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Methods of Isolation and Extraction of Anthocyanin's from *Hibiscus sabdariffa* L.: Literature Review

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

The classic method for extracting anthocyanins from plant tissues is the same as for other phenolics: the tissues are soaked and then extracted with a solvent in a process known as Solid Liquid Extraction. Modern approaches to anthocyanins extraction include;Supercritical fluid extraction, Ultrasound-assisted extraction, Pressurized liquid extraction, Microwave-assisted extraction, Ohmic heating-assisted extraction, Precipitation, and Solid phase extraction. The aim of this article is to review the methods used for Anthocyanins extraction from *Hibiscus Sabdariffa*.The study for the same has been done at Charak's Pharmacognosy Laboratory, MIT-WPU School of Pharmacy, Pune. Usually, modern methods seem to have an edge againsttraditional methods in extraction procedures. This article discusses these methodsand tries to identify if it is the case in the case of anthocyanins extraction from *Hibiscus sabdariffa* L.while individually commenting on the method's advantages or disadvantages too.

Keywords: Anthocyanins; Hibiscus Sabdariffa; Modern methods of extraction; colorant; pigments.

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1. INTRODUCTION

Richard Willstatter 1 investigated the origins of color in nature in the early 1900s, discovering that flowers that display various colors, these colors were dependent on mixtures of a variety of anthocyanins in various structural concentrations [1].

As anthocyanins are major constitutes which contributeto the color diversity in fruits and flowers, they have become the focus of intense investigation to see if they have the ability to establish the use of themselves as natural coloring agents and eliminating artificial colorants in cosmetic, food and other industries. Also, Anthocyanins have anti-cancer, antimicrobial, anti-diabetic, anti-inflammatory, anti-obesity, and anti-obesity properties, as well preventing cardiovascular diseases. Therefore, anthocyanin's ingredients derived from edible plants have the potential to be used in pharmaceuticals [2,3,4].

Anticancer activity of hibiscus was seen in the Phase 2 enzyme activation part by treating the rat liver clone 9 cells with 50 3M anthocyanin's and benign breast cells with 10–20 µg/ml anthocyanins [5]. The anthocyanin components Delphinidin-3-O-glucoside, Malvidin-3-O-glucoside and Petunidin-3-O-glucoside are responsible for the anti-diabetic effect found [6]. Isolation of Delphinine 3-sambubioside from the dried calyces of *Hibiscus sabdariffa* L show cytokinine production suppression when used as follows:- 50 to 200 mM of Delphinine 3-sambubioside or 100 mM of Delphinidin for 30 mins [7].

Anthocyanins are predominantly found in violet, blue and red flowers. Some of the common red flowers include hibiscus, rose, Indian shot, and many others and they come in a variety of colors. Violet flowers that are edible are horsemint, lavender and chaparral sage, blue flowers are blue dandelion and Himalayan blue poppy.

Hibiscus sabdariffa L. (hibiscus flower, roselle, bissap, jamaica flower) belongs to the Malvaceae family and thrives in tropical and subtropical climates. India, Mexico, Thailand, and Senegal are major growers of *Hibiscus sabdariffa* L., which is used for ages for its medical usage and human consumption in these countries. Mainly consumed through cold and hot beverages worldwide [1].

The flower of the *Hibiscus Sabdariffa* is a natural source of anthocyanins, which give it its rich red color and make it an attractive yet inexpensive source of natural colorants. Frimpong et al. (2014) found that using an aqueous extract of H. sabdariffa calyces at a concentration of 33 percent w/v solution to color three pediatric oral formulations was successful. The compositions of colored extracts were vulnerable to degradation when exposed to a higher temperature, high pH and light. In oral formulations of pediatrics, amaranth can be replaced by H. sabdariffa extracts of calyces as a coloring ingredient by keeping the extracts at pH 5 in buffer condition and protected from light and elevated temperatures [8].

Keyanthocyaninsfound in the *Hibiscus Sabdariffa* are Cyanidin-3-O-sambubioside (Cy-samb) and Delphinidin-3-O-sambubioside (Dip samb) [1].

Cyanidin is a pigment that is reddish-purple in color. It's the pigment that gives berries and other red-colored foods their color. The chemical properties of delphinidin are comparable to those of most anthocyanidins. In the plant, it shows as a bluish-red or purple pigment. The delphinidin pigment is responsible for the blue color of flowers.

The pH of the fluid affects the colour of anthocyanins. As anthocyanins' molecular structure is ionic, this is the fact. The color of the *Hibiscus Sabdariffa* is red as the anthocyanins present have acidic nature (pH 3.70). The pH of the aqueous phase plays an important during extraction of anthocyanins because they are highly dependent on pH. The chart below shows the relationship between the pH of the aqueous medium of the extract and the color obtained from the anthocyanin extract. It is easy to decipher that when the pH range is 1-4, the color of anthocyanins shifts from dark red to light red. Solution color becomes colorless at pH 5 and gradually, the rise in the pH to 7 gave the change in the color to blue [9]. Thenatural stabilization process for anthocyanins can be done by pH adjustment [10].

The toxicity effects of alcoholic solvents such as methanol and ethanol on the anthocyanin pigments during extraction limit its use. Therefore, water is used as a potential solvent for extraction as it is much compatible and gives a good yield.

The particle size of the mixture of calyces is also an important parameter. Intensities of color obtained from the extract is greater when the powdered mixture of calyces is fine in nature.

Anthocyanins are isolated as a crude combination from plants. As a result, specific types of anthocyanin must be separated or isolated. Various chromatographic methods such as thin-layer chromatography, high-performance liquid chromatography, cellulose column chromatography, reversed-phase ion-pair chromatography, high speed countercurrent chromatography, and gas chromatography are used for extraction.

Quantification of these compounds is also necessary along with separation and identification, for which High performance chromatography is a favored method.

For the purification of these phenolic pigments, macroporous adsorption resin is used, AB-8 resin is specifically invented for the purification of flavonoids. [5]

The present review seeks to compile the available information of variouson methods of anthocyanins extraction from *Hibiscus Sabdariffa* in an effort to facilitate a better understanding of each methodology's potential.

2. REVIEW RESULTS

2.1 Traditional Methods for Extraction of Anthocyanins

2.1.1 Methanol Extraction [11]

The most frequent method of obtaining anthocyanins from plant sources is methanol extraction. In this method, anthocyanins are obtained by plant material maceration or by soaking in methanol with a small amount of mineral acid. This method is easy, convenient, rapid and efficient for anthocyanin extraction. This method is widely employed because methanol has a low boiling point and this allows rapid concentration of the extract. However, a major disadvantage of this approach is that it produces a crude aqueous extract containing many impurities, and evaporation of methanol causes hydrolysis of volatile acyl bonds and this is exacerbated due to hydrochloric acid present

2.1.2 Extraction Using Ethanol Solutions [12]

Ethanol solution with some amount of water can be used for the extraction of anthocyanins from *Hibiscus sabdariffa* L. Because a little water quantity is necessary for extracting the hydrophilic anthocyanins, a 100% ethanol concentration could not be employed for the extraction. But on increasing the percentage of water to ethanol up to 50% significantly increases the anthocyanin content of the hibiscus in the extract. Further on increasing the concentration of alcohol results in the decrease in the anthocyanin content in the extract. This was due to the presence of a higher percentage of alcohol and a lower percentage of water which resulted in the non-extraction of hydrophilic anthocyanins present in the hibiscus flower. Maximum content of anthocyanin is obtained by using 50% of alcohol and the lowest content was obtained using 80% of alcohol. The antioxidant activity of anthocyanins was the highest using 50% of ethanol.

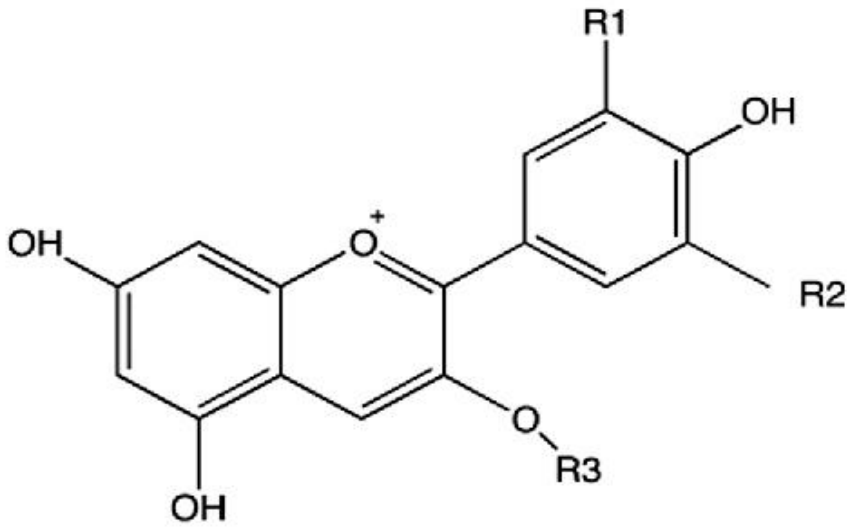

Fig. 1. Basic Anthocyanins Structure

Chart 1. Analysis of chemical compounds

	R1	R2
Cyanidin	OH	H
Delphinidine	OH	OH

Chart 2. Color measuring with pH range

pH range	Color obtained
1-4	*Dark red – Light red*
5-6	*Colorless*
7	*Blue*

2.1.3 Extraction Using Acidified Water (1% HCL) and Ethanol [12]

Anthocyanins from hibiscus can be efficiently extracted using acidified water and ethanol. HCL can stabilize the pigments present and helps to decrease the pH to the level. But the change in the pH changes the color of anthocyanin, therefore, 1% of HCL is used. Ethanol and Hydrochloric Acid were used very carefully and judiciously. This process resulted in the extraction of more anthocyanin content than 50% aqueous solution ethanol and pure water extraction technique. Highest anthocyanin content was obtained in 50% ethanol in acidified water as extracting media. Antioxidant capacity was the highest at 50% acidic ethanol extracts.

2.1.4 Extraction using acetic acid solution [12]

According to the methods for extraction in literature, it is mandatory to use weakly acid media for the prevention of hydrolysis of acylatedanthocyanins. Different types of anthocyanins can be obtained by using weakly acidic mediums. Acetic acid is most suitable for maintaining weakly acidic media. The growing concentration of acetic acid from 1-2%, significantly increases the amount of anthocyanin content in the extraction. Using 2% of acetic acid gives the highest amount of anthocyanin content. At this percentage, the antioxidant capacity of anthocyanin was the highest.

2.1.5 Extraction Using Ethanol Acidified With (0.5 – 2% Lactic Acid) [12]

Anthocyanin can be easily extracted using an acidified solution of ethanol with lactic acid. This method gave the highest yield of anthocyanin in contrast to the pure water extraction of anthocyanin. Lactic acid is used in the concentration of 0.5-2% in 80% of ethanol. The maximum yield of anthocyanins is obtained by using 2% of lactic acid in 80% ethanol, and eventually, this extract had the highest antioxidant activity. Also, this method is more convenient and gives the highest anthocyanin content w.r.t ethanol extraction, ethanol acidified water extraction and extraction using acetic acid.

Amount of extract obtained in each method [11]

Ethanol acidified with lactic acid > 50% Ethanol with acidified water (1% HCL) > Ethanol aqueous solution > 2% Acetic acid

2.1.6 Extraction with Acetone and Chloroform Partition of Anthocyanins [11]

Acetone helps in the extraction of anthocyanins easily and partition using chloroform helps in further isolation and partial purification of the pigments. Due to the addition of chloroform, aqueous phase containing anthocyanins, phenolics, etcetera is separated from the bulk phase which contains lipids, some amounts of solvents, etcetera. The greatest advantage of this method is that anthocyanin extract is obtained with no lipophilic contamination. The risk of pigment degradation is also minimized due to the absence of concentration step.

2.2 Modern Methods for Extraction of Anthocyanins

2.2.1 Ultrasound-assisted Extraction [13]

In this method, hydration of plant materials takes place because of the ultrasound frequencies and leads to enlargement of cell wall rupture. Mass transfer is promoted by this step, therefore increasing the extraction yield. Ultrasound-assisted extraction has the advantages of requiring fewer solvents, requiring no CO2, and consuming less energy. The only disadvantage of this technology is that it has relatively high production costs compared to other methods that produce equivalent yields at lower prices.

2.2.2 Microwave-assisted Extraction [13]

In this technique, both the target tissue and solvent are evenly heated. This technique requires alower amount of solvents, yields good results and consumes less time. Extraction takes place takes place because of disruption of the target cell as the water present in the hydrated tissues absorbs energy with the resulting heat. Another advantage is that compound diffusion form the matrix to the solvent enhances, which increases the yield of extraction and making nontargeted compound extraction easier. The major disadvantage of this technique is that anthocyanins are susceptible to degradation due to an increase in temperature.

2.2.3 Supercritical Fluid Extraction (SFE) [13]

This is a method for extracting anthocyanins that uses supercritical fluids (ata vapor liquid critical point). The traditional method of SFE is done using supercritical CO2 (scCO2), a polar compound is used for the extraction of anthocyanins. A mixture of CO2 and ethanol is most commonly used. Advantages of this method are inhibition of enzymes that degrade anthocyanins due to use of scCO2 and pressure, removal of nonpolar components by pretreatment of samples with supercritical CO2 and reduction of anthocyanin oxidation due to total absence of light and atmospheric O2. The major disadvantage is the production costs as they are very high.

2.2.4 Solid Phase Extraction of Anthocyanins using Capillary Electrophoresis [14]

This process helps in the extraction of Delphinidin-3-sambubioside, cyanidin-3-sambubioside along with minor components such as, delphinidin-3-O-glucoside, cyanidin-3-O-rutinoside, chlorogenic acid and cyanidin-3, 5-diglucoside [15]. This method is useful for the components which involve arduous and tedious methods for extraction. [16] This technique efficiently separates charges compounds. Extraction is carried out in the following way-

I. Calyces are sun dried and cleaned.
II. These are then reduced to powder by usinga mortar.
III. The resultant solution is then mixed with MeOH/HCL for 4h at room temperature.
IV. Then this is magnetically stirred and filtered.
V. At last, water is mixed (2ml) and this mixture is passed through a 5micrometer membrane filter and keep for Capillary Electrophoresis. [17-21]

3. CONCLUSION

Both the traditional/natural and modern methods were discussed above give good amount of anthocyanin extract from *Hibiscus sabdariffa* L. flowers. The variety of options is enormous, ranging from very simple procedures to more technical ones, and given the dearth of plain publications that evaluate various methods and techniques for a specific sample, it's difficult to say whichis a better methodology based just onthe yield of extraction. The use of acetone (acidified) as a solvent for extraction ensures a low-pH solution. Furthermore, the aqueous fraction will have a low pH, promoting anthocyanin stability. A higher proportion of chloroform in the aqueous phase decreases the quantity of acetone in the solution and may obviate the requirement for rotary evaporation to remove the acetone. A higher proportion of chloroform in the aqueous phase in the partition technique decreases the quantity of acetone in the solution and may obviate the requirement for rotary evaporation to remove the acetone. The acetone/chloroform technique reduces the possibility of hydrochloric acid concentration in the extract since the majority of the extract is water (higher boiling point) with tiny amounts of organic solvent. However, during methanol extraction (lower boiling point),frequently evaporation of alcohol to dryness, increasing the risk of anthocyanin breakdown. When compared to the methanol technique, the acetone/chloroform technique has the benefit of not contaminating the aqueous anthocyanin with lipophilic molecules. For obtaining a good amount of extract, Ethanol acidified with lactic acid technique is more suitable than other methods. However, other methods listed also give a good anthocyanin yield with better antioxidant properties. Percentages at which gives adequate amount of yield are 80% (for ethanol extraction), 50% (for ethanol with acidified water), 2% (for acetic acid) and 2% of lactic acid in 80% ethanol. These methods are time consuming is the major drawback but anthocyanins are less susceptible to degradation in these methods.

Modern methods are fast, sensitive and give accurate results as compared to the traditional methods. Supercritical Fluid Extraction is an

attractive strategy for obtaining relatively pure and clean extracts since it allows for the elimination of nonpolar solvents; however, if followed by an adequate purification process, a simpler and costly extraction approach may be of interest Without utilizing harmful solvents, ultrasonic aided extraction may effectively increase the extraction performance of the total anthocyanins and total phenolic compounds. Techniques that require higher temperature such as Microwave assisted extraction, can promote degradation during the extraction process as there is no specific temperature obtained which gives a good yield and also prevents degradation. Extraction using Capillary Electrophoresis involves the use of Capillary Electrophoresis technique along with the combination of Mass Spectrophotometers which makes this technique more effective. Glycoside group positions can be roughly determined by this technique and various compounds including proanthocyanidin and monomeric phenolic compounds can be analyzed. Charged based separation is achieved through this technique and this technique eliminates complicated pre-treatment steps. Modern methods are more reliable but the only drawback is that, cost is higher than other methods which provide adequate amount of yield at lower costs.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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