1- Introduction

1-1-Natural product

Natural products are biochemical compounds that are isolated from living organisms. Oil, carbohydrates, alkaloids, terpenoids, saponins and flavonoids are examples of natural products. They play important roles in both drug discovery and chemical biology because many approved therapeutics and drugs are derived from natural sources.

a_Tannins

Tannins are widely distributed in plants. The name tannins are derived from their ability to tan leather and are not based on a class of compounds with a common basic structure. There are two groups of tannins:

- The hydrolysable tannins, which are esters of Gallic acid and also glycosides of these esters.
- The condensed tannins, which are polymers derived from various flavonoids.

Tannins are colorless non-crystalline substances which form colloidal solutions in water; these solutions have an astringent taste. It is found in leaf tissues, bud tissues, seed tissues, root tissues and stem tissues. It does not only heal burns and stop bleeding, but they also stop infection while they continue to heat the wound internally.

Tannins can also be effective in protecting the kidneys. They are also used for immediate relief of sore throats, diarrhea, dysentery, hemorrhage, fatique, skin ulcers. It has antiviral effects and is used to pull out poisons from poison oak or from bee stings, causing instant relief. It is

also helps to draw out all irritants from the skin because it is an astringent that tightens pores and pull out liquids.

b – Saponins

Saponins are glycosides with a distinctive foaming characteristic. They are known as soap plants and are natural surfactants. They are found in many plants and act as active immune system. Saponins are used as cough remedy and for diuretics. They act as a natural antibiotic and also bind cholesterol and thus interfere with cell growth and division. They are highly toxic to some creatures such as fish.

c – Steroids

Steroids are lipids that are insoluble in water and can be extracted from cells by organic solvents of low polarity like ether or chloroform. Many plant steroids occur as glycosides and have the property of stimulating heart muscles. Steroidal glycosides have the property of forming foams in water.

d – Glycosides

Glycosides contain a phenolic group and they occur in most parts of the plants. The simple glycosides are colorless, soluble in water and are optically active. They do not reduce Fehling's solution.

e – Alkaloids

Originally, the name alkaloid (which means alkali-like) was given to all organic bases isolated from plants. Alkaloids can be defined as naturally occurring organic bases which contain a pyridine ring .It can also be defined as natural plant compounds having a basic character and containing at least one nitrogen atom in a heterocyclic ring.Alkaloids are very poisonous but are used medicinally in a very small quantity.

f – Flavonoids

Flavonoids are used to define all compounds whose structure is based on flavones. Thus the anthocyanins are one group of flavonoid compounds. They are natural plant pigments and are water-soluble, generally occur in the aqueous cell-sap and are responsible for the large variety of colours in flowers. These pigments are amphoteric; their acid salts are usually red, their metallic salts usually blue and in neutral solution, they are violet. Flavonoids show anti-allergic, anti-flammatory, anti-microbial and anti-cancer activity it also acts as powerful antioxidants.

The isolation of these compounds depends on the plant source. The earlier methods used solvent extraction, but nowadays, chromatography is the main method [(1)].

1-2 The flavonoids

Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics, found ubiquitously in plants. Therefore, flavonoids are part of the human diet. Over 4,000 different flavonoids have been identified within the major flavonoid classes which include flavonols, flavones, flavanones, catechins, anthocyanidins, isoflavones, dihydroflavonols, and chalcones. Flavonoids are absorbed from the gastrointestinal tracts of humans and animals and are excreted either unchanged or as flavonoid metabolites in the urine and feces. Flavonoids are potent antioxidants, free radical scavengers [(2)].

Flavonoids are low molecular weight polyphenolic substances based on the flavan [(3)].

The study of flavonoid chemistry has emerged, like that of most natural products, from the search for new compounds with useful physiological properties. Semisynthetic endeavors of oligoflavonoids are in

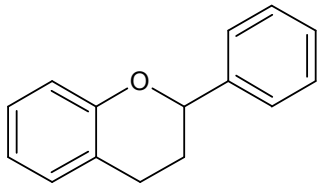
most instances confined to those substitution patterns exhibited by monomeric natural products that are available in quantities sufficient for preparative purposes. Synthesis of the desired enantiomer in optically pure forms remains a daunting objective and is limited to only a few types of compounds. Chalcone epoxides, α- and β-hydroxydihydrochalcones, dihydroflavonols, flavan-3-ols, flavan-3, 4-diols, isoflavans, isoflavanones, and pterocarpans thus far have been synthesized in reasonable yields and purity.

1-2-1 Nomenclature of flavonoids

The term "flavonoid" is generally used to describe a broad collection of natural products that include a C6-C3-C6 carbon framework, or more specifically phenylbenzopyran functionality. Depending on the position of the linkage of the aromatic ring to the benzopyrano (chromano) moiety, this group of natural products may be divided into three classes:

- Flavonoids (2- phenyl benzopyrans) **1**
- Isoflavonoids (3- benzopyrans) **2**
- Neoflavonoids (4- benzpyrans) **3**

These groups usually share a common chalcone precursor, and therefore are biogenetically and structurally related.



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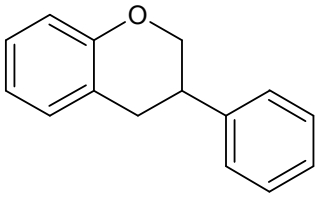

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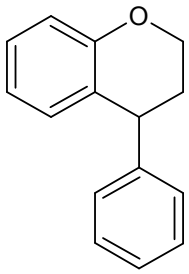

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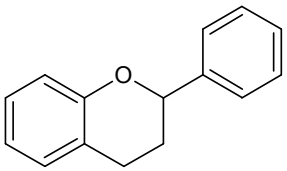
1-2-2 Classification of flavonoids

2-Phenylbenzopyrans (C6-C3-C6 Backbone)

Based on the degree of oxidation and saturation present in the heterocyclic C-ring, the flavonoids may be divided into the following groups:

- Flavan **4**
- Flavanone **5**
- Flavone **6**
- Flavonol **7**
- Dihydroflavol **8**
- Flavan-3-ol **9**
- Flavan-4-ol **10**
- Flavan-3,4-diol **11**



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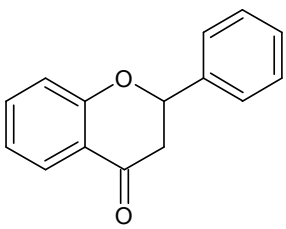

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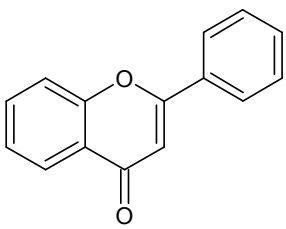

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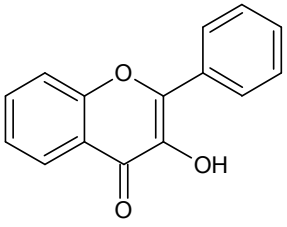

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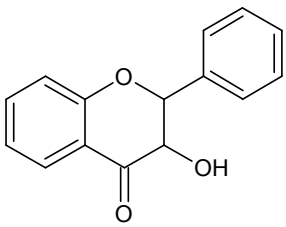

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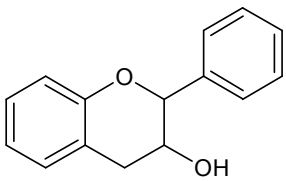

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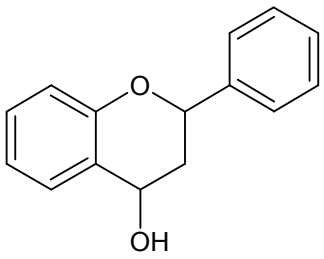

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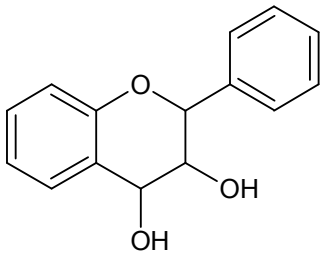

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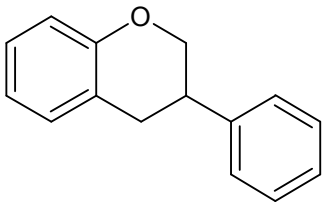
Isoflavonoids

The isoflavonoids are a distinctive subclass of the flavonoids. These compounds possess a 3-phenylchroman skeleton that is biogenetically derived by 1, 2-aryl migration in a 2-phenylchroman precursor. Despite their limited distribution in the plant kingdom, isoflavonoids are remarkably diverse as far as structural variations are concerned. This arises not only from the number and complexity of substituents on the basic 3-phenylchroman system, but also from the different oxidation levels and presence of additional heterocyclic rings. Isoflavonoids are subdivided into the following groups:

- Isoflavan **12**
- Isoflavone **13**
- Isoflavanone **14**
- Isoflav-3-ene **15**
- Isoflavanol **16**
- Rotenoid **17**
- Coumestane **18**
- 3- arylcoumarin **19**

- Coumaronochromene **20**
- Coumaronochromone **21**
- Pterocarpan **22**



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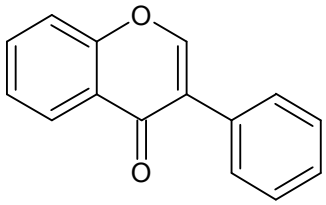

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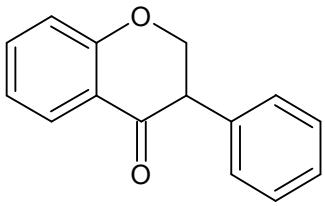

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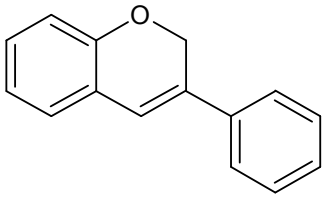

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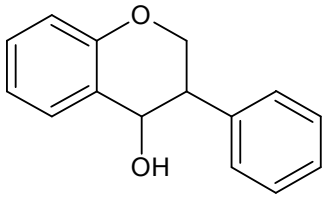

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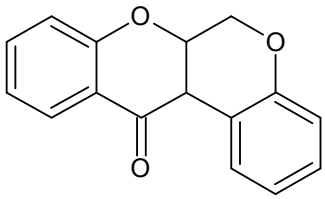

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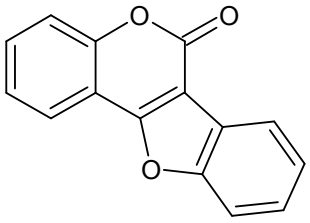

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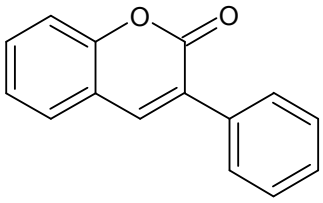

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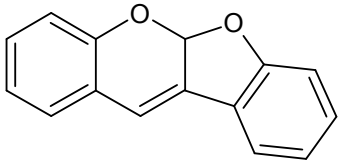

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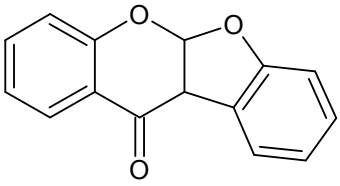

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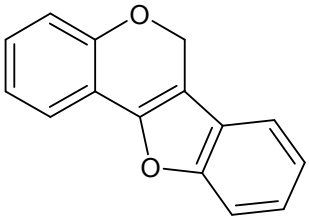

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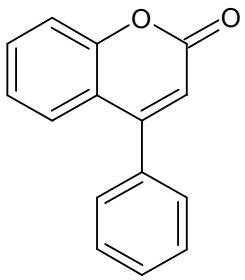
Neoflavonoids

The neoflavonoids are structurally and biogenetically closely related to the flavonoids and the isoflavonoids and comprise the:

- 4- arylcoumarin **23**
- 3,4- dihydro-4-arylcoumarin **24**
- Neoflavene **25**



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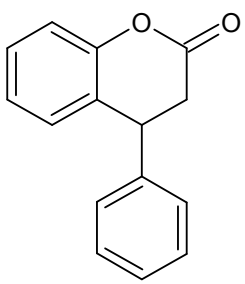

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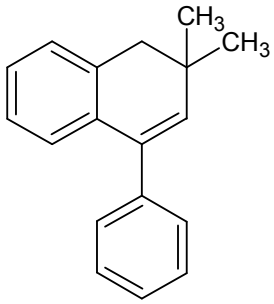

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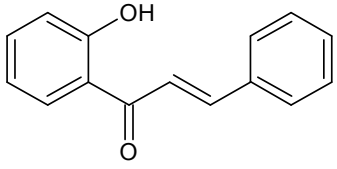
MinorFlavonoids

Natural products such as chalcones and aurones also contain a C6-C3-C6 backbone and are considered to be minor flavonoids. These groups of compounds include the

- 2- OH –chalcone **26**
- 2-OH- dihydrochalcone **27**
- 2-OH- retro-chalcone **28**
- Aurone **29**
- Auronols **30**



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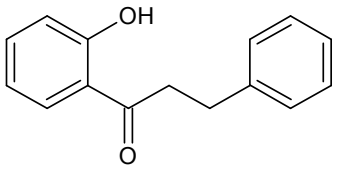

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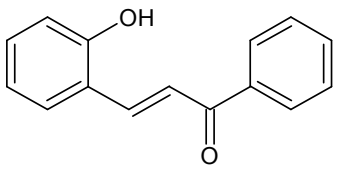

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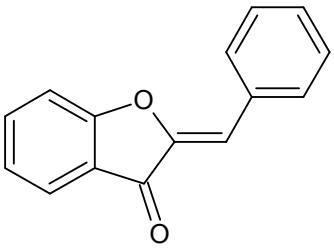

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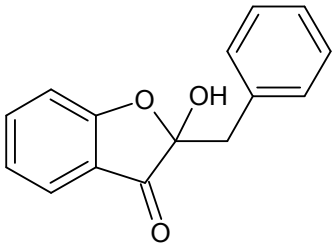

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1-2-3 Extraction of flavonoids

Flavonoids 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 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 procyanidins[4].

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.

Extraction is typically performed with magnetic stirring or shaking but other methods have recently been introduced to increase the efficiency and speed of the extraction procedure.

The first of these is called pressurized liquid extraction (PLE). By this method, extraction is accelerated by using high temperature and high pressure. There is enhanced diffusivity of the solvent and, at the same time, there is the possibility of working under an inert atmosphere and with protection from light. Commercially available instruments have extraction vessels with volumes up to about 100 ml. In a study involving medicinal plants, solvent use was reduced by a factor of two [5].

The optimization of rutin and isoquercitrin recovery from older flowers has been described. Application of PLE gave better results than maceration — and shorter extraction times and smaller amounts of solvent were required [6]. PLE of grape seeds and skins from winemaking wastes proved to be an efficient procedure for obtaining catechin and epicatechin with little decomposition, provided the temperature was kept below 130C[7] .

Supercritical fluid extraction (SFE) relies on the solubilizing properties of supercritical fluids. The lower viscosities and higher diffusion rates of supercritical fluids, when compared with those of liquids, make them ideal for the extraction of diffusion controlled matrices, such as plant tissues. Advantages of the method are lower solvent consumption, controllable selectivity, and less thermal or chemical degradation than methods such as Soxhlet extraction. Numerous applications in the extraction of natural products have been reported, with supercritical carbon dioxide being the most widely used solvent [8, 9].

Microwave-assisted extraction (MAE) has been described for the extraction of various compounds from different matrices [10]. It is a simple technique that can be completed in a few minutes. Microwave energy is applied to the sample suspended in solvent, either in a closed vessel or in an open cell. The latter allows larger amounts of sample to be extracted. A certain degree of heating is involved [11].

1-2-4 Separation and quantification of flavonoid

Essential to the study of flavonoids is having the means available for their separation and isolation.

Natural products are isolated in a pure form by techniques such as simple distillation, extraction with polar and non-polar solvent. The structure can be determined by spectroscopic techniques. Moon et al (2005) also reported that natural products can be identified by the simple comparison of the nuclear magnetic resonance (NMR) data. The identification of natural products usually involve the separation of each compound and subsequent analysis of mass (MS) and NMR spectroscopy but for separation analysis, thin layer chromatography (TLC) and column chromatography can be used.

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.

One feature that is of immense benefit for flavonoid analysis is the presence of the phenyl ring. This excellent chromophore is, of course, UV active and provides the reason why flavonoids are so easy to detect. Their UV spectra are particularly informative, providing considerable structural information that can distinguish the type of phenol and the oxidation pattern.

A number of techniques have been used for the preparative separation of flavonoids. These include HPLC, Diaion, Amberlite XAD-2 and XAD-7, and Fractogel TSK/Toyopearl HW-40 resins, gel filtration on Sephadex, and centrifugal partition chromatography (CPC) [12] .The choice of methods and strategies varies from research group to research group and depends often on the class of flavonoid studied.

- Preparative separation:
- Preliminary Purification

Once a suitably polar plant extract is obtained , a preliminary clean up is advantageous. The classical method of separating phenolics from plant

extracts is precipitate with lead acetate or extract into alkali or carbonate , followed by acidification. The lead acetate procedure is often unsatisfactory since some phenolics precipitate; other compound may co-precipitate and it is always easy to remove the lead salts.

Alternatively, solvent partition or counter current techniques may be isoflavonoid rich fraction from erythrina species for further purification work, an organic solvent extract was dissolved in 90% methanol and 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 mixtures was then chromatography to obtain pure compouns[13].

A short polyamide column, a sephadex LH-20 column, or an ion exchange can be used. Absorption of crude extracts on to Diaion HP-20 or Amberlite XAD-2(XAD-7)column, followed by elution with a methanol- water gradient, is an excellent way of preparing flavonoid-rich fractions.

- Preparative Methods:

One of the major problems with the preparative separation of flavonoids is sparing solubility in solvents improved 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 complication can also arise. For example, in the separation of anthocyanins and anthocyanin- rich fraction, it is advisable to avoid acetonitrile and formic acid-acetonitrile is difficult to evaporate and there is a risk of ester formation with formic acid.

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 [14].

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

Support materials include polyamide , cellulose ,silica gel, sephadex LH-20 and sephadex G-10,G-50. Sephadex LH-20 is recommended for exclusion, adsorption and partition mechanism operate in the presence eluents for proanthocyanidins ,acetone is better for displacing the high molecular weight polyphenols. Slow flow rates also recommended. Open – column chromatography with certain support suffers from a certain degree of irreversible adsorption of the solute on the column.

Preparative TLC is a separation method that required 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. Preparative TLC in conjunction with open-column chromatography remains a straight forward means of purifying natural products, although variants of planar chromatography, such as centrifugal TLC[15] have found application in the separation of flavonoids.

Other combination are, of course, possible , depending on the particular separation problem. Combining gel filtration or liquid –liquid

partition with liquid chromatography (LC) it is one solution. Inclusion of chromatography on polymeric support can also provide additional means of solving a difficult separation.

Several preparative pressure liquid chromatography 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/30psi)

g- High – performance liquid chromatography

HPLC is becoming by far the most popular technique for the separation of flavonoids, both on preparative and analytical scales. Improvement in instrumentation , making materials, and column technology are being introduced all the time, making the technique more and more attractive. The difference between the analytical and preparative methodologies is that analytical HPLC does not rely on the recovery of sample, while preparative HPLC is purification process and aims at the isolation of a pure substance from a mixture.

Semi-preparative HPLC separation(for 1 to 100 mg sample size) uses columns of internal diameter 8 to 20 mm, often packed with 20μm (or smaller) particles. Large samples can be separated by preparative (or even process- scale) installation but costs become correspondingly higher.

Optimization can be performed on analytical HPLC columns before trans-position to a semi preparative scale.

h- Medium – pressure liquid chromatography

The term "medium –pressure liquid chromatography" (MPLC) covers a wide range of column diameters, different granulometry packing materials, different pressure, and a number of commercial available systems. In its simplest form, MPLC is a closed column(generally glasses) connected to a compressed or reciprocating pump. It fulfils the requirement for a simple alternative method to open –column chromatography of flash chromatography , with both higher resolution an shorter separation times. MPLC column have a high loading capacity – up to 1:25 sample – to – packing –material ratio[16] – and are ideal for the separation of flavonoids.

In MPLC the columns are generally filled by the user. Particle size of 25 to 200 μm are usually advocated (15 to 25 ,25 to 40, or 43 to 60 μm are the most common ranges) and either slurry packing to dry packing is possible. Resolution is increased for a long column of small internal diameter when compared with a shorter column of larger internal diameter(with the same amount of stationary phase) [17]. Choice of solvent system can be efficiently performed by TLC [18] or by analytical HPLC .

Transposition to MPLC is straight forward and direct [19].

i- Centrifugal partition chromatography

Various counter current chromatographic techniques have been successfully employed for the separation of flavonoids . counter current chromatography is a separation technique that relies on the partition of a

sample between two immiscible solvents, the related proportion of solute passing into each of the phases determined by the partition coefficients of the components of the solute. It is an all –liquid method that is characterized by the absence of a solid support, and thus has the following advantages over other chromatographic:

- No irreversible adsorption of the sample.
- Quantative recovery to the introduced sample.
- Greatly reduced risk of sample denaturation.
- Low solvent consumption.
- Favourable economics .

It is obvious , therefore , that such a technique if ideal for flavonoids. Which often suffer from problems of retention on solid supports such as silica gel and polyamide .

1-2-5 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. 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, [20] . Have reported the separation of flavones, flavonols, flavanones, and chalcones by GC.

- Sample preparation :

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 [21] :-

- 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 .
- Provide robust and reproducible methods that are independent of variation 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.

As well as typical sample preparation methods such as filtration and liquid–liquid extraction [22], 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.

- Thin-Layer-Chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids [23], 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 [24].

Many different solvent systems have been employed for the separation of flavonoids using TLC. Highly methylated or acetylated flavones and flavonols require nonpolar 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 . With regard to detection, brief exposure of the TLC Plate to iodine vapour produces yellow –brown spots against a white background. And , as stated by [25] flavonoids appear as dark spots against afluores sent

green background when observed in UV light (254nn) on plates containing amv-fluorescent indicator. In 365 nm UV light depending on structural type, flavonoids show dark yellow, green or blue fluorescence, which is intensified and change by the use of spray reagent. One of the most important of these is "natural products reagent" which produces an intense fluorescence under 365 nm UV light after spraying with 1% solution of diphenyl boric acid-B-ethyl amino ester in methanol. Subsequent spraying with a 5% solution of polyethylene glycol- 4000(PEG) in ethanol lowers the detection limit from 10 µg to about 2.5 µg intensifying the fluorescence behaviour .

The colors observed in 365 nm UV light are as follows:

Quercetin , myricetin ,and their 3 and 7 O-glycosides :orange-yellow.

Kampferol , ISO yahamnetin, and their 3 –and-7 –O –glycosides: Yellow – green .

Luteolin and its 7 – O-glycoside: orange .

Apigenin and its 7-O-glycoside:yellow-green.

Aqueous or methanolic ferric chloride is general spray reagent for phenolic compounds and gives a blue – black coloration with flavonoids, similarly, fast blue salt B forms blue or blue –violet azodyes. For quantitative analysis, scanning of the TLC late with a densitometer provides good results. The flavonoids , both aglycoside in a vaccinium myrtillus and V. Vitis idea were determined after TLC and densitometry at 254 nm[26] with suitable spray reagent , detection limits 20ng can be achieved by densitometry [27].

- High-Performance Liquid Chromatography:

Analytical HPLC finds use in the quantitative determination of plant constituents, in the purity control of natural products, and in chemotaxonomic investigations. For the analytical HPLC of a given subclass of flavonoids, the stationary phase, solvent, and gradient have to be optimized.

Applications of HPLC to the analysis of flavonoids in medicinal and other plants are summarized by Cimpan and Gocan [28] . From the methods listed, it is noteworthy that 90% of the separations use C18 columns. The importance of flavonoids in foods means that it is indispensable to have suitable means of determining their content.
The big drawback is that solvent gradients cannot normally be run with normal phases.

Isoflavones chiefly found in the leguminosae in the plant kingdom are also successfully analyzed by HPLC on C_{18}columns[29].

The anthocyanins exist in solution as various structural forms in a equilibrium , depending on the PH and temperature. In order to obtain reproducible results in HPLC , it is essential to control the PH of the mobile phase and to work with thermostatically controlled columns. And anthocyanin equilbria have to be displaced toward their flavylium forms – peak tailing is thus minimize and peak sharpness improved flaylium are colored and can be selectively detected in the visible region at about 520 nm , avoiding the interference of the other phenolics and flavonoids .

All separation are performed on RP -18 columns with column length between 100 and 300 mm and with diameters between 2 and 5 mm.

Granulometries vary from 3 to 10 µm with most being 5 µm separation runs are generally up to 1 h in duration.

For a glycones and glycosides of Isoflavones , certain reported separation of soybean products have employed C_8 packings.

Some applications are given in which two or more subclasses are analyzed simultaneously, such as flavonones , flavones and flavonols in honey and anthocyanins, catechins and flavonols in fruits and wines.

The method allowed the determination of aglycones separately from glycosides .

Information could also be obtained about simple polyphenols in the presents of more complex polycyclic polyphenols.

Quantitive determination was achieved for a total of 63 different food samples.

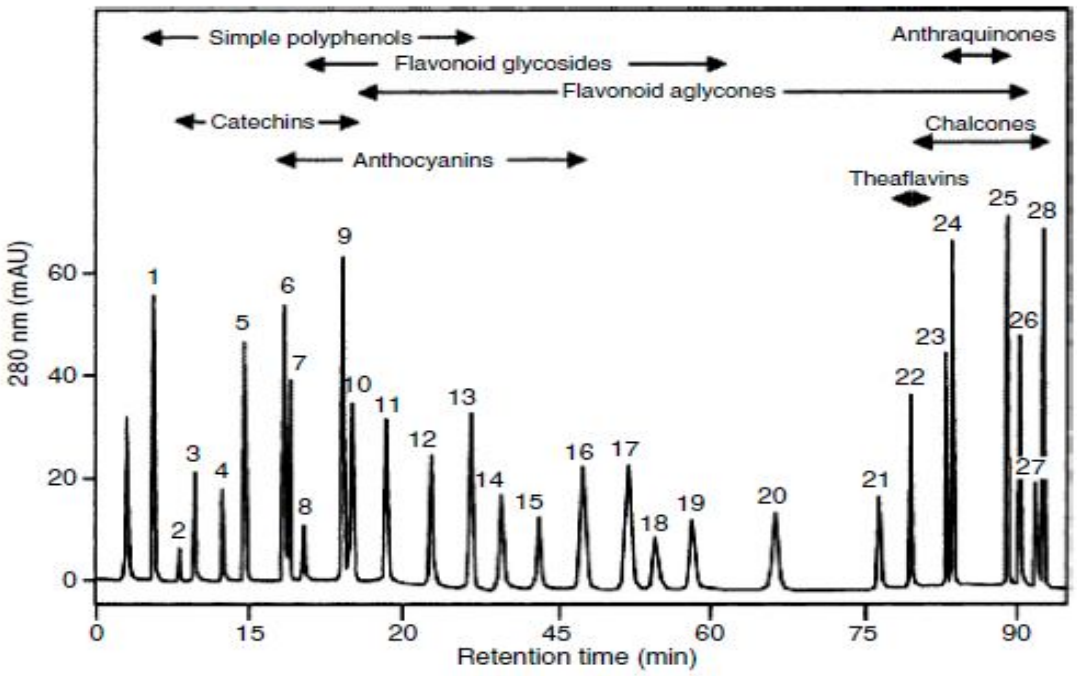


Figure : 1 HPLC profile for 28 different polyphenols on a C_{18} column classes of compound are shown in the upper part of the chromatogram[30].

Lactones and therefore the content of flavonoids terpene lactones is one of the important parameters to assess the quality of ginkgo products.

-High-PerformanceLiquidChromatography-Ultraviolet Spectrophotometry :

The most frequently used detection method for HPLC is UV Spectrophotometry. Routine detection is HPLC is typically based on measurement of UV absorption, or visible absorption in the case of anthocyanins. No single wave length is ideal for all classes of flavonoids since they display absorbance maxima at distinctly different wave length. The most commonly used wave length for routine detection has been 280 nm which represents a suitable compromise.

A further dimension is now possible because coupled LC –UV with diode array detection (DAD) allow the chromatographic eluent to be scanned or UV –visible spectral date which are stored and can later be compared with library for peak identification[31]. This increases the power of HPLC analysis because with the information from the UV spectrum , it may be possible to identify the compound subclass or perhaps even the compound itself.

UV spectral data of 175 flavonoids in several solvents can be found [32] LC–UV with DAD enables simultaneous recording of chromatograms at different wavelengths. . This improves the possibilities of quantification because detection can be performed at the wave length 365 nm for flavones and flavonols , at 290 nm for flavononeS, at 236 or 260 nm for isoflavones ,

at 340 to 360 nm for chalcones , at 280 for dihydrochalcones , at 502 or 520 nm for anthocyanins, and at 210 or 280 nm for catecnins.
Spectra of eluting peaks obtained at the a pex and both inflexion points of the peak can be compared in order to obtain a measure at the purity the particular component of the sample.

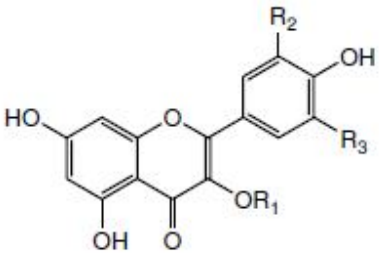
LC –UV is a valuable for the identification of isoflavones since their spectra differ in absorption properties from most of the other flavonoids . They have a C_2-C_3 double bond with B-ring at C_3, which prevents conjugation of the phenyl group with the pyrone carbonyl group. This reduces the contribution of the B-ring to UV spectrum an results in a peak of very low intensity in the 300 to 330 nm range.

The analysis of catechins and proanthocyanidins by LC- UV present certain problems. Only monomers and only oligomers up to tetramers can be separated and detected as defined peaks. Polymeric form which may constitute the bulk of proanthocyanidins in many plant materials are not well resolved. They give place to drift in the base line and the formation of characteristic humps in HPLC chromatograms. Furthermore the spectral characteristics of these compound do not allow easy detection and identification. Flavan-3 – ols give absorption maxima at none specific wave lengths (270 to 290 nm) and they have lower extinction coefficients than other a company in phenolics. Their quantification is thus not easy. The lack of reference – proanthocyanidins applies that results have to be expressed with respect to other reference substance. For reverse, phase HPLC of proanthocyanidins only oligomers, the percentage of methanol or acetonitrile does not exceed 20%.

The coupling of HPLC with DAD allows online quantification of flavonoids in samples analyzed [33] . have quantified flavones, flavones , and flavanones in fruits , vegetables and beverages in this fashion. While identification of the peak in a LC-UV chromatogram is possible by comparing retention times and UV spectra with authentic sample or data band, this might not be possible for compounds with closely related structure and wrong conclusions might be drown. It has been established that in order to complete the characterization of phenolic compound, reagents inducing a shift of the UV absorption maxim can be used.

A post column derivatization procedure based this technique is possible by adding suitably modified shift reagents to the elute leaving a HLPC column [34] . Direct information is provided about the flavonoid oxidation pattern and position of free phenolic hydroxyl group.

In the analysis of Gentiana extract , best results were obtained on a reversed- phase , column with a methanol – water eluent at a PH of around 3.5 to avoid peak tailing . classical shift reagents were adapted in order to be compatible with these conditions. Sodium mono hydrogen phosphate and potassium hydroxide were used as weak and strong bases respectively. Instead of sodium acetated and sodium methanolate in order to form a complex with the Keto function. An aqueous solution of aluminium chloride was passed with boric acid – sodium acetate. These shift reagents gave identical results to those obtained with classical shift reagents. The small amount of material required (50 to 100 µg of crude plant extract) in LC/UV post column derivatizaion allows the analysis of very rare and small species , as well as a single plant parts of herbarium samples[34].



	R1	R2	R3
1	Rha	H	H
2	Ara	H	H
3	GicA	H	H
4	Rha	OH	H
5	Ara	OH	H
6	Gal	OH	H
7	GIC	OH	H
8	GICA	OH	H
9	Gal^{6}-galloyl	OH	H
10	Rha	OH	OH
11	GIC	OH	OH
12	Gal^{6}-galloyl	OH	OH

Figure : 2 Flavonoid glycosides isolated from the aerial parts *of Epilobium angustifolium.*

To illustrate this approach , the online identification of flavonol glycosides in Epilobium species will be described. Certain of these willow-herbs have important implications in the treatment of being prostatic

hyperplasia and knowledge of their flavonoid contents is an aid to their identification. The aerial parts of 13 different species were extracted first with dichloromethane and then with methanol. The flavonoid containing methanol extracts were portioned between n- butanol and water : then butanol fractions were then analyzed on a novapak C_{18} column with an acetonitrile – water gradient.

TFA was added to give a PH of 3. Photodiode- array detection allow the online recording of UV spectra (200 to 500 nm) all typical of flavonoids.

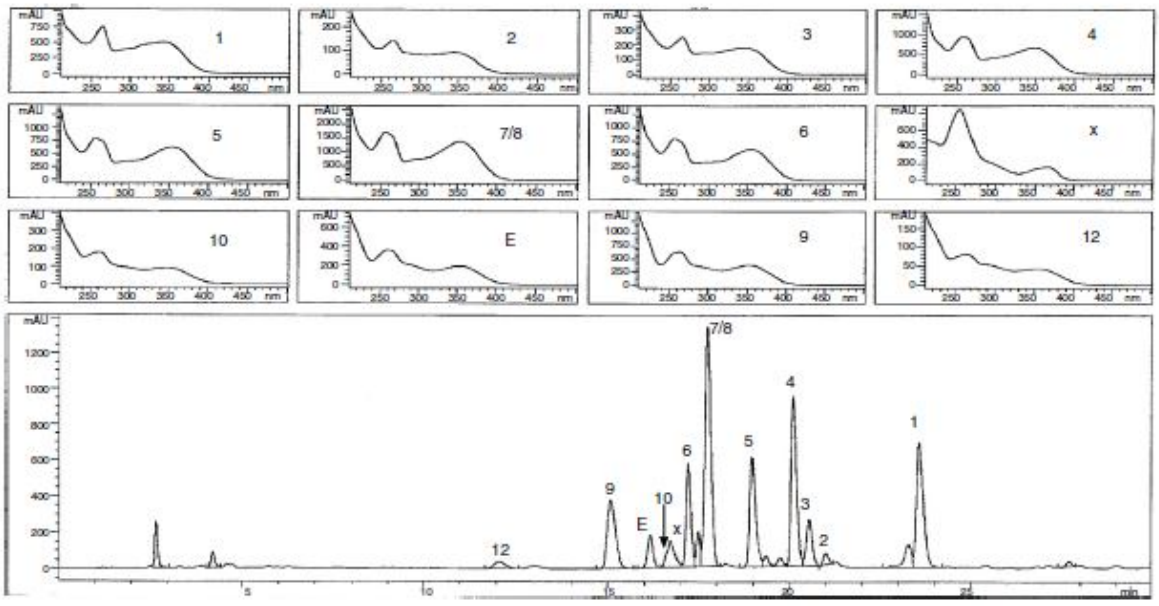


Figure : 3 LC.UV .chromatogram a methanolic extract of the aerial parts of Epilobium engustifolium by n- BuOH- H_2O partition [35].

In this figure structure elucidation of flavonoid E was incomplete. In combination with shift reagents added post column, LC –UV allowed determination of the hydroxylation pattern of flavonols and position of the sugars on the aglycone.

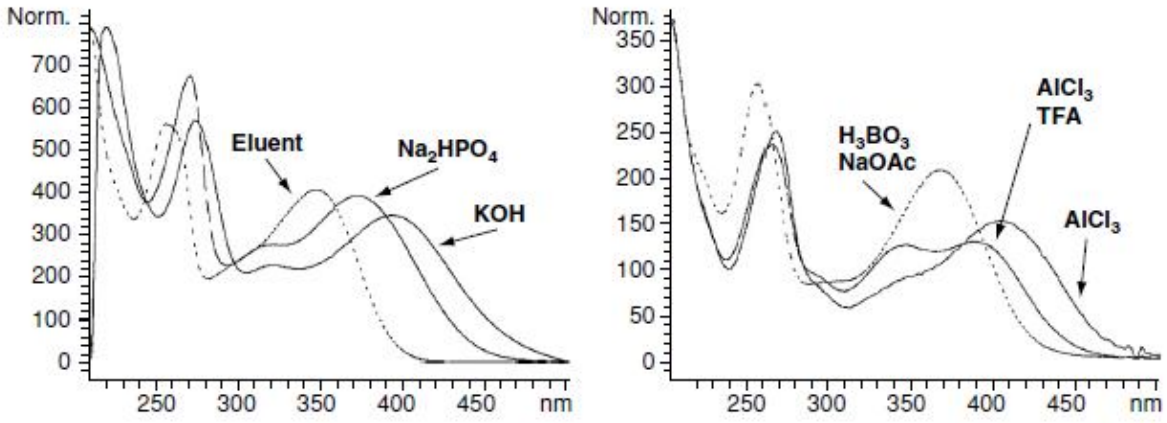


Figure : 4 Online UV spectra of 4 obtained after post column addition of different shift reagents [35] .

This figure shows the UV spectra obtained online for 4 after the addition of five different shift reagents.

The shift of 11 nm of band (II) with weak base, 0.1 M Na_2HPO_4, was characteristic for non substituted 7- hydroxyl group . A 15 nm shift with boric acid reagent was typical for ortho-dihydroxl group on the B- ring .The shift of 42 nm of band I obtained for aluminium chloride without neutralization of the elute was specific for A5 –hydroxyl substituent. Addition of aluminium chloride after neutralization gave a 56 nm shift of band I. This was due to a combination of an ortho-dihydroxyl group (C-3- and C-4-) and complex formation with the C -4 keto function and the 5 – hydroxyl group. These data confirmed the aglycone to be quercetin .A similar procedure was adopted for the identification of the other flavonol glycosides – showing the presence of three different aglycones :Kaempferol , quercetin, and myricetin. There mospray LC-MS provided additional information on the molecular weight of the flavonol aglycones[36] .

1-2-6 Transport of flavonoids

Flavonoid compounds are one of the most analyzed groups of secondary metabolites in higher plants. The main reason for the interest in flavonoids is that they are major constituents of plant pigments. Anthocyanins, a flavonoid subclass, have been of special interest because of their ability to confer red, orange, blue, and purple coloration to leaves, flowers, and fruits [37] . As pigments, flavonoids have facilitated the testing of hypotheses related to Mendel's law and transposable elements. Flavonoids have been the focus of attempts to modify flower color by genetic engineering [38]. There also is interest in using them as drugs or dietary supplements because of their strong antioxidant activities[39,40] in plants, flavonoids have several functions including attracting insects for pollination and dispersal of seeds, acting in defense systems , signaling between plants and microbes, and regulating auxin transport [41]. Many of these functions cannot occur unless flavonoids are properly localized within the cells. In the seed coat, proanthocyanidin precursors accumulate in the vacuole, followed by polymerization and oxidation to proanthocyanidin within this organelle. The oxidation of proanthocyanidins hardens the seed coat, which induces moderate dormancy in the seeds and limits the detrimental effects of physical and biological attacks [42,43]. Thus, the proper subcellular localization of these flavonoids is crucial for fulfilling their functions in plant cells. The subcellular localization of flavonols is still a matter of debate. Some flavonols have a protective role as UV-B filters, and they also could function as copigments for anthocyanins in specific tissues. The results of many experiments indicate that the sites at which flavonoids

accumulate are different from the sites at which they are synthesized. It thus is believed that plant cells have flavonoid transport mechanisms.

1-2-7 Flavonoid pigments as tools in molecular genetics

In the plant kingdom, the flavonoid biosynthetic pathway is ubiquitous and produces a variety of pigmented as well as nonpigmented compounds. Flavonoid compounds have been implicated in several biological processes and some of their functions include the attraction of pollinating agents via pigmentation of floral organs [44] , pollen tube germination [45], protection from UV exposure [46] , and defense against insects by acting as insecticides [47] and fungal pathogens by acting as phytoalexins [48,49] . Flavonoid pigments have been used as a convenient visible marker in molecular genetic experiments and to study regulation of gene expression [50].

Flavonoid biosynthesis takes place through the phenylpropanoid pathway, and depending on the genetic constitution of the plant naringenin can have several different fates leading to the formation of flavonoid metabolites that include anthocyanins, flavones, and anthocyanidins [51] .

It is clear that flower pigmentation attracts not only pollinators but also many plant biologists including geneticists. Because mutations in flavonoid pigmentation genes are nonlethal and confer easily scorable color phenotypes in stems, flowers, and seeds, various flavonoid pigments, including the anthocyanins, proanthocyanidins, and phlobaphenes described here, have served as important traits in elucidating genetic and epigenetic phenomena. Undoubtedly, these secondary metabolites will keep on making important and significant contributions as useful tools for molecular genetic studies in the future.

1-2-8 Flavonoids as nutraceuticals

The major active nutraceutical ingredients in plants are flavonoids. The flavonoids are a group of organic molecules ubiquitously distributed in vascular plants. Approximately 2000 individual members of the flavonoids group of compounds have been described. As is typical for phenolic compounds, they can act as potent antioxidants and metal chelators. They also appear to be effective at influencing the risk of cancer. Overall, several of these flavonoids appear to be effective anticancer promoters and cancer chemopreventive agents .

Within the subgroups of the flavonols and the flavones, the flavonol quercetin is the most frequently occurring compound in foods. Also common are kaempferol, myricetin, and the flavones apigenin and luteolin. Tea and onions are the main dietary sources of flavonols and flavones. In this review, we describe the recent developments on the biological activities of flavonoids that have provided the important basis for their nutraceutical functions.

- Antioxidant activity :

-Reactive oxygen species and antioxidant systems

Diets high in flavonoids, fruits, and vegetables are protective against a variety of diseases, particularly cardiovascular disease and some types of cancer[52]. Antioxidants and dietary fiber are believed to be the principal nutrients responsible for these protective effects. Reactive oxygen species (ROS) are formed *in vivo* during normal aerobic metabolism and can cause damage to DNA, proteins, and lipids, despite the natural antioxidant defense system of all organisms[53]. ROS contribute to cellular aging [54], mutagenesis [55], carcinogenesis [56], and coronary heart disease [57], possibly through the

destabilization of membranes [55] , DNA damage, and oxidation of low-density lipoprotein (LDL). Many *in vitro* studies have demonstrated the potent peroxyl radical scavenging abilities of flavonoids, which contribute to inhibiting lipid peroxidation and oxidation of LDL [58,59] . Since oxidation of LDL is implicated in the pathogenesis of coronary heart diseases [60] through its ability to decrease the susceptibility of LDL to oxidation .

-Reactive nitrogen species and inducible nitric oxide synthase

Reactive nitrogen species (RNS) also appear to contribute to the pathology of cardiovascular diseases. NO is one RNS produced by the action of nitric oxide synthase in endothelial cells, neurons, and other cell types. At the sites of inflammation, inducible nitric oxide synthase (iNOS) is also augmented, and NO synthesis is further activated. Peroxynitrite, a potent oxidant generated by the reaction of nitric oxide (NO) with superoxide in the vascular endothelium, induces LDL oxidation[61,62] and proinflammatory cytokine-mediated myocardial dysfunction [63,64] . Another potential source of RNS derives from dietary nitrite, which reacts with the acidic gastric juice to produce nitrous acid, which decomposes to oxides of nitrogen. Nitrous acid and its products are able to nitrosate amines, deaminate DNA bases, and nitrate aromatic compounds including tyrosine. several flavonoids and phenolic compounds including the epicatechin/gallate family of flavanols, are powerful inhibitors of nitrous acid-dependent nitration and DNA deamination *in vitro* [65] .

-Protective effects of flavonoids

The protective effects of flavonoids in biological systems are ascribed to their capacity to transfer free radical electrons, chelate metal catalysts [66],

activate antioxidant enzymes [67] , reduce alpha-tocopherol radicals [68] , and inhibit oxidases [69] .

Green tea is a rich source of flavonoids, primarily catechins and flavonols. In black tea, as a consequence of the fermentation process, catechins are converted to complex condensation products, the theaflavins Tea polyphenols show strong antioxidative effects and provide powerful scavengers against superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, and peroxynitrite produced by various chemicals and biological systems. With regard to *in vitro* LDL oxidation, gallate esters were found to be less efficient than the respective free forms in inhibiting the oxidation catalyzed by Cu(II). Their activity follows the order epigallocatechin gallate (EGCG) > epicatechin gallate (ECG) > catechin (C) > epicatechin (C) [70] . Anderson and collaborators reported that green tea polyphenols partially protect DNA from •OH radical-induced strand breaks and base damage [71] . Pulse radiolysis results support the mechanism of electron transfer (or H-transfer) from catechins to radical sites on DNA [71] . In black tea, all the theaflavins showed the same capacity to inhibit the production of superoxide. Green tea, black tea, and EGCG were shown to block the production of oxygen free radicals derived from the cooked meat mutagen 2-amino-3-methylimidazo[4,5 f]quinoline (IQ) in the presence of a NADPH-cytochrome P450 reductase [72] . These results support an antioxidant role of catechins in their direct interaction with DNA radicals. catechin polyphenols could also decrease the peroxynitrite-induced nitration of tyrosine and protect the apolipoprotein B-100 of LDL from peroxynitriteinduced modification of critical amino acids, which contribute to its surface charge [73] . Recently, our laboratory found that oral feeding of green tea leaves to rats resulted in enhanced SOD activity in serum and catalase activity in liver

and an increased concentration of glutathione in the liver [74] . We also established that theaflavins and EGCG inhibit xanthine oxidase (XO). They inhibit XO to produce uric acid and also act as scavengers of superoxides. Theaflavin 3,3′- digallate (TF-3) inhibited the superoxide production in HL-60 cells. Therefore, the antioxidative activity of tea polyphenols may be due not only to their ability to scavenge superoxides, but also because of their ability to block XO and relative oxidative signal transducers [75] . Other flavonoids such as quercetin, kaempferol, myristin, apigenin, and leuteolin also have antioxidative activity in many *in vitro* studies [76] .

1-2-9 Flavonoids as signal molecules

Plant-derived foods that are rich in flavonoids are regularly touted in the popular press for their benefits in ameliorating age-related diseases. A majority of these reports focus on the antioxidant characteristics of flavonoid-rich diets and their enhancement of cardiovascular health. However, a growing number of reports in the pharmacology literature characterize flavonoid interactions with cellular components implicated in neurological pathologies and cancer. As the effective flavonoid concentrations employed in pharmacological studies utilizing cell cultures are often orders of magnitude higher than the serum concentrations seen in humans, some discrimination is required when interpreting these reports.

Nearly every class of flavonoid has been shown to have biological activity, with a majority related to antioxidant properties. In plants, flavonoids appear to contribute to a general reduction of reactive oxygen species and therefore impact cellular processes sensitive to REDOX effects. However, flavonoids also have been implicated in more direct interactions with transport and signal transduction pathways. One well-documented

example is the role of flavonoids in fertility: while a few flavonoid-deficient plants are able to germinate, grow, and set fertile seed, most plants require flavonoids for fertility and normal pollen development. Another is flavonoid modulation of auxin transport as well as localized auxin accumulations observed during nodulation.

1-2-10 Flavonoids function in plants

The flavonoids are a remarkable group of plant metabolites. No other class of secondary product has been credited with so many—or such diverse—key functions in plant growth and development. Many of these tasks are critical for survival,flavonoids are known to enhance tolerance to a variety of abiotic stressors, they are employed as agents of defense against herbivores and pathogens, and they form the basis for allelopathic interactions with other plant species. The flavonoids are evidently extremely useful to plants, and it is not surprising, therefore, that species from all orders of the plant kingdom, from the basal liverworts to the most advanced angiosperms, invest significant amounts of metabolic energy into the production of these compounds. The past decade has witnessed resurgence in research activity on the functions of flavonoids in plants. There are several reasons for this. First, advances in molecular biology, coupled with an improved knowledge of the pathway for flavonoid biosynthesis, have led to the production of plant mutants that are deficient or superabundant in one or more flavonoid pigments. Comparisons of mutant and wild-type phenotypes have permitted hypotheses for flavonoid function to be tested directly. Second, improvements in analytical techniques (e.g., HPLC, LC, LC-MS , and NMR) for flavonoid compounds have stimulated the search

for novel compounds useful for the manipulation of flower color. These, in turn, prompted the discovery of hitherto unknown functions of flavonoids in plant reproduction. Third, concerns about the enlarging ozone hole and the increased exposure of biota to ultraviolet (UV) radiation led to the quest for sunscreens — and to the knowledge that some flavonoids play an important role in protecting plants from harmful UV-B levels. Fourth, there has been an explosive interest in flavonoids, particularly the anthocyanins, as potential nutritional supplements for humans. This contributed to the discovery of their antioxidant roles in planta. Finally, advances in field-portable instrumentation have enabled hypotheses for flavonoid function to be tested directly in the field. Despite the resurgence in research activity on flavonoid function, many questions remain unanswered. Some functions are only partially understood, and there are probably many others not yet uncovered. For example, there have been several intriguing reports that describe correlations between flavonoid content and morphology. In Antirrhinum, the intensity of anthocyanin pigmentation in the flowers depends upon the shape of cells in the petals [77] .In maize, mutant endosperm cells show both an abnormality in shape and a blockage in anthocyanin biosynthesis, indicating a possible connection between flavonoid precursors and cell morphology [78] . Although these may perhaps be explained by some of the activities described above (e.g., regulation of auxin), [79] . have postulated a more direct function for flavonoid intermediates in tissue development. Investigations into the medicinal properties of flavonoids have also revealed novel mechanisms of action ; for example, in mediating nucleic acid strand scission, and the inhibition or induction of certain enzymes. It is unclear, however, if these functions have any physiological significance in the plant [80] , have

suggested that flavonoids in plants may affect gene expression by acting on a putative hormone receptor in the nuclear membrane, or else they could change the activity of regulatory proteins, such as tyrosine kinase, that are involved in cell division. This is being investigated further. It is fascinating that this one class of secondary compounds has such a diversity of functions. Multigene families in the flavonoid pathway have presumably lead to specialization of flavonoid gene members in processes as disparate as signaling, defense, development, flower pigmentation, and cell wall modification [81,82].Different plant species may use different mechanisms to distribute flavonoids among subcellular compartments, and multiple mechanisms are used in individual species [83] .In addition to the flavonoids as a group displaying a diversity of functions, individual compounds also show multifarious functions. The different functions often share common mechanisms. For example, the ability of flavonoids to act as antioxidants is behind their role in combating many different types of stresses. Similarly, the role of flavonoids in regulation of auxin distribution influences plant responses to nodulating bacteria, mycorrhizal fungi, and Agrobacterium. Future applications of analytical methodology and molecular biology techniques are likely to reveal much more about flavonoid function in plants in the coming decades. A more complete understanding of flavonoid function would provide the foundation for further manipulating plants to cope with environmental stress.

2- Experimental procedure :

The plant was identified by my project supervisor (Mrs M-abd alkareem).

2-1 Sample Collection:

The plant Aprus precatorius was collected from Khordoufan state.

2-2 Preparation of the Sample:

The roots of Aprus precatorius were air-dried after collection . The dried roots were ground into fine powder and then weighed.

2-3 Extraction of the Sample:

Solvent-solid extraction was carried out on the weighed, air-dried and roots of Aprus precatorius the weighed sample was soaked with methanol (95%) for two days , with continuose shaking . The separation of the residue from filtrate was done by using filter paper. It was followed by the concentration of the filtrate by using distillation method.

2-4 Chromatography:

The type of chromatographic method was used to separate the constituents that were present in the extract is thin-layer chromatography.

2-4-1 Thin-Layer Chromatography (TLC):

TLC was used to ascertain the number of constituents present in the extract and to determine their purity. TLC was also used to determine the solvent mixture that will affect the separation of the components. The TLC were eluted with butanol :acetic acid : water (4 : 1.5 : 6) .

-Preparation of Silica Gel Plates:

50g of silica gel powder was weighed into a conical flask, 100ml of distilled water was added and the resulting solution was shaken vigorously in order to avoid lumps. The white smooth paste mixture was spread over the glass plate and was allowed to solidify. The coated glass plates were put inside oven for 1-2 hours at 1100C to ensure further solidification.

-Spotting of the Plates:

This is done with aid of capillary tubes to introduce few drops of the dissolved sample extract unto the coated plate, allowing each drop to dry before adding another drop.

-Developing of the Plates:

After the solvent had travelled some distance across the plate, the plate was removed and allowed to dry and then spray a clouration reagent which is vaanalin solution with concentrated sulfuric acid . The separated components appeared as dark braown-red spots .

3- Results and discutions :

3-1 discution

The flavonoid of Aprus precatorius were extracted by ethanol 95 % after removing the solvent acrude product was obtained which was fractionated by TLC (silica gel ,BAW 4 : 1.5 : 6) after the usual workup apure flavonoid compound (I) was isolated .

In the IR spectrum (**Figure : 5**) it gave ν (KBr) 700 **:**
(3415.70, O-H : H- bonded) (2925.81, sp^3 C-H stretching vibration) (1569.95 , 1506.30 , 1460.01 ,1415.65 , C=C aromatic stretching vibration) (1222.79 , 1107.06 ,C-O stretching vibration) . Since the IR revealed absence of C=O stretching then this flavonoid is either an Anthocyanins or flavan (catechins) .

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