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Extraction, Purification and Comparative Analysis of Anthocyanin from Grapes (*Vitis labrusca*)

Ms. Jeba Kezi J and *Dr. V. Judia Harriet Sumathy

Postgraduate & Research Department of Biotechnology, Women's Christian College, Chennai–600006, India
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Abstract

Grapes are small round or oval berries that feature semi-translucent flesh encased by a smooth skin. Some contain edible seeds while others are seedless. Like blueberries, grapes are often covered by a protective, whitish bloom. Grapes that are eaten as it is or used in a recipe are called table grapes and as opposed to wine grapes (used in viniculture) or raisin grapes (used to make dried fruit). Grapes are antimutagenic, antineoplastic and reduce human low-density lipoprotein (LDL). The high demand for grape products is due to the associated health benefits for consumers and this has motivated research for formulating ways to improve its quality and health effects. Grape peel and seeds are rich sources of functional components such as phenolics and anthocyanins which have antioxidant and radical scavenging activities. Grape is the single most abundant fruit harvested in the world from which a natural color is commercially obtained. Grapes are highly pigmented with Anthocyanins, a pool of colorants responsible for the purple, violet, blue, magenta, red and orange color of many fruits, vegetables and flowers. The present study was conducted to extract, purify and apply anthocyanin pigment of *Vitis labrusca* in food.

Keywords: *Grapes, Berries, Anthocyanins, Vitis labrusca and Health Benefits.*

Contents

***Corresponding author**
Dr. V. Judia Harriet Sumathy
Department of Biotechnology,
Women's Christian College, Chennai, India
E-mail: sbsj@rediffmail.com
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1. Introduction

GRAPE

Vitis labrusca is a species of grapevines belonging to the *Vitis* genus in the flowering plant family Vitaceae (**Kearsley MW and Katsaboxakis KZ, 2007)**. The vines are native to eastern North America and are the source of many grape cultivars, including Catawba and Concord grapes, and many hybrid grape varieties such as Agawam, Alexander and Onaka **(Figure 1).**

Figure 1. *Vitis labrusca*

Table 1 Taxonomy

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Vitales
Family	Vitaceae
Genus	*Vitis*
Species	*V.labrusca*

Anthocyanin Pigment from Grape Skin

Anthocyanins (Greek: anthos, flower and kyanos, blue) are water-soluble vacuolar pigments that may appear red, purple, or blue depending on the pH. They belong to a parent class of molecules called flavonoids synthesized via the phenyl propanoid pathway (**Stintzing FC and Carle R, 2004)**. They are odorless and nearly flavorless, contributing to taste as a moderately astringent sensation. Anthocyanins occur in all tissues of plant, including leaves, stems, roots, flowers, and fruits (**Gabriela Stanciu A, *et al*., 2010).** Anthoxanthins are clear, white to yellow counterparts of anthocyanins occurring in plants. Anthocyanins are derived from anthocyanidins by adding pendant sugars (**Gliszczynska-Swiglo, *et. al.*, 2006)**. Anthocyanins are considered secondary metabolites as a food additive with E number E163 (INS number 163). They are approved for use as a food additive in the EU, Australia and New Zealand (**Figure 2**).

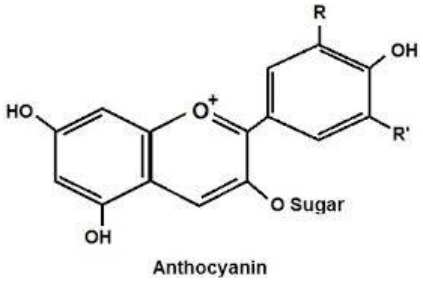

Figure 2. Structure of Anthocyanin

Anthocyanins act as a "sunscreen", protecting cells from high-light damage by absorbing blue-green and ultraviolet light, thereby protecting the tissues from photo inhibition, or high-light stress**.** The anthocyanins, anthocyanidins with sugar groups, are mostly 3-glucosides of the anthocyanidins. The anthocyanins are subdivided into the sugar-free anthocyanidinaglycones and the anthocyanin glycosides**.** As of 2003, more than 400 anthocyanins had been reported while more recent literature (early 2006), puts the number at more than 550 different anthocyanins **(Edmar Clemente, *et al.*, 2013).** The difference in chemical structure that occurs in response to changes in pH is the reason why anthocyanins are often used as pH indicators, as they change from red in acids to blue in bases. Although anthocyanins are powerful antioxidants *in vitro*, this antioxidant property is unlikely to be conserved after the plant is consumed**.** As interpreted by the Linus Pauling Institute and European Food Safety Authority, dietary anthocyanins and other flavonoids have little or no direct antioxidant food value following digestion. Unlike controlled test-tube conditions, the fate of anthocyanins *in vivo* shows they are poorly conserved (less than 5%), with

most of what is absorbed existing as chemically modified metabolites that are rapidly excreted (**Stintzing FC, *et.al.*, 2002)**.

Anthocyanins Stability and Medicinal use

Anthocyanins are thought to be subject to physiochemical degradation *in vivo* and *in vitro*. Structure, pH, temperature, light, oxygen, metal ions, intramolecular association, and intermolecular association with other compounds (copigments, sugars, proteins, degradation products, etc.) are generally known to affect the color and stability of anthocyanins. Indeed, significant portions of ingested anthocyanins are likely to degrade to phenolic acids and aldehyde *in vivo*, following consumption**.** Anthocyanins protect both large and small blood vessels from oxidative damage. They neutralize enzymes that destroy connective tissue and prevents oxidants from damaging connective tissue. Finally, they repair damaged proteins in the blood-vessel walls. Anthocyanins helps in maintaining microcapillary integrity by stabilizing capillary walls. Serious complication of diabetic is retinopathy, which can cause blindness. Anthocyanin occurs when the body attempts to repair leaking mainly by the overproduction of abnormal proteins. Anthocyanins may also improve eyesight by various mechanisms (**Gandia-Herrero, *et.al.*, 2005)**.

Benefits of Biocolourants

The use of bio-colorants may show benefits over synthetic colours**.** Natural dyes are less toxic, less polluting, less health hazardous, non-carcinogenic and non-poisonous and prevent chronic diseases such as prostrate cancer**.** In addition to this, they are harmonizing colours, gentle, soft and subtle, and create a restful effect. Most of them are water-soluble which facilitates their incorporation into aqueous food systems**.** These qualities make natural food colorants attractive. Above all, they are environment friendly and can be recycled after use. Thus, they attribute to food-both for aesthetic value and for quality judgment and also they tend to yield potential positive health effects, as they possess potent antioxidant and improve visual acuity properties (**Gaertner, V. L., and Goldman, I. L, 2005)**.

2. Materials and Methodology

Extraction of Anthocyanin

Aqueous ethanol (50:50) -100mL and Grapes skin -100g were the materials required. Extraction of pigment was achieved by homogenization of equal ratio of sample and solvents (1/1 w/v). 100 g of the peeled skin of grapes was weighed and macerated with 100 ml solvents (EtOH, aqueous ethanol 50:50) for 15 minutes. The aqueous mixture was centrifuged at 12,000 rpm at 4˚C for 20 min. The supernatant was taken and concentrated using rocker. The ethanol was removed after concentration process and samples were kept in a dark.

Phytochemical Analysis of Anthocyanin Pigment Extract

To 2ml of the extract 2ml of distilled water was added followed by few drops 1% lead acetate. Formation of white precipitate indicates the presence of tannins. 1ml of the extract was shaken vigorously with 1ml of distilled water in a test tube and warmed. The formation of stable foam, honey comb like shapes indicates the presence of saponins. To 2ml of the extract few drops of acetone was added .It was then kept in water bath to evaporate acetone. After evaporation boiling water was added and then it was cooled .It was followed by the addition of 5ml of 20% NaOH. Appearance of yellow colouration indicates the presence of flavonoids. To 0.5ml of the extract 1ml of distilled water and 5-8 drops of fehlings solution was added and heated over water bath. Appearance of brick red indicates the presence of reducing sugars. To 2ml of the extract 0.1ml of NaOH was added and shaken vigorously followed by addition of small amount of distilled water. Formation of white precipitate indicates the presence of volatile oil. 5ml of each extract was mixed in 2ml of chloroform and concentrated sulphuric acid (3ml) was carefully added to form a layer . A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

Estimation of Flavonoids

5%Sodium Nitrite, 10% Aluminium Chloride, Sodium Hydroxide, Distilled water and volumetric flask were the materials required. An aliquot (1 ml) of extract (concentration 1 mg/ml) was added to 10 ml volumetric flask containing 4 ml of distilled H_2O. To this 0.3 ml 5% $NaNO_2$ was added and 5 minutes later 0.3 ml 10% $AlCl_3$ was added. After 6 minutes, 2 ml of 1M NaOH solution was added. Finally the total volume was made up to 10 ml with distilled H_2O. The solution was well mixed. The absorbance was measured at 510 nm against the control. Control was prepared in the same manner only with replacing the extract with distilled water.

Evaluation of Antioxidant Capacity by Phosphomolybdenum Method

Test tubes, Colorimeter, 0.6M Sulphuric acid, 28mM Sodium phosphate, 4mM Ammonium molybdate and Distilled water were the required materials. An aliquot of 0.3 ml of sample solution was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in colorimeter. A typical blank solution containing 1 ml of reagent solution and the appropriate volume of the same solvent used for the sample was

incubated under same conditions as rest of the sample. For samples of unknown composition, water-soluble antioxidant capacity was expressed as equivalents of ascorbic acid.

Stability Tests of Anthocyanin Pigments

Stability yo Temperature

Heat stability was evaluated by exposing the color extracts to different temperatures such as 40°C, 50°C, 60°C 70°C, and 80°C for different time periods of 5, 10, 15 and 20 minutes. After every treatment the extract were immediately cooled. The absorbance of grape skin extract is read at 520nm.

Stability to pH

Materials Required

HCl -0.1M and NaOH -0.1M are the reagents required. The pH of the extracts was adjusted using 0.1M HCl and 0.1M NaOH. The pH ranges of 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 were attained. The absorbance of grape skin extract is read at 520 nm.

Purification of Pigments using Column Chromatography

When a mixture of mobile phase and sample to be separated are introduced from top of the column, the individual components of mixture move with different rates. Those with lower affinity and adsorption to stationary phase move faster and eluted out first while those with greater adsorption affinity move or travel slower and get eluted out last. A vertical glass column with a knob at the bottom end, cotton, Silica gel (120 mesh), Solvent (Ethanol : Water – 8 : 2) and sample were the materials required.

Preparation of the Crude Extract

The anthocyanin extacted using ethanol is concentrated using rocker before purification

Packing the Column for Chromatography

A cylindrical glass column was taken and was plugged with a small piece of cotton. The column was mounted on the stand. 25g of fresh silica gel (120 mesh) was taken in a 250 ml beaker. 100 ml of solvent was poured into the beaker and stirred well using a glass rod to make slurry of the silica. The slurry was poured into the column. The conical flask was placed below the mounted column and the excess solvent was drained out. The solvent was eluted for few times until the column gets well packed. The knob was closed when the level of the solvent reached just above the settled silica gel.

Loading Extract on to the Column

The sample was transferred into the solvent layer above the silica gel in the packed column. The column was continually filled with ethanol and was eluted until the pigment runs down the column. The elution was performed with the same binary solvent mixture at a flow rate of 0.7 mL/min. As the elution progresses the pigment will elute out of the column was collected in a conical flask. The silica gel 60 column was not regenerated.

Removal of Solvent

The pigments collected from the column are then concentrated by removing the solvents using rocker. The pigments left behind in the round bottomed flask after concentrating are transferred and stored.

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Confirmatory Test for Anthocyanin Pigments by TLC

TLC works on the principle of capillary action. Separation occurs as each component, being different in chemical and physical composition, interacts with the stationary and mobile phases to a different degree, creating the individual bands on the plate. The retardation factor, R_f value, is used to characterize and compare components of various samples. TLC plates, capillary tubes, beaker, sample, Solvent - n-Butanol : Acetic acid : Water - 4:1:5 for anthocyanin were the materials required. A TLC plate was taken and with a pencil a line was drawn approximately 1 cm from the short edge of the TLC plate. Care should be taken not to scrape the coating of the plate. With a capillary tube, the sample was spotted on the TLC plate. The spot was labeled at the top of the TLC plate. The sample was reapplied to the same place at least 3 times or until the spot is clearly visible. The chromatography

chamber was filled to a depth of approximately 0.5 cm with the Butanol: Acetic acid : Water - 4:1:5 for anthocyanin pigment. The TLC plate was placed in the chromatography chamber with the sample spot toward the bottom. The sample spot is ensured to be above the level of the solvent and the chamber was closed. The plate was allowed to remain undisturbed until the solvent reaches to within 1 cm of the top. The plate was removed from the chamber and immediately the solvent was marked in front using a pencil. The distance from the spotting line (origin) to the center of each spot and from the spotting line to the solvent front was immediately measured and recorded and each component was identified.

$$R_f \text{ value} = \frac{\text{distance from origin to component spot}}{\text{distance from origin to solvent front}}$$

Immobilization of Betalain Dye

Calcium Chloride -4%, Sodium Chloride -0.1N, Sodium Alginate -3.5g and Syringe were the materials required. 4% $CaCl_2$ by weight 4 gm of $CaCl_2$ is mixed into 100ml water. Then by using magnetic stirrer the solution is mixed properly. After that the solution is kept at 4 degree centigrade for 2 hours. Then 0.1N NaCl sodium alginate solution is prepared by adding 0.685gm of NaCl is mixed into 100 ml water. To this 3.5gm of Sodium Alginate is mixed. Then the solution is kept for incubation. After incubation 2 - 4% of the sample is added in 10ml of 0.1N NaCl sodium alginate solution. Then $CaCl_2$ is taken in beakers and by using a syringe Sodium Alginate solution of different colour is added drop by drop into different beakers.

Application of Natural Dye in Food

The pigment was removed from the ethanol using rocker. 1 – 2 ml of anthocyanin pigment was applied in the curd. The solution turns into violet color. It was then store in the refrigerator at 20˚C.

3. Results and Discussion

Plants extracts constitute an important source of active natural products which differ widely in terms of structures, biological properties and mechanisms of actions. Anthocyanin showed the presence of tannins, saponins, flavonoids, reducing sugars and terpenoids (**Figures 3 – 4**).

Figure 3. Anthocyanin Extracted From *Vitis Labrusca*

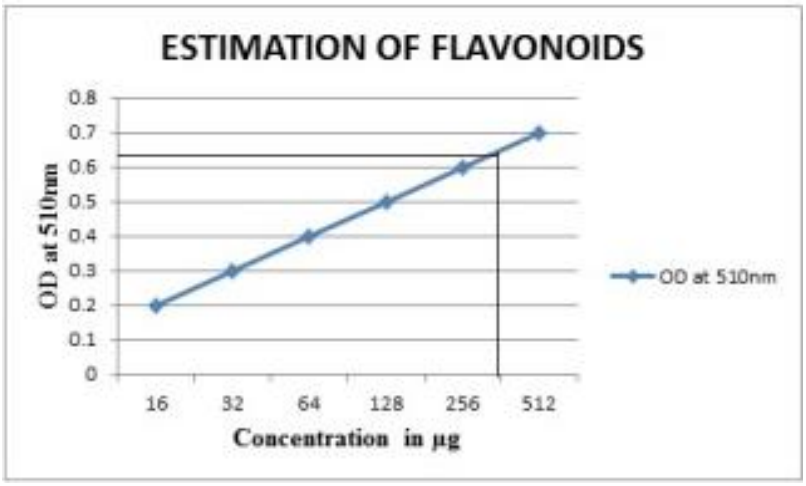
Figure 4. Phytochemical Test of Grape Skin
[Tannins, Saponins, Flavonoids, Reducing Sugar, Volatile Oil And Terpenoids]

Table 2

Phytochemical Test	Grape Skin *(Vitis labrusca)*
Tannins	+
Saponins	+
Flavonoids	+
Reducing sugars	+
Volatile oil	-
Terpenoids	+

Estimation of Flavonoids in Grape Skin *(Vitis labrusca)* Extract

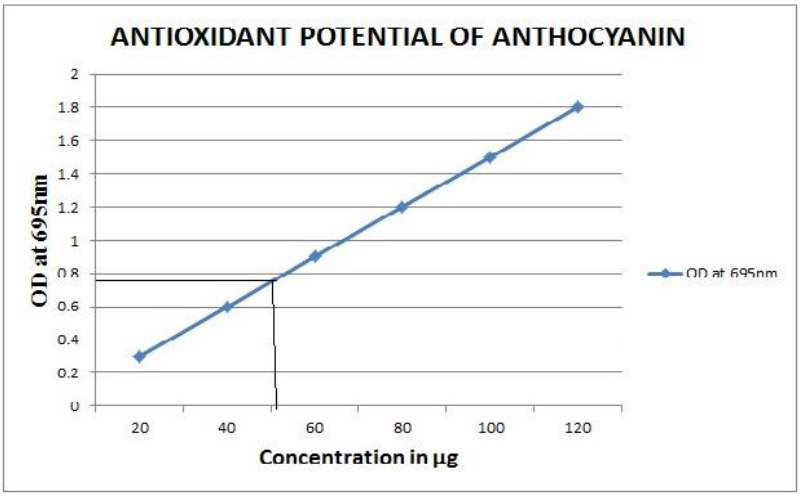
The flavonoid content in anthocyanin was 384 µg/ml. This is because anthocyanin comes under the group of flavonoids and it is rich in it (**Graphs 1 & 2**).



Graph 1. Flavonoids in Anthocyanin

Antioxidant Capacity of Grape Skin *(Vitis labrusca)* by Phosphomolybdenum Method

The antioxidant capacity of grape skin *(Vitis lambrusca)* to be 52µg/ml (**Graphs 2**).



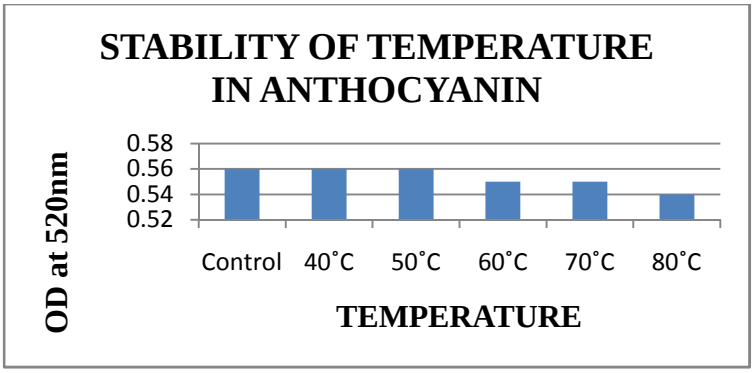
Graph 2. Antioxidant Potential of Anthocyanin

Stability Tests of Anthocyanin Pigments

Stability of Temperature in Anthocyanin

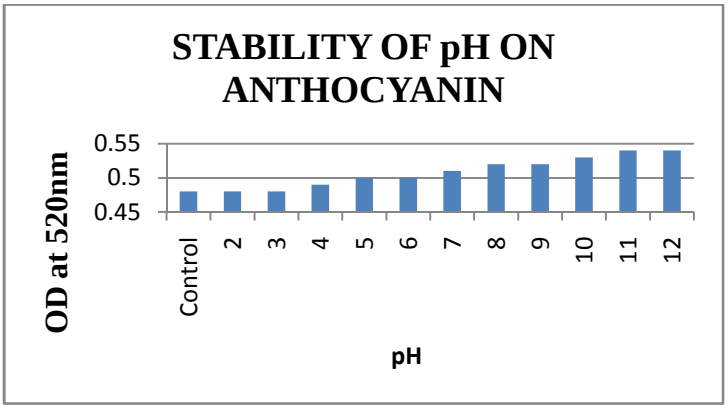
Anthocyanin

It is observed that there was no effect of temperature up to 80ºC on the pigment stability because the anthocyanin compounds decomposed only at a temperature higher than 80ºC **(Graph 3 – 4)**.



Graph 3. Stability of Temperature in Anthocyanin

Stability to pH in Grape Skin -Anthocyanin (*Vitis labrusca)*



Graph 4. Stability of pH on Anthocyanin

Purification of Anthocyanin from Grape Skin by Column Chromatography

Anthocyanin was purified using the grape skin extract. The colour of the purified was said to to be violet. Hence grape anthocyanin was purified from impurities using column chromatography (**Figures 5 - 6**).

Figure 5. Column Chromatography

Figure 6. Confirmatorytest of Anthocyanin

Thin Layer Chromatography of Anthocyanin Pigment From Grape Skin (*Vitis labrusca*)

$$R_f \text{ value} = \frac{\text{distance from origin to component spot}}{\text{distance from origin to solvent front}}$$

Table 3

Pigment	Distance migrated	R_f value
Anthocyanin	**2.5cm**	**0.55**

The compound was confirmed to be anthocyanin. Since the R_f value was found to be similar to that of the previous work done on anthocyanin the R_f value of anthocyanin determined is 0.55 (**Figure 7**).

Figure 7. Thin Layer Chromatography of Anthocyanin

Immobilisation of Anthocyanin

When different coloured Sodium Alginate solutions is added drop by drop into different beakers containing $CaCl_2$ different colours of beads are formed. Enzymes can be safely preserved by natural dyes. Using the chemical dye for enzyme preservation technique may affect the chemical nature of the preserving enzyme which leads to some changes in enzyme activity. But if we use dye extracted from natural materials it doesn't affect the nature of enzyme. Thus Natural dye has no harmful effect on health (**Figures 8**).

Figure 8. Immobilization of Anthocyanin

Application of Anthocyanin Pigment in Food (CURD)

The curd was coloured using anthocyanin pigments. Natural dyes can be used in various food materials because it is non-toxic and has no harmful effects .Since it is unstable to conditions such as temperature and pH it is maintained in the correct conditions (**Figure 9).**

Figure 9. Application of Anthocyanin Pigment in Food

4. Conclusion

Consumers are avoiding foods containing synthetic colourants, which lead food industries to replace them by natural pigments. Most often, the colorants are extracted from plant material, but other sources such as insects, algae, cyanobacteria and fungi are used as well. To consider the natural as early as the colorant, stability, yield and price are mostly the constrains. Most of them are sensitive to pH, heat and sunlight. Inspite of such factors biocolorants are gaining importance because of health and hygiene, nutrition, pharmaceutical activities, fashion environmental consciousness, indicates relative on natural products. Colors derived from minerals (lead chromate, copper sulphate)

may cause serious health problems. Anthocyanin is a stable at acidic pH. It does not get affected by the temperatue upto 80˚C after which it gets decomposed. The interest of the food industry in betalains and anthocyanin has grown since because of the antioxidant property which may protect against oxidation of low-density lipoproteins. Anthothocyanin exhibit various colour at different pH. So it can be use as natural source for pH indication. Anthocyanins have been used in organic solar cells because of their ability to convert light energy into electrical energy. The many benefits to using dye-sensitized solar cells instead of traditional ion junction silicon cells include lower purity requirements and abundance of component materials, such as titania, as well as the fact they can be produced on flexible substrates, making them amenable to roll-to-roll printing processes. Natural colors are suitable for a wide range of sugar confectionery products. Hard candies, tablets, gummies, pectin-based candies, panned candies, and gums are perfect applications for natural colors. Natural colors work well in sugar free applications, including in candies sweetened with stevia, and in confections fortified with health ingredients or vitamins. Thus the present study indicates that Anthothocyanin pigment has an immense impact and major contributing role in food industry which is yet to be unveiled.

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