Sudan University of Science and Technology

Graduate College

Chemical Characterization and Biological Activity of Flavonoids in Some Medicinal Plants

تحديد التركيب والفعالية الحيوية للفلافونيدات في بعض النباتات الطبية

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

By:

Mahmoud Mohamed Ali Mahmoud Idris

(B.Sc in Chemistry(International University of Africa, M.Sc.in Chemistry, Alnileen University)

Supervisor: Prof: Mohamed Abdel karim Mohamed

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كلية الدراسات العليا

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كلية الدراسات العليا

Approval Page

Name of Candidate: ...Mahmoud.....Mohamed.....Ali.....

Thesis title: .....Chemical....characterization.....and.....Biological....Activity....of.....Flavonoids.....from.....some.....Medicinal.....Plants.....

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Approved by:

1. External Examiner

Name: M.O.H. ALI HUSSEIN.....

Signature: .....Date: 9.12.2014.....

2. Internal Examiner

Name: Mohammed Elmubark Osman.....

Signature: .....Date: 9/12/2014.....

3. Supervisor

Name: ...Mohamed...Abdel Karim...Mahamed.....

Signature: .....Date: 9.12.2014.....

cgs @ sustech edu. البريد الالكتروني 83 769363 /فاكس ص.ب 407

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Praise to Allah, almighty for raising me to this point, it would not be possible without your help. You have watched me and always stood by me anytime anywhere.

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DEDICATION

To my parents

my Wife and Children

Abstract

In this study Three plants species from eastern and western Sudan were studied: Namely *Acacia nubica* ***benth***(Laot), *Citrus paradisi* (grapefruit) and *Combretum Hartmonnina* ***Schweinf*** (Habeel).

The plant material was extracted using 95% ethanol.The crude extracts were subjected to a phytochemical screening which revealed the presence of flavonoids among other phytochemicals .The crude extracts were purified by thin layer chromatography utilizing different solvent systems ,and characterized using : IR,UV,^{1}HNMR and Mass spectroscopy .

Aflalavonoid :7,5´,6´- trihydroxy-5-methyl-isoflavone was isolated from the bark of *Combretum Hartmonnina* ***Schweinf*** (Habeel),

5,7,4´,5´-tetrahydroxy-6,8,1´,2´,6´-pentamethoxyflavanone was isolated from the leaves of *Acacia nubica* and7-oglycosyl-5,4´ -dihydroxyflavanone was isolated from the leaves of *Citrus paradise*.

The isolated flavonoids revealed promising antimicrobial potential.

مستخلص البحث

في هذه الدراسة تم اختيار ثلاثة نباتات طبية من شرق وغرب السودان وهي اللعوت ، قريب فروت و الهبيل .

استخلصت المادة النباتية بستخدام الإيثانول 95% و أخضع الخام المستخلص لمسح فيتوكيميائي أوضح وجود الفلافونيدات بجانب نواتج طبيعية أخرى .

تمت تنقية المستخلصات الخام بكرماتوغرافيا الطبقة الرقيقة وتم تحديد التركيب بالطرق المطيافية : طيف الأشعة تحت الحمراء , طيف الأشعة فوق البنفسجية , طيف الرنين النووي المغنطيسي وطيف الكتلة وبهذه الطريقة :

1.من لحاء نبات الهبيل تم فصل المركب:

7,5ˉ,6ˉ- trihydroxy-5-methyl-isoflavone

2. من ورق نبات اللعوت تم فصل المركب :

5,7,4ˉ,5ˉ- tetrahydroxy-6,8,1ˉ,2ˉ,6ˉ-pentamethoxyflavanone

3.من ورق نبات القريب فروت تم فصل المركب :

7-o-glycosyl-5,4´ -dihydroxyflavanone

أظهرت نتائج المسح البيولوجي فعالية واعدة للمركبات المفصولة ضد بعض أنواع الميكروبات .

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1. Introduction

1.1- General approach

Flavonoids and their conjugates form a very large group of natural products. They are found in many plant tissues, where they are present inside the cells or on the surfaces of different plant organs. The chemical structures of this class of compounds are based on a C6-C3-C6 skeleton. They differ in the saturation of the heteroatomic ring C, in the placement of the aromatic ring B at the positions C-2 or C-3 of ring C, and in the overall hydroxylation patterns . Flavonoids may be modified by hydroxylation, methoxylation, or o-glycosylation of hydroxyl groups as well as direct C- glycosylation to carbon atom of the flavonoid skeleton. In addition, alkyl groups ; often prenyls may be covalently attached to the flavonoid moieties, and additional rings are sometimes condensed the basic skeleton of the flavonoid core. The last modification takes place most often in the case of isoflavonoids, where the B ring is condensed to the C-3 carbon atom of the skeleton(Harborne and Williams, 2000).

Flavonoid glycosides are, frequently acylated with aliphatic or aromatic acid molecules. These derivatives are thermally labile and their isolation and further purification without partial degradation is difficult. The multiplicity of possible

classes was known in the end of the last century and this number continues to increase (Harborne and Williams, 2000). Condensed tannins create a special group of flavonoid compounds formed by polymeric compounds built of flavan-3-ol units, and their molecular weights often exceeding 1,000 Da. In the plant kingdom, different plant families have characteristic patterns of flavonoids and their conjugates. All these compounds play an important biochemical and physiological roles in various cell types or organs (seed, root, green part, fruit) where they accumulate. Different classes of flavonoids and their conjugates have numerous functions during the interactions of plant with the environment, both in biotic and abiotic stress conditions. Additionally, flavonoid conjugates, because of their common presence in plants, are important components of human and animal diet. Due to the different biological activities of plant secondary metabolites, their regular consumption may have positive and negative consequences on health. (Beck, 2003) . For the mentioned reasons, methods for the efficient and reproducible analysis of flavonoids play a crucial role in research conducted in different fields of the biological and medical sciences.

The identification and structural characterization of flavonoids and their conjugates isolated from plant material, as single compounds or as part of mixtures of structurally similar natural products, create some problems due to the presence of

isomeric forms of flavonoid aglycones and their patterns of glycosylation. A number of analytical methods are used for the characterization of flavonoids. In many cases, nuclear magnetic resonance (NMR) analyses (^{1}H and ^{13}C) are necessary for the unambiguous identification of unknown compounds; other instrumental methods (mass spectrometry, UV and IR spectrophotometer) applied for the identification of organic compounds fail to provide the information necessary to answer all the structural questions. Utilization of standards during analyses and comparison of retention times as well as spectral properties, especially when compounds are present in a mixture, is critical. An important area of research on flavonoids is the identification of their metabolites in animal tissues and body fluids (urine, blood, spinal fluid). For this, investigators have to deal with different modifications of the flavonoid moieties, modifications often not found in plant tissues (Blaut,2003). The metabolism of flavonoids in human and animal organisms, among others, is based on glucosylation, sulfation, or methylation (Sfakianos, *2002*).

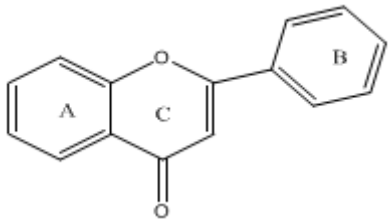
1.2- Classification of flavonoids

Flavonoids are a large family of compounds comprising about 10 classes, different from each other by the degree of oxidation of

the C ring (viz. flavones, flavonols, flavans, flavanones , chalcones, dihydrochalcones, isoflavones, aurones, anthocyanins , catechins). Both the oxidation state of heterocyclic ring and the position of ring B are important in thier classification (Dixon 2005)

1.2.1-Flavones

Flavones are a class of polyhdroxy derivatives based on flavone itself, the numbering of flavone (1) skeleton is shown below:



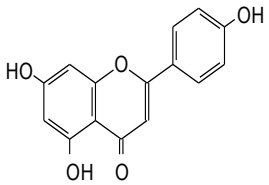
(1)

Flavones are closely related to the flavonols , but differ in their spectroscopic and chromatographic properties and can readily be distinguished by these means.

Chemically, flavones can be classified into groups according to whether they are: (a) hydroxylated; (b) O-methylated; (c) C-methylated; (d) methylenedioxy substituted; and (e) isoprenylated. Flavones occur in the free state as lipophilic components of leaves and in bud exudates. They occur much more frequently in polar forms glycosides or other conjugates. Two main glycosides are known; O-and C-glycosides

(Harborne, JB ,1999). Flavones constituent one of the major classes of naturally occurring products. Flavones occur as glycosides, a common type is the 7-glycoside exemplified by luteotlin-7-glycoside. Flavones unlike flavonols also occur remarkably, with sugar bound by a carbon-carbon bond. A carbon-carbon bond is very resistant to acid hydrolysis so that it is relatively easy to distinguish C-glycosides from O-Glycosides which are more readily hydrolysed. A Special group of flavone –based C- glycosides occur in plants(Harborne, 1999).

C -glycosides of flavones commonly occur as such or with further O-glycosylation. These glycosides readily lose their O-linked sugars on acid hydrolysis .Such O-glycosidic residues may be attached either to the hydroxyl of sugar carbon or directly linked to one of the free phenolic groups (Tasdemir,2006). Flavone is the root from which the word flavonoid is derived · The commonest flavones are apigenin (2) and luteolin (3). Flavones are common in angiosperms(Dixon,2010).



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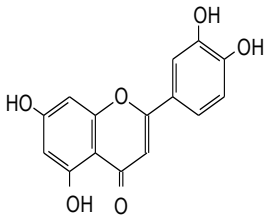

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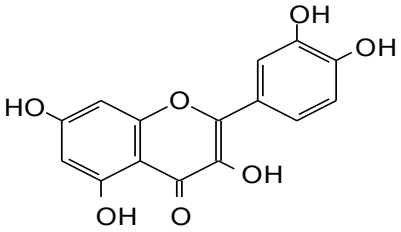
1.2.2- Flavonols

Flavonol are very widely distributed in plants, both as co-pigments to anthocyanins in petals and also in leaves of higher plants .They occur most frequently in glycosidic combination. Flavonols are of limited natural occurrences. Flavonol are flavones with a 3-hydroxy substituent and they share the same nomenclature (Tasdemir,2006).

The most widely distributed flavonols in nature are quercetin (4) kaempferol (5), myricetin (6) and methylated derivative isorhamnetin (7) (Dixon , 2010).



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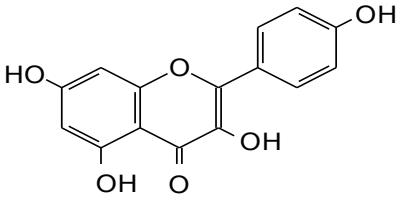

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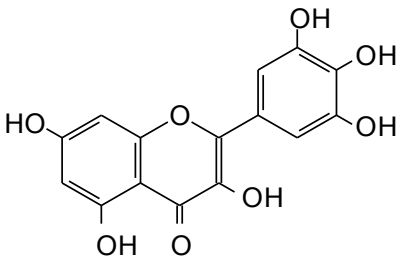

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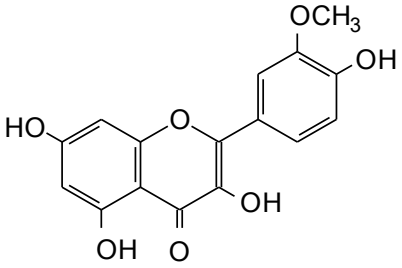

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Quercetin is the most abundant flavonol. It is present in many fruits, vegetables, and herbs, especially in onions, apples, berries and tea. Quercetin may be present either as a glycone or glycosides such as rutin, quercitrin, isoquercitrin, hyperoside

(Andersen, 2006). Quercetin is also, often, added as a soluble derivative to multi-vitamin tablets, with other common flavonols like myricetin and kaempferol.

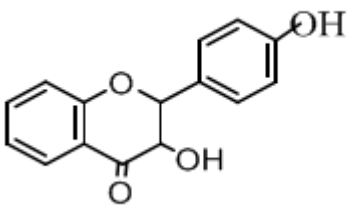
Flavonols have a wider range of biological activities than flavones. Quercetin, for example, is an effective inhibitor of many enzymic activities. Chrysosplenol C is an antiviral agent, while gossypetin is a yellow flower pigment in cotton plant and the primrose Flavonols play an important role in the protection of plant from UV damage. They also play a role in regulation of stem elongation; dormancy and fruit maturation. Flavonols appear to be important in regulating growth in the pea plant (Harbone,1967).

1.2.3- Flavanones

Flavanones also called dihydroflavones lack the double bond between carbons 2 and 3, which is present in flavones and flavonols .Thus, in flavanones, C-2 bears one hydrogen atom in addition to the phenolic B-ring, and C-3 two hydrogen atoms.

Two stereoisomeric forms of each flavanone structure are possible, since C-2 is a centre of assymmetry (epimeric center). Consequently, the B-ring can be either in the (2S) or (2R) configuration. The great majority of the flavanones isolated from plants are levorotatory (2R) or (2S), because the enzymatic reaction catalysing the conversion of chalcones to flavanones is stereospecific (Harbone,1967).

Flavanones are, mainly represented by narigenin (8), hespertin (9), and eriodictyol (10), while a number of minor compounds, including sakuranetin (11) and isosakuranetin (12) (Andersen, 2006). They are represented by the saturated heteorcyclic ring (C) and a carbonyl groups at the 4 position. They are, usually, glycosylated at position 7 by a disaccharide (neohesperidose, rutinose) or, by a monosaccharide (glucose).



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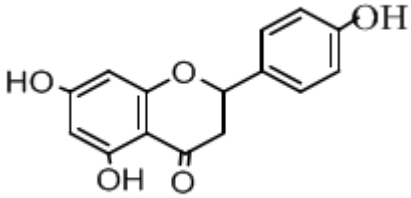

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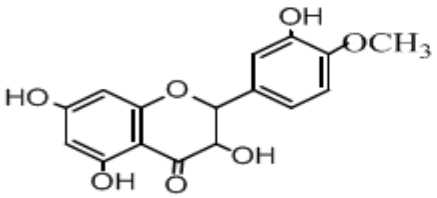

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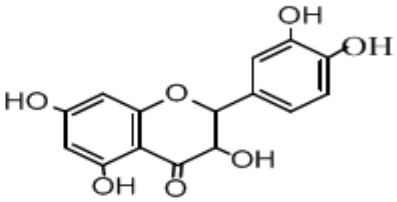

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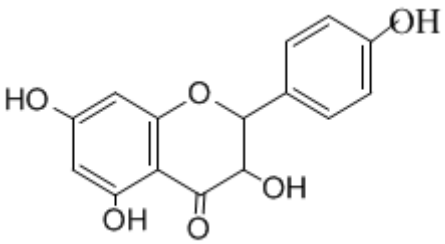

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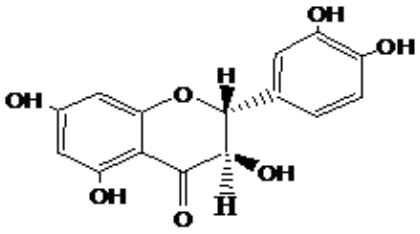
1.2.4- Dihydroflavonols

Dihydroflavonols or 3-hydroxyflavanones have wide distribution in the plant kingdom. The most common members of this group are: dihydroquercetin (13), dihydrokaempferol (14), dihydromyricetin (15))(Andersen and Markham, 2006)

Dihydroflavonols have two asymmetric carbons at C-2 and C-3.Most dihydroflavonols are polyhydroxylated and some are partially O-methylated. Dihydroquercetin, for example, has been found in 50 angiosperm families. The most significant biological property is their antimicrobial activity (Tasdemir ,2006).



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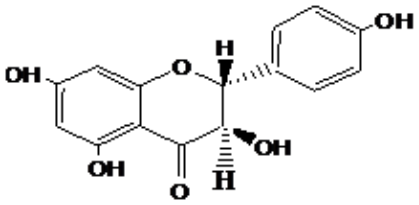

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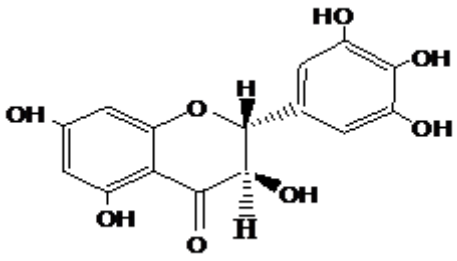

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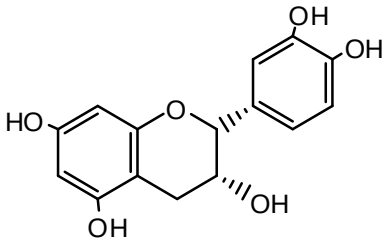
1.2.5- Flavan-3-ols

Flavan-3-ols constitute the largest class of monomeric flavans. The two compounds: catechin and epicatechin are the commonest known flavonoids. A notable feature of the, naturally, occurring flavan -3-ols is there occurrence in leaf of tea .They are also known to occur in glycosidic combination, with sugar attached to the 3`, 5`, or 7-hydroxyls. These compounds are used as a haemostatic drug in the treatment of liver disease (Spedding, 1989).

Flavan-3-ols represent the most common flavonoid consumed in the American and, most probably, the Western diet and are regarded as functional ingredients in various beverages, processed foods, herbal remedies, and supplements. Their presence in food affects quality parameters such as astringency, bitterness, sourness, sweetness, salivary viscosity, aroma, and color formation. Flavan-3-ols are structurally, the most complex subclass of flavonoids ranging from the simple monomers (+)-catechin (16) and its isomer (-)-epicatechin (17) to the oligomeric and polymeric proanthocyanidins which are also known as condensed tannins.

Flavan-3-ols are found abundantly in fruits such as apricots (*Prunus armeniaca*), sour cherries (*Prunus cerasus*), grapes and blackberries (Harbone,1967).



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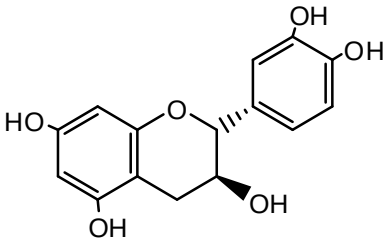

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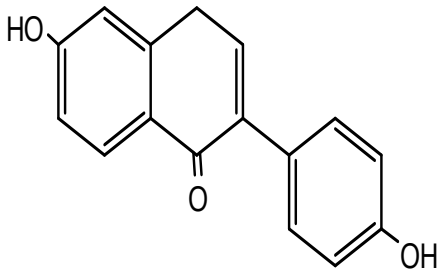
1.1.6- Isoflavones

Isoflavones differ from flavones in the position of the B-ring, linked to the C_3 position of the heterocyclic ring instead of the C_2 position as in most flavonoids. Isoflavones are also termed phytoestrogens because of their estrogenic activity in mammals derived from the structural similarity between oestrogens and isoflavones. The most common isoflavones are daidzein (21) and genistein (22), together with their 7-O-glucosides daidzin and genistin, respectively (Dixon , 2010).

In contrast to most other flavonoids, isoflavones are characterized by having the B-ring attached at C-3 rather than the C-2 position. They have a very limited distribution in the plant kingdom with substantial quantities being found only in leguminous species. Isoflavones are known for their estrogenic activity due to their ability to bind to estrogen receptor and have received much attention due to their putative role in the prevention of breast cancer and osteoporosis . In plants isoflavones occur predominantly as glucosides (genistin, daidzin, glycitin), or as acetyl-β-glucosides and malonyl-β-glucosides,and are therefore polar, water-soluble compounds.

Isoflavones also undergo various modifications, such as methylation, hydroxylation, or polymerization, and these modifications lead to simple isoflavonoids, such as isoflavanones, isoflavans, and isoflavanols, as well as more complex structures including rotenoids, pterocarpans, and coumestans.



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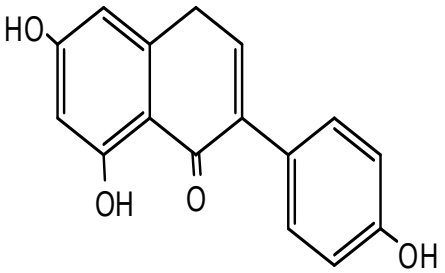

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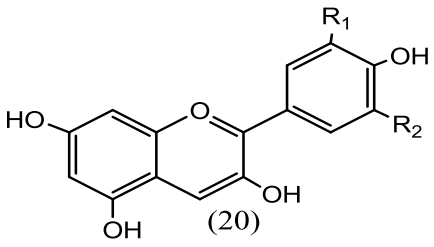
1.2.7- Anthocyanidins and anthocyanins

Anthocyanins are water-soluble plant pigments and are particularly evident in fruit and flower tissue where they are responsible for a diverse range of red, blue, and purple colors. They occur, primarily, as glycosides of their respective aglycone anthocyanidin , with the sugar moiety, typically, attached at the 3-position on the C-ring or the 5-position on the A-ring. They are involved in the protection of plants against excessive light by shading leaf mesophyll cells. They also have an important role in attracting pollinating insects.

There are about seven teen anthocyanidins found in nature, but only six cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin are, ubiquitously, distributed and of dietary importance. The variations of anthocyanins are due to: (i) the number and position of hydroxyl and methoxyl groups on the basic anthocyanidin skeleton; (ii) the identity, number, and positions at which sugars are attached; and (iii) the extent of sugar acylation and the identity of the acylating agent (Harbone,1967). Unlike other subgroups of flavonoids with the same C6–C3–C6 skeleton, anthocyanins have a positive charge in their structure at acidic pH. The most widespread anthocyanin in fruits is cyanidin-3-glucoside. However, malvidin glycosides are the characteristic anthocyanins in red grapes and their, derived, products (Dixon, 2010). Other anthocyanins that occur in grapes include petunidin-3-O-glucoside, malvidin-3-O-(6-O-p-coumaroyl) glycoside, malvidin-3-O-(6-O-acetyl) glycoside, delphinidin- 3-O-glucoside, and malvidin-3-, 5-O-diglucoside (Tasdemir,2006). The major anthocyanins are shown below:



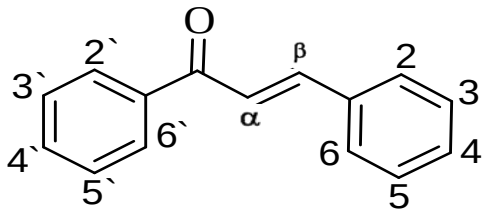
(20)

Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

They are all based chemically on a single aromatic structure, that of cyanidin, and all are derived from this pigment by addition or subtraction of hydroxyl group or by methylation or by glycosylation (Tasdemir,2006).Anthocyanins are those flavonoid pigments which provide intense cyanic colours from pink to blue in the flowering plant .Chemically ,anthocyanins are highly oxidised flavylium cation ,exemplified by the structure of the common anthocyanidin ,pelargonidin .The anthocynidin are unstable to light and are water –insoluble so that they do not occur usually in the free state,instead ,they are present in the cell vacuole linked to sugars ,which provide stability and water solubility. Some anthcyanidins have malonic acid (or other aliphatic dicarboxylic acid) residues linked through sugar and are zwitterionic in their properties(Tasdemir,2006).

1.2.8- Chalcones

Chalcones (1, 3- diaryl-2-propen-1-ones) are a group of naturally or synthetic organic compounds, containing at least two aromatic rings, linked by ketoethylenic group (-CO-CH=CH-), or they are α, β- unsaturated ketones, (Dixon , 2010). The general formula of chalcones is as illustrated below:

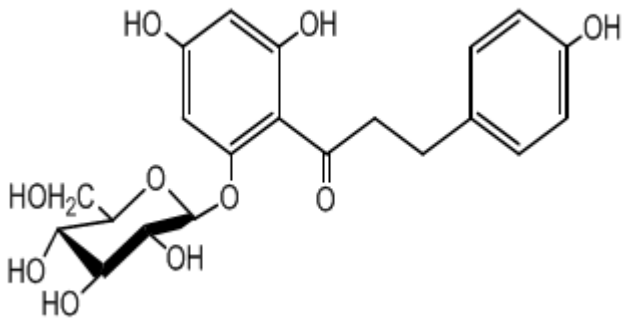


The chemistry of chalcones has generated intensive scientific studies throughout the world. Especial interest has been focused on the synthesis and biodynamic activities of chalcones. These compounds are also known as benzalacetophenone or benzylidene acetophenone. In chalcones, two aromatic rings are linked by an aliphatic three carbon chain. Chalcones bear very good synthons, so that variety of novel heterocycles with good pharmaceutical profiles can be designed (Ahluwalia,2009).

1.2.9- Dihydrochalcones

Dihydrochalcones are directly, related to the chalcones and are derived from them by reduction of the chalcones α, β- double bond. The best known dihydrochalcone is phloridzin (27), which occurs in the skin of apples. When taken orally, it causes glycosuria by interfering with tubular readsorption of

glucose in the kidney. It is therefore in demand in experimental physiology to study glucose transport.



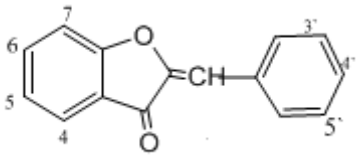
(27)

Dihydrochalcones are relatively small group of flavonoids and they have a somewhat erratic distribution (Dixon , 2010).

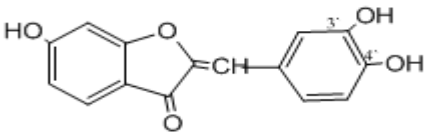
1.2.10- Aurones

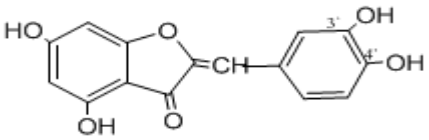
Aurones are small group of yellow pigment related to flavonoids. Aurones are limited occurrence, and the first aurones was discovered in 1943. Aurones are hydroxylated 2-benzylidenecoumaranones, the parent compound being (18) .The normal numbering system applies to this group of compounds: positions on the A ring are identified by unprimed numbers and B-ring positions by primed number ,it has to be notcid that, in aurones, position 4 corresponds biosynthetically to position 5 of other heterocyclic flavonoids (Koes and Quattrocchio,1994). The two most common structures are

sulfretin,6,`3,`4-triahydroxyaurone(19)and aureusidin 4, 6, `3, `4-tetrahydroxaurones (20) (Dixon , 2010).



(18)

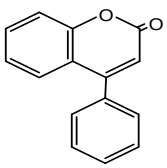


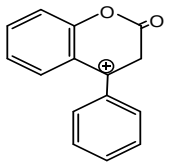


(19) **(20)**

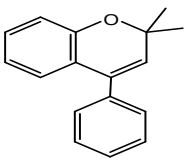
1.2.11- Neoflavonoids

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the 4-arylcoumarins (4-aryl-2H-1- benzopyran-2-ones) (23), 3, 4-dihydro-4-arylcoumarins (24), and neoflavenes (25).A typical neoflavonoids is melanin (26)(Dixon , 2010)..

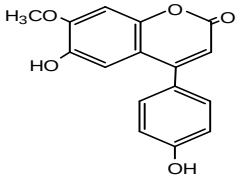




(23) (24)



(25)



(26)

1.3- Purification

Isolation of compounds in a pure state from natural sources is the most important, yet, it can be a difficult and time-consuming, step in natural product research. It begins with the process of extraction followed by various separation techniques. One such separation technique is solvent partitioning, which, usually, involves the use of two immiscible solvents in a separating funnel. In this method, compounds are distributed in two solvents according to their different partition coefficients. This technique is highly effective and is the first step of a fairly large-scale separation of compounds from crude natural product extracts (Akindele ,2006) .

A crude natural product extract is, generally, an extremely complicated mixture of several compounds possessing varying chemical and physical properties. The fundamental strategy for separating these compounds is based on their physical and chemical properties that can be, cleverly, exploited to initially separate them into various chemical groups. However,in some cases, from the literature in search of the related genera and families, it is possible to predict the types of compounds that

might be present in a particular extract. This tentative prediction on the possible identity of the classes of compounds may help choose suitable extraction and solvents partitioning methods, for extracting specific classes of compounds, for example, phenolics, saponins, alkaloids. Plant natural products are usually extracted with solvents of increasing polarity, for example, first n-hexane, diethylether, chloroform ($CHCl_3$), to name a few, followed by more polar solvents, i.e., methanol (MeOH), depending on the chemical and physical nature of targeted compounds. Alcoholic (MeOH or EtOH) extracts of plant materials contain a wide variety of polar and moderately polar compounds. By virtue of the cosolubility, many compounds, which are individually insoluble in pure state in MeOH or EtOH, can be extracted quite easily with these solvents. Hence, Nernst's partition law may not be applicable. A dried alcoholic extract can also be extracted directly with a suitable solvent.A typical partitioning scheme is presented in Fig. 1.1. A MeOH extract is concentrated, and the volume is reduced to an appropriate level that can be handled easily with a separating funnel. The concentrated extract is then extracted with an equal volume of n-hexane, usually three times, to give a fraction containing nonpolar compounds, such as lipids, chlorophylls, and so on. The process is, sometimes, referred to as "defatting." Although MeOH and n-hexane are not completely miscible (Takeda ,1988)

Sometimes, a small amount of water is added to MeOH to obtain a 95%-aqueous methanolic solution to get two distinct layers with similar volumes. The methanolic layer is evaporated to dryness and then dissolved in water. Occasionally, it is not a solution,but a suspension.

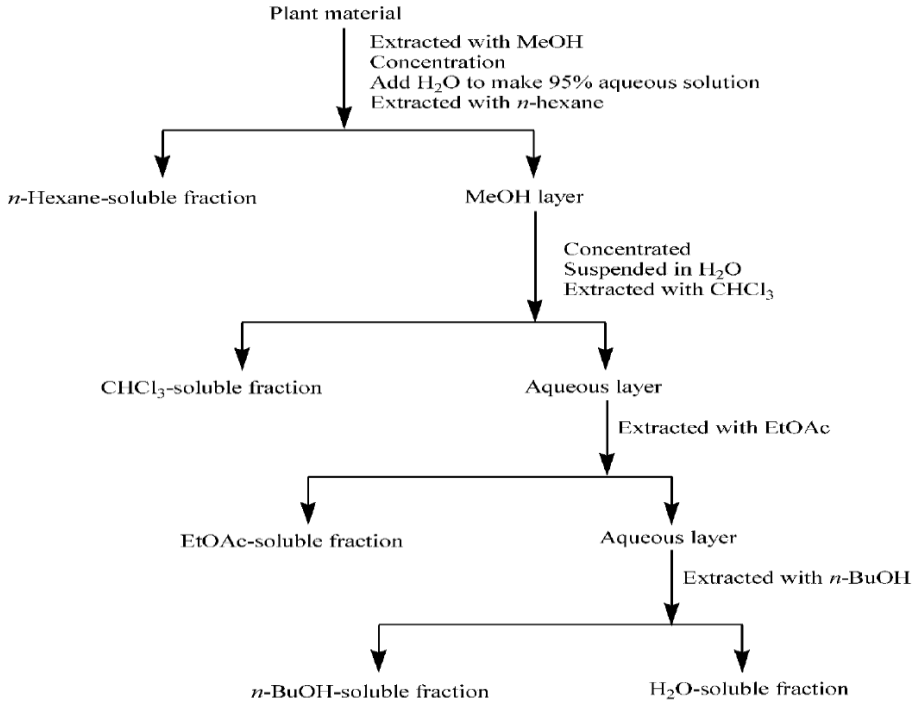


Fig.1.1:Atypical partitioning scheme using immiscible solvents

The solution (suspension) is partitioned between $CHCl_3$, ethylacetate (EtOAc), and n-butanol (n-BuOH), successively. Partitioning with $CHCl_3$ can be omitted depending on the chemical nature of targeted compounds. Less polar compounds are present in the $CHCl_3$- soluble fraction and polar compounds, probably up to monoglycosides, in the EtOAc-soluble one. The n-BuOH fraction contains polar compounds,

mainly glycosides. Evaporation of , remaining, water layer leaves polar glycosides and sugars as a viscous gum. However, separation by solvent partitioning cannot be always performed in a clear cut manner; overlapping of the compounds in successive fractions is usually found. When using EtOAc as an extraction solvent, especially the technical grade solvent, researchers must remember that it contains a trace amount of acetic acid , which may cause a trans-esterification of acetyl group to the hydroxyl groups, and have a catalytic effect on labile functional groups or delicate structures. When the acetates of some compounds are isolated from the EtOAc-soluble or subsequent n-BuOH-soluble fraction, it is suspected that trans-esterification may have produced the acetates of the original compounds as artifacts. Chloroform is an ideal solvent for extracting alkaloids owing to its slight acidic nature, because alkaloids tend to be soluble in acidic media. When water layer is to be extracted, thoroughly, with n-BuOH, water saturated, n-BuOH is, frequently, used. Although n-BuOH is not miscible with water, 9.1mLs of n-BuOH are soluble in 100mL of water at 25 **C**. Therefore, when the water layer is extracted with n-BuOH unsaturated with water many times, the volume of water layer drastically decreases. Usage of unbalanced volumes of solvents, sometimes, causes unexpected partitioning of compounds. When saponins are the major target, it is advisable that the glycoside fraction (n-BuOH

layer) is partitioned with a 1%-KOH solution to remove, widely, distributed phenolic compounds, such as flavonoids and related glycosides. Before concentrating the extract, the n-BuOH layer must be washed several times with water. In turn, re-extraction of the acidified alkaline layer gives a fraction rich in phenolic compounds. Some acylated saponins and flavonoids, present in plant extracts, are also hydrolyzed under alkaline conditions. Thus, at least a small-scale pilot experiment, such as tracing the fate of compounds by thin layer chromatography(Takeda,1988)is, strongly recommended.

1.3.1- Immiscible solvents

The separation technique using solvent partitioning involves, primarily, the use of two immiscible solvents in a separating funnel, and the compounds are distributed in two solvents according to their different partition coefficients. This method is relatively easy, to perform and, highly, effective as the first step of the fairly large-scale separation of compounds from crude natural product extracts.

Solvent partitioning methods, such as countercurrent distribution (Craig distribution).

1.3.2- Partitioning Between Immiscible Solvents

A crude natural product extract is generally an extremely complicated mixture of several compounds possessing varying chemical and physical properties. The fundamental strategy for separating these compounds is based on their physical and chemical properties that can be cleverly exploited to initially

separate them into various chemical groups. However, in some cases, from the literature search of the related genera and families, it is possible to predict the types of compounds that might be present in a particular extract. This tentative prediction on the possible identity of the classes of compounds may help choose suitable extraction and partitioning methods, and solvents for extracting specific classes of compounds, for example, phenolics, saponins, alkaloids. Plant natural products are usually extracted with solvents of increasing polarity, for example, first n-hexane, diethylether, chloroform ($CHCl_3$), to name a few, followed by more polar solvents, i.e., methanol (MeOH), depending on the chemical and physical nature of targeted compounds. Alcoholic (MeOH or EtOH) extracts of plant materials containing a wide variety of polar and moderately polar compounds. By virtue of their cosolubility, many compounds, which are insoluble individually in pure state in MeOH or EtOH, can be extracted quite easily with these solvents. Hence, Nernst's partition law may not be applicable. A dried alcoholic extract can also be extracted directly with a suitable solvent.A typical partitioning scheme is presented in Fig. 1.1. A MeOH extract is concentrated, and the volume is reduced to an appropriate level that can be easily handled with a separating funnel. The concentrated extract is then extracted with an equal volume of n-hexane, usually three times, to give a fraction containing nonpolar compounds, such as lipids,

chlorophylls, and so on. The process is sometimes referred to as "defatting." Although MeOH and n-hexane are not completely miscible,

Fig. 1.1. A typical partitioning scheme using immiscible solvents. Purification by Solvent Extraction (Takeda,1988)they are miscible to some extent..

1.3.3- Partitioning Between Miscible Solvents

Contrary to what has already been discussed earlier, miscible solvents are sometimes used for partitioning on addition of water (Takeda,1988). A plant material is extracted with MeOH and evaporated to obtain a residue. The residue is re-dissolved in 90% aqueous MeOH, and the resulting solution is extracted with n-hexane. This step seems to be similar to the previous partitioning example. In the next step, an appropriate amount of water is added to the 90%-aqueous MeOH to obtain an 80% aqueous solution, which is then extracted with CCl_4 (MeOH and CCl_4 are miscible). The final step is to make a 65%-aqueous MeOH solution with the addition of water, and the resulting solution is extracted with $CHCl_3$(MeOH and $CHCl_3$ are miscible). Evaporation of n-hexane, CCl_4, and $CHCl_3$ layers gives three fractions in order of polarity. Concentration of the 65%-aqueous MeOH layer gives the most polar fraction. This fraction is expected to contain glycosides as major constituents as well as a large amount of water-soluble sugars, (Takeda ,1988)-Fig1.1.

1.4- Medicinal properties of flavonoids

Flavonoids are increasingly,become the subject of medical research. They have been reported to possess many useful properties, including anti-inflammatory , oestrogenic , enzyme inhibition , antimicrobial activity (Havsteen, 1983),antiallergic , antioxidant ,vascular and cytotoxic antitumor activity (Harborne, 1999). For a group of compounds of, relatively, homogeneous structure, flavonoids inhibit a perplexing number and variety of eukaryotic enzymes and have ,a tremendously, wide range of activities. In the case of enzyme inhibition, this has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring (Havsteen , 1983). Several reviews have been written on the interaction between flavonoids and mammalian cells. An extensive review on the biochemistry and medical significance of flavonoids has also been produced (Havsteen ,2002).

For centuries, preparations that contain flavonoids as the principal , physiologically, active constituents have been used by physicians and lay healers in attempts to treat human diseases (Havsteen,1983).For example, the plant *Tagetes minuta* (containing quercetagetin-7-arabinosyl-galactoside) has been used extensively in Argentine folkmedicine to treat infectious disease (Tereschuk , 1997).

It has been suggested that because flavonoids are widely distributed in edible plants and beverages and have previously been used in traditional medicine, they are likely to have minimal toxicity. However, this family of compounds has a diverse range of activities in mammalian cells (Middleton,2000) and *in vivo* confirmation of their side effects would be necessary for a full evaluation of their practical usefulness in the field of modern medicine (Tsuchiy , 1996) given that the selectivity of flavonoids for eukaryotic enzymes appears to vary from compound to compound (Harborne , 1992), such a study would need to assess the toxicity of these phytochemicals on individual basis.

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 (Harborne , 1992). A prenylated flavanone isolated from the shrub *Eysenhardtia texana* has been identified as 5,7,4_-trihydroxy-8-methyl-6-(3-methyl-[2-butenyl])-(2*S*)-flavanone and was shown to possess activity against the opportunistic pathogen*Candida albicans* (Wachter,1999). The flavonoid 7-hydroxy-3_,4_-methylenedioxyflavan, isolated from *Terminalia bellerica* fruit rind, has also been shown to possess activity against *C.albicans* (Valsaraj ,1997).Two flavones from *Artemisia giraldi*, identified as 6,7,4-trihydroxy-3,5-dimethoxyflavone and 5,5- dihydroxy-8,2,4-trimethoxyflavone, together with 5,7,4- trihydroxy-3,5-

dimethoxyflavone have been reported to exhibit activity against *Aspergillus flavus* (Zheng, 1996), a species of fungi that causes invasive disease in immunosup pressed patients (Prescott,1999). The activity of propolis against dermatophytes and *Candida* spp. has been attributed at least partially to its high flavonoid content (Cafarchia, 1999). Galangin, a flavonol, commonly, found in propolis samples , has been shown to have inhibitory, activity against *Aspergillus tamarii*, *A. flavus* ,*Cladosporium sphaerospermum*, *Penicillium digitatum* and *Penicillium italicum* (Afolayan and Mandyer ,1997).

A recent area of research that is of particular interest is the apparent inhibitory activity of some flavonoids against human immunodeficiency virus (HIV). To date, most if not all investigations have involved work with the pandemic HIV-1 strain and its enzymes. *In vitro* studies have shown that the flavonoid baicalin inhibits HIV-1 infection and replication. Inhibition of HIV-1 entry into cells expressing CD4 and chemokine co-receptors, and antagonism of HIV-1 reverse transcriptase by the flavone baicalein- *O*-glycoside have been demonstrated by Li and colleagues. (Li ,1993). Robusta-flavone and hinokiflavone (Lin , 1997) have also been shown to inhibit HIV-1 reverse transcriptase, as have several catechins. However, catechins inhibit other DNA polymerases and their interaction with the HIV-1 enzyme is therefore thought to be non-specific in nature (Moore and Pizza ,1992). In addition, it

has been demonstrated that several flavonoids, including demethylated gardenin A and 3,2_-dihydroxyflavone, inhibit HIV-1 proteinase. Robinetin, myricetin, baicalein, quercetagetin and quercetin 3-*O*-(2-galloyl)-l-arabinopyranoside (Kim, 1998) inhibit HIV-1integrase, although there are concerns that HIV enzyme inhibition by quercetagetin and myricetin is no-specific. 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(Critchfield,1996). Interestingly, chrysin was reported to have the highest therapeutic index of 21 natural and 13 synthetic flavonoids against HIV-1 (Cushnie, and Lamb,2005). Several research groups have investigated the relationship between flavonoid structure and inhibitory activity against HIV-1 and its enzymes (Critchfield,1996). Furthermore, at least two groups have proposed mechanisms of action for HIV-1 enzyme inhibition (Kim , 1998]. Flavonoids also have inhibitory activity against a variety of other viruses. For example, (Selway,1986) reported that quercetin, morin, rutin, dihydroquercetin, dihydrofisetin, leucocyanidin,pelargonidin chloride and catechin possess activity against up to seven types of virus, including herpes simplex virus (HSV), respiratory syncytial virus, poliovirus and Sindbis virus. (Selway,1986) also proposed antiviral mechanisms of action including inhibition of viral polymerase

and binding of viral nucleic acid or viral capsid proteins. In addition to flavonoids mentioned above, three proanthocyanidins from *Pavetta owariensis* (with structural similarity to proanthocyanidin A2 and cinnamtannin B1 and B2) have shown activity against HSV and coxsackie B virus (Yamada , 1991). It has also been demonstrated that two of the flavonoids found in propolis- chrysin and kaempferol- inhibit viral replication of HSV, human coronavirus and rotavirus (Cheng and Wong ,1996). The flavonol galangin has been reported to have significant antiviral activity against HSV and coxsackie B virus. Although naturally occurring flavonoids with antiviral activity have been recognised since the 1940s, it is only in the last 25 years that attempts have been made to, synthetically, modify flavonoids for improved antiviral activity.One such synthesised compound is 6,4-dichloroflavan. However, despite showing strong *in vitro* activity, this compound proved unsuccessful in clinical trials (Havsteen ,2002). Synergism has been demonstrated, between various combinations of flavones and flavonols, to be active against HSV(Amoros,1992). Synergism has also been reported as antiviral agent. Quercetin, for example, potentiates the effects of 5-ethyl-2-dioxyuridine and acyclovir against HSV and pseudorabies infection. Apigenin also enhances the antiviral activity of acyclovir against these viruses (Mucsi,1992).

The antibacterial activity of flavonoids is being, increasingly documented. Crude extracts from plants with a history of use in folkmedicine have been screened *in vitro* for antibacterial activity by many research groups. Flavonoid-rich plant extracts from species of *Hypericum*, *Capsella* and *Chromolaena* (El-Abyad,1990) have been reported to possess antibacterial activity. Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity (Torrenegra and Ricardo ,1989). Propolis has been analysed on many occasions too, and samples containing high concentrations of flavonoids are frequently reported to show antibacterial activity (Park and Ikegaki,1998). Many research groups have gone one step further and either isolated and identified the structure of flavonoids that possess antibacterial activity, or quantified the activity of commercially, available flavonoids. Some researchers have reported synergy between naturally occurring flavonoids and other antibacterial agents against resistant strains of bacteria. Examples of these include epicatechin gallate and sophoraflavanone G (Hamilton and Shah, 2000).Others have, synthetically modified natural flavones and analysed them for antibacterial activity (Stapleton,2004). A group of researchers reported, increased, antibacterial activity of 3-methyleneflavanones when the B ring contained bromine or chlorine substituents (Ward,1981).More recently, Dastidar and co-workers reported that intraperitoneal

injection of either 1.58 mg/kg sophoraisoflavone A or 3.16 mg/kg 6,8- diprenylgenistein gave significant protection to mice challenged with ~9.5×108 colony-forming units (CFUs) of *Salmonella typhimurium* (Dastidar, 2004).

Several research groups have attempted to determine whether flavonoid activity is bacteriostatic or bactericidal by conducting time–kill studies. In such experiments, epigallocatechin gallate (Kono and Tatara ,1994), galangin (Cushnie and Hamilton ,2003) and 3-*O*-octanoyl- (+)-catechin (Stapleto , 2004) have been shown to cause a reduction of 1000-fold or more in viable counts of MRSA-YK, *S. aureus* NCTC6571 and EMRSA-16, respectively. This would, immediately appear to suggest that flavonoids are capable of bactericidal activity. However, it has been demonstrated that 3-*O*-octanoyl-epicatechin induces the formation of pseudomulticellular aggregates both in antibiotic-sensitive and antibiotic-resistant strains of *S. aureus* (Stapleton, 2004). If this phenomenon is induced by other compounds within the flavonoid class ,and liposomal studies suggest that this is the case for epigallocatechin . It may be that flavonoids are not killing bacterial cells but merely induce the formation of bacterial aggregates and thereby reduces the number of CFUs in viable counts.

1.5- Antioxidant Activities of Flavonoids

The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity.

1.6- Plants species

In this study three plants growing in Sudan were collected and some flavonoid compounds were isolated and identified using spectroscopy (IR, UV, NMR, MS).

1.6.1-*Acacia nubica* Benth(Laot)

Acacia nubica (Leguminosae) is widely distributed in Sudan, Egypt, Kenya,Ethiopia and Eriteria(Brenan, 1959) .*Acacia nubica* is used to treat body and joint pain. The plant is utilized as a tonic. (White, 1983)

1.6.2- *Combretum Hartmonnianum* Schweinf(Habeel)

This plant is distributed in Sudan, Egypt, Kenya,Ethiopia,Eriterea (Wickens, 1976)

Combretum hartmannianum is commonly used in Sudanese traditional medicine against jaundice, external skin infections, malaria and similar febrile diseases(Ali, . ,2002)

Leaves are used as an antipyretic, diuretic and for various diseases such as yellow fever, hepatic disorder (Maydell ,1990).

The methanolic extracts of different parts of *Combretum hartmannianum* possessed significant activity against the chloroquine-sensitive *Plasmodium falciparum* strain (El Ghazali, 1994).

1.6.3-*Citrus paradisi*

Scientific name: *Citrus paradisi*
Common name: grapefruit, toronja
Family: Rutaceae

Grapefruit leaves are bitter and contain a compound known as naringin, which does have medicinal properties, which include antioxidant, lowering of cholesterol and anticancer effects primarily by inhibiting angiogenesis(Armando,1997)

Fig.1.2: *Acacia nubica*

Fig.1.3:*Combretum hartmannianum* Schweinf(Habeel)

Fig.1.4:*Citrus paradisi*

1.7- Aim of this study

This study aims to :

- Extrac flavonoids from *Citrus paradisi* , *Combretum hartmannianum* and *Acacia nubica*
- Isolate flavonoids using chromatographic techniques.
- Elucidate structures of the isolated phytochemicals using sensitive spectroscopy.
- Evaluate antimicrobial potential of the targeted molecules .

2-Materials and Methods

2.1- Materials

2.1.1- Plant material

The leaves of *Acacia nubica* Benth(Laot) , *Citrus paradisi* (Grapefruit) and bark of *Combretum Hartmonnina* Schweinf (Habeel) were collected during 2009-2011 from Kassala state (Sudan) .The plant materials were kindly authenticated by Botany Department, University of Khartoum with the help of Prof. Seadat Eltigani. Fresh leaves and bark were dried and milled into fine powder.

2.1.2- Chemicals

Analytical grade solvents and chemicals were used in this study.

2.2- Methods

2.2.1- Extraction of flavonoids

Powdered air-dried samples (1kg) of *Acacia nubica*,*Citrus paradisi* and *Combretum Hartmonnina* were extracted with 95% ethanol (5L) at ambient temperature for 5 days. The solvent was evaporated *in vacu* to give a crude product.

2.2.2- Test reagents for phytochemical screening:

i) Preparation of Methanolic Aluminum Chloride

10 g of Aluminum chloride were dissolved in 100 ml of methanol (analytical grade) to prepare 10% aluminum chloride solution.

ii) Preparation of potassium acetate test reagent

(9.8g) of potassium acetate was added to 100 ml of distilled water to prepare 1 M potassium acetate.

iii) Preparation of ferric chloride solution

1.62 g of ferric chloride were dissolved in 100 ml of distilled water to give 1% iron (III) chloride solution which is neutralized with sodium hydroxide until a slight precipitate of FeO (OH) is formed. The mixture was filtered before use.

iv) Preparation of Dragendroff's reagents

Solution A: Bismuth nitrate (0.17g) in acetic acid (2mL) and H_2O (8mL).

Solution B: KI (4g) in acetic acid (10mL) and H_2O (20mL).

When used solutions. A and B are diluted to 100mL with H_2O.

2.2.3- Phytochemical Screening

Methanolic extracts from the three plants were screened for flavonoids, alkaloids , tannins, steroids, and glycosides .

(i) Test for Flavonoids

Methanolic extract of the three plants materials (bark and leaves) were evaporated to dryness on a water bath; the cooled residue were defatted by extraction with Pet. Ether, and dissolved in 30ml of 95% ethanol and filtered. The filtrate were used for the following tests:

1. To (3ml) of the filtrate few drops of methanolic aluminum chloride were added. Formation of a dark yellow color was taken as a positive test of flavonoids.
2. To (3ml) of the filtrate few drops of potassium hydroxide solution were added. A dark yellow color indicated the presence of flavonoids.
3. To (3ml) of the filtrate few drops of ferric chloride: ammonium ferrocyanide (1:1) solution was added. Development of blue coloration was taken as a positive test for flavonoids.

(ii)Test for alkaloids

Methanolic extract of the plant materials were evaporated to dryness on a water bath, (5ml) of 2N hydrochloric acid were added and the solutions were heated with stirring in a water bath for 10 minutes. The cooled solutions were filtered. To a portion (5ml) of the solution, few drops of Dragendroff's reagent were added. Formation of a precipitate(or turbidity)indicates the presence of alkaloids.

(iii) Test for Tannins

Methanolic extracts of the plant materials were separately evaporated to dryness on a water bath and the residues were extracted with n-hexane and filtered. Hexane –insoluble portions were stirred with 10ml of hot saline solution (0.9% w/v) of sodium chloride and freshly prepared distilled H_2O.

The mixtures were cooled and filtered and the volume adjusted to 10ml with more saline solution. 5ml of this solution was treated with, few drops of, ferric chloride solution, a blue color is considered positive for tannins.

(iv)Test for steroids

Few drops of the plant extracts were taken in a filter paper and a few drops of Rhodamin B reagent were added , a violet colour is considered positive for steroids.

(v)Test for glycosides

Ten grams of dried powdered plant materials were taken in a clean test tube , 10 ml of distillated water was added and the suspension was well shaken for about 30 seconds ,if a formed froth persisted for one hour then the test is considered positive for glycosides .

2.2.4- Preparation of UV shift reagents

Stock solution of sodium methoxide, aluminum chloride, boric acid and hydrochloric acid were prepared (Mabry, 1970) as follows:

(i) Sodium methoxide

Freshly cut metallic sodium (2.5g) cautiously was added in small portions to 100ml dry spectroscopic methanol. The solution was stored in a glass container with a tightly fitting, plastic.

(ii)Aluminum chloride

Five grams of fresh anhydrous aluminum chloride were cautiously added to spectroscopic methanol (100ml).

(iii) Hydrochloric acid

50ml of concentrated hydrochloric acid were mixed with 100 ml distilled water and stored in a glass stoppered bottle.

(iv) Boric acid

100mls of spectroscopic methanol were saturated with anhydrous boric acid.

(v) Sodium acetate

Anhydrous NaOAc was used.

2.2.5- Ultraviolet analysis

Pure samples dissolved in methanol, were subjected to UV spectrophotometric investigation in a 4 mls capacity quartz cells using Shimadzu UV Spectrophotometer -Model UV 240 .

The $AlCl_3$, $AlCl_3$/ HCl, fused NaOAc/ H_3BO_3 and NaOMe reagents were, separately, added to methanolic solutions of the investigated materials and UV measurements were then recorded according the following steps:

(i) A stock solution of the flavonoid was prepared by dissolving a small amount of the compound (about 0.1 mg)in10 ml spectroscopic methanol.

(ii)The methanol spectrum was measured using 2 – 3 ml of the stock solution of the flavonoid.

(iii)Three drops of a freshly prepared solution of sodium methoxide were added to the methanolic solution and, immediately, recorded. After 5 minutes , the spectrum was rerun to check for flavonoids decomposition.

(iv) $AlCl_3$ spectrum was measured, immediately, after the addition of the six drops of the aluminum chloride stock solution to 2 – 3 ml of fresh stock solution of the flavonoid.

(v) $AlCl_3$/ HCl spectrum was recorded immediately after the addition of three drops of the stock solution of HCl to the solution containing $AlCl_3$ (step iv)

(vi) NaOAc spectrum was determined by the addition of excess, coarsely, powdered anhydrous sodium acetate to 2-3 ml fresh stock solution of the flavonoid and shaking the cuvette (about 2 mm layer of NaOAc remained at the bottom of the cuvette) and the spectrum was recorded immediately and then after 10 min. to check for flavonoid decomposition.

(vii) NaOAc/ H_3BO_3 spectrum was recorded after the addition of powdered anhydrous H_3BO_3 to give a saturated solution to the cuvette from (step vi)).

2.2.6-IR Spectroscopy

IR spectra of the isolated compounds were recorded using infra red spectrophotometer (FTIR-84005 Shimadzu Japan).

2.2.7-Nuclear magnetic resonance

NMR spectrometry was performed on a Bruker 300 MHz and 600MHzBruker Ultra shield spectrophotometer at 300K (25C^0). The solvent used was DMSO . Chemical shifts were reported in parts per million (ppm) on the δ- scale and coupling constant are given in Hz, chemical shifts are expressed relative to tetramethylsilane (TMS 0 ppm) as internal reference. The data analysed by Spectra –Software Cairo University Micro analytical Center .

2.2.8-Fast atom bombardment (FAB) Mass Spectrometry

All (FAB) mass spectra were recorded on DI analysis Shimadzu Qp 2010 Plus , Mass Spectrometer .

2.2.9-Thin layer chromatography

Qualitative thin layer chromatography (TLC) was performed on pre-coated Loba silica gel sheets (loba H05704). After developing the plates in the suitable solvent system, the plates were sprayed with ferric chloride solution. Preparativescale thin layer chromatography (PTLC) was performed on glass plates (20x20cm) coated with silica gel (Loba H05704) which were dried overnight at room temperature. The plate (loaded with 10-15) mg of material per plate were developed with an appropriate solvent system and dried in a stream of air. The bands were distinguished by UV (334nm) light and scratched off. Compounds were eluted from the adsorbent (silica gecl) with acetone which was removed on a rotary evaporator under reduced pressure at 40-50 C^{o}. The following abbreviations were used in the description of the solvent systems used during the development of TLC plates: Butanol : acitic acid : water (4:1:2)for *Acacia nubica benth* ; Butanol : acitic acid : watet (5:1:2)for *Citrus paradise* and chloroform : methanol (4:1) for *Hartmonnina Schweinf*.

2.2.9.1-Spray reagents for TLC

(i) Ferric Chloride / sulfuric acid

All TLC plates were sprayed lightly with a Spray with $FeCl_3$ solution of 2g $FeCl_3$ in 83ml n-butanol and 15ml conc sulfuric acid.The plates were then heated 110°C and Viewed at 5 min intervals to see if spots appear at 254 and 360nm.

(ii) Vanillin-sulphuric acid

TLC plates were sprayed with a (1%w/v) solution of vanillin in concentrated sulphuric acid (96%) and left to dry in air.

2.2.10-Antimicrobial assay

The isolated flavonoids were screened for their antimicrobial activity against five standards human pathogens (*Bacillus cereus, Bacillus subtilis, staphylococcus aureus, Escherichia coli, Pseudomonas aeruginose* and *Candida albicans*) using the cup agar method with some minor modifications.

2.2.10.1-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto hutment agar slopes and incubated at 37 c° for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100ml of normal saline to produce a suspension containing about $10^8 - 10^4$ colony forming units per ml. The suspension was stored in the

refrigerator at 4° C until used. The average number of viable organism per ml of the saline suspension was determined surface viable counting technique.

Serial dilution of the stock suspension were made in sterile saline in tubes and one drop volumes (0-20ml) of the appropriate dilution were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for drop to dry, and then incubated at 37c° for 24 hours.

2.2.10.2- Preparation of fungal suspensions

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

2.2.10.3-Testing for antibacterial activity

The cub-plate agar diffusion method was adopted ,with some minor modifications, to assess the antibacterial activity of the isolated phytochemicals. 2ml of the standardized bacterial stock suspension were mixed with 200ml of sterile molten nutrient agar which was maintained at 45 c° in water bath.

20ml Aliquots of the incubated nutrient agar were distribution into sterile Petri dishes; the agar was left to settle

in each of these plates which were divided into two halves. Two cubs in each half (10mm in diameter) were cut using sterile cork borer (No.4). Each of the halves was designed for each test. Separate Petri dishes were designed for standard antibiotic (ampicillin and gentamycin).

The agar discs were removed, a hamates cup were filled with 0.1ml samples of each compound using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37c° for 24 hours.

The above procedure was repeated for different concentrations of the isolated compounds and the standard antibiotics. After incubation, the diameters of resultant growth inhabitation zones were measured.

2.2.10.4-Testing for antifungal activity

The above mentioned method was adopted for antifungal activity, but instead of nutrient agar saturated dextrose agar was used at the extracted concentrations.

3-Results and Discussion

3.1- Phytochemical screening

The leaves of *Acacia nubica* Benth(Laot) , *Citrus paradisi* (grapefruit) and bark of *Combretum Hartmonnina* Schweinf (Habeel) were subjected to a phytochemical screening and the results are depicted in Table 3.1.

3.2- Spectral data of compound I

Compound I was isolated from *Combretum Hartmonnina* (Habeel), and its structure was elucidated using spectroscopy . Fig. 3.1 showed the IR spectrum of compound1 ν (KBr) 3423 (OH), 1656 (C=O), 1585,1515 and 1423 (C=C, Ar), 1103 (C-O), 2852, and 939 cm^{-1} (C-H, Ar, bending).

Compound 1 cannot be an anthocyanin or a catechin since the IR spectrum gave carbonyl stretching at 1656cm^{-1}. It could be one of (flavone, flavonol, flavanone, chalcone or aurone).

The UV spectrum of flavonoids usually consists of two absorption maxima in the ranges of 240-285 nm (Band II, due to the A-ring absorption), and 300-400 nm (Band I, due to the B-ring absorption). The precise position and relative intensities

of these maxima give valuable information regarding the nature of the flavonoid and its oxygenation pattern (Mabry ,1970). (Table 3.2).

Table 3.1 : Phytochemical Screening of studied species

Plants	Flavonoid	Alkaloid	Tannin	steroid	glycoside
Acacia nubica	+	+	+	+	+
Citrus paradisi	+	+	+	+	+
Combretum Hartmonnina	+	+	+	+	+

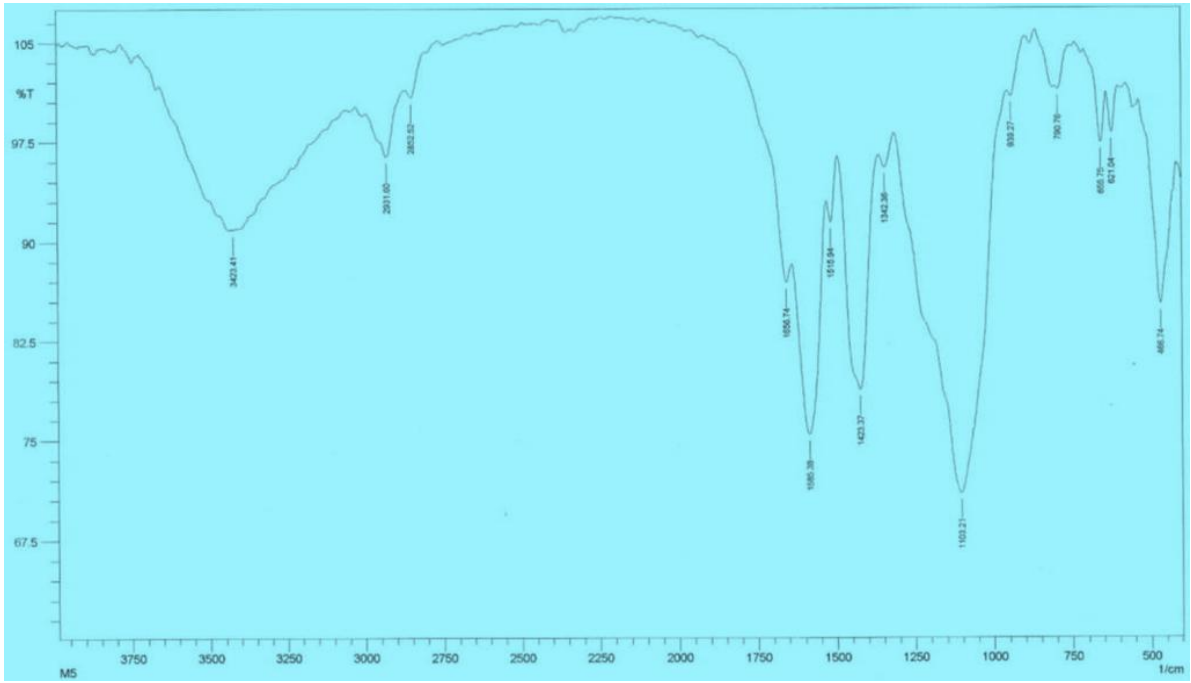


Fig. 3.1 : IR spectrum of compound I

Table 3.2: the UV absorption of flavones, flavonols chalcones and aurones

Flavonoid class	Band I	Band II
Flavones	304- 350	240 - 280
Flavonol(3-OH substituted	330 -360	250 - 280
Flavonols(3-OH free)	350-390	250-280
Isoflavones	shoulder	245 - 270
Flavanones	shoulder	270 - 295
Dihydroflvonols	shoulder	270 - 295
Chalcones	365-390	240-260
Aurones	390-430	240-270

Fig. 3.2 shows a UV spectrum pattern characteristic of isoflavones(Mabry ,1970) ; it revealed λ_{max} (MeOH) 266 nm (Band II) together with a shoulder at 310nm.

Next the hydroxylation pattern of the isolated isoflavone was investigated using UV shift reagents : sodium methoxide, sodium acetate, aluminium chloride and boric acid. These reagents locate the hydroxyl groups in the flavonoid nucleus , since they afford bathochromic shifts diagnostic of a specific hydroxylation pattern.The strong base , sodium methoxide, is diagnostic of 3- and 4`-hydroxyl grou. It reveals bathochromic shifts in the presence of such hydroxyl group , but in case of a 3-OH group, the shift is accompanied by a decrease in

intensity. In case of a 7-OH group , the shift reagent sodium acetate can provide a diagnostic bathochromic shift.

Aluminium chloride can chelate the 4-keto and 3-OH(or 5-OH) groups giving bathochromic shifts indicative of such complexes. It also chelates catechol moieties giving bathochromic shifts, catechol complexes may be differentiated from the 3- (or 5) OH complexes by their decomposition in acidic media. Boric acid is, like aluminium chloride, diagnostic of catechol systems , where it gives bathochromic shifts in the presence of such moieties.

Sodium methoxide spectrum of compound1 did not reveal any bathochromic shift (Fig. 3.3) indicating absence of a 3-OH , 5-OH as well as catechol systems.

The sodium acetate spectrum, gave a bathochromic shift (Fig.3.4), indicating the presence of a 7-OH group.

The boric acid spectrum (Fig.3.5) revealed a bathochromic shift, indicating the presence of a catechol system .

When $AlCl_3$ was added to a methanolic solution of compound I ,a bathochromic shift was observed (Fig. 3.6), indicating a catechol moiety.

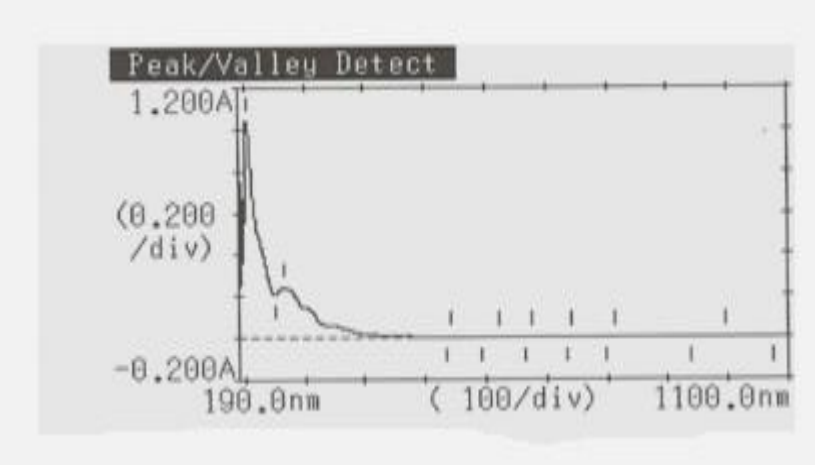


Fig.3.2 : UV spectrum of compound I in MeOH

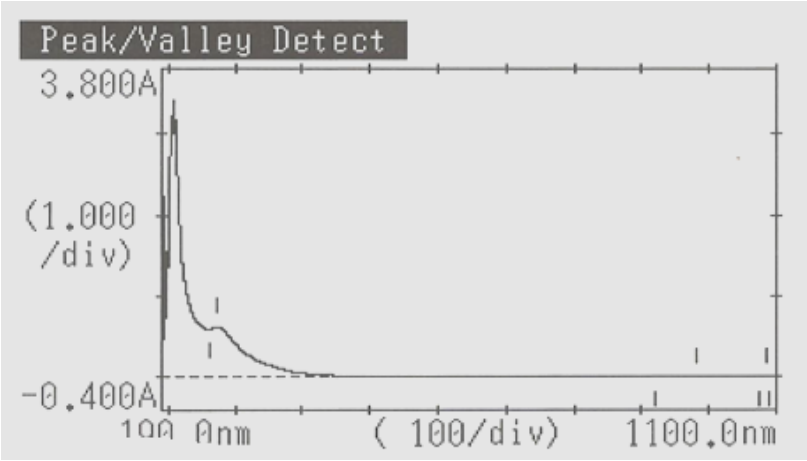


Fig.3.3 : Sodium methoxide uv spectrum of compound I

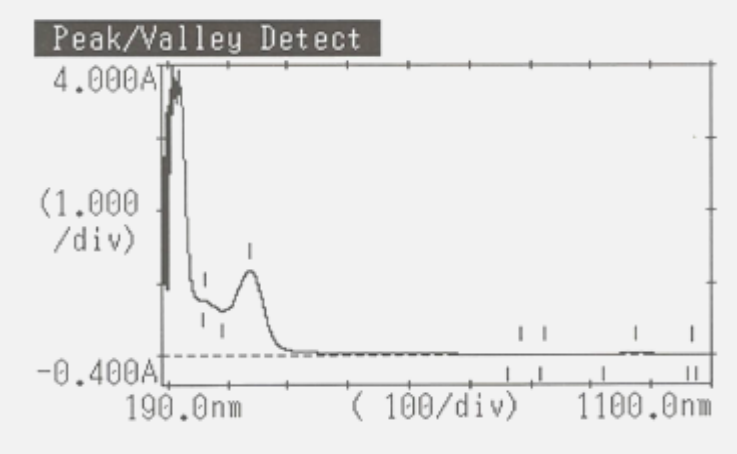


Fig.3.4 : UV spectrum of compound I in NaOAc

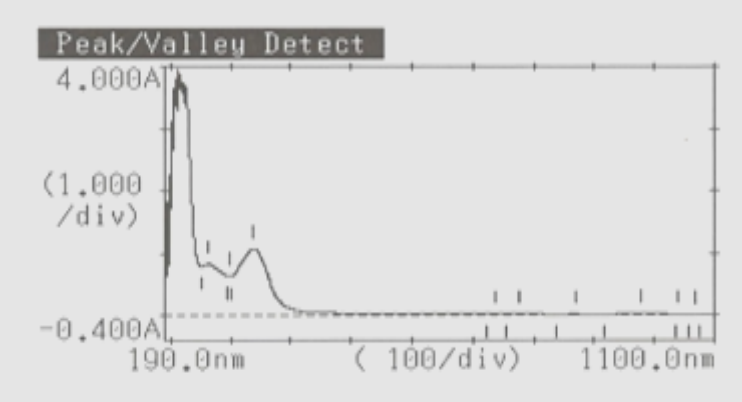


Fig.3.5 : UV spectrum of compound1 in NaOAc+H_3BO_3

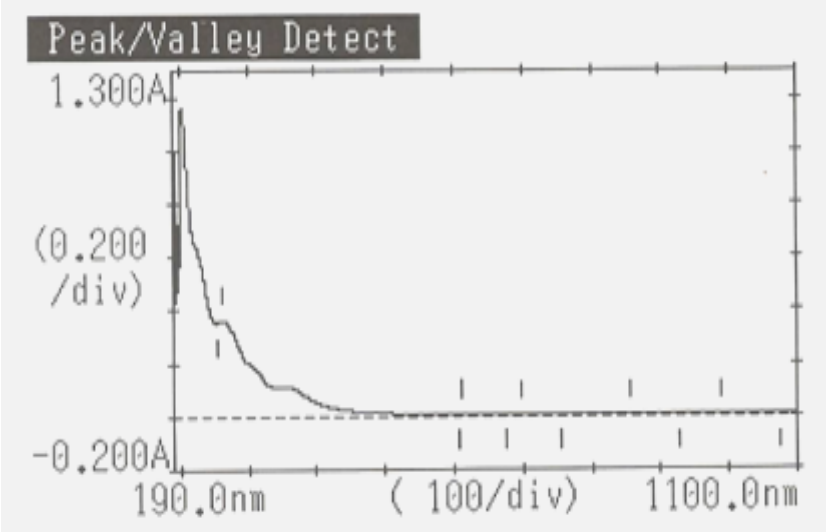
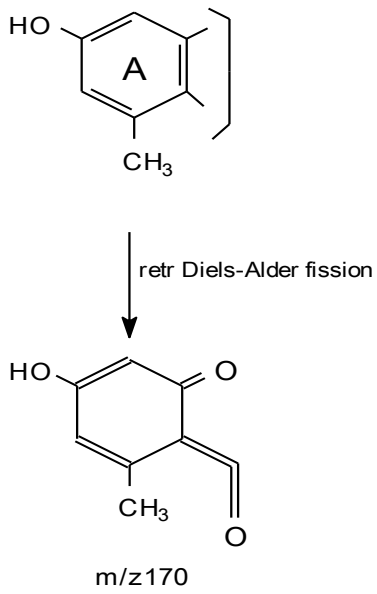


Fig.3.6 : The aluminium chloride spectrum of compound1

Fig.3.7 shows the ^{1}HNMR of compound1is shows a singlet at δ 1.2 ppm (3H),which is characteristic of a methyl group assigned to C_5.This was based on(i) the absence of a C_5 –H signal which, usually, resonates around 8ppm due to the deshielding influence of the 4-keto group. (ii) the retro Diels-Alder fission (scheme 3.1). The multiplet at δ3.45- 3.70 ppm (10H) accounts for a glucosyl moiety. C_6 proton resonates as a doublet at δ6.3(1H), while C_8 proton resonates at δ6.40(d,1H)ppm.The signal at δ6.74 was assigned C_5 –proton. The multiplet at δ6.5-6.8(3H) was assigned to ring B protons. C_2 vinylic proton of the isoflavone resonates downfield at δ7.72 ppm due to the deshielding influence of the neighbouring O atom.

Fig.3.8 show the mass spectrum which gave m/z284 for the molecular ion.The fragment at m/z134 , which results from the retro Diels –Alder cleavage (Scheme 3.2) indicates, clearly, that the catechol moiety- dictated by the aluminium chloride UV spectrum- is located at ring B. Furthermore the appearance of ring B protons as a multiplet at δ6.3-6.8(3H) together with the absence of a 4`-OH group,(sodium methoxide UV spectrum),this suggest that the catechol is cited either at the 2`,3` or the 5`,6`- positions.Citation of catechol at the 3`,4`-positions is not supported by the methanol UV spectrum in which suggests splitting of band II in presence of such hydroxylation pattern.

Another important fragment (m/z 170) resulting from the retro Diels – Alder fission suggests the following substitution pattern for the A ring:



Scheme 3.1

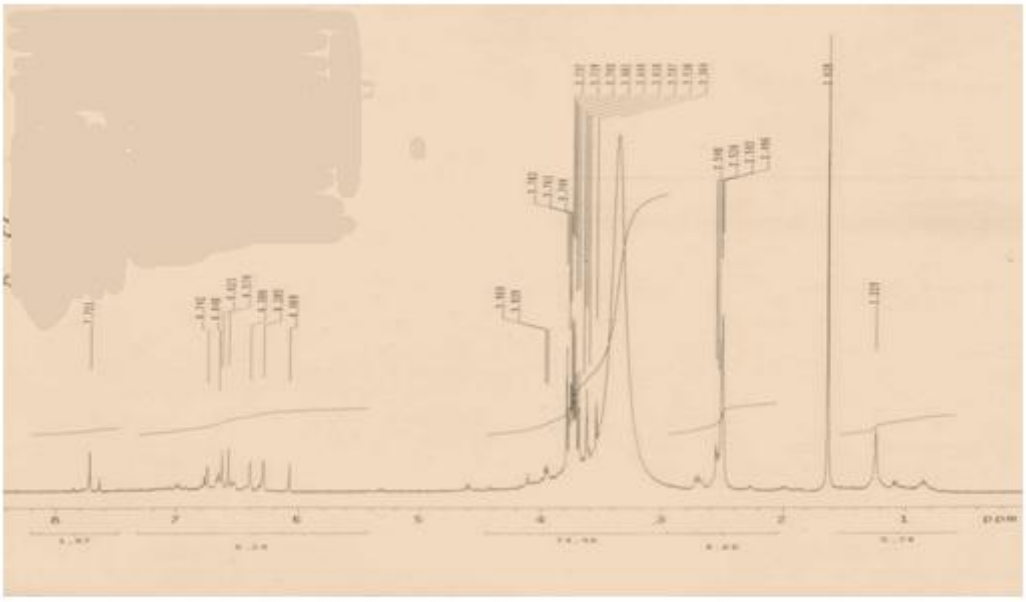


Fig.3.7: ^{1}HNMR of compound I

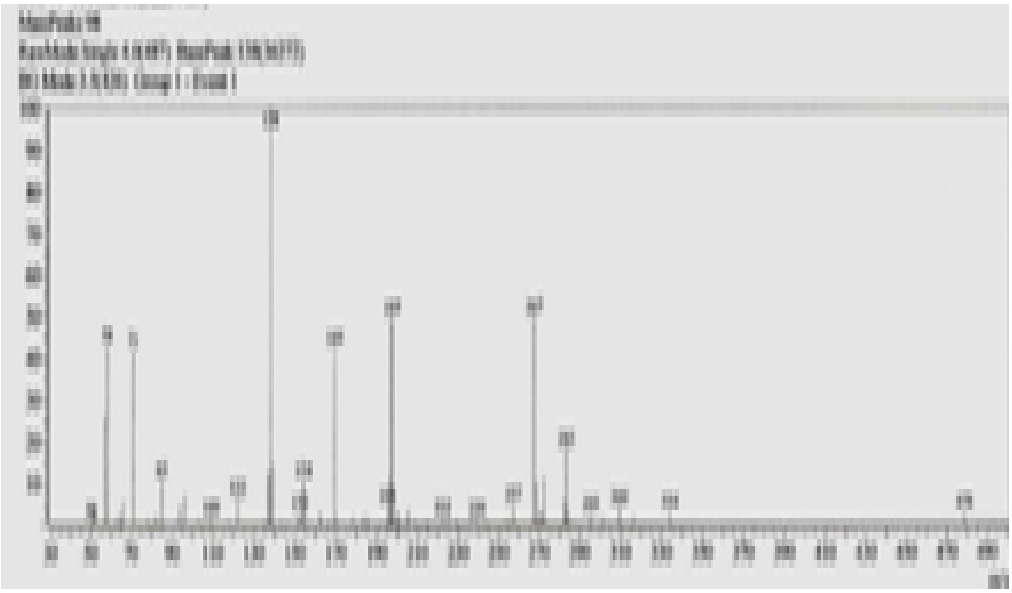
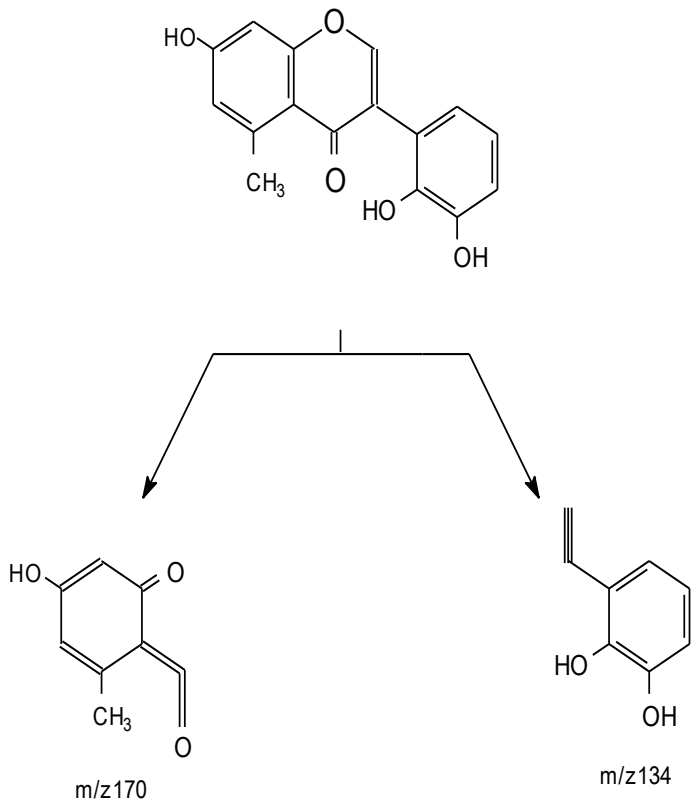
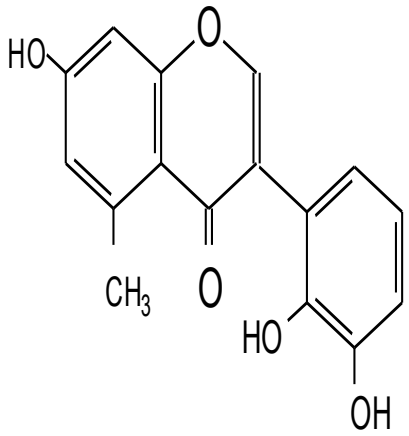


Fig.3.8: Mass spectrum of compound I



Scheme 3.2 : Retro Diels-Alder fission of compound I

On the bases of the above cumulative data the following structure was suggested for **compound I** :



3.3- Spectral data of compound II

Compound II was isolated from *Acacia nubica*, and its structure was elucidated using spectral tools. The IR spectrum (Fig.3.9) showed ν (KBr) 3392 (OH), 1619 (C=O), 1575 (C=C, Ar), 1091 (C-O), 780, and 651 cm^{-1} (C-H, Ar, bending).

Compound II cannot be an anthocyanin or a catechin since the IR spectrum gave carbonyl stretching at 1419cm^{-1}. It could be : a flavone, flavonol, flavanone, chalcone or aurone.

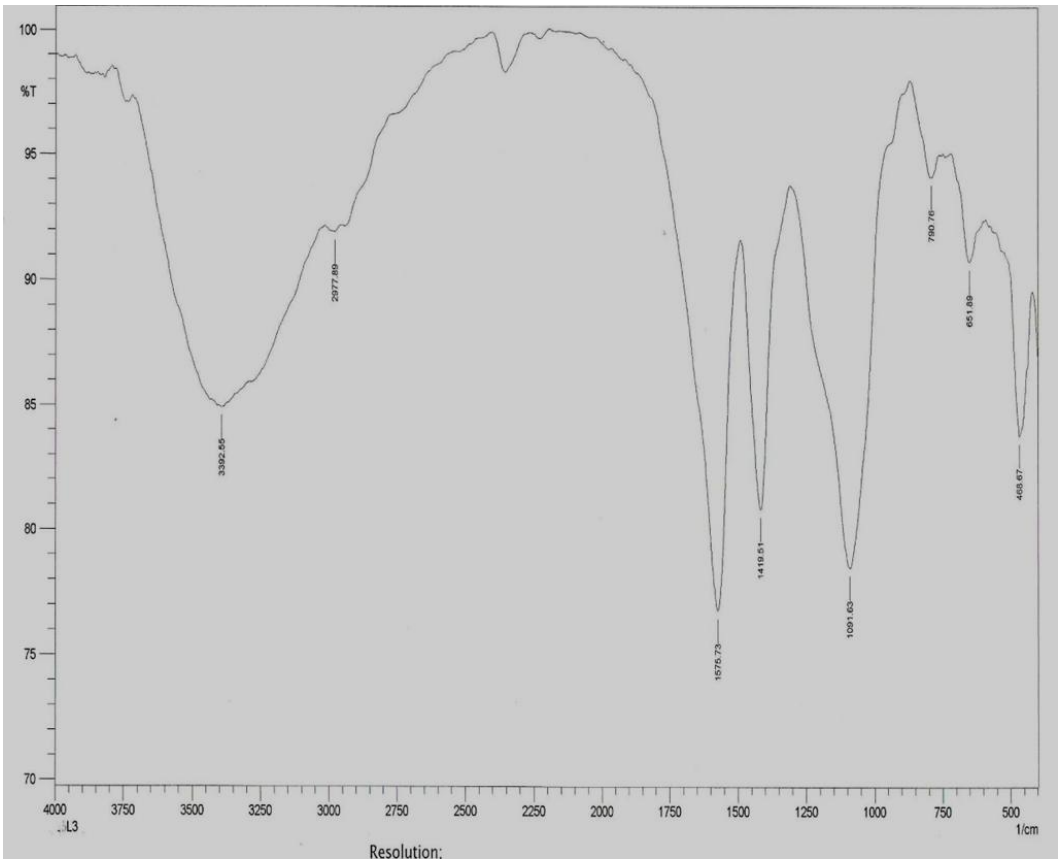


Fig. 3.9 : IR spectrum of compound II

The UV spectrum revealed λ_{max} (MeOH) 273 nm (Band II) (Fig. 3.10).Such absorption is characteristic of : isoflavones, flavanones and dihydroflavonols.

However, no shoulder in the region 300-340nm –which is characteristic of flavanones was detected in the UV spectrum. Also C_3 is not bearing a hydroxyl function as indicated by the sodium methoxide spectrum (see below). Such data suggests a flavanone skeleton.

Addition of NaOMe to a methanolic solution of compound II gave a 61 nm bathochromic shift (Fig. 3.11) with increase in intensity indicating a free 4`-OH group.

Next the UV shift reagents : sodium acetate, boric acid , aluminium chloride we employed to elucidate the hydroxylation pattern on the flavanone nucleus.

Fig. 3.12 the sodium acetate spectrum of compound II which show a 61 nm bathochromic shift indicative of a 7-OH group.

The boric acid spectrum (Fig.3.13) revealed a bathochromic shift which is diagnostic of a chetechol moiety.

When $AlCl_3$ was added to a methanolic solution of compound I a bathochromic shift was observed (Fig. 3.14). This indicates the presence of a 5-OH function.

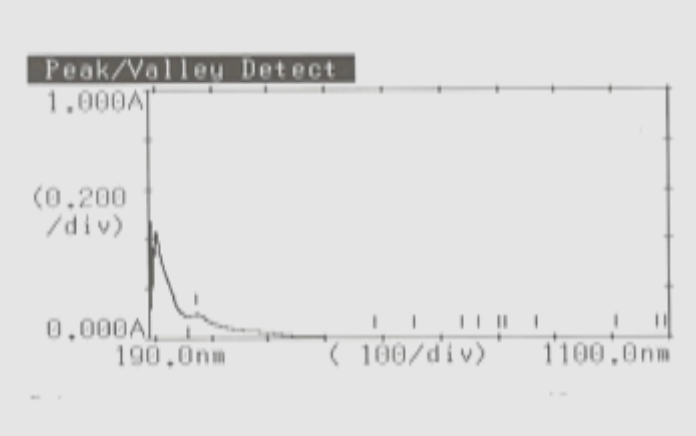


Fig3.10 : UV spectrum of compound11 in MeOH

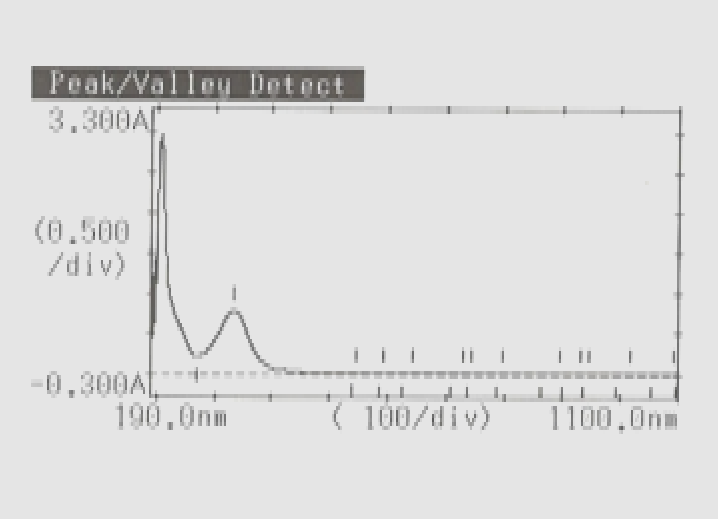


Fig.3.11 : Sodium methoxide spectrum of compound II

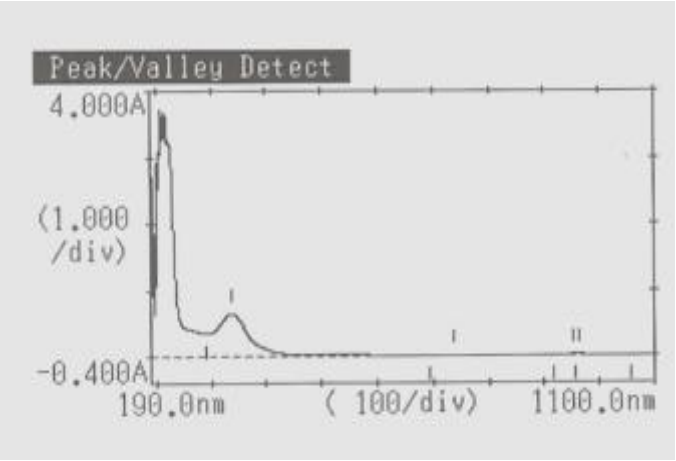


Fig.3.12 : Sodium acetate spectrum of compound II

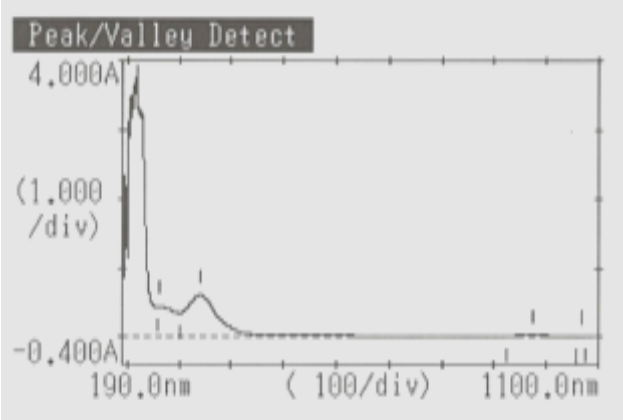


Fig.3.13 :Boric acid uv spectrum of compound II

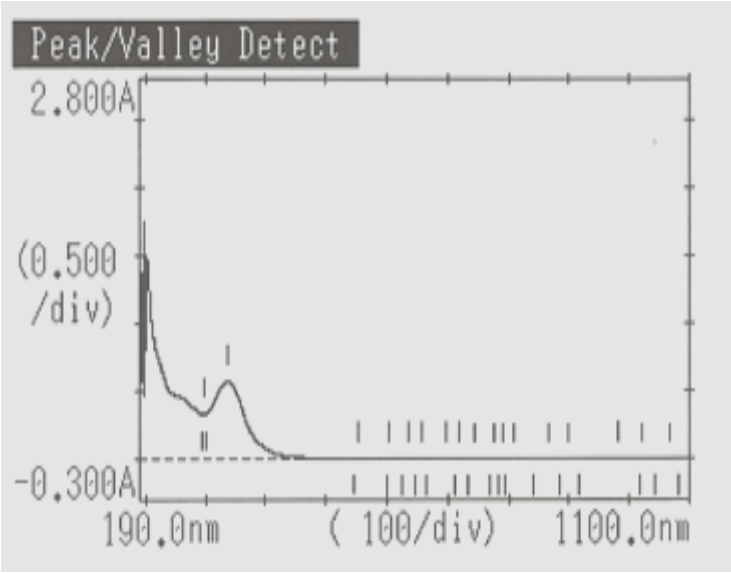


Fig.3.14 : Aluminium chloride uv spectrum of compound II

The ^{1}HNMR ,Fig.3.15, showed a pattern characteristic of flavonones. The signal at δ1.62 (s,2H) is characteristic of C_3 methylene.The resonance at δ3.42(s,12H) accounts for five methoxyls , while the singlet at δ5.60 was assigned for the methine proton at C_2.

The mass spectrum ,Fig.3.16, gave m/z438 for the molecular ion.The pattern of substitution in A and B rings was further confirmed by the retro Diels-Alder fission, (Scheme 3.2) where the ions ,corresponding to intact A and B rings ,were detected in the electron beam at m/z166 and m/z212 respectvely.

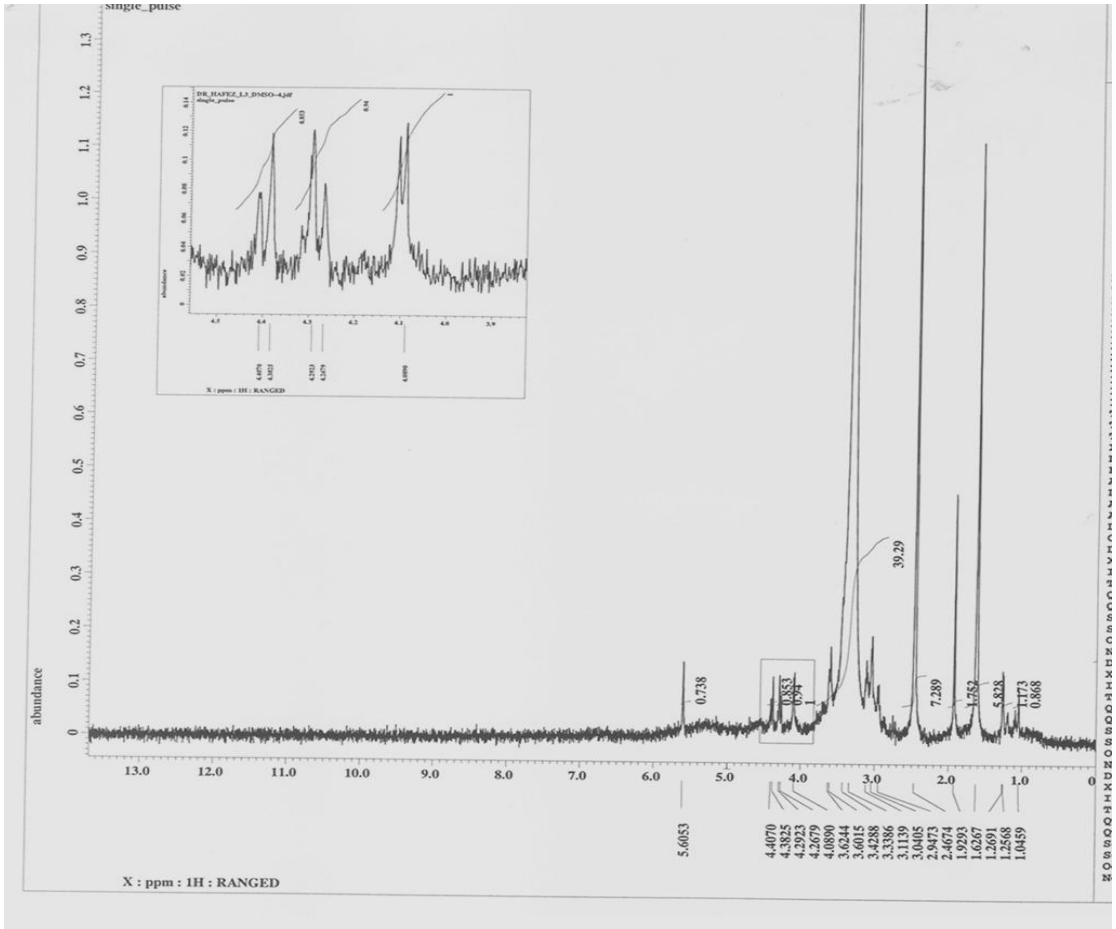


Fig.3.15 : 1HNMR of compound II

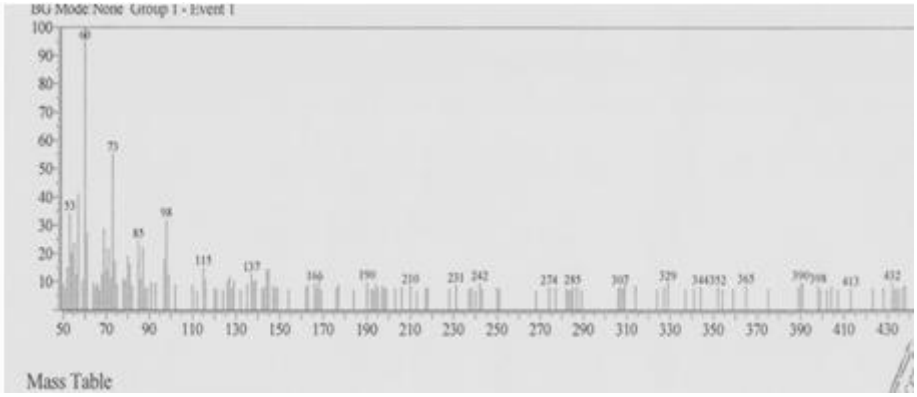
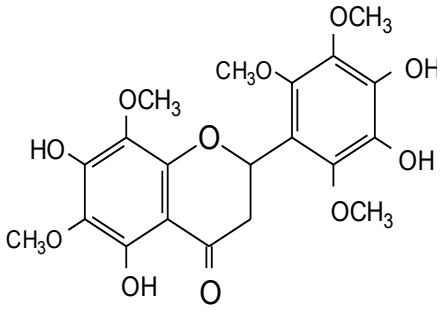
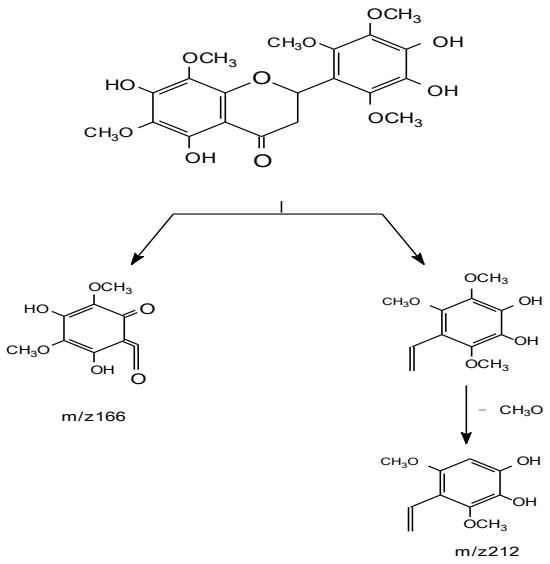


Fig.3.16: Mass spectrum of compound II

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



5,7,4´,5´-tetrahydroxy-6,8,1´,2´,6´-pentamethoxyflavanone



Scheme 3.2: Retro Diels-Alder fission of compound II

3.4- Spectral data of compound III

Compound III was isolated from *Citrus paradisi* and its structure elucidated using spectral tools. The IR (KBr disc) spectrum (Fig.3.17) showed ν (KBr) 3375 (OH), 1641 (C=O), 1515 (C=C, Ar), 1027 (C-O), 833, and 686 cm^{-1} (C-H, Ar, bending).

Compound III cannot be an anthocyanin or a catechin since the IR spectrum gave carbonyl stretching at 1641cm^{-1}. It could be : flavone, flavonol, flavanone, chalcone or aurone.

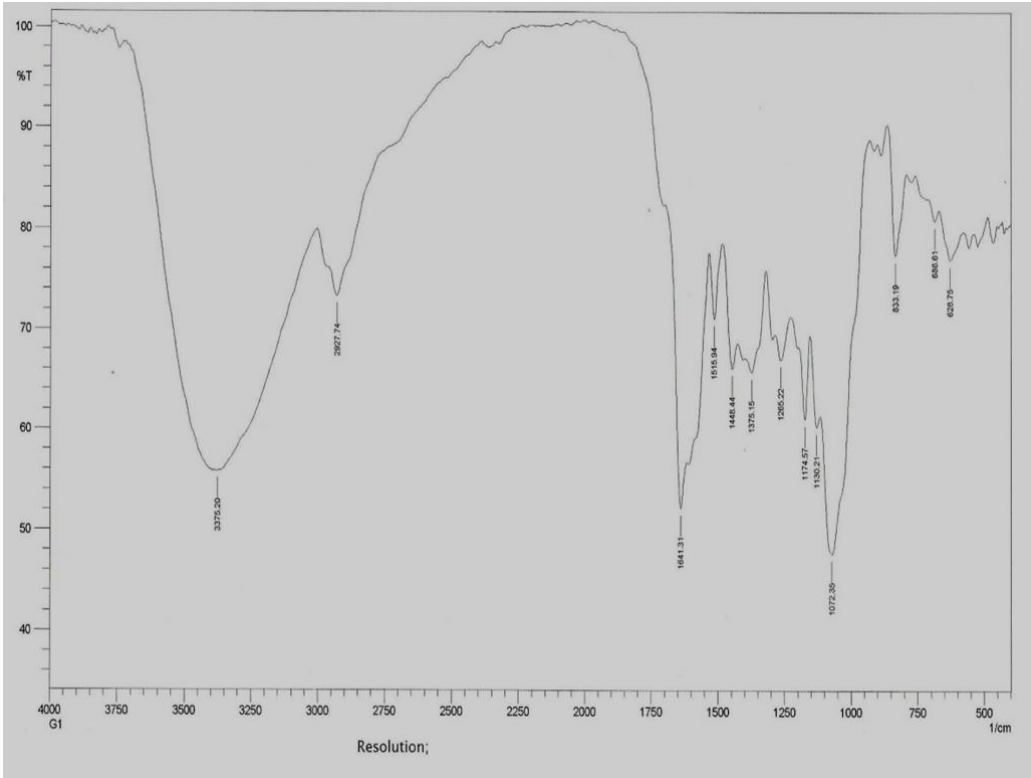


Fig.3.17: IR spectrum of compound III

The UV spectrum gave a pattern characteristic of isoflavones since it revealed λ_{max} (MeOH) 283 nm (Band II) together with a shoulder at 320nm (Fig.3.18).

Next the hydroxylation pattern of the isoflavone was investigated by using UV shift reagents.

Addition of sodium methoxide to a methanolic solution of compound III gave a 41 nm bathochromic shift (Fig. 3.19) without decrease in intensity indicating a free 4`-OH.

The sodium acetate spectrum gave no bathochromic shift band II (Fig. 3.20) . This indicates absence of a 7-OH function.

The boric acid spectrum (Fig. 3.21) did not reveal a bathochromic shift indicating absence of catechol systems.

When $AlCl_3$ was added to a methanolic solution, a 21 nm bathochromic shift (Fig. 3.22) was observed . This indicates the presence of a 5-OH function.

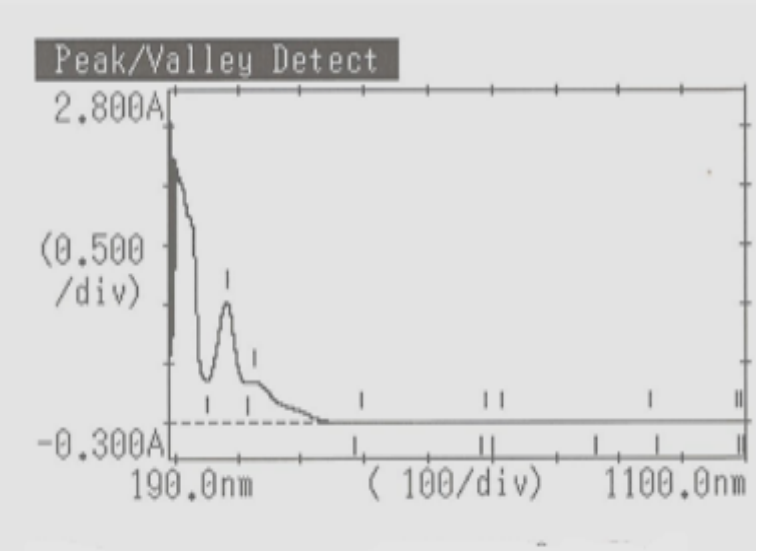


Fig.3.18 :The UV spectrum of compound III

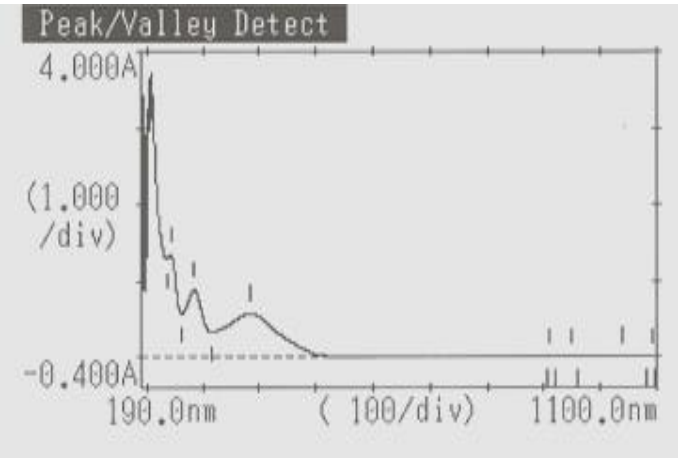


Fig.3.19 :Sodium methoxide spectrum of compound III

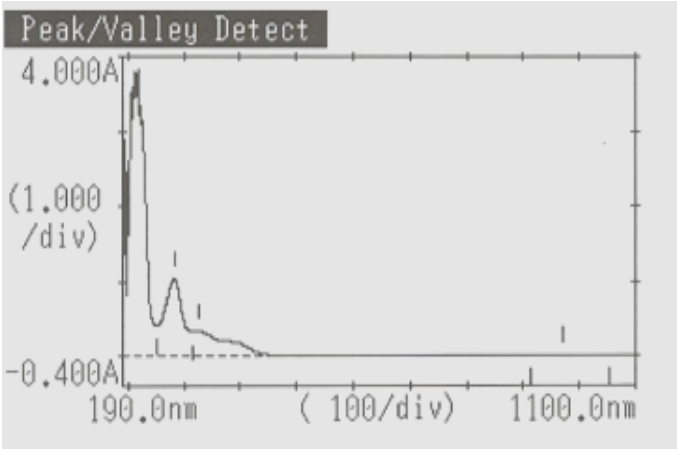


Fig.3.20: Sodium acetate spectrum of compound III

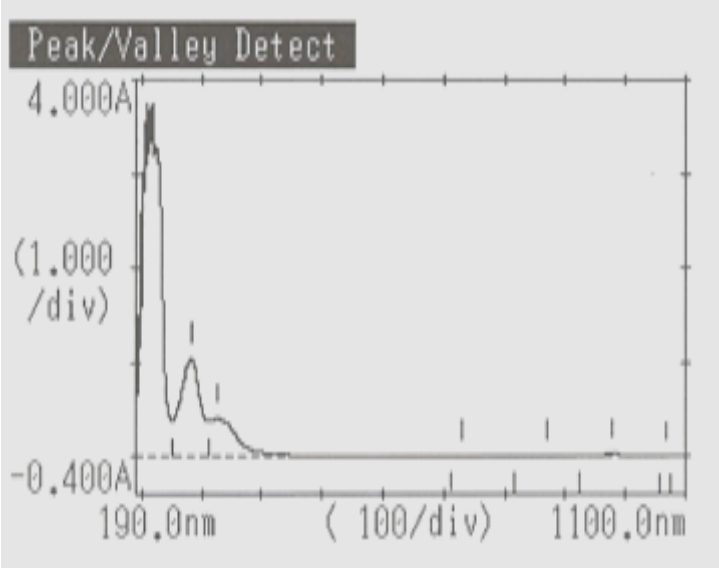


Fig.3.21: Boric acid spectrum of compound III

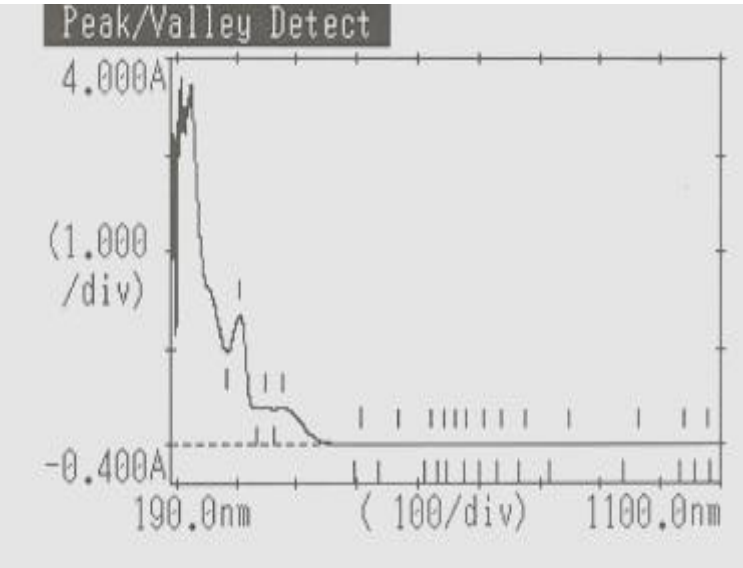


Fig.3.22: Aluminium chloride spectrum of compound III

The ^{1}HNMR of compound III (Fig. 3.23) showed a singlet at δ5.08 (1H), characteristic of C_2 vinylic proton.The signal was shifted downfield due to the deshielding influence of the neighbouring O atom. The C_6 proton resonates as a doublet at δ6.75 (1H), while the doublet at δ 6.76 accounts for C_8 proton. The double doublet at δ7.45(1H) and δ7.50 (1H) was assigned for $C_{2`,3`}$ and $C_{5`,6`}$ protons respectively. The signal at δ3.39(6H) is characteristic of a glucosyl moiety.

The mass spectrum (Fig.3.24) gave m/z448 for the molecular ion. Other important fragments resulting from the retro Diels-Alder fission(scheme 3.3) were shown in the electron beam at m/z118,m/z119 and m/z136. Such fragments result from intact A and B rings

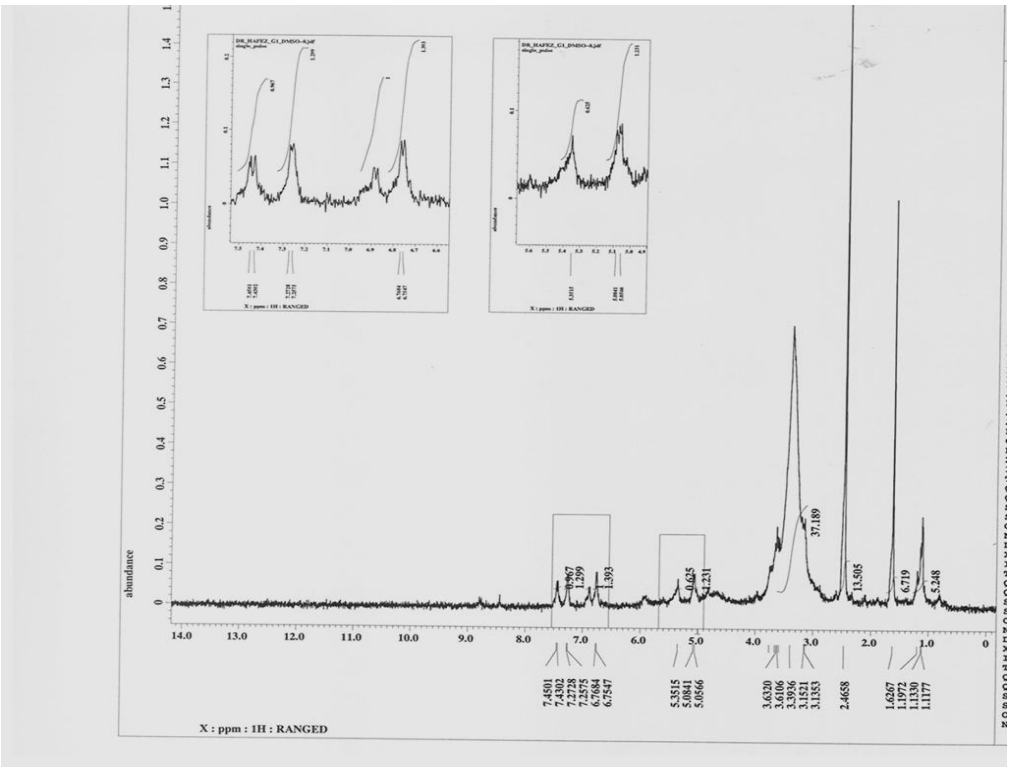


Fig.3.23 : ^{1}HNMR of compound III

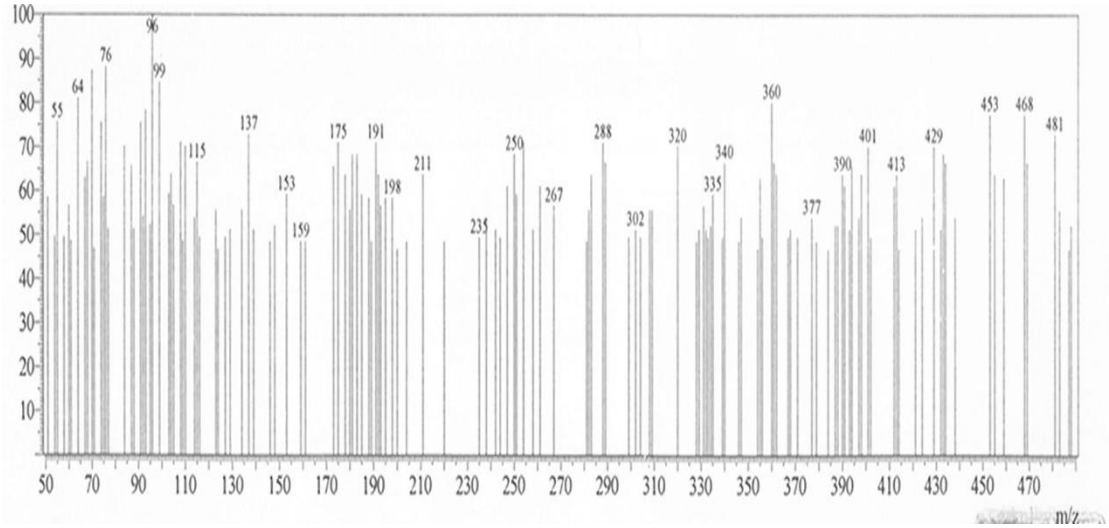
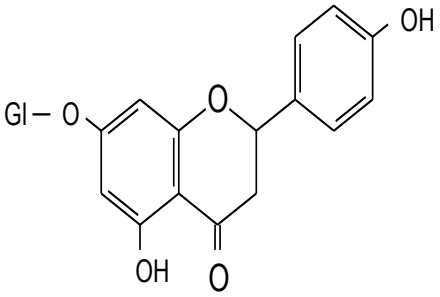
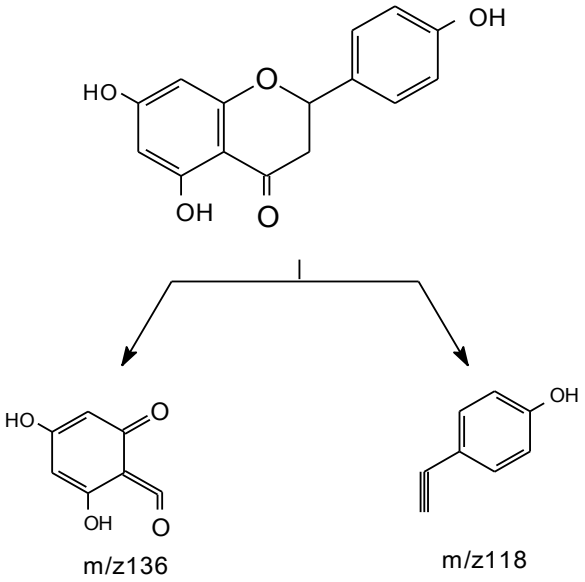


Fig. 3.24: Mass spectrum of compound III

On the basis of the above argument the following structure was assigned for compound III:



7-o-glycosyl-5,4´-dihydroxyflavanone



Scheme 3.2: Retro Diels –Alder fission of compound III

3.5. Biological Activity

The antimicrobial activity of the test compounds was examined with Gram positive bacteria *Bacillus subtilis* , *Bacillus cereus*, and *staphylococcus aureus*, Gram negative *bacteria Escherichia coli*, *pseud*om*onas aeruginose* and fungus *candida albicans* .The results obtained are compared with a reference antibiotic (gentamycin).

Table 3.3 reveals the variation of antimicrobial activity of compounds (I ,II and III) have different antimicrobial responses.

As shown in Table 3.3, compound I exhibited antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* . Compound II was active against all tested organisms except Gram positive *Bacillus cereus*. Compound III showed antibacterial activity , but it was inactive against *Bacillus cereus* and the Gram positive bacteria *Staphylococcus aureus* , moreover all tested compounds showed antifungal activity against the human pathogen *Candida albicans*.

Table *3.3* The antimicrobial activities of the tested compounds (inhibition zone diameter mm/mg sample).

Microorganism	Gram stain reaction	Compounds		
		I	II	III
Bacillus cereus	positive	13	11	14
Bacillus subtilis	positive	21	15	17
Escherichia coli	negative	10	22	15
Pseudomonas aeruginosa	Positive	13	22	20
Staphylococcus aureus	Positive	20	18	14
Candida albicans	fungus	17	18	20

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