْأَلُونَكَ عَنِ الرُّوحِ قُلِ الرُّوحُ مِنْ أَمْرِ رَبِّي وَمَا أُوتِيتُمْ مِنَ الْعِلْمِ إِلَّا قَلِيلًا)).

سورة الإسراء الآية (85)

صدق الله العظيم

DEDICATIO

To:

My parents

My brothers and sisters

My wife

My son (Mohamed)

ACKNOWLEDGEMENT

In the name of Allah, Almighty who taught man about matter that he does not know, prayers and peace be upon our Prophet Mohammed and his good followers till the Day of Judgment. I am most grateful to Allah, Almighty for helping and guiding me throughout my life.

With respect and gratitude, I would like to extend my thanks to my supervisor Professor, Dr. Mohamed Abdel Karim Mohammed for his continuous encouragement, excellent supervision, valuable advice and constructive criticism that help me geting through many intricate phases during the preparation of this thesis. Thanks are also due to my co-supervisor Dr. Mohamed Al-Mubarak Osman for his help, support, advice and cooperation.

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Finally, I would like to thank my family who tolerated my absence during the preparation of this thesis and for making my life so wonderful. May Allah bless them All.

Abstract

Phytochemical screening of methanol extract of leaves of Albizia Amara revealed the presence of secondary metabolites: saponins, alkaloids, tannins, glycosides and flavonoids. Powdered shade- dried leaves (1kg) of A. amara. were extracted with 95% ethanol at room temperature for 48 hours, The crude extract was suspended in water and partitioned with organic solvents in order of increasing polarity, petroleum ether, chloroform ethyl acetate and n-butanol, three compounds were isolated from n-butanol fraction. These compounds were isolated by chromatographic techniques and identified via spectroscopic tools: IR, UV, 1HNMR and Mass spectroscopy.

The isolated compounds were tested for antimicrobial potential against four bacterial strains: Gram-positive (Staphylococus aureus and Bacillus subtilis), gram-negative (Pseudomonas aeruginosa and Escherichia coli) and two Fungal species (Candida albicans and Aspergillus niger). Agar well diffusion method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) at concentrations of 100 mg /ml to 6.25mg /ml.

Compounds II and III showed significant antimicrobial activity at 100mg/ml and 50mg/ml. Compound I exhibited promising activity at these doses. The results of the Table 4 suggests that these flavonoids could serve as leads for further optimization.

المستخلص

أوضح المسح الفيتوكيميائي لأوراق نبات العرد وجود مركبات الأيض الثانوية: الصابونينات، القلويدات، التنينات، الجليكوسيدات والفلافونيدات. تم أستخلاص الأوراق الجافة بالإيثانول 95% لمدة 48 ساعة، ثم أخضع المستخلص الخام لكروموتوغرافيا الورق حيث تم فصل ثلاثة فلافونيدات من مستخلص البيوتانول العادي، تم توضيح التركيب بعدد من الطرق المطيافية : طيف الأشعة فوق البنفسجية، طيف الأشعة تحت الحمراء، طيف الرنين النووي المغنطيسي وطيف الكتلة.

أجري إختبار مضاد الميكروبات للمركبات المعزولة ضد بعض الميكروبات القياسية (اربعة انواع من البكتريا موجبة الصبغة وسالبة الصبغة ونوعان من الفطريات) واعطت المركبات **II** و **III** فعالية عالية عند التراكيز 100 ملغم/مل و 50 ملغم/مل اما المركب **I** فقد اعطي فعالية واعدة عند هذه التراكيز.

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List of Abbreviations

HIV	Human Immunodeficiency Virus.
ORAC	Oxygen radical absorbance capacity.
ROS	Reactive oxygen species.
MRSA	Methicilline Resistant Staphylococcus Aureus.
EBS	Estrogen Binding Sites.
HSV	Herpes Simplex Virus.
CCC	Counter Current Chromatography.
HSCCC	High speed counter current chromatography.
CCD	Current distribution method.
DAD	diode array detector.
FAB	Fast Atom Bombardment.
MALDI	Matrix Assisted Laser Desorption/Ionization.
IT	Ion Trap.
FT–ICR	Fourier Transform Ion Cyclotron Resonance.
ESI	Electro Spray Ionization.
TQ	Triple Quadrupole.
QTOF	Quadrupole Time-Of-Flight.
MS/MS	Tandem Mass Spectrometry.
CID	Collision induced dissociation.
SRM	Selected/single reaction monitoring,
MRM	Multiple reaction monitoring.
ESI	Electron Spray Ionization.
COSY	Correlation Spectroscopy.
TOCSY	Total correlation spectroscopy.
HSQC	Heteronuclear single quantum correlation.
HMBC	Heteronuclear multiple bond correlation.
DMSO	Dimethyl Sulfoxide.

CHAPTER ONE

INTRODUCTION

1. Introduction

1.1-General approach

Since ancient times, nature has been an important source of medicinal agents. This fact is illustrated by the large number of natural products currently in use in medical practice. The value of natural products in this regard can be assessed using three criteria: first, the rate of introduction of new chemical entities of wide structural diversity including natural products serving as templates for semi-synthetic and total synthetic analogues. Secondly, the number of diseases treated or prevented by these natural substances[1]. Thirdly, their frequent use in treatment of diseases. In recent years, there has been growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants. This interest in drugs of plant origin is due to several reasons, namely, the frequent inefficiency of conventional medicine, possible development of side effects of synthetic drugs, and that a large percentage of the world's poor population doesn't have access to conventional pharmacological treatment. In addition, the long history of use of folk medicine suggests that "natural" products are usually harmless. The plant kingdom offers a unique and renewable resource for the discovery of potential new drugs and important leads against various pharmacological targets including pain, cancer, HIV/AIDS, Alzheimer's and malaria[1].Medicinal plants are widely spread in Sudan

and their biological and phytochemical properties are not yet thoroughly evaluated[1].

1.2-Family Fabaceae

The legume family *(Fabaceae)* is the third largest family of flowering plants with more than 18,000 described species. It is surpassed in size only by the orchid family *(Orchidaceae)* with about 20,000 species and the sunflower family *(Asteraceae)* with about 24,000 species[2]. The family includes herbs, shrubs, trees and vinthies distributed throughout the world, especially the tropical rain forest.Fruits of plants belonging to this family are technically called legumes or pods. Fruits are composed of a single seed-bearing carpel that splits open along two seams. Legume fruits come in an enormous variety of shapes and sizes, including indehiscent pods that do not split open[2]. The leaves are usually alternate and compound. Most often they are even- or odd-pinnately compound (e.g. *Caragana* and *Robinia* respectively), often trifoliate (e.g. *Trifolium medicago*) and rarely palmately compound (e.g. *Lupinus*), in the Mimosoideae and in the commonly bipinnate Caesalpinioideae (e.g. *Acacia Mimosa*). They always have stipules, which can be leaf-like (e.g. *Pisum*), thorn-like (e.g. *Robinia*) or be rather inconspicuous. Leaf margins are entire or, occasionally, serrate. Both the leaves and the leaflets often have wrinkled pulvini to permit nastic movements. In some species, leaflets have evolved into tendrils (e.g. Vicia). Many species have leaves with structures that attract ants that protect the plant from herbivore insects (a form of mutualism). Extrafloral nectariesare common among the

Mimosoideae and the Caesalpinioideae are also found in some Faboideae (e.g. *Vicia sativa*). In some *Acacia*, the modified hollow stipules are inhabited by ants[3]. The flowers always have five generally fused sepals and five free petals. They are generally hermaphrodite, and have a short hypanthia, usually cup- shaped. There are normally ten stamens and one elongated superior ovary, with a curved style. They are usually arranged in indeterminate inflorescences. Fabaceae are typically entomophilies' plants (i.e. they are pollinated by insects), and the flowers are usually showy to attract pollinators[3].

Fruits of legume family have ovaries which most typically develop into legumes. A legume is a simple dry fruit that usually dehisces (opens along a seam) on two sides. A common name for this type of fruit is a "pod", although that can also be applied to a few other fruit types. A few species have evolved loments, follicles, indehiscent legumes and berries from the basic legume fruit[4].

Many Fabaceae host bacteria in their roots within structures called root nodules. These bacteria have the ability to take nitrogen gas (N_2) out of the air and convert it to a form of nitrogen that is usable to the host plant (NO_3- or NH_3). This process is called nitrogen fixation. The legume, acting as a host, and bacteria, acting as a provider of usable nitrate, form a symbiotic relationship[4].

There are three subfamilies of the legume family : Papilionoideae, Caesalpinioideae and Subfamily Mimosoideae. Members of the subfamily Mimosoideae have flowers with radial symmetry, small, inconspicuous corollas and numerous, showy stamens. The flowers

are typically in many flowered heads or spikes. This subfamily includes *Acacia* (wattle), *Albizia* (silk tree), *Samanea* (monkeypod), *Prosopis* (mesquite) and *Calliandra* (powder puff)[2].

The genus *Albizia* comprises approximately 150 species, mostly trees and shrubs native to tropical and subtropical regions of Asia and Africa. Leaves are bipinnate with leaflets in numerous pairs or larger in fewer pairs. Petiolar glands are conspicuous. Flowers are in globes heads or spikes. Stamens elongated, usually white. Corolla is funnel-shaped, connate beyond the middle. Fruit is broadly linear indehiscent or valved, valves not twisted[5].

Albizia amara is a deciduous tree, often rounded or spreading crown, reaching 10 m in height but often smaller. Bark dark brown and roughly cracked. Leaves compound with numerous small leaflets, feathery. Leaves and twigs covered with distinctive soft, golden hairs. Numerous small creamy-white flowers crowded together at the end of branches. The large pods are brown and papery, up to 20 x 3 cm, bulging over the few seeds.

The leaves of Albizia amara

Taxonomists classify the plant as follows:

kingdom : Plantae, **division**: Magnoliophyta, **class**: Magnoliopsida, **subclass**: Rosidae, **order**: Fabales, **family**: Fabaceae, **subfamily**: Mimosoideae, **genus**: *Albizia*, **species**: *amara*, **Common name**: Bitter *albizia* (English), **Local name**: Arrad, Botanical name: *Albizia amara* subsp. *Amara*, and synonyms: *Mimosa amara*.

The genus Albizia has been known to contain many classes of bioactive compounds including bioactive saponins, alkaloids, flavonoids…etc. The following are some representative examples of flavonoids isolated from genus Albizia.

A novel flavonol glycoside was isolated from the acetone soluble fraction of ethanolic extract of stem of *A. procera* which was identified as 5,2', 4'-trihydroxy-3,7,5'-trimethoxyflavonol-2'-*O*-β-D-galactopyranosyl (1→4)-*O*-β-D-glucopyranoside**.** The acetone fraction showed moderate anti-inflammatory action on albino rats by using carrageenan -induced hind paw oedema method[6].

Two flavonol glycosides, quercitrin and isoquercitrin were isolated from the flowers of *A. julibrissin* and showed sedative activity. Both compounds quercitrin and isoquercitrin increased pentobarbital-induced sleeping time in dose-dependent manner in mice. These results supported the use of the flowers of this plant as a sedative agent in oriental traditional medicine[7].

In addition, a new biologically active flavonol glycoside 3, 5, 4'-trihydroxy, 7, 3`- dimethoxy-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-xylopyranoside was isolated from the chloroform-soluble fraction of

the seeds of *A. julibrissin*. The antibacterial activity of the chloroform-soluble fraction of the methanolic extract was fairly active against Gram positive and Gram negative bacteria[8].

Oxygen radical absorbance capacity (ORAC) values showed that the methanolic extracts of *A. julibrissin* foliage displayed antioxidant activity. The analysis confirmed the presence of three compounds in *A. julibrissin* foliage methanolic extract: an unknown quercetin derivative, hyperoside (quercetin-3-*O*-galactoside)**,** and quercitrin (quercetin-3-*O*-rhamnoside)[9].

The seeds of *A. amara* was reported to contain spermine alkaloids referred to as budmunchiamines[10,11] as well as some flavonoids (e.g. melacacidin) that have also been extracted from the heartwood[12], budmunchiamines from other Albizia species have been shown to exhibit anti-plasmodial activities[13]. Seeds of *Albizia amara* are regarded as astringent, and used in the treatment of piles, diarrhoea and gonorrhea[14]. The leaves and flowers have been applied to boils, eruptions, and swellings,they are also regarded as an emetic and as a remedy for coughs, ulcer, dandruff and malaria[15].

The current literature revealed that some plants belonging to genus Albizia have great medicinal values. Recently, an antitumor triterpene saponin julibroside J28 was isolated from the stem bark of *A. Julibrissin*[16].

It is also reported in Indian traditional medicine that *A. lebbeck* has antiseptic, antidysentric and anti-tubercular activities[17]. Moreover,

saponins of *A.lebbeck* have been claimed to be useful in treatment of Alzheimer,s and Parkinson,s diseases[18].

Functional uses of *A. amara* include deriving tannin or dyestuff from the bark. However, it is alleged that the seeds are poisonous. In traditional medicine the roots are chewed and applied to an eye infection of cattle. Fruits are also used as an emetic and for treating coughs and malaria. Saponins are extracted from the roots and leaves. Tannins and gums are extracted from the bark and the gum is used against ulcers; fruits are said to cure malaria and coughs. Bark stem decoction taken three times aday serves as an emetic to induce vomiting and to treat malaria[19,20]. Leaves are said to be used in the treatment of wounds[19]. *A. amara* is attractive and can be planted in urban areas as an ornamental and avenue tree. It could also be more widely grown as boundary marker. Many streets of Harare are lined with *A. amara* plants[20].

1.3-Phytochemicals

Phytochemicals (from the *Greek* word *phyto*, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients[21]. They protect plants from disease and damage and contribute to the plant's colour, aroma and flavour. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called phytochemicals[22,23]. Recently, it is clearly known that they have roles in the protection of

human health, when their dietary intake is significant. More than 4,000 phytochemicals have been catalogued[24], and are classified by protective function, physical characteristics and chemical characteristics[25], and about 150 phytochemicals have been studied in detail[24].In wide-ranging dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi and herbs[23]. Broccoli, cabbage, carrots, onions, garlic, whole wheat bread, tomatoes, grapes, cherries, strawberries, raspberries, beans, legumes, and soy foods are common sources[26]. Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds[27]. Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. Levels vary from plant to plant depending upon the variety, processing, cooking and growing conditions[28]. Phytochemicals are also available in supplementary forms, but evidence is lacking that they provide the same health benefits as dietary phytochemicals[24]. These compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals. It is well-known that plants produce these chemicals to protect themselves, but recent researches demonstrate that many phytochemicals can also protect human against diseases[29].

Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common diseases. Many of these benefits suggest a possible role for phytochemicals in the prevention and treatment of disease, Because of this property; many researches have been performed to reveal the beneficial health effects of phytochemicals[29].

The exact classification of phytochemicals could have not been performed so far, because of the wide variety of them. In resent year phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides[30].

Literature survey indicate that phenolics are the most numerous and structurally diverse plant phytocontituents. Phenolics 45%, alkaloids 18%, terpenoids and steroids 27%, others 10%. Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom. The three most important groups of dietary phenolics are flavonoids, phenolic acids, and polyphenols. Phenols are hydroxyl group (-OH) containing class of chemical compounds where the (-OH) bonded directly to an aromatic hydrocarbon group. Phenol (C_6H_5OH) is considered the

simplest member of this group. Phenolic compounds are a large and complex group of chemical constituents found in plants[31]. They are plant secondary metabolites, and they have an important role as defence compounds. Phenolics exhibit several properties beneficial to humans and their antioxidant properties are important in determining their role as protecting agents against free radical-mediated disease processes. Flavonoids are the largest group of plant phenols and the most studied[32]. Phenolic acids form a diverse group that includes the widely distributed hydroxybenzoic and hydroxycinnamic acids. Phenolic polymers, commonly known as tannins, are compounds of high molecular weight that are divided into two classes: hydrolysable and condensed tannins.

The term “phenolic acids”, in general, designates phenols that possess one carboxylic acid functional group. Naturally occurring phenolic acids contain two distinctive carbon frameworks: (see table 1) the hydroxycinnamic and hydroxybenzoic structures. Hydroxycinnamic acid compounds are produced as simple esters with glucose or hydroxy carboxylic acids. Plant phenolic compounds are different in molecular structure, and are characterized by hydroxylated aromatic rings[33]. These compounds have been studied mainly for their properties against oxidative damage leading to various degenerative diseases, such as cardiovascular diseases, inflammation and cancer. Indeed, tumour cells, including leukaemia cells, typically have higher levels of reactive oxygen species (ROS) than normal cells so that they are particularly sensitive to oxidative stress[34]. Many papers and

reviews describe studies on bioavailability of phenolic acids, emphasizing both the direct intake through food consumption and the indirect bioavailability deriving by gastric, intestinal and hepatic metabolism[35].

Table 1: The major classes of phenolic compounds in plants

S.N.	Number of carbon atom	Basic skeleton	Class
1-	6	C_6	Simple phenols Benzoquinones
2-	7	C_6-C_1	Phenolic acids
3-	8	C_6-C_2	Acetophenones Tyrosine derivatives
4-	9	C_6-C_3	Hydroxycinnamic acid, Coumarins
5-	10	C_6-C_4	Naphthoquinones
6-	13	C_6- C_1-C_6	Xanthones
7-	14	C_6- C_2-C_6	Stilbenes
8-	15	C_6- C_3-C_6	Flavonoids
9-	18	$(C_6$- $C_3)_2$	Lignans
10-	30	$(C_6$- C_3-C_6 $)_2$	Bioflavonoids
11-	N	$(C_6$- C_3-$C_6)_n$	Condensed tannins

Phenolic acids have been the subject of a great number of agricultural, biological, chemical and medical studies. In recent years, the importance of antioxidant activities of phenolic compounds and their potential usage in processed foods as a natural antioxidant compounds has reached a new level and some evidence suggests that the biological actions of these compounds are related to their antioxidant activity[36].

Flavonoids are polyphenolic compounds are ubiquitous in nature. More than 8,000 flavonoids have been recognised, many of which

occur in vegetables, fruits and beverages like tea, coffee and fruit drinks[37].

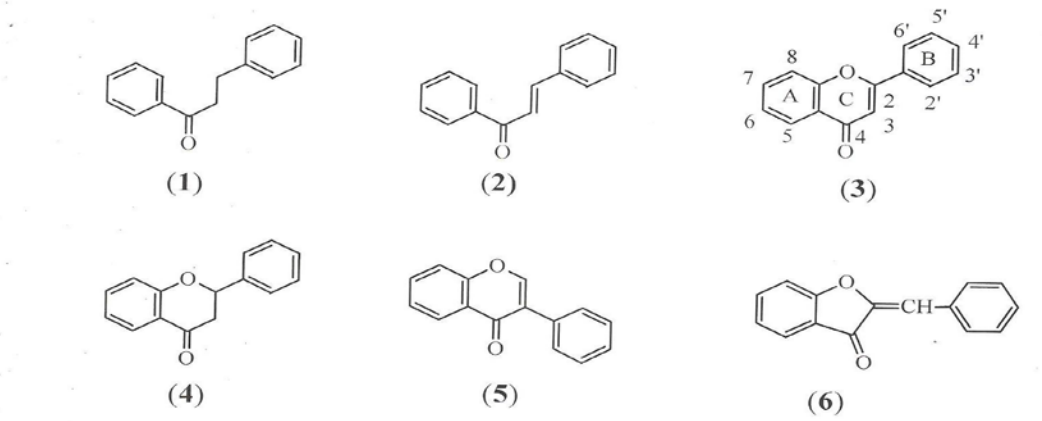
Flavonoids are a group of common naturally occurring polyphenolic compounds that are widely found in the plant kingdom. They occur naturally as plant pigments in a broad range of fruits and vegetables as well as beverages such as tea, red wine, coffee and beer[38,39]. Many of the known flavonoids to date are part of our regular diet[39].

Flavonoids are polyphenolic compounds comprising 15 carbons, with two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle (C_6-C_3-C_6) . Flavonoids are found throughout the plant kingdom and in particular in the epidermis of leaves and in the skin of fruits. Based on the variation in the type of heterocycle involved, flavonoids are divided into different sub-classes: anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols, isoflavones, dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones. The basic C_6-C_3-C_6 flavonoid skeleton can have numerous substituents (e.g. hydroxyl, acetyl, methoxy and methyl groups) and the majority of the flavonoids exist naturally as glycosides e.g., conjugated to one or several different sugar moieties such as glucose, rhamnose and galactose[40-44].

The basic structure of flavonoids consists of two benzene rings (A and B) connected through central pyrone ring (C).The number of hydroxy groups and the presence of a 2,3-double bond and orthodiphenolic structure enhance antiradical and antioxidative activity of flavonoids[41]. The glycosylation, blocking the 3-OH group in C ring,

lack of a hydroxy group or the presence of only a methoxy group in B-ring has a decreasing effect on anti-radical or anti-oxidative activity of these compounds[41]. Tannins show strong antioxidative properties because of the high number of OH groups. Some tannins of gallate esters were proved to have antioxidative effect *in vivo*. The numbers of hydroxy groups connected with the aromatic ring, in ortho or para position relative to each other, enhance antioxidative and antiradical activity of phenolic acids[41].

Flavonoids can be subdivided into: dihydrochalcone(1),flavones(3),flavanones(4),isoflavones(5),aurones(6)



1.4-Flavones

The flavones structure is planar. It consists of a double bond between positions 2 and 3 of the C ring. The C-ring is connected to the B-ring at position 2. The B ring has a hydroxyl group at position 4`. Flavones are found in red peppers, apple skins and celeries[42]. Examples of flavones are luteolin and apigenin.

1.5-Flavonols

The flavonols have a planar ring structure. The B ring is connected to the pyrone ring at position 2. The C- ring fission occurs at positions

between bonds 1 and 2, 3 and 4 as well as between 4 and 10. Examples of flavonols are quercetin, kaempferol and myricetin. These are found in onions, apples, red wine, broccoli and tea[42].

1.6-Flavanone

The Flavanone structure is not planar and lacks a double bond between positions 2 and 3 of the central pyrone ring. Flavanones are degraded through C-ring fission at positions between 1 and 2 as well as 4 and 10. Citrus fruits, grape fruits are abundant in flavanone. Examples of flavanone are hesperitin and naringenin[42].

1.7-Flavan-3-ols

The flavan-3-ols ring structure is not planar. The C ring of flavan-3-ols does not contain a carbonyl group. Flavan-3-ols are found in fruits and tea. Some examples are : catechin, epicatechin and epigallocatechin[42].

1.8-Anthocyanins

The anthocyanin structure is planar. The central C ring lacks the carbonyl group found in flavones. There are double bonds between positions 1 and 2 as well as 3 and 4 of the C-ring. These are present in fruits and flowers and are responsible for fruit and flower coloration. Cherries, grapes, plums, raspberries, strawberries, peaches and apples are abundant in anthocyanins[42].

1.9-Isoflavones

The isoflavone structure is planar. It differs from the rest of the flavonoids since A-ring is connected to the central C-ring at position

3. Soybeans are rich in such flavonoids. Examples of isoflavones are daidzein and genistein[42].

1.10-Isoflavonoids

The isoflavonoids are a distinctive subclass of the flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by 1,2-aryl migration in a 2-phenylchroman precursor. Despite their limited distribution in the plant kingdom, isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises not only from the number and complexity of substituents on the basic 3-phenylchroman system, but also from the different oxidation levels and presence of additional heterocyclic rings. Neoflavonoids which are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1- benzopyran-2-ones), 3,4-dihydro-4-arylcoumarins, and neoflavenes[43].

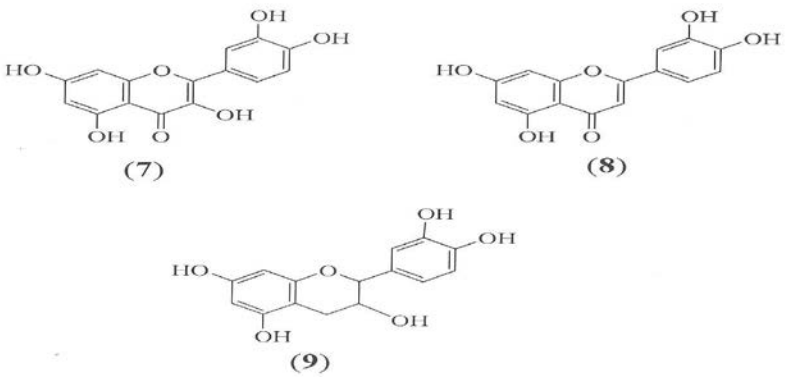
1.11-Minor Flavonoids

Natural products such as chalcones and aurones also contain a C_6-C_3-C_6 backbone and are considered to be minor flavonoids. These groups of compounds include the 2′-hydroxychalcones, 2′-OH-dihydrochalcones, 2′-OH-retro chalcone, aurones (2-benzylidenecoumaranone), and auronols. chalcones, dihydrochalcones, and racemic flavonoids,chalcones and dihydrochalcones are considered to be the primary C_6-C_3-C_6 precursors and constitute important 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[4] and benzaldehydes [5,43]. The base-catalyzed aldol condensation is usually the preferred route towards chalcone[6] formation, since under acidic conditions cyclization of the ensuing chalcone leads to formation of corresponding racemic flavanones[7,44,45]. Dihydrochalcones[8] are generally obtained via reduction (H_2/Pd) of the preceding chalcones.

Flavonoids have gained recent interest because of their broad biological and pharmacological activities. Flavonoids have been reported to exert multiple biological effects including antimicrobial[46], cytotoxicity[47], anti-inflammatory[48] as well as antitumor activities[49].

The work of Hertog and co-workers showed the inverse correlation between flavonoids intake and coronary heart disease mortality[50]. Flavonoids have attracted the interest of researchers because they showed promise of being powerful antioxidants which can protect the human body from free radicals[51,52]. Many flavonoids such as quercetin (7), luteolin (8) and cathechins (9), are better antioxidants than the nutrients antioxidants such as vitamin C, vitamin E and β-carotene[53]. The function of an antioxidant is to intercept and react with free radicals at a rate faster than the substrate. Since free radicals are able to attack at a variety of target including lipids, fats and proteins, it is believed that they may damage organisms, leading to disease, poisoning and including aging[54].



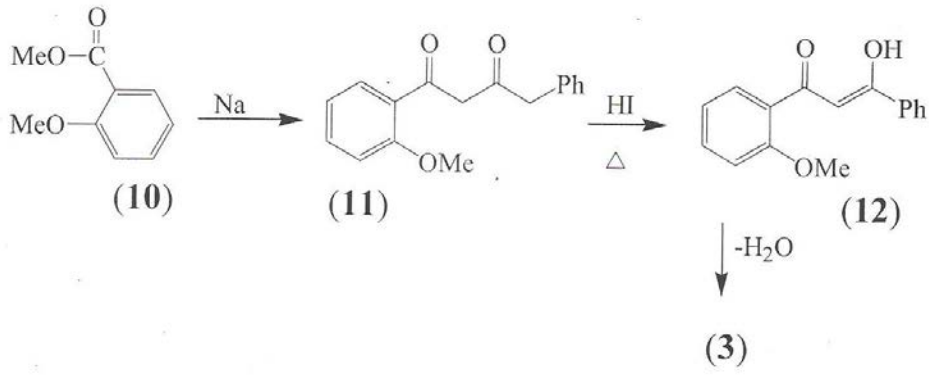
The interest in the biological properties of flavones has resulted intense synthetic efforts towards the synthesis of various flavones.

1.12- Synthesis of flavonoids

There are a number of methods reported for the synthesis of flavones:

The Von-Konstanecki method

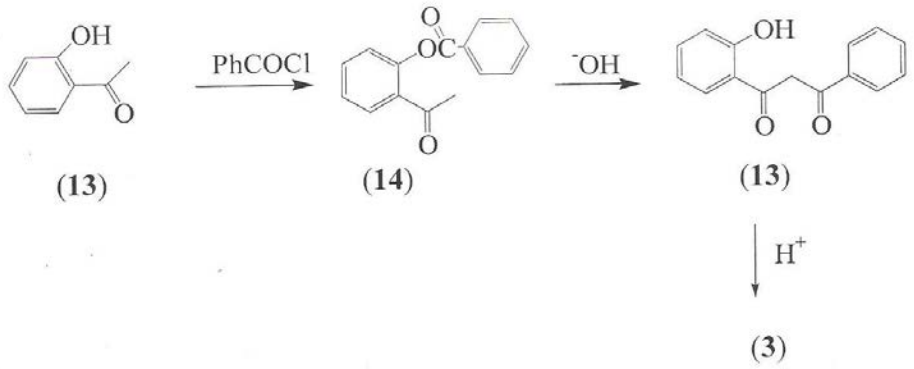
This is a general method for synthesizing flavones which involves a reaction of o-methoxybenzoate (10) and acetophenone in the presence of sodium to form (11) as shown in Scheme I. The reaction occurring is the Claisen condensation. This is followed by treatment of (11) with an acid to form compound (12) followed by elimination of water in order to form the flavone (3)[55].



Scheme I: The Von-Konstanecki synthesis

The Baker-Venkataraman method

The Baker-Venkataraman approach would be the most convenient route to the synthesis of flavone as shown in Scheme II. In Baker-Venkataraman reaction, 2- hydroxyacetophenone wis converted to ester, which then undergoes rearrangement by intramolecular Claisen condensation in the presence of potassium hydroxide and pyridine to afford 1,3-diketone. 1,3-diketone which are then cyclised to flavone (3) under rather harsh conditions either by treatment with concentrated sulfuric acid or heating with glacial acetic acid[56].

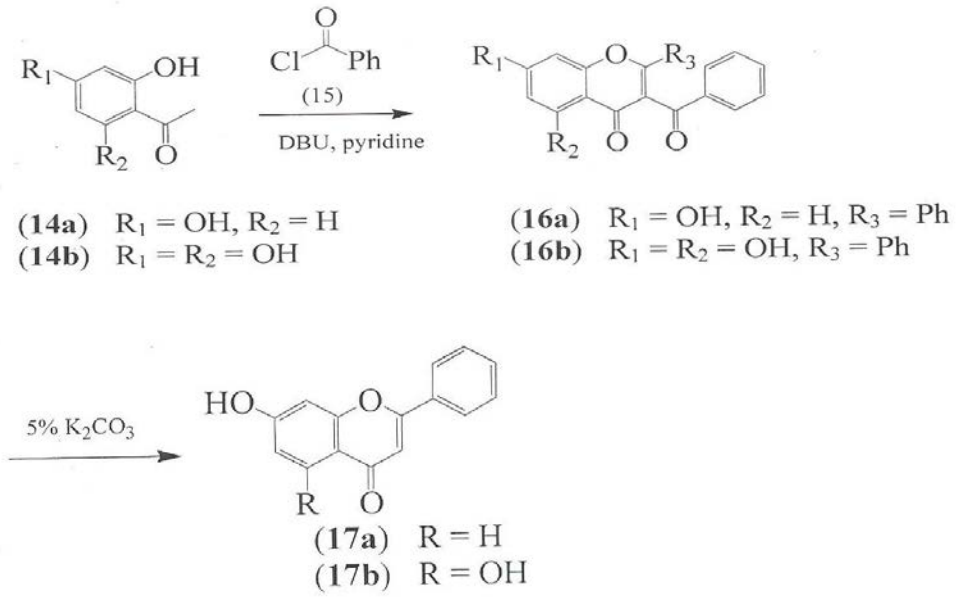


Scheme II : The Baker-Venkataraman method

Ganguly's synthesis

Over a period of years several groups have investigated and improved experimental conditions of Baker–Venkataraman reaction amongst which the work of Ganguly and colleagues[56]. Using modified Baker–Venkataraman reaction, a novel class of 3-acylflavones which is the precursors to flavones have been synthesised. In their procedure, compounds such as 2`,4`-dihydroxyacetophenone (14a) and 2`,4`,6` trihydroxyacetophenone (14b) were heated with acyl chloride (15) in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and pyridine to obtain 3-acylflavones (16a) and (16b). On heating under

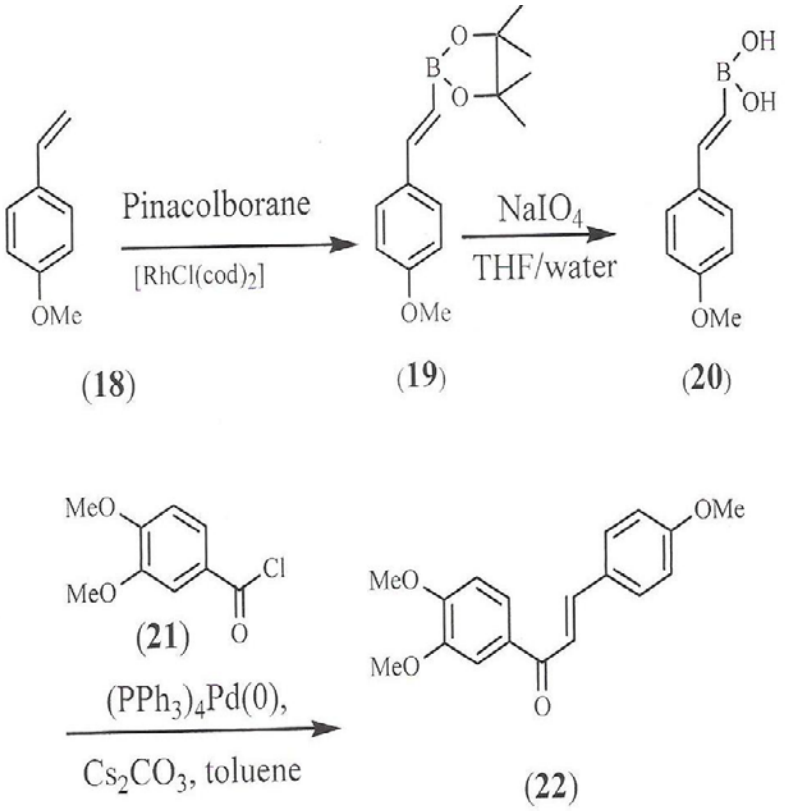
reflux with an aqueous solution of 5% potassium carbonate, compound (16a) and (16b) yielded (17a) and (17b), respectively as shown in Scheme III.



Scheme III : Ganguly's synthesis of flavones

Synthesis of Chalcones via Suzuki coupling reaction

Chalcone (2) or 1, 3-diphenyl-2-propen-1-one and especially chalcones bearing a hydroxyl function on the aromatic rings are the precursors of all the flavonoids[57]. In 2003, Edrarir and co-workers reported an efficient synthesis of chalcones based on the Suzuki coupling reaction between benzoyl chloride and phenyl vinylboronic acid as shown in Scheme IV.



Scheme IV : Synthesis of calcones via Suzuki coupling reaction

1.13-Biological activity of flavonoids

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 [58, 59, 60, 61]. Several flavonoids including apigenin, galangin, flavone and flavonol glycosides, isoflavones, flavanones, and chalcones have been shown to possess potent antibacterial activity[62].

Antibacterial flavonoids might be having multiple cellular targets, rather than one specific site of action. One of their molecular actions

is to form complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesion, enzymes, cell envelope transport proteins, and so forth. Lipophilic flavonoids may also disrupt microbial membranes[63, 64]. 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[65, 66]. The catechins have been shown to inactivate cholera toxin in *Vibrio cholera* and inhibit isolated bacterial glucosyltransferases in *S.mutans*, probably due to complexing activities[65, 67]. Robinetin, myricetin, and (−)-epigallocatechin are known to inhibit DNA synthesis in *Proteus vulgaris*. Mori et al[68], suggested that the B ring of the flavonoids may intercalate or form hydrogen bond with the stacking of nucleic acid bases and further lead to inhibition of DNA and RNA synthesis in bacteria. Another study demonstrated inhibitory activity of quercetin, apigenin, and 3,6,7,3′,4′-pentahydroxyflavone against *Escherichia coli* DNA gyrase[69].

Naringenin and sophoraflavanone have intensive antibacterial activity against methicilline resistant *Staphylococcus aureus* (MRSA) and *streptococci*. An alteration of membrane fluidity in hydrophilic and hydrophobic regions may be attributed to this effect which suggests that these flavonoids might reduce the fluidity of outer and inner

layers of membranes[70]. The correlation between antibacterial activity and membrane interference supports the theory that flavonoids may demonstrate antibacterial activity by reducing membrane fluidity of bacterial cells. The 5,7-dihydroxylation of the A ring and 2′,4′-or 2′,6′-dihydroxylation of the B ring in the flavanone structure is important for anti-MRSA activity[71]. 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 anti-staphylococcal activity of flavonoids belonging to the flavan-3-ol class[72]. 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*[73].

Haraguchi and colleagues[71], studied antibacterial activity of two flavonoids, licochalcones A and C, isolated from the roots of *Glycyrrhiza inflata* against *S. aureus* and *Micrococcus luteus*. They observed that licochalcone A inhibited incorporation of radioactive precursors into macromolecules (DNA, RNA, and protein). This activity was similar to the mode of action of antibiotics inhibiting respiratory chain, since energy is required for active uptake of various metabolites as well as for biosynthesis of macromolecules. After further studies it was suggested that the inhibition site of these flavonoids was between CoQ and cytochrome in the bacterial respiratory electron transport chain[71]. There are many examples that

lend support to the prowess of phytoconstituents derived from edible and medicinal plants as potent antibacterial agents[74,75].

Anticancer Activity

Dietary factors play an important role in the prevention of cancers. Fruits and vegetables having flavonoids have been reported as cancer chemo- preventive agents[59, 76]. Consumption of onions and/or apples, two major sources of the flavonol quercetin, is inversely associated with the incidence of cancer of the prostate, lung, stomach, and breast. In addition, moderate wine drinkers also seem to have a lower risk to develop cancer of the lung, endometrium, esophagus, stomach, and colon[77]. The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented. It has been suggested that major public health benefits could be achieved by substantially increasing consumption of these foods[78].

Quercetin is known to produce cell cycle arrest in proliferating lymphoid cells. In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included leukemia cells, gastric cancer cells, colon cancer cells, human breast cancer cells and ovarian cancer cells[79]. Markaverich et al[80]. proposed that tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS). It has been experimentally proved that increased signal transduction in human breast cancer cells is markedly reduced by quercetin acting as an antiproliferative agent[81].

Barnes[82] 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. Neonatal administration of genistein (a flavonoid) exhibited a protective effect against the subsequent development of induced mammary cancer in rats[83]. Hesperidin, a flavanone glycoside, is known to inhibit azoxymethanol induced colon and mammary cancers in rats[84]. The anticancer properties of flavonoids contained in citrus fruits have been reviewed by Carroll et al[85]. Several flavonols, flavones, flavanones, and the isoflavone biochanin A are reported to have potent antimutagenic activity[86]. 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[87]. In earlier studies ellagic acid, robinetin, quercetin, and myricetin have been shown to inhibit the tumorigenicity of BP-7, 8-diol-9, and 10-epoxide-2 on mouse skin[88].

Higher consumption of phytoestrogens, including isoflavones and other flavonoids, has been shown to provide protection against prostate cancer risk[89]. 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[58, 59, 90]. Therefore diets rich in

radical scavengers would diminish the cancer-promoting action of some radicals[91].

Antiviral Activity

Natural compounds are an important source for the discovery and the development of novel antiviral drugs because of their availability and expected low side effects. Naturally occurring flavonoids with antiviral activity have been recognized since the 1940s and many reports on the antiviral activity of various flavonoids are available. Search of effective drug against human immunodeficiency virus (HIV) is the need of hour. Most of the work related with antiviral compounds revolves around inhibition of various enzymes associated with the life cycle of viruses. Structure function relationship between flavonoids and their enzyme inhibitory activity has been observed. Gerdin and Srensso[92] 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[93]. 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[94]. Catechins are also known to inhibit DNA polymerases of HIV-1. Flavonoid such as demethylated gardenin A and robinetin are known to inhibit HIV-1 proteinase[93]. 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[95].

Various combinations of flavones and flavonols have been shown to exhibit synergism. Kaempferol and luteolin show synergistic effect against herpes simplex virus (HSV). Synergism has also been reported between flavonoids and other antiviral agents. Quercetin is reported to potentiate the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection[93]. 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[93].

Zandi et al.[96] 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[92]. Inhibition of viral polymerase and binding of viral nucleic acid or viral capsid proteins have been proposed as antiviral mechanisms of action[96].

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[97]. A new prenylated flavanone recently 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[98]. The flavonoid 7-hydroxy-3,4-(methylenedioxy)flavan, isolated from *Terminalia bellerica* fruit rind, has also been shown to possess activity against *C. albicans*[99].Two new 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*[100], a species of fungi that causes invasive disease in immuno-suppressed patients[101]. The activity of *Propolis* against dermatophytes and *Candida* spp. has been attributed at least partially to its high flavonoid content[102]. Galangin, a flavonol commonly found in *Propolis* samples[103], has been shown to have inhibitory activity against *Aspergillus tamarii*, *A. flavus*, *Cladosporium sphaerospermum*, *Penicillium digitatum* and *Penicillium italicum* [104].

The use of plant extracts in medicine, for example against microbial infections[105,106], is still very widespread and based on knowledge from traditional medicinal practice[107]. Flavonoids seem to be very important in this respect as they were found to have anti-inflammatory, antiproliferative, antiviral, antithrombotic, antimutagenic, anticarcinogenic, hepatoprotective, oestrogenic, antibacterial and antioxidant activities[108,109]. Antibacterial activity is

widely found in chemically different groups of flavonoids. Morusin, kuwanon C, sanggenon B and D, which were isolated from Morus root bark showed for example strong antimicrobial activity against many microorganisms[110].

1.14-Isolation techniques of flavonoids

Flavonoids and other phenolics are usually present in plants in complex mixtures. Isolation of flavonoid compounds involves two separate procedures: (a) extraction and (b) isolation and separation into pure compounds.

1.14.1-Extraction of flavonoids

Solvents used for extraction are chosen according to the polarity of the flavonoids being studied. The less polar solvents are particularly useful for the extraction of flavonoid aglycones. Whilst the more polar solvents are used if flavonoid glycosides, sulphates or anhocyanins are involved[111,112].

In general 70-80% ethanol is the solvent of choice for the extraction of the dried plant material of all classes of flavonoids. 50% ethanol is recommended when water- soluble derivatives (glucuronides or potassium bisulphate's) are present. For anthocyanins, hydrochloric acid (0.1%) should be present to prevent their conversion to the pseudobase form[113].

The crude extract is then subjected to two dimensional papers or thin layer chromatography. This is followed by the separation of the flavonoids using the suitable method for each class[114,115].

1.14.2-Separation techniques of flavonoids

When flavonoids of varying types are to be extracted from a single

batch of plant, a method for preliminary separation is sequential extraction with a number of solvents of varying polarity. This can lead to the separation of glycosides from aglycones and to the separation of polar from non-polar aglycones. Although a certain degree of overlap could occur.

More conventional methods are the use of column, paper and thin layer chromatography, high performance liquid chromatography (HPLC) and counter current chromatography (CCC)[116].

Column chromatography

Column chromatography remains the single most useful technique for the isolation of large quantities of flavonoids from crude plant extracts. The absorbents of choice are generally silica gel, cellulose, polyamide and sephadex.

Silica gel column has been used for the separation of isoflavones, flavanones, dihydroflavonols and highly methylated flavones and flavonols using chloroform and chloroform - methanol for the separation of flavonoid glycosides.

Column chromatography using cellulose can be considered as a scale - up from paper chromatography.It is suitable for the separation of all classes of flavonoids and their glycosides. In principle, the full range of solvents developed for use in paper chromatography is available for partition column chromatography and many have been used with success[117].

Polyamide column chromatography is suitable for the separation of all types of flavonoids[118]. It has a high capacity for phenolic materials

which form strong hydrogen bonds with phenolic hydroxyl groups via their amide carbonyl function[119].

Another suitable absorbent is the special Sephadex LH -20 which can be used with organic solvents or water/solvents mixture‘. Sephadex is a highly cross linked dextran on which separation is ideally obtained on the basis of molecular size[119].

Paper chromatography

Column chromatography often yields pure flavonoids or simple mixtures which may be further separated by paper chromatography. Chromatography on paper usually involves either partition or adsorption chromatography. Paper chromatography is suitable for the separation of all types of flavonoids and their glycosides[120-122].

Most flavonoids appear as coloured spots on paper chromatograms viewed in UV light, and fuming with ammonia often produces significant changes in their colour. For majority of flavonoids, separation is first effected by the use of n-butanol-acetic acid-water (4:1:5) (BAW) which produces a separation based largely on partitioning. The second dimension is commonly run using an aqueous solvents such as water and 2 -60% aqueous acetic acid[123].

Flavonoid aglycones and highly methylated flavonoids are best separated by using BAW, choloroform -acetic acid -water (13:6:1), phenol- water (4:1), or forestall (acetic acid-Water-conc HCl, 30:10:3). Solvents containing HC1 or acetic acid are required for the chromatography of anthocyanins and anthocyanidins[123,124].

Thin layer chromatography

Thin layer chromatography (TLC) is commonly used for the analysis of mixtures and/or the isolation of pure flavonoids. The absorbents of choice for the separation of flavonoids are silica gel, polyamide cellulose and sephedex[118]. The greater speed of TLC is due to the more compact nature of the absorbent when spread on a plate which is an advantage when working with labile compounds.

This method is preferably used: a) when the compounds are obtained from small quantities of plant material, b) resolving mixtures of glycosides or acylated glycosides which will not separate on paper, c) resolving the highly methylated flavones and flavonols, d) resolving the bioflavonoids which do not separate on paper chromatography, e) test the purity of glycoside sample and, f) identification by chromatography.

The highly methylated flavones and flavonols are separated on cellulose, polyamide and silica gel plates using solvent system: t-butyl alcohol- actic acid-water (upper phase) (4:1:5), benzene-petroleum ether (65-110C) - methyl ethyl ketone - methanol (60:26:7:7), toluene – methyl ethyl ketone - methanol (60:25:15), toluene - methyl ethyl ketone - methanol -acetyl acetone(40:20:30:10)and (13:3:3:1), chloroform - formic acid - acetone (9: 1: 2), benzene - pyridine - formic acid (36: 9: 5), and benzene - dioxane - acetic acid (90: 25: 4)[111,122].

Silica gel TLC of flavonoid glycosides requires a polar solvent such as ethyl acetate - methyl ethyl ketone - formic acid - water (5:3:1:1),

or benzene - pyridine - formic acid (36: 9:5)[109].

The acylated glycosides are separated on polyamide and silica gel plates with the solvent systems: bezene - ethyl acetate - formic acid (5:4:2), benzene - methanol - ethyl methyl ketone- water (55:20:22:3), benzene - pyridine - formic acid (36: 9: 5), toluene – ethyl acetate - formic acid (5:4:1), toluene – methyl ethyl ketone - methanol (60:25:15), methanol-acetic acid- water (18:1:1), methylene chloride-benzene-methanol(15:l:l), methylene chloride- ethyl acetate-acetic acid (80:10:10), chloroform-methanol-methyl ethyl ketone-50% acetic acid (65:20:11:4) and ethyl acetate-pyridine-water-methanol (80:12:10:5) [109,123.124].

The C-glycosides are separated on cellulose plates with 15% acetic acid. Glycosides and aglycones of aurones, chalcones, flavanones, flavones, flavnols and isoflavonoids are separated on TLC polyamide plates with the solvent mixtures: acid acid-water (90:5) and Egger's solvent, chloroform-methanol-2-butanone (12:2:1)[125].

Thin layer chromatography has also been used for separation of biflavones mixtures. Silica gel and polyamide were used as TLC support. In case of silica gel as support, the solvents used are: benzene-pyridine- ethylformate-dioxane (5:1:2:2) benzene-pyridine-formic acid (36:9:5), toluene-ethyl formate-formic acid (5:4:1), and benzene-ethyl acetate-acetic acid (8:5:2). In case of polyamide as support the solvents used are: methanol- acetic acid-water (18:1:1), and chloroform-methanol (3:2)[111,126,127].

Separated constituents can be recovered by scraping off and eluting

the powder with an appropriate solvent and finally centrifuging to remove the absorbent.

Paper electrophoresis

Paper electrophoresis is a technique of limited application in flavonoid analysis science. To be mobile; a flavonoid must be in an ionized state at the pH of the electrolyte. Thus pH 2.2 (formate-acetate buffer) on Whatman 3MM, flavonoid bisulphate's migrates towards the anode whereas other flavonoids do not[128,129]. The distinction of glucuronoides from glycosides at low levels is conveniently accomplished at pH 4 (0.01M potassium hydrogen phthalate) on cellulose acetate sheets. Under this condition the glucuronoides migrate towards the anode but the glycosides do not[129,130].

High performance liquid cartography

HPLC is akin to GLC except that the carrier gas replaced by a solvent or solvent mixture. In principle it is ideally suited to the chromatographic analysis, qualitative and quantitative, of non-volatile compounds[109' 129].

Advantages claimed for HPLC analysis include (a) short analysis time, (b) high resolution, (c) no derivatisation required, (d) no risk of thermal decomposition and (e) easy quantification.

HPLC of flavonoids has been described previously in several publications utilizing both reversed-phase [131,132] and silica column[133]

High speed countercurrent chromatography (HSCCC)

Countercurrent chromatography (CCC) is a support-free partition

chromatography and offers the advantage over liquid chromatography of eliminating all complication arising from the use of solid support. Countercurrent distribution method (CCD) and countercurrent chromatography (CCC) can yield pure fraction at a high reproducibility rate without the risk of adsorptive loss of samples, while it permits continuous elution, monitoring and fraction are performed as in liquid chromatography[134]. A countercurrent extraction scheme with a coil planet centrifuge, based on the dual countercurrent system, has the capability of efficiently performing both clean up and sample enrichment in one stop operation.

Recently, high speed countercurrent chromatography has been successfully developed by utilizing a new coil configuration called a multi- layer coil, which compactly accommodates a numbe of coiled layers around a spool-shaped holder[135]. The isolation of water soluble and high polar derivatives is also achieved by the application of countercurrent, techniques[136].

1.15-Identification technique of flavonoids

The identification technique of flavonoids involves both chemical methods and physical tools.

1.15.1-Chemical methods

If the pure flavonoid is suspected to be a glycoside, the standard procedure is to hydrolyse it, normally by acidic and/or enzymatic hydrolysis. Complete acidic hydrolysis is carried out with 2N hydrochloric acid, while mild acid hydrolysis is carried out with 0.2N hydrochloric acid. Enzymatic hydrolysis is carried out with specific

enzymes such as β- glucuroidase.

The sugar moiety obtained by hydrolysis of flavonoid glycosides is identified by paper and thin layer chromatography. Identification of the aglycone obtained by hydrolysis of flavonoid glycoside involves such data as R_f values, spot colour under UV light, UV data, nuclear magnetic resonance (1H-NMR and 13C-NMR) and mass spectrometry.

Demethylation is also applied in case of a methylated flavonoid suspected of being present. This is carried out with anhydrous $AICI_3$. Pyridinium chloride alkaline degradation could also be used to identify the basic nucleus of the flavonoid under study[111,121,129].

1.15.2-Physical methods

Complete elucidation of the flavonoids should include physical methods such as ultraviolet (UV), nuclear magnetic resonance (^{1}H-NMR and ^{13}C NMR) and mass spectrometry[109,121,125].

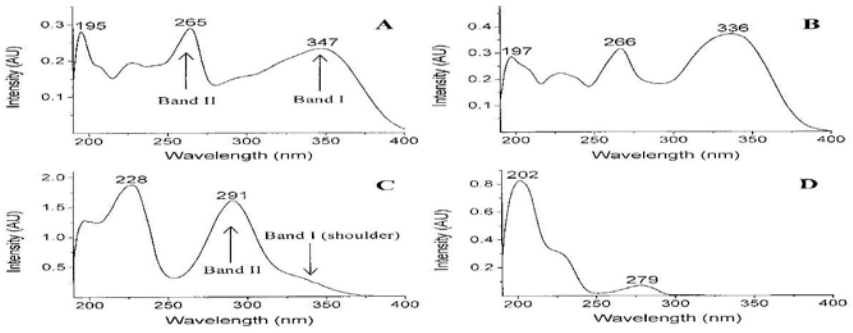
Ultraviolet-visible spectroscopy

UV spectroscopy has become a major technique for the structural analysis of flavonoids for two reasons. First, the UV spectra of different types of flavones are usually different, and thus these spectra can be used to identify the structure class. Second, the information from the UV spectra of flavonoids can be considerably enhanced by the use of certain UV-shift reagents. The commonly used UV-shift reagents are aluminium chloride ($AlCl_3$), sodium methoxide (NaOMe), sodium acetate (NaOAc), and boric acid (H_3BO_3). The preparation and use of these reagents has been described by Mabry et. al. in 1970[125].

All phenolic compounds contain aromatic conjugated systems that absorb light in the UV–Vis spectral region enabling their detection with UV–Vis detectors. A traditional UV–Vis detector monitors only one or several wavelengths at the same time, whereas a diode array detector (DAD) simultaneously measures a range of wavelengths (e.g., 200– 500 nm), which enables the measurement of UV–Vis spectra of phenolic compounds.

The UV–Vis spectra of flavonoids are comprised of absorptions arising from the benzene rings: A and B and their possible conjugations to ring C. The UV–Vis spectra of different subclasses of flavonoids give tentative information about the structures of flavonoid aglycones[126] (Scheme V). All flavonoids have an absorption maximum at around 240–290 nm (Band II), which is mostly affected by the conjugation of ring A and its substitution pattern[126]. Some flavonoids have another absorbance maximum at around 300–550 nm (Band I), which is detected in flavonoids where rings B and C are conjugated (via a double bond between carbons 2 and 3 in ring C). This absorption maximum is at around 460–560 nm for anthocyanins and at 310–370 nm for flavones and flavonols[127]. The Band I maximum is generally at a longer wavelength in flavonols than in flavones, which can help to differentiate between these two types of flavonoids (Figure 4 A and B). In addition, chalcones and aurones have an elongated conjugation system from their ring B and, thus, these compounds show a second absorption maximum, which is at around 345–390 nm for chalcones and at 390–430 nm for aurones[128].

Flavan-3-ols, proanthocyanidins and dihydrochalcones show mainly one absorption maximum at around 270–290 nm (Band II), while flavanones and dihydroflavonols also have a small shoulder (Band I) at around 320 nm (Figure 4C and D)[127,128]. The absence or low absorption maximum of Band I is probably caused by the lack of conjugation between ring B and the rest of the molecule (no double bond beside the ring B). For most flavonoids, except for anthocyanins and some aurones, the above-mentioned Band I and II absorption maxima lie in the range of UV radiation (i.e. below 400 nm), so the term UV spectrum of flavonoids is often used instead of the term UV–vis spectrum(Scheme V).



Scheme V: Example UV spectra of flavonoids (acquired from UHPLC–DAD chromatograms with 0.1 % formic acid and acetone in the solvent mixture): a flavonol (kaempferol monohexoside) (A), a flavone (apigenin glucuronide) (B), a dihydroflavonol (dihydromyricetin) (C), a flavan-3-ol ((+)-catechin) (D).

The locations of Band I and II maxima are affected by the number of hydroxyl groups in rings A and B as well as by the glycosylation of the flavonoid. For example, the glycosylation of a hydroxyl group can cause a decrease in the Band I absorbance maximum[127]. However, the actual sugar molecule has no effect on the UV spectrum of flavonoid glycosides. Acyl substituents in glycosyls cause an additional maximum in the UV spectrum of flavonoid glycosides. For example, a

coumaroyl substituent (a conjugated system) produced an extra absorption maximum at around 310–320 nm in the UV spectrum of kaempferol and quercetin glycosides[129,1130]. When interpreting UV spectra from LC– DAD data, it should be noted that the UV absorption maxima of flavonoids might be slightly shifted depending on the solvent composition by which they were eluted from the LC column[130].

In addition to structure elucidation, UV–vis detectors are used in the quantitative analyses of flavonoids and other phenolics. The sensitivity of detection is improved by choosing a detection wavelength or wavelengths where the phenolics in question show maximum absorption[128]. UV–vis quantitation has good reproducibility, because the absorbance of a particular compound stays virtually unchanged between different analysis times. For the best accuracy, phenolic compounds should be quantified with standards that have the same or similar chemical structures as the quantified compounds. The use of unsuitable standards produces quantification errors[128] because these standards have different UV–vis absorption properties than the quantified compounds. Detection limits of phenolic compounds are often higher with UV–vis than with MS techniques, and the co-elution of phenolics hampers the quantitation of individual compounds[132].

Mass spectrometry

Similarly to UV–vis detectors, mass spectrometers have a crucial role in detecting and analyzing flavonoids and other phenolic compounds.

While UV–vis detectors only detect compounds with a UV–vis absorbing chromophore, mass spectrometers detect virtually all kinds of chemical compounds that are ionized in the used analytical conditions. These two instruments are often coupled in line, so that the eluent flow from LC first passes through a UV–vis detector, after which the eluent is directed to MS (LC– DAD–MS).

In MS, analyte molecules are first ionized, after which the ions are analyzed according to their mass-to-charge ratios (m/z)[133]. Mass spectrometers are divided into different groups according to their ionization techniques and mass analyzers. Ionization techniques used in flavonoid analyses include, for example, fast atom bombardment (FAB) and matrix assisted laser desorption/ionization (MALDI). In FAB, the flavonoid sample is dissolved in a liquid matrix that is bombarded with a beam consisting of fast atoms (e.g. Ar or Xe), which causes the desorption of ions from the sample matrix[131]. In MALDI, the sample is dissolved in a matrix that absorbs energy from laser pulses, which causes the formation of plasma that produces ions from the analyte molecules[131]. In addition to different ionization techniques, also several mass analyzers exist, such as, ion trap (IT) and Fourier transform ion cyclotron resonance (FT–ICR) analyzers and electrospray ionization (ESI) technique. ESI is used in combination with two different mass analyzers: triple quadrupole (TQ) and quadrupole time-of-flight (QTOF) analyzers.

ESI functions as an interface between LC and a mass spectrometer. In ESI, the eluent and analyte molecules from LC are sprayed through a

needle that is kept at a high potential or there is a potential difference between the needle and the inlet (nozzle) of the mass spectrometer. The applied potential causes the formation of charged droplets that move towards the inlet of the mass spectrometer[134]. The charged droplets shrink as the assisting drying gas flow helps to evaporate the solvent and the repulsion of positive or negative charges breaks down the droplets until ions are formed from the analyte molecules. The ionization process in ESI source functions at atmospheric pressure, while the inner parts of the mass spectrometer are kept at strong vacuum. Only gas phase ions move inside of the mass spectrometer, while solvent molecules evaporate in the ESI source, or they are prevented from entering the mass spectrometer by a gas flow (e.g., a curtain gas) at the nozzle of the mass spectrometer. Inside the mass spectrometer, the ions are transferred to a mass analyzer, where they are analyzed according to their m/z ratios.

In TQ analyzers, the ion spray from the ion source is guided through quadrupoles that are comprised of four rods that are placed in the corners of a square. The diagonally opposite rods are electrically connected to radio frequency and direct current sources, which causes an electric field between the rods[133]. An oscillating electric field is maintained between the rods by changing the radio frequency amplitude and direct current potential, which puts ions in a spiral path through the quadrupole[133,135]. The electric field is adjusted so that only ions with a particular m/z ratio pass through the quadrupole at a certain time, while other ions collide with the rods. A selected range

of m/z values can be scanned with the quadrupoles by gradually changing the parameters of the electric field. After the quadrupoles, the ions reach a detector, which converts the ion signals into electric current signals that are further converted into a mass spectrum by a computer system.

The TQ analyzers enable analyses with tandem mass spectrometry (MS/MS). In these studies, the first quadrupole is used to select or scan ions with the desired m/z ratios. The selected precursor ions move to the second quadrupole, i.e., the collision cell, where the ions are fragmented with the aid of a collision gas. The fragmented ions, i.e., the product ions, are formed by collision induced dissociation (CID) reactions, where part of the kinetic energy formed in the collision of precursor ions and collision gas is converted to internal energy of the precursor ions[135]. The excess internal energy breaks down the chemical bonds in the precursor ions and consequently the product ions are formed. The product ions are transferred to the third quadrupole where they are analyzed according to their m/z values. Several different types of MS/MS analyses can be performed with TQ analyzers. For example, in product ion analysis a precursor ion is first selected and fragmented, after which all the formed product ions are scanned at the third quadrupole. In selected/single reaction monitoring (SRM), a precursor ion or ions are selected at the first quadrupole and their specific product ions are scanned at the third quadrupole. If multiple product ions from one or more precursor ions are studied, a term multiple reaction monitoring (MRM) can be used[137].

Another way of conducting fragmentation in some instruments is by the so called in-source CID fragmentation[132]. This means that the newly formed ions are fragmented in the ESI source before they enter the high vacuum region inside the mass spectrometer. The kinetic energy of ions can be adjusted, for example, by the cone voltage of the system. The accelerated ions collide mostly with nitrogen gas that is present in the residual air of ionization chamber, or with the nebulizer or drying gas. Contrary to MS/MS, the use of in-source CID is unselective, because basically all ions present are susceptible to possible fragmentations.

TQ analyzers are sensitive and they are especially suitable for quantitative analyses because they have wide linear response ranges and rapid duty cycles (10–50 ms), which enable the detection of several data points for each chromatographic peak [132,134]. TQ analyzers produce low-resolution mass spectra, and the maximal m/z range is limited to 4000 or less[133,136]. A QTOF analyzer is a hybrid technique that combines a quadrupole analyzer (Q) with a time-of-flight analyzer (TOF). The ions first pass through the quadrupole, which resembles the first quadrupole in TQ. Next to the quadrupole is a collision cell that functions in MS/MS measurements in a similar way as in TQ. Sometimes, QTOF is denoted as QqTOF which takes into account the collision cell[135]. Finally, the ions move to the flight tube that is placed orthogonally to the quadrupole. The ions are accelerated with high-voltage pulses at the beginning of the flight tube after which the ions fly towards the detector (often via a reflector or

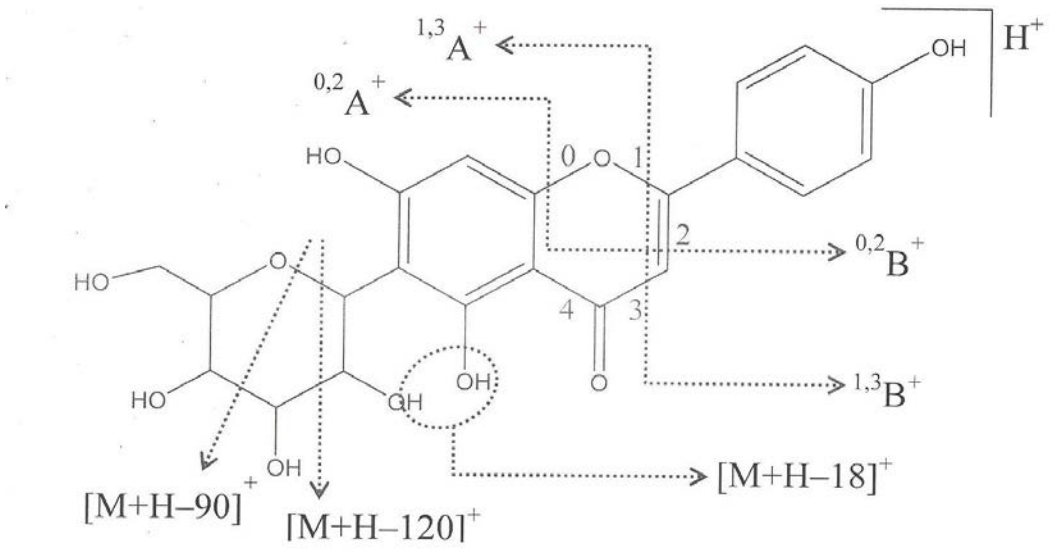
several reflectors). All ions with a similar charge have a similar kinetic energy at the beginning of the flight, but their velocities are dependent on their masses[133]. This causes ions with similar charges but different masses to reach the detector at slightly different times, which is used to determine the mass spectrum. The linear dynamic range of QTOF analyzers for quantitative analysis is lower than in TQ analyzers[135]. However, QTOF analyzers produce high-resolution mass spectra and, thus, can be used to measure the accurate masses of analyte ions and their fragments. The accurate masses are used to estimate the molecular composition of ions, which is important when the structures of unknown compounds are determined[136]. The high mass accuracy of QTOF is a combination of several factors[133]. First, the flight tube is placed orthogonally to the ion beam coming from the quadrupole region. This causes the diminution of longitudinal dispersion of ions (forward and backward) when the ions are accelerated in the TOF tube, which increases the resolution of ions. Secondly, the reflector equalizes the flight times of ions with the same m/z values but slightly different kinetic energies. This means that ions with higher kinetic energies will penetrate deeper into the electric field of the reflector, while similar ions with less kinetic energy will penetrate less in the reflector. Thus, ions with higher kinetic energy will have a slightly longer flying path than ions with lower kinetic energy, so that ions with the same mass reach the detector simultaneously, thereby enhancing mass accuracy. The reflector also

increases the flying path of ions enhancing the resolution of ions with different masses.

Both positive and negative ionization modes can be used in the analysis of flavonoids with ESI. The negative ionization often gives better sensitivity for flavonoids, probably because their ionization is enhanced by the deprotonation of the acidic hydroxyl groups[126,131]. However, the positive ionization mode can sometimes give more structural and fragmentation information about the flavonoids[138]. MS is used, first of all, to elucidate the molecular masses of flavonoids. This can be done by detecting the deprotonated or protonated molecular ions ($[M–H^+]$ or $[M+H]^+$) of the analytes. The ESI conditions may produce adducts both in the positive and negative ionization modes (e.g., a Na+ adduct [M+23]+ or a formic acid adduct $[M+46–H]^+$)[126,131]. In addition to molecular ions, also cluster ions (e.g., $[2M–H^+]$) can be formed[138,139], and they can be used in identifying the molecular masses of unknown compounds. Fragmentation of flavonoids in their first-order mass spectra gives further details about their structures. For example, the cleavage of O-glycosidic bonds between the flavonoid and the sugars can be used to elucidate the sugar residues. The cleavage of a hexose sugar is detected as an ion that has a 162 Da smaller m/z value than the molecular ion, whereas the cleavage of a pentose represents a corresponding ion with a decrease of 132 Da[138,139].

The first order mass spectrum of a flavonoid normally reveals only the molecular mass of the flavonoid aglycone and possible acidic or

glycosidic residues. More structural information can be gained with MS/MS analyses of flavonoids[140]. In a fragmentation reaction, two C–C bonds in the ring C can be cleaved, which cuts the flavonoid aglycone into two fragments: the first fragment includes atoms on the side of ring A (^{i,j}A+ or ^{i,j}A– ion), and the other ion represents atoms on the side of ring B (^{i,j}B+ or ^{i,j}B–, Figure 5). The masses of the fragments can be used to estimate how many hydroxyl groups or other substituents are attached to rings A and B[128,138,140]. Ions 1,3A+ and 1,3A– are especially significant MS/MS fragments of flavonoids (Figure 5)[138]. Further fragments deriving from the loss of small molecules or radicals (e.g., water, carbon monoxide, or methyl group) are also useful in structural elucidation of flavonoids[140]. MS/MS may also be used to determine the structures of acylated flavonoid glycosides, and the losses of acidic moieties are observed as neutral losses, such as –206 Da (sinapic acid) and –176 Da (ferulic acid)[141,142](see Scheme VI below).



Scheme VI : Examples of possible MS/MS fragmentation pathways of a flavonoid with a C-glycosyl unit

Flavonoids with C-glycosyls show only molecular ions without any further fragments in their first-order mass spectra. More structural information about flavonoids with C-glycosyls can be achieved with MS/MS. In addition to the ^{i,j}A and ^{i,j}B ions, fragment ions resulting from the loss of water molecules and the cross-ring cleavage of glycosyl units can be observed (Figure 5)[138,143].

NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy has an important role in the structural elucidation of flavonoids and other phenolic compounds. In NMR spectroscopy, a sample is placed in a homogenous magnetic field after which radio frequency pulses are directed to the sample. Different NMR active nuclei have characteristic frequencies at which they absorb energy from these pulses, which is used to measure an NMR spectrum. NMR spectra provide chemical shifts and coupling constants of NMR active nuclei (most often ^{1}H and ^{13}C). Chemical shifts give information about the chemical environment and neighboring groups and atoms of the NMR active nuclei, while coupling constants show how these nuclei are coupled to each other. Thus, NMR spectra provide information on how individual atoms are spatially situated in the molecule, which can be used to elucidate the chemical structures of unknown compounds. There are a variety of NMR experiments that all give different kinds of structural information. Standard ^{1}H NMR experiment provides the

relative number of hydrogen atoms (protons), their chemical shifts and spin-spin couplings to neighbouring hydrogens[131]. Similarly to ^{1}H NMR, a ^{13}C NMR spectrum provides the chemical shifts of carbon atoms and the relative number of carbon atoms in the molecule.

In the case of flavonoid glycosides, ^{1}H NMR spectra also show the signal (or signals) of an anomeric sugar proton whose coupling constant to the neighboring sugar proton determines whether the glycosyl is in α- or β-anomeric configuration[144]. Further, the chemical shifts and coupling constants of anomeric and other protons help to determine the structure of the glycosyl unit. This is important, since the MS analyses of flavonoid glycosides are unable to differentiate between two different glycosyls that have same molecular masses (e.g. glucose and galactose). The position of an acyl group attached to a sugar unit can sometimes be deduced from the ^{1}H NMR spectrum, as the chemical shift of the sugar proton next to the acyl group often shows a marked downfield shift[144].

Two dimensional NMR experiments show how different atoms are bonded or spatially situated in relation to each other. For example, correlation spectroscopy (COSY) is used to determine which hydrogen atoms are coupled to each other. Heteronuclear single quantum correlation (HSQC) experiment determines which two nuclei of different atoms (most often ^{1}H and ^{13}C) are directly bonded to each other. Heteronuclear multiple bond correlation (HMBC) spectra show correlations between two different atoms (for example ^{1}H and ^{13}C) that usually are separated by 2–4 bonds. In flavonoid research, HMBC

is especially useful in elucidating the attachment sites of glycosyl and acyl groups, which cannot be determined by the first-order mass spectra of flavonoids[131,145,146,147]. Total correlation spectroscopy (TOCSY) experiments are useful in the assignment of glycosyl protons, especially in flavonoid oligoglycosides where the proton signals of different glycosyls are often overlapping[131]. The positions of glycosyl units and other structural details of flavonoids can also be determined with $^{1}H–^{1}H$ experiments with nuclear overhauser enhancement spectroscopy (NOESY) and rotating frame overhauser effect spectroscopy (ROESY) that show protons that are spatially close to each other in the molecule[145,147]. An advantage of NMR is that it keeps analyte compounds intact for their possible further use, whereas in MS the analyte compounds are degraded. However, a rather high amount of an analyte (often a few milligrams) is needed for NMR experiments, and the NMR spectra of mixtures can be difficult to interpret[131]. Eluent flow from LC can be coupled to NMR (LC–NMR). This technique could be used to analyze mixtures of phenolic compounds without the time consuming purification of individual compounds, but the instrumentation and the use of deuterated solvents can be too expensive for routine use[148]. As noted above, the need for rather large sample sizes in NMR is another limiting factor for the use of LC–NMR as compared to LC–MS.

Aim of this study

This study was designed to:

- Extract flavonoids from the Sudanese material of *Albizia amara*.
- Isolation of pure flavonoids via different chromatographic techniques.
- Elucidation of isolates structures using a combination of spectral techniques.
- Evaluation of antimicrobial potential of isolates via well diffusion assay.

CHAPTER TWO

MATERIALS AND METHOD

2. Materials and Methods

2.1- Materials

2.1.1- Plant material

The leaves of *A. amara* were collected in April 2013 and June 2015 from Maleet (western Sudan) . The plant was identified and kindly authenticated by the Dept. of Botany , University of Khartoum.

2.1.2- Solvents

Analytical grade solvents were used. Methanol HPLC grade is used for spectroscopic purposes (BDH , England).

2.1.3- Chromatographic materials

Thin layer chromatographic plates

(TLC Pre-coated silica gel 60 F254 Merck and pre-coated RP-18 Merck, Germany). Different solvent systems were used in different ratios such as: petroleum ether-ethyl acetate, chloroform-acetone, dichloromethane -methanol, hexane-ethyl acetate, hexane-acetone, dichloromethane-acetone.

Sheets of Whatman paper (No.1 and No.3 mm-46x57cm) from Whatman Ltd. Kent, England.

Glass jars, 10x20x24 cm, fitted with covers, were used for TLC (Camag).

2.1.4- Equipments

The ultraviolet lamp used in visualizing TLC plates and paper chromatography was a multiband UV λmax (254 / 365 nm) portable ultaviolet, a product of Hanovia lamps (6 watt S/Y and L/W).

Ultraviolet spectral analysis

Ultraviolet absorption spectra were obtained in spectroscopic methanol on UV - Visible Spectrophotometer(Shimadzu).

Infrared spectral analysis

Infrared spectra were generally obtained in potassium bromide (KBr) discs using Perkin-Elmer, FTIR, model 1600-Jasco.

Mass spectrophotometer analysis

The electron impact ionization (EIMS) mass spectra were obtained on a solid probe using Shimadzu QP-class-500.

Nuclear Magnetic Resonance (NMR)

^{1}H spectra were obtained on a Bruker AM 500 spectrophotometer (Germany) operating at 500 MHz in spectroscopic grade DMSO-d_6. The chemical shifts values are expressed in δ (ppm) units using (TMS) as an internal standard and the coupling constants (J) are expressed in Hertz (Hz).

2.2- Methods

2.2.1- Preparation of reagents for phytochemical screening

Flavonoids test reagents

a- Aluminium chloride solution

(1g) of $AlCl_3$ was dissolved in 100 ml methanol.

b- Potassium hydroxide solution

(1g) of KOH was dissolved in 100 ml water.

c- Ferric chloride solution

(1g) of $FeCl_3$ was dissolved in 100 ml methanol.

Alkaloids test reagents

Mayer's reagent

(1.358 g) of $HgCl_2$ was dissolved in 60 ml of water and poured into a solution of (5 g) of KI in (10 ml) of H_2O,then sufficient water was added to 100 ml.

Wagner's reagent

(2 g) of iodine and (6 g) of KI were dissolved in 100 ml of water.

Glycosides test reagents

Molisch reagent

(2 g) α-naphthol dissolved in 20 ml EtOH 96%

2.2.2- Shift Reagents

The diagnostic reagents used for the UV spectral measurements of the isolated flavonoids were prepared as follows:

Sodium methoxide (NaOMe)

Freshly cut metallic sodium(2.5 gm.) was dissolved in100 ml spectroscopic methanol.

Aluminum chloride

Anhydrous $AlCl_3$(5 gm) was cautiously dissolved in100 ml spectroscopic methanol and filtration was carried out after about 24 hours.

Hydrochloric acid

Fifty ml. concentrated HC1 were mixed with 100 ml. distilled water.

Sodium acetate

(NaOAc) Anhydrous sodium acetate was melted and allowed to stand for about 10 minutes. The material was then powdered and stored in a dry bottle.

Boric acid

Anhydrous powdered reagent grade H_3BO_3 was used

2.2.3- Stepwise procedure for use of shift reagents for UV

- The UV spectrum of the compound in methanol was first recorded.
- 3 drops of NaOMe reagent were added to the sample and the NaOMe spectrum was recorded, and after 8 minutes the NaOMe spectrum was re-recorded.
- 6 drops of $AlCl_3$ reagent were added to the fresh sample and the $AlCl_3$ spectrum was recorded, 3 drops of HCl were added and after mixing , the $AlCl_3$/ HCl spectrum was recorded.
- Powdered NaOAC was then added to the fresh sample, the mixture was shaked and the NaOAC spectrum was recorded. NaOAC/ H_3BO_3 spectrum was then recorded after adding H_3BO_3.

2.2.4- Preparation of plant extract for phytochemical screening

(100g) of powdered shade-dried leaves of *A. Amara* were extracted with 80% methanol (soxhlet) for 6 hours. The cooled solution was filtered and evaporated to dryness. This prepared extract(PE) was subjected to preliminary

phytochemical screening for the presence of flavonoids, saponins, tannins, alkaloids, carbohydrates and/or glycoside, and triterpenes and/or sterols.

-Test for saponins (Froth test)

The dried extract was dissolved in water, transferred into a test tube and shakend vigorously, then it was left to stand for 10 minutes, when a thick persistent froth appears and persists for one hour saponins are present.

-Test for alkaloids

(50 mg) of extract was dissolved in (50 ml) of methanol in a water bath for 20 minutes, the extract was then filtered off and allowed to cool. Two ml of the extract was poured into test tubes. Dragendorff's or Mayer's reagent was added to the tube and the presence or absence of colours or any precipitates was noted.

- Test for tannins

(50 mg) of extract was dissolved in (50 ml) of water .The aqueous extract was then treated with a 15% ferric chloride solution. A blue color indicates condensed tannins, a green color indicates hydrolysable tannins.

- Test for glycosides (Molisch's test)

(1 ml) of the extract solution was pipetted into a test tube, 3 drops of Molisch reagent were added . After mixing, (1ml) of concentrated sulfuric acid was added to the wall of test tube. A positive test for carbohydrates is indicated by a violet ring forming at the interface between the denser sulfuric acid and the less dense test solution above

-Test for sterols and triterpenes (Liebermann-Burchard test)

(1 ml) of glacial acetic acid was added to (1 ml)chloroform and cooled to 0°C, then one drop of concentrated sulphuric acid was added to the cooled mixture followed by the extract.

- Test for flavonoids

(50 mg) of extract was defatted with petroleum ether and the residue was dissolved in(30ml) 80% methanol and filtered. The filtrate was used for the following tests:

(1) To (30 ml) of the filtrate few drops of methanolic aluminium chloride were added . A dark yellow colour was observed .

(2) To (30 ml) of the filtrate few drops of potassium hydroxide solution were added . A dark yellow colour was observed .

(3) To (30 ml) of the filtrate few drops of ferric chloride solution were added . A blue coloration was observed .

2.2.5- Extraction of flavonoids

Powdered shade- dried leaves (1kg) of *A. amara*. were extracted with 95% ethanol at room temperature for 48 hours, The extract was filtered and evaporated to dryness under reduced pressure. Then it was suspended in water and partitioned with organic solvents in order of increasing polarity, petroleum ether, chloroform ethyl acetate and n-butanol .

2.2.6-Paper chromatography(PC)

The n-butanol fraction was rich in phenolics. It was dissolved in methanol and applied to the Whatman paper (No. 3 mm – 46x 57cm). The bands were irrigated with BAW (n-butanol acetic acid-water, 4:1:5). The developed chromatograms were air-dried and examined under both visible and UV light (ʎ 366,245nm). The chromatograms were exposed to ammonia vapour

for about 2-3 seconds and immediately re-examined to observe possible changes that may eventually appear in colour or fluorescence under along wavelength UV lamp. The equivalent bands from each PC were then cut out, combined, cut into small strips and slurred with methanol. After several hours of contact occasional shaking, the liquid was filtered and evaporated to dryness. In this way compound I(R_f 0.89) , compound II(R_f 0.61) and compound III(R_f 0.33) were isolated in chromatographically pure forms.

2.2.7- Antimicrobial assay

The isolated flavonoids were screened for their antimicrobial activity against four human pathogenic bacterial strains: Gram-positive (*Staphylococus aureus* and *Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa* and

Escherichia coli) and fungal species **(***Aspergillus niger*, *Candida albicans*). The cup plate agar diffusion assay was used.

2.2.7.1- Preparation of bacterial suspensions

One ml aliquots of a 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37º C for 24 hours. The bacterial growth was harvested and washed off with 100 ml sterile normal saline solution to produce a suspension containing about 10^8- 10^9 C.F.U/ ml. The suspension was stored in the refrigerator at 4° C till used. The average number of viable organisms per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micro pipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drops to dry and then incubated at 37 °C for 24 hours. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and by the dilution factor to give the viable count of the stock suspension, expressed as the number of colony forming units per ml suspension.

Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.2.7.2- Preparation of fungal suspension

The fungal cultures were maintained on dextrose agar, incubated at 25 °C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100ml of sterile normal saline and the suspension was stored in the refrigerator until used.

2.2.7.3- Testing of antibacterial susceptibility

The cup plate agar diffusion assay was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA). The experiment was carried out according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS, 1999). Bacterial suspension was diluted with sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial suspension were swabbed uniformly on surface of MHA and the inoculum was allowed to dry for 5 minutes. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of the MHA and soaked with 20 µl of a solution of each plant extract.The inoculated plates were incubated at 37 °C for 24 h in the inverted position. The diameters (mm) of the inhibition zones were measured in duplicates and averaged.

2.2.7.4-Testing of antifungal susceptibility

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

CHAPTER THREE

RESULTS AND DISCUSSION

3-Results and Discussions

3.1-Phytochemical sceening

The preliminary phytochemical screening of methanolic extract of *A.amara* leaves revealed the presence of saponins, alkaloids, tannins , glycosides and flavonoids Table(2).

Table 2: Phytochemical screening of the methanolic extract of of *A. Amara* leaves

Class of compounds	Reagent used	Methanolic extract
Saponins	Froth test	+
Alkaloids	Mayer's reagent	+
Tannins	Ferric chloride test solution	+
Glycosides	Molisch's test	+
Sterols and triterpenes	Liebermann-Burchard test	-
Flavonoids	(1) methanolic aluminium chloride (2) potassium hydroxide solution (3) ferric chloride solution	+ + +

The crude extract was then purified by paper chromatography. After the usual workup compounds: I , II and III were isolated.

3.2- Characteization of compound I

The IR (KBr) spectrum of compound I (Fig 1) showed *v*(KBr) :575.04, 781.12 (C-H, bending Ar.), 10513.13-1107.06 (C-O, ether),

1600(C=O), 1730.03(α, β-unsaturated carbonyl group) and 3411.84 cm^{-1}(OH).

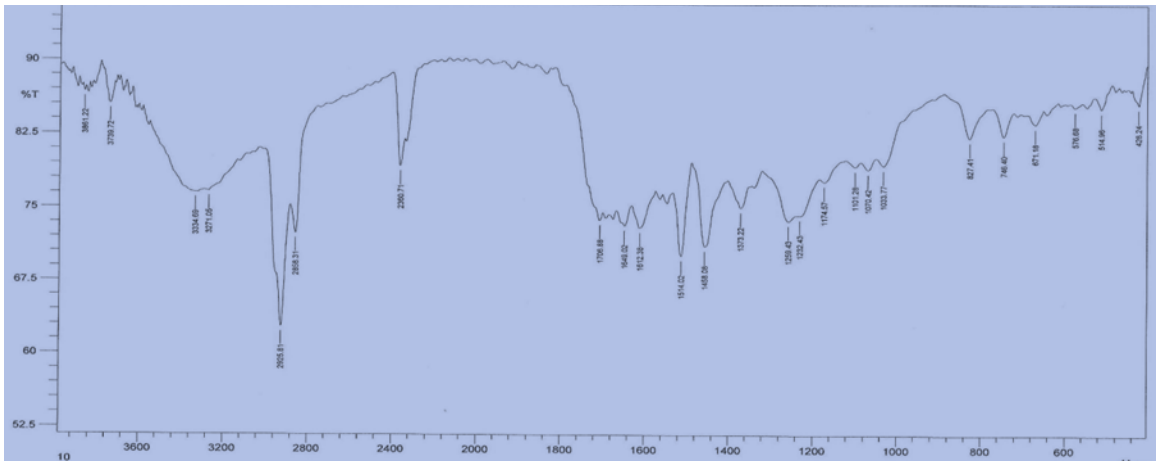


Fig.1: IR spectrum of compound I

In UV , compound I absorbs at λ_{max} (MeOH) 278nm(Fig.2).Such absorption indicates absence of conjugation between the 4 keto function and the B aromatic ring of the flavonoid nucleus.Compounds which reveal such absorption belong to one of the following classes: isoflavone, flavanone, dihydrochalcone or dihydroflavonol. Other classes , namely, flavones, flavonols , chalcones and aurones afford two peaks originating from two chromophores; the benzoyl system and the cinnamoyl system. These two peaks are commonly referred to as band I (usually 300 – 400nm) and band II (usually 240 – 280 nm)[11]

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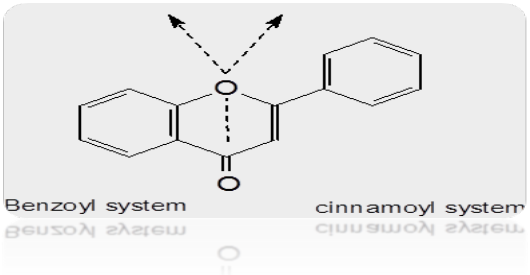


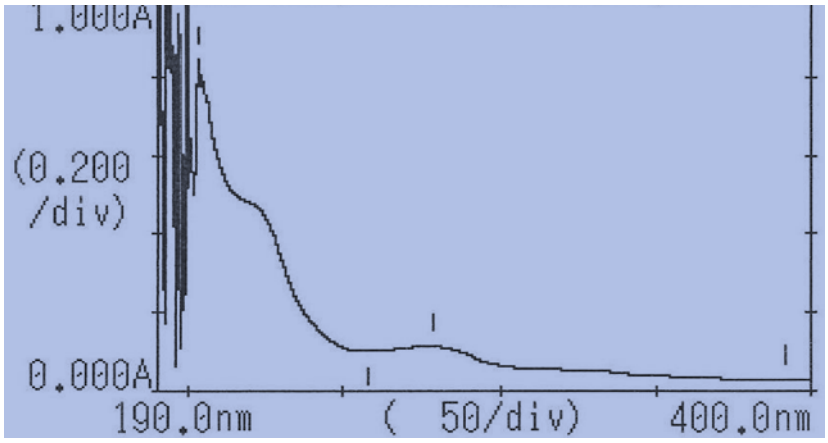
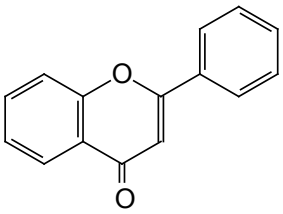
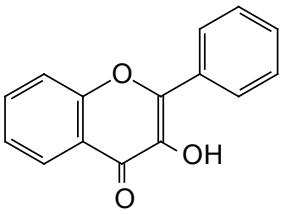


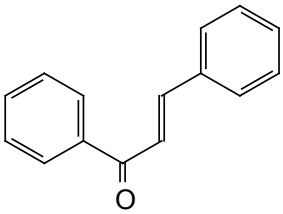
Fig.2 : UV spectrum of compound I



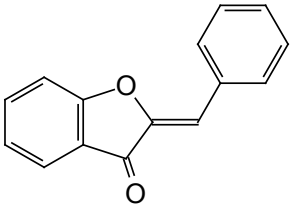
Flavone



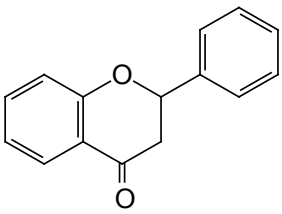
Flavonol



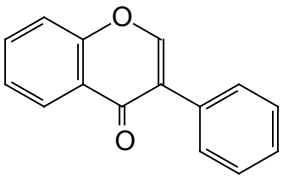
Chalcone



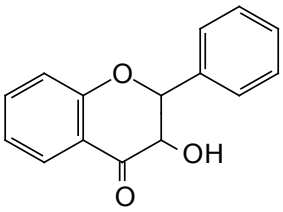
Aurone



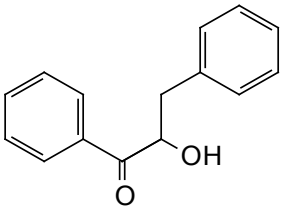
Flavanone



Isoflavone



Dihydroflavonol



Dihydrochalcone

The classes of flavonoids known as :Isoflavones, flavanones and dihydroflavonols all give only band II due to lack of conjugation between the carbonyl function and the aromatic B ring The UV absorption of some classes of flavonoids is depicted in Table 3.

Table 3: The UV absorption of flavones, flavonols , chalcones and aurone.

Flavonoid class	Band I	Band II
Favone	330-350	250-270
Flavonol	350-390	250-280
Chalcone	365-390	240-260
Aurone	390-430	240-270

Very significant structural features have been obtained by utilizing the so called UV shift reagents which produce shifts in the UV absorption maxima in accordance with the location of the various hydroxyl functions in flavonoid nucleus.These shift reagents are : sodium methoxide(strong base which is diagnostic of 3- and 4`-OH functions);sodium acetate (a weak base , diagnostic of 7-OH function); aluminium chloride (chelating agent , diagnostic of 3- and

5-OH and caatechol systems) and boric acid (diagnostic of catechol systems).

Figure 2 did not reveal any shoulder characteristic of isoflavones[111] in the range : 300-340nm. Also the absence of a 3-OH function as revealed by sodium methoxide spectrum(Fig.3) suggests absence of dihydroflavonol. Consequently the isolated flavonoid is probably a flavanone.

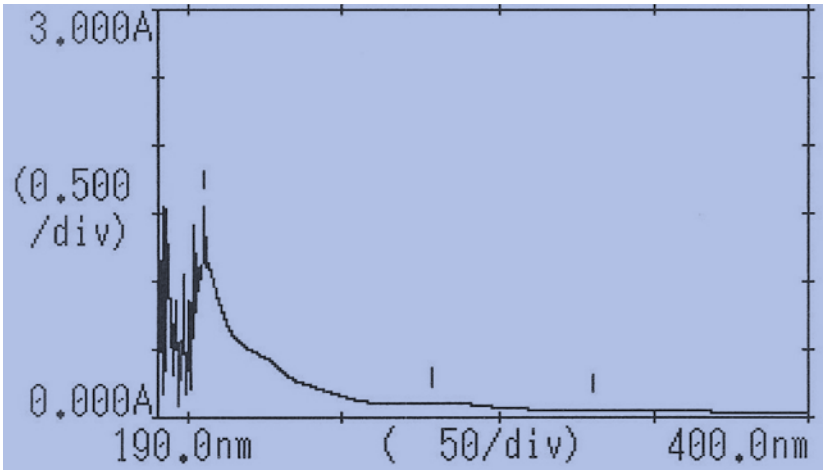


Fig.3 : Sodium methoxide spectrum of compound I

Such flavanone lacks 3-, 5-hydroxyls and catechol systems as evidenced by absence of bathochromic shift in aluminium chloride spectrum(Fig4) ; 4`-hydroxylation as indicated by sodium methoxide spectrum(Fig.3) and 7-hydroxylation as suggested by sodium acetate spectrum(Fig.5) which did not show any detectable bathochromic shift

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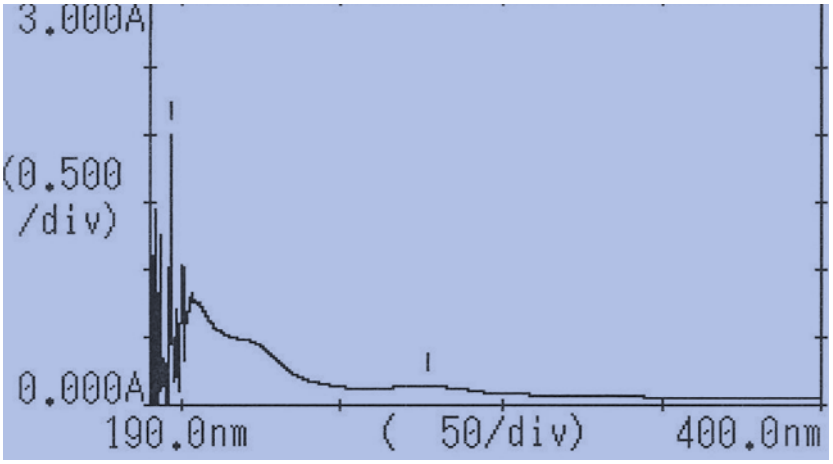


Fig.4 : Aluminium chloride spectrum of compound I

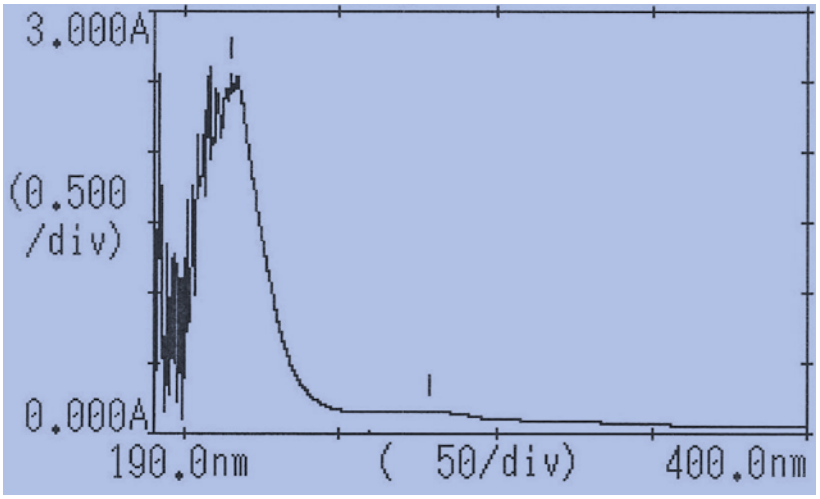


Fig.5 : Sodium acetate spectrum of compound I

The ^{1}HNMR spectrum(Fig.6) and as anticipated revealed a double multiplets centered at δ2.80ppm and 5.20 ppm characteristic of flavanones.These multipletes are due to mutual spin-spin splitting of the magnetically unequivalent C_2 – protons , which undergo further splitting with the C_2 proton. The signals at δ0.80 , 1.23 and 1.45ppm were assigned to three methyl groups , while the resonance at δ 3.39 ppm accounts for a methoxyl function.The aromatic prons appeared at δ 6.90 and δ7.39ppm. The mass spectrum(Fig.7) gave m/z312 for M^{+} (aglycone).Peaks > 312 seems to originate from glycoside.

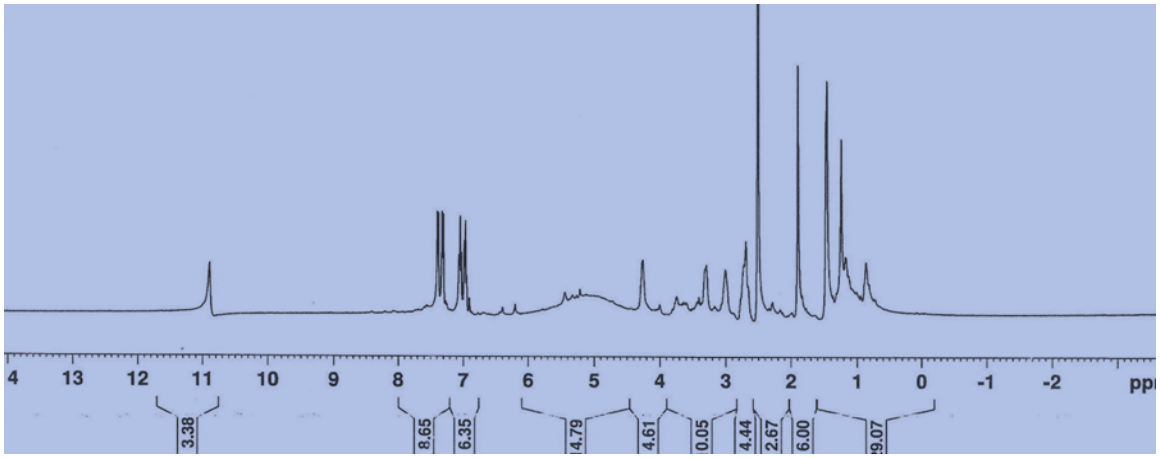


Fig. 6 :^{1}HNMR spectrum of compound I

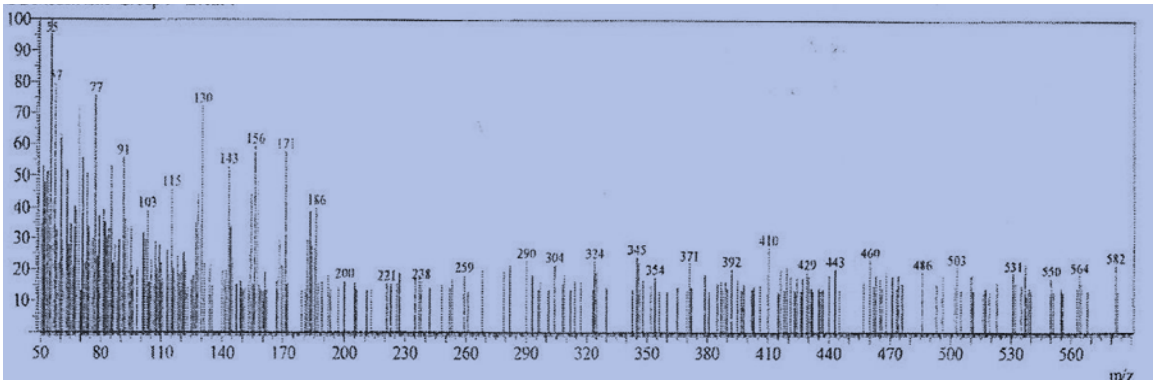
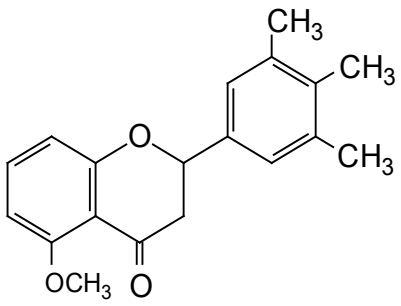


Fig. 7: Mass spectrum of compound I

The substitution pattern of A ring was deduced from (i) no downfield signal (around 8.00 ppm) was observed for C_5 proton and (ii) the NMR spectrum revealed a 3 proton signal for A ring protons at δ7.00ppm.The appearance of a B ring signal for meta-coupled protons at δ7.50ppm provide evidence for the following proposed structure for compound I:



Compound I

3.3-Characterization of compound II

Compound II was isolated as yellow amorphous powder from *Albizia amara* leaves. The structure of this flavonoid was deduced on the basis of its spectral data (IR,UV,HNMR and MS).

The IR (KBr) spectrum of compound I (Fig.8) showed *v* 615,744 (C-H, bending Ar.), 1024 (C-O, ether),1456-1558(C=C, Ar.), 1650(C=O),2923(C-H,alkanes) and 3382 cm^{-1}(OH).

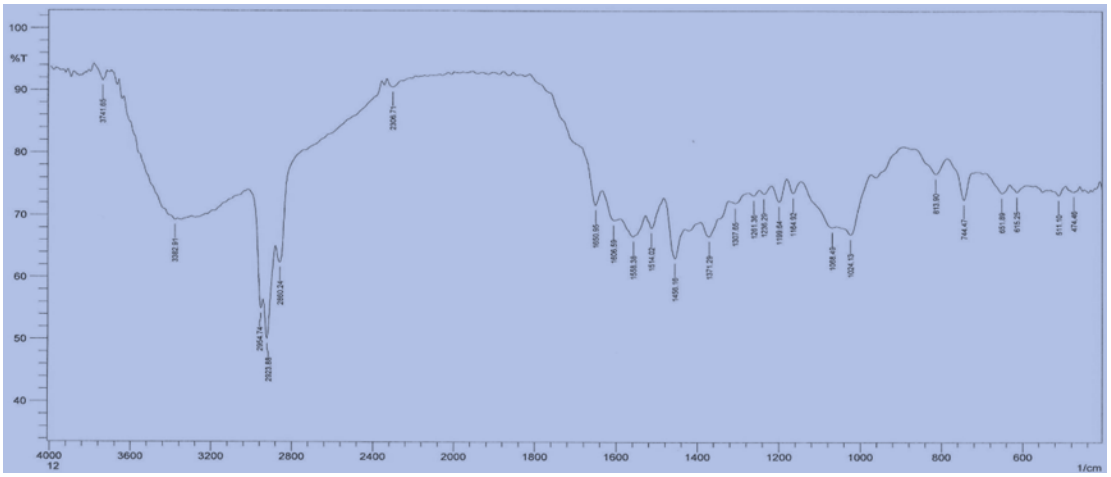


Fig. 8: IR spectrum of compound II

In UV , compound II absorbs at λ_{max} (MeOH) 267nm(Fig.9). No shoulder characteristic of isoflavones appeared in the range :300-340nm.

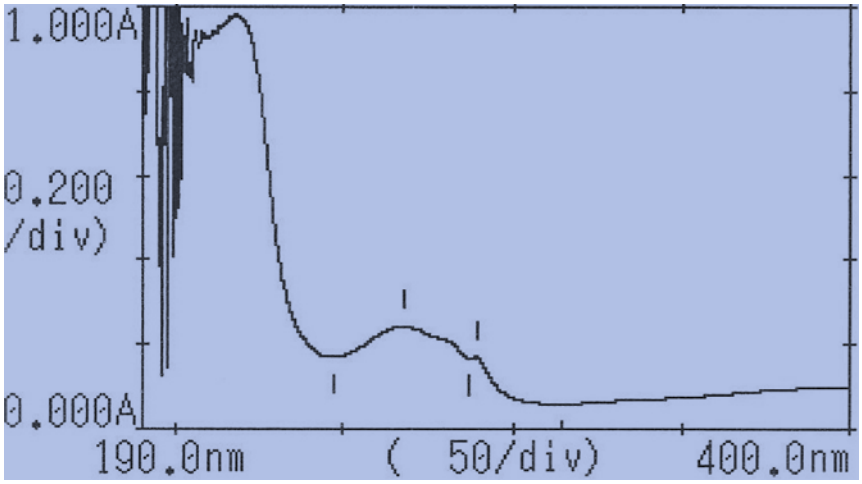


Fig.9: UV spectrum of compound II

The sodium methoxide spectrum (Fig.10) revealed a bathochromic shift with decrease in intensity diagnostic of a 3-OH function.Thus compound II is probably a dihydroflavonol.

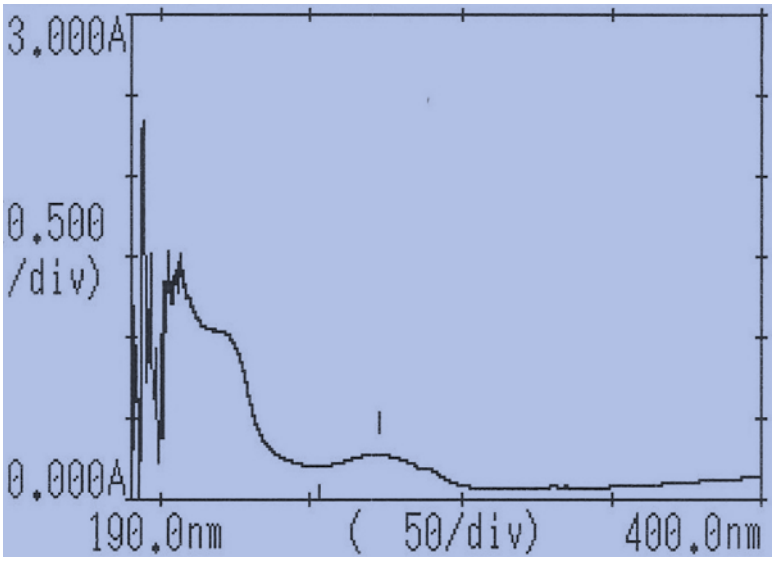


Fig.10 : Sodium methoxide spectrum of compound I

No detectable bathochromic shifts were observed in the sodium acetate,aluminium chloride and boric acid spectra(Figures:11-13).

This clearly indicates absence of 5-,7-OH functions as well as catechol systems.

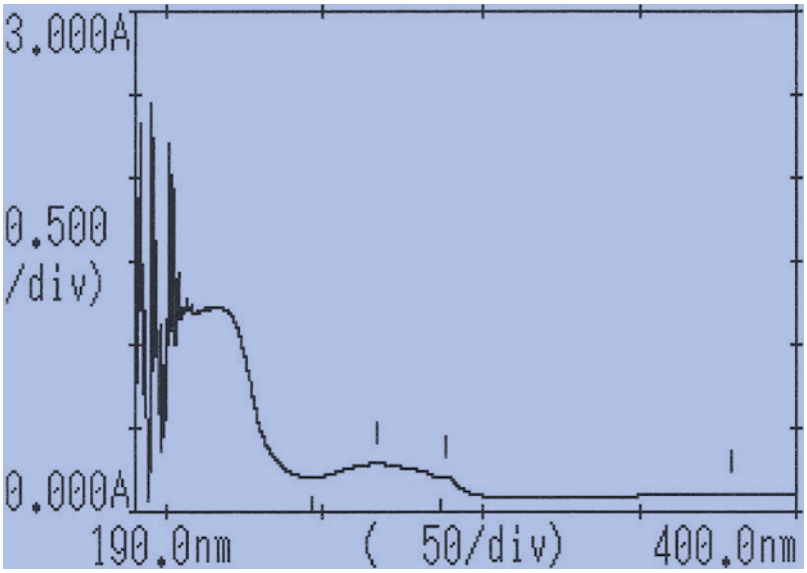


Fig.11: Sodium acetate spectrum of compound II

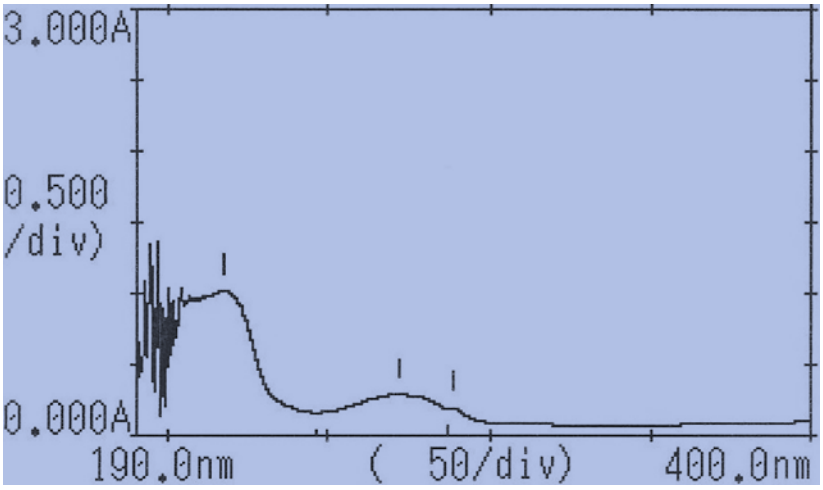


Fig.12: Aluminium chloride spectrum of compound II

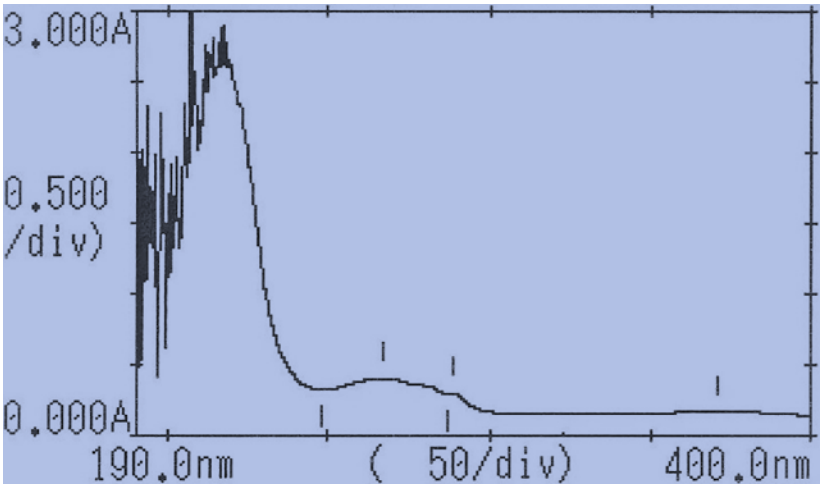


Fig.13: Boric acid spectrum of compound II

The ^{1}HNMR spectrum(Fig.14) showed two singlets at δ0.85 and δ1.2.5 each integrating for 3 protons assigned for two methyl groups. The C_2 proton resonates at δ3.00 due to the deshielding influence of the C_1 oxygen atom..The signal at δ 1.90(3H) was attributed to an acetyl function, while the resonance at δ3.80 (3H)accounts for a methoxyl function. The multiplet at δ3.30-3.70ppm accounts for sugar protons. The signals at δ6.1091H) and δ6.30(1H) were assigned for C_6 and C_8 protons respectively The latter usually resonates at lower field relative to the former due to the deshielding effect of the neighboring oxygen at C_1 .The aromatic protons appeared as multiplet at δ 6.95-7.80ppm. The mass spectrum(Fig.15) gave m/z340 for M^+ (aglycone).Signals > m/z340 are associated with the glycoside which rarely afford discernable molecular ion[111] .

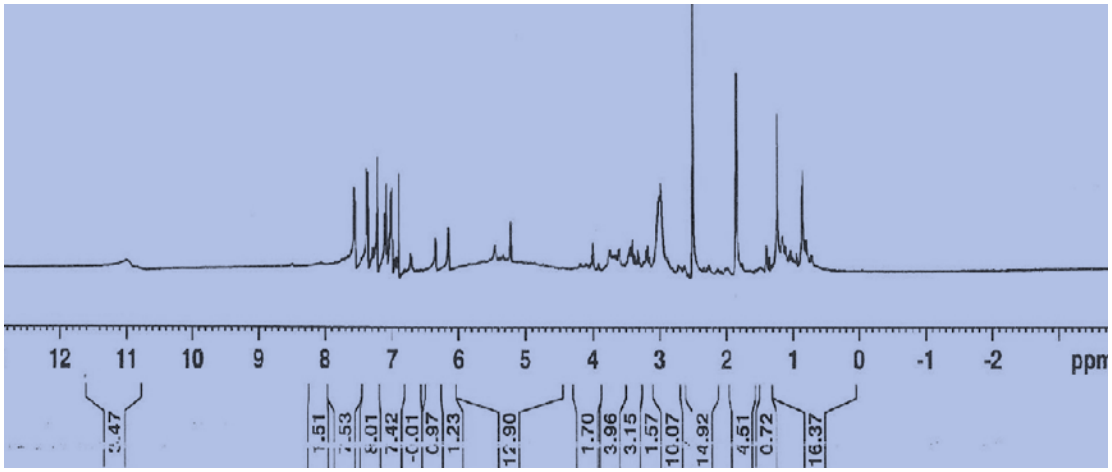


Fig. 14 : ^{1}HNMR spectrum of compound II

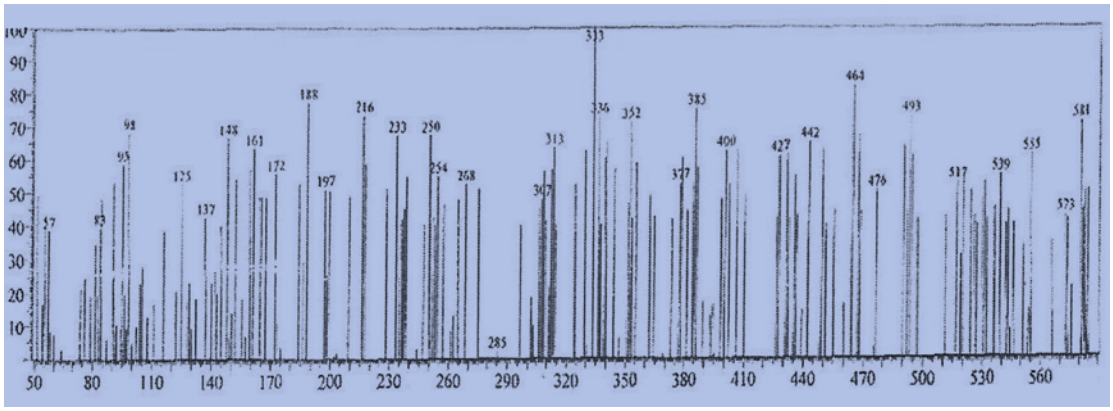
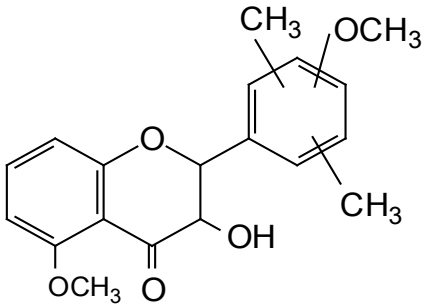
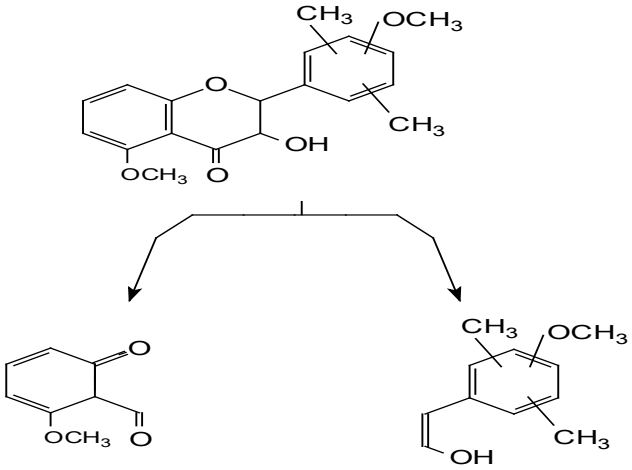


Fig.15: Mass spectrum of compound II

The substitution pattern of A ring was deduced from (i) no downfield signal (around 8.00 ppm) was observed for C_5 proton and (ii) the NMR spectrum revealed a 3 proton signal for A ring protons and (iii) the retro Diels-Alder fission (Scheme I) gave m/z 148 for intact A ring.The assignment of two methyl and an acetyl function for B ring was based on the retro Diels-Alder cleavage which revealed a signal at m/z190 for intact B ring. Comparison of the above cumulative data with data published on literature resulted in the following partial structure for compound II:



Compound II



m/z148 m/z190

Scheme I : Retro Diels –Alder fission of compound II

3.4-Characterization of compound III

Compound III was isolated as pale yellow powder from *Albizia amara* leaves structure of this isolate was elucidated on the basis of its spectral data (IR,UV,HNMR and MS).

The IR (KBr) spectrum of compound III (Fig 16) showed *v*575.04, 781.12 (C-H, bending Ar.), 10513.13-1107.06 (C-O, ether), 1600(C=O), 1730.03(α, β-unsaturated carbonyl group) and 3411.84 cm^{-1}(OH).

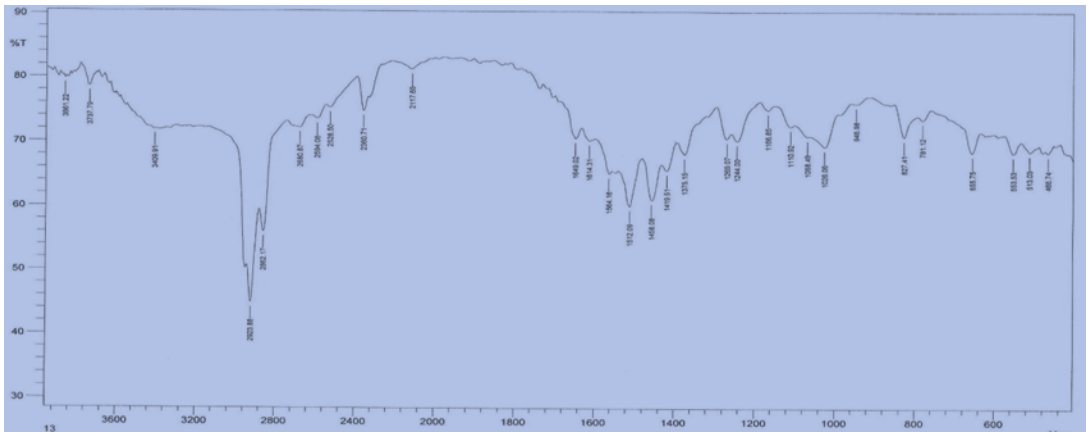


Fig.16 : IR spectrum of compound III

The UV spectrum of compound III gave λ_{max} (MeOH) 221,278nm(Fig.17). No shoulder characteristic of isoflavones was detected in the range :300-340nm.The sodium methoxide spectrum (Fig.18) did not reveal any bathochromic shift diagnostic of a 3- , 4`-OH functions.The 3-OH is a characteristic feature of dihydroflavonols.

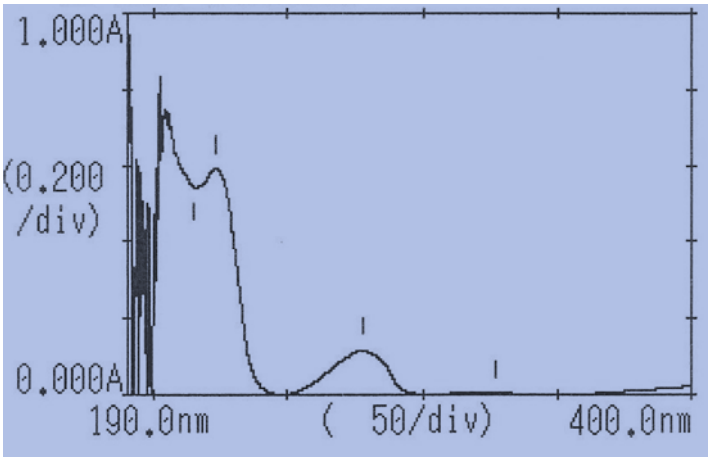


Fig.17 : UV spectrum of compound III

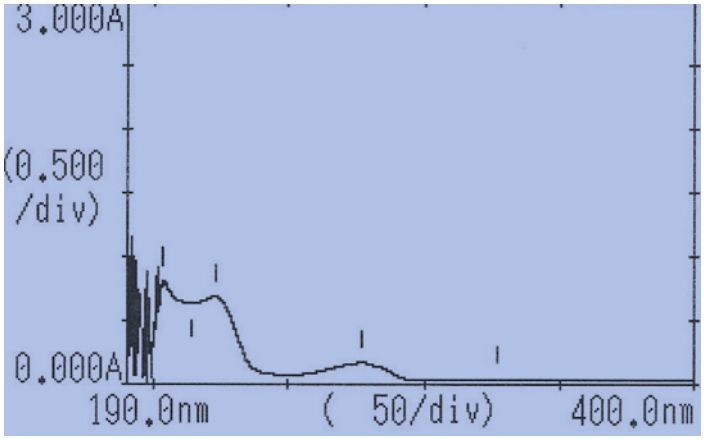


Fig.18 : Sodium methoxide spectrum of compound III

Also the ^{1}HNMR spectrum(Fig.22) did not show a double multiplet at δ2.80 and δ5.20ppm characteristic of flavanones. Thus compound III is probably a dihydrochalcone.

No detectable bathochromic shifts were observed in the sodium acetate(Fig.19),aluminium chloride(Fig.20) and boric acid spectra(Fig.21). This clearly indicates absence of 3-, 5-,7-OH functions as well as catechol systems.

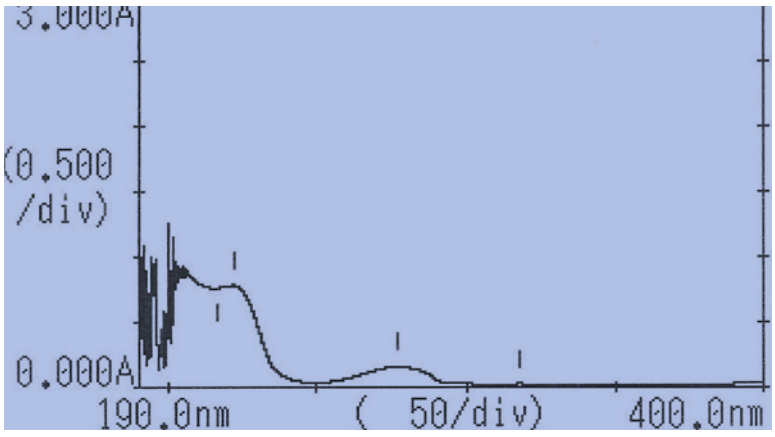


Fig.19 : Sodium acetate spectrum of compound III

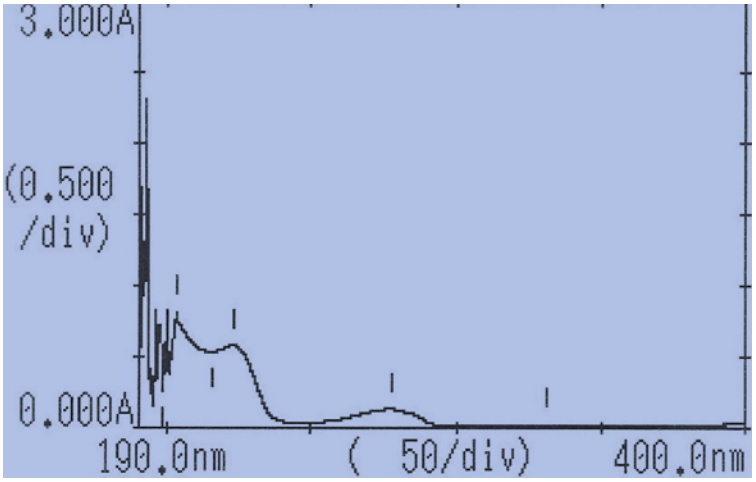


Fig.20 : Aluminium chloride spectrum of compound III

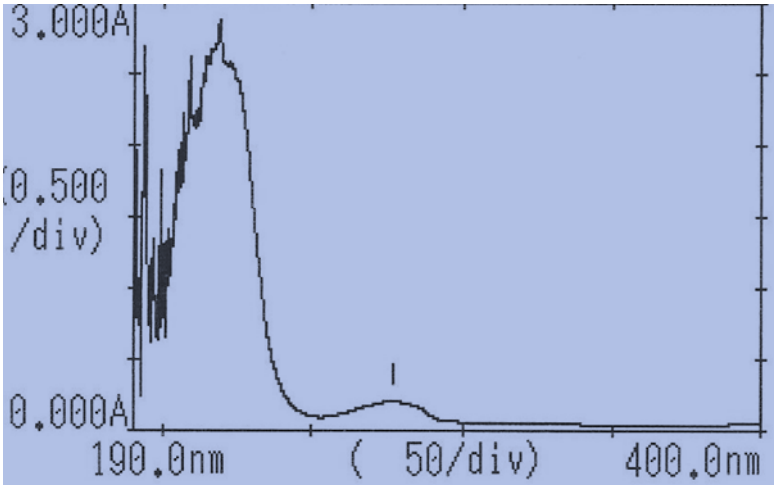


Fig.21 : Boric acid spectrum of compound III

The ^{1}HNMR spectrum(Fig.22) showed a singlets at δ01.23 integrating for 3 protons assigned for a methyl group. The resonances at δ2.74(2H) and δ2.92(2H)ppm account for the α- and β-protons of the dihydrochalcone moiety.The multiplet centered at δ3.50 accounts for sugar protons, while the resonances at δ6.73, δ7.04 were assigned for the aromatic protons.

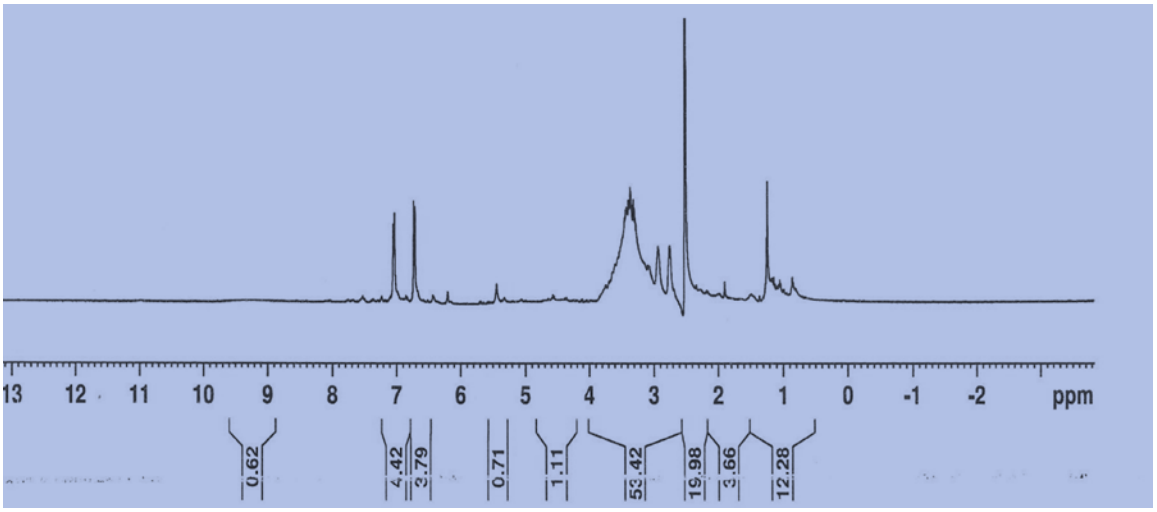


Fig.22 :[1]HNMR spectrum of compound III

The mass spectrum(Fig.23) gave m/z254 for M^+ (aglycone). The assignment of the methyl function for A ring was based on the retro Diels-Alder cleavage(Scheme II) which revealed a signal at m/z120 for intact A ring.

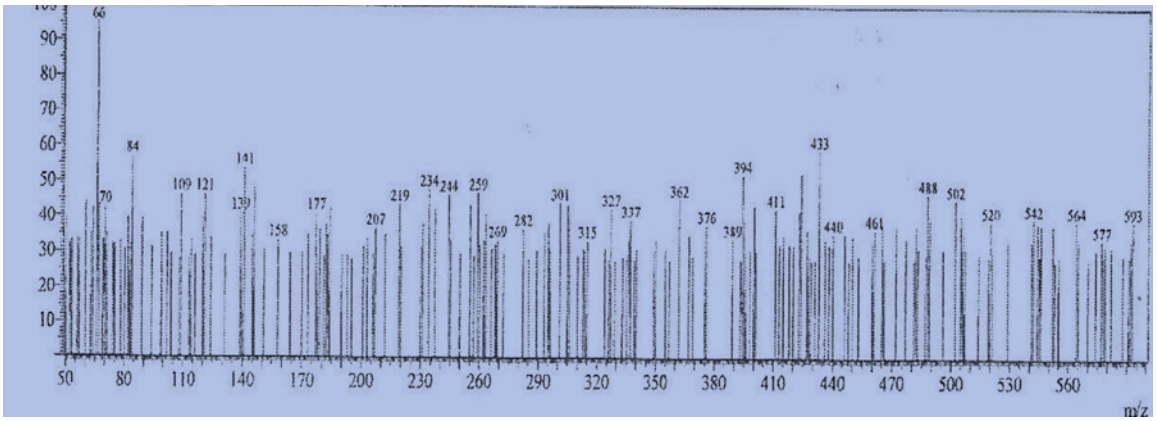
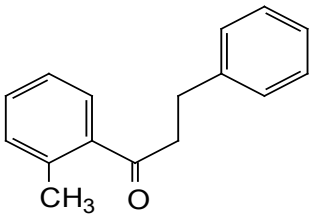


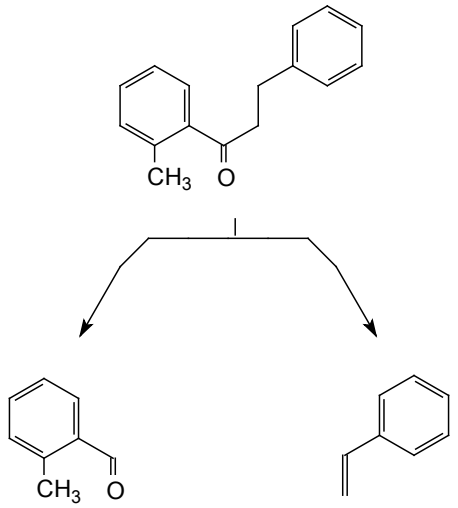
Fig.23 : Mass spectrum of compound III

The methyl function was assigned positon 5 due to the absence of a downfield signal (δ8.00) characteristic of C_5 –proton. Another important fragment resulting from intact B ring appeared at m/z104(Scheme II). Comparison of the above cumulative data with

data published on literature resulted in the following structure for compound III:



Compound III



m/z120 m/z104

Scheme II : Retro Diels-Alder fission of compound III

3.5-Antibacterial activity

In cup plate agar diffusion assay ,the isolates were screened for antimicrobial activity against six standard human pathogens. The average of the diameters of the growth of inhibition zones are depicted in Table (4) .The results were interpreted in commonly used terms (<9mm: inative;9-12mm:partially active; 13-18mm: active; >

18mm:very active) .Tables (5) and (6) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table 4: Antimicrobial Activity of the isolated compounds .

Concentration		Antibacterial activity				Antifungal activity	
		Gram-positive		Gram -negative			
compound I	Control Methanol	*B.subtilis*	*St.auerus*	*E. coli*	*Ps. aeruginosa*	*Ca. albicans*	*As. niger*
100mg/ml	0.0	15	15	13	13	15	15
50mg/ml	0.0	14	14	12	12	14	15
25mg/ml	0.0	12	13	14	13	13	14
12.5mg/ml	0.0	9	12	10	11	12	12
6.25mg/ml	0.0	9	13	10	11	13	13
compound II							
100mg/ml	0.0	12	17	15	15	17	16
50mg/ml	0.0	11	15	12	12	15	15
25mg/ml	0.0	14	14	14	12	14	14
12.5mg/ml	0.0	13	13	15	11	13	13
6.25mg/ml	0.0	11	12	8	10	12	13
compound III							
100mg/ml	0.0	16	16	13	16	16	16
50mg/ml	0.0	11	15	13	15	15	11
25mg/ml	0.0	12	15	12	12	15	12
12.5mg/ml	0.0	10	13	14	11	13	10
6.25mg/ml	0.0	10	13	13	10	13	11

Table 5:- Antibacterial activity of standard chemotherapeutic agents

Drug	Conc.(mg/ml)	Bs	Sa	Ec	Ps
Ampicilin	40	15	30	-	-
	20	14	25	-	-

	10	11	15	-	-
Gentamycin	40	25	19	22	21
	20	22	18	18	15
	10	17	14	15	12

Table 6:- Antifungal activity of standard chemotherapeutic agent

Drug	Conc.(mg/ml)	An	Ca
Clotrimazole	30	22	38
	15	17	31
	7.5	16	29

Sa.: *Staphylococcus aureus*
Ec.: *Escherichia coli*
Pa.: *Pseudomonas aeruginosa*
An.: *Aspergillus niger*
Ca.: *Candida albicans*
Bs.: *Bacillus subtilis*

Compounds II and III showed significant antimicrobiali activity at 100mg/ml and 50mg/ml . Compound I exhibited promising activity at these doses. The results of Table 4 suggest that these flavonoids could serve as leads for further optimization.

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