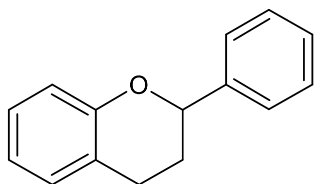
1-Introduction

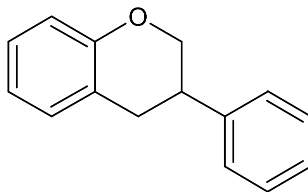
1.1-General approach

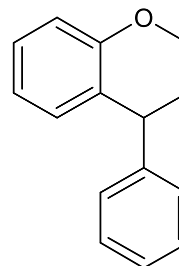
Flavonoids are a group of polyphenolic compounds, diverse in chemical structure and characteristics .they occur naturally in fruits, vegetables, nuts, seeds, flowers, bark and an integral part of human diet [1-3]. They have been reported to exhibit a wide range of biological effects. Including antibacterial, antiviral [4], anti-inflammatory, antiallergic [1, 4, 5,] and vasodilatory action[6].

The term flavonids is generally used to describe a broad collection of natural products that include C_6-C_3-C_6 backbones ,it's nomenclature depending on the position of the linkage of aromatic ring to benzopyrano (chromano)moiety .This group of natural products may be divided in to four classes : the flavonoids(2-phenylbenzopyrans) **1,** isoflavonoids(3-benzopyrans) **2,**neoflavonoids(4-benzopyrans) **3,** and minor flavonoids.

The first three classes usually share a common chalcone precursor and there are biogenetically and structurally related.

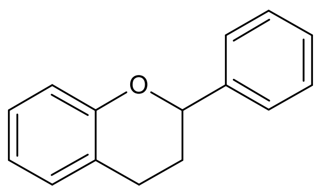






1 **2** **3**

Based on the degree of oxidation and saturation present in heterocyclic ring it divided in to: flavans **4**, flavanones **5**, flavones **6**, flavonols **7**, dihydroflavonols **8**, anthocyanins **9**, chalcones **10**, aurones **11**, dihydrochalcones **12**, isofavones **13**.



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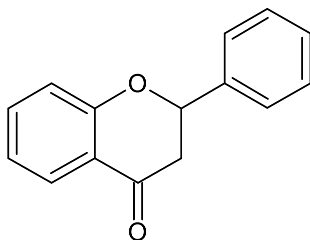

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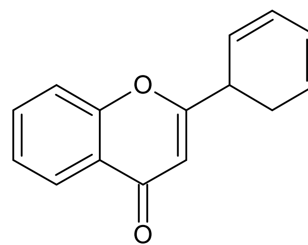

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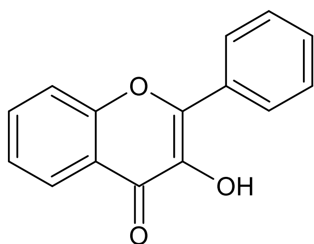

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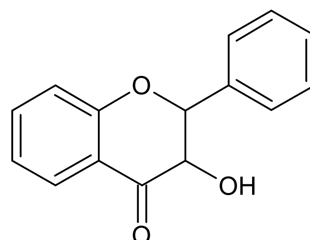

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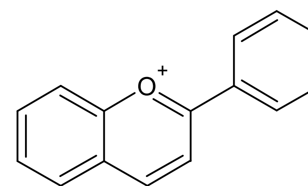

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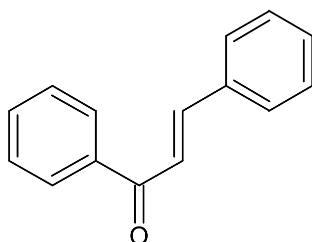

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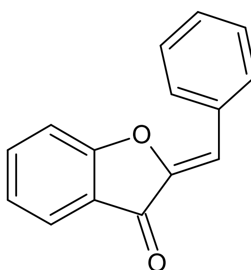

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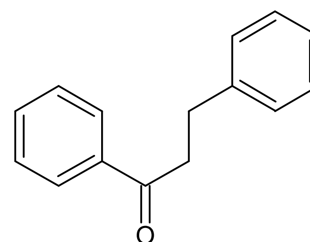

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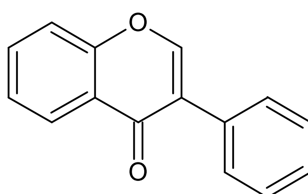

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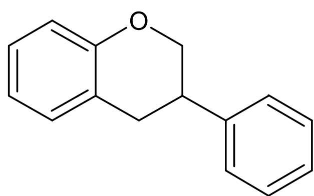
1.2-Isoflavonoid

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 plant kingdom, isoflavonoids are very divers in structures due to different in oxidation levels and presence of additional heterocyclic rings it divided in to: isoflavones **13**, isoflavans

14, isoflavanones **15**, isoflav-3-enes **16**, isoflavanols **17**, rotenoids **18**, coumestanes **19**, 3-arylcoumarins **20**, coumaronochromenes **21**, coumaronochromones **22** and petrocarpans **23**.



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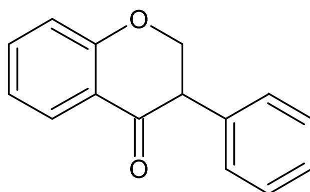

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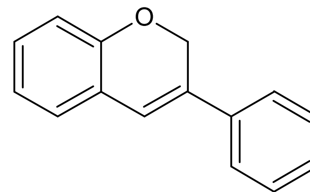

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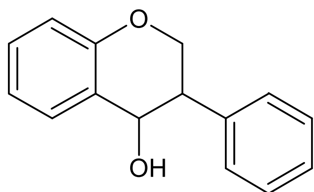

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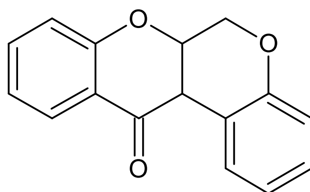

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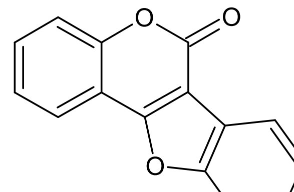

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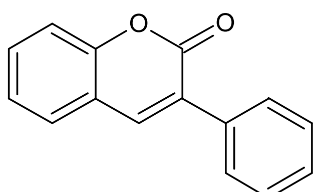

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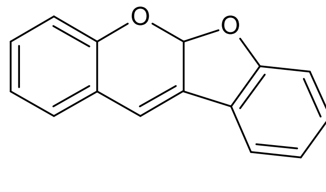

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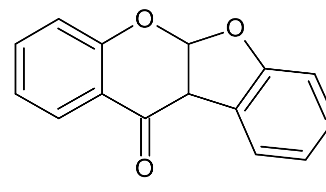

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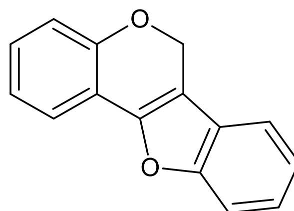

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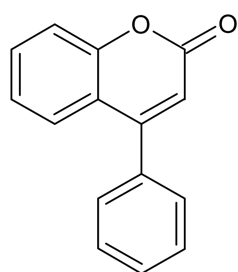
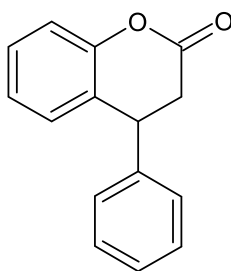
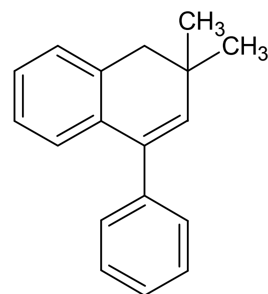
1.3-Neoflavonoids

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



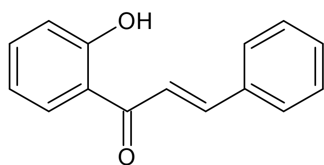


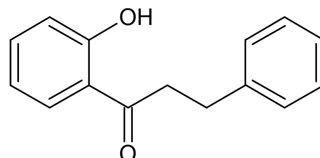


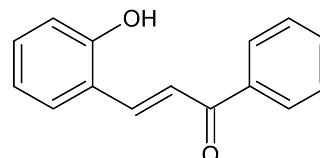
24 **25** **26**

1.4-Minor flavonoids

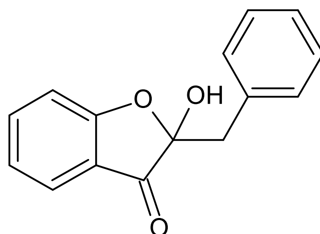
Natural products such as chalcones and aurones also contain C6-C3-C6 backbone and are considered minor flavonoids. These groups of compounds include the 2-hydroxychalcones **27**, 2-OH-dihydrochalcones **28**, 2-OH-retro-chalcone **29**, aurones (2-benzylidenecoumaranone) **11**, auronols **30** [7].







27 **28** **29**



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1.5-Extraction

Flavonoids can be degraded by enzyme action when collected plant material is fresh or nondried. It is thus advisable to use dry, lyophilized, or frozen samples. When dry plant material is used, it is generally ground 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 procyanidins [8].

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 method 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[9]. Application of PLE gave better results than maceration — and shorter extraction times and smaller amounts of solvent were required[10]. 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 130 ^{0}C[11].

As its name suggests, 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 extraction solvent[12, 13] .However, to allow for the extraction of polar compounds such as flavonoids, polar solvent (like methanol) have to be added as modifiers. There is consequently a substantial reduction in selectivity. This explains why there are relatively few applications to polyphenols in the literature. Even with pressures of up to 689 bar and 20% modifier (usually methanol) in the extraction fluid, yields of polyphenolic compounds remain low[14].

Ultrasound-assisted extraction is a rapid technique that can also be used with mixtures of immiscible solvents: hexane with methanol–water

(9:1), the hexane phase concentrated less polar sesquiterpene lactones and hydrocarbons, while the aqueous alcohol phase concentrated flavonoids and more polar sesquiterpene lactones[15].

Microwave-assisted extraction (MAE) has been described for the extraction of various compounds from different matrices[16]. 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[17].

1.6-Preparative separation

1.6.1-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 counter current techniques may be applied. In order to obtain an isoflavonoid-rich fraction from *Erythrina* species (Leguminosae) for further purification work, an organic solvent extract was dissolved in 90% methanol and first partitioned with 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[18].

A short polyamide column, a Sephadex LH-20 column, or an ion exchange resin can be used. Absorption of crude extracts onto Diaion HP-20 or Amberlite XAD-2 (or XAD-7) columns, followed by elution with a

methanol–water gradient, is an excellent way of preparing flavonoid-rich fractions.

1.6.2-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. For example, in the separation of anthocyanins and anthocyanin-rich fractions, 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.[19]

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. Support materials include polyamide, cellulose, silica gel, Sephadex LH-20, and Sephadex G-10, G-25, and G-50. Sephadex LH-20 is recommended for the separation of proanthocyanidins. For Sephadex gels, as well as size exclusion, adsorption and partition mechanisms operate in the presence of organic solvents. Although methanol and ethanol can be used as eluents for proanthocyanidins, acetone is better for displacing the high molecular weight polyphenols. Slow flow rates are also recommended. Open-column chromatography with certain supports (silica gel, polyamide)

suffers from a certain degree of irreversible adsorption of the solute on the column.

Modifications of the method (dry-column chromatography, vacuum liquid chromatography, VLC, for example) are also of practical use for the rapid fractionation of plant extracts. VLC with a polyamide support has been reported for the separation of flavonol glycosides.[20]

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. Preparative TLC in conjunction with open-column chromatography remains a straightforward means of purifying natural products, although variants of planar chromatography, such as centrifugal TLC, [19] have found application in the separation of flavonoids.

Other combinations are, of course, possible, depending on the particular separation problem. Combining gel filtration or liquid–liquid partition with liquid chromatography (LC) is one solution. Inclusion of chromatography on polymeric supports[19] can also provide additional means of solving a difficult separation. 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)

1.6.2.1-High-performance liquid chromatography

HPLC is becoming by far the most popular technique for the separation of flavonoids, both on preparative and analytical scales. Improvements in instrumentation, packing 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 a sample, while preparative HPLC is a purification process and aims at the isolation of a pure substance from a mixture.

Semipreparative HPLC separations (for 1 to 100 mg sample sizes) use columns of internal diameter 8 to 20 mm, often packed with 10 µm (or smaller) particles. Large samples can be separated by preparative (or even process-scale) installations but costs become correspondingly higher.

Optimization can be performed on analytical HPLC columns before transposition to a semipreparative scale.

1.6.2.2- Medium-Pressure Liquid Chromatography

The term ‘‘medium-pressure liquid chromatography’’ (MPLC) covers a wide range of column diameters, different granulometry packing materials, different pressures, and a number of commercially available systems. In its simplest form, MPLC is a closed column (generally glass) connected to a compressed air source or a reciprocating pump. It fulfils the requirement for a simple alternative method to open-column chromatography or flash chromatography, with both higher resolution and shorter separation times. MPLC columns have a high loading capacity — up to a 1:25 sample-to-packing-material ratio[21] — and are ideal for the separation of flavonoids.

In MPLC, the columns are generally filled by the user. Particle sizes 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 or 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).[22] Choices of solvent systems

can be efficiently performed by TLC[23] or by analytical HPLC. Transposition to MPLC is straight forward and direct.[24]

1.6.2.3- Centrifugal Partition Chromatography

Various countercurrent chromatographic techniques have been successfully employed for the separation of flavonoids.[19] Countercurrent chromatography is a separation technique that relies on the partition of a sample between two immiscible solvents, the relative proportions of solute passing into each of the two 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 techniques:

. No irreversible adsorption of the sample

. Quantitative recovery of the introduced sample

. Greatly reduced risk of sample denaturation

. Low solvent consumption

. Favorable economics

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

Countercurrent distribution, droplet countercurrent chromatography, and rotation locular countercurrent chromatography are now seldom used but CPC, also known as centrifugal countercurrent chromatography, finds extensive application for the preparative separation of flavonoids. In CPC, the liquid stationary phase is retained by centrifugal force instead of a solid support (in column chromatography). Basically, two alternative designs of apparatus are on the market[25]: (a) rotating coil instruments; (b) disk or cartridge instruments.

Although most CPC separations are on a preparative scale, analytical instruments do exist.[26] However; these are mostly used to find suitable separation conditions for scale-up.

The technique of CPC was also employed as a key step in the purification of 26 phenolic compounds from the needles of Norway spruce (*Picea abies*, Pinaceae). An aqueous extract of needles (5.45 g) was separated with the solvent system $CHCl_3$–MeOH–i-PrOH–H_2O (5:6:1:4), initially with the lower phase as mobile phase and then subsequently switching to the upper phase as mobile phase. Final purification of the constituent flavonol glycosides, stilbenes, and catechins was by gel filtration and semipreparative HPLC.[27]

1.7-Identification

1.7.1-Sample preparation

The analysis of flavonoids and their conjugates is one of the most important areas in the field of instrumental analytical methods, helping to solve problems in biological and medical sciences. Different methods of isolation of the natural products may be applied, and the utilization of various strategies is dependent on the origin of the biological material from which the target natural products are to be extracted (plant or animal tissue or body fluids). In the case of polyphenolic compounds, it often is important to initially determine whether the researchers are interested in the identification of individual components present in a mixture of target compounds or whether they would like to estimate the total amount of phenolic compounds in the biological material investigated. This second approach most often takes place during the nutritional studies on different foods or fodders, mainly of plant origin.

The presence of carbohydrates and/or lipophylic substances may influence the profile of the qualitative and quantitative composition of

flavonoids and their derivatives in the obtained extracts. One has to consider the above-mentioned selection of the methods for sample preparation and extraction, and in many cases additional cleaning based on solid-phase extraction (SPE) of the extracted samples is required.

1.7.1.1- Preparation of Plant or Animal Tissue and Foodstuffs for Flavonoid Analysis

The utilization of dried plant material for extraction may cause a substantial decrease in the yield of flavonoid conjugates. Acylated flavonoid glycosides are especially labile at elevated temperatures and are frequently thermally degraded during the process of drying plant tissues. This is important during the profiling of this class of natural products in research directed toward the investigation of their physiological and biochemical roles in plants under the influence of environmental factors, or in studies of genetically modified plants for the elucidation of changes in metabolic pathways.

Free flavonoid aglycones exuded by plant tissues (leaf or root) may be washed from the surface with non polar solvents, such as methylene chloride, ethyl ether, or ethyl acetate. However, more polar glycosidic conjugates dissolve in polar solvents (methanol and ethanol), and these organic solvents are applied for extraction procedures in Soxhlet apparatus. Mixtures of alcohol and water in different ratios are applied for the extraction of flavonoids and their conjugates from solid biological material (plant or animal tissues and different food products). The extraction efficiency may be enhanced by the application of ultrasonication[28, 29] or pressurized liquid extraction (PLE), a procedure performed at elevated temperature ranging from 60°C to 200°C [30]. Supercritical fluid extraction with carbon dioxide also may be used[31] procedures have to be carefully adjusted because of the possibility of thermal degradation of the flavonoid derivatives. In many cases, further purification and/or preconcentration of

the target compound fraction is necessary. In these cases, liquid–liquid extraction (LLE) or SPE are most commonly used. For estimation of the extraction yield it is necessary to spike biological materials with proper internal standards. Most suitable are compounds structurally similar to the studied analytes but not present in the sample. Compounds labelled with stable isotopes (^{2}H or ^{13}C) are useful when mass spectrometric detection is applied. In the case of the extraction of flavonoids from biological materials, different classes of phenolic compounds are often added. On the other hand, quantitative analysis of consecutive components of the analyzed flavonoid mixture needs reference standard compounds necessary for preparation of calibration curves essential for a precise quantification.

The choice of the extraction procedure for obtaining flavonoid conjugates from biological material is very important and depends on the goals of the conducted research. The evaluation of the spatial distribution of target compounds on the organ, tissue, cellular or even sub cellular level is of special interest in some projects. In these situations, the amount of biological material for the isolation of natural products may be extremely small, and the application of micro extraction techniques is necessary[32]. In many cases, it is necessary to avoid the chemical and/or enzymatic degradation of the metabolites. This is of special importance in the profiling of flavonoid glycosides in research directed toward plant functional genomics or during physiological and biochemical studies that need information about all classes of flavonoid conjugates present, even the thermally labile acylated derivatives. On the other hand, in the phytochemical analysis of plant species or phytopharmaceutical studies of plant material, the repeatable isolation of all biologically active flavonoid aglycones with a good yield is more important. In these cases, more drastic extraction conditions are acceptable. Excellent reviews have been published

on isolation strategies for the determination of active phenols in plants tissue or food and foodstuff [33, 34].

Robust multistep chromatographic methods are necessary for the isolation of individual components from plant extracts containing new uncharacterized compounds. Various stationary phases are used in column chromatography, including polyamide, Sephadex LH-20, and different types of silica gels (normal and reversed phase with chemically bonded functional groups). The proper choice of solvent systems is necessary, often requiring the application of gradients of more polar (normal phases) or more hydrophobic solvents (reverse phases), together with the above-mentioned chromatographic supports in different chromatography systems. The sequence and kind of separation methods used depends on the composition of the sample and the experience of the researcher. However, minor flavonoid components are difficult to obtain as pure compounds. In cases of analysis of samples containing a number of compounds present in small amounts, the application of an analytical chromatographic systems enhanced by proper detectors (UV, NMR, and/or MS) gives spectrometric information sufficient for establishing the structure of minor target components. When liquid chromatography is used for separation of compounds, multiple detector systems are available (UV diode array detector, mass spectrometers, and nuclear magnetic resonance spectrometer). It is possible to achieve complete structural information about isomeric flavonoids and their conjugates in this way.

1.7.1.2-Preparation of Body Fluids

For the isolation of flavonoids and their derivatives from liquid samples like beverages (wine or fruit juice) and physiological fluids (blood or urine) , two different approaches are usually applied. The first one is based on liquid–liquid extraction and the second one on solid-phase

extraction of target natural products mainly on RP C-18 silica gel cartridges. In the case of body fluids, special procedures have to be considered to avoid degradation of target compounds due to the activity of different enzymes present [35].However, in some cases, flavonoid conjugates can be enzymatically hydrolyzed with external glucuronidases and sulfatases prior to the isolation and analysis of products.

1.7.2-Analysis methods

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, Schmidt et al[36].Have reported the separation of flavones, flavonols, flavanones, and chalcones (with frequent substitution by methyl groups) by GC.

Quantification aspects are discussed under individual techniques

1.7.2.1- Thin-layer chromatography

Paper chromatography and paper electrophoresis were once extensively used for the analysis of flavonoids[37], 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.

Many different solvent systems have been employed for the separation of flavonoids using TLC. Highly methylated or acetylated flavones and flavonols require non polar solvents such as chloroform–methanol (15:1). Widely distributed flavonoid a glycones, such as apigenin, luteolin, and quercetin, can be separated in chloroform–methanol (96:4) and similar polarity solvents. One system that is of wide spread 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. [38] Careful choice of solvent system also allows separation of flavonoid glucosides from their galactosidic analogs[39]. 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).[39]

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 Markham, [40] flavonoids appear as dark spots against a fluorescent green background when observed in UV light (254 nm) on plates containing a UV-fluorescent indicator (such as silica gel F_{254}). In 365 nm UV light, depending on the structural type, flavonoids show dark yellow, green, or blue fluorescence, which is intensified and changed by the use of spray reagents. One of the most important of these is the ‘‘natural products reagent,’’ which produces an intense fluorescence under 365 nm UV light after spraying with a 1% solution of diphenylboric acid-β-ethylamino ester (diphenylboryloxyethylamine) in methanol. Subsequent spraying with a 5% solution of polyethylene glycol-4000 (PEG) in ethanol lowers the detection limit from 10 μg (the average TLC detection limit for flavonoids) to about 2.5 μg, intensifying the fluorescence behaviour.

Aqueous or methanolic ferric chloride is a general spray reagent for phenolic compounds and gives a blue-black coloration with flavonoids. Similarly, Fast Blue Salt B forms blue or blue-violet azo dyes.

For quantitative analysis, scanning of the TLC plate with a densitometer provides good results.

Better resolution is obtained by chromatographing flavonoids on high-performance TLC (HPTLC) plates. Silica gel $60F_{254}$, RP-18, or, less frequently, Diol HPTLC plates are used for separation purposes. Methanol–water eluents are indicated for HPTLC on RP-18 chemically bonded silica gel but some acid is generally added to avoid tailing. Polar glycosides require eluents containing a high percentage of water. Special HPTLC plates have been designed for this purpose, since normal plates can only accommodate aqueous methanol mixtures with up to about 40% water.

The European Pharmacopoeia stipulates TLC fingerprint analysis for the identification of plant drugs.

1.7.2.2- High-performance liquid chromatography

The method of choice for the qualitative and quantitative analysis of flavonoids is HPLC. HPLC has been used for all classes of flavonoids and hundreds of applications have been published.

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 (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_{18}) phases. Some reported analyses use octasilyl bonded (RP-8 or C_8) 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 analogs. 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_{18} columns for the separation of flavonoid aglycones and glycosides. While some columns give good results, others produce substantial band broadening and peak asymmetry.[41]

Octadecylsilyl stationary phases with hydrophilic endcapping 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.[42]

Normal phases (unmodified silica gel) are rarely employed, except for the occasional separation of weakly polar flavonoid aglycones, polymethoxylated flavones, flavanones, or isoflavones. The polymethoxylated flavones present in citrus fruits can, for example, be separated on silica gel columns. The big drawback is that solvent gradients cannot normally be run with normal phases.

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.

Flavanones elute before their corresponding flavones due to the effect of unsaturation between positions 2 and 3.

Isoflavones, chiefly found in the Leguminosae (such as soy, *Medicago sativa*, and red clover, *Trifolium pratense*) in the plant kingdom, are also successfully analyzed by HPLC on C_{18} columns.[43]

The anthocyanins exist in solution as various structural forms in 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. For the best resolution, anthocyanin equilibria have to be displaced toward their flavylium forms — peak tailing is thus minimized and peak sharpness improved. Flavylium cations are colored and can be selectively detected in the visible region at about 520 nm, avoiding the interference of other phenolics and flavonoids that may be present in the same extracts. Typically, the pH of elution should be lower than 2. A comparison of reversed-phase columns (C_{18}, C_{12}, and phenyl-bonded) for the separation of 20 wine anthocyanins, including monoglucosides, diglucosides, and acylated derivatives was made by Berente et al.[44] It was found that the best results were obtained with a C_{12} 4 µm column, with acetonitrile–phosphate buffer as mobile phase, at pH 1.6 and 500 ^{0}C.

In general, though, there is not a single HPLC method that can solve all flavonoid separation problems.

1.7.2.2.1-High-performance liquid chromatography-ultraviolet spectroscopy

The most frequently used detection method for HPLC is UV spectrophotometry. Routine detection in HPLC is typically based on measurement of UV absorption, or visible absorption in the case of anthocyanins. No single wavelength is ideal for all classes of flavonoids

since they display absorbance maxima at distinctly different wavelengths. The most commonly used wavelength for routine detection has been 280 nm, which represents a suitable compromise.

With the introduction of diode-array technology in the 1980s, a further dimension is now possible because coupled LC–UV with diode array detection (DAD) allows the chromatographic eluent to be scanned for UV–visible spectral data, which are stored and can later be compared with a library for peak identification.[45] 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. This improves the possibilities of quantification because detection can be performed at the wavelength maximum of the compound in question. These are typically to be found[43] at 270 and 330 to 365 nm for flavones and flavonols, at 290 nm for flavanones, at 236 or 260 nm for isoflavones, at 340 to 360 nm for chalcones, at 280 nm for dihydrochalcones, at 502 or 520 nm for anthocyanins, and at 210 or 280 nm for catechins.

Peak purity can also be determined. The spectra of eluting peaks obtained at the apex and both inflexion points of the peak can be compared in order to obtain a measure of the purity of the particular component of the sample.

LC–UV is valuable for the identification of isoflavones since their spectra differ in absorption properties from most of the other flavonoids. They have a C2–C3 double bond, with the B-ring at C3, which prevents conjugation of the phenyl group with the pyrone carbonyl group. This reduces the contribution of the B-ring to the UV spectrum and results in a peak of very low intensity in the 300 to 330 nm range.

The analysis of catechins and proanthocyanidins by LC–UV presents certain problems. In general, only monomers and oligomers up to tetramers can be separated and detected as defined peaks. Polymeric forms, which may

constitute the bulk of proanthocyanidins in many plant materials, are not well resolved. They give place to a drift in the baseline and the formation of characteristic humps in HPLC chromatograms. Furthermore, the spectral characteristics of these compounds do not allow easy detection and identification. Flavan-3-ols give absorption maxima at nonspecific wavelengths (270 to 290 nm) and they have lower extinction coefficients than other accompanying phenolics. Their quantification is thus not easy. The lack of reference proanthocyanidins implies that results have to be expressed with respect to other reference substances, normally catechin or epicatechin. This causes concomitant errors of quantification caused by the different extinctions shown by the individual flavan-3-ols. For reverse-phase HPLC of proanthocyanidin oligomers, the percentage of methanol or acetonitrile usually does not exceed 20%.

The coupling of HPLC with DAD allows online quantification of flavonoids in samples analyzed. Justesen et al.[46] have quantified flavonols, flavones, and flavanones in fruits, vegetables, and beverages in this fashion. The food material was extracted and then hydrolyzed to produce the corresponding flavonoid aglycones. These were analyzed on a Phenomenex C_{18} column (250_4.6 mm, 5 μm) using a mobile phase of methanol–water (30:70) with 1% formic acid (solvent A) and 100% methanol (solvent B). The gradient was 25 to 86% B in 50 min at a flow rate of 1 ml/min. UV spectra were recorded from 220 to 450 nm. For each compound, peak areas were determined at the wavelength providing maximal UV absorbance. Quantification was performed based on external standards. A mixture of standards of known concentrations was analyzed in duplicate before and after the batch of samples. Peak areas were used to calculate the hydrolyzed food sample flavonoid aglycone content. Method validation indicated good day-to-day variability (reproducibility) and recoveries in the range of 68 to 103%. There was low recovery of myricetin standard, presumably because of

degradation during hydrolysis. Detection by online mass spectrometry (MS) was also included to check possible interferences between flavonoids eluting at similar retention times.

While identification of the peaks in a LC–UV chromatogram is possible by comparing retention times and UV spectra with authentic samples or a databank, this might not be possible for compounds with closely related structures, and wrong conclusions might be drawn. It has been established that in order to complete the characterization of phenolic compounds, reagents inducing a shift of the UV absorption maxima can be used.[47]

A postcolumn derivatization procedure, based on this technique, is possible by adding suitably modified shift reagents to the eluate leaving a HPLC column[48] .Direct information is provided about the flavonoid oxidation pattern and position of free phenolic hydroxyl groups. In the analysis of *Gentiana* (Gentianaceae) extracts, 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 monohydrogenphosphate and potassium hydroxide were used as the weak and strong bases, respectively, instead of sodium acetate and sodium methanolate. In order to form a complex with the keto function, an aqueous solution of aluminum chloride was passed with the eluate through a reaction coil at 60 ^{0}C. The presence of *ortho*-hydroxyl groups was shown 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 postcolumn derivatization allows the analysis of very rare and small species, as well as single plant parts of herbarium samples.[48]

1.7.2.2.2- High performance liquid chromatography mass spectroscopy

Coupled HPLC–MS is one of the most important techniques of the last decade of the 20th century. The combination offers the possibility of taking advantage of chromatography as a separation method and MS as an identification tool. The amazing number of applications and the rapid drop in price (and size) of MS instruments has meant that the use of LC–MS is now extremely widespread. MS is one of the most sensitive methods of molecular analysis. Due to its high power of mass separation, very good selectivities can be obtained. However, the coupling between HPLC and MS has not been straightforward since the normal operating conditions of a mass spectrometer (high vacuum, high temperature, gas-phase operation, and low flow rates) are diametrically opposed to those used in HPLC, namely liquid-phase operation, high pressures, high flow rates, and relatively low temperatures.

In LC–MS, there are three general problems: the amount of column effluent that has to be introduced in the MS vacuum system, the composition of the eluent, and the type of compounds to be analyzed. Many interfaces have been developed in order to cope with these factors.[49] The interfaces must accomplish nebulization and vaporization of the liquid, ionization of the sample, removal of excess solvent vapour, and extraction of the ions into the mass analyzer. To date, no real universal interface has been constructed; each interface has characteristics that are strongly dependent on the nature of the compounds for which they are used. In LC–MS, the same rules that govern the ionization of pure compounds in the direct insertion mode are roughly preserved. Most interfaces work with reversed-phase HPLC systems, with a number of them suitable for the analysis of plant secondary metabolites. These include thermospray (TSP), continuous-flow fast-atom

bombardment (CF-FAB), and electrospray (ES) [50] .They cover the ionization of relatively small non polar products (aglycones, MW200) to highly polar molecules (glycosides, MW2000). Contrary to TSP or CF-FAB, where the source is in the vacuum region of the mass spectrometer, in ES the ion source is at atmospheric pressure. Atmospheric pressure ionization (API) has rendered LC–MS more sensitive and easy to handle. An API interface or source consists of five parts: (a) the liquid introduction device or spray probe; (b) the actual atmospheric pressure ion source region, where the ions are generated by means of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), or by other means; (c) an ion sampling aperture; (d) an atmospheric pressure-to-vacuum interface; (e) an ion optical system, where the ions are subsequently transported into the mass analyser.[49] ESI and APCI are soft ionization techniques that generate mainly molecular ions for relatively small plant metabolites such as flavonoids.

Mass spectral data provide structural information on flavonoids and are used to determine molecular masses and to establish the distribution of substituents between the A- and B-rings. A careful study of fragmentation patterns can also be of particular value in the determination of the nature and site of attachment of the sugars in *O*- and *C*-glycosides. For flavonoid aglycones and glycosides with a limited number of sugar units (not more than three), TSP LC–MS analysis leads to a soft ionization, providing only intense $[M+H]^+$ ions for the aglycones and weak $[M+H]^+$ ions of glycosides (mono- or disaccharide), together with intense fragment ions due to the loss of the saccharide units, leading to the aglycone moiety $[A+H]^+$.

In general, HPLC coupled with diode array and mass spectrometric detection provides an efficient method of rapid identification of flavonoids in a mixture. This technique now finds widespread application. By this means, 14 xanthone and flavonol glycosides were characterized online in a prepurified extract of mango (*Mangifera indica*, Anacardiaceae) peels, using

ESI MS.[42] LC–UV–MS profiles of several medicinal plant extracts, including red clover (isoflavonoids), sour orange (flavanones), and astragalus (isoflavonoids and isoflavans), have been described by He.[51]

Both ES and TSP are soft ionization methods and do not typically produce many fragments. This is useful in quantitative analysis or molecular mass determination but is of little use in structure elucidation. In this case, collision-induced dissociation (CID) or collision- activated dissociation methods can be employed.[52] Fragmentation is induced in one of the high-pressure regions of the ion passageway from source to mass analyzer. Fragment ions produced by CID are very efficiently transported into the mass analyzer, providing a simple MS–MS method. With LC–MS, one analysis without CID and one with CID can be performed to obtain fragments of all components. CID is carried out to enhance fragmentation of the analytes either at the ES source (in-source CID) or in conjunction with tandem MS. In tandem MS, the first operation is to isolate a parent ion and the second is to determine the mass-to-charge ratio of the product ions formed after CID of the parent ion. The sequence of ion isolation and CID can be repeated many times in MS^n. Tandem MS and in-source CID give very similar product-ion mass spectra.

Since molecular weight information alone is insufficient for online structural determination of natural products, the fragmentation pathways of flavones and flavonols by fast-atom bombardment CID MS–MS have been documented.[53-56]

The CID MS–MS and MS^n spectra of flavonoids have been systematically studied using hybrid quadrupole time of flight (Q-TOF) and ion trap (IT) mass analyzers, under various energy conditions, to generate fragment ions.[57] These two instruments were chosen because the CID process in beam and trap systems is not generated in the same way. The results demonstrated that, if for hydroxylated flavonoids the CID MS–MS

spectra generated on both instruments were similar, for partially methoxylated derivatives, there were important differences. This is a hindrance to the creation of MS–MS databases exchangeable between instruments. Generally, fragments issued from C-ring cleavage were easier to observe on a QTOF instrument, while losses of small molecules were favored in IT-MS. MS–MS recorded in the positive ion mode were more informative than those obtained from negative ions. Online accurate mass measurements of all MS–MS fragments were obtained on the Q-TOF instrument, while the multiple-stage MS^n capability of the IT was used to prove fragmentation pathways. Molecular formulae with an accuracy of 1.8 ppm could be produced for isovitexin on the Q-TOF instrument.[57] It is worthy of note that high-resolution MS allows molecular formulae of compounds to be assessed directly; these can then be cross-checked with spectral libraries to provide identification of unknown components.

The application of tandem MS (LC–TSP MS–MS) can be illustrated for the online characterization of flavonoids from *Gentianella cabrerae*. The LC–UV–MS of the methanol extract indicated the presence of different flavonoid *C*-glycosides.

In a study of the Guinean medicinal plant Dissotis *rotundifolia* (Melastomataceae) by hyphenated HPLC techniques, online data showed the presence of isomeric pairs of *C*-glycosylflavones in the alcoholic or hydroalcoholic extracts but these could not be distinguished either by TSP LC–MS or their UV spectra

While typical flow rates for the HPLC analyses of flavonoids lie in the 1.0 to 1.5 ml/min range, the introduction of short columns containing stationary phases with smaller pore sizes (allowing narrower peaks to be obtained in shorter separation times) means that considerably lower flow rates are the trend. Not only is there a decrease in solvent consumption but coupling to mass spectrometers or NMR instruments is facilitated.

1.7.2.2.3-High performance liquid chromatography nuclear magnetic resonance

Careful and critical use of the hyphenated techniques LC–UV–MS and LC–MS–MS can provide sufficient online information for the identification of small molecules such as flavonoids. However, in many cases, more data are required for an in-depth structural investigation and this can be supplied by the addition of an LC–NMR analytical capability. For practical purposes, LC–UV–MS and LC–UV–NMR are generally run as separate operations. The coupling of HPLC with NMR spectroscopy, introduced around 1978, is one of the most powerful methods for the combined separation and structural elucidation of unknown compounds in mixtures.[58, 59]

At first, LC–NMR was little used because of its lack of sensitivity. However, recent progress in pulse field gradients and solvent suppression, improvement in probe technology, and the construction of high-field magnets have given a new impulse to the technique. While HPLC–NMR coupling is relatively straightforward (the samples flow in a non rotating 60 to 180 µl glass tube connected at both ends with HPLC tubing) compared to LC–MS, the main problem of LC–NMR is the difficulty of observing analyte resonances in the presence of the much larger resonances of the mobile phase. This problem is magnified under typical reversed-phase HPLC operating conditions, where more than one protonated solvent is used and where the resonances change frequencies during analysis in the gradient mode. Furthermore, the continuous flow of sample in the detector coil complicates solvent suppression. These problems have now been overcome by the development of fast, reliable, and powerful solvent suppression techniques, such as WET (water suppression enhanced through T_1 effects), [60] which produce high-quality spectra in both on-flow and stopped-flow modes. These techniques consist of a combination of pulsed-field gradients,

shaped radiofrequency pulses, shifted laminar pulses, and selective ^{13}C decoupling, and are much faster than classical presaturation techniques previously used in the field. Thus, for typical reversed-phase HPLC analyses, non deuterated solvents, such as methanol and acetonitrile, can be used, while water is replaced by D_2O.

The information provided by LC–NMR consists mainly of ^{1}H NMR spectra or ^{1}H–^{1}H correlation experiments. Access to ^{13}C NMR is possible but is restricted only to a very limited number of cases where the concentration of the LC peak of interest is high and ^{13}C NMR data can be deduced indirectly from inverse detection experiments. Due to the low natural abundance of the ^{13}C isotope (1.1%), the sensitivity for direct measurement in the LC–NMR mode is insufficient.

LC–NMR can be operated in two different modes: on-flow and stopped-flow. In the on-flow mode, LC–NMR spectra are acquired continuously during the separation. The data are processed as a two-dimensional (2D) NMR experiment. The main drawback is the inherent low sensitivity. The detection limit with a 60 μl cell in a 500 MHz instrument for a compound with a molecular weight around 400 amu is 20 μg. Thus, on-flow LC–NMR runs are mainly restricted to the direct measurement of the main constituents of a crude extract and this is often under overloaded HPLC conditions. Typically, 1 to 5 mg of crude plant extract will have to be injected on-column[61] .In the stopped-flow mode, the flow of solvent after HPLC separation is stopped for a certain length of time when the required peak reaches the NMR flow cell. This makes it possible to acquire a large number of transients for a given LC peak and improves the detection limit. In this mode, various 2D correlation experiments (COSY, NOESY, HSQC, and HMBC) are possible.

The combination of HPLC with online UV, MS, and NMR detection has proved to be a very valuable tool for the analysis of natural products in

extracts or mixtures[61, 62] .The field of flavonoids is no exception. The LC–NMR information obtained comes from the ^{1}H NMR spectra of selected peaks in the HPLC chromatogram. From LC–MS, A- or B-ring substitution can be deduced from the fragmentation pattern but the exact location of the substituent cannot be determined. However, for a flavonoid like apigenin, where only one hydroxyl group is located on the B-ring, ^{1}H NMR will give the substitution position because each of the three possibilities of localization of the hydroxyl group will give a unique splitting pattern. Much information can be derived about the nature and linkage positions of sugars. However, since D_2O is present in the eluent, exchangeable signals are not observed in the NMR spectrum.

If full metabolite profiling of a plant extract has to be performed, LC–NMR can be run in the on-flow mode. In order to obtain adequate NMR spectra of all constituents, the amount of sample injected has to be increased — this produces overloading when compared with normal analytical HPLC conditions but gives the possibility of testing for biological activity (in conjunction with a microfractionation procedure). This was the approach adopted for the investigation of new antifungal constituents from *Erythrina vogelii* (Leguminosae), a medicinal plant of the Ivory Coast.[63] In order to rapidly identify the active principles from the antifungal dichloromethane extract of the roots, preliminary analysis by LC–UV and Q-TOF LC–MS was performed. Approximately 12 major peaks were observed in the HPLC chromatogram and from UV, MS, and MS–MS online data; these were shown to be prenylated isoflavones and isoflavanones. In order to obtain more information, on-flow LC–^{1}H NMR was performed by injecting 10 mg of crude extract onto an 8 mm C_{18} radial compression column connected to the NMR instrument. At a low flow rate (0.1 ml/min), acquisition of ten LC–NMR spectra was possible. Of these ten peaks, five were found by simultaneous HPLC microfractionation to be associated with the antifungal

activity of the extract. Interpretation of all online data, with emphasis on LC–NMR, allowed the identification of eight flavonoids, including a known isoflavone with antifungal activity and two putative new isoflavanones, also with antifungal activity. This dereplication procedure allowed the targeted isolation of the new antifungal compounds.[63]

Applications of LC–NMR for the online identification of flavonoids are still few and far between, one reason probably being the high cost of the apparatus. However, several other examples do exist, in addition to those mentioned above. The technique has been successfully applied to the analysis of *Hypericum perforatum* (Guttiferae). Online identification of quercetin, several of its glycosides, and the biflavonoid 15, 118-biapigenin in an extract was possible.[64]

1.7.2.3- 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 isotachophoresis, (f) capillary electrochromatography (CEC), and (g) non aqueous 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.[65] Anions and cations are separated in CZE by electrophoretic migration and electro-osmotic 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.[66, 67] CE is especially suitable for the separation of flavonoids as they are negatively charged at higher pH values.[66, 68] Suntornsuk[68] 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. For example, while TLC and HPLC analyses of passion flower do not provide adequate separation of all identified flavonoids, CE can fulfil the necessary requirements.[69] Separations of Passiflora incarnata (Passifloraceae) flavonoid glycosides were performed on a 50 µm internal diameter uncoated fused-silica capillary with 25 m*M* sodium borate buffer with 20% methanol (pH 9.5). The voltage was 30 kV and the temperature of the capillary maintained at 35C. The CE instrument was equipped with a diode array detector. Twelve glycosides were satisfactorily separated within 13 min .For quantification; quercetin 3-*O*-arabinoside was used as internal standard. Calibration curves for internal standardization were established. The method was applied to the analysis of ten commercial samples of Passiflorae herba. They showed similar flavonoid patterns but differed quantitatively in individual flavonoid glycosides. Reproducibility was good, with a coefficient of variation (CV) of 2.83% for interday precision and a mean CV of 1.26% for migration time.[69]

Other applications include the online coupling of capillary isotachophoresis and CZE for the quantitative determination of flavonoids in *Hypericum perforatum* (Guttiferae) leaves and flowers. This method involved the concentration and preseparation of the flavonoid fraction before

introduction into the CZE capillary. The limit of detection for quercetin 3-*O*-glycosides was 100 ng/ml.[70]

1.8-Flavonoids as nutraceuticals

"Nutraceutical" is defined "as a food or parts of food that provide medical or health benefits, including the prevention and treatment of disease." Subsequently, several other terms (medical food, functional food, and nutritional supplements) were used. A nutraceutical is any nontoxic food extract supplement that has scientifically proven health benefits for both the treatment and prevention of disease [71].

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. 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. 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. The presentation in this chapter is designed to provide the reader the tools to understand the biological and molecular role of plant flavonoids, including their antioxidant and antiproliferative activities and their role in intracellular signaling cascades.

The flavonoids, ubiquitous in plants, are the largest class of polyphenols, with a common structure of diphenylpropanes (C6-C3-C6), consisting of two aromatic rings linked through three carbons. The six major

subclasses of flavonoids include the flavones , flavonols , flavanones , flavanols , anthocyanidins, and isoflavones [72] they are widely distributed in foods and beverages of plant origin, such as fruits, vegetables, tea, cocoa, and wine. Numerous publications report their content in various foods[73, 74]. 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.

1.8.1- Antioxidant activity

1.8.1.1-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 [75]. 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 [76]. ROS contribute to cellular aging [77], mutagenesis [78], carcinogenesis [79], and coronary heart disease [80] possibly through the destabilization of membranes [78], 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 [81, 82]. Since oxidation of LDL is implicated in the pathogenesis of coronary heart diseases [83] through its ability to decrease the susceptibility of LDL to oxidation, a number of

researches have undertaken investigations examining the activity of dietary agents rich in flavonoids in inhibiting LDL oxidation *ex vivo*[84, 85, 86].

1.8.1.2-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[87, 88] and proinflammatory cytokine-mediated myocardial dysfunction[89, 90]. 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* [91].

1.8.1.3-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 [92], activate antioxidant enzymes [93], reduce alpha-tocopherol radicals [94], and inhibit oxidases [95].

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) [96]. Anderson and collaborators reported that green tea polyphenols partially protect DNA from •OH radical-induced strand breaks and base damage [97]. Pulse radiolysis results support the mechanism of electron transfer (or H-transfer) from catechins to radical sites on DNA [97]. 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 [98]. 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 peroxynitrite-induced modification of critical amino acids, which contribute to its surface charge [99]. 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 [100]. 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 [101]. Other flavonoids such

as quercetin, kaempferol, myristin, apigenin, and leuteolin also have antioxidative activity in many *in vitro* studies [102].

The Caerphilly Study began in 1979 with the overall objective of examining the determinants and predictive ability of new and classic risk factors for incident CHD. During the initial recruitment phase (1979–83) 2512 men aged 45–59 years were examined, representing 90% of the population of men in this age group from the town of Caerphilly, South Wales, UK, and its surrounding villages (total population 40,000). Since then they have been examined at 5-year intervals. At the first reexamination between 1984 and 1988, when the men were aged 49–64 years, men of the same age who had moved into the defined geographical area also were deemed to be eligible. A total of 2398 men were recruited into the reconstructed cohort and they form the baseline population for the current study. This Caerphilly Study, which investigated flavonols and ischemic heart disease in Welsh men, found that quercetin can inhibit LDL oxidation and therefore inhibit platelet aggregation *in vitro* [103]. The positive effect of red wine phenolics on the modulation of human LDL resistance against oxidative modification has been demonstrated *in vitro* [104]. These authors showed that catechin oligomers, anthocyanidin dimers and trimers, as well as myricetin were main antioxidant components in red wine [105]. Ghiselli and collaborators observed that anthocyanins were the most effective, both in scavenging ROS and in inhibiting lipoprotein oxidation [106]. In soybean, the isoflavone genistin inhibited the oxidation of LDL by metal anions, superoxide/nitric oxide, and endothelial cells [107]. The feeding of a soy protein high-fat diet (21g isoflavone in 100 g protein) to C57BL/6 mice result in the reduction of plasma cholesterol levels, increased the resistance of LDL against oxidation, and decreased the atherosclerotic lesion area [108]. Humans who eat three soy bars (containing 12 mg genistein and 7 mg daidzein each) daily for 2 weeks could increase the plasma isoflavone level

and the resistance of LDL to Cu-catalyzed oxidation [109]. Other studies in humans found that chronic red wine consumption (400 ml/day) reduced the susceptibility of LDL to lipid peroxidation catalyzed by Cu [110]. Similarly, a small but significant increase in the lag time of LDL oxidation was observed after 4 weeks of black tea consumption (600 ml/day) [84].

1.8.2-Anticarcinogenesis

Studies on cancer prevention have assessed the impact of a wide variety of flavonoids and a selected few isoflavones for their efficacy in inhibiting cancer in a number of animal models. These studies demonstrated that flavonoids inhibit carcinogenesis *in vitro* and substantial evidence indicates that they also do so *in vivo* [111, 112]. Flavonoids may inhibit carcinogenesis by affecting the molecular events in the initiation, promotion, and progression stages. Animal studies and investigations using different cellular models suggested that certain flavonoids could inhibit tumor initiation as well as tumor progression[113, 114, 115, 116].

1.8.2.1-Quercetin

Dietary quercetin inhibited DMBA-induced carcinogenesis in hamster buccal pouch [117] and in rat mammary gland [118]. When given during the initiation stage, quercetin also inhibited DEN induced lung tumorgenesis in mice [119]. In a medium-term multiorgan carcinogenesis model in rats, quercetin (1% in the diet) inhibited tumor promotion in the small intestine [120]. Feeding rats with quercetin, during either the initiation or promotion stage, inhibited 4-NQO-induced carcinoma formation in the tongue [114]. Siess and coworkers investigated the effects of feeding rats with flavone, flavanone, tangeretin, and quercetin on two steps of aflatoxin B1 (AFB1)-induced hepatocarcinogenesis (initiation and promotion) and found that flavones, flavanones, and tangeretin administered through the initiation period decreased the number of gamma-glutamyl

transpeptidasepreneoplastic foci [121]. Quercetin decreased oxidative stress-induced neuronal cell membrane damage more than vitamin C. These results suggest that quercetin, in addition to many other biological benefits, contributes significantly to the protective effects of neuronal cells from oxidative stress-induced neurotoxicity, such as Alzheimer's disease [122]. On the other hand, the suppressive effects of flavones, such as chrysin and apigenin, on the expression of the high affinity IgE receptor FcεRI, which plays a central role in the IgE-mediated allergic response [123] has been demonstrate.

1.8.2.2-Genistein and daidzein

Genistein and daidzein (isoflavones derived from soybeans) have been shown to inhibit the development of both hormone- and non-hormone-related cancers, including mouse models of breast, prostate, and skin cancer. Treatment of TRAMP mice with 100–500 mg genistein/kg diet reduced the incidence of advanced-stage prostate tumors, in a dose-dependent manner [124]. A high-isoflavone diet also was shown to inhibit methylnitrosourea-induced prostate tumor in Lobund-Wistar rats [125]. Topically applied genistein reduces the incidence and multiplicity of skin tumors in the DMBA-initiated and TPA-promoted multiplicity of skin mouse model by 20% and 50%, respectively [125] the UVB light-induced complete carcinogenesis model, topical pretreatment of SKH-1 mice with 10 μM genistein significantly reduced the formation of H_2O_2 and 8-bydroxy-2'-deoxyguanosine, but not of pyrimidine dimmers in the epidermis [125].

1.8.2.3-Anthocyanins

Only a few studies have been performed to elucidate a potential anticarcinogenic activity of anthocyanins, despite their presence and importance in the human diet. Hagiwara et al. performed an animal experiment in 1, 2-dimethylhydrazine (DMH) - initiated f344/DuCrj rats.

Anthocyanins from purple sweet potato and red cabbage were given at a dietary level of 5.0% in combination with 0.02% 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), a promoter in the diet until 36 weeks. Lesion development induced by DMH and PhIP was suppressed by the anthocyanins. The marked inhibitory effects on colon carcinogenesis were apparent for the anthocyanins comprising cyanidin, but not peonidin, as the main constituent, suggesting that the inhibition of anthocyanins on carcinogenesis may be related to the number of hydroxyl groups on the B-ring [126]. Hou and coworkers used JB6 mouse epidermal cells to study the molecular mechanisms of anticarcinogenesis by anthocyanins [127]. Their data indicated that both TPA-induced cell transformation and AP-1 transactivation were significantly inhibited by delphinidin, petunidin, and cyanidin, but not by pelargonidin, peonidin, or malvidin. These results suggest that the orthodihydroxyphenyl structure on the B-ring of anthocyanins may be essential for the inhibitory action, because pelargonidin, peonidin, and malvidin, having no such orthodihydroxyphenyl structure, failed to show the inhibitory effects. The molecular mechanism of delphinidin inhibition of TPA-induced AP-1 transactivation was due to a suppression of TPA-induced phosphorylation of ERK and JNK/SAPK.

Because strawberries are shown to contain higher concentrations of phytochemicals, including anthocyanins, and have a antioxidant capacity when compared with other common fruits, their neuroprotective activity was tested *in vitro* on PC12 cells treated with H_2O_2. Their protective effect and antioxidant capacity also were compared with those of banana and orange, which are the fresh fruits consumed at highest levels in the United States. The overall relative neuronal cell protective activity of these three fruits followed the decreasing order strawberry> banana > orange. The protective effects appeared to be due to the higher phenolic contents including anthocyanins, which are the major contributors in strawberries [128].

1.8.2.4-Tea and tea polyphenols

The anticarcinogenesis effects of EGCG, green tea, and black tea extracts on various organs and animal model have been reported. Studies by Khan and collaborators showed that green tea polyphenols have a potent inhibitory effect on skin tumorigenicity in Sencar mice[129]. In recent years, many studies demonstrated that topical application or oral feeding of a polyphenolic fraction from tea extract or of individual catechin derivatives had anticarcinogenesis effects in animal skins and other organs [130]. Tea extracts were found to be effective in inhibiting 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-incluced lung tumorigenesis in A/J mice with any of three dosing schedules[131]. In the NNK-induced lung tumorigenesis model, administration of black tea extracts to adenoma-breeding mice significantly inhibited tumor cell proliferation and the progression of adenoma to carcinoma[132, 133]. Inhibition of tumor invasion and metastasis in transplanted and spontaneous metastasis models by orally administered green tea infusion of EGCG also was reported [134, 77]. Most of the studies were conducted with chemical- or ultraviolet light-induced tumorigenesis models. For example, administration of 1% or 2% freshly brewed green or black tea significantly inhibited the spontaneous development of lung adenoma and rhabdomyosarcoma in A/J mice [135]. The bioavailability of tea constituents is apparently a key factor determining the effectiveness of tea in inhibiting tumor formation. In this respect, the oral cavity and digestive tract, which have direct contact with orally administrated tea, may represent good targets for chemoprevention. In the 7, 12- dimethylbenz[a]anthracene (DMBA)-induced oral carcinogenesis hamster model, treatment with 0.6% green tea as the sole source of drinking fluid reduced the number of visible tumors by 35% and reduced tumor volume by 57%. In addition, immunohistochemical analyses showed that tea increased the apoptotic index of the tumors while decreasing the proliferation index and microvessel

density [136]. Purified tea constituents, EGCG and theaflavins, also have been reported to inhibit tumorigenesis. For example, EGCG inhibited lung tumorigenesis in A/J mice induced by NNK and cisplatin [137]. Theaflavins (a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3, 3′-digallate) reduced NNK-induced lung tumor multiplicity and volume in A/J mice[132, 133] .These findings are interesting, give the extremely poor bioavalibility of theaflavins, and may suggest that the theaflavins are metabolized to a more-bioavailable active metabolite.

The membrane protective effects of the phenolics determined by LDH release and trypan blue exclusion assays demonstrated that epicatechin, catechin, and their mixture protect cellular membrane from β-amyloid-induced cytotoxicity. It has been demonstrated that the major flavonoids of cocoa, epicatechin and catechin, protect PC12 cells from Aβ-induced neurotoxicity, and suggest that cocoa may have an antineurodegenerative effect in addition to other known chemopreventive effects [138].

An ester of catechin, 3-*O*-octanoyl-(+)-catechin (OC), was synthesized from (+) - catechin by the incorporation of an octanoyl chain into catechin in the light of (-) - epicatechin gallate (ECG) and (-)-epigallocatechin-3-gallate (EGCG). OC was found to inhibit the response of ionotropic GABA receptors and Na+/glucose cotransporters expressed in *Xenopus* oocytes in a noncompetitive manner, more efficiently than catechin. OC also induced a nonspecific membrane current and decreased the membrane potential of the oocyte. This newly synthesized catechin derivative OC possibly binds to the lipid membrane more strongly than doe’s catechin, ECG, or EGCG and as a result perturbs the membrane structure [139].

1.8.3-Suppression of cancer growth

1.8.3.1-Antiproliferative effects

Deregulated proliferation appears to be a hallmark of increased susceptibility to neoplasia. Cancer prevention generally is associated with inhibition, reversion, or delay of cellular hyperproliferation. Most flavonoids have been demonstrated to inhibit proliferation in many types of cultured human cancer cell lines, whereas they have little or no toxicity to normal human cells. For example, Kandaswami and coworkers reported antiproliferative effects of four citrus flavonoids (quercetin, taxifolin, nobiletin, and tangeretin, at 2-8 μg/ml for 3–7 days) on squamous cell carcinoma HTB43 [140] .Kuo showed antiproliferative potency of five flavonoids and two isoflavonoids (0–100 μM) on colon carcinoma HY29 and Caco-2 cell lines, with the induction of apoptosis [141]. Le Bail and coworkers suggested antiproliferative activity of certain flavonoids and genistein at high concentrations (50 μM) on breast cancer MCF-7 through a mechanism independent of the estrogen receptor [142]. Twentyseven citrus flavonoids were investigated for antiproliferative activities, at 40 μM, on several tumor cell lines, including lung carcinoma A549 and gastric TGBC11TKB cancer cells and were found to inhibit proliferation of cancer cell lines. However, they did not significantly affect proliferation of normal human cell lines [143]. Studies on the inhibition of cell proliferation and angiogenesis by flavonoids in six different cancer cell lines had been reported and noted that the IC50 of active flavonoids were in the low micromolar range, physiologically available concentrations [144]. Genistein and synthetic isoflavone analogues, at 0.1–25 μg/ml, inhibited intestinal epithelia cell proliferation and induced apoptosis *in vitro* [145]. An inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids also was found [146]. Over 30 flavonoids had been screened for their effects on cell proliferation and

potential cytotoxicity in two human colon cancer cell lines. All compounds tested, including specific flavones, flavonols, flavanones, and isoflavonones, demonstrated antiproliferative activity in the absence of cell cytotoxicity [147]. No notable structure–activity relationships were found on the basis of flavonoid subclass. Other studies evaluated how the core structure of the flavones, 2-phenyl-4H-1-benzopyran-4-one, affects proliferation, differentiation, and apoptosis in a human colon cancer cell line [148]. In particular, this study evaluated the effect of the flavone on the expression of cell cycle and apoptosis-related genes in the cell line, reporting dramatic changes in mRNA levels of specific genes including cyclooxygenase-2, NF-κ, and BCL-X. Further, there was a high selectively for apoptosis of the transformed cells. The authors concluded that flavones could furnish a new chemopreventive agent.

1.8.3.2-Inhibition of cell cycle progression

Perturbations in the progression of the cell cycle may account for the anti-carcinogenetic effects of many flavonoids. Mitogenic signals commit cells to entry into a series of regulated steps allowing the progression of the cell cycle. Synthesis of DNA (S phase) and separation of two daughter cells (M phase) are main features of cell cycle progression. The time between the S and M phases is known as the G2 phase. This phase is important to allow cells to repair errors that occur during DNA duplication, preventing the propagation of these errors to daughter cells. In contrast, the G1 phase represents the period of commitment to cell cycle progression that separates M and S phases as cells prepare for DNA duplication upon mitogenic signals.

CDKs and CDKIs have been recognized as key regulators of cell cycle progression. Alternation and deregulation of CDK activity are pathogenic hallmarks of neoplasia. A number of cancers are associated with hyperactivation of CDKs as a result of mutations of the CDK or CDKI

genes. Therefore, compounds that function as inhibitors or modulators of these enzymes are of interest as novel potential therapeutic agents in cancer [149,150]. Flavonoids and tea polyphenols have been found to perturb the cell cycle in certain cancer cell lines. Genistein produces cell cycle arrest at both G1/S and G2/M phase in the human myelogenous leukemia HL60 cells and in the lymphocytic leukemia MOLT-4 cells [151]. Furthermore, it has been shown that genistein induced G2/M arrest through p21Waf1/Cip1 up-regulation and apoptosis induction in a non-small-cell lung cancer cell lines[152]. Isoflavonones (genistein, genistin, daidzein, and biochanin A) also inhibit growth of murine and human bladder cancer cell lines by inducing cell cycle arrest, apoptosis, and angiogenesis [153]. Quercetin blocks the cell cycle at G1/S phase in human colonic COLO320 DM cells [154] and leukemic T cells [155]. Another widely distributed flavonoid, apigenin, significantly induced a reversible G2/M arrest in keratinocyte, fibroblasts, and colonic carcinoma cell lines[156, 157, 158]. Studies on silibinin found that perturbations in cell cycle progression may account for the anticarcinogenic effects on human prostate carcinoma cell lines and colon carcinoma cell line [159,160,161]. Anthocyanins block the cell cycle progression through CDK inhibition in many cancer cell lines [162, 163, 164]. Tea polyphenols inhibit cell proliferation and suppress tumor growth activity. Our laboratory has investigated the effects of EGCG and other catechins on the cell cycle progression [165] .The results suggest that EGCG either exerts its growth-inhibitory effects through modulation of the activities of several key G1 regulatory proteins such as CDK2 and CDK4 or mediates the induction of the CDK inhibitors p21Waf1/Cip1 and p27Kip1.

2-Materials and Method

2.1-Materials

95% ethanol, n-butanol, glacial acitic acid and disteld water.

2.2-Apparatus

Separatory funnel 500ml, 20x20 cm glass plaets, coverd tank, stand, measuring cylinder and capillary tubs.

2.3-Method

The plant was identified by D. Ekram / Alkhartom university / Science College /department of plant science.

2.3.1- Sample Collection

The plant *Anastatica hiecochuntica* was collected from Al Bahr al Ahmar state (Tokar).

2.3.2- Preparation of the Sample

The plant was air-dried after collection. The dried plant was ground into fine powder and then weighed.

2.3.3- Extraction of the Sample

Solvent-solid extraction was carried out on the weighed, air-dried *Anastatica hiecochuntica* .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.

2.3.4- Chromatography

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

2.3.4.1- Thin-Layer Chromatography (TLC)

TLC was used to ascertain the number of constituents present in the extract 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) .

2.3.4.2-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 110^0C to ensure further solidification.

2.3.4.3-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.

2.3.4.4-Devolping 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 colouration reagent which is vanalin solution with concentrated sulfuric acid. The separated components appeared as dark braown-red spots.

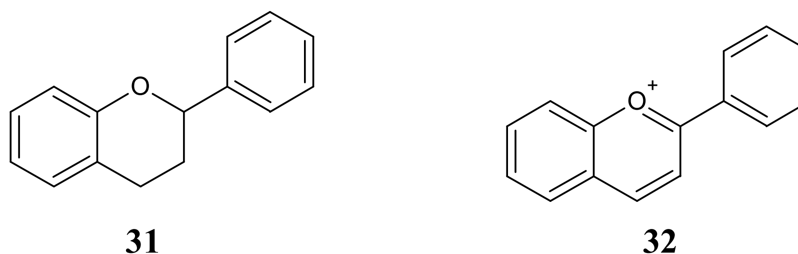
3-Result and Discussion

3.1-Result and Discussion

The flavonoids of *Anastatica hiecochuntica* were extracted by ethanol. After removing the solvent acrude product was obtained which was fractionated by TLC (silica gel, butanol: acetic acid: water (4: 1.5: 6)). After the usual workup a pure flavonoid-compound I was isolated.

In the IR spectrum (fig-1) it gave ν(KBr) 660.5cm^{-1}(C – H, Ar), 1046.5 cm^{-1}(C – O), 1456.96cm^{-1} (C=C ,Ar), 3360.37 cm^{-1}(OH).

Since the IR revealed absence of the carbonyl(C=O) Stretch vibration then this flavonoid is either flavan **31**or an anthocyanines **32**.



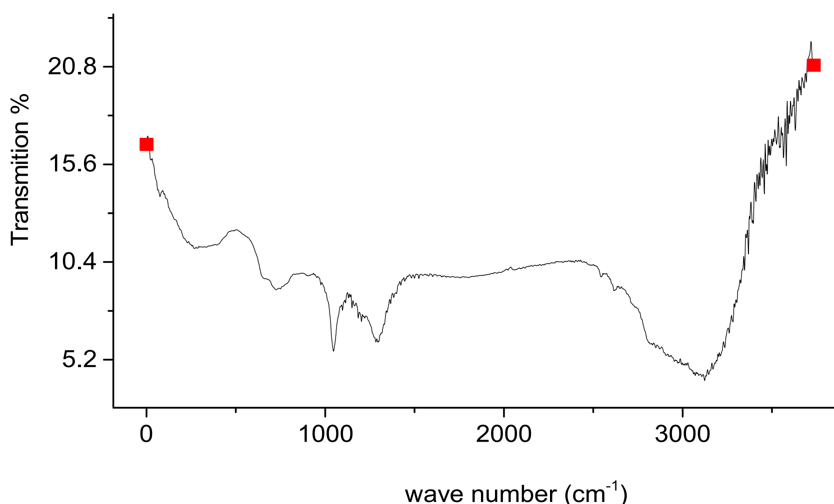


fig-1

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