A brief overview on the methods for extraction and identification of flavonoids

Aidai Turatbekova[1*], *Dilbar* Mirzarakhmetova[1,2,3], *Jumaniyoz* Jumaniyozov[4], *Erkaboy* Khudayberganov[4], *Nusratillo* Toshpulatov[5], *Abdugani* Rakhmatov[5], and *Shavkat* Muzafarov[5]

[1]Kimyo International University in Tashkent, 100121 Tashkent, Uzbekistan
[2]Tashkent State Technical University, Tashkent, Uzbekistan
[3]National University of Uzbekistan named after Mirzo Ulugbek, Tashkent, Uzbekistan
[4]Urgench State University, 220100 Urgench, Uzbekistan
[5]"Tashkent Institute of Irrigation and Agricultural Mechanization Engineers" National Research University, 100000 Tashkent, Uzbekistan

Abstract. Flavonoids are active metabolites of plant cells. The significant biological role of these compounds is evidenced by the nature of their distribution in the plant. Flavonoids play an important role in plant reproduction, development and accumulation of pollen, and their content also determines the color of flowers, fruits, and plant seeds. Most flavonoids are found in actively functioning organs: leaves, flowers, fruits (color, aroma), seedlings, as well as in integumentary tissues that perform protective functions. Different organs and tissues differ in the quantity and qualitative composition of flavonoids. Relatively recently, it was found that flavonoids also influence signaling processes occurring in living systems due to specific interactions with proteins that perform regulatory functions. Numerous data have been accumulated on the influence of these compounds on other protein and non-protein structures, which can lead to changes in the functional state of cells and the entire organism as a whole. Despite the fact that currently the redox properties of phenolic compounds are not given so much importance, their comprehensive study still remains an important task. In addition, numerous studies confirm that flavonoids act as effective phytoalexins, exhibiting a wide range of antibacterial and antifungal activities. After all, a significant role of flavonoids is their function of protecting against various unfavorable factors for plants, such as temperature fluctuations, the influence of ultraviolet rays, attacks by viruses, bacteria, and parasites. A correlation has been established between their content in plants and plant resistance to fungal diseases and some pests. The appearance of these compounds in plants in response to the action of parasitic fungi of cultivated plants and inhibition of the growth of fungal mycelium is considered a protective reaction. Flavonoids with antimicrobial activity are found in large quantities in the seed coat. This review paper highlights the basic technologies for extraction and identification of flavonoids from different type of plants.

1. Introduction

Flavonoids represent the largest class of phenolic compounds. They belong to secondary metabolites of higher plants, however, compared to other secondary metabolites, they are involved in many processes of plant growth and development. The term "flavonoid" was proposed in 1949 by the English scientist Geissman more than a century after the isolation of the first flavonoid quercetin (Quercus) not only for flavones - yellow substances, but also for other compounds of flavonoid nature that have different colors - white or colorless (flavanones), orange (aurons, chalcones), red, crimson, blue (anthocyanins). Flavonoids are the largest group of water-soluble natural phenolic compounds, heterocyclic oxygen-containing compounds of predominantly yellow, orange, and red color. They belong to a series of C6-C3-C6 compounds - their molecules have two benzene rings connected to each other by a three-carbon fragment. Most flavonoids can be considered flavone or flavan derivatives [1, 2].

*Corresponding author: turatbekova888@gmail.com

Flavonoids are natural heteroaromatic compounds that are products of secondary metabolism of plant tissues. It is obvious that this class of biologically active substances, numbering up to 10,000 registered representatives, is characterized by great structural diversity and is of great value as a subject of study. The interest of scientists of various specialties in the study of flavonoids is caused by the variety of biological and pharmacological effects that these compounds exhibit in humans and animals. The biological effect of flavonoids is explained by the regulation of redox processes, stabilization of cell membranes, modulation of the activity of enzymes and receptors [3-7]. To date, the spectrum of action of these compounds in the human body has been determined: capillary-strengthening, spasmolytic, anti-stress, anti-inflammatory, antifungal, antibacterial, antiviral, antiulcer, antitoxic, antiallergic, antiatherosclerotic, antiarrhythmic, antihypertensive, immunomodulatory, anticarcinogenic, nephroprotective, estrogen-like, hepatoprotective [8-12]. The established properties of flavonoids open up wide opportunities for their use as medicines that do not have serious side effects, unlike synthetic analogues.

Along with this, the question of the functions of flavonoids in plants, the direct place of their biosynthesis, is poorly covered in modern literature, which, in our opinion, is a gap in the general biological understanding of their role. In this regard, we analyzed the available information on the physiological role of flavonoids in the plant body.

2. Methods for extracting of flavonoids

Currently, in the scientific world there are two types of methods for extracting plant materials: the first is traditional, which uses simple and inexpensive equipment, a large amount of solvent, and increased extraction time at relatively high temperatures and low pressures, and the second is unconventional methods, which are modern, environmentally friendly , and using expensive and complex equipment that saves time and, as a rule, the ability to work at higher pressures and temperatures. Essentially, the general procedure for obtaining flavonoids has not changed; broadly speaking, it involves exposing plant material to a solvent that allows the solubilization of phytochemicals [13, 14]. The most widely used traditional methods for obtaining bioactive compounds are decoction (boiling), recirculation (refluxing), maceration (simple infusion), and percolation. Unconventional extraction methods can be broadly classified into four categories: mechanical force methods, electromagnetic force methods, electrical force methods, and enzymatic extraction methods for specific biocatalytic reactions.

2.1 Maceration

Maceration is an extraction method consisting of a liquid (water, oil, alcohol, etc.) in which the plant is immersed inside an airtight container (Fig. 1). The process is carried out at room temperature with different periods of time depending on the liquid and plant material used (a mixture of ethanol and water (30–90%), a mixture of methanol and water (60–90%), from 20–60°C, from 2– 72 hours). Environmentally Friendly Methods for Flavonoid Extraction from PlantMaterial: Impact of Their Operating Conditions on Yield and Antioxidant Properties The infusion method in this form has a number of disadvantages: extraction takes a long time; evaporation of the extractant, which causes a decrease in the volume of the tincture; the swollen plant mass cakes at the bottom of the vessel, which requires additional action to eliminate sticking.

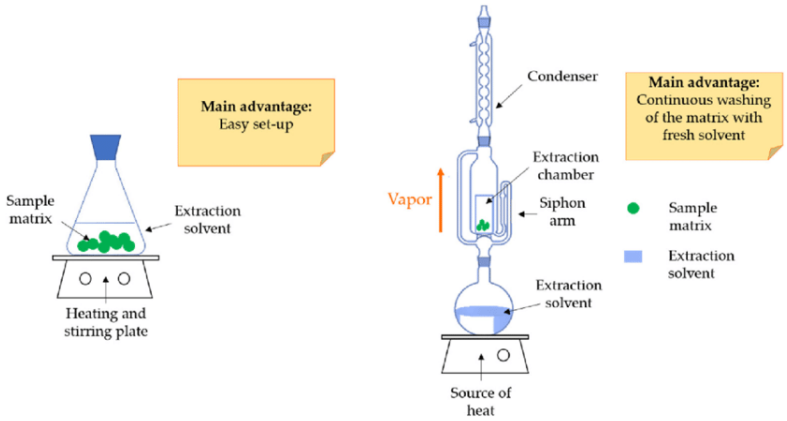

Fig. 1. Maceration extraction (ME) with a source of heat (left) and Soxhlet extraction (right) [15]

2.2 Percolation

Percolation is an extraction method in which the plant is placed in a narrow cone-shaped vessel, open on both sides, called a percolator extractor, and a liquid [16] (solvent: water; ethanol; methanol; a mixture of ethanol and water (50–95%) is passed through it; mixture of methanol and water (80%), from 10–25°C for 24–72 hours) (Fig. 2). This process is carried out at room temperature by passing liquid between solid substances drop by drop, while the extracted substances pass from the raw material to the extractant as a result of their dissolution and diffusion. The percolation rate must be such that the diffusion of the extracted substances into the extract has time to occur. Extracts obtained by infusion or percolation require mandatory purification.

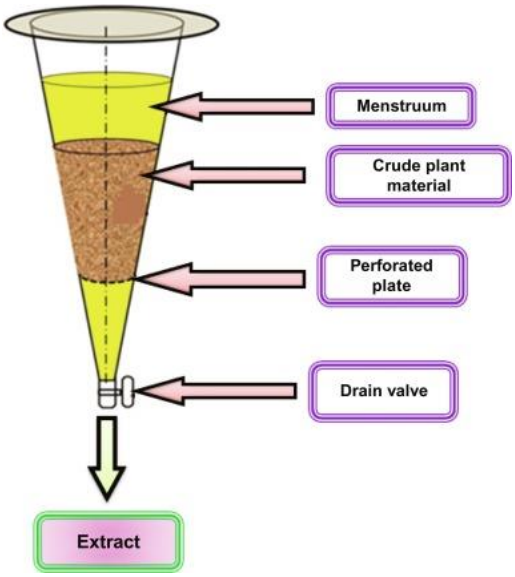

Fig. 2. Percolation extraction method [17]

2.3 Soxhlet extraction

Soxhlet extraction is a method that uses the principles of recirculation and siphoning to continuously extract plant materials with fresh solvent (Figure 3). Compared to other extraction methods such as reflux and percolation, the Soxhlet extractor has several advantages. This is a highly efficient automated method that requires less solvent and time than percolation and maceration [18]. A good example of this method is the extraction of flavonoids from Vernonia cinerea leaves using ethanol (60%) in just 2 hours. However, it is important to note that high temperatures and long extraction times can increase the likelihood of thermal degradation, such as the degradation of catechins in tea.

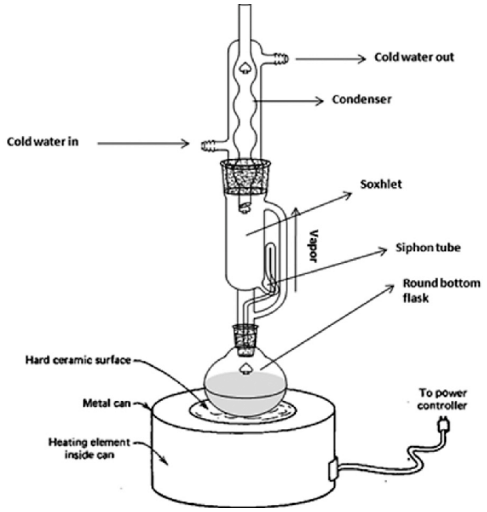

Fig. 3. Soxhlet extraction method

2.4 Extraction of flavonoids in the presence of surfactants

The creation of drugs based on the most complete natural complex of biologically active substances of medicinal plants seems to be a promising and relevant area of research. The introduction of a surfactant into a two-phase extractant system can significantly affect its extraction properties. It can be assumed that in this case the range of extracted substances will expand and their quantity will increase, because during the emulsification process in the presence of a surfactant, the surface tension decreases, and the interface between the phases through which mass transfer of lipophilic biologically active substances occurs increases. Solvents used in the compositions of medicinal and cosmetic products (DMSO, PEO 400, PG, their mixtures with water) have a high extracting ability with respect to flavonoids. The use of the studied extractants makes it possible to obtain extracts that, without removing solvents, can be introduced into soft dosage forms and cosmetic creams. DMSO as an extractant exhibits a monotonous increase in extraction ability in relation to both flavonoids and AP as its concentration in aqueous solutions increases [19, 20, 21]. Apparently, this is explained by the high dissolving properties of DMSO, as well as its ability to diffuse into the cell and desorb BAS. However, the nature of solvation of plant materials and desorption of biologically active substances for DMSO is different than for alcohol, because DMSO is an aprotic solvent. Therefore, the total extraction capacity of DMSO does not exceed that for aqueous solutions of PG and PEO 400.

2.5 Extraction under microwave irradiation

One of the effective methods for extracting plant materials is microwave processing in a microwave field. Microwave radiation is taken to be a section of the electromagnetic spectrum with oscillation frequencies from 30 MHz to 3000 GHz (wavelength from 10 m to 0.1 mm).

Extraction using a microwave field allows you to achieve high degrees of extraction in a shorter time (15 - 30 minutes), while the consumption of solvents is significantly reduced. The gain in time is achieved by increasing the boiling point of the solvent, which makes it possible to increase the temperature of the reaction, as well as constant stirring [22-25]. More precise control over reaction parameters (temperature, time) allows obtaining more reproducible results. In addition, microwave extraction makes it possible to isolate a larger number of components from the analyzed samples, which makes the analyzed sample more representative.

Heating when using the microwave irradiation method occurs for two reasons: the electrical component of the microwaves accelerates the movement of the molecules having a dipole moment, and the magnetic field strength created by the microwaves promotes the vibrational movements of the molecules having a dipole moment. Intermolecular friction leads to the absorption of electromagnetic radiation and an increase in the temperature of the substance. The dielectric constant of the solvent has a great influence on the extraction process. In polar extractants (with a high dielectric constant), substances containing polar molecules dissolve well, and in nonpolar extractants, substances with nonpolar molecules dissolve well.

3. Methods for identifying flavonoids

To isolate flavonoids, plant material is extracted, usually with ethyl, methyl alcohol or aqueous alcohols (most often, 70% alcohol is one of the optimal extractants). Identification of flavonoids is carried out based on their physicochemical properties. The melting point, specific rotation of glycosides are determined, and UV, IR, and mass PMR spectra are compared with the spectra of known samples.

3.1 UV spectroscopy

Spectrophotometric determination based on intrinsic absorption maxima in the form of direct or differential spectrophotometry is one of the most common methods for the analysis of flavonoid compounds. Recently, a large amount of material has been accumulated on UV spectroscopy of flavonoid compounds, with the help of which it is possible to identify not only the basic structure of flavonoids, but also to determine the number and position of hydroxyl groups and sugar residues. The spectrophotometric method of analysis is based on the selective absorption of monochromatic light by a solution of the substances under study. Absorption is caused by electronic transitions from the orbit of the donor substituent to the vacant orbit of the benzene ring or acceptor substituent. Flavonoids in the UV spectrum are characterized by two intense absorption bands in the long-wave region 320-380 nm (I band) and in the short-wave region 240-270 nm (II band), and for flavonols 350-390 nm and 250-270 nm, respectively, an additional maximum at 300 nm. The distance between the main maxima is more or less constant and for flavonols it is 93-125 im, which can serve as a distinctive feature. Spectral studies of alcoholic solutions of flavonoid compounds

show that hydroxyl groups have a significant bathochromic to hypsochromic effect on absorption maxima depending on their positions. The greatest influence is exerted by hydroxy groups conjugated with carbonyl; other groups are of auxiliary importance [26]. Therefore, when taking UV spectra, various reagents are used that affect the chromophoric system of flavonoids, which manifests itself in the form of bathochromic or hypsochromic shifts of the main absorption maxima. The working wavelength ranges include both long-wave and short-wave maxima. Sodium ethoxide, sodium acetate, sodium acetate with boric acid, aluminum chloride and zirconyl chloride with citric acid are widely used as ionizing and complexing additives. In the presence of a hydroxyl group at position C-7, a bathochromic shift of band I is observed under the influence of sodium acetate. In flavones and flavonols, the free hydroxyl group in the 4-position is established in the presence of sodium ethoxide by a bathochromic shift of the first band by 40-64 nm without a decrease in intensity. The hydroxyl group at position C-3 in flavonols in the absence of hydroxyl at C-4" also causes bathochromia of the first band at 50-60 nm, but with a decrease in intensity. If hydroxyl groups are present at C-3 and C-4" hypsochromic is observed simultaneously shift, which is due to the oxidation and destruction of 4"-hydroxyflavonols in an alkaline environment. The ortho-dioxyl group in the side phenyl radical is established by the bathochromic shift of the first band in the presence of anhydrous sodium acetate with boric acid by 25 nm. Aluminum chloride and zirconyl salts make it possible to determine free hydroxyl groups at position C-3 and C-5 according to the bathochromy of band 1. If, when citric acid is added, the bathochromic shift disappears, then that is due to the 5-hydroxy group. A stable shift to citric acid is exerted on the free hydroxyl group at position 3. Free hydroxyls at C- 3 and C-5 cause a double bathochromic shift of 1 band under the influence of zirconyl chloride, reaching 100 nm or more. Flavonols in the presence of aluminum chloride and hydrochloric acid cause bathochromia of the first band by 50-60 nm, and a shift of the second band by 20-26 nm.

3.2 IR spectroscopy

When analyzing flavonoid compounds, spectral studies in the IR region are widely used to establish and confirm the structure of the molecules of substances. IR spectroscopy also allows one to determine the configuration and conformation of molecules. IR spectra are caused by vibrations of the atoms of the molecule. In this case, the vibrations have different energies and can be directed along the valence bond between the atoms. Vibrations of all atoms of a molecule determine absorption bands individual for a given substance. The fingerprint region (1400-650 cm-1) is mainly used for identification purposes. This region is most often used to establish identity by side-by-side comparison of the IR spectra of the test and known compounds. The presence of functional groups in a flavonoid molecule is determined by the characteristic absorption in a certain region of the spectrum [27, 28]. Thus, it was found that in the IR spectra of flavonoids the unsubstituted carbonyl group of flavanone absorbs at 1660-1690 cm-1. The C=O stretching vibrations of the Flavonol group are in the region of 1637-1650 cm-1. The presence of a hydroxyl group in the C-7 position reduces the frequency of stretching vibrations of this group by 15-10 cm-1. The formation of a hydrogen bond between the C=O and OH groups at the C-5 position explains the decrease in the C=O frequency to 1640 cm-1. Stretching vibrations of double bonds appear in the form of several intense absorption bands in the region of 1600-1470 cm-1. Vibrations of the CH group of aromatic rings with a double bond conjugated to C=0 appear in the region of 3130-3110 cm-1. Free aliphatic hydroxyl groups absorb in the range of 3625-3600 cm-1. And phenolic hydroxyls of the aglycone are determined in the region of 31 3300-2700 cm-1. The 0H groups of carbohydrate substituents appear in the region of 3600-3300 cm-1. Using IR spectra, L- and B-anomers of monosaccharides and their derivatives are distinguished. The L-configuration of the C-0 bond is characterized by a band at 844 ± 8 cm-1, and the L-configuration is characterized by a band at 891 ± 7 cm-1. The combination of IR spectroscopy with chromatography in thin layers of sorbent makes it possible to increase the selectivity of qualitative detection of substances in a mixture. The amount of the test compound that can be removed from a thin layer chromatogram is sufficient to obtain IR spectra of small samples. This allows the use of IR spectroscopy in the analysis of biologically active substances, the content of which in plants is usually low.

3.3 NMR spectroscopy

NMR spectroscopy makes it possible to determine the structure of flavonoid molecules, their conformational structure and electron density distribution. When combined with UV and IR spectroscopy, it provides very valuable information on the structure of flavonoids.

By the number of signals in the NMR spectrum, you can determine how many types of protons are present in the molecule, and by the position of the signals, you can determine the type of protons. Due to the low solubility of

glycosides in low-polar and non-polar solvents used to obtain spectra, they are in most cases studied in the form of acetyl or trimethylsilyl derivatives. Using NMR spectra, it is possible not only to quickly and accurately determine the position of substituents in rings A and B of a flavonoid [29, 30], but also to decipher the structure of the carbohydrate component, determine the configuration of the glycosidic bond, and the nature and conformation of the carbohydrate.

4. Methods for quantitative determination of flavonoids

Currently, various physicochemical and spectral methods of analysis are becoming increasingly widespread, which have a number of significant advantages in comparison, for example, with gravimetric and titrimetric methods, namely the speed and accuracy of determination, the detection of even small quantities and, most importantly, the ability isolation of individual flavonoids from plant materials. Such methods include HPLC [31], chromatography spectrophotometry, spectrophotometry, photoelectrocolorimetry, densitometry using chromatography on paper and in a thin (fixed and unfixed) layer of sorbent. If it is necessary to use a chromatospectrophotometric method, then chromatography (BC, TLC, column) is used both for purification and for separating the sum of flavonoids into individual components.

4.1 Spectrophotometric method

The spectrophotometric method is based on determining the optical density of a solution of analytes at a certain wavelength. For example, in the case of milk thistle fruits and poplar buds, direct spectrophotometry is used, but most often, due to the possible contribution of other aromatic substances to the optical density of the analyzed solutions, it is necessary to resort to purification of the sum of flavonoids (without chromatography) or to a complexation reaction. For pharmacopoeial analysis, a solution of aluminum chloride (St. John's wort herb, etc.) is usually used. Based on the data of the State Pharmacopoeia, in more than 60% of medicinal plant materials containing flavonoids, the quantitative determination of flavonoids occurs through the use of a spectrophotometric method after a complexation reaction.

For example, flavones and flavonols, in particular, rutin, are characterized by two absorption maxima - short-wavelength (260 nm) and long-wavelength (362 nm), which can be used not only for the purpose of identifying substances, but in terms of quantitative assessment, especially under conditions of differential spectrophotometry [31, 32]. In this case, in the presence of AlC13, a bathochromic shift of the long-wavelength band is formed with the formation of a maximum at a wavelength of 412 nm (analytical wavelength). This approach is one of the most used in the analysis of medicinal plants containing flavonoids, since it allows minimizing the contribution of accompanying substances to the optical density of the solutions under study (see St. John's wort herb).

4.2 Chromatospectrophotometric method

The name of the method means that it combines two approaches - chromatographic purification of the total or individual flavonoids and subsequent spectrophotometric determination of the target substances. The chromatospectrophotometric method can be carried out in various modifications, but in general they can be divided into 2 groups:

1. Chromatographic separation of flavonoids from accompanying substances by TLC or HD (for example, determination of rutin in medicinal plants) [33, 34]. Methods for the quantitative determination of rutin in buckwheat grass and Sophora japonica buds are based on chromatospectrophotometry, and in the first case, paper chromatography is used, and in the second, TLC. In both cases, the technique of separating rutin from accompanying flavonoids is used, and then the optical density of the eluate is measured. The methods use GSO routine.
2. Chromatographic separation of flavonoids from accompanying substances using column chromatography (for example, Lespedeza kopeechnikova). The developed method for quantitative determination of the amount of flavonoids in the aerial part of Lespedeza is based on isolating the amount of flavonoids and determining the optical density of a solution in ethyl alcohol at a long-wave absorption maximum (353 nm), followed by calculating the percentage based on the specific absorption indicator of pure homooretin (luteolin-6-C- c-0-glucopyranoside).

4.3 Photocolorimetric method

The photocolorimetric method is based on the diazo coupling reaction, as well as on the basis of color reactions of flavonoids with salts of various metals (aluminum, zirconium, titanium, chromium, antimony), with a lemon-boron

reagent and on the reduction reaction with zinc or magnesium in an acidic environment. Methods of more theoretical significance:

1. Polarographic method. It is based on the ability of flavonoids, such as flavonols and flavones, to be reduced on a mercury drop electrode [35].
2. Method of acid-base titration in non-aqueous solvents. The method is based on the ability of flavonoids to exhibit mild acidic properties (due to the presence of phenolic hydroxyls in the molecule, especially the 7-OH group). The acid-base titration method is carried out in non-aqueous solvents - dimethylformamide, dimethyl sulfoxide, acetone.
3. Densitometric method. The method is based on color reactions, and it does not require additional operations to isolate substances from chromatograms.

5. Conclusions

This succinct study covered the many techniques for extracting and identifying flavonoids from natural sources as well as the key factors of influence (temperature, solvent, sample size, and extraction time, among others). In-depth discussions of current developments and their industrial significance also gave an understanding of their potential. Thus, in an effort to provide a wider perspective on the understanding of flavonoid extraction from various plant matrices, this review examined the advances of compound extraction strategies, highlighting in each of them their merits and disadvantages.

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