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Effect of Different Acids in the Extraction Procedure of Anthocyanin from Locally Available Banana Bract (Bhimkol) by two Different Conventional Methods

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A B S T R A C T

Anthocyanin, a coloured natural pigment possesses many phytochemical activities along with various health benefits, so can be used as colourant in food and as drug in pharmaceutical industry. In this study anthocyanin was extracted from flower bracts of 'Bhimkol', confirmed by TLC and coloured tests; TAC (Total Anthocyanin Content) was determined using pH-differential method and calculated by using UV-Visible Spectrophotometer under different extraction conditions. The findings of the study reveals that TAC is found to be 40.35 mg/100g in the studied flower bracts using 50% ethanol acidified with 3% citric acid, a naturally occurring antioxidant. The extraction conditions used in this study is mild, avoids degradation of anthocyanin and environmentally benign too. The solvents used are cheap, easily available in common laboratory and can be reused by recovering it using simple distillation technique.

***Key words*:** Bhimkol, Flower bracts, Anthocyanin, TLC, pH-differential method, TAC

Banana is one of the most important and popular agricultural food crops available all over the world that provides numerous health benefits [1]. The banana flower or banana blossom, also known as banana heart or banana inflorescence is a byproduct of banana plant. The cream-colored inner bracts of banana flower are used in the preparation of variety of dishes in our country whereas, the purple-red to maroon-colored outer bracts are generally disposed off after harvesting the banana plant. Different research groups have investigated the presence of different type of pigments in banana bract by acid extraction and paper chromatography [2-3] revealed that the colors of bracts are due to the presence of glycoconjugated anthocyanidin pigments. Anthocyanins are the glycosides of anthocyanidins which belong to the phenolic group known as flavonoids and possess many health benefits documented by researchers [4]. Now a days the demands for natural food colorant derived from variety of edible sources is high as they have antioxidant and antimicrobial properties and possessing additional health benefits. They show important pharmacological activity due to the presence of various beneficial phytochemicals. It is reported that natural food colorants are more expensive than synthetic ones, with a cost that can be two to ten times higher than synthetic colorants [5]. So, to minimize the cost of production of natural colorants, use of byproducts of agro food is found beneficial which inter alia effectively utilize the waste of the byproducts of agro food. In our study, we used banana flower bracts, a byproduct of 'Bhimkol' or 'Aathiakol' (*Musa balbisiana Colla*) belonging to the genus *Musa* from the family *Musaceae* which is an indigenous variety of banana found in Assam and NE region as a source of anthocyanin.

Various types of extraction methods have been carried out to extract anthocyanin content which are mainly divided into two main groups: Conventional techniques (viz. maceration and Soxhlet extraction) and modern techniques (extraction by ultrasound, microwaves, pressurized liquid, supercritical fluid, enzyme assistance, matrix solid-phase dispersion, etc. [6]. It is reported that the conventional extraction techniques are very much popular from long back and are still being used by using solvents at higher temperature. The conventional methods are easy to perform, need no special laboratory equipment's and therefore can be easily performed in a common laboratory. However, in conventional extraction techniques the selection of suitable operational conditions is important for efficient extraction of natural pigments. The factors that have significant influence on the extraction techniques are the type of solvents or other reagents, acids used, extraction temperature and extraction time. As such lack of proper parameter optimization may cause incomplete extraction or degradation of anthocyanin.

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The objective of this work is to investigate the effect of factors like mild solvent composition, inorganic and organic acids used, extraction time as well as the extraction temperature on the TAC from banana flower bracts and to determine the optimum extraction conditions using conventional extraction techniques.

MATERIALS AND METHODS

Plant samples

Banana flower blossom of "Bhimkol" (*Musa balbisiana* or *Musa BB*) were collected from local market near Pragjyotish College, Bharalumukh, Assam (Fig 1).

Fig 1 (a) 'Bhimkol' (b) Bracts of 'Bhimkol'

The banana bracts were separated from flower and washed several times with tap water followed by distilled water to remove dirt and other surface impurities, dried under partial sunshade for 2 to 3 days, semi dried bracts are then cut into small pieces followed by grinding in a mortar and pestle to obtain a coarse mixture of flower bracts with a small amount of solvents used in the extraction.

Chemicals and Reagents

Ethanol, Hydrochloric acid, Sodium Hydroxide, Citric acid, Acetic acid, Butanol, Potassium Chloride, Sodium acetate, Silica Gel H are purchased from Qualigens and CDH and all reagents and solvents are of analytical grade. Distilled water was used throughout the study.

Apparatus

Soxhlet apparatus, pH meter (Elico LI 120), UV-VIS Spectrophotometer (Agilent Technologies Cary Co.), Rotavapor (Buchi Rotavapor R-3).

Experimental

In this study, the effect of different solvents (ethanol and water) and acids (HCl, citric acid, acetic acid) combinations upon the extraction of anthocyanins are studied. Soxhlet (for 2 hours and 4 hours) and maceration (24 hours) techniques were used during the extraction process of anthocyanins.

Anthocyanin Extraction

In this study, we mainly use of two conventional methods namely, maceration method and the Soxhlet method for the extraction of anthocyanin from the flower bracts of 'Bhimkol'.

Extraction of Anthocyanin by Soxhlet method

10g of the banana flower bracts were placed in a Soxhlet apparatus with 150 ml of solvent mixture and was kept in the normal Soxhlet for 2 hours and 4 hours. After the scheduled time, the contents of the flask were collected, filtered the extract using Whatman filter paper No.1 and concentrated the extract in a rotavapor. The concentrated extracts were then stored in refrigerator at 4°C until being used.

Extraction of anthocyanin by maceration technique

5g of the banana sample in 75 ml of solvents ware put into a conical flask covered with aluminum foil. The flask was then kept in a lightless place at room temperature for 24 hours. After that, the extract was filtered by Whatman filter paper No. 1, concentrated in a rotavapor and stored in refrigerator at 4°C until being used for further analysis.

Identification and qualitative analysis of anthocyanin pigment

After extraction, two simple tests were performed with each extract to identify and confirm the presence of the anthocyanin pigments [7].

(a) *Chromatographic analysis by TLC*

After concentrating the extract from banana bracts by rotavapor, TLC was done by using the solvent system of butanol: acetic acid: water in the ratio (4:1:5).

(b) *Coloured tests with acid and base*

0.5 ml of concentrated extract was mixed with 2M HCl and 2M NaOH solution separately, instant appearance of orange-red and blue-violet colour respectively, confirms the presence of anthocyanin.

Preparation of buffer solutions

Preparation of 0.025 M KCl buffer

0.025M HCl –KCl buffer was prepared by dissolving 1.86g KCl in 980 ml of distilled water.

Preparation of 0.4 M CH_3COONa buffer

0.4 M Sodium acetate buffer was prepared by mixing 54.43 g $CH_3COONa.\ 3H_2O$ in 960 ml distilled water.

Analysis of total anthocyanin content (TAC)

TAC was determined according to the spectrophotometric pH-differential method [8-9]. The amount of TAC was calculated by analyzing the UV-visible spectroscopy at pH =1 and pH =4.5. Each extract was separately diluted with 0.025M HCl-KCl buffer and with 0.4M CH_3COONa and the pH was adjusted to pH =1.0 and pH =4.5 respectively with concentrated HCl.

The maximum of absorbance was found from the UV-Visible spectra recorded in Agilent Technologies Cary Co UV-Visible Spectrophotometer. The concentration of each anthocyanin was calculated according to the following formula [8-9] and expressed as Cy-3-glc equivalents.

$$TAC = \frac{(A \times MW \times DF \times 10^3)}{\varepsilon \times 100}$$

Where, A is the absorbance = $[A_{\lambda max}]_{pH\ 1.0} - [A_{\lambda max}]_{pH\ 4.5}$.
MW is the molecular weight = 449.2 g/mol for Cy-3-glc
DF is the diluted factor
Ɛ is the extinction coefficient (L× cm^{-1}× mol^{-1}) = 26900 for Cy-3-glc
L is the path length = 1 cm

The amount of anthocyanin in our study is expressed in mg of anthocyanins per 100g flower bracts of 'Bhimkol'.

Statistical analysis

All the experiments were replicated twice to increase the precision and the results are expressed as mean ± SD. All estimated data were statistically analysed by using SPSS 23. Paired t-test and independent one sample t test is performed to analyse the data comparisons between groups.

RESULTS AND DISCUSSION

Identification and qualitative analysis of pigments

(a) Chromatographic analysis by TLC

The observed R_f value of the spot was found to be 0.72 revealing the presence of anthocyanin (Fig 2a).

(b) Coloured Tests with 2M HCl and 2M NaOH

Table1 Coloured tests of anthocyanin pigment extracted

Coloured Tests	Observation	Inference
Extract (0.5 ml) + 2M HCl (0.2 ml)	Orange-red colour (Fig 2(b) A)	Anthocyanin confirmed
Extract (0.5 ml) + 2M NaOH (0.2 ml)	Blue-violet colour (Fig 2(b) B)	

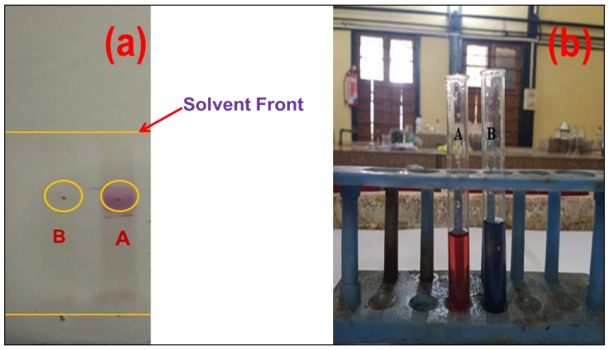

Fig 2 (a) TLC Chromatogram (A) Soxhlet extraction and (B) Maceration: (b) in HCl (A) and in NaOH (B)

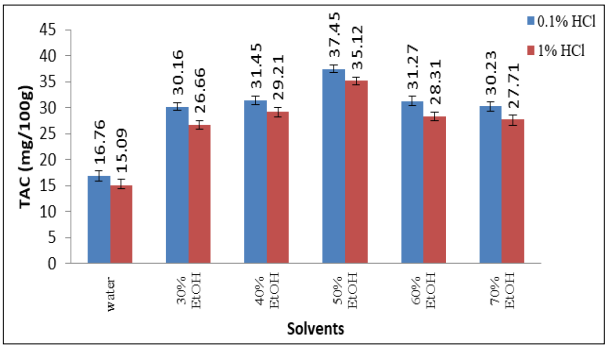

Fig 3 Effect of solvent concentration on TAC from bracts of 'Bhimkol' acidified with 0.1% HCl and 1% HCl

Optimization of extraction condition

The anthocyanins were extracted with various combinations of water and ethanol (30%, 40%, 50%, 60% and 70%) acidified with 0.1%, 1% HCl using Soxhlet apparatus for 2 hours. Acidified aqueous mixtures of ethanol, methanol or acetone are commonly used for the extraction of phenolics and anthocyanins from fruits and vegetables [10-11]. Since methanol is more toxic than ethanol and considering the immense importance of anthocyanin in food and in pharmaceutical industries, we carried out extractions with water and ethanol only. The extraction capacities of different ethanol-water concentrations and also with water on TAC (Fig 3).

The highest yield of TAC extracted from bracts of 'Bhimkol' was obtained from 50% ethanol acidified with 0.1% HCl which is 37.45 mg/100g of flower bracts. It is observed that extraction with 1% HCl irrespective of the solvent used gives lower anthocyanin content than 0.1% HCl. Paired t-test is computed and ($p= 0.001<0.01$) is observed which indicates that the difference is statistically significant. (Fig 3) shows that, as the ethanol level increased from 30% to 50%, TAC increased considerably both in extracts acidified with 0.1% HCl and 1% HCl. In contrast, when the alcohol content was further increased to 70%, the anthocyanin content decreased. This may be because at higher alcohol concentrations, undesirable impurities such as mucus and resin are formed [12]. In our study, good recovery of anthocyanins from banana flower bracts of 'Bhimkol' were obtained with 50% ethanol, so this solvent system was chosen to be used for further work.

Acids were used to weaken the membrane structures of the cells in which the anthocyanin were stored in vacuole which stabilizes anthocyanins in the form of flavylium cation but higher concentration of acids may lead to the partial hydrolysis of glycosidic bond or breaking linkages with metals or co-pigments leading to lower anthocyanin content in the extract [13-14]. Some studies carried out with citric acid were also reported [15-17]. The degradation of

anthocyanin can be reduced by using weaker organic acids such as citric acid and acetic acid as they might destroy the cell membranes, simultaneously dissolving the phenolics and anthocyanins and stabilize them [18-19]. Moreover, extraction of natural pigments may be improved by increasing the extraction time and temperature. For that purpose, solvent mixtures containing 50% ethanol in combination with 1-5% citric acid and 1-5% acetic acid using Soxhlet method for 2 hours and 4 hours as well as maceration for 24 hours was used to evaluate the effect of the extraction time and extraction temperature on the yield of anthocyanins. The experiment conducted shows that out of the all-experimental conditions, the solvent containing 50% ethanol-water acidified with 3% citric acid in Soxhlet apparatus for 2 hours give good recovery of anthocyanin (Fig 4).

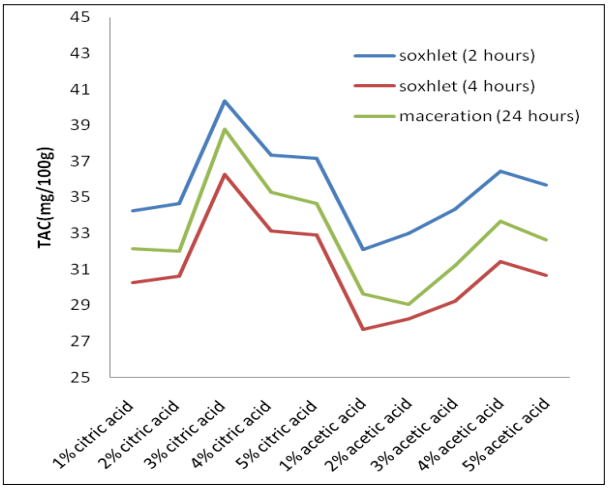

Fig 4 TAC (mg/100g) vs. different concentrations of organic acids by Soxhlet (2 hours, 4 hours) and maceration (24 hours)

Among the all studied methods, Soxhlet method for 2 hours gives us the best result followed by maceration (Table 2). This indicates the influence of temperature on the amount of anthocyanins. A study carried out with grapes skin reported that increase in temperature increases the amount of TAC in extracts by reducing the viscosity which in turn increases the diffusion of the molecules in the solvent [20]. Soxhlet extraction for 4 hours causes decrease in TAC. Heating even with mild organic acids (citric acid and acetic acid) for a longer period of time i.e., 4 hours causes decrease in TAC. Studies carried out in this regard also reported that the total anthocyanin concentration decreased with extraction times [21-22]. This can be explained that degradation of anthocyanins or polymerization reactions may occur which decreases the anthocyanin content. Extraction using maceration for more than 24 hours may yield more anthocyanin content but will be time consuming so was not performed in our study.

Table 2 TAC in extract acidified with 3% citric acid

Extraction methods	Total anthocyanin content (mg/100g) (Mean ± SD)
Soxhlet (2 hours)	40.35 ± 0.27
Soxhlet (4 hours)	36.25 ± 0.45
Maceration (24 hours)	38.76 ± 0.21

50% ethanol acidified with 3% citric acid during Soxhlet for 2 hours and maceration showed higher TAC than solvent acidified with 0.1% and 1% HCl. This finding may be because of degradation of pigment due to the use of solvent containing HCl. Citric acid (pH between 3-6) being a weaker organic acid than HCl (pH of 1.5-3.5), minimizes the degradation of anthocyanin. Citric acid, a naturally occurring antioxidant is also less corrosive and less hazardous than mineral acids, so it can be used to acidify the solvents instead of HCl. Low anthocyanin content was reported in 1-5% acetic acid in 50% ethanol than in 1% HCl in 50% ethanol. This can be explained from the fact that acetic acid being weaker than citric acid could not break the cell membranes effectively resulting in less anthocyanin content in the extracts. The contents of anthocyanins in the extracts obtained by acetic acid and HCl did not differ significantly ($p= 0.253>0.05$). Acetic acid (1.74D) has a higher dipole moment than HCl (1.08D) but, since HCl has a higher hydrolyzing ability of cell membrane thereby leading to almost same results. A similar result has been reported by Hosseini et al [23]. Based on the results of the study, it can be concluded that the 50% ethanol acidified with 3% citric acid using Soxhlet method for 2 hours shows good results on the yield of anthocyanins.

Effect of extraction time and organic acid concentration on TAC

(a) *Effect of extraction time during Soxhlet extraction*

Anthocyanin in the banana flower bract tends to decrease with increase of time in Soxhlet extraction method (Fig 5).

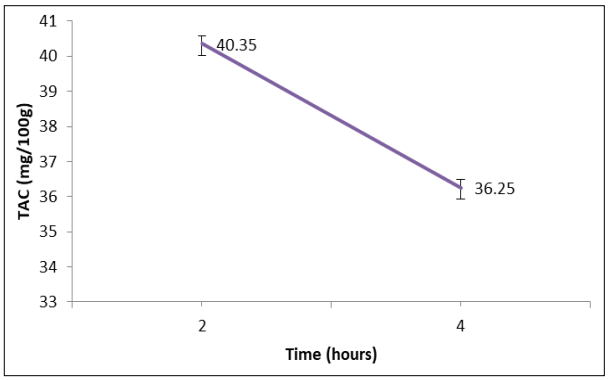

Fig 5 TAC (mg/100g) vs extraction time in Soxhlet method

The extraction yield of anthocyanin is found to be 40.35 mg/100g at 2 hours but decreases with time (Fig 5) which is also found to be statistically significant ($p=0.034<0.05$). This finding shows that the extraction time significantly influences TAC along with other factors. TAC decreases markedly due to the degradation of the extracted anthocyanins, since longer extraction increases the risk of anthocyanin degradation [21].

(b) *Organic acid concentration*

Anthocyanin was extracted using 50% ethanol in the Soxhlet apparatus for 2 hours using 1-5% citric acid and 1-5% acetic acid (Fig 6).

50% ethanol acidified with 3% citric acid in Soxhlet apparatus for 2 hours give good recovery of anthocyanin which is also found to be statistically significant ($p=0.03<0.05$). On the other hand, extractions carried out with 4% acetic acid give more anthocyanin content but is weakly significant ($p=0.057 > 0.05$).

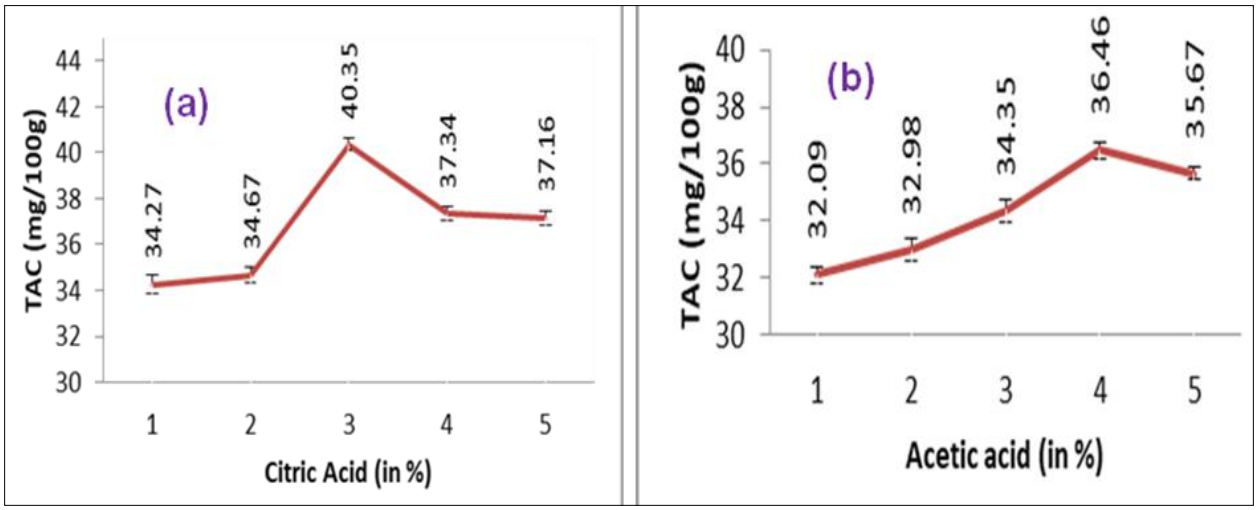

Fig 6 TAC in extracts acidified with (a) citric acid (b) acetic acid concentrations

In this study, pH-differential method is used for calculating the total anthocyanin content. Anthocyanin pigmentation is mainly because of the formation of flavylium cation. The positive charge of flavylium cation however is pH dependent. At pH 1.0, due to the formation of the cation, the molecule is pigmented whereas at pH 4.5 and higher, the positive charge is neutralized so the anthocyanins become colorless. The pH-dependent chemical reaction of anthocyanins is shown in (Fig 7).

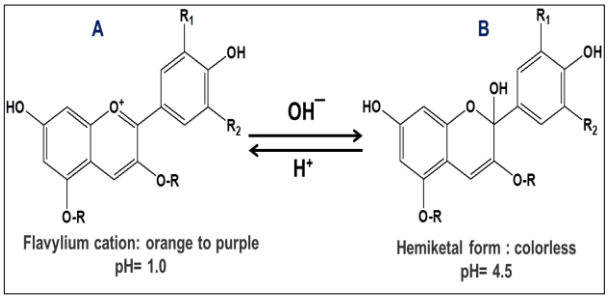

Fig 7 Structures of flavylium cation (A) and hemiketal form (B) of Anthocyanin

The spectrum (Fig 8) clearly shows its pH dependency which is based on the unique pH-dependency of anthocyanin light absorption.

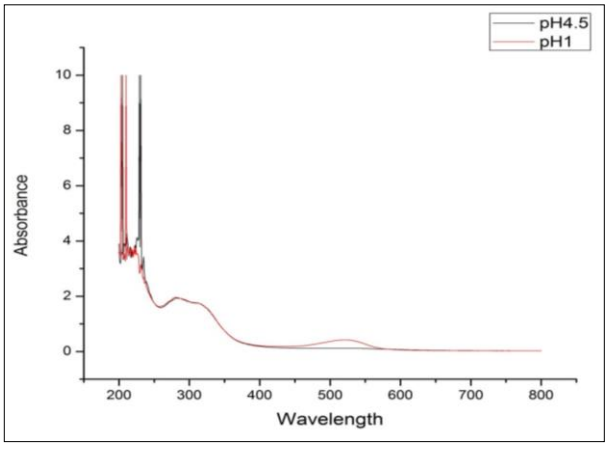

Fig 8 UV –Visible Spectra of Anthocyanin in pH 1 and 4.5 buffers of extracts acidified with 3% citric acid from Soxhlet extraction (2 hours)

Data in (Fig 8) shows the spectra of extract acidified with 3% citric acid from Soxhlet extraction (2 hours). The maximum wavelength of extract acidified with 3% citric acid using different extraction procedures is in the range of 519.31–522.89 nm (Table 3), which is consistent with the absorption spectrum of the anthocyanin color group [8-9].

Table 3 Maximum wavelength of extract acidified with 3% citric acid

Extraction method	Maximum wavelength (nm)
Soxhlet (2 hours)	519.31
Soxhlet (2 hours)	522.89
Maceration (24 hours)	521.32

The highest anthocyanin content extracted in our study from 'Bhimkol' was found to be 40.35 mg/100g which is in agreement with study carried out by Kitdamrongsont *et al*. i.e., 39.35 mg/100g in Thailand [24]. Another study carried out in Bangladesh [25] with 'Bhimkol' using HCl reported TAC to be 22.441 mg/100g (221.41 mg/kg) which is less than our results. In our study the pretreatments which includes drying and making the flower bracts coarse smaller was done which increases the surface contact between the flower bracts and the solvents thereby increases the extraction efficiency [26]. Moreover, other factors like soil pH, growth conditions and climatic conditions also play important role in TAC. Though conventional extraction methods involve large amount of solvents but in our study, the solvents were recovered and reused after distillation.

CONCLUSION

The optimal condition for extraction of anthocyanin from flower bracts of 'Bhimkol' amongst the studied conventional extraction method is 50% ethanol (v/v) acidified with 3% citric acid in a Soxhlet apparatus for 2 hours which give TAC 40.35 mg/100g flower bracts. The content of anthocyanin decreases with increasing extraction time using Soxhlet extraction procedure. The solvent systems for extraction used in this study involves less toxic as well as non-hazardous acidified with mild organic acids, thereby increases its potential use in food and in pharmaceutical industries. Further studies regarding identification and isolation of anthocyanins in flower bracts of 'Bhimkol' (*Musa* BB) is needed as the variation in

composition of anthocyanins depends upon other factors like soil pH, climatic conditions.

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