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ULTRASONIC EXTRACTION OF ANTHOCYANIN'S AS NATURAL DYES FROM *HIBISCUS SABDARIFFA* (KARKADE) AND ITS APPLICATION ON DYING FOODSTUFF AND BEVERAGES IN KINGDOM OF SAUDI ARABIA

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ABSTRACT

Dyes derived from natural resources have appeared as an important alternative to synthetic dyes. Therefore, there is a need to develop the techniques of extracting the best ingredients of solid and liquid for natural dyes colorants from plant materials and their application in the field of research. This is done through boiling and extraction of the dye anthocyanin from *Hibiscus sabdariffa* plant by solvents, such as: acidified methanol, water, ethanol and acetic acid. In this chemical survey, the extraction of natural colorant from *Hibiscus sabdariffa* plant has been studied. This study has relied on the extraction process. The extracted dye was then concentrated by rotary evaporator. Then analytical studies of the material extracted were conducted by UV-VIS spectroscopy and gravimetrical analysis so as to determine the structure of natural dyes extracted from *Hibiscus sabdariffa* plant. Through the analysis of the results, it was found that the dye extracted from *Hibiscus sabdariffa* plant of intense red color is anthocyanin (3, 5, 7- trihydroxyflavylium**)**, then the plant-origin-dye, which is considered as one of the most multi-colored dyes, gain their importance from their very low percentage of toxics, as well as their bright and shiny colors. These natural dyes could also be used as an alternative source of artificial pigments that are characterized by their bright and multiple types and colors and their lack of stability when exposed to light, washing and contaminations from the environment. In addition to harmful effects of these dyes as some organic materials which are embedded in the synthetic dyes have side effects. The dying process depends on the type of bonds that arise between the dye and the material to be stained. Therefore some dyes exhibit defined affinities for binding with some specific materials, for example; animal tissues, cellulose fibers, silk or synthetic fibers, foodstuff and beverages. During staining the dye enters into the interspaces of the material to be stained and be absorbed through Van der Waals' forces, hydrogen bonding or some other chemical bonds, by eliminating some molecules such as H_2O, HX, and NH_3 .… etc. Therefore, we recommend extracting natural dyes from plant because it is effective in using, safely and anti-environmental application and it can be used in the industrial field especially for colorant of foodstuff and beverages in Kingdom of Saudi Arabia.

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INTRODUCTION

Nowadays, the consumer preference for natural products instead of artificial ones and this has acquired an important role in the food industry, at least in part, due to nutritional supplementation and healthy properties associated with them. Among them, karkade (*Hibiscus sabdariffa* L.), an annual herb that belongs to malvaceae family [1-2], the plant is known locally in Kingdom of Saudi Arabia as ajar and it is widely known as a result of its calyces have been widely used to prepare cold and hot beverages in many of the world's tropical and subtropical countries as well as in phyto-therapeutic applications [3-8]. Many biological activities have been extensively reported, such as antihypertensive [9] and cardio protective agents [10,11], hepatoprotector [12], inhibitor against porcine pancreatic amylase [13,14], as well as sedative [15] and antioxidant capacity [16-18]. Moreover, karkade has gained an important position in the soft drink market and commercial preparations of *Hibiscus sabdariffa* extract are currently marketed as supplements due to their apparent potential health benefits and as a colorant to replace some synthetic dyes.

The intense red pigments in the red calyces of karkade are anthocyanin's, which are *O*-glycosides derivatives of 3, 5, 7- trihydroxyflavylium cation and their analysis is usually carried out and detected by mass spectroscopy [19,20]. Differentiation found on anthocyanin's are related by the number of hydroxyl groups, the nature, position, and number of sugars (*e.g* D-glucose and D-GA lactose) attached to the molecule and the number of aliphatic acids (*e.g.*, acetic, malic and malonic) or aromatic acids (*e.g.*, caffeic and *p*-coumaricorferulic acid) linked to sugars in the molecule [21]. Among eighteen known naturally occurring aglycones are common in higher plants, they are named as pelargonidin (Pg), peonidin (Pn), cyaniding (Cy), malvidin (Mv), petunidin (Pt) and delphinidin (Dp) [22-24]. The glycosides of the three non-methylated anthocyanin's (Cy, Dp and Pg) are the most prevalent in nature [25] and their structures were elucidated using spectroscopic and chemical transformation methods [26,27].

Organic dyes, whether natural or synthetics, are compounds of molecular structure that contain π- electrons [28] which can be affected by light beams that make the electrons oscillate between lower and higher energy levels. The appearance of the color depends on the following [29-32].

- Presence of chromophoric groups within the molecular structure.
- Presence of conjugate double bonds that help the polarization of the molecule
- Presence of auxochromophoric groups.

Anthocyanin's pigments are responsible for the red, purple and blue colors of many fruits, vegetables, cereal grains and flowers. They have long been the subject of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents [33,34]. They have also been very useful in taxonomic studies to study these compounds because of their obvious importance to the color quality of fresh and processed fruits and vegetables [35,36]. Our laboratory first started studying these compounds because of color degradation of strawberry preserves, fruit juice concentrates and wine. Subsequent projects were designed to determine their utility as natural food colorants and to authenticate fruit juice which may prevent disease.

Edible colors

Some edible plants contain colored ingredients that could be applied to color foodstuffs and other materials. These colored ingredients are natural phenol compounds, generally characterized by especial structural formula that contains more than one aromatic ring, which includes some functional groups. Each of these compounds, is characterized by a specified core structure, with some other substituent's, such as rings or OH, OR radicals.

These colored ingredients; which include the following group of compounds, are present in many plants, with various complicated structural forms:

1- Flavones
2- Coumarins
3- Xanthones
4- Anthraquinones

and also include some other carotenoids of high terpenes.

The structural formula of these colored compounds includes some functional groups that can be used to bind into the matrices of the materials to be colored. In the binding process, the colored molecules would be bonded to the matrices, by elimination of some small molecules, through the binding process. Many fruits, vegetables and other edible plants, have characteristic colors and odors, that very familiar to us. Therefore it is very interesting to add these colors or odors to our food. In this study, the colored anthocyanin's which has been extracted from *Hibiscus Sabdariffa* (karkade) would be used in the coloring foodstuffs.

Research Objectives

The aim of this work to develop a method for these selective extraction, separation and identification using spectroscopic methods in order to determine qualitative determination anthocyanin's in *Hibiscus sabdariffa* plant.

The scope and purpose of the project are defined by the objectives which were:

1. To utilize waste plants for the production of natural dyes
2. To produce natural dyes in powder form
3. To apply the anthocyanin's extracts from *Hibiscus sabdariffa* (karkade), for coloring the foodstuff and beverages

4. To study of the optimum conditions required for the stability of the colors on the colored foodstuff and beverages
5. To use the finding dyeing in industry
6. To compare the results of our study with the previous study done

Literature Review

Natural dyes can be sorted into three categories: natural dyes obtained from plants for example indigo, those obtained from animals for example cochineal and those obtained from minerals for example ocher [29]. Natural dyes colorants derived from flora and fauna are believed to be safe because of its non-toxic, non-carcinogenic and biodegradable in nature [30]. However, the use of natural dyes to color textiles declined rapidly after the discovery of synthetic dyes in 1856 [31]. The main reason for the replacement of natural dyes by their synthetic counterparts is that most natural dyes have poor to moderate wash and light fastness, while synthetic dyes represent the full range of wash and light fastness at moderate costs [32]. Lately however, there is a growing interest in the revival of natural dyes in textile coloration [33-34]. This is as a result of the worldwide concern over the carcinogenic effects, toxicity and allergic reactions associated with synthetic dyes. Moreover, many countries already imposed stringent environmental standards over synthetic dyes. Germany, for instance banned the azo dyes [28]. In contrast, natural dyes are environmental friendly, exhibit better biodegradability and generally have a higher compatibility with the environment than synthetic dyes, with a tropical climate, is home to a very large number of plant species; many of them are used by natives in folk medicine. Malaysia is among the world's mega biodiversity - rich countries in terms of number of plant species [24] which also included *Mel stoma malabathricum* L. and *Dicranopteris linearis* plant. Although it is unlikely all dyestuffs will be produced solely from plants, it is an interesting and exciting prospect that one day a percentage of every day colors could be naturally derived [34].

EXPERIMENTAL METHODS

Plant Materials

The fresh plant *Hibiscus sabdariffa* (karkade) were collected from gabbier (Al-khurma Province), the plant collected was further shelled and dried organs were ground to a fine powder using Thomas-Willey Milling Machine.

Extraction procedure of anthocyanin's

The karkade samples used in this study is known locally as ajar and the calyces sun-dried were cleaned. The dried calyces (50.0g) were reduced to powder with a mortar and 25.0 g of homogenized dry leaves was mixed with one liter of Me OH/HCl (99:1 v/v) for 48 hours with magnetic stirring at room temperature. The solution was filtered by Buchner and then passed through a 5 mm membrane filter to remove solid particles. The filtrate was mixed with 40.0 g Amberlite XAD-2 (pore size 9 nm, particles size 0.3-1.2 mm) and stirred in a magnetic stirrer for 40 minutes at room temperature, in order to absorb enough anthocyanin's quantity. The Amberlite particles were then packed into a glass column (64 cm×42cm) and the column was washed with deionized water until a colorless solution was obtained. The anthocyanin's remained absorbed on the column was eluted with one liter of ethanol (70% v/v) and acetic acid (1% v/v). The intense red solution was concentrated to dryness in a rotary evaporator under reduced pressure at40℃. During the last step, 2 ml of water were added to dissolve the extract, which was then passed through a 5 mm membrane filter and keep before the analysis by using spectroscopic methods. The method of dying procedure in each case depends on the type of the foodstuff and the dying conditions that is done by varying temperature, pH and addition of other additives and duration of treatments. The dying of samples is done in test tubes and vetri dishes. Each time 20.0 g of samples are used adding ingredients and controlling the required conditions (Figure1).

Coloring of foodstuffs

The colored ingredients dyes could be mixed with the foodstuffs to form homogeneous mixtures. Either colloidal or true solutions, by physical mixing, where like dissolves likes, or by forming permanent bonds, through chemical reactions. Formation of permanent covalent chemical bonds, by eliminating some small molecules, like H_2O, NH_3 …. etc. through controlled mild reaction conditions, if the dye and the foodstuff are of like features. The reaction of the dye with the substrate is favored by two factors the close proximity of the dye and the substrate fiber.

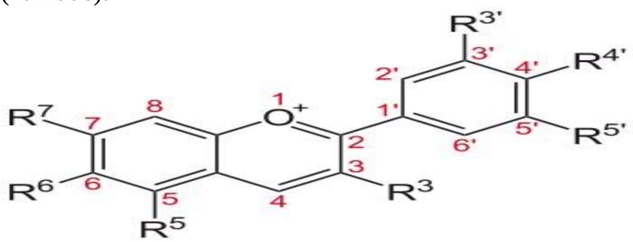
The dyes characterized by hydroxyl groups are more suitable for coloring carbohydrates and some proteins of the foodstuff, with similar radicals, for example the hydroxyl groups. The molecular structure, the size and the oxidation – reduction behavior of the dye are the major factors in the dying process. The PH of the dying process could be adjusted by adding carbonate or hydroxide ions.

Dying process

1- Forming homogenous mixture from the dye and the foodstuffs by physical mixing using a mixer, heater …. etc.
2- Forming permanent covalent chemical bonds, by eliminating some small molecules, like H_2O, NH_3 …. etc. through controlled chemical reaction, in mild conditions. Using a mixer, heater, $NaHCO_3$, Na_2CO_3 to adjust the PH of the reaction …. etc.

RESULTS AND DISCUSSIONS

UV–VIS spectrum of natural dye obtained from *Hibiscus Sabdariffa* (karkade) was obtained. Here, the dye extracted was analyzed at the wave length of 480 nm. The absorbance values for natural dye extract obtained by ultrasonic and magnetic stirring control are shown in figure 2. The results indicate that there is about 12.5% improvement in the % yield of extract due to the use of ultrasonic as compared to the control process. The effect of ascorbic acid and anthocyanin's for tification on the stability of anthocyanin's was shown in figure 3. Dyes extracted through boiling and solvent extraction process. The same source of dye was mixed with different mordant to give different colors. For instance, fabrics dyed using iron as mordant gave the dark color in comparison with other mordrants for both dyes from *Hibiscus Sabdariffa* (karkade).



Anthocyanin's are belong to a parent class of molecules called flavonoids compounds [27], they are odorless and nearly flavorless, contributing to taste as a moderately astringent sensation that produce plant colors ranging from orange and red to various shades of blue and purple depending on the pH [28,29]. This unit describes methods for extraction, isolation and purification of anthocyanin's from plant, it occur in all tissues of higher plants, including leaves, stems, roots, flowers and fruits.

This method is essential laboratory operation to subsequent experimental work involving extraction, separation and characterization. The polar character of the anthocyanin's molecule allows for its solubility in many different solvents such as alcohols, acetone, dimethyl sulfide and water [30]. The choice of extraction method should maximize pigment recovery with a minimal amount of adjuncts and minimal degradation or alteration of the natural state. This procedure permits concentration of anthocyanin's in the aqueous phase while removing lipids, chlorophylls and other water-insoluble compounds. The alternate method describes the extraction of anthocyanin's with acidified methanolic solutions. Methanol is the most commonly used solvent for anthocyanin's extraction because its low boiling point allows for rapid concentration of the extracted material [31-32]. However, the resultant extract contains low-polarity contaminants and further purification may be necessary.

Comparison of extraction yield

Extraction of anthocyanin's as natural dyes from *Hibiscus Sabdariffa* (karkade) was done. The results indicate that there it is a significant improvement in the % yield of coloring matter extract obtained due to the use of ultrasonic. The difference in the enhancement in extraction yield with ultrasonic for different plant material could be due to different degree of binding of coloring matter attached to plant cell membranes. Moreover, another important factor is chemical constituents present in plant material responsible for the color chromosphere group and their solubility nature. Considering the example of the plant, the basic chromosphere group is flavonoids having hydroxyl group, which is expected to be extracted better with aqueous solvents such as organic solvents. Whereas, in the case of Green wattle, the basic chromosphere is poly phenolic (Kaempferol) which is expected to be extracted better with water [33,34]. Hence, better yields are observed for *Hibiscus Sabdariffa* (karkade) as compared to Green wattle using water as solvent. These aspects are planned for our future study.

Figure 1. Preparation the materials and extraction method

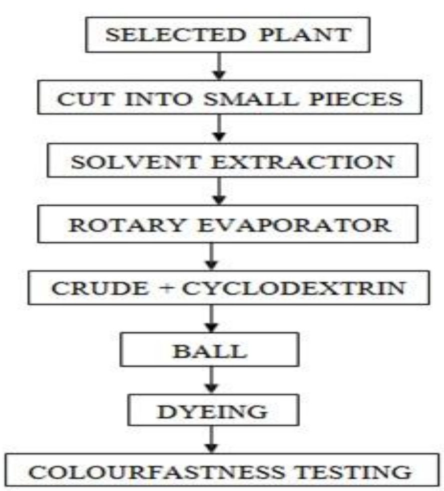


Figure 2. The absorbance values for anthocyanin's obtained by ultrasonic and magnetic stirring

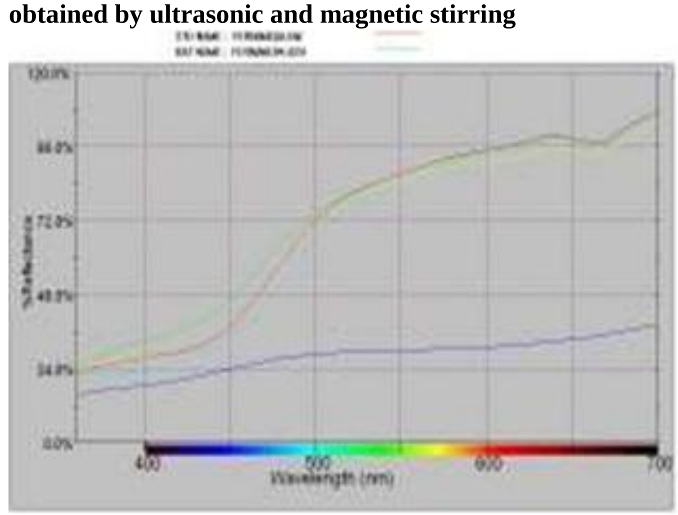


Figure 3. Effect of ascorbic acid and anthocyanin's fortification on the stability of anthocyanin's

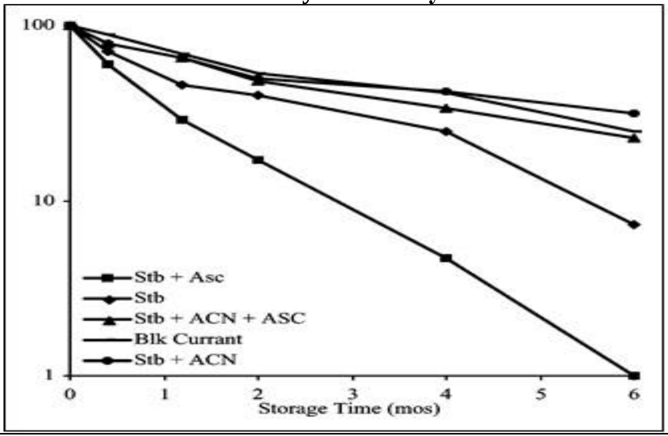


Figure 4. Structural transformations of anthocyanin's with change in pH

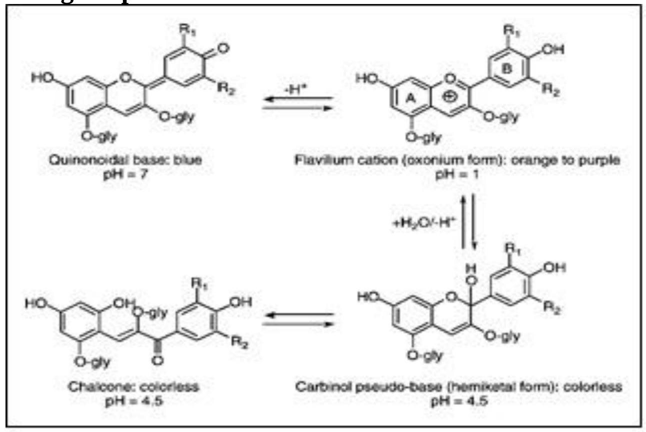
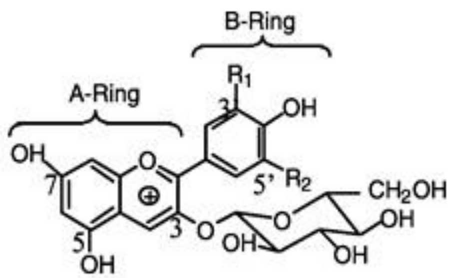


Figure 5. General structure for anthocyanin's R_1&R_2=H, OH, or OMe. Glycosidic substitution can occur on 3,5,or7, acylation with cinnamic and aliphatic acids mayoccuron sugar hydroxyls



CONCLUSIONS

Natural dyes provide an environmentally safe option for coloring of foodstuff and beverages. It was found that the application of ultrasonic can increase the extraction of dyes from different parts of various plant resources. Extraction was done using ultrasonic as well as magnetic stirring methods and the kinetics and the extraction efficiency were compared. The reason for the improvement could be due to better leaching of natural dye material from plant cell membranes and mass transfer to solvent assisted by acoustic cavitation provided by ultrasonic. The results indicate that there is about 12-100 % improvement in % yield of extract obtained due to the use of ultrasonic as compared to magnetic stirring at 45°C. Various process parameters such as solvent system, temperature, ultrasound power and amount of dye material are interesting for our study as future work. One would expect better extraction efficiency with solvents like n-hexane for those dye materials better soluble in organic solvents. But, our objective is to develop sustainable effective process with aqueous system without using organic solvents. Extraction efficiency may decrease if temperature is lowered than 45°C; however, higher temperatures could affect plant material itself as they are sensitive to the same. This novel technique can be employed effectively for the extraction of coloring matter from various plant resources even dispensing with conventional heating requirements. This process provides effective utilization of natural resources as eco-friendly method in current situation of global environmental concern.

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