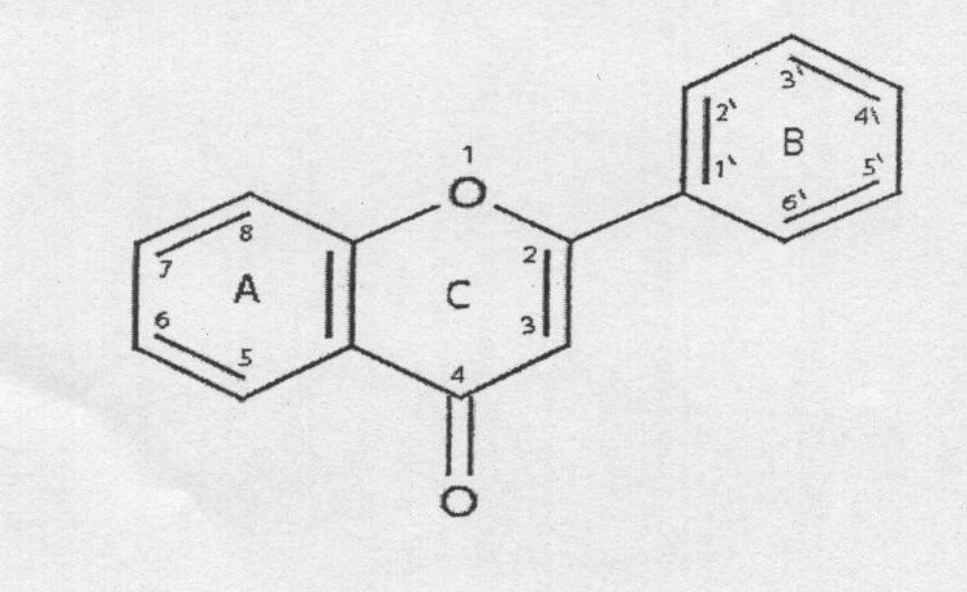
1-Introduction

1.1General approach

Flavonoids (2-phenyl benzopyrone) (1) are a large group of polyphenolic compounds occur commonly in plant [1] . The name (flavonoid) is derived from Greek word “Flavus” :it means yellow [2] .



(1)

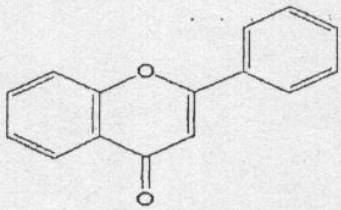
These phytochemicals are distributed widely in higher plant parts (barks, roots, stems, flowers) but also found in some lower plants including algae [3,4] .Many of these compounds are responsible for the attractive colours of flowers and fruits and leaves [5] .flavonoids mainly occur as aglycones (consisting af a benzene ring condensed with a six membered ring which possesses a phenyl ring at the 2,3 or 4 position) Glycosides which carry one or more sugar residues [6]and methylated derivatives are very common.

1.2 Structure and classification of flavonoids:

The chemical structure of these compounds are based on a (C_6-C_3-C_6) skeleton. They differ in saturation of hetero atomic ring C, in the placement of the aromatic ring B at the positions 2,3 0r 4 . According to the modifications of the central C-ring, they can be divided into different structural classes including flavones (2) flavonols (3) flavan-3-ols (4), flavonones (5), isoflavones (6), and anthocyanine (7) . In a few cases, the 6-membered heterocyclic ring C occurs in an isometric open form or is replaced by a 5-membered ring as in the case of chaclones (8) and aurones [7,8,9]. In plants, flavonoid aglycones (flavonoids without attached sugars) occur in a variety of structures .

Most frequently encountered groups of flavonoid aglycones included flavones, flavonols, anthocyanidins, isoflavones, flavanones, dihydroflavonols, biflavonoids, chalcones, and aurones. Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slightly acidic .

Those possessing a number of un substituted hydroxyl groups, or sugar moieties, are polar substances and soluble in polar organic solvents. The presence of sugar makes flavonoid more water soluble, while less polar aglycones like isoflavones, flavanones, and highly methoxyalted flavonols tend to be more soluble in ether or chloroform [10,11].



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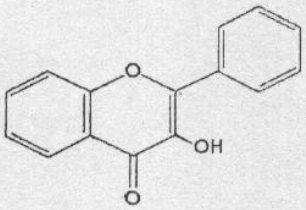

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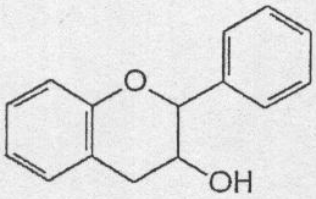

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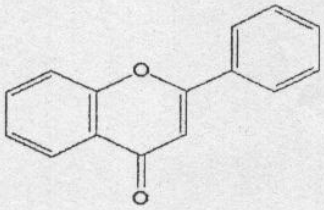

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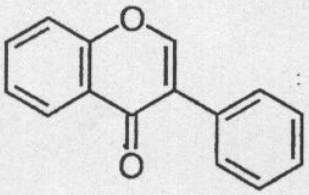

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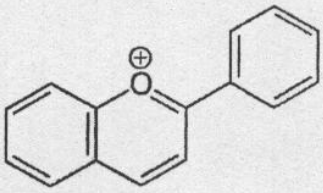

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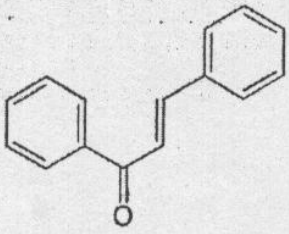

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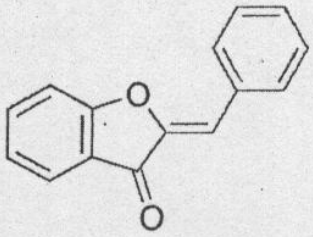

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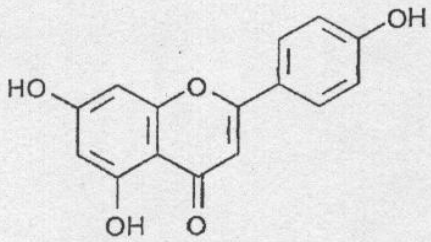
1.2.1 Flavones

The ring C of flavones (2-phenyl-chromen-4-one) (2) contains a double bond between positions 2 and 3, and a keto function on position 4. Most flavones of fruits and vegetables hold a hydroxyl groups on position 5 of ring A, whereas hydroxylation on other positions, most often position 7 of ring A or position 3` and 4` of ring B, can vary depending on the taxonomic classification of a particular fruit or vegetable.

The flavones are of widespread occurrence and they include apigenin, (5,7,4`-trihydroxy flavones ) (10), luteolin (5,7,3`,4` tetra hydroxyl flavones) (11) and chrysin (12). Apigenin and luteolin are the most widespread flavones aglycones. However they diverse substitution patterns make this group the largest. Glycosylation occur mostly at positions 5 and 7 , and methylations and acylation usually occur on hydroxyl groups of ring B. Some flavones are poly methoxylated, such as tangeretin (13) nobiletin(14), scutellarein (15) and sinensetin (16) and they are found almost exclusively in the peels of citrus fruits which contain a number of compounds including C-flavone glycosides in the form of isoorientinxz (luteolin-6-glucoside)(17) and orientin(luteonlin-8-C-glucoside) (18) .

Orientin and isoorientin also occur in cymbopogencitrata along with two other flavones C-glycosides , chrysoeriol -6- C glucoside (isoscoparin) (19) and 7-O-methyl- luteonlin-6-glucoside (swertiajaponin) (20) [12,13,14]



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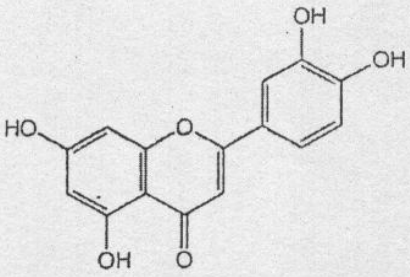

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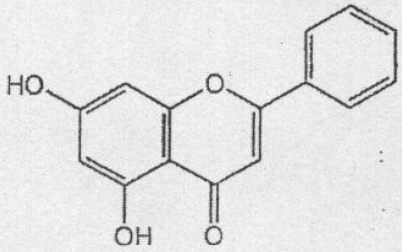

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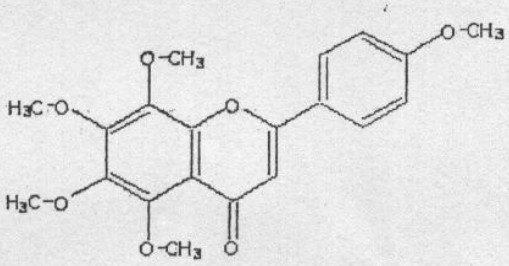

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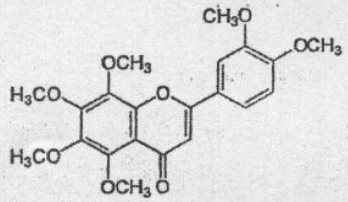

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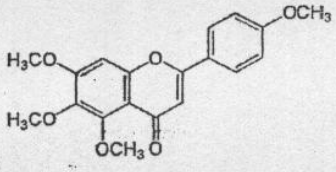

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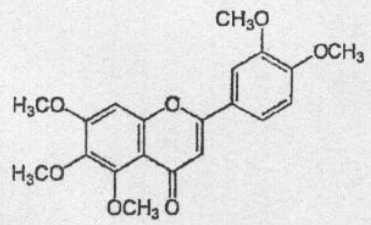

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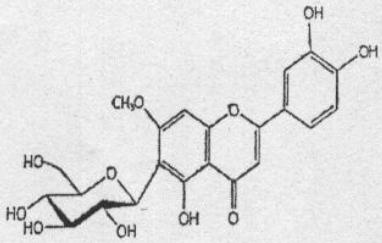

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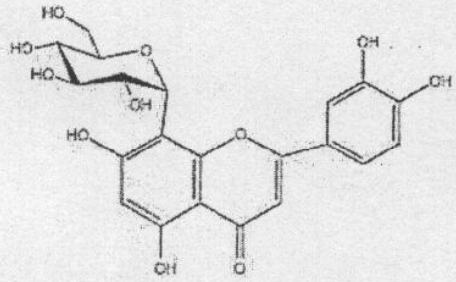

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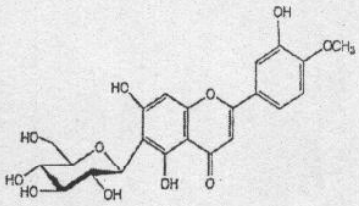

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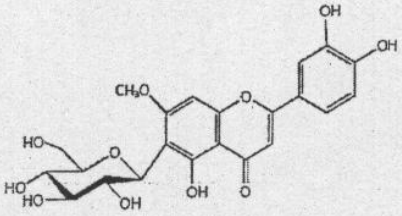

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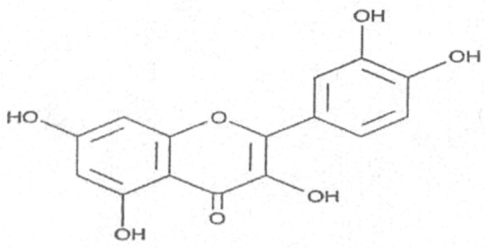
1.2.2 flavonols

The flavonols (3-hydroxy-2-phenyl-chromen -4-one) (3) are the most widespread flavonoids in plant . They vary in colour from white to yellow. Flavonoies have a double bond between C2 and C3 and an oxygen atom at the C4 position. Furthermore flavonols also have a hydroxyl group at the C3 position. They are represented mainly by quercetin (21) kaempferol (22) and myricentin (23) and methylated derivative isorhamnetin (24).They are frequently found as 0-glycosides, in which glycosylation occurs mainly at the 3- position of the C-ring, but substitution can also occur at the 5', 7', 4' or 3' positions. Many types of glycosides are derived from flavonolaglycones because various sugar groups of flavonols can conjugate to the hydroxyl group of flavonols at different positions8' 15 16) Flavan -3- ols or flavonols have a saturated three —carbon chain with a hydroxyl group in the C3position. In foods they are present as monomers or as pro anthocyanidins, which are polymeric flavanols (4 to 11 units) known also as condensed tannins. In foods they are never gIycosylate[17].



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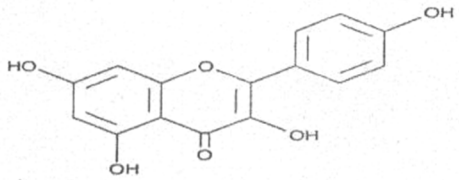

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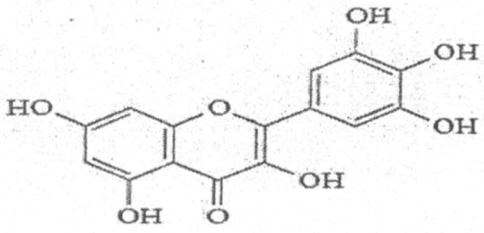

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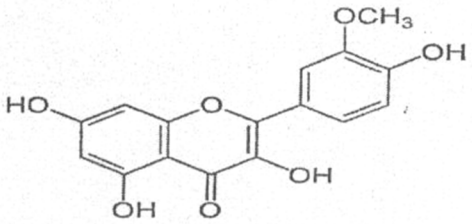

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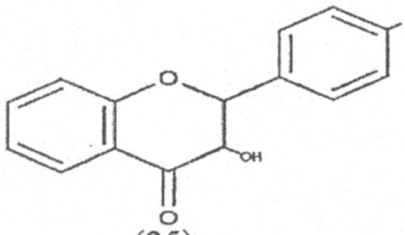
1.2.3 Flavanones

Flavanones (2-phenyl- chroman- 4- one) (5) (also called di hydroflavones) lack the double bond between carbons 2 and 3 in the C- ring of the flavone skeleton, which is present in flavones and flavonols.

Two stereo isomeric forms of each flavanone structure are possible, since C-2 is a center of asymmetry (epimeric center). Consequently, the B-ring can be either in the (2S) or (2R) configuration. The great majority of the flavanones isolated from plants are levorotatory (2R) or (2 S) flavanones, because the enzymatic reaction catalyzing the conversion of chalcones to flavanones is stereo specific. The C-3 atom of di hydro flavonols bears both the a hydrogen atom and a hydroxyl group, and is therefore an additional center of asymmetry. Thus, four stereo isomers are possible for each di hydro flavonol structure, (2R, and 3R), (2R, 3S), (2S, 3R), and (2S, 3S). All four configurations have been found in naturally occurring di hydro flavonols, but the (2R, 3R) configuration is by far the most common[18].

Flavanones are mainly represented by narigenin (25), hespertin (26), and eriodyctol (27), while a number of minor compounds, including sakuranetin (28) and iso sakuranetin (29) also occur [8]. Flavanones are represented by the saturated heterocyclic ring (C) and a carbonyl function at the 4 position.

Flavanones are usually glycosylated at position 7 by a disaccharide (neohespiredose, rutinose) or, by a monosaccharide (glucose) [19].



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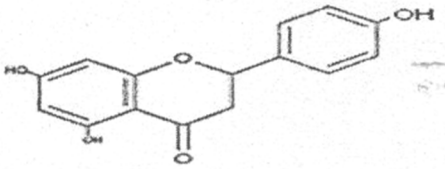

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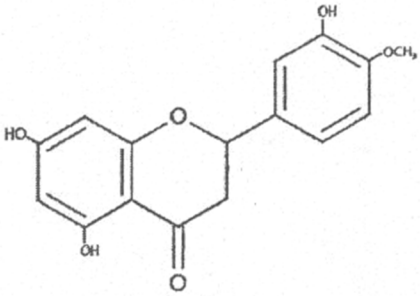

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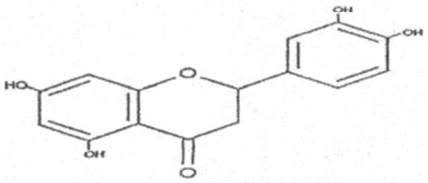

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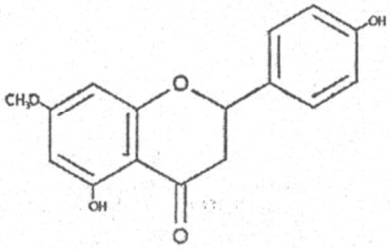

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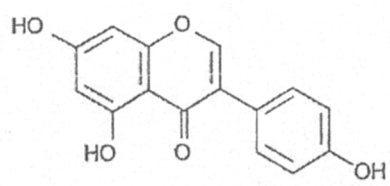
1.2.4 Isoflavones

Isoflavones (3-phenyl-chromen- 4- one) (6) also have a diphenyipropane structure in which the B ring is Iocated in the 3 position.

Common iso flavones naglycones such as genistein (30), daidzein (31), and glycitein (32) occur, ii low levels, in black beans (Phaseolus vulgaris) and green beans (Pisumsafivum) together with formononetin (33) and biochanin A (34)(8)• They have structural analogues to estrogens, such as estradiol, with hydroxyl groups at the C7 and C4 positions[20].



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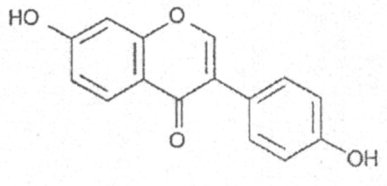

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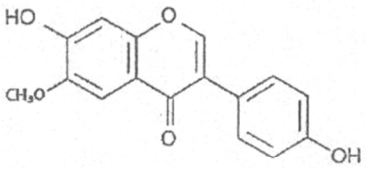

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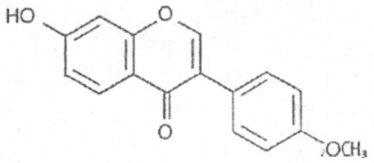

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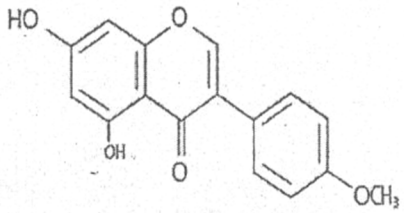

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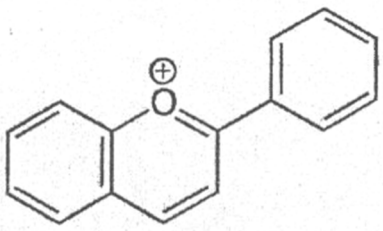
1.2.5 Flavanols

Flavanols (4) are often referred to as flavan-3-ols, as the hydroxyl group is almost always attached to the position 3 of ring C, but it may occupy position 4 or the 3,4- positions giving flavan-3,4-diols. Flavanols - are based on the flavylium salt (35) structure and are water- soluble pigments in plants. Flavanols are also interchangeable with the term catechins. Flavanols do not have the ketone feature as the flavonols. Catechins have two epimers depending on the stereo configurations of the bond between ring B and position 2, and the hydroxyl group on position 3. These two epimers are: (+)-cachetin (36) and (-) —epicatechin (37). Their respective derivatives are gallocatechin (38) and epigallo catechin (39). (+) epigallocatechinegallate (40) and (-)- epicatechingallate (41) are together categorized as catechins (-)-. Gallocatechin and epigallocatechin contain an extra hydroxyl group on ring B. Flavanols or catechins are often found in the skins of fruits and certain vegetables. Many commonly consumed fruits and vegetables are found to contain flavanols and their gallic acid esters [17].



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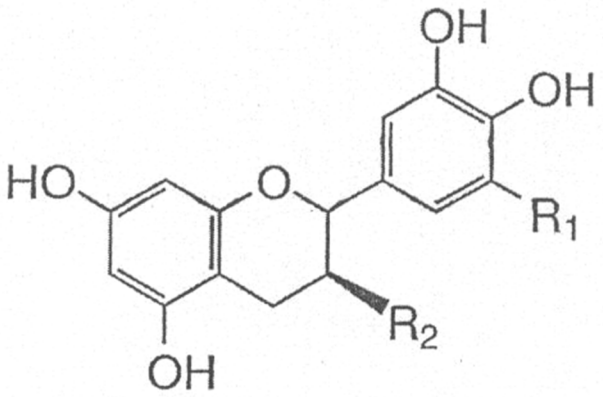
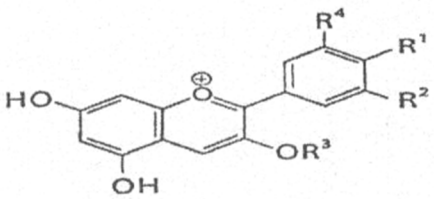


(36) R_1 = H,R_2 =OH
(37) R_1 = H,R_2 =O
(38) R_1 = OC- Ph (OH), R_2 = H
(39) R_1 = OH , R_2 = OH
(40) R_1= OH , R_2= OO-Ph (OH)
(41) $_{R1}$ = H, R_2 = OC- Ph (OH)

1.2.6 Anthocyanins

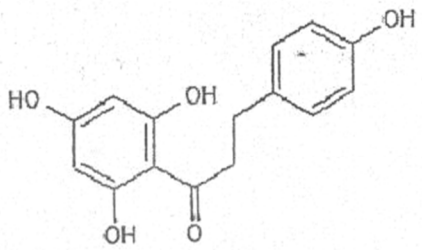
Anthocyanins (3-hydroxy-2phenylchromenylium) (7) are one of the most important plant pigments visible to the human eye. Anthocyanins are the largest group of colourful plant pigments and are responsible for colours ranging from red to violet and blue [21]. These plant pigments accumulate richly in the epidermal or subepidermal cell vacuoles of flowers, fruits, vegetables and foliage. Generally, anthocyanins belong to the widespread class of poly phenolic compounds, which are collectivity [22] named flavonoids. Anthocyanins are the glycosides of flavonoids with poly hydroxy and poly methoxy derivatives of 2- phenyl benzopyrylium or flavyliumcation. Anthocyanins are conjugated aromatic systems, which are often positively charged. Anthocyanins can absorb in the visible range, hence each anthocyanin can be represented by their unique colour. An anthocyanidin, termed aglycone , does not have a sugar at the 3 position.



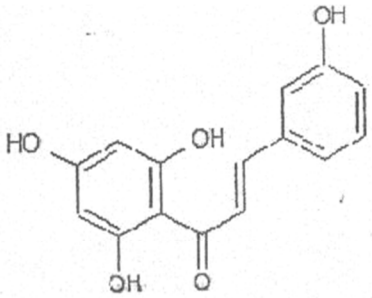
(42)

1.2.7 Chalcones

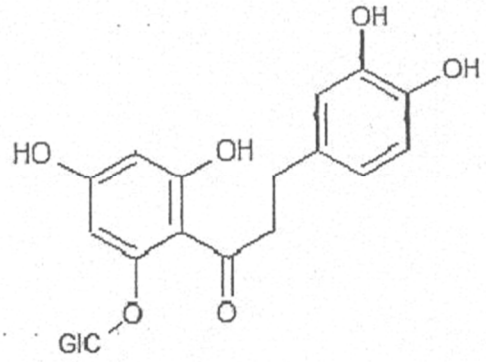
Chalcones 1-(2Hydroxyl-pheflYl)-3- phenyl—propenone(8)and di hdro chalocones (43) have a linear C_3-chain connecting the two aromatic rings. The C3—chain of chalcones contains a double bond, whereas the C_3- chain of di hydrochalcones is saturated. Chalcones, such as butein (44), are yellow pigments in flowers. An example of a dihydrochalcone is phloridzin (45), a compound found in apple leaves, which has been reported to have anti-tumor[(9)] activity. Chalcones are flavonoids lacking a heterocyclic C-ring. Generally, plants do not accumulate chalcones. After its formation, narigenin chalcone is rapidly isomerized by the enzyme chalcone isomerase to form the flavanon enarigenin. The most common chalcones found in foods are phioretin and its 2-O-glucosides: chalconarigenin and are butin[(17)].



(43)



(44)



(45)

1.2.8Aurones

Aurones (9) were first described from flower of *Coreopsis grandfora* by Geissman and Heaton 1934. These conspicuously colored compounds have been found in a variety of yellow flowered species since that time. However, the aurones are not restricated to floral tissue but have been obtained from bark, wood and leaves as well.

Chemically aurones are based on the 2-benzylidene –coumaranone or 2-benzylidine -3 (2H)- benzo furanone system (9). The term "aurone" recognized both yellow golden colour and their isomeric relationship with the flavones. Structures of natural aurone are most easily discussed by grouping them according to the number of hydroxyl groups present in the B-ring [(23)].

1-3 Separation and quantification of flavonoids

Essential to the study of flavonoids is having the means available for there separation (analytical and preparative) and isolation. The importance of this aspect of flavonoid research can be seen in the number of review articles that refer to their chromatography [(24-29)]**.** In earlier times, thin-layer chromatography (TLC), polyimide chromatography, and paper electrophoresis were the major separation techniques for phenolics. Of these methods, TLC is still the workhorse of flavonoid analysis. It is used as a rapid, simple, and versatile method for following poly phenolics in plant extracts and in fractionation work. However, the majority of published work now refers to qualitative and qualitative applications of high performance liquid chromatography (HPLC) for analysis. Flavonoid can be separated, quantified, and identified in one operation by coupling HPLC to ultraviolet (UV) , mass or nuclear magnetic resonance (NMR) detectors . Recently, the technique of capillary electrophoresis (EC) has been gaining attention.

1-3-1 Extraction

Flavonoids (particularly glycosides) can be degraded by enzyme action when collected plant material is fresh or nondried. It is thus advisable to use dry, lyophilized, or frozen samples. When dry plant material is used, it is generally ground in to a powder. For extraction, the solvent is chosen as a function of the type of flavonoid required. Polarity is an important consideration here. Less polar flavonoids (e.g.,isoflavones , flavanones , methylated flavones , and flavonoids) are extracted with chloroform , dichoromethane , diethyl ether , or ethyl acetate ,

while flavonoid glycosides and more polar aglycones are extracted with alcohols or alcohol-water mixtures . Glycosides have increased water solubility and aqueous alcoholic solution are suitable. The bulk of extraction of flavonoid-contaning material are still performed by simple direct solvent extraction.

Powdered plant material can also be extracted in a Soxhlet apparatus , first with hexane , for example , to remove lipids and then with ethyl acetate or ethanol to obtain phenolics . This approach is not suitable for heat-sensitive compounds.

A convenient and frequently used procedure is sequential solvent extraction. A first step, with di chloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid aglycones and polar constituents.

Certain flavanone and chalcone glycosides are difficult to dissolve in methanol, ethanol, or alcohol-water mextures . Flavanone solubility depends on the PH of water-containing solutions.

Flavn-3-ols (catechins , pro anthocanidins , and condensed tannins) can often be extracted directly with water. However, the composition of the extract dose vary with the solvent – whether water , methanol , ethanol , acetone , or ethyl acetate . For example it is claimed that methanol is the best solvent for catechins and 70% acetone for pro cyanidins **(30)**

Anthocyanins are extracted with cold acidified methanol . The acid employed is usually acetic acid (about7%) or tri fluoro acetic acid (about 3%)the use of mineral acid can lead to the loss of attached acyl groups .

1-3-2 Preparative separation

1-3-2-1 preliminary purification

Once a suitably polar plant extract is obtained, a preliminary cleanup is advantageous. The classical method of separating phenolics from plant extracts is to precipitate with lead acetate or extract into alkali or carbonate, followed by acidification. The lead acetate procedure is often unsatisfactory since some phenolics do not precipitate other compounds may coprecipitate and it is not always easy to remove the lead salts.

Alternatively, solvent partition or countercurrent techniques may be applied. In order to obtain an isoflavonoid-rich fraction from Erythrina species (leguminosae) for further purification work, an organic solvent extract was dissolved in 90% methanol and first partitioned with hexan The residual methanol part was adjusted with water to30% and partitioned with t-butyl methyl ether-hexan(9:1) This letter mixture was than chromate graprphed to obtaion pure compounds[31] .

1-3-2-2 Preparative methods

One of the majer problem with the preparative separation of flavoids is their sparing solubility in solvents employed in chromatography. More Over, the flavonoids become less soluble as their purification proceeds. Poor solubility in the mobile phase used for a chromatographic separation can induce precipitation at the head of the coiumn,leading to poor resolution ,decrease in solvent flow,or even blockage of the column .Other complications can also arise.

There is no single isolation strategy for the separation of flavonoids and one or many steps may bantitye necessary for their isolation .The choice of method depenge on the polarity of the compounds and the quantity of sample available.

Most of the preparative methods available are described in a volume by Hostettmann et al [32]

Conventional open-column chromatography is still widely used because of its simplicity and its value as an initial separation step. Preparative work on large quantities of flavonoids from crude plant extracts is also possible.

Preparative TLC is a separation method that requires the least financial outlay and the most basic equipment..It is normally employed for milligram

quantities of sample, although gram quantities are also handled if the mixture is not too complex.

1.3.3Analytical methods

Knowledge of the flavonoid content of plant-based food is paramount to understanding their role in plant physiology and human health.Anlytical methods are also important to identify adulteration of beverages, for example . And flavonoids are indispensable market for chemotaxonomic purposes.

Various analytical methods exist for flavonoids. These range from TlC to CE. With the introduction of hyphenated HPLC techniques, the analytical potential has been dramatically extended.

Gas chromatography (GC) is generally impractical,due to the low volatility of many flavonoid compound and the necessity of preparing derivatives. However, Schmidtet al have reported the separation of flavones, flavonols, flavanones, and chalcones (with frequent substitution by methyl groups) by GC.

1.3.3.1 Sample preparation

Sample preparation is included in sample handling and is rapidly becoming a science in it self . The initial treatment of the sample is critical step in chemical and biochemical analyses;it is usually the critical step in analysis. In the case of food and plant sample, the number and diversity of analytes is very high and efficient pretreatment is required to obtain enriched phenolic fractions.

Sample preparation methods should [36]:

- Remove possible interferents (for either the separation or detection stages)from the sample , there by in creasing the selectivity of the analytical method
- Increase the concentration o the analyte and hence the sensitivity of the assay

- Convert the analyte into a more suitable form for detection or separation(if needed)
- Provide robust and reproducible method that are independent of variations in the sample matrix.

The aim of sample preparation is that the components of interest should be extracted from complex matrices with the least time and energy comsumption but with highest efficiency andreprodcitiy. condition should be mild enough to avoid oxidation, thermal degradation ,and other chemical and biochemical changes .Sample procedures –CE, for example – neessitate more rigorous sample pretreatment than other .On the other hand, TLC requiresan absolute minimum of sample preparation.

1.3.3.2 Thin layer chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids [28] . But now the method of choice for sample an in expensive analytical runs is TLC. The advantages of this technique are well known: short separation times, amenability to detection reagents , and the possibility of running several sample at the same time. TLC is also ideally suited for the preliminary screening of plant extracts before HPLC analysis. An excellent general text on TLC methodology has been written by Jork . A good discussion presented by markham in one of the earlier volumes [40]**.** On flavonoid describes TLC on silica gel and also two other supports , cellulose and polyamide (which now find less application) [41] . In his chapter, solvent systems and spray reagents are described.

Many different solvent systems have been employed for the separation of flavonoids using TLC. Some solvent systems cited by markham [41] are reproduced here because they still find application in the separation of flavonoids .Highly

methylated or acetylated flavones and flavonols require nonpolar solvents solvent such as chloroform-methanol (15:1) widely distributed flavonoid aglycones, such as apigenin, luteolin, and quercetin, can be separated in chloroform-methanol (96:4) and similar polarity solvents. One system that is of widespread application for flavonoid glycosides is ethyl acetate – formic Acid – glacial acetic acid – water (100 – 111126) By the addition of ethyl methyl ketone (ethyl acctate – ethyl methyle ketone – formic acid – glacial acetic – water, 50:30:7:3:10), rutin and vitexin-2 – O – rhamnoside can be separated[42]

1.3.3.3 High performance liquid chromatography

The method of choice for the qualitative and quantitative analysis of flavonoids is **HPLC.** since is introduction in the 1970s, **HPLC** has been used for all classes of flavonoids and hundred of applications have been published. Numerous reviews have also appeared, such as those by hostettmann and hostettmann[26]Merken and Beecher[44]He,[45]and cimpan and Gocan[46]

For the Analytical **HPLC** of a given subclass of flavonoids (flavones, flavonols, isoflavones, anthocyanins, etc), the stationary phase, solvent, and gradient have to be optimized .

1-3-3-4 Capillaryelectrophoresis

CE is an analytical technique that provides high separation efficiency and short run times. When compared to HPLC, however, CE generally exhibits much lower sensitivity, a tendency to overload with samples, and less reproducible data. In contrast to HPLC, method development is more time consuming in CE – involving investigation of types, PH and concentration of electrolytes, types and concentrations of surfactants and organic modifiers, temperatures, and applied voltages.

1-4 Spectroscopic Techniques Applied to Flavonoids

1-4-1 NMR spectroscopy

NMR spectroscopy is an extremely powerful analytical technique for the determination of flavonoid structures,[54-57]

But it is limited by poor sensitivity , slow through put, and difficulties in analysis of mixtures. Recent developments have , made NMR arguable the most important tool for complete structure elucidation of flavonoids .Today , it is possible to make complete assignment of all proton and carbon signals in NMR spectra of most flavonoids isolated in the low milligram range. These assignments are based on chemical shift (δ) and coubling constant (*J*) observed in 1D H^{1} and C^{13} NMR spectra combined with correlations observed as cross peaks in homo_ and hetero nuclear 2D NMR experiments. Other nuclei like O^{17} NMR spectroscopy has been used to study flavonoids only in a few cases. Natural abundance O^{17} NMR Spectra have been recorded for 11 methoxy flavones [58] and [17] ONMR data for some 3-arylidenechromanones and flavaones have recently been discussed in terms of mesomeric and steric substituent interaction .[59] ^{17}O NMR spectroscopy has also been used to study the effect of sugar on anthocyanin degradation and water mobility in a rosell anthocyanin model system.[60]

1.4.2 Mass spectrometry

Modern mass spectrometric techniques are very well suited for the analysis of flavonoids isolaed from plants and food stuffs and in their in vivo metabolite forms. Progress during the last tow decades has made MS the most Sensitive method for molecular analysis of flavonoids. MS has the potential to yield information on the exact molecular mass, as wellas on the structure and quantity of compounds with the nature and within the mass range of flavonids. Furthermore,

due to the high power of mass separation, very good selectivities can also be obtained.

The purpose of the MS techniques is to detect charged molecular ions and fragments sparated according to their molecular masses. Most flavonoid glycosides are polar, nonvolatile, and often thermally labile Conventional MS ionization methods like electron impact (EI) and chemical ionization (CI) have not been suitable for MS analyses of these compounds because they require the flavonoid to be in the gas phase for ionization. To increase volatility, derivatization of the flavonoids may be performed. However, derivatization often leads to difficulties with respect to interpretation of the fragmentation patterns. Flavonoid glycoside without derivatization become possible with introduction of desorption ionization techniques. Field desorption, which was the first technique employed for the direct analysis of polar flavonoid glycoside, has provided molecular mass data and little structural in formation [(67)].

1.4.3 Vibrational Spectroscopy (IR and Raman)

Two different types of spectroscopic techniques are most frequently used to view the fundamental modes of molecular vibrations, namely mid-IR spectroscopy and Raman spectroscopy.[(68)]

The first method measures the absorption, transmission, or reflection of IR radiation with wavelengths in the range of 2.5 to 25 m. The Raman method irradiates the sample with radiation of much shorter wavelengths and measures the fraction of scattered radiation for which the energy of the photon has changed. The vibrational spectra serve as fingerprints of structure, compositions, and dynamics. The reciprocal of wavelength, wavelength wave number (cm^{-1}), is commonly used to characterize the energy in the field of vibrationol spectroscopy.

Systematic vibrationol spectroscopy studies on flavonoids have occurred since the early1950s, and most of them have been limited to a discussion on the hydroxyl and carbonyl absorption frequencies.[69]

Hawever, with the technical advances of the last two decades, the application of vibrational spectroscopy has become much more relevant in the field of flavonoid analysis[70,71] .The implementation of FTIR spectroscopy has significantly enhanced the sensitivity, and Raman spectroscopy has benefited the availability of holographic notch filters, which efficiently suppress the strong signal from elastically scatter (Rayleigh) radiation while maintaining the Raman –shifted intensity with minimal attenuation. Furthermore, high-powered NIR semiconductor lasers and charge coupled devices have replaced inconvenient gas lasers and light –detection technologies. Ordinary Raman spectroscopy has drawbacks in that it requires high compound concentrations, and the recorded spectrum will correspond to all molecules present in the sample. In resonance Raman spectroscopy, this I over come through the use of laser light with a frequency corresponding to the absorption maximum of the compound to be characterized. Finally, the increase in the computing power of stander computers has facilitated more so phisticated data evaluation of both IR and Raman spectra.

1.4.4 Ultraviolet-visible absorption spectroscopy

The application of standardized UV (or UV-VIS) spectroscopy has for years been used in analyses of flavonoids .These poly phenolic compound reveal two characteristic UV absorption bands with maxima in the 240 to 285 and 300 to 550 nm rang .The various flavonoid classes can be recognized by their UV spectra ,[74] and UV _spectral characteristics of individual flavonoids including the effects of the ,and nature of aromatic acyl groups have been reviewed in several excellent books .[75,74,76] today, the major use of UV_VIS spectroscopy applied to flavonoids is in quantitative analyses ,and the value of this method for some structural

analyses is diminishing compared to the level of information gained by other modern spectroscopic techniques like NMR and MS.

1.5 Absorption, Bioavailability, and Metabolism of Flavonoids

1.5.1 Absorption of Flavonoids

Before dietary flavonoids can be absorbed from the gut, they must be released from plant foods by chewing , action of the digestive juices in the gas trointestin ltract, and finally the microorganisms of the colon. Itcan. be envisaged that this release from the plant tissues , the so - called food matrix, depends on the type of plant food ,its processing conditions ,and the presence of other dietary components ,its physicochemical properties such as molecular size and configuration, lipophilicity , solubility ,and p Ka . To date only fragmentary is available on the effect of the plant food matrix on absorption. Well-desingned studies that addressed this issue have not been reported.

1.5.1.1 Role of the Flavonoid structure: Glycoside and oligomers

I. Glycoside

Most flavonoids except catechins , are usually present in the diet as b-glycosides . Glycosides were considered too hydrophilic for absorption by passive diffusion in the small intestine, thus only aglycones were likely to be absorbed . Studies with germ-free rats showed that large amounts of unchanged glycosides were excreted with feces , whereas only small amounts of glycosides were found in feces of rats with a normal micro flora [(86)]thus, it was thought that the glycosylated flavonoids were only marginally absorbed . However, this view on absorption of glycosides had to be revised.

II. Oligomers

In contrast with other flavonoids , catechins occur as aglycones and galloy lated forms in foods . Pharmacokinetic data point to absorption from the small intestine of both the glycones and the galloylated forms [87].

Human data on the quantity of catechins that are absorbed are lacking However, besides aglycones , catechins occur in plant foods as oligomers of up to 17 catechin units : pro anthocyanidins . In vitro studies with caco-2 cells showed that only dimmers and trimers were able to pass across mono layers of these cells [88] . Indeed pro cyanid dimmer B2{epicatehcin-(4b!8)-epicatechin } was detected in human plasma after in gesti of a cocoa beverage [89] .It has been suggested that oligomers can be hydrolyzed to monomers and dimmers due to the acidic conditions in the stomach [90] · However, sampling of human gastric juice showed that hydrolysis of pro anthocyanidins does not occurin vivo [91]· It can be concluded that only pro anthocyandin up to three catechins are absorbable form the colon . lager molecules will reach the colon where they will be degraded by bacteria.

1.5.2 Bioavailability

Dietary Flavonol glycosides showed very rapid to very slow absorption in man. Times to reach peak concentration (T max) were between <0.5 and 9 h>. The bioavailalbitiy of quercetin glucosides from onions was superior . Bioavailalbitiy of various quercetin glycosides (b-galactosides and b- xylosides) from apples and of pure quercetin rutinoside was only 30% of that from onions [92]. Thus, the sugar moiety of quercetin glycosides seemed to be an important determinant of their bioavailability , which was confirmed when purequercetin-b-glucoside or pure quercetin-b-rutinoside was administered to healthy human volunteers [93] The peak concentration of quercetin (C max) in plasma was 20-times higher and reached (T max) more than 11110-times faster after intake of the glucoside than after the

rutinoside . These pharmacokinetic data suggest that quercetin glucoside was absorbed form the small intestine, whereas quercetin rutinoside was absorbed from the colon after deglycosylation . Evidently, the sugar moiety played no role in the elimination of quercetin from plasma : elimination half-five was about 20 for all glycosides . This is consistent with the observation that quercetin glucosides do not circulatein the blood [(94)]. Apparently, the sugar part only plays a role upon absorption.

1-5-3 Metabolism

In the metabolism of flavonoids , two compartments are considered the first compartment consists of tissues such as the small intestine , liver and kidneys . The colon constitutes the second compartment.

Flavonoids that are un absorbable from the small intestine and flavonoids that have been absorbed and then secreted with bile will reach the colon .

The significance of biliary secretion in humans remains to be determined, but in rats about 40% of the absorbed (+)-catechin was secreted with bile into the small intestine [(95)].

1.6 Flavonoids as nutraceuticals

"Nutraceutical" is a term coined in 1979 by Stephen De Felice [**98**]. It is defined "as a food or parts of food that provide medical or health benefits including the prevention and treatment of disease."Nutraceuticals may from isolated nutrients. Dietary suppliments , and diets to genetically engineered "designer" food ,herbal products , and processed products such as cereals , soups, and beverages. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease [**(99)**]. The increasing interest in nutraceuticals reflects the fact a specific diet or component of the diet is associated with a lower risk for a certain disease. The

major active nutraceutical ingredients in plant are flavonoids. As is typical for phenolic compounds, they can act as potentantioxidants and metal chelators. They also have long been recognized to possess anti-inflammatory, anti allergic, hepato protective, anti thrombotic, antiviral, and anti carcinogenic activities, as discussed in the subsections that follow:

1.6.1 Antioxidant activity

The best-described property of almost every of flavonoids is their capacity to acts as antioxidants .The flavonoids and catechins seem to be the most powerful flavonoids for protecting the body against reactive oxygen species (ROS) .Body cells and tissues are continuously threatened by the damage caused by free radicals and ROS which are produced during normal oxygen metabolism or are induced by exogeneous damage [100,101] .free radicals and ROS have been implicated in a large number of human diseases[102 ,103] .

1.6.2 Antimicrobial activity

Flavonoids and esters of phenolic acids have also been investigated for their antibacterial, antifungal and antiviral activities.

1.6.3 Antibacterial activity

Antibacterial activity has been displayed by a number of flavonoids .Qurecetin has been reported to completely inhibit the growth of *Staphylococcus aureus* .Most of the fiavononeshaving no sugar moiety showed antimicrobial activities whereas none of the flavonols and flavonolignans tested showed inhibitory activity on microorganisms [107].

1.6.4 Antifungal activity

A number of flavonoids isolate from the peelings of tangerine orange when tested for fungistatic activity towards *Deuterophoma tracheiphila* were found to be activity ;nobiletin and langeritin strong and weak activities respectively ,while hesperidin could stimulate fungal growth slightly.Chlorflavonin was the first chlorine-conaining flavonoid –type antibiotic by strains of *Aspergillus candidus*[108]

1.6.5 Antiviral activity

Naturally occurring flavonoids with antiviral activity have been recognized since the 1940 sbut only recently have attempts been made to make synthetic modification of natural compounds to improve antiviral activity .Qurecetin, morin, rutin dihydroquercetin (taxifolin), apigenin, catechin ,and hesperidine have reported to possess antiviral activity against some of the 11 type of viruses[109

1.6.6 Antiulcer activity

Exert significant anti-inflammatory activity in the animal model of both acute chronic inflammatory when given orally or topically [110,111]. Hesperidin, a citrus flavonoids , possesses significant anti-inflammatory and analgesic [112] . Recently apigenin , luteolin and quercetin have been reported to exhibit to anti-inflammatory activity[113] .

1.6.7 Hepatoprotective activity

The liver is subject to acute and potentinally lethal injury by several substances including phalloidin (the toxic constituent of the mushroom, Amaita phalloides),CC14 ,galactosamine ,ethanol and other compounds Flavonoids activity .In a study carried out to investigate the flavonoid derivatives silymarin, apigenin , quercetin ,and naringenin, as putative therapeutic agents microcrystin LR-induced hepatotoxicity , silymaein was found to be the most effective one [114].The flavonoid ,rutin and venoruton ,showed regenerative and hepatoptrotective effects in experimental [115].

1.6.8 Anti-inflammatory activity

The anti –inflammatory activity of flavonoids in many animal models have been reported .flavon/ flavonol glycosides as well as flavonoid aglycons have been reported to flavonoes / flavonls kaempferol, quercetin myriceetin, fisetin were reported to possess LO and COX inhibitory activity [116,117].

1.6.9 Antidiabetic effects

Flavonoid, especially quercetin, has been reported to to possess Antidiabetic activity. Vessal et al reported that quercetin brings about the regeneration of pancreatic islets and proprably increases insulin release in strptozotocin – induced

diabetic rast [118-119] .Also in another study , Hif and Howell reported that quercetin stimulate insulin release and enhanced Ca2+uptake from isolated islets cell which suggest a place for flavonoids in noninsulin-dependent diabetes [120-121].

1.6.10 Effect on central nervous system

_3_nitro flavones were shown to displace [3H] flumazenil binding to membranes from rat cerebellum but not from spinal cord, indicating selectivity for the BZOmega receptor subtype, but the latter was more potent than 6_bromoflavne. Results from tow conflict tests in rats shows that these synthetic flavonoids possess anxiolytic like properties similar or superior to that of diazepam **(121).**

1.7 Toxicity of flavonoids

Flavonoids are ubiquitous in plant foods and drinks and, therefore, a significant quantity is consumed in our daily diet. The toxicity of flavonoids is very low in animals. For rats, the LD50 is 2_10 g per animals for most flavonoids. Similar doses in humans are quite un realistic.As a precaution, doses less than 1mg per adult per day have been recommended for humans **(122)**. Dunnick and Hailey reported that high doses of quercetin over several years might result in the formation of tumors in mice **(122)**. However, in other long-term studies, no carcinogenicity was found **(124)**. Moreover, as described earlier, quercetin has been reported to be anti-mutagenic in vivo.

1.8 Aim of this study

This study was aimed to :

-Extract flavonoid from the targeted plant species

- Isolate flavonoids via chromatographgic

- Conducting UV and IR studies on the isolates

2- Material and Methods

2.1 Materials

2.1.1 Chemicals and Instruments

Analytical grade reagents were used .The UV –Visible spectra were The on a Perkin – Elmer Lambda –2UV- Visible Spectrophotometer .The IR spectra were run on a Perkin –Elmer 1310 Infrared Spectrophotometer .

2 .1.2 Plant material:

The peels of *ponica granatum* collected from Omdurman, Khartoum state province and kindly authenticated by Aromatic and Medicinal Plant Institute- Khartoum - Sudan.

2.2-Methods

2.2.1- Preparation of test reagents for phytochemical screening

2.2.1.1 Flavonoid test reagents

(i)Aluminum chloride solution

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

(ii) **Potassium hydroxide solution .**

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

(iii) Ferric chloride solution.

(0.5g) of ferric chloride was dissolved in (100 ml) 95% ethanol**.**

2.2.1.2 Alkaloidal test reagents

(i) Wagner reagent:

(5g) iodine and (10g) potassium iodide was dissolved in (100)distilled water.

2.2.2- Preparation of plant extract for phytochemical screening

(100g) of powdered air – dried peels of *ponica granatum* were extracted with (200ml) 95% ethanol for five days. The cooled solution was filtered and its volume was adjusted to (100ml) by addition of enough 95% ethanol .This prepared extract (PE) was used for the following tests.

2.2.3 – Phytochmical screening

The plant specices were screened for steroids, flavonoids ,alkaloids and glycoside .

I)Test for steroides

(40ml) Aliquot of the prepared extract was evaporated to dryness on a water bath .The cooled residue was stirred with petroleum ether to remove most of the colouring matter .The residue was extracted with (20ml) chloroform.The chloroform solution was dehydrated over anhydrous sodium. Sulphate.

(50ml) portion of the solution was mixed with (0.5ml) acetic anhydride , followed by two drops concentrated sulphuric acid .No development of a green colour.

II)Test alkaloids

(5ml) 0f 2N hydrochloric acid was added to the crude extract and the solution was heated with stirring in a water bath for 10 minutes.To the cooled solution, few drops of reagent were added. No formation of precipitate.

III) Test for flavonoids

(85ml) aliquot of the prepared extract was evaporated to dryness on a water bath .The cooled residue was decated and the residue was dissolved in (30ml) 95% ethanol and filtered .The filtered was used for the following tests.

- To(3ml)of the filtrate few drops of methanolic aluminum chloride were added. Adark yellow colour soon developed.
- To(3ml) of the filtrate few drops of potassium hydroxide solution were added . Adark yellow colour was observed.
- To (3ml) of filtrate few drops of ferric chloride solution were added . Ablue colouration was observed.

IV) Test for glycosides

(20ml) of the persisted extract was vigorously shaken in a test tube .a froth that persisted for one hour was observed.

2.2.4-Extraction of flavonoids from the peel of *poncia granatum*

Powere air –dired peels (1kg) of *ponica granatum* extracted with 95% ethanol at ambient temperature for five days .The solvent was removed giving a brown solid (5g)

2.2.5 – TLC chromatography of the crude products

Part of the crude product of *ponica granatum* (0.1g) was dissolved in 95% ethanol (2ml) and applied as concentrated spots on TLC plates .The plates were irrigated with the solvent system BAW (4:1:5) .The chomotograms were located under UV light .

2.2.6 –UV shift reagents

Stock solution of sodium methoxide and aluminum chloride.

(i) Sodium methoxide

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

(ii) Aluminum chloride stock solution

(5g) of fresh anhydrous aluminum chloride were added cautiously to spectroscopic methanol (100ml).

2.2.7 – The UV spectra of compound in presence

Three drops of sodium methoxide were added to a solution of compound in methanol (2ml) and the UV spectrum was immediately.

2.2.8 –The UV Spectra of compound in presence of aluminum chloride

Six drops of the stock solution of aluminum chloride were added to a solution of compound in methanol (2ml) and the UV spectrum was recprded immediately.

2.2.9 – UV spectra of compound in presence of sodium acetate

Excess coursley powdered anhydrous NaOAc was added with shaking to a cuvette containing (2ml) of the solution of compound in methanol and the UV spectrum was recorded after two minutes.

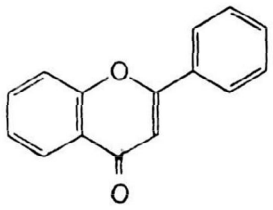
3-Results and Discussion

3.1 Extraction and purification of flavonoids

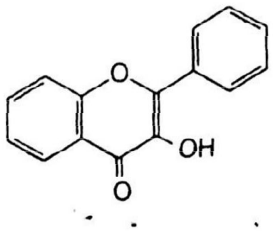
The peels of *ponica granatum* were extracted with 90% ethanol and the crude product obtained was subjected to phytochemical screening where qualitative tests were positive for flavonoids, steroids, but negative for
The crude extract was then fractionated by thin layer chromatography where plates were irrigated with BAW (4:1:5) after the usual workup tow flavonoids were isolated-compounds .

3-2 spectral data of compound

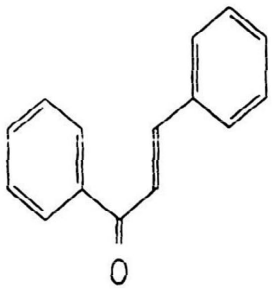
The IR (kBr) spectrum of compound (Fig. 1) showed V 675.04,794.62 (C-H bending, Ar) , 1107.06 (C-O , ether) , 1741.60 (C=O) , 1631.67 (α, β-unsaturated carbonyl group) and 3425.34 cm^{-1} (OH) compound cannot be an anthocyanin or a catechin since the IR spectrum gave a carbonyl stretching at 1741.60 cm^{-1} . It could be : a flavone, flavonol, chalcone, aurone, isoflavone, flavanone, dihydrochalcone or dihydroflavonol .



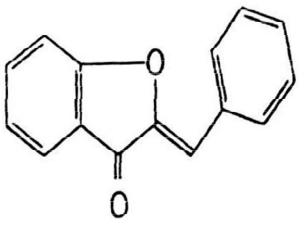
Flavone



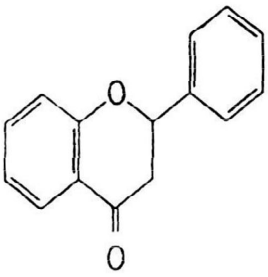
Flavonol



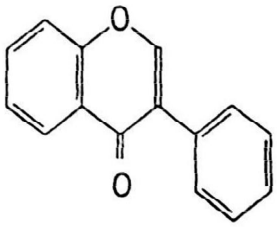
Chalcone



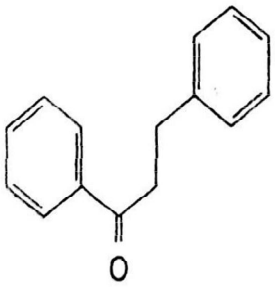
Aurone



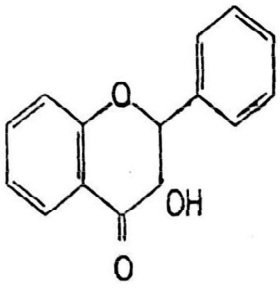
Flavanone



Isoflavone



Dihydrochalcone



Dihydroflavonol

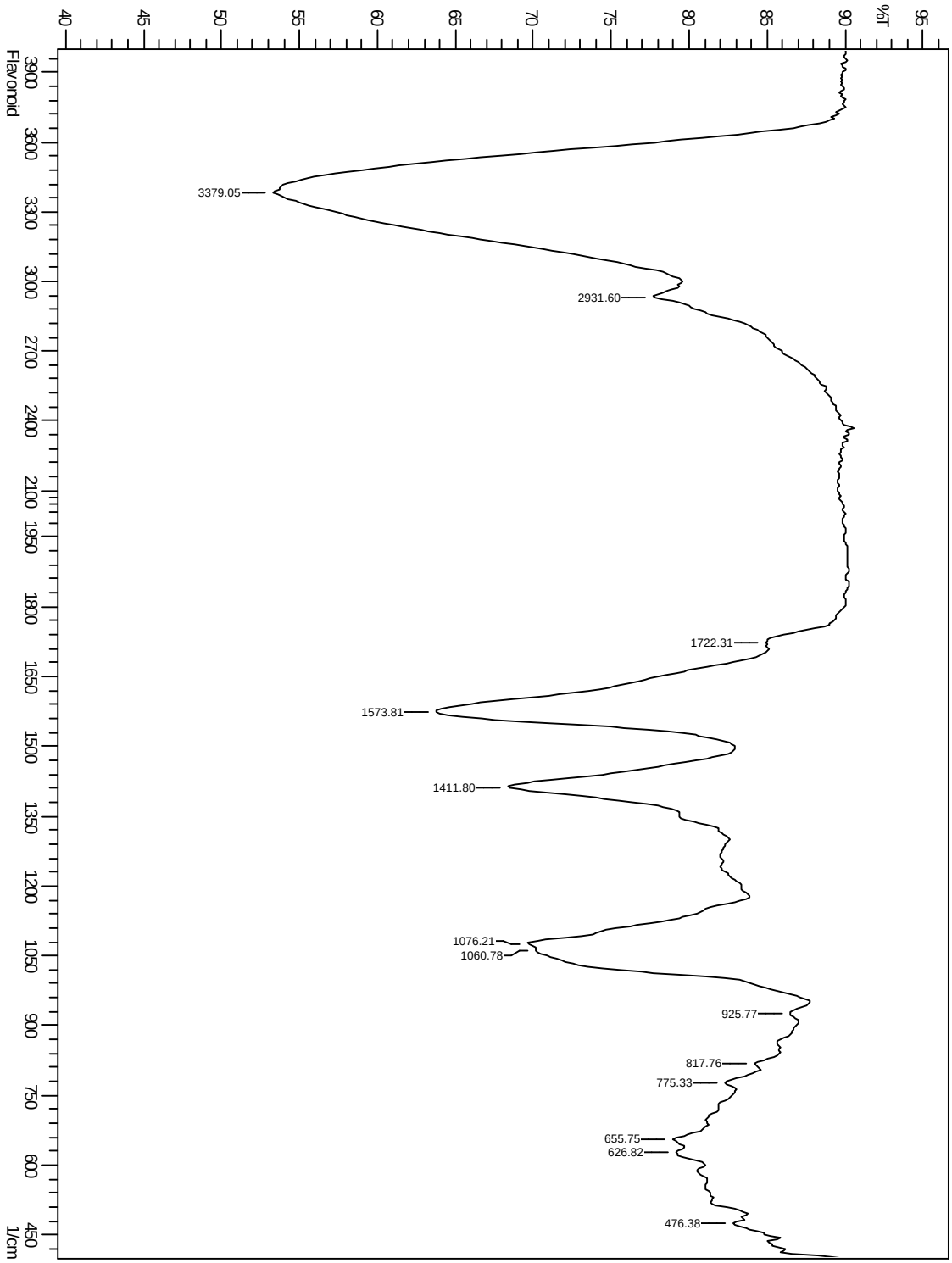
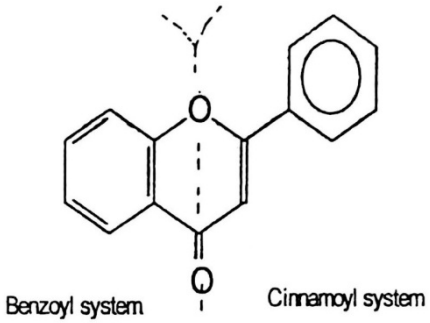


Fig . (1) IR spectrum of compound

In their UV spectra, most flavonoids exhibit two major bands in the region 240-400 nm. These two peaks are commonly refered to as band I (usually 300-400 nm) and band II (usually 240-280nm) [11] Band I is considered to be associated with absorption due to the B-ring cinnamoyl system, and band II with absorption involving the-A-ring benzoyl system [11].



Isoflavones, flavanone and dihydroflavonols all give only band I due to lack of conjugation between the carbonyl function and the aromatic B ring , while flavones , flavonols, aurones and chalcones give similar UV spectra as a result of conjugation between the A-and B-rings . Thus they exhibit both band I and II. The UV absorption of some classes of flavonoids is depicted in table 3.1.

Table (3.1) : The UV absorption of flavones, flavonols, chalcones and aurone

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

In the UV, compound absorbs (Fig.2) at λ_{max} (MeOH) 270, 330nm (sh.). Such absorption indicates loss of conjugation between the A and B rings which is characteristic of: isoflavones, flavanones, dihydrochalcones and dihydroflavonols .However, the shoulder which appeared in the spectrum at 330 nm claearly indicates that this phytochemical is an isoflavone **[125]**

Considerable structural feature has also been obtained by UV shift reagens sush as sodium methoxide, sodium acetate, aluminum chloride.

These reagents produce shift in the UV absorption maxima in accordance with the location of the various functional groups in the flavonoids.

Sodium methoxide is strong base and ionizes to some extent all hydroxy Groups on the flavonoid nucleus. However, use has been made of the effect of sodium methoxide on the UV spectra.

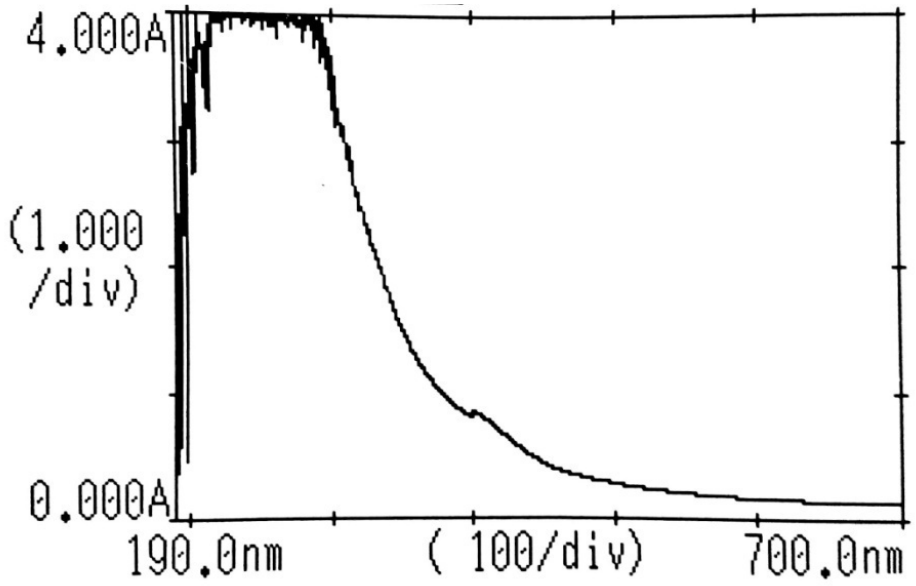


Fig.(2): UV sample of compound

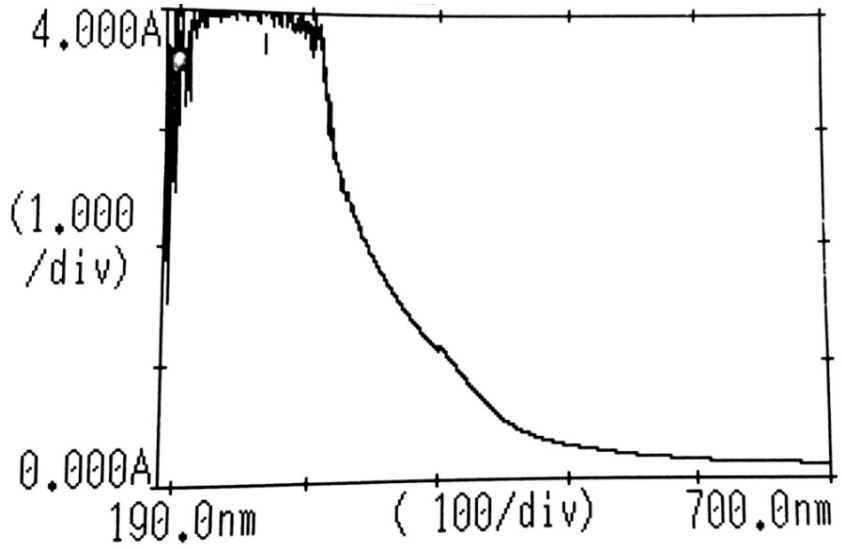


Fig.(3) sodium methoxide of compound

Of flavonoids for detection of free 3-and/or4`-hydroxy function.

When sodium methoxide was added a methanolic solution of compound no bathochromic shift (fig 3) was observed indicate in absence of 3-and/or4`-OH functions.

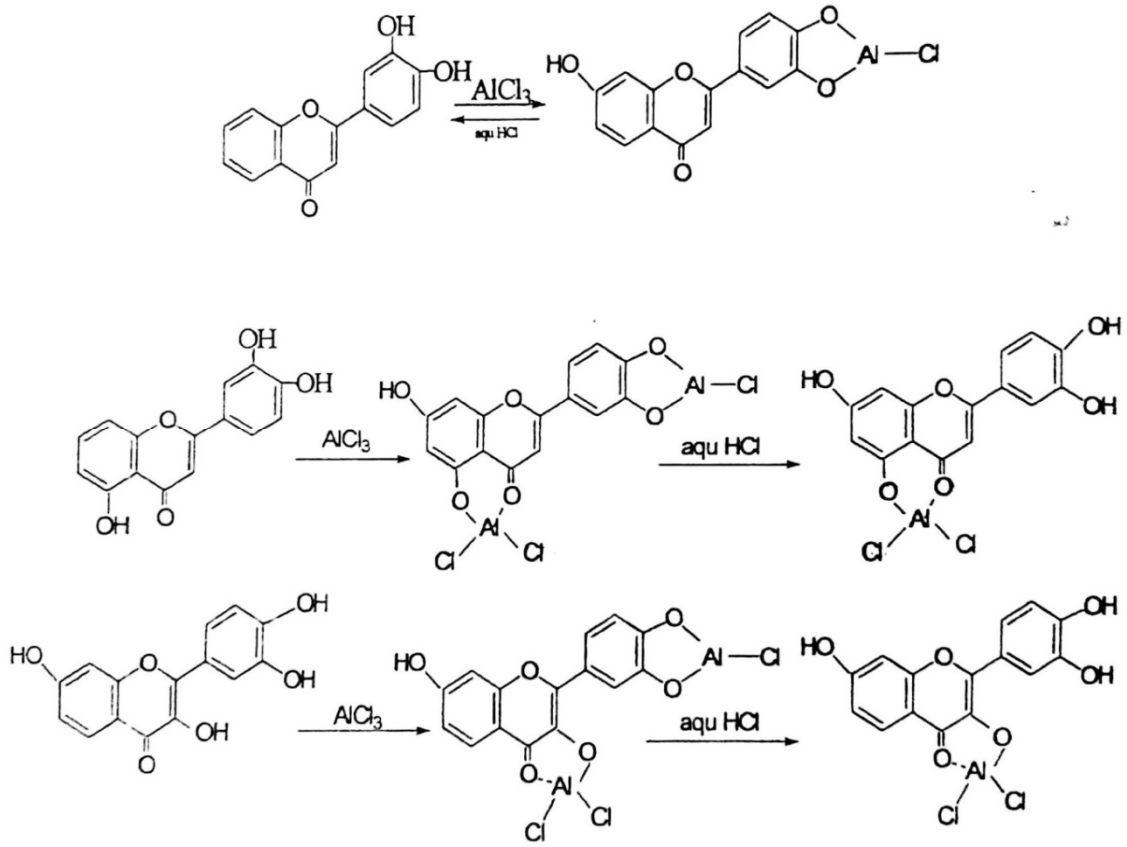
Sodium acetate is a weaker base than NaOMe , and as such , ionizes only the more acidic hydroxyl groups i.e. the 7-OH function.

A ten nm bathochromic shift was observed when sodium acetate was added to a methanolic solution of compound (fig.4).

Flavones and flavonols which contain hydroxyl groups at C-3 or C-5 form acid – stable complexes with aluminium chloride, where as the aluminium chloride complexes with ortho-dihydroxy groups are not stable in acidic media. Such acid-stable and acid- stable complexes are shown in scheme 3.1.

The presence of an ortho-hidroxy group in the B-rings of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in the presence of $ALCL_3$ with that obtained in $ALCL_3$/HCL. 30-40 nm hyposchromic shift observed in band I OH $ALCL_3$ spectrum on the addition of acid results from the decomposition of the complex of $ALCL_3$ with the ortho- dihydroxy group. The

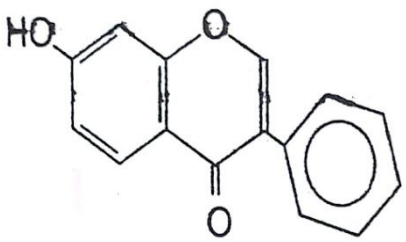
presence of the adjacent hydroxyl groups in the B-ring gives only a 20 nm hyposchromic shift on the addition of acid to $ALCL_3$ solution.



Scheme 3.1: Aluminum chloride-falconoid complexes

Figure (5) illustrates the $ALCL_3$ spectrum of compound where no bathochromic shift was observed indicating absence of 3-,5- OH functions and catechol moieties.

On the basis of the above argument compound I is an isoflavone hydroxylated at the 7-position.



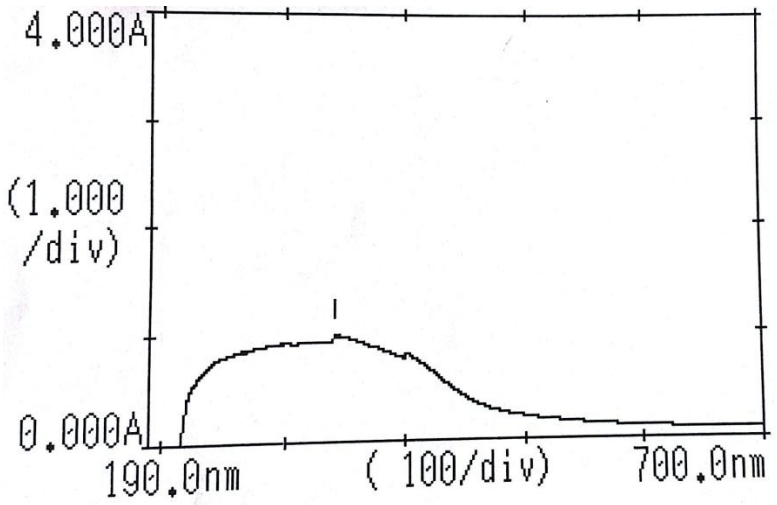


Fig.(4): Sodium acetate compound

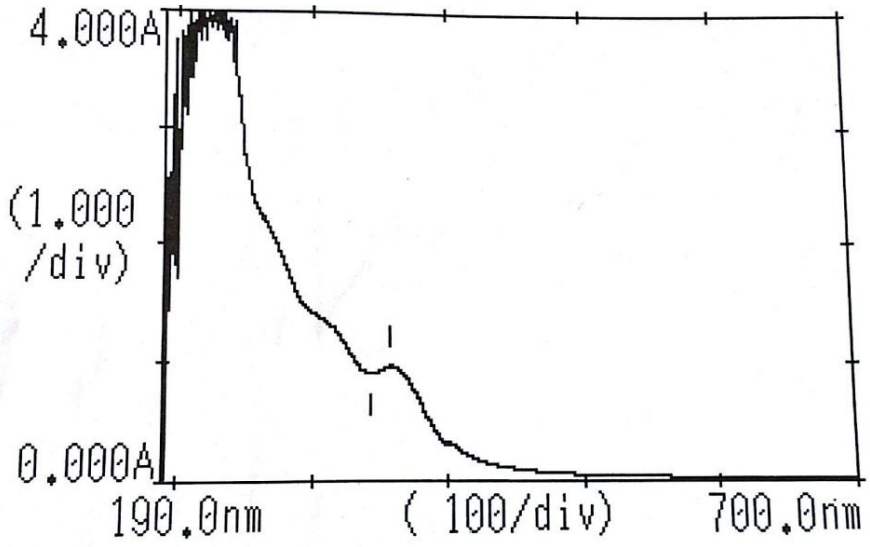


Fig.(5): Aluminum chloride of compound

Recommendations:

- The structure of the isolated flavonoid may further be elucidated by Employing ^{1}H NMR, ^{13}C NMR, HMBC, HSQC and MC spectroscopy.
- The isolated phytochemical may be evaluated for the its anti-inflammatory, antibacterial, antifungal, antimalarial and antioxidant.
- Other phytochemical (glycosides) existing in *ponica granatum* may also be isolated and its structure elucidated.

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