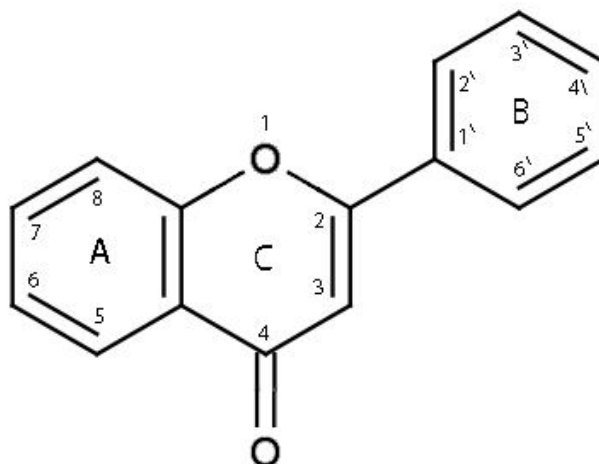


## 1. Introduction

### 1.1 General approach

Flavonoids (2- phenylbenzpyrone)-(1) are a large group of polyphenolic compounds that occur commonly in plant<sup>1</sup>. The name "Flavonoid" is derived from Greek word "Flavus": it means yellow<sup>2</sup>.



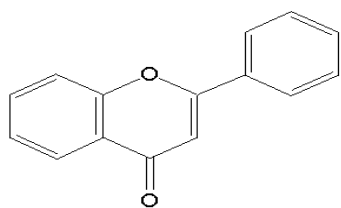
(1)

These phytochemicals are distributed widely in higher plant parts (barks, roots, stems, flowers) but also found in some lower plants including algae<sup>3, 4</sup>. Many of these compounds are responsible for the attractive colours of flowers and fruits and leaves<sup>5</sup>. Flavonoids mainly occur as aglycones (consisting of 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<sup>6</sup> and methylated derivatives are very common.

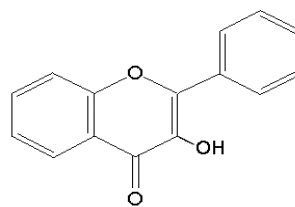
## 1.2 Structure and Classification of flavonoids

The chemical structure of these compounds are based on a ( $C_6 - C_3 - C_6$ ) skeleton. They differ in saturation of heteroatomic ring C, in the placement of the aromatic ring B at the positions 2,3 or 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 anthocyanins (7). In a few cases, the 6-membered heterocyclic ring c occurs in an isomeric open form or is replaced by a 5- membered ring as in the case of chalcones (8) and aurones<sup>7, 8, 9</sup>(9). In plants, flavonoid aglycones (flavonoids without attached sugars) occur in a variety of structures. Most frequently encountered groups of flavonoid aglycones includes flavones, flavonols, anthocyanidins, isoflavones, flavanones, dihydroflavonols, biflavonoids, chalcones, and aurones.

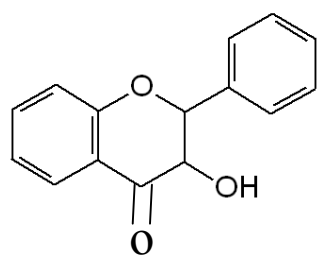
Flavonoid aglycones possess the chemical properties of phenolics, and thus they are slightly acidic. Those possessing a number of unsubstituted hydroxyl groups, or sugar moieties, are polar substances and soluble in polar organic solvents. The presence of sugar makes flavonoid more water soluble, while less polar aglycones like isoflavones, flavanones, and highly methoxylated flavones and flavonols tend to be more soluble in ether or chloroform<sup>10, 11</sup>.



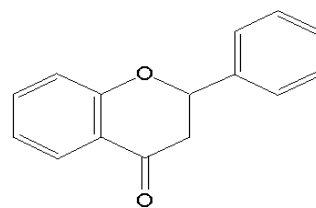
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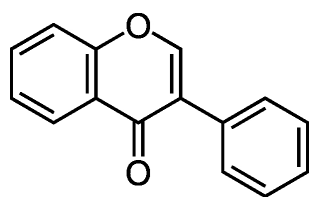
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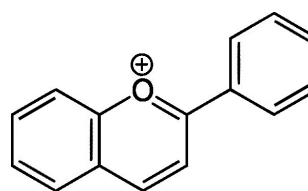
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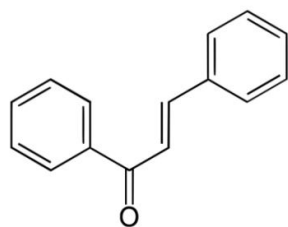
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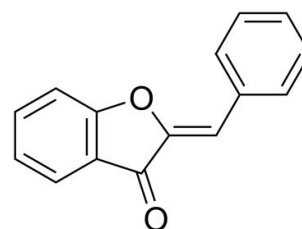
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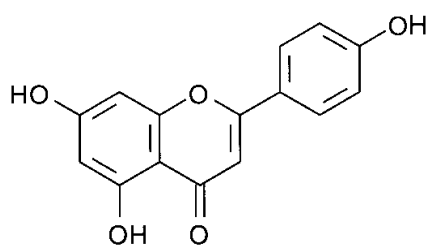


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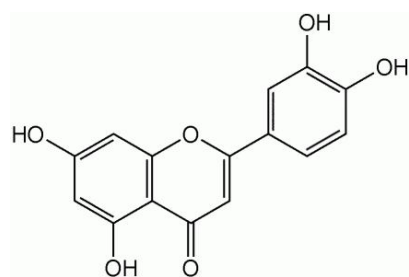
### 1.2.1 Flavones

The Ring C of the 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 group 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.

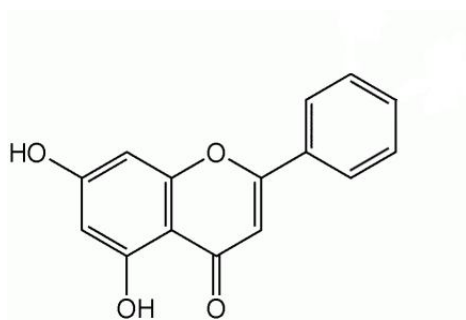
The flavones are of widespread occurrence and they include apigenin, (5, 7, 4'-trihydroxyflavone) (10), luteolin (5, 7, 3', 4' tetrahydroxyflavone) (11) and chrysin (12). Apigenin and luteolin are the most widespread flavones aglycones. However the diverse substitution patterns make this group the largest. Glycosylation occurs mostly at positions 5 and 7, and methylation and acylation usually occur on hydroxyl groups of ring B. Some flavones are polymethoxylated, 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 isoorientin (luteolin -6- C- glucoside) (17) and orientin (luteonlin -8- C- glucoside) (18). Orientin and isoorientin also occur in *Cymbopogon citratus* along with two other flavones C-glycosides, chrysoeriol- 6- C glucoside (isoscoparin) (19) and 7-O- methyl- luteonlin- 6- C – glucoside (swertiajaponin) (20)<sup>12, 13, 14</sup>



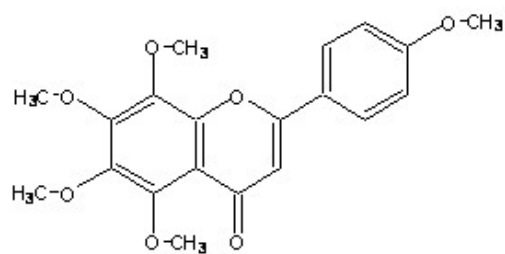
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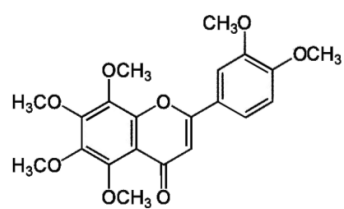
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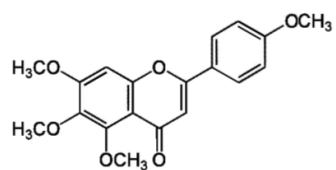
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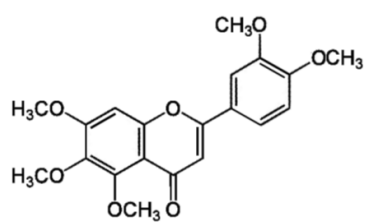
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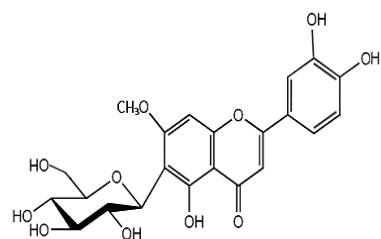
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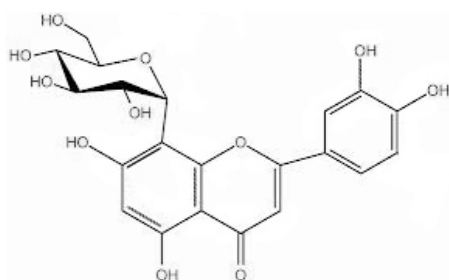
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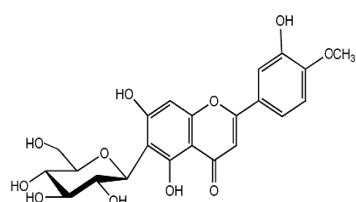
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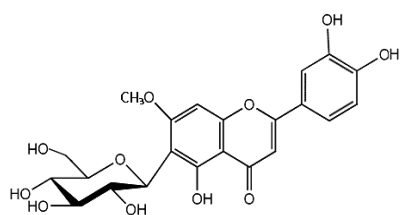
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(18)



(19)

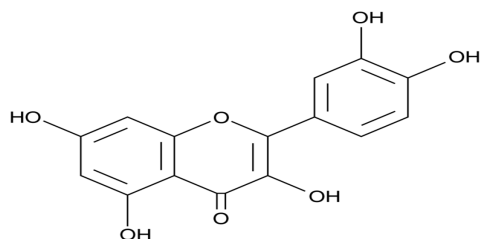


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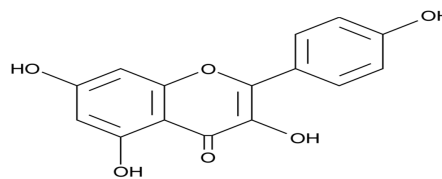
### 1.2.2 Flavonols

The flavonols (3- hydroxy-2- phenyl – chromen- 4 –one) (3) are the most widespread flavonoids in plant. They vary in colour from white

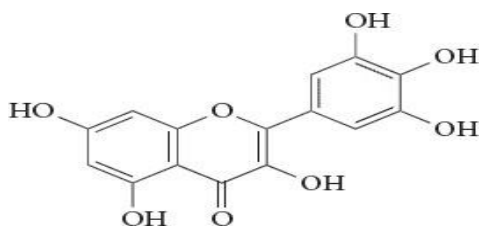
to yellow. Flavonols have a double bond between C<sub>2</sub> and C<sub>3</sub> and an oxygen atom at the C<sub>4</sub> position. Furthermore flavonols also have a hydroxyl group at the C<sub>3</sub> position. They are represented mainly by quercetin (21) kaempferol (22) and myricetin (23) and methylated derivative isorhamnetin (24). They are frequently found as O-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 flavonol aglycones because various sugar groups of flavonols can conjugate to the hydroxyl group of flavonols at different positions<sup>8, 15, 16</sup>. Flavan-3-ols or flavanols have a saturated three-carbon chain with a hydroxyl group in the C<sub>3</sub> position. In foods they are present as monomers or as proanthocyanidins, which are polymeric flavanols (4 to 11 units) known also as condensed tannins. In foods they are never glycosylated<sup>17</sup>.



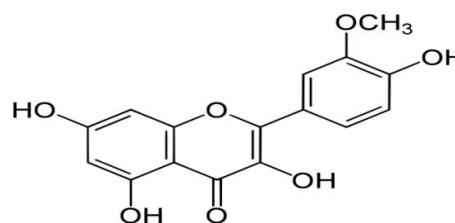
(21)



(22)



(23)



(24)

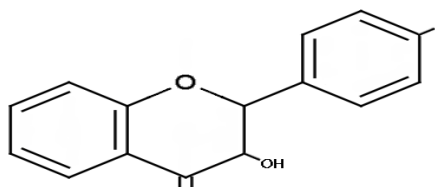
### 1.2.3 Flavanones

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

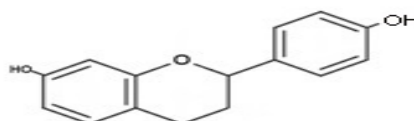
Two stereoisomeric forms of each flavanone structure are possible, since C-2 is a center of asymmetry (epimeric center). Consequently, the B-ring can be either in the (2S) or (2R) configuration. The great majority of the flavanones isolated from plants are levorotatory (2R) or (2S)- flavanones, because the enzymatic reaction catalyzing the conversion of chalcones to flavanones is stereospecific. The C-3 atom of dihydroflavonols bears both the a hydrogen atom and a hydroxyl group, and is therefore an additional center of asymmetry. Thus, four stereoisomers are possible for each dihydroflavonol structure, (2R, and 3R), (2R, 3S), (2S, 3R), and (2S, 3S). All four configurations have been found in naturally occurring dihydroflavonols, but the (2R, 3R) – configuration is by far the most common<sup>18</sup>.

Flavanones are mainly represented by narigenin (25) , hespertin (26), and eriodictol (27), while a number of minor compounds, including sakuranetin (28) and isosakuranetin (29) also occur<sup>8</sup>. 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 (neohespirose, rutinose) or, by a monosaccharide (glucose)<sup>19</sup>.



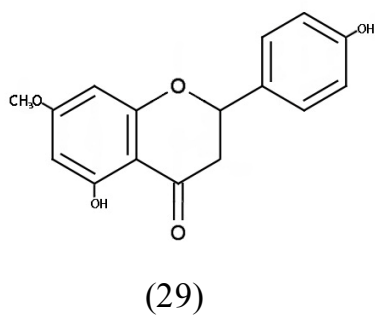
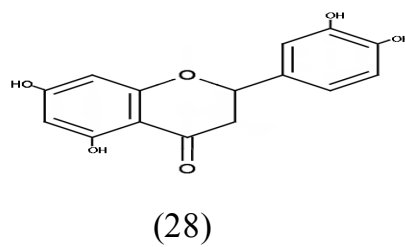
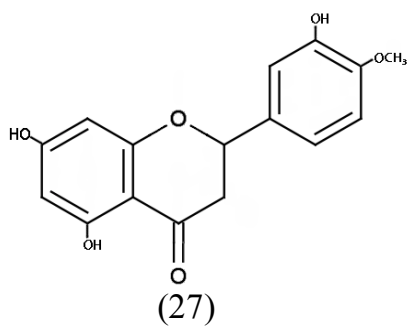
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(25)

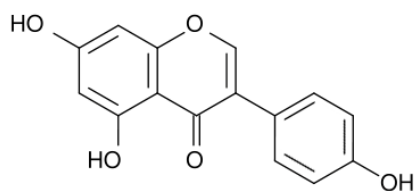
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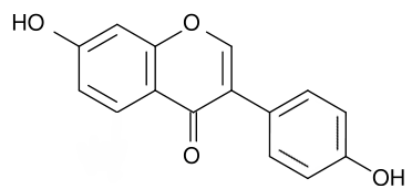
#### 1.2.4 Isoflavones

Isoflavones (3-phenyl-chromen- 4- one) (6) also have a diphenylpropane structure in which the B ring is located in the 3 position. Common isoflavones aglycones such as genistein (30), daidzein (31), and

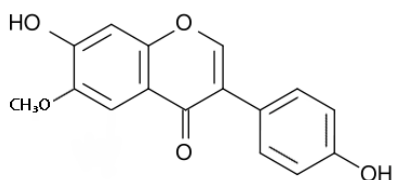
glycitein (32) occur, in low levels, in black beans (*Phaseolus vulgaris*) and green beans (*Pisumsativum*) together with formononetin (33) and biochanin A (34)<sup>8</sup>. They have structural analogues to estrogens, such as estradiol, with hydroxyl groups at the C<sub>7</sub> and C<sub>4</sub> positions<sup>20</sup>.



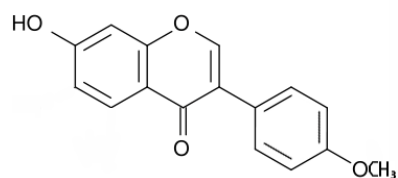
(30)



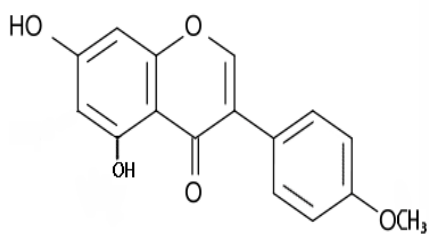
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(32)



(33)

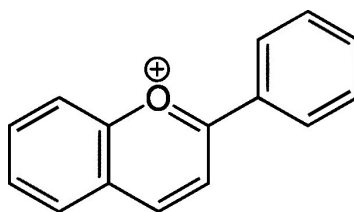


(34)

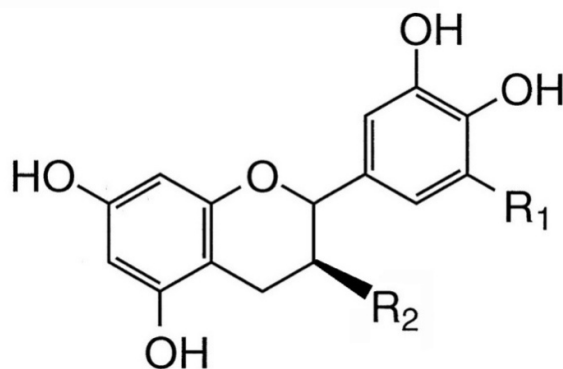
### 1.2.5 Flavanols

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 flavylum 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 epigallocatechin (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<sup>17</sup>.



(35)



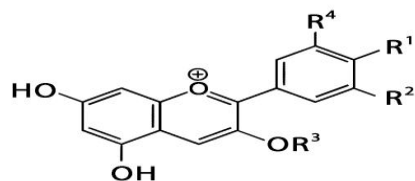
- (36)  $R_1 = H, R_2 = OH$   
 (37)  $R_1 = H, R_2 = O$   
 (38)  $R_1 = OC-Ph(OH), R_2 = H$   
 (39)  $R_1 = OH, R_2 = OH$   
 (40)  $R_1 = OH, R_2 = OO-Ph(OH)$   
 (41)  $R_1 = H, R_2 = OC-Ph(OH)$

### 1.2.6 Anthocyanins

Anthocyanins (3-hydroxy-2-phenylchromenylium) (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<sup>21</sup>. These plant pigments accumulate richly in the epidermal or subepidermal cell vacuoles of flowers, fruits, vegetables and foliage. Generally, anthocyanins belong to the wide spread class of polyphenolic compounds, which are collectively<sup>22</sup> named flavonoids. Anthocyanins are the glycosides of flavonoids with polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium 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. Glucose (glc), galactose (gal), arabinose (arab), rhamnose (rham) and xylose (xyl) are the most common sugars that are bonded to anthocyanidins as mono-, di- or trisaccharide forms. Except for the 3-deoxyanthocyanidins, such as

luteolinidin and apigeninidin in sorghum<sup>21</sup>, aglycones are rarely found in fresh plant materials. Only six anthocyanidins: cyanidin (Cy), delphinidin (Dp), petunidin (Pt), peonidin (Pn), pelargonidin (Pg) and malvidin (Mv), are ubiquitously distributed. For example, the distributions of anthocyanidins in the edible parts of plants are: Cy (50%), Pg (12%), Pn (12%), Dp(12%),Pt (7%) and Mv (7%). The presence of sugar in anthocyanins is important for water solubility; if their sugar (s) are hydrolyzed or lost, their solubility might decrease or be lost and the anthocyanin molecules will be destabilized and lost. The differences in function between the individual anthocyanins relate to the number of hydroxyl groups, the kinds and number of sugars attached to the molecule, the position of the attachment, and the kinds and number of aliphatic or aromatic acids attached to the sugars in the anthocyanin molecule.

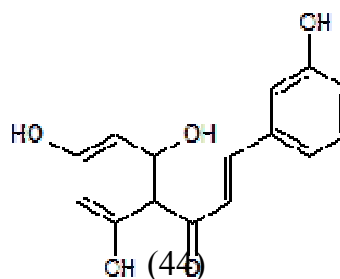
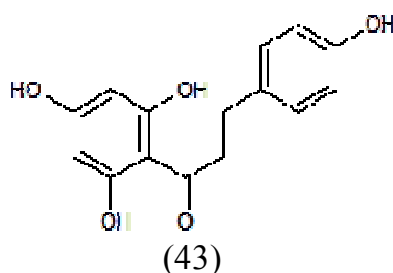
The following four classes of anthocyanin glycosides are very common: 3-monosides, 3-biosides, 3,5- diglycosides and 3,7 – diglycosides. 3-Glycosides occur about two and half times more frequently than 3,5-diglycosides. The most widespread anthocyanin is cyanidin -3- glucoside. Three non-methylated anthocyanidins of cyanidin (Cy), delphinidin (Dp) and pelargonidin (Pg) are the most widespread in the plant kingdom, being present in 80% of pigmented leaves, 69% of fruits and 50% of flowers<sup>21</sup>.

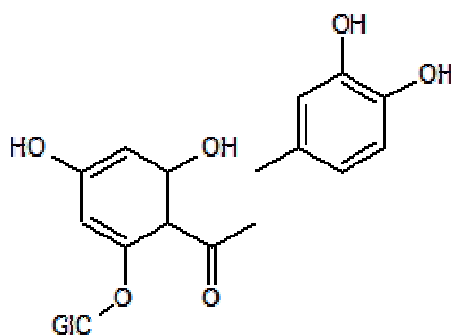


(42)

### 1.2.7 Chalcones

Chalcones 1-(2Hydroxyl-phenyl)-3- phenyl –propenone (8) and dihydrochalocones (43) have a linear C<sub>3</sub>-chain connecting the two aromatic rings. The C<sub>3</sub>-chain of chalcones contains a double bond, whereas the C<sub>3</sub>-chain of dihydrochalcones 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<sup>9</sup> activity. Chalcones are flavonoids lacking a heterocyclic C-ring. Generally, plants do not accumulate chalcones. After its formation, narigeninchalcone is rapidly isomerized by the enzyme chalcone isomerase to form the flavanone-narigenin. The most common chalcones found in foods are phloretin and its 2-O-glucosides: chalconarigenin and arbutin<sup>17</sup>.





(45)

### 1.2.8 Aurones

Aurones (9) were first described from flower of *Coreopsis grandiflora* 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 restricted to floral tissue but have been obtained from bark, wood and leaves as well.

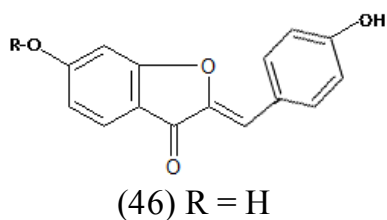
Chemically aurones are based on the 2-benzylidene –coumaranone or 2-benzylidene -3 (2H)- benzofuranone system (9). The term "aurone" recognized both yellow golden colour and their isomeric relationship with the flavones. Structures of natural aurone are most easily discussed by grouping them according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

#### 1.2.8.1 Aurone Lacking B-Ring Hydroxyls

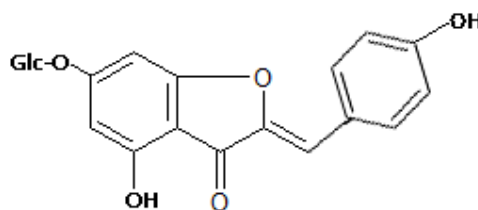
There are known naturally occurring aurones which lack B-ring hydroxyl function. The aurone which was isolated from *Helichrysum arenarium*, with unsubstituted B-ring, for which an aurone structure was tentatively put forward, have been reinvestigated and was found to be 5-hydroxy-6,7,8-trimethoxyflavonol<sup>23</sup>.

### 1.2.8.2 Aurone Having One B-Ring Hydroxyl

Hispidol (46) and its 6-O-glucoside (47) occurs in *Glycine max* seedlings. The aurone: 4,6,4 – trihydroxyaurone was isolated as the 6-O-glucoside (48) from a *Limonium* cultivar<sup>23</sup>.



(47) R = Glc



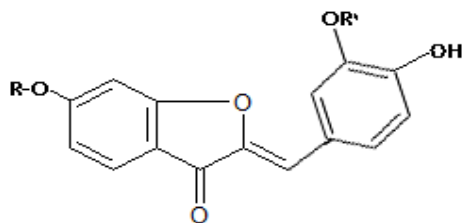
(48)

### 1.2.8.3 Aurones Having Two B-Ring Hydroxyl

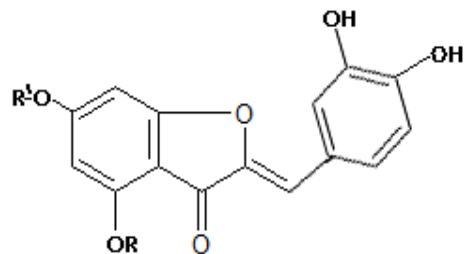
Sulfuretin (49): 6, 3', 4' - trihydroxyaurone has been reported to occur in *Bidens macrocarpa* flowers. Sulfuretin 6-O-glucoside (50), commonly known as sulfurein, and sulfuretin 6-O-diglucose (51) occur in *Coreopsis mutica* together with palasitrin -6,3' - diglucoside of sulfurretin (52). However, the most widely distributed aurone is aureusidin (53) which is: 4,6,3',4',- tetrahydroxyaurone. The free phenol has been reported in snapdragon flowers but the cernuside- 6- O- glucoside (54) is probably the natural constituent. Other derivatives: aureusin (55), rengasine , maritimetin, and its 6-O-glucoside, were reported from *Bident*.



One methyl ether of maritimetin– (leptosidin) along with its 6-O-glucoside (leptosin), were isolated from flowers of *Coreopsis grandiflora*<sup>23</sup>.



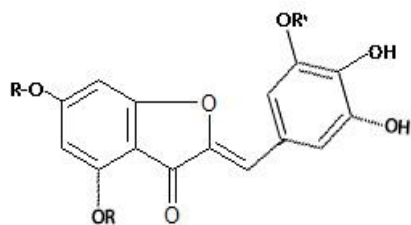
- (49) R = R' = H  
 (50) R = Glc, R' = H  
 (51) R = Glc- OGlc, R' = H  
 (52) R = R' = Glc



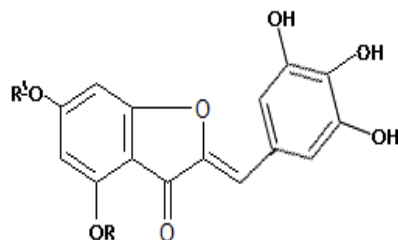
- (53) R = R' = H  
 (54) R = Glc, R' = H  
 (55) R = Me, R' = H

#### 1.2.8.4 Aurone Having Three B-Ring Hydroxyl

The first one of this type is bracteatin : 4,6,3',4', 5`pentahydroxyaurone (56), which occurs in *Helichtymus* as the 4-O-glucoside – bractein (57). The 6-O-glucoside (58) has been found in flowers of *Linaria*<sup>23</sup>.



- (56) R = H  
 (57) R = Glc



- (58)

### **1.3 Separation and quantification of flavonoids**

Essential to the study of flavonoids is having the means available for their separation (analytical and preparative). The importance of this aspect of flavonoid research can be seen in the number of review articles that refer to their chromatography<sup>24-29</sup>. Aim of these reviews is to in earlier times, thin-layer chromatography (TLC), polyamide 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 polyphenolics in plant extracts and in fractionation work. However, the majority of published work now refers to qualitative and quantitative applications of high performance liquid chromatography (HPLC) for analysis. Flavonoids 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 (CE) has been gaining attention.

#### **1.3.1 Extraction**

Flavonoids (particularly glycosides) can be degraded by enzyme action when collected plant material is fresh or non-dried. It is thus advisable to use dry, lyophilized, or frozen samples. When dry plant material is used, it is generally ground into 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 flavonols) are extracted with chloroform, dichloromethane, 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 solutions are suitable. The bulk of extractions of flavonoid-containing 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 dichloromethane, for example, will extract flavonoid aglycones and less polar material. A subsequent step with an alcohol will extract flavonoid glycosides and polar constituents.

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

Flavan-3-ols (catechins, proanthocyanidins, and condensed tannins) can often be extracted directly with water. However, the composition of the extract does 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 proanthocyanidins<sup>30</sup>.

Anthocyanins are extracted with cold acidified methanol. The acid employed is usually acetic acid (about 7%) or trifluoroacetic acid (TFA) (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 co precipitate 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 alcoholic extract (90% methanol) was first partitioned with hexane. The residual methanol part was adjusted with water to 30% and partitioned with t-butyl methyl ether–hexane (9:1). This latter mixture was then chromatographed to obtain pure compounds<sup>31</sup>.

#### **1.3.2.2 Preparative methods**

One of the major problems with the preparative separation of flavonoids is their sparing solubility in solvents employed in chromatography. Moreover, 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 column, 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 be necessary for their isolation. The choice of method depends 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<sup>32</sup>.

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.

Several preparative pressure liquid chromatographic methods are available. These can be classified according to the pressure employed for the separation:

- High-pressure (or high-performance) LC (>20 bar/300 psi)
- Medium-pressure LC (5 to 20 bar/75 to 300 psi)
- Low-pressure LC (<5 bar/75 psi)
- Flash chromatography (ca. 2 bar/30 psi)

### **1.3.3 Analytical methods**

A herbal product contains multiple constituents that might be responsible for its therapeutic effects. It is thus necessary to define as many of the constituents as possible in order to understand and explain the bioactivity. The concept of “phytoequivalence” has been introduced in Germany to ensure consistency of phytotherapeutics<sup>33</sup> According to this concept, a chemical profile for a herbal product is constructed and compared with the profile of a clinically proven reference product. Since many of these

preparations contain flavonoids, it is essential to have adequate analytical techniques at hand for this class of natural product.

Knowledge of the flavonoid content of plant-based foods is paramount to understanding their role in plant physiology and human health. Analytical methods are also important to identify adulteration of beverages, for example. And flavonoids are indispensable markers 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 compounds and the necessity of preparing derivatives. However, Schmidtetel-al<sup>34</sup> 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<sup>35</sup> and is rapidly becoming a science in itself. The initial treatment of the sample is a critical step in chemical and biochemical analyses; it is usually the slowest step in the analysis. In the case of food and plant samples, the number and diversity of analytes is very high and efficient pretreatment is required to obtain enriched phenolic fractions.

Sample preparation methods should<sup>36</sup>:

- Remove possible interferents (for either the separation or detection stages) from the sample, thereby increasing the selectivity of the analytical method.

- Increase the concentration of 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 methods 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 consumption but with highest efficiency and reproducibility. Conditions should be mild enough to avoid oxidation, thermal degradation, and other chemical and biochemical changes. Some procedures — CE, for example — necessitate more rigorous sample pretreatment than others. On the other hand, TLC requires an absolute minimum of sample preparation.

As well as typical sample preparation methods such as filtration and liquid–liquid extraction<sup>36</sup>, newer developments are now extensively used. The first of these is solid-phase extraction (SPE). This is a rapid, economical, and sensitive technique that uses several different types of cartridges and disks, with a variety of sorbents. Sample preparation and concentration can be achieved in a single step. Interfering sugars can be eluted with aqueous methanol on reversed-phase columns prior to elution of flavonoids with methanol.

Among the numerous applications of SPE are separations of phenolic acids and flavonoids from wines and fruit juices. Sep-Pak C18 cartridges have been used for the fractionation of flavonol glycosides and phenolic compounds from cranberry juice into neutral and acidic parts before HPLC analysis<sup>37</sup>. Antimutagenic flavonoids were identified in aqueous extracts of dry spinach after removal of lipophilic compounds by SPE<sup>39</sup>.

### 1.3.3.2 Thin layer chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids<sup>28</sup>, but now the method of choice for simple and inexpensive 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 samples 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<sup>40</sup>. A good discussion presented by Markham in one of the earlier volumes<sup>41</sup>: which describes TLC on silica gel and also two other supports, cellulose and polyamide.

Many different solvent systems have been employed for the separation of flavonoids using TLC. Some solvent systems cited by Markham<sup>41</sup> are reproduced here because they still find application in the separation of flavonoids. Highly methylated or acetylated flavones and flavonols require non polar solvents 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 (100:11:11:26). By the addition of ethyl methyl ketone (ethyl acetate–ethyl methyl ketone–formic acid–glacial acetic acid–water, 50:30:7:3:10), rutin and vitexin-2''-O-rhamnoside can be separated<sup>(42)</sup>. Careful choice of solvent system also allows separation of flavonoid glucosides from their galactosidic analogues<sup>43</sup>. This is especially important for the distinction of C-glucosides from C-galactosides. As an illustration, 8-C-glucosylapigenin (vitexin) can be separated from 8-C-galactosylapigenin with the solvent ethyl acetate–formic acid–water (50:4:10)<sup>43</sup>.



### 1.3.3.3 High performance liquid chromatography

The method of choice for the qualitative and quantitative analysis of flavonoids is HPLC. Since its introduction in the 1970s, HPLC has been used for all classes of flavonoids and hundreds of applications have been published. Numerous reviews have also appeared, such as those by Hostettmann and Hostettmann<sup>26</sup>, Merken and Beecher,<sup>44</sup> He,<sup>45</sup> and Cimpan and Gocan.<sup>46</sup>

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.

A very high proportion of separations are run on octadecylsilyl bonded (ODS, RP-18, or C<sub>18</sub>) phases. Some reported analyses use octasilyl bonded (RP-8 or C<sub>8</sub>) phases but these are becoming increasingly rare. Flavonoid glycosides are eluted before aglycones with these phases, and flavonoids possessing more hydroxyl groups are eluted before the less substituted analogues. As solvents for application, acetonitrile–water or methanol–water mixtures, with or without small amounts of acid, are very common. These are compatible with gradients and UV detection. Occasionally, other solvents such as tetrahydrofuran, isopropanol, or *n*-propanol are used. Acid modifiers are necessary to suppress the ionization of phenolic hydroxyl groups, giving sharper peaks with less tailing. A study has shown that there are large differences in the effectiveness of C<sub>18</sub> columns for the separation of flavonoid aglycones and glycosides. While some columns give good results, others produce substantial band broadening and peak asymmetry.<sup>47</sup>

Octadecylsilyl stationary phases with hydrophilic end capping have been developed for the separation of very polar analytes, which are not sufficiently retained on conventional reversed-phase columns. Among

numerous other applications, they have been demonstrated to be suitable for the separation of flavonol and xanthone glycosides from mango (*Mangifera indica*, Anacardiaceae) peels.<sup>58</sup>

Flavone C-glycosides generally elute with shorter retention times than the corresponding O-glycosides. Thus, vitexin (8-C-glucosylapigenin) elutes with a shorter retention time than apigenin 7-O-glucoside. Furthermore, 8-C-glycosylflavones elute with shorter retention times than the corresponding 6-C-glycosylflavones. Thus, apigenin 8-C-glucoside elutes earlier than apigenin 6-C-glucoside.

Applications of HPLC to the analysis of flavonoids in medicinal and other plants are summarized by Cimpan and Gocan.<sup>46</sup> From the methods listed, it is noteworthy that 90% of the separations use C<sub>18</sub> columns. The importance of flavonoids in foods (fruits, vegetables, and grains) means that it is indispensable to have suitable means of determining their content. The review by Merken and Beecher<sup>39</sup> gives an excellent summary (including full details of separation conditions) of applications of HPLC to the determination of flavones, flavonols, flavanones, isoflavones, anthocyanidins, catechins, and their respective glycosides in foods. Here again, virtually all separations are performed on RP-18 columns, with column lengths between 100 and 300 mm and with internal diameters between 2 and 5 mm. Granulometries vary from 3 to 10  $\mu\text{m}$ , with most being 5  $\mu\text{m}$ . Separation runs are generally up to 1 h in duration. For aglycones and glycosides of isoflavones, certain reported separations of soybean products (e.g., the work of Barnes et al.<sup>49</sup>) have employed C<sub>8</sub> packings, but these are rare. Some applications are given in which two or more subclasses are analyzed simultaneously, such as flavanones, flavones, and flavonols in honey, and anthocyanins, catechins, and flavonols in fruit and wines.

#### 1.3.3.4 Capillary electrophoresis

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 quantitative data. In contrast to HPLC, method development is more time consuming in CE — involving investigation of types, pH and concentrations of electrolytes, types and concentrations of surfactants and organic modifiers, temperatures, and applied voltages. Several modes of CE are available: (a) capillary zone electrophoresis (CZE), (b) micellar electrokinetic chromatography (MEKC), (c) capillary gel electrophoresis (CGE), (d) capillary isoelectric focusing, (e) capillary isotachopheresis, (f) capillary electrochromatography (CEC), and (g) nonaqueous CE. The simplest and most versatile CE mode is CZE, in which the separation is based on differences in the charge-to-mass ratio and analytes migrate into discrete zones at different velocities.<sup>50</sup> Anions and cations are separated in CZE by electrophoretic migration and electroosmotic flow (EOF), while neutral species coelute with the EOF. In MEKC, surfactants are added to the electrolyte to form micelles. During MEKC separations, non polar portions of neutral solutes are incorporated into the micelles and migrate at the same velocity as the micelles, while the polar portions are free and migrate at the EOF velocity.

Applications of CE for the analysis of phytochemicals have been well documented.<sup>(51,52)</sup> CE is especially suitable for the separation of flavonoids as they are negatively charged at higher pH values.<sup>51-53</sup> Suntornsuk<sup>(53)</sup> has reviewed quantitative aspects and method validation of CE for flavonoids. Compared with HPLC, CE can provide an alternative analytical method when higher efficiency or higher resolution is required.

## 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,<sup>54-57</sup> but it is limited by poor sensitivity, slow throughput, and difficulties in analysis of mixtures. Recent developments have, however, made NMR arguably the most important tool for complete structure elucidation of flavonoids. Today, it is possible to make complete assignments of all proton and carbon signals in NMR spectra of most flavonoids isolated in the low milligram range. These assignments are based on chemical shifts ( $\delta$ ) and coupling constants ( $J$ ) observed in 1D  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra combined with correlations observed as crosspeaks in homo- and heteronuclear 2D NMR experiments. Other nuclei like  $^{17}\text{O}$  NMR spectroscopy has been used to study flavonoids only in a few cases. Natural abundance  $^{17}\text{O}$  NMR spectra have been recorded for 11 methoxyflavones,<sup>58</sup> and  $^{17}\text{O}$  NMR data for some 3-arylidenechromanones and flavanones have been discussed in terms of mesomeric and steric substituent interactions.<sup>59</sup>  $^{17}\text{O}$  NMR spectroscopy has also been used to study the effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system.<sup>60</sup>

Excellent compilation of NMR data on individual flavonoids has previously been presented,<sup>54-56</sup> and some useful reviews in this field have also been published.<sup>57, 61, 62</sup> Based on a database containing 700  $^{13}\text{C}$  spectra of flavonoids obtained from the literature, pattern recognition has been used to assemble compatible substructures according to related spectra.<sup>(63)</sup> Some recent publications reporting flavonoid coupling constants include: NMR studies on flavones after the incorporation of  $^{13}\text{C}$  at the carbonyl group, which allowed the measurement of two- and three-bond carbon-carbon coupling constants, ranging from 1.4 to 3.5 Hz, and the measurement of two-

, three-, and four-bond carbon–hydrogen coupling constants, which ranged from 0.3 to 3.8 Hz;<sup>64</sup> complete assignment of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of several flavones and their proton–proton and carbon–proton coupling constants, including the extreme seven-bond long-range coupling between H-7 and H-3 in 6-hydroxyflavone (0.52 Hz) and flavone (0.27 Hz).<sup>(65)</sup> Typical one-bond  $^1\text{H}$ – $^{13}\text{C}$  coupling constants of monosaccharides in anthocyanins have been observed within magnitudes of 125 and 175 Hz.<sup>66</sup>

#### 1.4.2 Mass spectrometry

Modern mass spectrometric techniques are very well suited for the analysis of flavonoids isolated from plants and foodstuffs and in their *in vivo* metabolite forms. Progress during the last two 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 well as on the structure and quantity of compounds with the nature and within the mass range of flavonoids. 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 separated according to their molecular masses. Most flavonoid glycosides are polar, non volatile, 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. Analysis of flavonoid glycosides without derivatization became possible with the introduction of desorption ionization techniques. Field desorption, which was the first technique employed for the

direct analysis of polar flavonoid glycosides, has provided molecular mass data and little structural information.<sup>(67)</sup>

### **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.<sup>68</sup> The first method measures the absorption, transmission, or reflection of IR radiation with wavelengths in the range of 2.5 to 25  $\mu\text{m}$ . 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 may serve as fingerprints of structure, composition, interactions, and dynamics. The reciprocal of wavelength, wavenumber ( $\text{cm}^{-1}$ ), is commonly used to characterize the energy in the field of vibrational spectroscopy.

Systematic vibrational spectroscopy studies on flavonoids have occurred since the early 1950s, and most of them have been limited to a discussion on the hydroxyl and carbonyl absorption frequencies.<sup>69</sup> However, 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.<sup>70,71</sup> The implementation of FTIR spectroscopy has significantly enhanced the sensitivity, and Raman spectroscopy has benefited from the availability of holographic notch filters, which efficiently suppress the strong signal from elastically scattered (Rayleigh) radiation while maintaining the Raman-shifted intensity with minimal attenuation. Furthermore, high-powered NIR semiconductor lasers and sensitive 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 is

overcome through the use of laser light with frequency corresponding to the absorption maximum of the compound to be characterized. Finally, the increase in the computing power of standard computers has facilitated more sophisticated data evaluation of both IR and Raman spectra.

#### **1.4.3.1 IR and Raman spectroscopic techniques in studies of complexes involving flavonoids**

In recent years, IR and Raman spectroscopic techniques have been applied for the characterization of flavonoid-containing systems with rather complex composition. A rapid analytical method involving attenuated total reflection (ATR) mid-IR spectroscopy and UV–Vis spectroscopy, combined with multivariate data analysis, has been applied for the discrimination of some beverages.<sup>72</sup> By analyzing phenolic extracts (obtained by C<sub>18</sub> solid-phase extractions followed by elution with acidified methanol) of some beverages by mid-IR spectroscopy, almost complete discrimination of all samples was achieved. Furthermore, it was possible to establish class models for five different beverages and to classify the test samples correctly. In another study, the Raman spectrum of *Artocarpus heterophyllus* heartwood was shown to exhibit two characteristic bands at 1247 and 745 cm<sup>-1</sup>.<sup>73</sup>

#### **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 polyphenolic compounds reveal two characteristic UV absorption bands with maxima in the 240 to 285 and 300 to 550 nm range. The various flavonoid classes can be recognized by their UV spectra,<sup>74</sup> and UV-spectral characteristics of individual flavonoids including the effects of the number of aglycone hydroxyl groups, glycosidic substitution pattern, and nature of aromatic acyl

groups have been reviewed in several excellent books.<sup>75,74,76</sup> 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.

#### **1.4.4.1 Online UV absorption spectroscopy in chromatography**

The combination of HPLC equipped with a UV–Vis DAD has for the two last decades been the standard method for the detection of flavonoids in mixtures. This type of detector allows the simultaneous recording of chromatograms at different wavelengths. The HPLC–DAD (alternatively called LC–UV) method has been used for isolation, identification, screening, measurement of peak purity, or quantitative determinations of flavonoids in numerous studies, and there exist several excellent views in the field.<sup>77-79</sup>

#### **1.4.5 X-Ray crystallography**

X-ray crystallography is the most accurate method for structural elucidation of flavonoids in the solid state. The method can only be applied to crystallized compounds, which has limited the number of flavonoid crystal structures reported.

##### **1.4.5.1 X-Ray studies on flavonoids involving complexes**

Flavocommelin is a flavonoid component of the blue pigment commelin, which has been isolated from the petals of *Commelina communis*. Commelin is composed of six molecules each of the anthocyanin malonylawobanin and flavocommelin, and two atoms of magnesium.<sup>(80)</sup> The crystal structure of the octaacetate derivative of flavocommelin has been determined by x-ray diffraction.<sup>81</sup> In the crystal, the molecules were arranged parallel to each other according to the periodicity of the crystal lattice.



However, intermolecular stacking of the flavanone skeletons was not observed, which suggested that the hydrophilicity of the glucose moieties was one of the important factors governing the self-association. X-ray diffraction data collected at low temperature (130 K), using synchrotron radiation, have recently been used for determination of crystal architecture and conformational properties of the inclusion complex, neohesperidin dihydrochalcone- $\beta$ -cyclodextrin.<sup>82</sup> The complex was characterized by one aromatic part of neohesperidin dihydrochalcone deeply inserted into the hydrophobic cavity of  $\beta$ -cyclodextrin through the primary OH rim. The formation of other  $\beta$ -cyclodextrin inclusion complexes involving various flavanones,<sup>83</sup> as well as the inclusion behavior of both 2-hydroxypropyl  $\beta$ -cyclodextrin and  $\beta$ -cyclodextrin in solution and solid-state toward quercetin,<sup>84</sup> have also been subjected to x-ray diffractometric analysis. Genistein and its amine complexes with morpholine and piperazine have been studied in the solid and liquid states by x-ray crystallography and <sup>13</sup>C and <sup>15</sup>N NMR spectroscopy.<sup>85</sup>

## **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 gastrointestinal tract, and finally the microorganisms of the colon. It can 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. The absorption of the flavonoid liberated from the food will depend on its physicochemical properties such as molecular size and configuration, lipophilicity, solubility, and pKa. To date, only fragmentary information is available on the effect of the plant food matrix on

absorption. Well-designed studies that addressed this issue have not been reported.

#### **1.5.1.1 Role of the flavonoid structure: glycosides and oligomers**

Most flavonoids, except catechins, are usually present in the diet as  $\beta$ -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 microflora<sup>86</sup>. Thus, it was thought that the glycosylated flavonoids were only marginally absorbed. However, this view on absorption of glycosides had to be revised.

In contrast with other flavonoids, catechins occur as aglycones and galloylated forms in foods. Pharmacokinetic data point to absorption from the small intestine of both the aglycones and the galloylated forms<sup>87</sup>. 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: the proanthocyanidins. In vitro studies with Caco-2 cells showed that only trimers were able to pass across monolayers of cells<sup>88-89</sup>. It has been suggested that oligomers can be hydrolyzed to monomers and dimers due to the acidic conditions in the stomach<sup>90</sup>.

However, sampling of human gastric juice showed that hydrolysis of proanthocyanidins does not occur in vivo<sup>91</sup>. It can be concluded that only proanthocyanidins up to three catechins are absorbable from the colon. Larger molecules will reach the colon where they will be degraded by bacteria.

### **1.5.1.2 Bioavailability**

Dietary flavonol glycosides showed very rapid to very slow absorption in man. Times to reach peak concentrations (T<sub>max</sub>) were between <0.5 and 9 h. The bioavailability of quercetinglucosides from onions was superior. Bioavailability of various quercetin glycosides (β-galactosides and β-xylosides) from apples and of pure quercetinrutinoside was only 30% of that from onions<sup>92</sup>. Thus, the sugar moiety of quercetin glycosides seemed to be an important determinant of their bioavailability, which was confirmed when pure quercetin-β-glucoside or pure quercetin-β-rutinoside was administered to healthy human volunteers<sup>93</sup>. The peak concentration of quercetin (C<sub>max</sub>) in plasma was 20-times higher and reached (T<sub>max</sub>) more than 10-times faster after intake of the glucoside than after the rutinoside. These pharmacokinetic data suggest that quercetinglucoside was absorbed from the small intestine, whereas quercetinrutinoside was absorbed from the colon after deglycosylation. Evidently, the sugar moiety played no role in the elimination of quercetin from plasma: elimination half-life was about 20 h for all glycosides. This is consistent with the observation that quercetinglucosides do not circulate in the blood<sup>(94)</sup>. Apparently, the sugar part only plays a role upon absorption.

### **1.5.1.3 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 not 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<sup>95</sup>.

In the first compartment, mainly the small intestine and liver, biotransformation enzymes act upon flavonoids and their colonic metabolites. The kidney also contains enzymes capable of biotransformation of flavonoids. Conjugation of the polar hydroxyl groups with glucuronic acid, sulfate, or glycine has been reported for flavonoids and for their colonic metabolites<sup>96</sup>. In addition, O-methylation by the enzyme catechol-O-methyltransferase plays an important role in the inactivation of the catechol moiety, that is, the two adjacent (ortho) aromatic hydroxyl groups, of flavonoids and their colonic metabolites. Recently, deglycosylation of glycosides in the brush border membrane of the small intestine was demonstrated<sup>97</sup>.

## **1.6 Flavonoids as nutraceuticals**

“Nutraceutical” is a term coined in 1979 by Stephen DeFelice<sup>(98)</sup>. 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 range from isolated nutrients, dietary supplements, 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<sup>99</sup>. The increasing interest in nutraceuticals reflects the fact that consumers hear about epidemiological studies indicating that a specific diet or component of the diet is associated with a lower risk for a certain disease. The major active nutraceutical ingredients in plants are flavonoids. As is typical for phenolic compounds, they can act as potent

antioxidants and metal chelators. They also have long been recognized to possess antiinflammatory, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities<sup>99</sup>.

### **1.6.1 Antioxidant activity**

The best-described property of almost every group of flavonoids is their capacity to act as antioxidants. The flavones 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 exogenous damage<sup>100, 101</sup>. Free radicals and ROS have been implicated in a large number of human diseases<sup>102, 103</sup>. Quercetin, kaempferol, morin, myricetin and rutin, by acting as antioxidants, exhibited beneficial effects such as anti-inflammatory, antiallergic, antiviral, as well as anticancer activity. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases. Quercetin and silybin, acting as free radical scavengers, were shown to exert a protective effect in liver reperfusion ischemic tissue damage<sup>104, 105</sup>. The scavenging activity of flavonoids has been reported to be in the order: Myrcetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavone<sup>106</sup>.

### **1.6.2 Antimicrobial activity**

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

Antibacterial activity has been displayed by a number of flavonoids. Quercetin has been reported to completely inhibit the growth of *Staphylococcus aureus*. Most of the flavonones having no sugar moiety showed antimicrobial activities whereas none of the flavonols and flavonolignans tested showed inhibitory activity on microorganisms<sup>107</sup>.

### **1.6.3 Antifungal activity**

A number of flavonoids isolated from the peelings of tangerine orange, when tested for fungistatic activity towards *Deuterophomatracheiphila* were found to be active; nobiletin and langeritin exhibited strong and weak activities, respectively, while hesperidin could stimulate fungal growth slightly. Chlorflavonin was the first chlorine-containing flavonoid-type antifungal antibiotic produced by strains of *Aspergillus candidus*<sup>108</sup>.

### **1.6.4 Antiviral activity**

Naturally occurring flavonoids with antiviral activity have been recognized since the 1940s but only recently have attempts been made to make synthetic modifications of natural compounds to improve antiviral activity. Quercetin, morin, rutin, dihydroquercetin (taxifolin), apigenin, catechin, and hesperidine have been reported to possess antiviral activity against some of the 11 types of viruses<sup>109</sup>.

### **1.6.5 Antiulcer activity**

Some flavonoids exert significant anti-inflammatory activity in the animal model of both acute and chronic inflammation when given orally or topically<sup>110, 111</sup>. Hesperidin, a citrus flavonoid, possesses

significant anti-inflammatory and analgesic effects<sup>112</sup>. Recently apigenin, luteolin and quercetin have been reported to exhibit anti-inflammatory activity<sup>113</sup>.

#### **1.6.6 Hepatoprotective activity**

The liver is subject to acute and potentially lethal injury by several substances including phalloidin (the toxic constituent of the mushroom, *Amanita phalloides*), CCl<sub>4</sub>, galactosamine, ethanol, and other compounds. Flavonoids have also been found to possess hepatoprotective activity. In a study carried out to investigate the flavonoid derivatives silymarin, apigenin, quercetin, and naringenin, as putative therapeutic agents against microcrystin LR-induced hepatotoxicity, silymarin was found to be the most effective one<sup>114</sup>. The flavonoid, rutin and venoruton, showed regenerative and hepatoprotective effects in experimental cirrhosis<sup>115</sup>.

#### **1.6.7 Anti-inflammatory activity**

The anti-inflammatory activity of flavonoids in many animal models have been reported. Flavone/flavonol glycosides as well as flavonoid aglycones have been reported to possess anti-inflammatory. Kaempferol, quercetin, myricetin, fisetin were reported to possess LO and COX inhibitory activities<sup>116, 117</sup>.

#### **1.6.8 Antidiabetic effects**

Flavonoids, especially quercetin, has been reported to possess antidiabetic activity. It was reported that quercetin brings about the regeneration of pancreatic islets and probably increases insulin release in

streptozotocin-induced diabetic rats<sup>118, 119</sup>. Also in another study, Hif and Howell reported<sup>120, 121</sup> that quercetin stimulate insulin release and enhanced  $\text{Ca}^{2+}$  uptake from isolated islets cell which suggest a place for flavonoids in noninsulin-dependent diabetes.

#### **1.6.9 Effect on central nervous system**

Synthetic flavonoids, such as 6-bromoflavone and 6-bromo-3'-nitroflavones, were shown to displace [3H] flumazenil binding to membranes from rat cerebellum but not from spinal cord, indicating selectivity for the BZO mega receptor subtype, but the latter was more potent than 6-bromoflavone. Results from two conflict tests in rats showed that these synthetic flavonoids possess anxiolytic like properties similar or superior to that of diazepam<sup>121</sup>.

#### **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 animal for most flavonoids. Similar doses in humans are quite unrealistic. As a precaution, doses less than 1mg per adult per day have been recommended for humans<sup>122</sup>. Dunnick and Hailey<sup>123</sup> reported that high doses of quercetin over several years might result in the formation of tumors in mice. However, in other long-term studies, no carcinogenicity was found<sup>124</sup>. 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 chromatographic techniques
- 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 recorded on a Perkin- Elmer Lambda -2 UV- Visible Spectrophotometer. The IR spectra were run on a Perkin- Elmer 1310 Infrared Spectrophotometer.

#### 2.1.2- Plant material

The leaves of *Clematis vitalbawere* were 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 (100 ml) methanol .

**(ii) Potassium hydroxide solution .**

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

**2.2.2- Preparation of plant extract for phytochemical screening** (100g) of powdered air – dried leaves of *Clematis vitalba* were extracted with (200 ml ) 95% ethanol for five days . The cooled solution was filtered and it's volume was adjusted to (100ml) by addition of enough 95% ethanol . This prepared extract (PE) was used for the following tests .

**2.2.3- Phytochemical screening**

The plant species were screened for steroids, flavonoids, alkaloids and glycosides .

**i) Test for steroids**

(40 ml) 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 (20 ml) chloroform . The chloroform solution was dehydrated over anhydrous sodium sulphate .

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

## **ii) Test for alkaloids**

(5 ml) of 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 wagner reagent were added. No formation of precipitate.

## **iii) Test for flavonoids**

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

- (i) To (3 ml) of the filtrate few drops of methanolic aluminum chloride were added. A dark yellow colour soon developed.
- (ii) To (3 ml) of the filtrate few drops of potassium hydroxide solution were added. A dark yellow colour was observed.
- (iii) To (3 ml) of filtrate few drops of ferric chloride solution were added. A blue colouration was observed.

## **iv) Test for glycosides**

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

### **2.2.4- Extraction of flavonoids from the leaves of *Clematis vitalba***

Powered air-dried leaves (1 kg) of *Clematis vitalba* were extracted with 95% ethanol at ambient temperature for five days. The solvent was removed giving a green solid (5g)

### **2.2.5- Paper chromatography of the crude products**

Part of the crude product of *Clematis vitalba* (0.1 g) was dissolved in 95% ethanol (2 ml) and applied as concentrated spots on Whatman No. 3 sheets. The sheets were irrigated with the solvent system B:A:W(4:1:5). The chromatograms 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 (100 ml) . The solution was stored in a glass container with tightly fitting 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 compounds I (or II) in presence of sodium methoxide**

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

### **2.2.8- The UV Spectra of compounds I and II in presence of aluminum chloride**

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

### **2.2.9- The UV spectra of compounds I and II in presence of sodium acetate**

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

### 3-Results and Discussion

#### 3.1 Extraction and purification of flavonoids

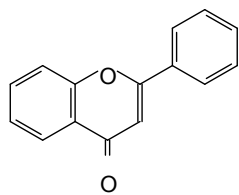
The leaves of *Clematis Vitalba* were extracted with 90% ethanol and the crude product obtained was subjected to phytochemical screening where qualitative tests were positive for flavonoids, glycosides, but negative for steroids and alkaloids.

The crude extract was then fractionated by paper chromatography where Whatman number 3 sheets were irrigated with BAW (4:1:5). After the usual workup two flavonoids were isolated- compounds I and II.

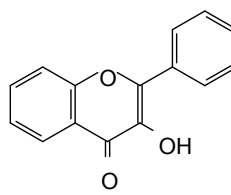
#### 3.2- Spectral data of compound I

The IR (KBr) spectrum of compound I (Fig. 1) showed  $\nu$  667.32 , 779.19 ( C-H bending, Ar ) , 1099.35 (C- O , ether ) , 1649.02 ( C = O ) , 1685.67 (  $\alpha, \beta$  -unsaturated carbonyl group) and  $3417.63\text{ cm}^{-1}$  ( OH ).

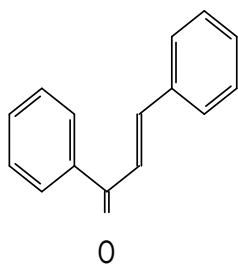
Compound I can not be an anthocyanin or a catechin since the IR spectrum gave a carbonyl stretching at  $1649.02\text{ cm}^{-1}$  . It could be : a flavone, flavonol, chalcone , aurone , isoflavone , flavanone, dihydrochalcone or dihydroflavonol .



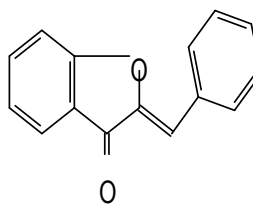
Flavone



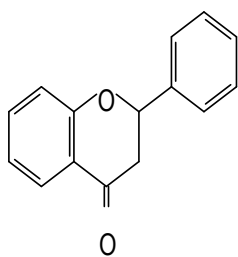
Flavonol



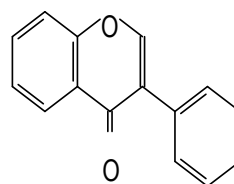
Chalcone



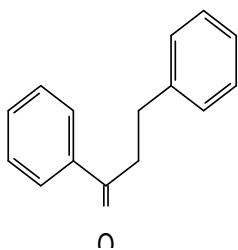
Aurone



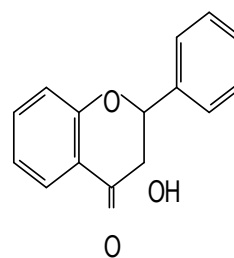
Flavanone



Isoflavone



Dihydrochalcone



Dihydroflavonol

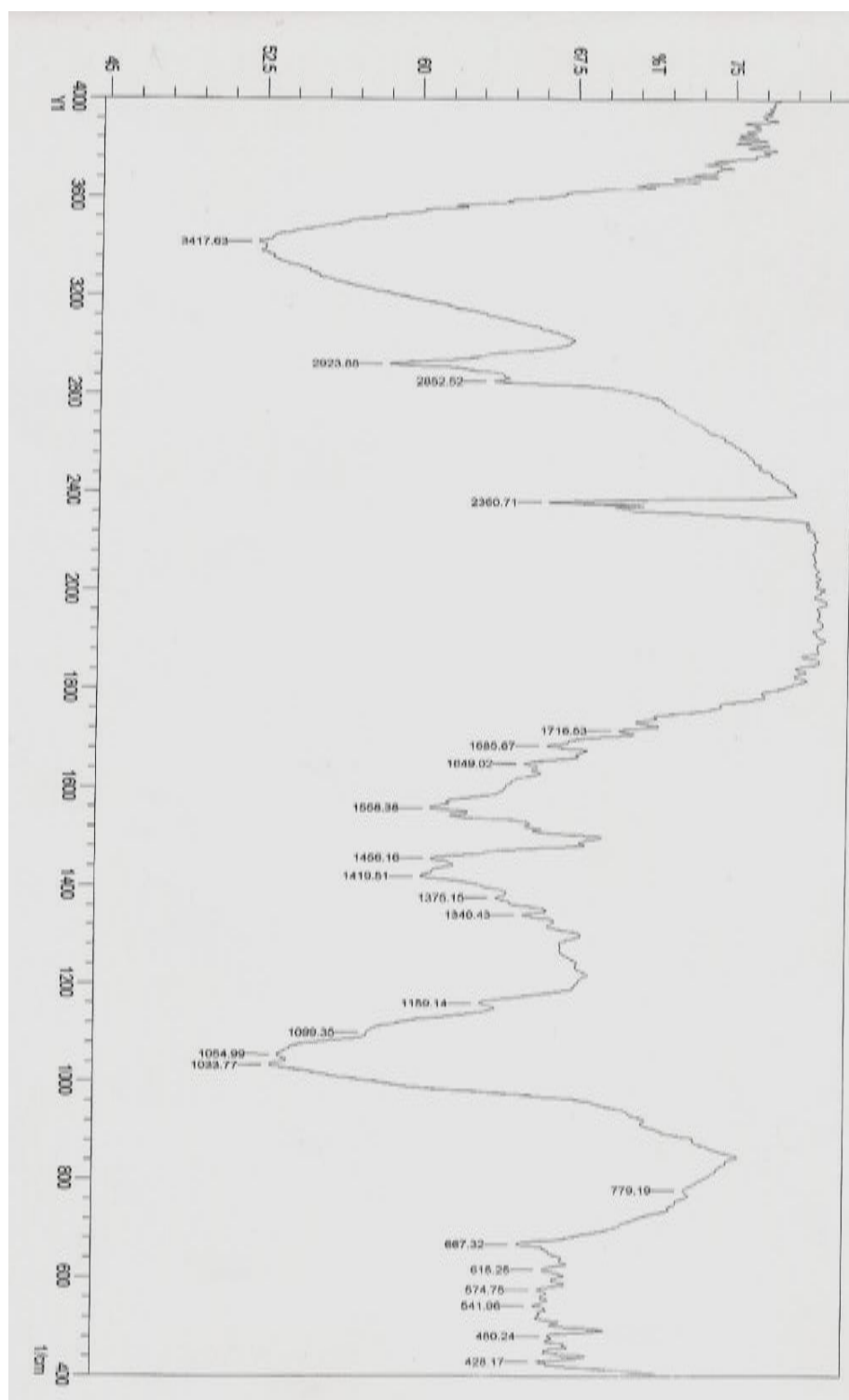
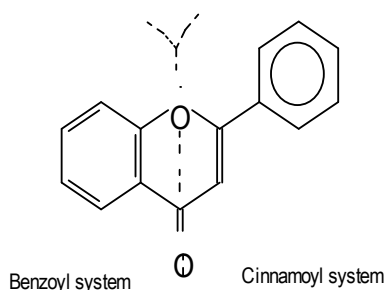


Fig.1: IR spectrum of compound I



In their UV spectra, most flavonoids exhibit two major bands in the region 240-400 nm. These two peaks are commonly referred to as band I (usually 300-400nm) and band II (usually 240-280 nm)<sup>11</sup>. 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<sup>11</sup>.



Isoflavones, flavanone and dihydroflavonols all give only one band 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 I absorbs( Fig.2) at  $\lambda_{\text{max}}$  (MeOH)270, 330nm (sh.). Which is characteristic of : isoflavones, flavanones, dihydrochalcones and dihydroflavonols. However, the shoulder which appeared in the spectrum at 330 nm clearly indicates that this phytochemical is an isoflavone<sup>73</sup>

Considerable structural feature has also been obtained by UV shift reagents such as sodium methoxide, sodium acetate, aluminum chloride. These reagents produce shifts 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

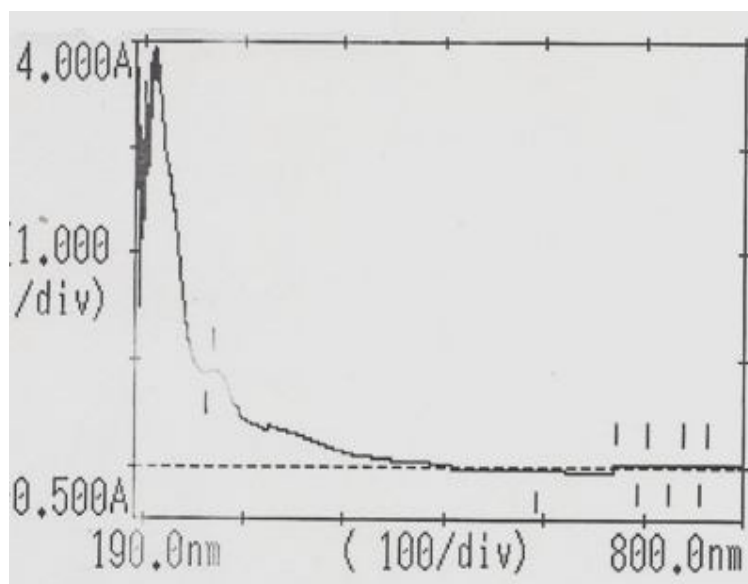
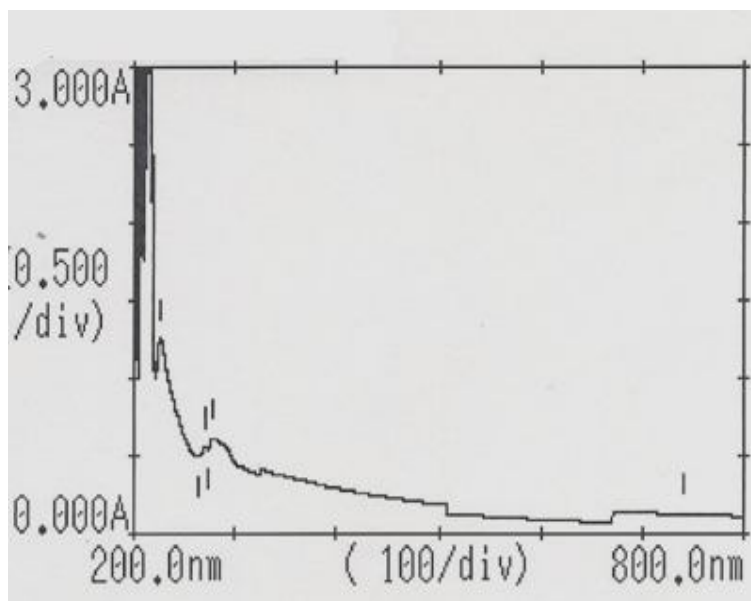


Fig.2: UV spectrum of compound I



**Fig.3: Sodium methoxide spectrum of compound I**

of flavonoids for detection of free 3-and/or 4'-hydroxy function.

When sodium methoxide was added to a methanolic solution of compound I no bathochromic shift (Fig 3) was observed indicating absence of 3- and 4'-OH functions.

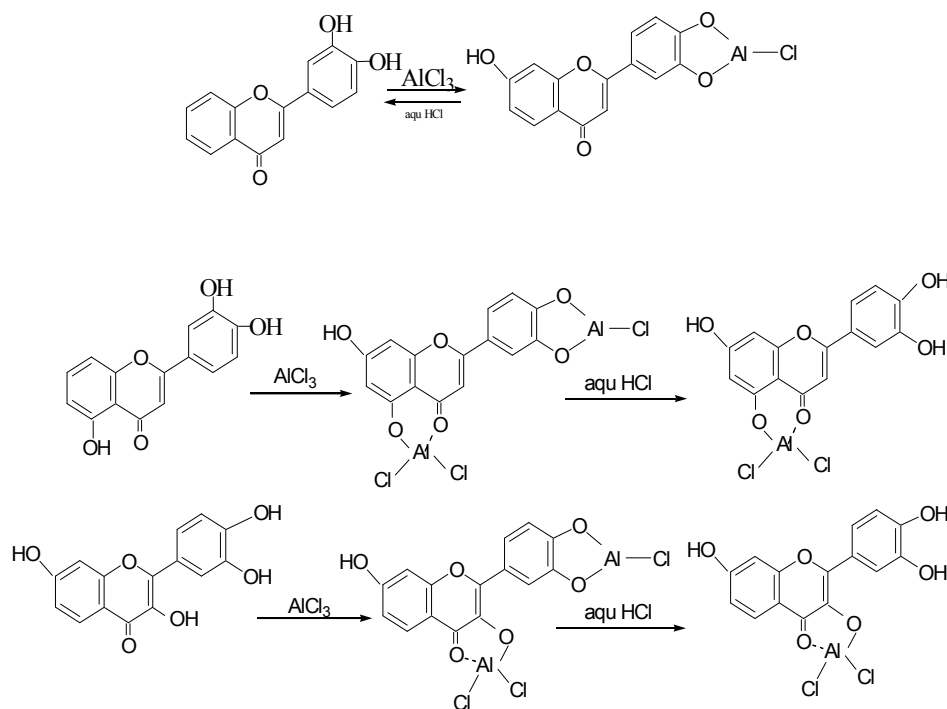
Sodium acetate is a weaker base than NaOMe, and as such, ionizes only the more acidic hydroxyl groups i.e. the 7-OH function.

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

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

The presence of an ortho-dihydroxy groups in the B-ring of flavones and flavonols can be detected by comparison of the spectrum of the flavonoid in the presence of  $\text{AlCl}_3$  with that obtained in  $\text{AlCl}_3/\text{HCl}$ . 30-40 nm hypsochromic shift observed in band I of  $\text{AlCl}_3$  spectrum on the addition of

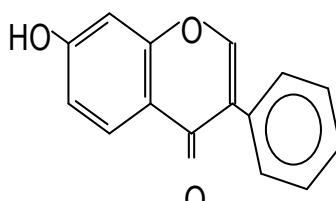
acid results from the decomposition of the complex of  $\text{AlCl}_3$  with the ortho-dihydroxy group. The presence of the adjacent hydroxyl groups in the B – ring gives only a 20 nm hypsochromic shift on the addition of acid to  $\text{AlCl}_3$  solution .

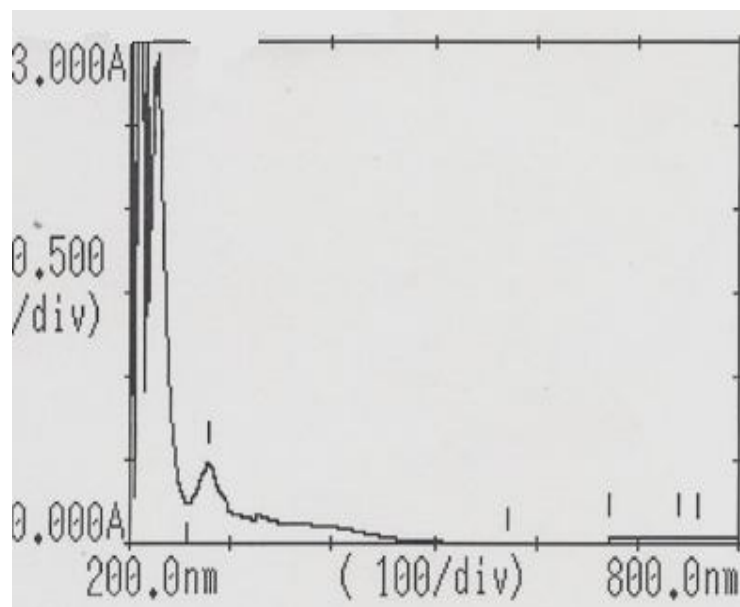


**Scheme 3.1: Aluminium chloride-flavonoid complexes**

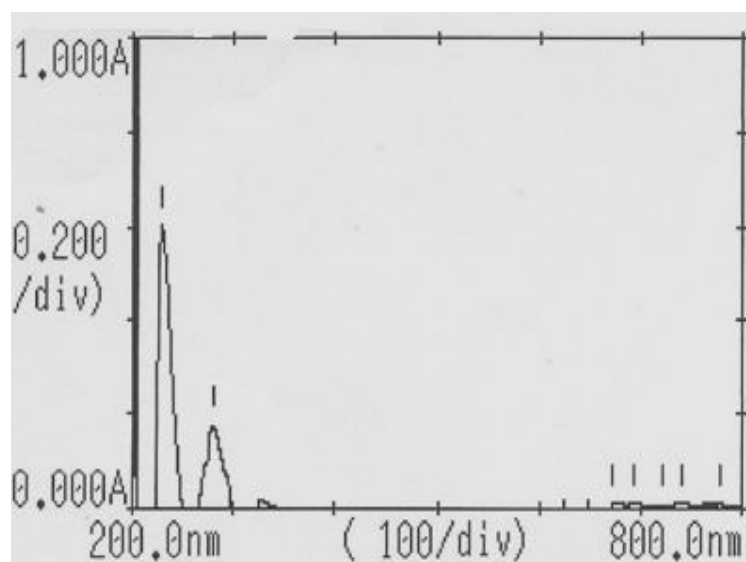
Figure 5 illustrates the  $\text{AlCl}_3$  spectrum of compound I where no bathochromic shift was observed indicating absence of 3-,5- OH functions and catechol moieties. .

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





**Fig.4: Sodium acetate spectrum of compound I**



**Fig.5: Aluminium chloride spectrum of compound I**

### 3.3-Spectral data of compound II

The IR (KBr) spectrum of compound II (Fig. 6) showed  $\nu$  661.54 , 896.84 ( C-H bending, Ar ) , 1074.28 (C- O , ether ) , 1649.02 ( C = O ) , 1685.67 (  $\alpha,\beta$  -unsaturated carbonyl group) and 3398.34  $\text{cm}^{-1}$  ( OH ).

Compound II can not be an anthocyanin or a catechin since the IR spectrum gave a carbonyl stretching at  $1685.67\text{ cm}^{-1}$ . It could be : a flavone, flavonol, chalcone , aurone , isoflavone , flavanone, dihydrochalcone or dihydroflavonol .

The presence of a carbonyl function in compound II indicates absence of catechins and anthocyanins . These classes are characterized by absence of a carbonyl function.

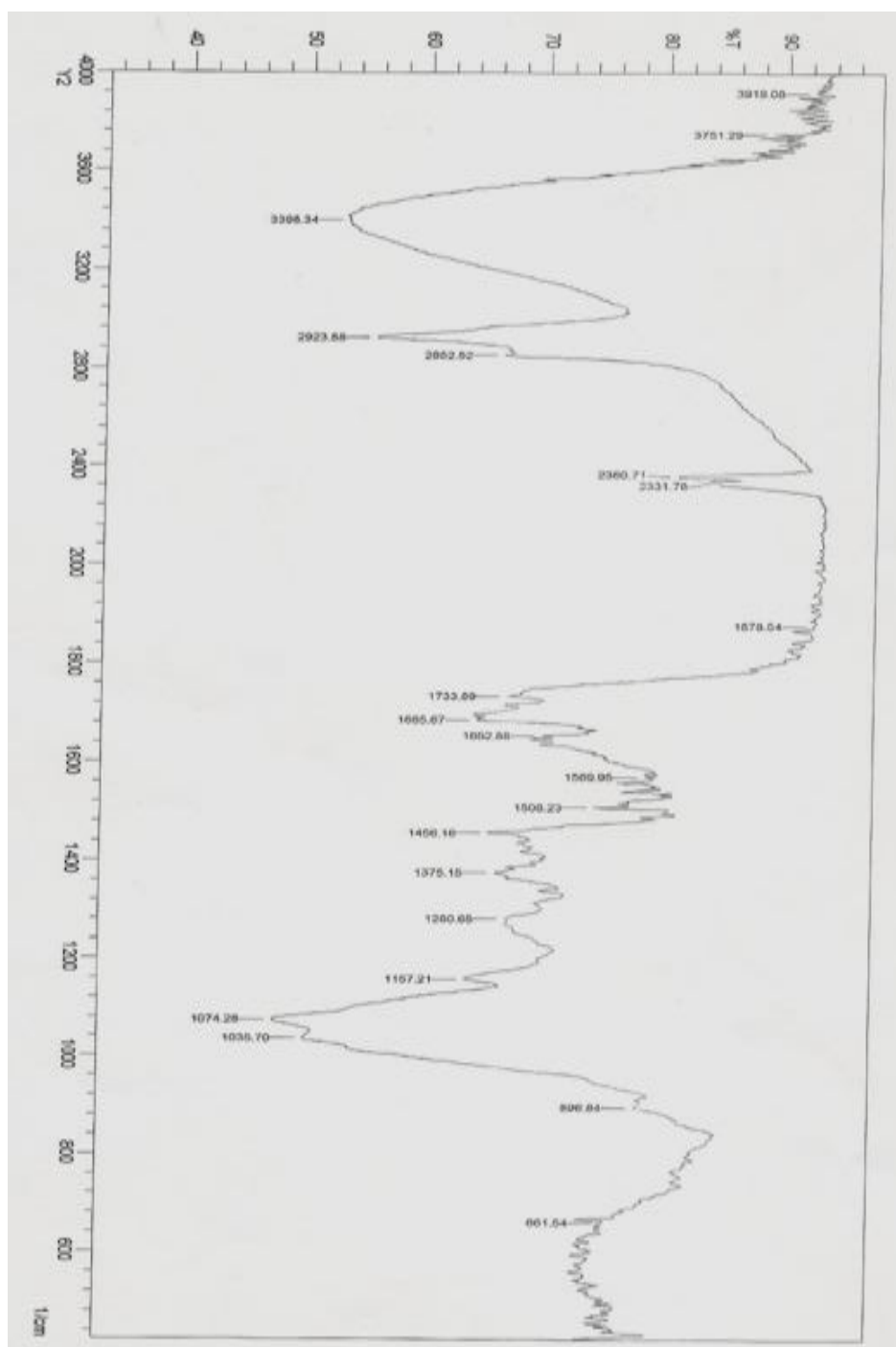
In the UV compound II absorbs (Fig.7) at  $\lambda_{\text{max}}$  248, 310(sh.)nm. The presence of a shoulder in this spectrum indicates that compound II is an isoflavone.

Next UV shift reagents were employed to investigate the hydroxylation pattern in the isoflavone nucleus.

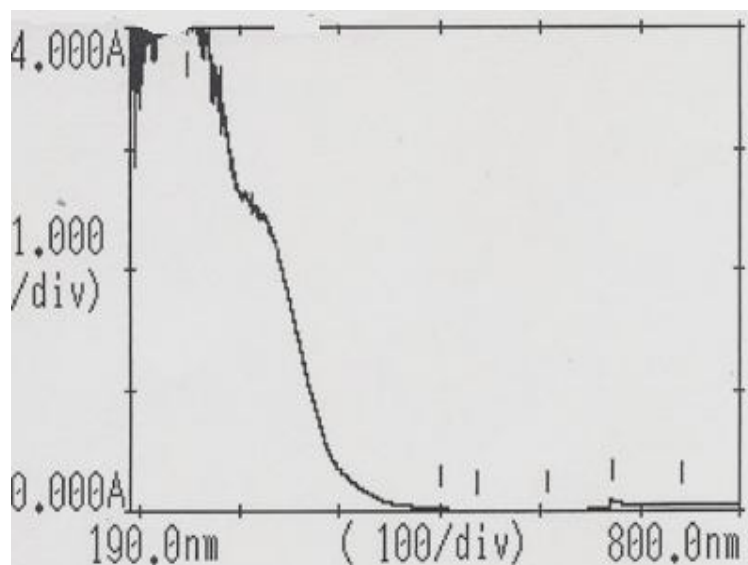
The sodium methoxide spectrum of compound II (Fig.8) gave a 23 nm bathochromic shift without decrease in intensity indicating the presence of a 4'-OH function.

The sodium acetate spectrum (Fig.9) a 9 nm bathochromic shift without decrease in intensity which is indicative of a 7- OH function.

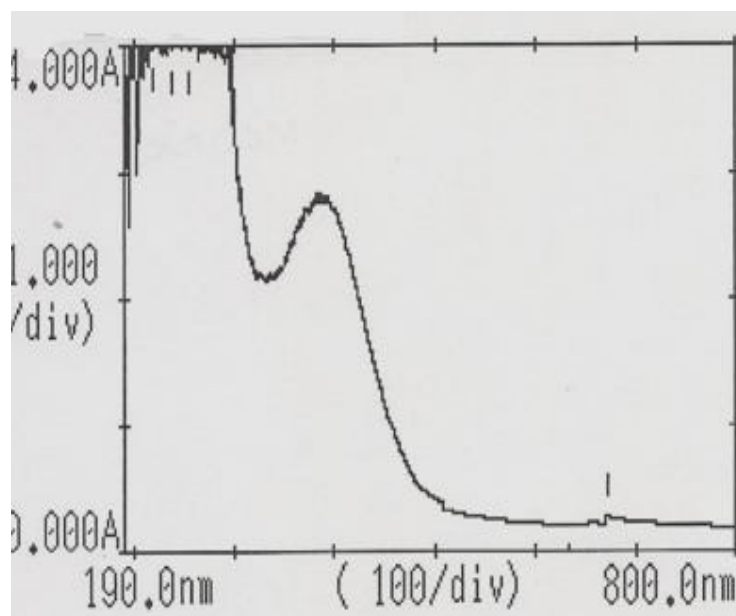
No bathochromic shift was observed in the aluminium chloride spectrum suggesting absence of a 3- and 5-OH functions as well as catechol moieties.



**Fig.6: IR spectrum of compound II**

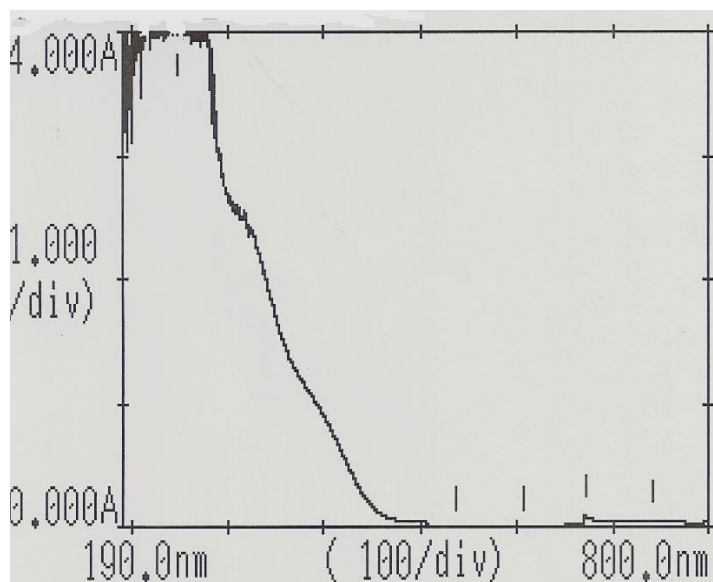


**Fig.7: UV spectrum of compound II**



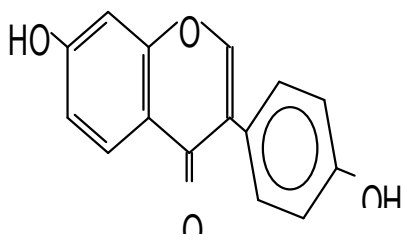
**Fig8: Sodium methoxide spectrum of compound II**





**Fig.8: Sodium acetate spectrum of compound II**

The above argument suggests that compound II is an isoflavone hydroxylated at the 7- and 4'-positions:



**Recommendations:**

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

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