بسم الله الرحمن الرحيم

Sudan University of Science and Technology

College of Post Graduate Studies

Chemical Characterization of Some Flavonoids from *Trianthema prot ulacstrum* and *phoenix dactylefrera* and Their Biological Activity

التوصيف الكيميائي لبعض فلافونيدات نباتي الربعة والنخيل والفعالية البيولوجية للمستخلصات

A Thesis in Fulfillment of the Requirement of the Ph.D . Degree in Chemistry

By

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الآية

(قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيم)

سورة البقرة (32)

Dedication

To

My Parents,

My brothers and my sisters

Acknowledgement

First of all, I would like to thank Almighty Allah, Most Merciful for giving me health, to complete this study.

I would like to thank my supervisor Prof... Mohamed Abdel K arim, for his

Close supervision , continuous and valuable assistance and close gui dance,

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I am also grateful to the academic staff and technicians of the M edicinal and Aromatic Plants Research Institute for all facilities .

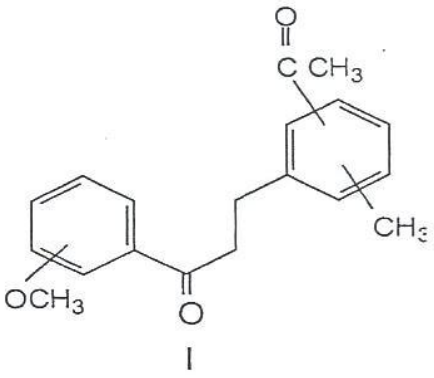
Thank are also due to the National Research Center, Cairo for t he Spectral data .

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

Abstract

In the present work, the stems of *Trianthema portulacastrum* and *Phoenix d actylefera* pollens were extracted with 95% ethanol at ambient temperature. Qualitative tests on alcoholic extract of *Trianthema portulacastrum* stems re vealed the presence of flavonoids, alkaloids, tannins, saponins, steroids, carb ohydrates and glycosides.

The crude extracts of *Trianthema portulacastrum* and *Phoenix daclylefera* w ere initially purified by column chromatography. Further purification was ac complished by TLC technique. In this way *Trianthema portulacastrum* gave two flavonoids (compounds I and II) while *Phoenix dactylefera* gave compo unds III and IV. The structures of these compounds were deduced on the bas is of their spectral data (UV, ^{1}HNMR and MS). The isolated compounds wer e assigned the following partial structures:



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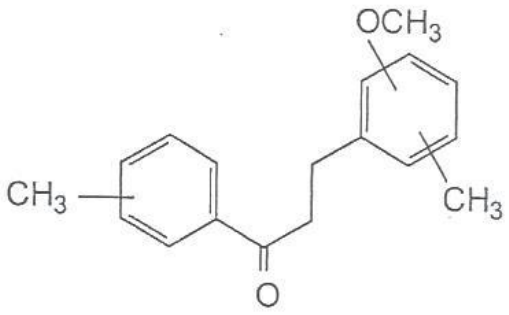

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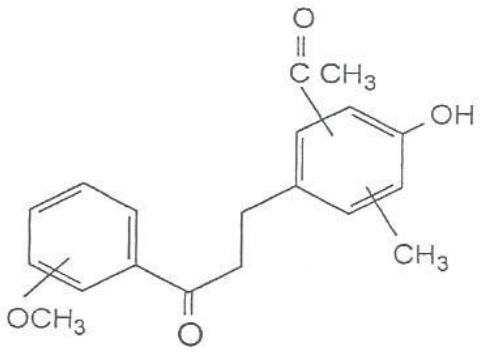

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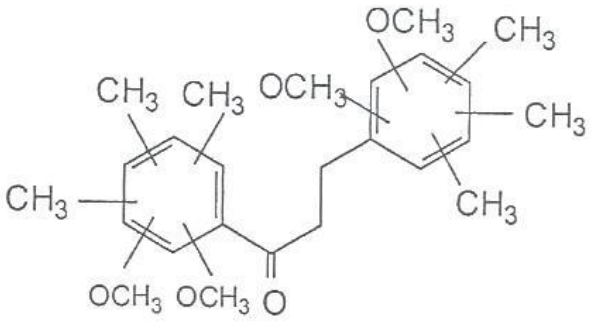


IV

Different fractions (ethanol, chloroform, ethyl acetate and n- butanol) of the *Trianthema portulacastrum* stems and *Phoenix dactylefera* pollens were assessed for antimicrobial activity against six standard pathogenic bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudo monas aeruginosa*, *Aspergillus niger* and *Candidia albacans*.

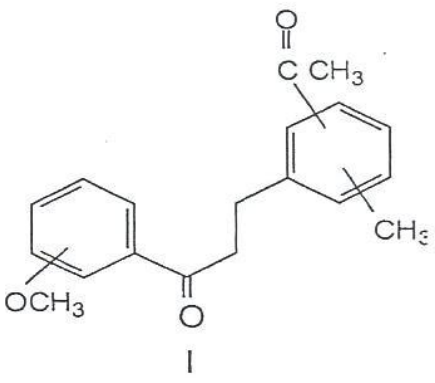
For *Trianthema portulacastrum* all fraction, except that of ethanol, show ed good antifungal activity. All fractions ,except that of n-butanol, exhibi ted good activity against *Bacillus subtilis*.

For *Phoenix dactylefera*, the ethyl acetate and n-butanol fractions showe d excellent activity against *Bacillus* subtilis and the yeast *Candidia albac ans* respectively. Also the butanol fraction gave good activity against *Pse udomonas aeruginosa*, *Aspergillus niger* and *Bacillus subtilis.*

المستخلص

تم اختيار اثنين من النباتات الطبية التي تنمو في السودان لدراسة المكونات الكيميائية الفعالة التي تـ حتويها تلك النباتات وهي: نبات الربعة وطلع النخيل.

استخلصت سوق نبات الربعة ولقاح التمر بواسطة الاثانول (95%)، ثم اجرى مسح فيتوكيميائي اثـ بت وجود الفلافونيدات، القلويدات، التنينات، الصابونينات، الاسترويدات، الكابوهايدريتات والانثراكوينـ انات. المستخلصات الكحولية للنباتين بكروموتوغرافيا العمود ثم عن طريق كروموتوغرافيا الطبقات الـ رقيقة. وبهذه الطريقة تم فصل مركبين من نبات الربعة (المركبين I, II) بينما اعطى طلع النخيل المر كبات (III, IV) وقد اقترح التراكيب المبدئية التالية لها:



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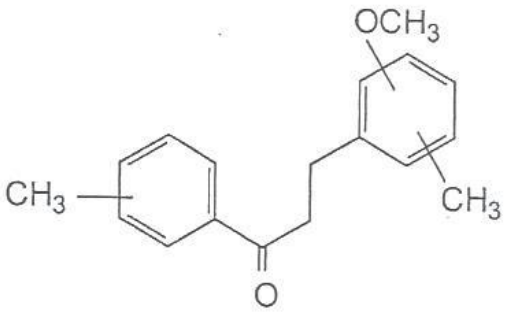

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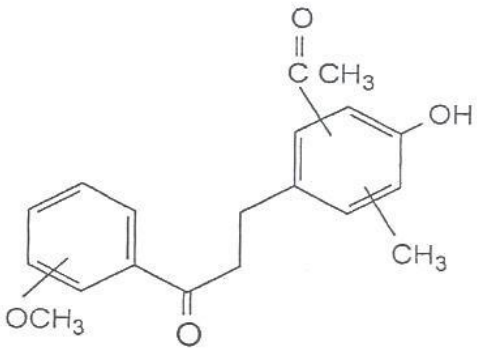

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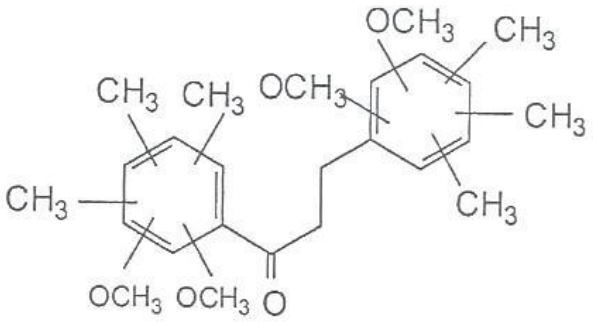

IV

جميع المستخلصات فعالية جيدة *Bacillus subitilis*. اما مستخلصات طلع النخيل فقد اعطى ومسد تخلصي اثيل استات والبيوتانول العادي فعالية ممتازة ضد*Bacillus subitilis* و *candidia alb cans*. ايضا ابدى المستخلص البيتونالولي فعالية ضد *Pseudomonas ae Bacillus subitilis, ruginosa* and *Aspergillus niger*.

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Chapter One
Introduction

1- Introduction

1-1 General approach

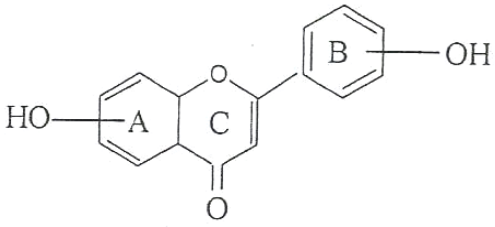
The study of flavonoids chemistry has emerged, like that of most natural products, from the search for new compounds with useful physiology properties. Semisynthetic endeavors of oligoflavonoid are in the most instances confined to those substitution patterns exhibited by monomeric natural products that are available in quantities sufficient for properties purposes. In order to alleviate these restrictions, served programs focusing on synthesis of enantiomeric pure flavonoids monomers have been undertaken[1] However, synthesis of the desired enantiomer in optically pure forms – remains a daunting objective is limited to only few types of compounds ; chalcones, epoxides, dihydroflavonoids, flavan-3-ols, flavan-3,4-diols, isoflavans, isoflavonones, and pterocarpans which have been synthesized in reasonable yield and purity.

The term flavonoids is generally used to describe abroad collection of natural product that include a C_6-C_3-C_6 carbon framework, or more specifically a phenylbenzopyran functionality.

Depending on the degree of oxidation and saturation present in the heterolytic ring, the flavonoids may be divided into seven classes: the flavones, flavonols, flavanones, isoflavones, chacones aurones, and anthocyanin .[1]

1.2 Flavonoids occurrence and importance

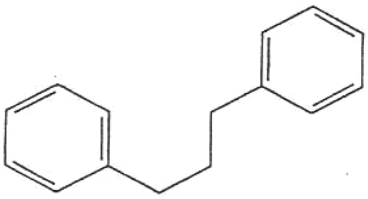
Polyphenols are compounds consisting of more than one aromatic ring wit h each containing at least one hydroxyl group as shown in {1} below[2].



{1}

Flavonoids are group of polyphenolic compounds. They are widely distrib uted through the plant kingdom.[3]the basic nucleus of flavonoids has 15 car bon atoms the three rings labeled A, B and C[4]. They have a characteristic backbone ring structure C_6- C_3 – C_6, namely biphenyl propane. The arom atic ring generally contain a number of phenolic hydroxyl groups[5] the "A "benzene ring arises by condensation of acetate units and "B" benzene rin g is constructed of phenyl propane units via shikmic acid pathway[6]

A and B rings are joined by a linear carbon chain, which may be open {2} or closed {3} as shown below.



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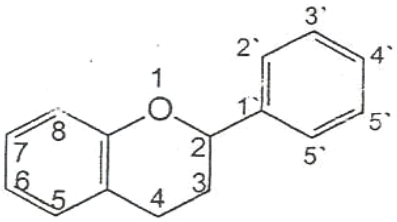
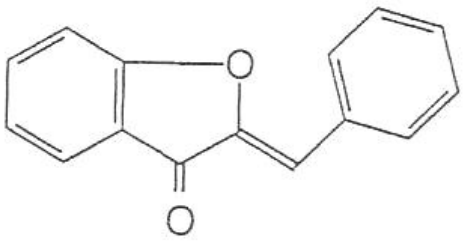


{3}

Flavonoid have a six –membered ring “C”, which in the 2 position carries a benzene ring “B” as substituent. Ring “C ”may be a heterocyclic pyrane, or pyrone ring[7] .In a few cases, the six membered heterocyclic ring “C” exist in an isomeric open form or is replaced by five membered ring, giving aurones (2-benzeylidenecoumaranone){4}[8] as shown below



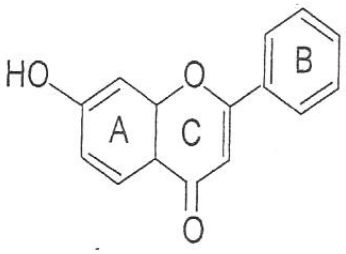
{4}

The flavonoid nucleus is normally linked to sugar. They occur naturally as water – soluble glycosylated and methylated derivatives.[9] the glycosidic l

inked are normally located in the 3- or 7- position, and are frequently hydr oxylated at the 4- position[10].

Generally the presence of sugars bound as glycoside in flavonoids may oc cur in a single plant in several glycosidic combinations[11].

For this reason when analyzing flavonoids it is usually better to examine t he a glycones present in hydrolysed plant extracts before considering the c omplexly of glycosides that may be present in the original extract. Flavon oids are structurally derived from the parent compound flavon e {5}. They bear phenolic hydroxyls and hence change in cooler when treated with ba se or ammonia. Thus flavonoids are easily detected on chromatograms or i n solution[4].



{5}

Flavonoids contain conjugated aromatic system and thus show intense abs orption band in UV light and visible regions of spectrum [4,12], for this reaso n some flavonoids are intensely colored, providing a spectrum of color fro m red to blue in most plant parts. Essentially colorless flavonoids produce, "whiteness"

of white flower. Beside their contribution to plant color, flavonoids have a variety of other roles in the growth, development, texture and taste of pla nt food[13].

Flavonoids form a class of water –soluble plant pigments. They are broken down into categories. One system breaks into flavones, flavonols, flavano nes, isoflavones, anthocyanins, chalcones[14,15], and seventh group. the auro nes[16] (table 1). All subclasses of flavonoids possess additional phenolic hy droxyl group at different position in ring A and B[17]. Flavonoids can also o ccur as dimers, in which two classes of flavonoids, mostly flavones and fla vanones are joined together.

Coupling may be composed of the same or different types of flavonoids, s uch as flavones- flavones or flavones – flavanones complex. They may be bounded together directly through their carbons and most often by C-8 and C-6 by C-O-C inter flavonyl link[18].

Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigment in most families (flowering plant). However their occurrence is not restrict ed to flower but include all parts of the plant[19] , root, heartwood, sap wood , bark, stem, leaf, fruit and seed. Some kinds of flavonoids are more chara cteristic of certain tissues[20] (table 2) which are present in all vascular plant s. Some classes are more widely distributed than others, while flavones an d flavonols are almost universal. Isoflavones and biflavonyls are found in few plant families[21].

The presence of flavonoids in plant is largely influenced by genetic factors and environmental condition. Other factors such as germination ,Degree of ripeness, processing and storage also influence the content of plant phe nolics[22,23].

Flavonoids are synonymous with the world color. They are responsible for the coloration of the flowers, fruits and sometimes the leaves. Colourless flavonoids are also abundant and many function as co-pigments[11]. The yel low colour of flower and fruit are derived from chalcones and aurones, w hilst the anthocyanin give rise to the red, blue and violet colors. These flav onoids also play an important role in the protection of the plant against the harmful and damaging effect of uv- radiation[24,25]. Flavonoids pigments fo und in plant play an important metabolic role[26,27]. also considered as impo rtant nutrients[28,29]. The function of the flower pigment in pollination is fai rly clear, insects and birds pollinators are attracted by coloured petals[30]. Fl avonoids have significant impact on various species of plant biology. The y exhibit a wide range of functions in physiology, biochemistry, and ecolo gy. Moreover, for long time flavonoids constitute useful tool in phytogene tic studies[31]. Flavonoids are believed to protect humans by providing prot ection against certain forms of cancer and reduction of cardiovascular dise ase[32]. Flavonoids are among the commonest of the secondary metabolites found in food plants possessing a wide spectrum of physiological effects[33] .the presence of flavonoids in foods and other compounds is thought to be

one of the reasons for the beneficial influence on human health[34]. They ha ve long been recognized in folkmedicine[35].

Finally, flavonoids possess strong anti-oxidative activity[36].as well as othe r potential beneficial effects including anti- inflammatory[37], anti-viral[38], a nti- atherosclerotic[39], anti-cancer[40],and anti- osteoptic effects[41].

1.3 Classification of flavonoid compounds

Flavonoids are sub-category of plant phenolics, they are widely distributed and characterized by the same basic structural elements[42,43]. Flavonoids ar e present in highly diversed classes of secondary plant metabolites with ab out 9000 structures[44].

Classification of flavonoids is based initially on study of solubility propert ies and color reaction. This is followed by a one –dimensional chromatogr aphic examination of hydrolyzed plant extract and a two –dimensional chr omatographic separation of direct alcoholic extract[4]. Flavonoids can be di vided into at least 7 different classes depending on their basic chemical str ucture. Flavonoids within a certain group, can be further subdivided into c lasses[4,45]. Flavonoids are classified by several methods. On the first metho ds, flavonoids are divided into two major groups according to the degree o f saturation of central heterocyclic ring, the unsaturated groups have plane r geometry and include flavones, flavonols and anthocyanins. The saturat ed groups is characterized by the absence of the 2,3- double bond and incl udes flavanones and flavans. These molecules normally have one or more chiral center[11]

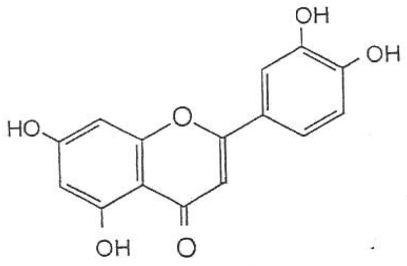
The remaining classes are: the chalcones, aurones and isoflavonoids[46]. in t he second method , flavonoids are classified according to the substitution pattern of ring C[47]., Both the oxidation state of the heterocyclic ring –C an d the position of the ring B are important in the classification. The major s ubgroups of flavonoids are: flavones, flavonols, flavanones, isoflavanone s, isoflavones, anthocyanines, chalcones and aurones[48].

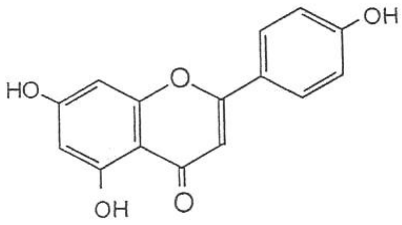
In general flavonoids occur in plant either in the form of aglycones or gluc osides. The aglycones are capable of being absorbed freely from gut by pa ssive diffusion,while the flavonoid glycosides are usually hydrolyzed to th e corresponding molecules prior to its gastrointestinal absorption[49].

Flavonoids are present in plant as mixture and it is very rare to find only a single flavonoid component in plant tissue. In addition , there are often mixture of different classes and they are generally present in all vascular p lants, but some classes are more widely distributed than others[4].

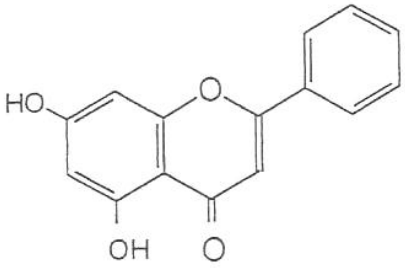
1.1.1Flavones

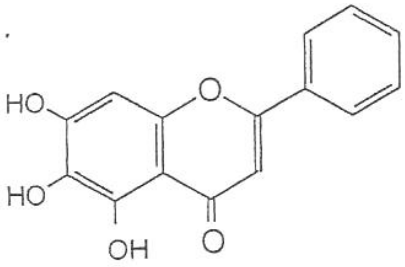
Flavones are the root from which the word flavonoid is derived. They are also known as anthoxanthins. They are yellow pigments which are widesp read in leaves and flowers of angiosperm[50]. The commonest flavones are i uteoline{6}[51], apigenin{7}[52], chrysin{8}, and baicalin{9}[53].





{6} {7}



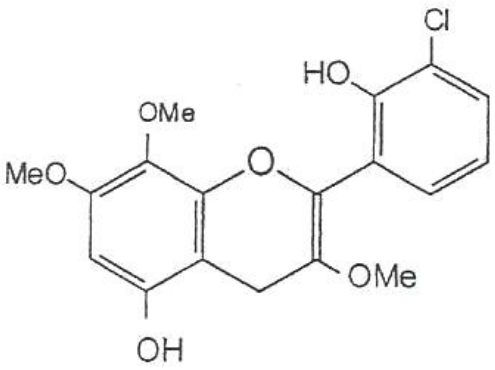


Flavones can be found in all part, above and below ground, in vegetable a nd generative organs. They are in stems, leaves, buds, barks, heartwood, t horns, roots, rhizomes, flowers and also in root and leaf exudates or resins. Flavones –producing plant species belong to more than 70 different famili es within the plant kingdom[20] . Representing an abundant class of phytoch emicals in our daily diet. Lastly, flavones attracted considerable scientific and therapeutic interest, because of the assumed beneficial health effects o f flavones in the prevention of some human diseases.

Besides their physiological importance they have important function in the biochemistry, physiology and ecology of plant[54,55]. Flavones occur naturally in the plant in Free State a glycone, glycosides, or associated with tannins[10].

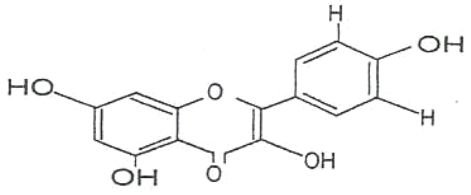
1.1.2 **Flavonols**

Flavonols (5,R=OH) are simply flavones (5,R=H) in which the 3- position is substituted by hydroxyl function. Flavonols are practically ubiquitous in woody angiosperms and appear less frequently in herbaceous angiosperms. There are no records of flavonoids occurring in
bacteria and algae, and chlroflavonin {10} is, so far, the only fully characterized flavonol isolated from a fungus. It was isolated from a strain of aspergillum's Niger and candida albacans.



{10}

The hydroxylated pattern of chloroflavonin, although not a common one, i ncorporates features usuallyassociated with flavonols from higher plant. K aempferol {11}, quercetin {12} and less frequently myrcetin {13} occur si ngly or jointly in a vast preparation of analyzed species[21].



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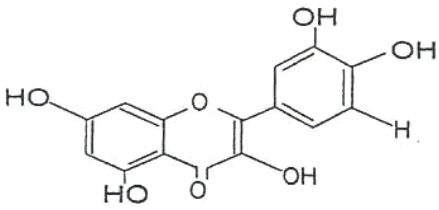

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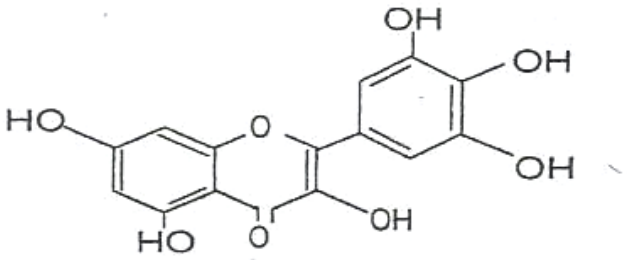
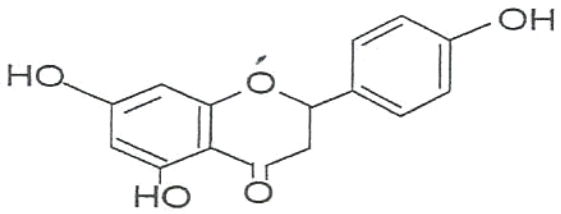


{13}

1.3.3 Flavanones

Flavanones are colorless substance . However, hydroxylated flavanones oc cur either in the free form or in combination with sugars as glycosides in fl owers, fruits, leaves, barks and roots and appear to be of fairly general dist ribution, especially in higher plants[56].

Chemically flavanones differ from flavones in being saturated between 2- and 3 position and thus laking the conjugation of the 2-phenyl group (B-ri ng)[9,56]. There are many naturally occurring flavanones e.g. naringenin {14 }, eriodictyol {15} and hesperidin {16}.



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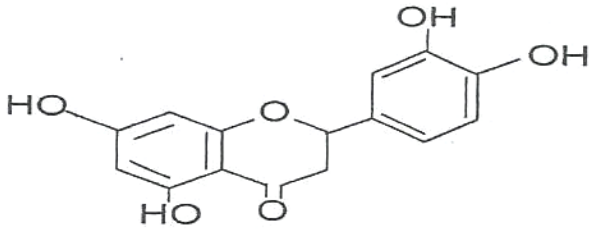

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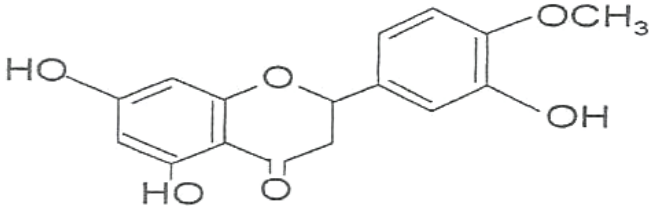


{16}

Flavanones absorb at short wavelength in contrast to flavones and hydroxy lation in the 2- phenyl group has very little influence on the position of ma ximum absorption of flavanones. The dihydropyrane ring

of the flavanones is more unstable than the pyrone ring of flavones or fla vonols and may be opened between O(1) and CH(2), giving rise to chalco ne compounds. The ring opening of flavanone to chalcone occur when fla vanones are treated with acetic anhydride[57].

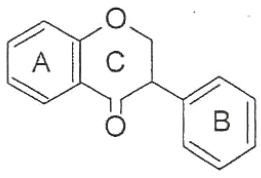
Flavanones in an alkaline solution are readily converted to the correspondi ng chalcones by ring –fission. Also flavanones decompose into benzaldeh yde, acetic acid and phenol when a strongly alkaline reaction mixture is he ated drastically.

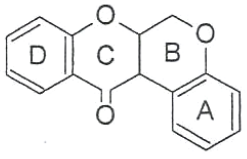
Some flavanones give bright yellow –green or light –blue colors on paper when viewed in UV-light with the help of ammonia vapour, but this is not reliable enough to be used as diagnostic test[58].

Flavanones have different spectral properties from other flavonoids, with one intense peak at about 225 nm, and another one at either 278nm or 288 nm, and a weak peak or inflection above 300nm. In some cases, flavanone s undergo ring opening or ring fission in alkaline solution and are readily c onverted to the corresponding chalcones[4].

1.3.4 Isoflavones

The isoflavones {17} form one of the larger classes of natural products. T he relationship of their structure to the skeleton of the rotenoids structure { 18}[56] is shown below.





{17} {18}

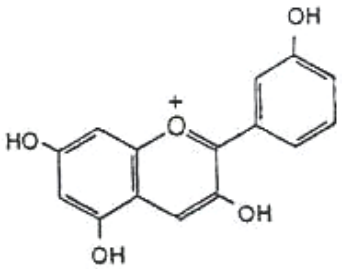
Isoflavones occur naturally, but are not as widespread as the flavones. The y are occurring either in the Free State or as glycosides[59].Glycosides of iso flavones have been known since a very early date. Natural glycosides, ho wever, have been reported, mainly as a result of more systematic analysis of plant extractives. The majority of glycosides are (7-glucosides) or (7-rh amnosylglucoside) and (4-glucosides) or (4- rhamnosylglucoside)[20]. Isofla vones are difficult to characterize since they do not respond specifically to any one color reaction. Some isoflavones give a light blue color in UV- li ght in the presence of ammonia, but most other appear as dull- purple abso rbing spot, changing to dull-brown with ammonia[21]. In fact in some early i nvestigations,

isoflavones have been mistaken for flavones owing to their similar behavi or in certain color tests. Various color reaction may be used to test for ben zopyrone structure, but they do not apparently differentiate between flavo nes and isoflavones[56].

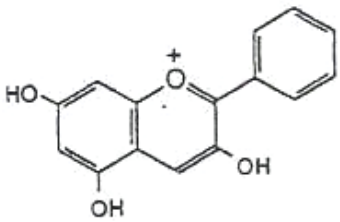
1.3.5 Anthocyanins

Anthocyanins are the most important and widespread group of coloring m atter in plant. These intensely –colored, water- soluble pigments are respo nsible for nearly all the pink, scarlet, red, mauve, violet and blue color in p etals, leaves and fruits of higher plant.There are six common anthocyanidi ns, (anthocyanin aglycone which are formed when anthocyanins are hydro lyzed with acid): the magenta colored cyanidin {19} being by far the most

common. The anthocyanin are all based chemically on a single aromatic s tructure, that of the cyandin and all are derived from this pigment by additi on or subtraction of hydroxyl groups or by methylation or glycosylation . orange- red are due to pelargonidin {20}, with one less hydroxyl group tha n cyandin , while mauve, purple and blue color are generally due to delph inidin {21}, which has one more hydroxyl group than cyanidin as shown b elow



{19}



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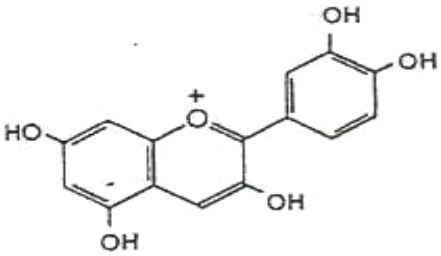
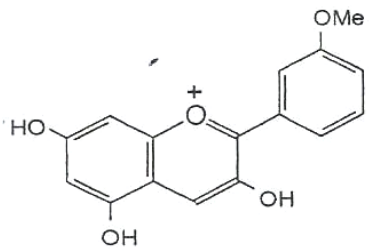
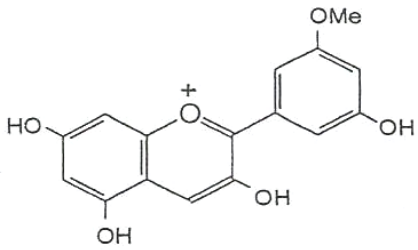


{21}

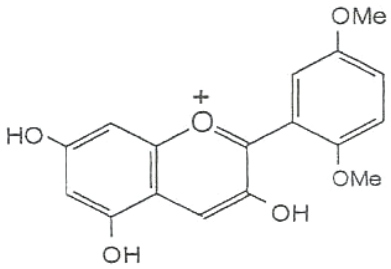
Three anthocyanidin methyl ethers are also quite common:

peonidin{22} derived from cyanidin, petunidin {23} and malvidin {24} based on delphindin as shown below:





{22} {23}



{24}

Each of these six anthocyanidins occur with various sugars attached as a r ange of glycosides, the main variation is in nature of sugar (glucose, galact ose, rhamnose ….), the number of sugar (mono, di, tri, glycoside) and the position of attachment of the sugar (usually at position 3 and 5)[48].Anthocy anins are typically the pigment of fruit, flowers and leaves, when they do occur in other parts of plant they are often confined to, or occur in highest concentration in one kind of tissue. Deeply colored flowers may be born o

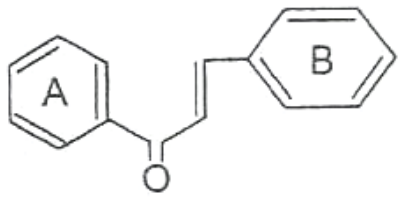
n plant with essentially anthocyanin in tree stems and leaves. In general, th e capacity of plant to synthesize anthocyanin result in the formation of at least traces of pigment in the green parts of plant. Occasionally, heavy ant hocyanin pigmentation cause plant leaves and stems to a quire red or brow n color, examples are found in the conspicuous coloration of many autum leaves, and in the color of young leaves of some plants[56].

Anthocyanins are characterized by two absorption bands. , 475-560 nm (v isible region), and , 275-280nm (ultraviolet region). Band 1 depends on th e number and position of hydroxyl and methoxyl groups[59].

1.3.6 Chalcones

Chalcones are open chain flavonoids, in which the Tow aromatic rings are joined by a three carbon , alpha and beta unsaturated carbonyl system[60]. C halcones are characterized by their possession of a C_6 (A)- CO- CH = CH- C_6 (B) structure . two aromatic rings (A and B) are linked by an aliphatic t hree carbon chain which does not participate in forming a hetero ring as is usually found in other types of flavonoids compounds.

Naturally occurring chalcones are all hydroxylated to greater or lesser exte nt: the parent compound chalcone (25) itself is not yet known as natural pr oduct[20].



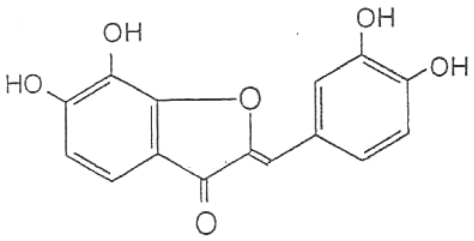
(25)

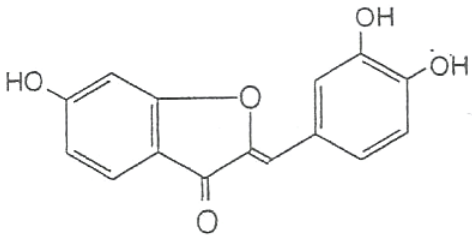
The chalcones play an ecological role in nature, in relation to plant color. These bright yellow colored compounds are found in many plant organs, b ut most conspicuously in flower. Most yellow flower color is due to the pr esence of carotenoids, but in the case of certain member of composites, co uthaceae, oxalidaceae, etc. the chalcones contribute significantly to the cor olla pigmentation. The yellow phenolic pigments give intense deep UV co lor when chromatographed on paper. On fuming the paper with ammonia, the color may change to rich deep red, although a few chalcones fail to res pond in this way. Chalcones are easily separated by paper chromatography in the usual solvents. In the uv- visible spectrum chalcones exhibit a broa d peak between 365- 390nm, which distinguishes them from aurones 390-430nm[61].

1.3.7 Aurones

Aurones are conspicuously coloured compounds found in a variety of yell ow flowered species[20]. Aurones are secondary metabolites belonging to th e flavonoid family and structurally isomeric with flavones, which are widl y distributed in fruits and flowers, and play significant role in the pigment

ation of the part of plant in which they occur. they are not restricted to flor al tissues, but have been obtained from bark, wood and leave as well .The first aurones was discovered only in 1943. Aurones however, have a limite d occurrence and limited method of synthesis[11,22]. Chemically aurones are on the (2-benzylidene- coumaranone) or (2- benzylidene-3 (2H)- benzofu ranone) system[20]. In aurones there is a 5- membered C- ring: exemplified by maritimetin (26) and resorcinol (27)[48].





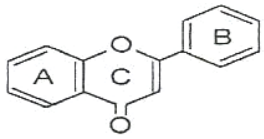
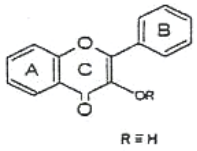
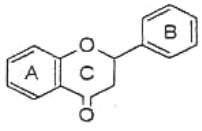
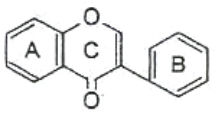
(26) (27)

The naturally occurring chalcones and aurones and their glycosides show an intense absorption band in the region 260-420nm and the absorption sp ectra can differentiate between the type of pigments since chalcones and a urones show quite different absorption spectra. The study of the spectra o f acetates of chalcones and aurones is particularly instructive. The absorpti on spectra of two series of aurones pigment and their derivatives have bee n determined and compared[62].

Aurones appear on paper chromatograms as yellow spots in day light,how ever, in the uv-light, they are very different, the color of aurones is an inte

nse bright yellow, changing with ammonia to bright orange- red. Analogy with flavonoids suggests that aurones could have interesting biological pro perties[63]. A series of naturally occurring aurones were synthesized and test ed for the ability to inhibit erythrocyte stages of plasmodium falciparum st ains. Some of these compounds exhibit antiplasmodial activity in the micr o range[64]. Clearly, flavones, flavonols, isoflavones and chalcones have bee n studied largely for their +therapeutical potentials[65].

Table 1: Characteristic properties of the different flavonoid classes.

Sub-class of Flavonoid	**Basic structure Of flavonoid**	**Characteristic Properties**
Flavone		Represent the root of the Flavonoid, differ from flavonol In lacking a 3-OH substitution.
Flavonol		Represent the most common compou nd: differ flavones in having a 3-OH s ubstitution.
Flavanone		Differ from all classes of flavonoid in lacking the double bond in 2,3 positi on.
Isoflavone		Isomeric of flavones, having the B-rin g attached at the 3-position in flavone s instead of 2-position in flavones.

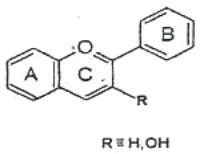
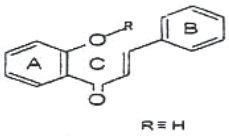
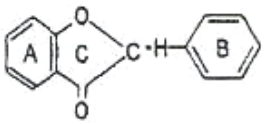
Anthocyanin		Differ from all classes of flavonoid in lacking the carbonyl group at 4- posit ion.
Chalcone		Isomeric with flavanone having the o pen chain instead of close chain in fla vanone (ring –c).
Aurone		Differ from all classes of flavonoids h aving 5-membered ring –c instead of six- membered ring in certain classes

Table (2): color properties and Occurrence of the different flavonoid Classes.

Flavonoid	**Occurrence**	**Colour in nature**
Flavone	Found in all parts plant, Widespread flower and l eaves	Yellow color
Flavonol	The same as flavones	Yellow color
Flavanone	The same as flavones	colorless
Isoflavone	Found in root, or commo n in one family legumina	colorless substance

	cea	
Anthocyanin	Flower pigment also leaves, fruits, petals and other tissues.	Scarlet, mauve, blue, pink and violet.
Chalcone	Flower pigment occasionally present in other tissues.	Bright yellow color.
Aurone	Flower pigment wide spread in leaves, fruits and bark wood.	Bright yellow color

1.4 Medicinal uses of flavonoid compounds

Pharmacological and chemical investigations of medicinal plants have provided important advances in the therapeutic approach to several pathologies. A number of medicinal plant containing flavonoids and alkaloids are used in traditional medicine and are known to contain important therapeutic agents[66].

A great number of plant medicine contain flavonoids having anti-inflammatory, anti-allergic, anti-thrombotic and vasodilatory activities[67]. Also some have anti-viral and anti- bacterial properties[68]. flavonoids could be found in many formulation of alternative medicine .ranging from treatment for cardiovascular disease to peripheral vascular disease, stroke and cancer[69]. Several epidemiological studies provided support for a protective effect of the consumption of fresh fruit and vegetable against cancer[70]. Heart disease and stroke[68].

Flavonoids are also considered to be the active ingredient in some medicinal plants, because ingested flavonoids enter the plasma, to elevate the redox and anti-oxidant levels. The physiological benefits of flavonoid are generally thought to be due to their anti-oxidant and free radical scavenging properties[71].

Quercetin (12) is considered a good antioxidant. It also exhibits anti-flammatory and was found to inhibit both tumor promoter and human cancer cell[72].

One of the main function of flavonoids is their accumulation as phytoalexins, which protect plant from microbial invasion[73]. Phytoalexins are compounds that are formed in response to microbial or other invasions. Nringenin (14), found in the heartwood of trees from the rosacea is an anti- fungal agent[74].

It can also function as stress protectants in plant cell by scavenging reactive oxygen species (ROS) produced by the photosynthetic electron transport system[75]. Furthermore, because of their UV absorbing properties, flavonoid protect plants from the UV radiation[76]. The fungicidal properties of flavonoids are effected by phenolic substitution and in many cases it has been shown to decrease with increasing substitution[24]. Isoflavonoids, flavanone and flavones are the most effective anti-microbial agents[68].

Anthocyanin are also used to treat skin diseases specially dermatological hypersensitivity. Flavonoids may inhibit the enzyme involved in the glycos

ylation process, which gives rise to sorbitol which causes swelling compli cation in diabetes[77].

The biological activities of chalcone is including anti- bacterial, anti- canc er, anti-ulcer, anti- protozoa, amoebicidal, cytotoxic and immune suppress ive activates[78].

Flavonoids are usually found alongside vitamin C in nature. Studies have shown that vitamin C alone may not be effective as being supplemented w ith flavonoids. Flavonoids may correspondingly increase the amount of vit amin C in tissues, by preventing the break- down of this vitamin[79].

1.5 Techniques used in flavonoids analysis

The separation and purification of plant constituents is mainly carried out using one or other or combination of five chromatographic techniques. Pa per chromatography (pc), thin –layer chromatography (TLC), gas- liquid c hromatography (GLC), high performance liquid chromatography (HPLC)a nd high speed counter current chromatography (HSCCC). The choice of th e technique depends largely on the solubility properties and volatility of c ompound to be separated[21].

1.5.1 Paper chromatography (PC)

The technique paper chromatographic (pc) is a common one in the field of flavonoid analysis and separation[80,81]. pc is suitable for the separation co mplex mixture of all type of flavonoids and their glycosides. It is convenie

nt for isolating of both small and relatively large amounts and is associate d with the low cost of the necessary equipment and material[82].

One of the main advantage of pc is the great convenience of carrying out s eparation simply on sheets of filter paper, which serve both as the medium for separation and as the support. Another advantage is the considerable r eproducibility of R_f determined on paper, so that such measurements are v aluable parmenters for use in describing new plant compounds. Indeed, for substance such as the anthocyanin, which do not have other clearly define d physical properties, the R_f is the most important means of describing an d distinguishing the different pigments[83].

Most flavonoids appear as colored spot on paper chromatogram when vie wed in UV- light, and fuming with ammonia often produces
significant changes in these colors. Often reaction with chromogenic reage nt which is used as spray is extremely usefull[26].

1.5.2 Thin- layer chromatography (TLC)

TLC is a technique which has developed rapidly . however , it is comple mentary to (pc) in that it provided new media for separation of flavonoids on small scale and permits the use of a wider variety of detecting reagent[84] . The special advantage of TLC compared to (pc) include speed, versatilit y and sensitivity . The greater speed of TLC is due to the more compact na ture of adsorbent when spread on plates and is an advantage when workin g with labile compounds. The sensitivity of TLC is such as separation on l

ess than milligram amount of material can be achieved if necessary detecti on of compounds on TLC plates is normally carried out by spraying proce dure. One advantage over (pc) is that glass plates may be sprayed with con c.H_2SO_4, an extremely useful detecting reagent for steroids and lipids[21]. T hin layer chromatography is a technique, which has replaced paper chrom atography in analytical and small scale separation of flavonoids[4,85].

As in column chromatography, the adsorbents of choice for the separation of flavonoids are silica, polyamide and cellulose[86]. Thin layer chromatogr aphy is essentially a technique for the separation of milligram quantities of material. However, it can be upgraded to handle up to a gram when layers of 1-5 mm thick are used in conjunction with plates of up to 20x 100 cm i n size[87].

Apart from the anthocyanin's and some of more intensity colored chalcon es and aurones, flavonoids are not sufficiently coloredproduce visible to th e naked eye on a thin- layer plate , thus some form of visualization is nece ssary for spot detection[88]. UV- light is often used is detecting agent. Anoth er useful method of detection is brief exposure of the plate to iodine vapou r where flavonoids appear as yellow- brown spots against a white backgro und. These techniques all have the advantages that they are non- destructiv e[86]. Most flavonoids are detectable by one or other of flavonoid spray reag ents, and some of the most frequently used are listed in table (3).

Highly methylated or acetylated flavones and flavonols require relatively non-polar solvents for thin layer chromatography on silica gel. Flavonol p

olyacetate and polymethyl ethers have been successfully chromatographed by using benzene : acetone (9:1) and toluene : acetone(19:1). More polar f lavones and flavonols require more polar solvents[57].

Flavone and flavonol glycosides are not commonly chromatographic on S iO_2[86]. However , when this has been done, polar solvents such as ethyl ace tate : butanone: formic acid :water(5:3:1:1), have been used[89,90]. Isoflavon es, flavanones and dihydroflavonols are generally chromatographed using less polar solvents than those required for the common flavones and flavonols.

Anumber of synthetic aurones have been chromatographed on SiO_2[91]. Ant hocyanine and anthocyanidines which are usually fractionated on paper or cellulose were successfully fractionated on silica[87,92].

TLC on cellulose layer has to some extent replaced paper chromatography in analytical work, since the high surface area, fine-grained cellulose thin layers offer the advantage of greater speed[93]. Cellul ose, when admixed with 3% by weight of polyamide, was found useful for the chromatography of a wide range of flavones and flavonols and their m ono- and diglycosides using 15,40 or 60% acetic acid as solvents[94].

Good separation of anthocyanidins and anthocyanins have been achieved us is cellulose TLC[95]. Commercially available polyamide have various ph ysical and chromatographic properties. An excellent polyamide powder, h owever , may be prepared from polyamide pellets. Polyamide separates fla vonoids either by partition or adsorption processes depending upon the sol

vent used[96]. The adsorption process is favoured with water – alcohol mixtures . Polyamide are generally considered unsatisfactory for the chromatographic separation of anthocyanins and anthocyanidins[96,97] .

TLC is an ideal technique for the screening of drugs because of its low cost, easy maintenance and selectivity of detection reagent. TLC on silica gel is very favorable for the analysis of flavonoids[4].

1.5.3 Gas chromatography (GC)

Two types of gas chromatography are encountered: gas – solid chromatography (GSC) and gas- liquid chromatography (GLC). GLC finds widespread use in all fields of science, where its, name is usually shortened to GC[98]. GC is no more complicated than other chromatographic procedures. The main variable are the nature of stationary phase of column and the temperature of operation, these one varied according to the polarity and volatility of compound being separated.

Most frequently, GC is automatically linked to mass spectroscopy (MS) and the combined GC-MS apparatus has emerged as one of the most important techniques for phytochemical analysis[21].

1.5.4 High performance liquid chromatography (HPLC)

HPLC is analogous to GLC in its sensitivity and ability to provide both quantitative and qualitative data in a single operation. HPLC is mainly used for those classes of compounds which are non- volatile, e.g. higher terpenoids, phenolic of all types, alkaloids, lipids and sugas[21]. Twenty six various t

echniques have been used for the determination of plant extract. The qualit ative analysis which produce a “finger print” chromatogram obtained unde r standard concentration can be very useful for quality control of phytoche micals. Although TLC is powerful and simple technique used for this purp ose, there are situations in which it can produce doubtful results. HPLC c an be also a useful tool In chemosystematics helping, for example, to char acterize species on the basis of their secondary metabolite contents[99]. HPL C has been used in a number of occasion for the analysis of flavonoids in plant. In one study[100], HPLC was used to distinguish species based on the quantitive variation of flavonoids among them. In other study[101], it was us is for the quantitive analysis of flavonoid aglycones. The literature is reple te with chromatographic systems which are capable of measuring flavonoi d content across one, two and three of the five common sub- classes of fla vonoids found in foods. However, many food and mixed diets, in particular, contain member of all five sub- classes of flavonoids[102]. Advant age for HPLC analysis includes. (i) short analysis time, (ii) high resolution . (iii) no derivatization is required, (IV) no risk of thermal decomposition a nd (v) easy quantification[20].Where twenty years ago the principal chromat ographic methods for analysis of flavonoids were TLC and PC, in last dec ade HPLC has taken over almost completely. Separation of widely differi ng compounds (for example aglycones and their conjugates) is a common problem in both phytochemical and clinical studies. This is now routinely resolved by use of HPLC on reversal- phase column with gradient elution[1]

[03]. Natural products are often obtained by some conventional protocols of extraction and separation technique, such as using organic solvent to extra ct and column chromatography, including silica gel and HPLC, to isolate. However , some organic solvent are unfriendly to our environment and the conventional separation methods are usually tedious, time consuming and needing multiple steps and the samples are adsorbed on the stationary pha se irreversibly[100].

1.5.5 High speed counter current chromatography (HSCCC)

Recently, two techniques, supercritical fluid extraction (SFE) and high spe ed counter current chromatography (HSCCC) are widely used to extract a nd separate natural products from medicinal plants[104].

(HSCCC) is a major tool for the fast separation of natural products from pl ants. It was used for the preparative isolation of the flavonoid monoglucos ides present in aerial parts of plants[104,105]. Several classes of natural produ cts were already isolated using (HSCCC), including flavonoids[106].

Separation using (HSCCC) provide natural products with a very efficient method for the separation of compounds derived from plant origin in short separation time and with the possibility of large range of aromatic solvent [107]. The solvent system selection is the first and most important step in per forming (HSCCC) separation[104]. Conventional methods such as column ch romatography requires several steps resulting in low recoveries of the prod uct. (HSCCC) is a liquid- liquid partition chromatographic technique with excellent sample recovery compared to some conventional methods, and is

widely used for separation and purification of various natural and syntheti c products[108].

1.5.6 Column chromatography

Column chromatography remains one of the most useful techniques for th e isolation of large quantities of flavonoids from crude plant extract. Adso rbents commonly used for the separation of flavonoids include silica gel, k ieselguhr, magnesol, cellulose, alumina, polyamide, and sephadex and ion exchange resins. The adsorbents of choice have generally been silica gel, c ellulose and polyamide[86].Silica gel has traditionally been used for the sepa ration of isoflavones, flavanones, dihydroflavonol and highly methylated f lavones and flavonol. Occasionally, even flavonoid glycosides have been purified on silica, for example the C- and O-glycosides of daidzein were s eparated on silica using ethyl acetate and ethyl acetate : methanol (19:1) as solvents[109], and glycosides of flavone naringenin were eluted from silica using benzene : ethanol(9:1)[110].

In is clear that silica gel is a useful adsorbent for the separation of flavonoi ds of quite a wide range of polarities. In general terms, this may be extended to include many of the more polar flavonoids simply by deact ivation through the addition of water. Many of the variable chromatograph ic properties observed with silica adsorbents from different sources are un doubtedly attributable to the water content of the gel[86]. An additional fact or is the presence of metal ions in the silica. It was observed that a number of the commercially available silica gels contain iron causing the flavonoi

ds to adhere strongly to the column. This impurity is removed by treatmen t of the silica with warm, conc HCL thus making the adsorbent much more useful for the separation of polar compounds[111].

Magnesol, a dehydrated magnesium acid silicate, and florisil, mixture of magnesium oxide and silica are somewhat more basic than silica. They ha ve not been used extensively for the separation of flavonoids probably bec ause they offer little or no advantage over silica. Separation of a number o f simple mixture of flavones, flavonols, flavanones and their glycosides w as achieved using magnesol[112]. In particular, good separation of flavonoid aglycones from the more strongly adsorbed glycosides was obtained. Wate r – soluble flavonoids could be extracted by water containing up to 5% of an organic solvent such as ethanol, acetone or ether. However, individual f lavonoids were not separated by this procedure[113].Florisil has been used fo r the partial separation of flavonoids obtained by counter current distributi on extracts[114]. As with silica gel, alumina may be deactivated by addition of water. Alumina has generally found little use in the separation of flavon oids, largely because of the problem of complex formation. Aluminum-III ions are known to complex strongly with the 4- keto -5-
hydroxyl and 4- keto-3-hydroxyl systems found in most flavones and flav onols[115].

Neutral alumina has been used successfully for separation of completely methylated and fully substituted flavonols[116]. It is clear that alumina is bes t suited to the purification of fully derivatized flavonoids[117]. In principle fl

avonoids which form only acid- labile complexes with aluminum, should be selectively removed from alumina with acid- containing solvents[118]. C ellulose column chromatography can be considered as a scaled- up form o f paper chromatography. As such, it is suited to the separation of all classe s of flavonoids and their glycosides. It is used for separations based on bot h absorption and partition, though a distinction between the two is often di fficult to make[119]. Cellulose powder has a low capacity[92] and limited resol ving power and although this may be compensated for to some extent by ti ght column packing, many workers favor scaling up of paper chromatogra phy itself for large scale separations. As in paper chromatography, the sol vents most favoured for use with cellulose columns are of the aqueous alc ohol and acid types.

Cellulose column chromatography has been used for the separation of anth ocyanin‘s in the past,but variable results, limited column capacity صتand production of dilute elutes, led to the conclusion that the method offered li ttle, if any advantage, over paper chromatography[86].

Polyamide commercially available for chromatography are mainly of the p erlon- type (polycaprolactam) , nylon –type(polyhexamethylenediamine) a diapate, or polyvinylpyrolidone (PVP) all have a high capacity for phenoli c material and all form strong

hydrogen bonds with phenolic hydroxyl group via their amide function[120].

Like cellulose, polyamide is suitable for the separation of all types of flav onoids[121]. However , it has the advantage over cellulose of higher capacit y and higher resolution.

Sephadex is highly cross- linked dextran on which separations are ideally obtained on the basis of molecular size[122]. Adsorption on dextran gels is known to occur with aromatic compounds, and phenols in particular, and i t is thus not surprising that this is commonly encountered with flavonoids especially the aglycones[86].

Ion-exchange resins have been used very little for the isolation of flavonoi ds. Early work[123], which has been thoroughly reviewed is involving the u se of cation –exchange resins entirely. These resins were generally used i n preliminary clean – up procedure in which flavonoids were held on the c olumn while other water- soluble impurities were washed off with water.

Table (3): spray reagents for the detection of flavonoids on TLC

No	**Reagent**	**Flavonoids type detected**
1	Boric acid- ethanol- arin complex(1% in ethano l	Most flavonoids
2	Ferric chloride (methanolic)	Most flavonoids
3	Ferric chloride- potassium ferricyanide (1%aq-s lons mixed (1:1)	Most flavonoids

4	Ferric chloride then alpha-alpha dispyridyl	Most flavonoids
5	Antimony chloride(in$CHCL_3$),UV	Most flavonoids
6	Lead acetate- basic, UV	Most flavonoids
7	Aluminum chloride (2%in methanol)	Most flavonoids
8	Zirconium oxychloride (in methanol),UV	Most flavonoids
9	Ceric sulphate	Isoflavones
10	Conc sulphuric acid	Isoflavones
11	Hydrogen chloride (Gas)	Fully methylated flavones
12	Zinc-hydrochloric acid	Dihydroflavonols
13	Oxalic acid (10% in acetone :H_2O,1:1)	Anthocyanineand anthocyanidins
14	Diazotized sulphuric acid	Most flavonoids
15	Diazotized p-ntroailine	Most flavonoids
16	Fast blue salt B(terazotized di-o- anisidine)0.5 %, then 0.1N NaOH	Most flavonoids

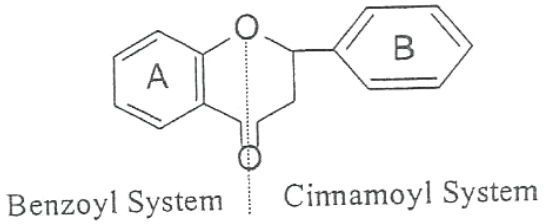
17	Fast red salt B (diazotized 5- nitro-2-mino aniso le)0.5%, then 0.1N NaOH	Most flavonoids
18	Bis- diazotized benzidine	Flavones and flavonols
19	Sodium borohydride (1% in isopropanol and H CL) or $ALCL_3$)	Flavanones, isoflavanones
20	Ammonical silver nitrate	Most flavonoids
21	Sodium hydroxide solution (1% in methanol)	Most flavonoids

1.6 Spectroscopic Methods

In identifying a plant constituent , once it has been isolated and purified , i t is necessary first to determine the class of compound and then to find out which particular substance it is within that class. It should travel as a singl e spot in several TLC and or PC systems. Complete identification within t hat class depend on measuring other properties and then comparing these data with those in the literature. These properties include melting point, bo iling point, R_f and optical reaction. However, equally informative data on plant substance are its spectral characteristics. These include ultraviolet (UV), infrared (IR), nuclear magnetic resonance(NMR) and mass spectra (MS). A known plant compound can usually be identified on the above ba sis If a new compound is present all the above data should be sufficient to characterize it[21].

1.6.1 The Ultraviolet / Visible Spectroscopy (UV/Vis)

The UV spectra of most flavonoids consist of two major absorption max ima, one of which occurs in the range of 240-285nm(band II)and other in t he range 300-400nm (band I). Band II occur from A-ring benzoyl system, while band I originates from B-ring cinnamoyl system



(28)

Flavonoids containing conjugated aromatic systems show intense absorpti on bands in UV region of spectrum[4].(table 4)

Table (4) spectral characteristics of main flavonoid classes

Class of flavonoid	**Band 1 nm**	**Band 11 nm**
Flavones	300-350	250-285
Flavonol	330-385	240-280
Flavanone	300-330(sh)	275-295

isoflavones	310-330(sh) 230 (one beak)	270-280
anthocyanin	465-550	270-280
Chalcone	340-390	220-270(low intensity
Aurone	390-430	230-270 (low intensity

UV spectrum has become a major technique for the structure analysis of f lavonoids for two reasons: the first is that only a small amount of pure mat erials is required, often a single flavonoid spot on paper chromatogram wil l yield sufficient compound to several UV studies. The second reason is th at the amount of structural information gained from a UV spectrum is cons iderably enhanced by the use of specific reagents (shift reagent), which rea ct with one or more functional groups on the flavonoid nucleus.

The addition of each of these reagent separately to an alcoholic solution of the flavonoid induces structurally significant shifts in the UV spectrum.

Shifts are commonly induced by the addition of sodium methoxide, sodiu m acetate, sodium acetate\ boric acid, aluminum chloride and aluminum c hloride \hydrochloric acid[124].

Sodium methoxide is a strong base and ionizes to some extent all hydroxy l groups on flavonoid nucleus. However, use has been made of the effect

of sodium methoxide on the UV spectra of flavones and flavonols for dete ction of 3 and or 4 -hydroxyl groups, The addition of sodium methoxide to flavones and flavonols in methanol usually produces bathochromic shifts in all absorption bands[125].

The absence of shift in the major band indicates the absence of isoflavones of A-ring hydroxylation, while the presence of 5, 6, 7 and 5, 7, 8- hydrox yl system is evidenced by degeneration of sodium methoxide spectra with time[126,127].

Flavanones and dihydroflavonols with 5,7- dihydroxyl system exhibit a co nsistent 35-40 nm bathochromic shift of band (II). Flavanones in particula r, those laking a free 5- hydroxyl group, isomerize to chalcones and have b and (I) peak in 400nm region[125].

4-hydroxyl groups in aurones and 4-hydroxyl groups in chalcones are evid enced, by band (I) bathochromic shift of 80-96 nm and 60-100 nm respecti vely each with an increase intensity, while 6-hydroxy- aurones give a smal ler shift (60-70nm) than 4-hydroxyaurones. Chalcones give a 60-100 nm b athochromic shift[128].

The only anthocyanidine that give stable spectra in sodium methoxide are the 3- deoxyanthocyanidine [129]. The bathochromic shift in band (I) being 5 0-60 nm.

Sodium acetate is weaker base than sodium methoxide, and as such ionize s only the more acidic hydroxyl groups. In flavones and flavonols it is dia gnostic of the 3,7- and 4- hydroxyl groups. Ionization of the 7-hydroxyl gr

oups mainly effect (band II), where as ionization of 3- and or 4-hydroxyl g roups mainly effect (band I). In the presence of sodium acetate the UV spe ctra of flavones and flavonols containing free 7- hydroxyl groups, with fe w exception exhibit a diagnostic 50- 20 nm bathochromic shift. Sodium ac etate is particularly useful diagnostic reagent for the specific detection of 7 - hydroxyl group. If within few

Minutes the flavonoid has decomposed then this is due to the presence of an alkali- sensitive grouping[130].

The presence of 7-hydroxyl group in isoflavones is evidenced by a band (I I) bathochromic shift of 6-20 nm, in 5,7- hydroxyl flavanones and dihydr oflavonols by a shift of 35 nm. Alkali- sensitive grouping in the A – ring c ause the spectrum to degenerate with time.

Hydroxyl groups at position 4- and\ or 4 in chalcones and at 4¯and \or 6 i n aurones are evidenced[127]. by a bathochromic shift of band (I) or by the a ppearance of along wavelength shoulder.

In the presence of sodium acetate, boric acid will chelate with ortho-dihyd roxyl groups at all location on the flavonoid nucleus, except at C-5,C-6. Fl avones and flavonols containing ortho- dihydroxyl group show a consisten t 12-30nmn bathochromic shift of band (I) in the presence of (NaOAc \$H_3$ BO_3). A-ring cetecholes at C-6, 7 and C-7, 8 in flavonoids are also detecta ble by the effect of ((NaOAc \$H_3BO_3$) on UV spectra. a band (I) bathochro mic shift of 5-10nm is observed[131].

Isoflavones, flavanones and dihydroflavonols exhibit a bathochromic shift of 10-15 nm for band (II). Aurones and chalcones give bathochromic shift of 28-36 nm, ortho-dihydroxyl groups in the A-ring give rise to smaller s hift.

With aluminum chloride, flavones and flavonols which contain hydroxyl g roups at C-3 or C-5. form acid- stable complexes, in addition $ALCL_3$ from acid- label complexes with flavonoids which contain ortho-dihydrox yl system. The complex formed between $ALCL_3$ and the A- B-ring ortho-dihydroxyl group; decompose in the presence of acid. In contrast, the AL CL_3 complex between the C-4 Keto function and either 3- or 5-hydroxyl g roup is stable in presence of acid. The presence of ortho- dihydroxyl group in the B- ring of flavones and flavonols can be detected by a comparison of the spectrum of the flavonoids in the presence of $ALCL_3$ with that obtai ned in ($ALCL_3$\HCL).

The presence of 5- hydroxyl group is revealed, by a 10- 14 nm, band (II) b athochromic shift in the spectra of flavones and a 20-26 nm in the spectra of flavonones and dihydroflavones. Ortho- dihydroxyl group are detectabl e only when present in the A-ring, and the spectrum shows a band (II) bat hochromic shift of 11-30 nm. The presence of 2¯hydroxyl group in chalco nes and a 4¯hydroxyl group in aurones is evidenced [127]by band (II) bathoc hromic shift of 48-64 nm.

B-ring ortho-dihydroxyl groups give rise to a 40-70 nm bathochromic shif t of band (I) with $ALCL_3$. A-ring ortho- dihydroxyl group give rise to a sm

aller shift. Anthocyanidines and anthocyanins containing ortho- dihydroxy l groups give band (i) bathochromic shift of 25-35 nm, longer shifts are ob served with 3-deoxyanthocyanidins[124,129].

1.6.2 Mass Spectroscopy

Mains value of the technique is that requires only microgram amounts of material. It can provide an accurate molecular weight and may yield a com plex fragmentation pattern, which is often characteristic of that particular compound[21]. Mass spectram has been applied successfully to all classes of flavonoids aglycones, and more recently to a number of different types of glucosides incloding mono and di-c glycosyl flavones and mono-tetra-o -glycosides[123].Electron impact spectroscopy of both flavonoid aglycones a nd glycosides serve as a vailable aid in determing other structures speciall y when only small quantities (i.e less than 1mg) of the flavonoid are available. Most flavonoid glycones yield intense peaks for the molecular i on $[M]^+$ and indeed this is often the base peak[134].In addition to the molecu lar ion ,flavonoid glycone usually afford major peak for $[M-H]^+$and when methoxylated $[M-CH_3]^+$. Perhaps the most useful fragmentation in team of flavonoid identification is those which involve cleavage of intact A-ring a nd B-ring fragments[135].

1.6.3 Nuclear Magnetic Resonance (NMR)

The major use of proton NMR is for structural determination , in combinat ion with other spectral technique . its use for determing the class of compo und is quite considerable[4].

The application of NMR spectroscopy to the structure analysis of flavonoi d is now well established. Most naturally occurring flavonoids , including all of the flavonoid glycosides , have low solubility in deuterio chloroform (CD CL_3). The dimethyl sulfoxid (DMSO-d_6) has been used as solvent fo r number of extensive investigations of flavonoid structure by NMR spectr oscopy[136,137] . Some of the advantage of this method ,most flavonoid gly cone and glycoside are sufficiently soluble in DMSO-d_6.

It occurs as narrow band between δ 2.4-2.6 ppm, out side the region wher e most flavonoid proton absorb. DMSO-D_6 can be used for observing prot on on phenolic hydroxyl group . It is anhydrous the hydroxyl proton signa ls are readily distinguishable . Water in the solvent ,however, cause the fla vonoid hydroxyl proton signals to broaden , thus making their detection di fficult [138].Proton of B- ring usually appear in the rang δ 6.7-7.6 ppm, whic h is downfield form the A- ring protons. . Considerable variation is found in the chemical shift of C-ring protons among the different flavonoid class es depending upon the oxidation level of the C-ring. The chemical shift of the proton of suger directly attached to the flavonoid hydroxyl group depe nds both on the nature of the flavonoid and on the position and stereochem istry of attachment .Methoxyl proton single with few exceptions. Appear i

n the region δ3.5-4.1 ppm. While most aromatic acetyl protons occur in th e rang δ 2.25-2.50ppm[139].

2. Literature review:

2.1 Trianthema portulacastrum l:

2.1.1 Taxonomy:

Kingdom: Plantae

Sub Kingdom: Tracheobionta

Division : Spermatophyta

Sub Division: Magnoliophyta

Class: Magnoliopsida

Sub class: Caryophyllidae

Order: Caryophyllales

Family: Aizoaceae

Genus: *Trianthema* Linnaeus

Species: *Trianthema portulacastrum* L.

2.1.2. Morphology:

Plants are diffuse, prostrate, branched herbs. glabrous or papillose; thicken ed and flattened at the nodes; Root- a taproot system with fibrous hairs; St em - more or less angular, glabrous or pubescent, much branched; Leaves - petioled, opposite, unequal, one of the lower pair much smaller than the

other , entire, sub-fleshy; leaf blade obovate to orbicular, or oblong, 1.5-3. 5 × 1-3 cm, sub-succulent, purplish on

margins, base cuneate, margin entire, apex obtuse, apiculate, petioles of ea ch pair connected at the base by stipuliform membranous; Flowers - small, white or bright pink, axillary, solitary in pouch or between forks of branc hes , bracts membranous as are the 2 bracteoles; calyx tube short or long; l obes 5, coloured within, mucronate on the back near the tip; petals 0; stam ens 5, 10, or 15, inserted near the top of the calyx-tube, filaments white, gl abrous; Ovary free, sessile, usually truncate at apex, 1-2 celled; ovules 1 o r more in each cell, from a basal placenta; styles 1 or 2, papillose. Fruit - a capsule, capsules circumscissile, glabrous, partly concealed in the petiolar hood; the upper part carrying away 1-2 seeds, the lower 2- many seeded. F lowering - June to October; Fruiting - July to December; Seeds are renifor m, muriculate and dull black in colour with epigeal germination.. The prod uction of flowers and seeds of *T. portulacastrum* starts 20 - 30 days after g ermination of the seeds. Enormous seeding capacity or very little dormanc y allows the mature seed to germinate immediately thus, producing multip le generations in the same season. Cotyledons are elliptic and have epigeal germination. Seeds of *T. portulacastrum* germinate between 20- 45 °C, w ith an optimum at 35 °C. More than 50% of fresh seeds germinate within 4 –8 days of incubation[140].

2.1.3. Photochemistry:

Photochemical screening has revealed the presence of alkaloids, steroids, f lavonoid, tannins, terpenoids, glycosides, flavonoids, phenolic compounds , fats, carbohydrates water soluble bases and potassium salts. Punarnavine and a new alkaloid, trianthemine and ecdysterone are present in the aerial parts. Roots contain saponin glycoside. The principal constituent of *T. portulacastrum* is ecdysterone and the other con stituents are trianthenol, 3acetylaleuritolic acid, 5, 2' dihydroxy 7 methoxy 6, 8 dimethyl flavone, leptorumol, 3,4dimethoxy cinnamic acid, 5hydroxy2 methoxy benzald ehyde, pmethoxy benzoic acid, and betacyani n. The red and white flowers contain an alkaloid trianthemine, also punarn avine. The plant contains nicotinic acid (Vitamin B), ascorbic acid (Vitami n C). The mineral profile of *T. portulacastrum* was reported as calcium (0. 3%), magnesium (0.2%), iron (50 ppm), copper (8 ppm), zinc (30.0 ppm), and manganese (50 ppm), whereas the phosphorus content at 0.13% ± 0.1 % and crude protein 1.5% ± 1.2%. The plant is rich in phosphorous and ir on but poor in calcium. The high content of oxalate affects the assimilatio n of calcium. Carotene (2.3 mg/100 g)[140].

2.1.4. Medicinal uses:

Several anatomical parts of T. portulacastrum are traditionally used as ana lgesic, alexiteric, alterative, laxative and stomachic and also valuable for t he treatment of alcohol poisoning, anemia, ascites, asthma, beri-beri, bron

chitis, corneal ulcers, dropsy, edema, heart diseases, inflammation, liver ai lments, migraine, night blindness, piles and rheumatism[141]. Though the wh ole plant is used medicinally from the ancient period of time in Unani syst em of medicine; however its leaves are more commonly used as therapeuti c agent for divers pathological conditions, viz. as *Mudirre Baul* (diuretic), *Mudirre haiz* (emmenagogue) *jali* (detergent), *muqawwie baah* (aphrodisi ac), *musakkhin* (calorific), used
in colitis, jaundice and ascites. Its juice is used in corneal ulcer, night blin dness and dribbling of urine[141].

2.1.5 Pharmacological studies:

Many studies regarding hepatoprotective effect of Biskhapra has been carr ied out by different researchers they found that the ethanolic leaves extract of *Trianthema portulacastrum* Linn exhibit a significant dose dependent protective effect in rats and mice. Similarly methanolic extract of Biskhap ra also play an important role in nephroprotection .Also evaluated that the drug has effect in adriamycin induced nephrotic syndrome in rats[142]. Anot hor study regarding diuretic effect of Biskhapra was found significant resu lt like standard drug frusemide. The remarkable effect of chloroform extra cts of *Trianthema portulacastrum* Linn. Were seen in DENA induced rat h epatocarcinogenesis. The ethanolic extract was also evaluated for analgesi c activity and was found that, the extract has significant antinociceptive ac tion in hot plate reaction time method in mice and this effect was compara

ble to that of standard drug aspirin treated controls, suggesting the central activity of EETP. Ethanolic extract of the whole plant of *Trianthema port ulacastrum* Linn. Showed the antipyretic activity, analgesic activity, anti-i nflammatory activity, *in vitro* antibacterial activity (against gram positive bacteria) and CNS depressant activity[142].

Photo of Trainthema portulacastrum l

2.2. Phoenix dactylefera l:

Phoenix dactylifera L is one of the species of date palm that grow abundan tly in countries around the Arabian Gulf. It belongs to family Arecaceae. T he plant is considered as one of the oldest cultivated fruit trees in the Mid dle East since 6000 BC. Due to its abundance and historical tradomedical applications, it has been described as “tree of life” among the Arabian nati ons. P. dactylifera L is identified by several names in different areas of the globe; the Arabs term it as „nakhla‟, the Brazilians call it „tamareira‟, wh ile the Chinese and Japanese refer to it as „wu low zi‟ and „natsumeyashi[1] [43] . Date palm (*Phoenix dactylifera* L., Palmate) is native to the Middle Ea st region over centuries ago[140]. In Folkloric practice, date represents an ess ential meal in some Arab area. Extracts of fruits, pits and edible kernels sh owed improvement of vital activities and increased the hormonal concentr ation in rat and the pollen has been used by Egyptians to improve fertility i n women; . Some reports on the previous phytochemical studies on the Eg yptian palm pollen indicated the presence of cholesterol, estrone, diosgeni n, β- amrin, β- sitosterol, ruin, quercetin. This work represents the first isol ation of estradiol, hplc detection of esteriol and first spectral data for sever al compounds from date palm pollen[144].

2.2.1 Taxonomy:

Kingdome: Plantae

Subkingdom: Tracheobionta

Super division : Spermatophyta

Division : Magnoliophyta

Class : Liliopsida

Subclass: Arecidae

Order : Arecales

Family: Arecaceae

Genus: *Phoenix*

Species: *Phoenix dactylifera* L

Common name: Date palm pollen

2.2.2. Distribution of date palm

Today the date palm is found in both the Old World (Near East, North Afr ica, Spain) and the New World (Australia and American continent) where dates are grown commercially in large quantities[145] . The date belt stretche s from the Indus valley in the east to the Atlantic in the west. In order to h ave a clear picture on the geographical distribution of date palm, it is wort h looking at it from the following aspects: (A) distribution according to lat itude, (B) distribution according to altitude and (C) number of date palms i n the world[145] . The distribution of date palm according to latitude for both northern and southern hemispheres are between 10°N (Somalia) and 39°

N (Elche/Spain or Turkmenistan). Favorable areas are located between 24 ° and 34°N (Morocco, Algeria, Tunisia, Libya, Egypt, Iraq, Iran). In USA date palm is found between 33° and 35°N. Because of climatic factors, the date palm will grow, but will not fruit properly outside the above defined geographical limits[145] . Altitude is very important since it imposes the avai lability of water and the temperature limits which largely determine the di stribution of date palm in the world. In fact, date palm grows well from 39 2 m below sea level to 1500 m above with an altitude range of 1892 m[145].

2.2.3 Phytochemical compositions of the date palm:

Numerous studies have been conducted to study the benefits of the date pa lm, either from its fruit or seed, and it has been found that the date palm p ossesses several highly beneficial properties such as antiviral, antifungal, a ntioxidant, antihyperlidimic activity and hepatoprotective activity[146] . The se are attributed to the rich contents of antioxidant in date fruit such as the coumaric acid and ferulic acid.

Moreover, it contains flavonoids, sterols, procyanidins, carotenoids, antho cyanin's, sugar (glucose, sucrose and fructose) with low GI, dietary fibers, less protein and fats, vitamins such as riboflavin, biotin, thiamine, ascorbi c and folic acid, and minerals for example calcium, iron, copper, cobalt m agnesium, fluorine, manganese, phosphorus, potassium, sodium, boron, su lfur, zinc and selenium within the date palm itself[146] . In the date seed, it majorly consists of fatty acids including capric, lauric, myristic, myristolei c, palmitic, stearic, oleic, linoleic, linolenic, arachidic[146] . Constituents of t

he date fruit and date seed may contribute to synergistic effects in the afor ementioned bioactivities. While these studies have been successful to inve stigate the benefits of the date palm generally, only a few of them explore the benefits of the date palm on the brain. In the next few paragraphs, the benefits of the date palm physically and psychologically to the brain will b e explored[146].

2.2.4 Medicinal Uses of Phoenix dactylefera:

P. dactylifera are widely used in traditional medicine for the treatment of various disorders which include memory disturbances, fever, inflammatio n, paralysis, loss of consciousness and nervous disorders Abedi in medicin al uses of P. dactylifera as evidenced by the huge research works conducte d in the last few decades[147] .

An opioid is defined as any psychoactive chemical that is characterized by morphine or other opiates in its pharmacological effects. The opioid drugs have the potential to produce profound analgesia, mood change, physical dependence, tolerance and a rewarding effect which may lead to compulsi ve drug use. It exerts its effect by binding to opioid-specific receptors, whi ch are principally localized in central and peripheral nervous systems, as well as in the gastrointestinal tract . The receptors in those organ systems mediate both the beneficial effects and the side effects of the drugs. Opiu m and its derivatives have been used for centuries, both in a medicinal and leisure manner. Indeed, findings of opium poppy seeds dating as far back

as 30,000 years ago suggest the use of opium by Neanderthal man, the birt h of opioid pharmacology can be traced in 1799, when Friedrich Serturner discovered morphine as the major active ingredient of opium. Morphine a nd its derivatives are not only used today for the treatment of acute and ch ronic pain, but also participate in modulation of gastrointestinal, endocrine and autonomic function, as well as a possible role in altered cognitive fun ction P. dactylifera are widely used in traditional medicine for the treatme nt of various disorders which include memory disturbances, fever, inflam mation, paralysis, loss of consciousness and nervous disorders[147].

2.2.5 Pharmacological activity:

Anumber of studies have been carried out on phoenix dactylefera l in rece nt years showing the diverse pharmacological effect that it possessed antio xidant , hepatoprotective , anticancer , nephroprotective anti-diabetic , ant imicrobial, antihyperlipidemic, and sexual improvement activity[148].

2.2.6 Pollination

Date palms are dioecious and bisexual, that means the male and the femal e flowers are born on separate palms. The male flowers produce the pollen and the female flowers produce the fruits. Pollination is one of the essential agricultural practices for date fruit production and quality. Th e pollen from different male cultivar may have different effect on the prod uction and quality of the fruits. The selection of certain male cultivar has e

ffect on the set, quality, size, and color of the fruit[145] . Some of the factors which play an important role in the pollination of date palm are the recepti vity of the stigma for pollen grains and the suitable temperature for the ger mination of pollen grains which is 35°C. The rain fall and the wind have n egative effect on the fruit set. There are many methods for pollination; ma nual and mechanical. Different devices and equipments are used for manu al and mechanical pollination[145] .

Photo of Phoenix dactylefera

Aim of this study

This study was designed to :

- Extract the flavonoids from target species.
- Elucidate the structures of the isolated phenolics.
- Evaluate the isolates for antimicrobial activity.

Chapter two
Material and methods

2. Material and Methods

2.1 Materials

2.1.1 Plant material

The *Trianthema Portulacustrum* stems were collected from Aloubid, Western Sudan , whi**le** *Phoenix Dactylefera* pollens were co llected from Khartoum state- Sudan. The plants were authenticated by th e Department phytochemistry and Taxonomy, National Research Center , Khartoum.

2.1.2 Instruments

Uv- visioble spectrophotometer (Shimadzu UV – 2401PC); joel ECA 50 0 NMR Spectrophotometer.

Joel Mass Spectrometer (JMS- AX500).

2.2 Methods

2.2 Preperations of reagents for phytochemical screening

i) Flavonoid and phenolic test reagents.

- Alumnium chloride solution

1 g of aluminum chloride was dissolved in 100 ml methanol

- **Potassium hydroxide solution**

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

- **Ferric chloride solution**

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

ii) Alkaloid test reagents

Mayer reagent

- **M**ercuric chloride solution : 1.36 g in 60 ml distilled water.
- Potassium iodide solution : 5 g in 10 ml distilled water.

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

-Wagner reagent

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

2.2.2 Preparation of plant extract for phytochemical screening

Powdered air – dried plant material (100g) was extracted with 80 % aque ous methanol (soxhelt) until exhaustion . this prepared extract (PE) was u sed for phytochemical screening.

2.2.3 Phytochemical screening

The prepared extract of target species (PE) was used for following test:

i-Test for unsaturated sterols and for triterpenes

Ten mls of the (PE) was evaporated to dryness on water bath, and th e cooled residue was stirred with petroleum ether to remove most of the c oloring materials. The residue was then extracted with 10 ml chloroform . the chloroform solution was dehydrated over sodium sulphite anhydrou

s. 5 ml portion of the solution was mixed with 0.5 ml of acetic anhydride , followed by two drops of concentrated sulphuric acid. Two separate la yers (green , red) were observed.

ii- Test of flavonoids

Twenty mls of (PE) was evaporated to dryness on water bath. The coo led residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered . the filtrate was used for the follo wing test:

- To 3ml of filtrate afragment of magnesium ribbon was added , shaken and then few drops of concentrated hydrochloric acid were added . red colour was osserved.
- To 3 ml of the filtrate few drops of aluminium chloride solution were added . dark yellow colour was formed.
- To 3 ml of the filtrate few drops of potassium hydroxide solution were added . A dark yellow colour wasobserved.

iii- Test of alkaloids

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

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

iv- Test for tannins

Ten mls of (PE) was evaporated to dryness and the residue was extracted with n- hexane and then filtrated . the insoluble residue was stirred with n- hexane and 10 ml of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added . the mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution . 5 ml of this solution was treated with few drops of ferric chloride solution . A dark blue colour was observed.

v- Test of Saponins

One gram of dried powdered plant material was placed in a clean test tube . 10 ml of distilled water were added and the tube was stoppered and vigorously shaken for about 30 seconds , and allowed to stand. Honey comb was formed.

vi- Test for anthraguinone glycosides

Five gram of powdered were boiled with 10 ml of 0.5 N Potassium hydr oxide containing 1 ml . or 3 % hydrogen peroxide solution. The mixture was extracted with 10 ml . of benzene 5 ml of benzene solution was shak en with 3 ml of 10% ammonium hydroxide solution and the lower layer was allowed to separate , there was no change in colour.

2.2.4 Extraction of flavonoids

Powdered air- dried *Trianthema Portulacustrum* stems 1Kg (or *Phoenix Dactylefera* pollen) were macerated with 80% aqueous ethanol (4 L) for 24hr . at room temperature with occasional shaking and then filtered off. The extraction process was repeated two more times with the same solve nt. Combined filtrates were concentrated under reduced pressure using ro tary evaporator at 40 C until all ethanol was removed yielding a crude pr oduct.

2.2.5 Isolation of flavonoids

Open column (80 x 4 cm) was used for fractionation the total extracts of *Trianthema Portulacustrum and Phoenix Dactylefera* Silica gel with pa rticle size 100 -200 mesh from LOBA chemicals was used as stationary p hase . the column was successively eluted chloroform : methanol (4:1; 3: 2 and 1:4 ;v:v) for both plants , the ratio : chloroform :methanol (1:4) gav e a fraction being riech in phenolics . this fraction was further purified b y TLC.

The fraction (1:4 ; chloroform : methanol) was dissolved in the minimu m amount of ethanol and applied as narrow strips on silica gel TLC plat es (20x20 cm) . the composition of the mobile phase (chloroform : meth anol; 1:4) was determined by TLC analysis.

For both species, the plates were developed with chloroform : methanol ; 1 :4 , v/v) . the chromatograms were viewed under UV light and similar zones were poled together. The pure flavonoids were eluted from silica b y methanol . in this way , I and II were

Isolated from of *Trianthema Portulacustrum* while compounds III and IV were isolated from *Phoenix Dactylefera.* The purity of -

2.2.6 Antimicrobial assay

Different fractions (ethanol, chloroform , ethyl acetate and n- butanol) of *Trianthema Portulacustrum and Phoenix Dactylefera* were evaluated f or their antimicrobial activity against six standard human pathogens (: B acillus subtilis , Staphylococcus aureus , Escherichia Coli , Pseudomon as oeruginos , Aspergillus' Niger and Candidia albacans .) using the cup plate agar diffusion bioassay with some minor modifications.

a-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were d istributed onto agar and incubated at 37 C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal sa line , and finally suspended in 100 ml of normal saline to produce suspen

sion containing about $10^8 – 10^4$ colony forming units per ml . the suspens ion was stored in refrigerator at 4 C until used . the average number of vi able organism per ml of the saline suspension was determined by means of the surface viable counting technique.

Sterial dilutions of the stock suspension were made in sterile normal salin e in tubes and one drop volume (0-02 ml) of the appropriate dilutions we re transferred by adjustable volume micropipette onto the surface of drie d nutrient agar plates. The plates were allowed to stand for two hours at r oom temperature to dry, and then incubated at 37 C for 24 hours.

b- preparation of fungal suspensions

Fungal cultures were maintained on dextrose agar incubated at 25 C for four days. The fungal growth was harvested and washed with sterile nor mal saline, and the suspension was stored in the refrigerator until used.

c- Testing for antibacterial activity

The cup agar diffusion method was adopted with some minor modificati on , to assess the antibacterial activity of the methanolic extract and ethy l acetate fraction of *Trianthema Portulacustrum and Phoenix Dactyle fera* . two ml of the standardized bacterial stock suspention were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45 C in water bath .

(20 ml) Aliquots of the incubated nutrient agar were distributed into steril e petri dishes and the agar was left to settle in each of these plates which were divided into two halves. Two cups in each half (10mm in diameter) were cut using sterile cork borer (NO4). Each of the halves was designed for one of the extracts .

The agar discs were removed and cups were filled with (0.1) ml of each e xtract using adjustable volume microtiter pipette and allowed to diffuse a t room temperature for two hours. The plates were then incubated in the u pright position at 37 C for 24 hours.

The above procedure was repeated for different concentrations of the extr acts and the standard antimicrobial chemotherapeutics . After incubation the diameters of the resultant growth inhibition zones were measures. th e above mentioned method was adopted for antifungal activity , but inste ad of nutrient agar dextrose agar was dextrose agar was used . Samples w ere used here by the same concentrations used above.

Chapter Three
Results and Discussion

3-Results and Discussion

3.1- *Trianthema portulacustrum*

3.1.1-Phytochemical screening

The alcoholic extract of *Trianthema portulacustrum* stems was assessed for major secondary metabolites and the results are depicted in Table (3.1) .

Table 3.1: Phytochemical screening of *Trianthema portulacustrum* stems

Clas	Reagent used	ethanolic extract
Saponins	Froth test	+
Alkaloids	-Mayer's reag	++
Tanni	Ferric chloride	+
Glycosides	Borntrager te	+
Sterols and triterp	Liebermann-Burchar	+
Flavonoids	-lead acetate t est	+
Phenols	Ferric chloride test	+
Carbohydrates	Molich,s test	+

3.1.2-Biological activity

Different fractions of *Trainthema Portulacastrum* stems (ehanol, chlor oform, ethyl acetate and n-butanol) were assessed for antimicrobial pote ntial against six standard bacterial strains using the cup plate agar diffus ion method with some minor modifications. Test organisms are: *Bacillu s subtilis* (Bs.), *Staphylococcus aureus* (Sa.) , *Escherichia Coli* (Ec.) , *Pseudomonas aeruginosa* (Ps.), *Aspergillus niger* (As.) and *Candidi a albacans* (Ca.) . The results are displayed in Table (3.2).

Table (3.2): Antimicrobial activity of *Trainthema portulacastrum*

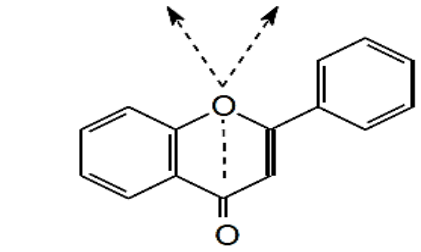
Fraction	MIZD(mm)					
	Standard micro-organism					
	Bacterial strains				Fungal strains	
	Ec.	Ps.	Bs.	Sa.	Ca.	An.
Crude extract	-	12	14	12	12	12
Chloroform frac	-	-	15	-	14	14
Ethyl acetate fra	-	-	14	-	15	16
n-butanol fractio	-	-	13	-	15	15

3.1.3-Isolation of flavonoids

The crude ethanolic extract of *Trianthema portulacustrum* stems was fractionated by column chromatography and then further purified by t hin layer chromatography . After the usual work up, two flavonoids-compounds I and II were isolated in chromatographically pure form. The structures of these isolates were partially elucidated by a combination of spectral tools(UV,1HNMR and MS).

3.1.4-Identification of compound I

Flavonoids with conjugation between A and B rings show two absorption band in the UV spectrum: band 1 in the range : 300-400 nm , arising fro m (B) ring cinnamoyl system. Band II in the 220- 285 nm range , arising from (A) ring benzoyl system.



The UV spectrum of compound I (Fig.1) showed λmax (MeOH) 269 nm. Such absorption – which originates from a benzoyl system- is revealed by : flavanones, isoflavones , dihydrochalcones and dihydroflavonols. But isoflavones give a shoulder in the range 300 -340nm – such shoulder was not found in the spectrum of compound I

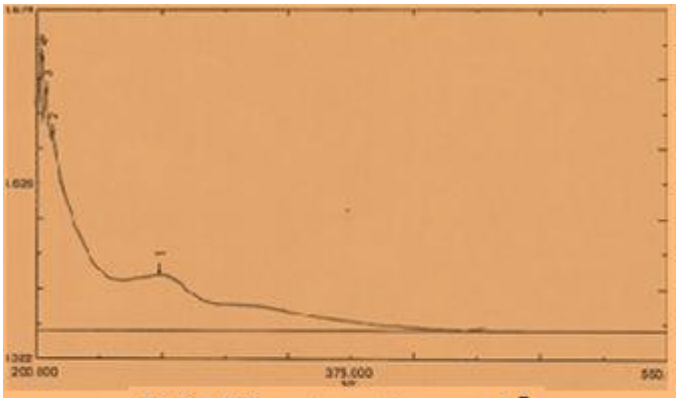


Fig.1 : UV spectrum of compound I

The shift reagent sodium methoxide is a strong base . it is diagnostic of 3- and 4`- OH . In both cases it affords a bathochromic shift , but with decrease in intensity in case of a 3-OH function. The presence of a 3-OH function is a characteristic feature of dihydroflavonols.

Addition of sodium methoxide to a methanolic solution of compound I caused (Fig.2) no shift . This shows absence of dihyflavonols which are characterized by their 3-OH function.

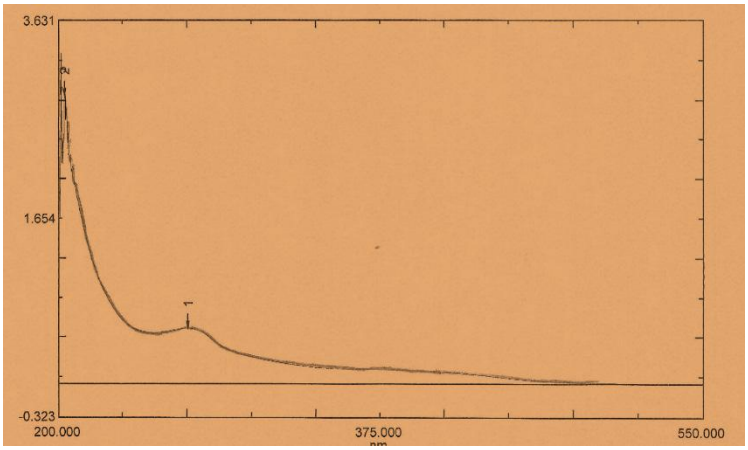


Fig. 2 : Sodium methoxide spectrum of compound1

Dihydrochalcones and flavanones are distinguished via their NMR spect ra. Flavanones exhibit a double multiplet around δ2.80 and δ5.20 due to mutual splitting of the magnetically equivalent protons at C3. The doubl e doublet resulting from such spin-spin splitting afford a pair of double d oublet (usually appearing as pair of multiplets) at δ2.80ppm and δ5.20pp m due to further splitting by the neighboring C2 proton.

However , the 1HNMR spectrum of compound I (Fig. 6) did not reveal such multiplets indicating that this compound is a dihydrochalcone.

The hydroxylation pattern of this dihydrochalcone was investigated using the shift regents: sodium acetate, aluminium chloride and boric aci d. Sodium acetate is a weaker base than sodium methoxide and as su ch ionizes only the more acidic hydroxyl group in flavonoids. It is partic ulary useful diagnostic reagent for the specific detection of a 7 – hydrox yl function. When the shift reagent sodium acetate was added to

amethanolic solution of compound I (Fig.3) no bathochrmic shift was observed this indicates absence of 7- OH group.

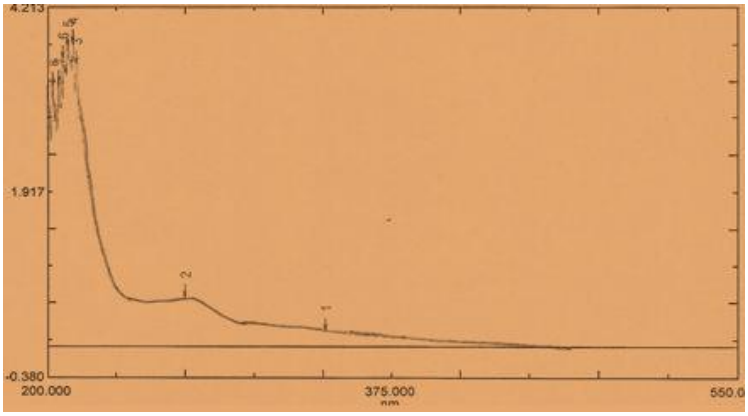
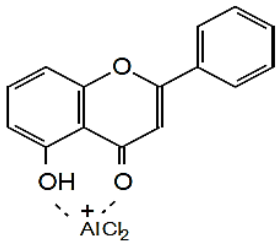


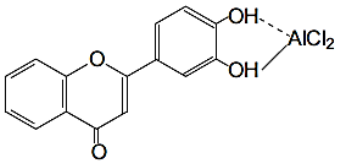
Fig. 3: The sodium acetate spectrum of compound I

Aluminium chloride chelates with functional groups such as the 5 – hyd roxyl – 4- keto , 3- hydroxyl -4- keto systems and this is evidenced by bathochromic shift of one or both bands in the spectrum . It also chelate s with catechol systems giving bathochromic shifts .The aluminium chlo ride complexes involving 3-OH, (5-OH) and the 4- keto

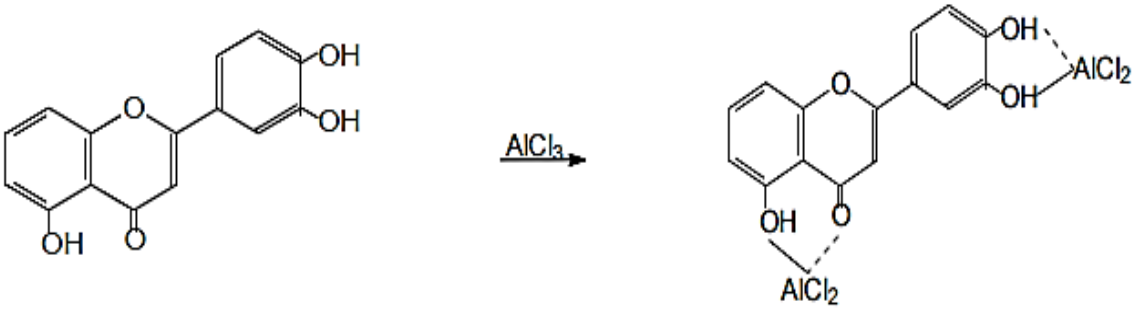
function are of the following type:

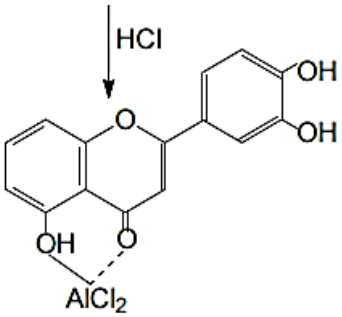


With catechol moieties complexes are of the following type:



While the 3- and 5-OH functions form acid-stable complexes, catechol systems form acid-labile complexes.





When the shift reagent aluminum chloride was added to a methanolic sol ution of compoundI (Fig.4) no bathochromic shift was observed indicat ing absence of 3- , 5-OH and catechol systems. Also the boric acid s pectrum did not reveal any bathochromic shift indicative of catechol moi eties (Fig.5).

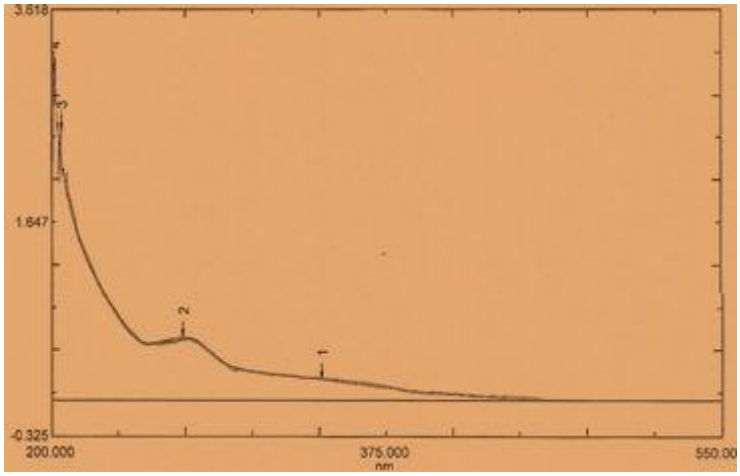


Fig. 4: Aluminium chloride spectrum of compound I

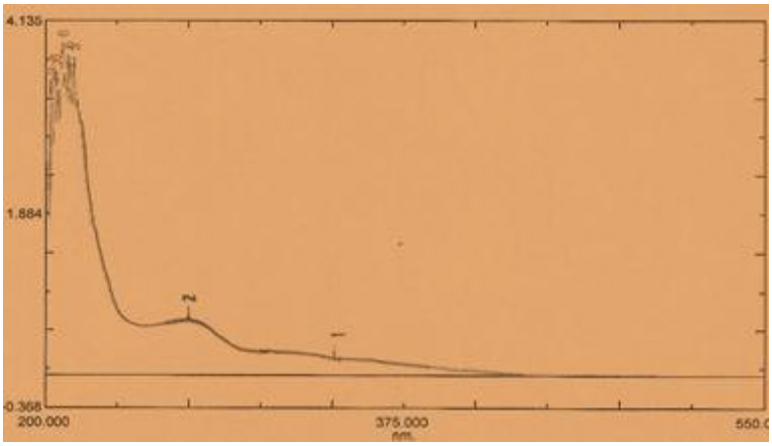


Fig. 5: Boric acid spectrum of compound I

The H1 NMR spectrum (Fig.6) showed a signal at δ 1.23 ppm assigned a methyl function .The resonance at δ1.67 is due to an acetyl function , w hile the peak at δ 4.26 accounts for a methoxyl group.. The multiplet at δ 3.40-3.9 was assigned for a sugar moiety. The sugar anomeric proton re sonated downfield relative to the bulk of the sugar protons at δ 5.18 ppm .This sugar was not identified in this study . The multiple at δ 8.480 ppm was attributed for the aromatic protons.

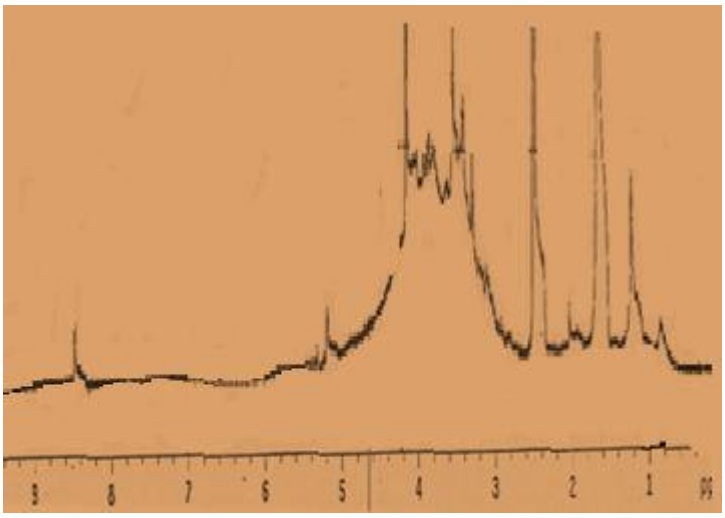
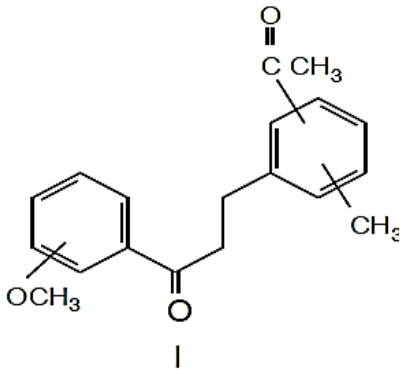


Fig.6 : 1H NMR spectrum of compound I

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



I

A future 2D NMR experiments (1H- 1H COSY NMR , HMBC , HSQC) may fully elucidate the structure of this flavonoid.

3-1-5 Charactrization of compound II

The UV spectrum of compound II (Fig.7) showed λmax (MeOH) 271 nm . Such absorption – which originates from a benzoyl system- is reveale d by : flavanones, isoflavones , dihydrochalcones and dihydroflavonols . But isoflavones give a shoulder in the range 300 -340nm – such shoulder was not found in the spectrum of compound II .

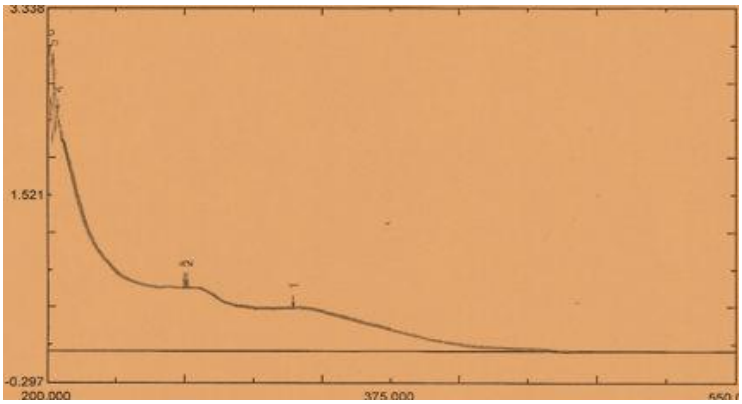


Fig. 7: UV spectrum of compound II

Addition of sodium methoxide to a methanolic solution of compound II did not reveal any bathochromic shift (Fig.8) . This shows absence of di hydroflavonols which are characterized by their 3-OH function.

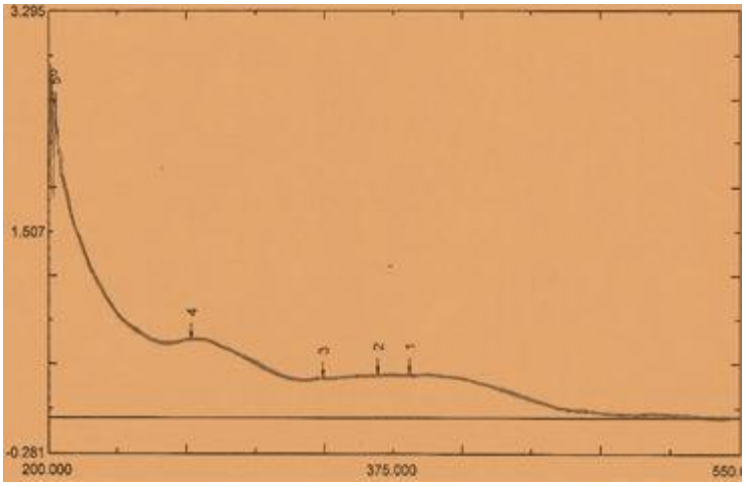


Fig. 8 : Sodium methoxide spectrum of compound II

Flavanones exhibit a double multiplets around δ2.80 and δ5.20ppm.The 1HNMR spectrum of compound II (Fig. 12) did not reveal such m ultiplets indicating that this compound II is a dihydrochalcone.

The hydroxylation pattern of this dihydrochalcone was investigated using the shift regents: sodium acetate, aluminium chloride and boric acid. Sodium acetate is a weaker base than sodium methoxide and as such ionizes only the more acidic hydroxyl groups in flavonoids. It is particularly useful diagnostic reagent for the specific detection of a 7 – hydroxyl function. When the shift reagent sodium acetate was added to amethanolic solution of compound II(Fig.9) no bathochrmic shift was observed this indicates absence of 7- OH group.

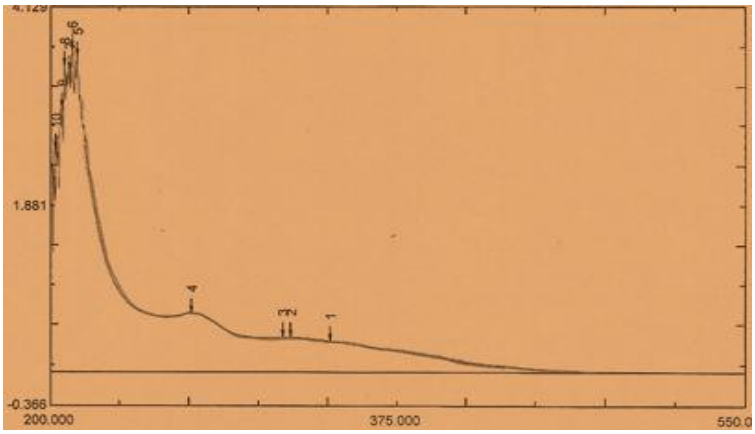


Fig. 9: The sodium acetate spectrum of compound II

When the shift reagent aluminum chloride was added to a methanolic solution of compound II (Fig.10) no bathochromic shift was observed indicating absence of 3- , 5-OH and catechol systems. Also the boric acid spectrum did not reveal any bathochromic shift indicative of catechol moieties (Fig.11).

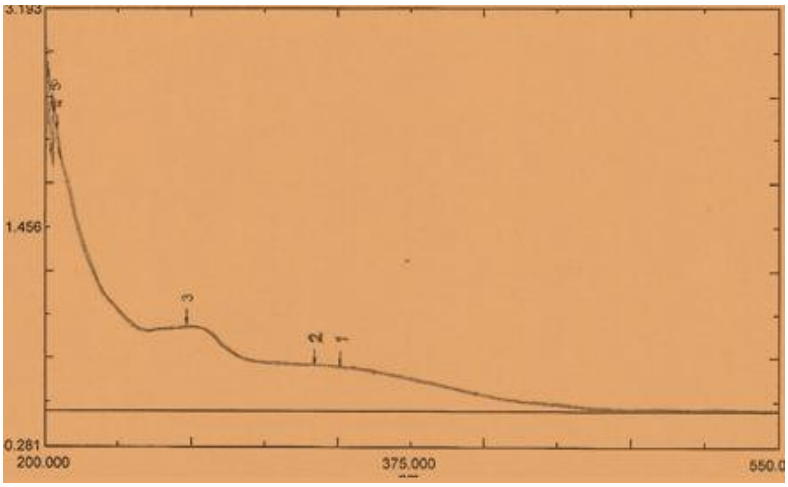


Fig. 10: Aluminium chloride spectrum of compound II

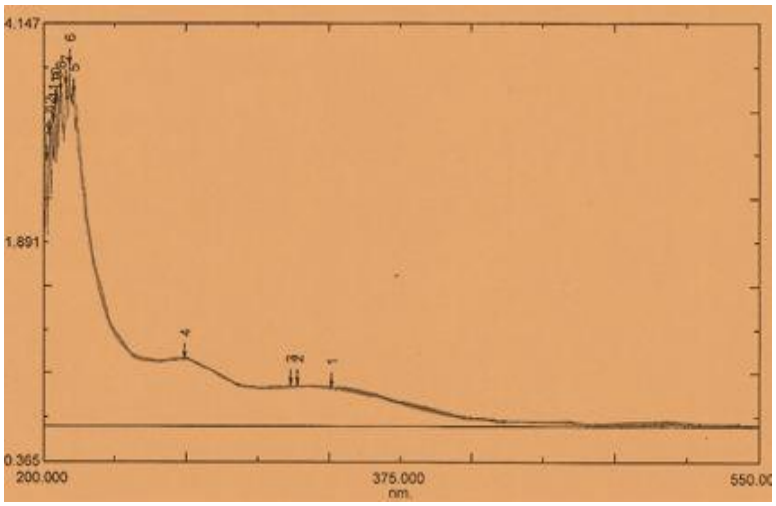


Fig. 11: Boric acid spectrum of compound II

The H1 NMR spectrum (Fig.12) showed a signal at δ 1.23ppm, 1.25ppm, assigned two methyl function .The resonance at δ2.50ppm is due to solv ent(DMSO) residual protons , while the peak at δ 3.6ppm accounts for a methoxyl group. The multiplet at δ4.12-4.14 ppm was assigned for a su

gar moiety(not identified in this study) . The multiple at δ 7.7 – 8.5 ppm was attributed for the aromatic protons.(DMSO water appears usually ar ound δ3.30).

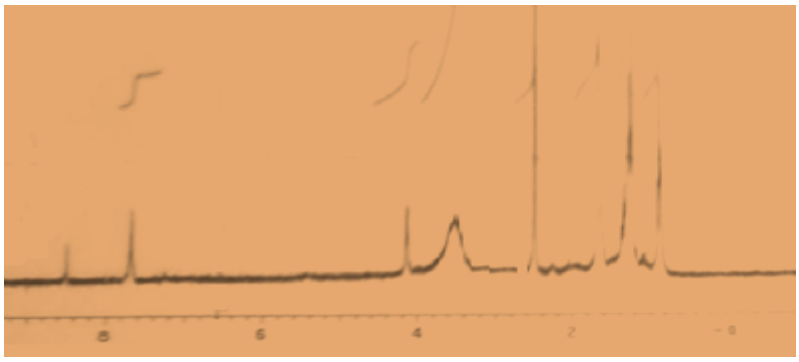
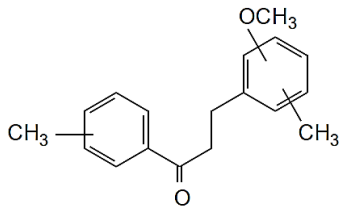


Fig.12 : 1H NMR spectrum of compound II

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



II

A future 2D NMR experiments (1H- 1H COSY NMR , HMBC , HSQC) may fully elucidate the structure of this flavonoid.

3.2- *Phoenix Dactylefera*

3.2.1-Phytochemical screening

The alcoholic extract of *Phoenix Dactylefera* pollen was assessed for major secondary metabolites and the results are depicted in Table (3.3)

Table 3.3: Phytochemical screening of *Phoenix Dactylefera* ethanolic extract

Class	**Reagent us ed**	**ethanolic extract**
Saponins	Froth test	+
Alkaloids	Mayer's reagent 2-Hajer,s test	+
Tannins	Ferric chloride test	+
Glycosides	Borntrager tes*t*	+
Sterols and triterpenes	Liebermann-Burchard test	+
Flavonoids	1-lead acetate test	+
Carbohydrates	Molich,s test	+
Phenols	Ferric chloride test	+

3.2.2-Antimicrobial susceptibility

Different fractions of *Phoenix dactylefera* pollen were evaluated for their antimicrobial activity against six standard human pathogens and the results are depicted in Table **3.4.**

Table (3.4): Antimicrobial activity of. Whole plant of Phoenix dactylefera**.**

fraction	MIZD(mm)					
	Standard micro-organism					
	Bacterial strains				Fungal strain	
	E.c	P.s	B.s	S.a	C.a	A.n
Crude extract	-	-	-	-	13	13
Chloroform fraction	-	-	-	-	12	-
Ethyl acetate fraction	-	-	18	12	13	-
n-butanol fraction	-	15	16	14	18	16

3.2.3-Identification of compound III

The crude ethanolic extract of *Phoenix Dactylefera* was fractionated by column chromatography and then by thin layer chromatography. After the usual work up, compounds III and IV were isolated in chromatogr aphically pure form. The structures of these isolates were partially eluci dated by a combination of spectral tools(UV,1HNMR and MS).

The UV spectrum of compound III (Fig.13) showed λmax (MeOH) 268nm . Such absorption – which originates from a benzoyl system- is r evealed by: flavanones, isoflavones , dihydrochalcones and dihydrofla vonols. No shoulder characteristic of isoflavones in the range 300 -340nm was found in the UV spectrum of compound III (Fig.13).

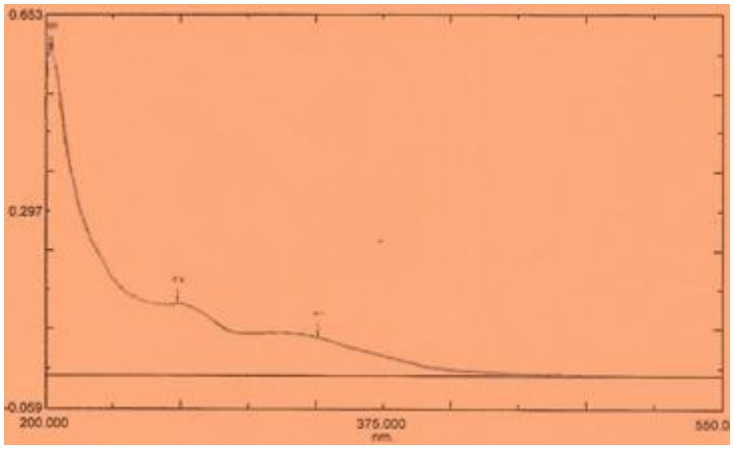


Fig. 13: UV spectrum of compound III

Addition of sodium methoxide to a methanolic solution of compound III caused (Fig.14) a 7nm bathochromic shift without decrease in int ensity indicating a 4`-OH group. The absence of a 3-OH –as revealed by the sodium methoxide spectrum suggests that compound III is either a f lavanone of a dihydrochalcone.

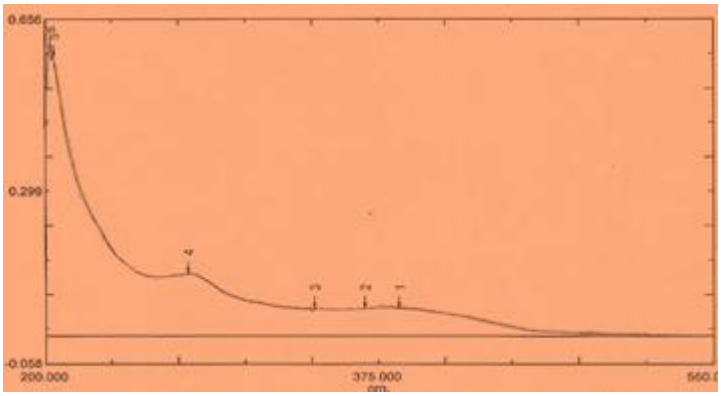


Fig.14 **:**Sodium methoxide spectrum of compound III

However, flavanones exhibit a double multiples around δ2.80 and δ5.20 ppm. , the 1HNMR spectrum of compound III (Fig. 18) did not reveal su ch multiples indicating that compound III is a dihydrochalcone.

The hydroxylation pattern of this dihydrochalcone was investigated using the shift regents: sodium acetate, aluminum chloride and boric aci d.. When the shift reagent sodium acetate was added to a methanolic sol ution of compound III (Fig.15) no bathochromic shift was observed this indicates absence of 7- OH group

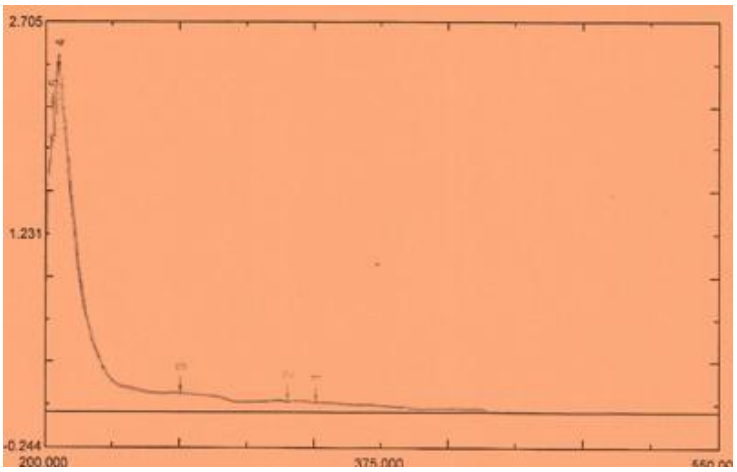


Fig. 15: The sodium acetate spectrum of compound III

When the shift reagent aluminum chloride was added to a methanolic sol ution of compound III (Fig.16) no bathochromic shift was observed in dicating absence of 3- , 5-OH and catechol systems. Also the boric a cid spectrum did not reveal any bathochromic shift indicative of catecho l moieties (Fig.17).

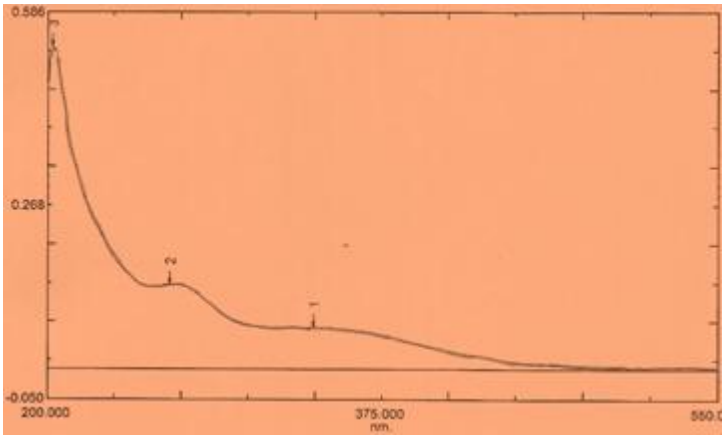


Fig. 16: Aluminium chloride spectrum of compound III

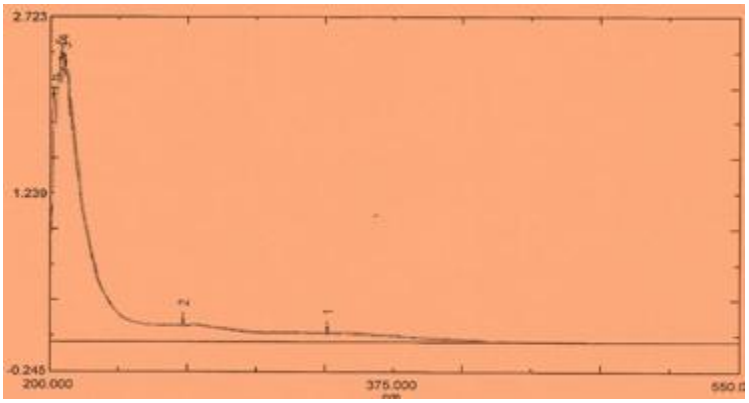


Fig. 17: Boric acid spectrum of compound III

The H1 NMR spectrum (Fig.18) showed a signal at δ 0.95ppm assigned a methyl function .The resonance at δ1.90 is due to an acetyl group , wh ile the peak at δ 3.74 accounts for a methoxyl group. The multiplet at δ4. 20-4.43 was assigned for a sugar moiety. The sugar anomeric proton res onated downfield relative to the bulk of the sugar protons at δ 5.65 ppm. This sugar was not identified in this study . The multiple at δ 6.60 – 6.80 ppm was attributed for the aromatic protons (signal at δ2.50 ppm is due to residual solvent(DMSO) protons. DMSO water appears usually a round δ3.30).

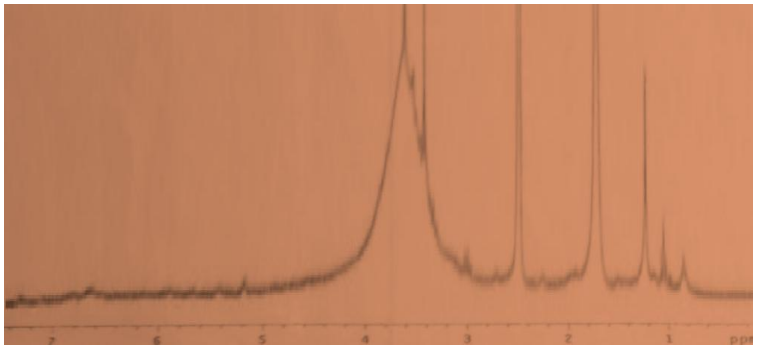
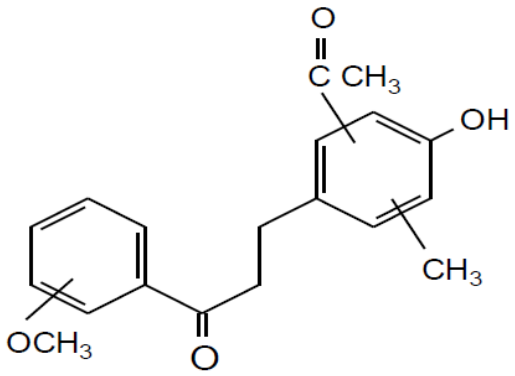


Fig.18 : 1H NMR spectrum of compound III

On the basis of the above cumaltive spectral data, the following partial structure was assigned for the aglycone of compound III :



III

A future 2D NMR experiments (1H- 1H COSY NMR , HMBC , HSQC) may fully elucidate the structure of this flavonoid.

3.2.4-Characterization of compound IV

The UV spectrum of compound IV (Fig.19) showed λmax (MeOH) 272 nm. Such absorption – which originates from a benzoyl system - is revealed by : flavanones, isoflavones , dihydrochalcones and dih ydroflavonols.No shoulder in the range 300-340nm characteristic of isof lavones was detected in the spectrum of compound IV .

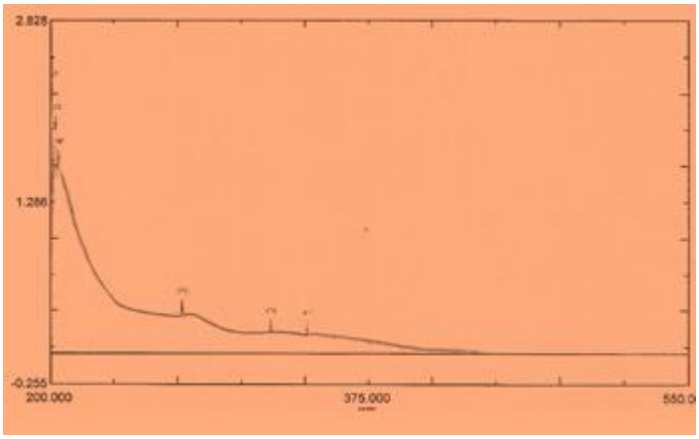


Fig. 19: UV spectrum of compound IV

Addition of sodium methoxide to a methanolic solution of compound IV caused (Fig.20) no bathochromic shift characteristic of the 3-OH gro up of dihydroflavonols.

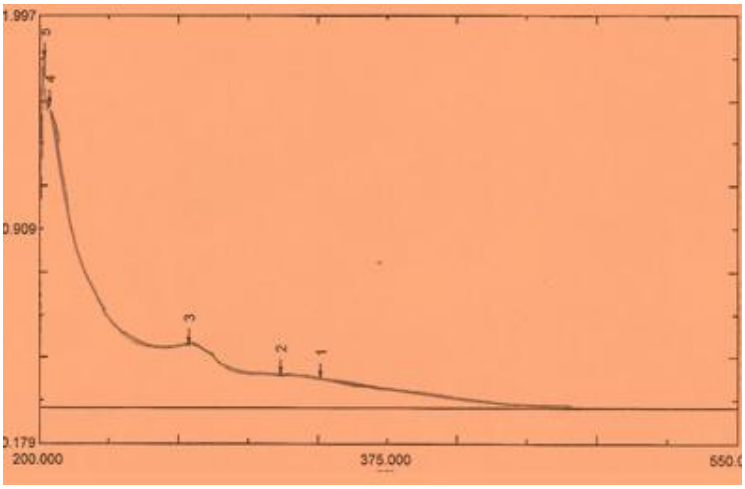


Fig.20 **:**Sodium methoxide spectrum of compound IV

the 1HNMR spectrum of compound IV (Fig. 24) did not reveal m ultiplets at 2.8 and 5.2ppm characteristic of flavanones indicating that th is compound is a dihydrochalcone.

The hydroxylation pattern of this dihydrochalcone was investigated using the shift regents: sodium acetate, aluminium chloride and boric aci d. When the shift reagent sodium acetate was added to a methanolic solu tion of compound IV(Fig.21) no bathochrmic shift was observed indic ating absence of 7- OH group.

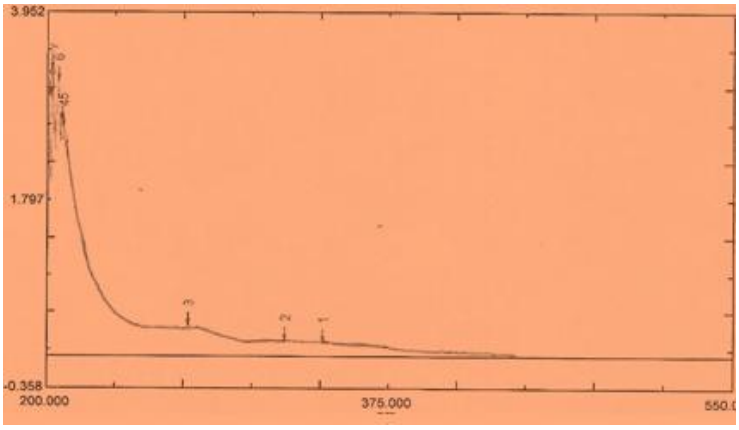


Fig. 21: The sodium acetate spectrum of compound IV

The aluminum chloride spectrum(Fig.22) showed no bathochromic shift characteristic of 3- , 5-OH and catechol systems. Also the boric acid spe ctrum did not reveal any bathochromic shift indicative of catechol moiet ies (Fig.23).

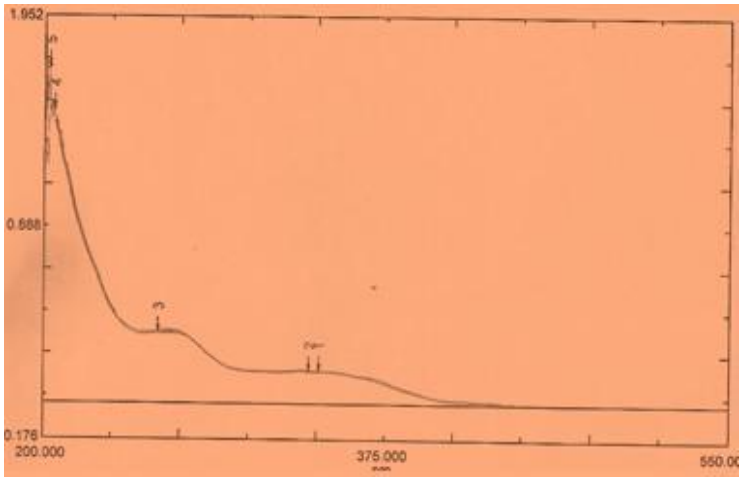


Fig. 22: Aluminium chloride spectrum of compound IV

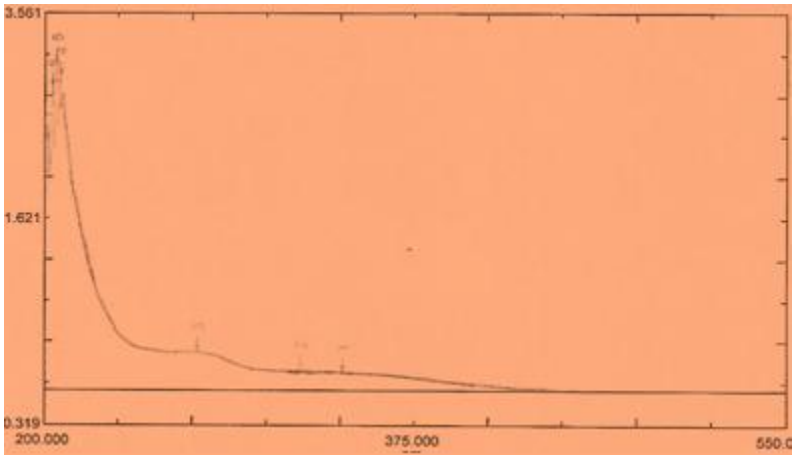


Fig. 23: Boric acid spectrum of co mpound IV

The H1 NMR spectrum (Fig.24) showed a signal at δ 1.12(9H)ppm ,1.63(9H)ppm assigned six methyl functions .The resonances at at δ 3.86(6H) and δ 4.20(6H)ppm accounts for four methoxyl groups. The m ultiplet at δ4.27-4.24ppm was assigned for a sugar moiety. The sugar a

nomeric proton resonated downfield relative to the bulk of the sugar prot ons at δ 5.18 ppm.This sugar was not identified in this study .

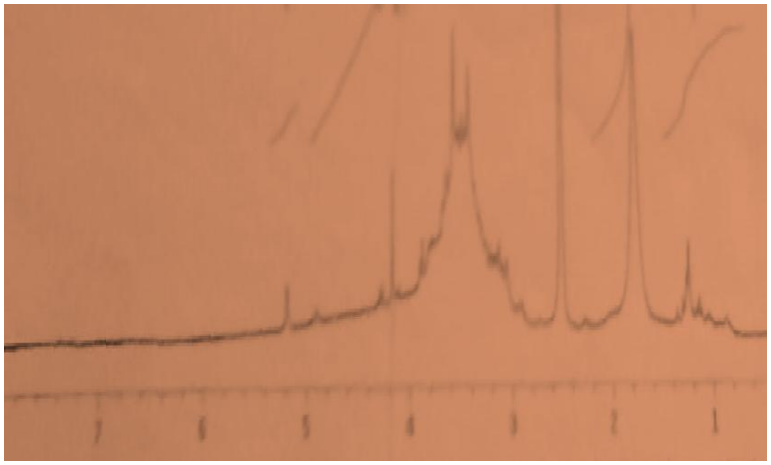
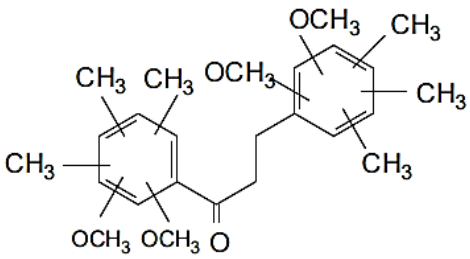


Fig.24 : 1H NMR spectrum of compound IV

On the basis of the above cumulative spectral data, the following partial structure was assigned for the substituted aglycone of compound IV:



IV

A future 2D NMR experiments (1H- 1H COSY NMR , HMBC , HSQC) are necessary for complete structural elucidation.

Conclusion:

The flavonoids of Trianthema *Portulacustrum and Phoenix Dactylefera* . Were extracted by aqueous ethanol. The crude extracts of both plants purifie d by column and further by TLC chromatography . in this way compounds I and II were isolated from *Trianthema Portulacustrum* while compounds III and IV were isolated from *Phoenix Dactylefera* . the partial structures of the se flavonoids were deduced on the basis of their spectral data (UV, 1HNMR and MS).

Different fractions of the target species were evaluated for their antimicrobia l potential and promising results were obtained.

Recommendations

i) Afuture 2D NMR (1H-1H COSY NMR, HMBC and HSQC)

experiment may fully elucidate the structures of the isolated compounds .

ii) The isolates may be evaluated for other biological activities i.e. as antimalarial , antinflammatory, antilaeshmenial ……etc.

iii) In vivo antimicrobial activity is also recommended.

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