EFFECTS OF DIFFERENT EXTRACTION CONDITIONS ON YIELD OF ANTHOCYANIN CONTENT FROM *Moringa oleifera* LEAVES

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ABSTRACT

This study was aimed to identify the better extraction conditions for anthocyanin from fresh and dry leaves of Moringa oleifera. The variables used in this study were solvents (aqueous, ethanol, methanol) and each solvent added with acidified agents (1% citric acid, 0.1% HCl and 1% acetic acid), temperatures (40, 50, 60, 70 and 80ºC), steeping times (60, 90, 120, 150, 180 and 210 min) and pH (1, 3, 5, 7 and 9). Study showed that the anthocyanin content was higher for fresh leaves of moringa extracted with test solvent methanol acidified with 0.1% HCl. From the results, extraction at 60ºC for 120 min at pH of 3 was observed as the optimum combination for the yield of maximum anthocyanin content. However, for food purposes, moringa fresh leaves are recommended to be extracted with ethanol (acidified with 1% citric acid). The yield of anthocyanin, at optimised extraction conditions, was found to be 0.359 ± 0.0005 mg C3G/g.

Key words: Anthocyanin, *Moringa oleifera*, pH, Solvents, Temperature, Time

INTRODUCTION

Moringa oleifera L. commonly referred as "*moringa*" is the most widely cultivated species of the family Moringaceae (Vergara-Jimenez *et al*., 2017). It is a fast-growing multipurpose miracle tree extensively grown in tropics and subtropics of India and Africa (Padayachee and Baijnath, 2012). Moringa contains β-carotene, anthocyanins and lycopene pigments in their leaves (Vats and Gupta, 2017). It is one of the nutritious crops and almost every part of the plant has food and medicinal value (Lin *et al*., 2018). Moringa leaves are rich sources of polyphenols, flavonoids and antioxidants. Their leaves are found to be good sources of vitamin – A and anthocyanin (Shm *et al*., 2017). *Moringa oleifera* shows antioxidant activity from their pigments to combat different diseases associated with oxidative stress (Udikala *et al.,* 2017).

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Health and environmental concern over the consumption of antioxidant rich foods has increased by leaps and bounds over the years. Anthocyanin (in Greek *"anthos"* meaning "flower" and *"kyanos"* meaning "blue") is a flavonoid which is a sub class of polyphenols. It is widely known for its pigments responsible for purple, red, blue and black colour of various plant parts (Wallace and Giusti, 2015). Anthocyanin is explored for its profound health benefits of antidiabetic, improving sight acuteness, reducing lipid lowering insulin secretion, inhibiting oxidation, easing platelet aggregation, anti-inflammatory and anticancer properties (Miguel, 2011).

Anthocyanin present in plant varieties was found to exhibit greater antioxidant activity compared to vitamins E and C (Bagchi *et al*., 1997) which has a better ability to scavenge free radicals by donating its hydrogen atoms (Rice-Evans *et al*., 1996). In the recent days, anthocyanin gains attention because of its health benefits owing to its anti-oxidizing properties. Thus, moringa leaves can be considered as a potential source of anthocyanins that exhibit exceptional antioxidant activity which might attract many researchers to find it as a natural antioxidant that can be employed in foods, pharmaceuticals, and cosmetics.

Several parameters that could affect the extraction of anthocyanin compound from the complex plant matrix includes solvent composition, solvent to solid ratio, temperature, time and pH (Swer *et al*., 2018). Anthocyanin is traditionally extracted by aqueous or mixture of aqueous solutions with organic solvents such as ethanol, methanol, and acetone (Kano *et al.*, 2005). Besides, anthocyanin is a sensitive pigment that may degrade or alter from its original state when subjected to a certain temperature, pH and solvents (Martin *et al.,* 2017). Hence it is very important to find precise extraction conditions for maximum recovery of anthocyanin from moringa leaves. The present investigation is an attempt to investigate the effect of different extraction conditions on the anthocyanin content from fresh and dry leaves of moringa. The crude anthocyanin extract obtained from moringa leaves can be used as natural antioxidant in food industry.

MATERIALS AND METHODS

Sample collection

Moringa leaves, procured from the local market, Chennai, were used for the study. The glassware used in the study were of either class A or B and reagents used were of analytical grade.

Extraction of anthocyanins

For extraction, fresh leaves separated from the stalk were subjected to immediate soaking in solvents. For the dried leaves, analysis, leaves were shade dried for 15 hours at room temperature until the moisture content reaches below 10%. To optimize the well suited condition for anthocyanin extraction, various parameters were considered. It included solvents, temperature, time and pH.

Extraction procedure

Crude anthocyanin (mg C3G/g) extraction from moringa leaves (*Moringa oleifera)* using various solvents

Fresh and dried materials were extracted using twelve solvent variations involving, aqueous (water), ethanol and methanol. Each solvent was added with acidifying agents such as 0.1 % HCl or 1 % citric acid or 1 % acetic acid. Fifty percent of each solvent (50 % water + 50 % alcohol/ aqueous) was taken for the study as the test solvents. The samples were mixed with 50% of test solvents, in the ratio of 1:10, as per the modified procedure of Rasha *et al*. (2016). Solvents with extract mixture were shaken in the rotary shaker at 100 rpm for 2 h under room temperature to evaporate the solvent. The resulting extracts were filtered using Whatmann No.1 filter paper. The combination of solvent and acidifier which yielded maximum anthocyanin was selected for further extraction experiments using different temperatures, steeping times and pH.

Crude anthocyanin extraction (mg C3G/g) from moringa leaves (*Moringa oleifera)* at different temperatures

The anthocyanin extraction was tried at temperatures of 40, 50, 60, 70 and 80ºC using the selected food grade solvent to optimize extraction temperature as per the modified procedure of Nayal and Babarb (2017).

Crude anthocyanin extraction (mg C3G/g) from moringa leaves (*Moringa oleifera)* at varying steeping time

To optimize the steeping time, same procedure was repeated and extraction was carried out at optimized temperature with the selected solvent at different time interval viz., 60, 90, 120, 150, 180 and 210 min as per the modified procedure of Nayal and Babarb (2017).

Effect of varying pH on total anthocyanin content of moringa leaves

Different pH buffer solutions (1, 3, 5, 7 and 9) were prepared and used for the study as per the procedure of Wahyuningsih *et al*. (2017).

Estimation of anthocyanins

Total monomeric anthocyanin content in the crude extracts was determined using pH differential method spectrophotometrically. Buffer solutions used were 0.025 M potassium chloride (pH 1.0) and 0.4 M sodium acetate (pH 4.5). Samples were diluted with buffer solution (1:10) and the absorbance was measured at the wavelength of 520 nm and 700 nm. The content of total anthocyanins was expressed as cyanidin-3-O-glucoside (mg) equivalent per gram of the mass of the sample according to the following equation (Lee *et al.*, 2005).

$$\text{Anthocyanin pigment (mg/L)} = \frac{A \times MW \times DF \times 1000}{\varepsilon \times 1}$$

Where,

A	=	(A520nm – A 700nm) $pH_{1.0}$ – (A520nm – A700nm) $pH_{4.5}$
MW	=	449.2 g/mol for cyanidin-3-glucoside (molecular weight)
DF	=	Dilution factor
ε	=	26,900 (molar absorptivity of cyanidin-3-glucoside)
1	=	cell path length in (1cm)
1000	=	factor for conversion from g to mg.

Statistical analysis

All experiments were conducted in six trials. Analysis of variance and t – test were performed using VETSTAT software. The results were expressed as mean ± SE and the least significant difference at $P < 0.01$ was calculated using Duncan's multiple range test to determine the significant differences on effect of solvents, time, temperature and pH on anthocyanin extraction quantity.

RESULTS

Crude anthocyanin (mg C3G/g) extraction from moringa leaves (*Moringa oleifera)* using various solvents

Table 1 shows the effect of different solvents on the yield of total anthocyanin content. The results obtained evidently showed that methanol and its acidified solvents represented high significant ($P<0.01$) difference over other solvents followed by ethanol and its acidified solvents. However, ethanol and its citric acid solvent was chosen for further study with the reasoning discussed later. The findings also showed a significant difference ($P\leq0.01$) on varying solvents between fresh and dry leaves.

Crude anthocyanin extraction (mg C3G/g) from moringa (*Moringa oleifera)* leaves using selected solvent (ethanol acidified with 1% citric acid) at different temperatures

Results of the study on varying temperature on anthocyanin yield from fresh and dry leaves is given in Table 2. The results of the study revealed a significant difference ($P\leq0.01$) in the anthocyanin content of fresh and dry leaves. Among varying temperatures studied, 60 ºC showed significantly ($P<0.01$) high anthocyanin content compared to others and chosen for further study.

Crude anthocyanin extraction (mg C3G/g) from moringa (*Moringa oleifera)* leaves using selected solvent (Ethanol acidified with 1% citric acid) and temperature (60ºC) at varying steeping times

From Table 3, it was observed that highly significant difference ($P\leq0.01$) in anthocyanin content was found between fresh leaves and dry leaves at varying times. Among all the steeping times attempted, 120 m (t3) showed high anthocyanin content and chosen for pH study.

Effects of varying pH on total anthocyanin content of moringa (*Moringa oleifera)* leaves

Table 4 shows the results of effect of different pH (1, 3, 5, 7 and 9) of buffer solution on the yield of anthocyanin. A significant (P≤0.01) difference in total anthocyanin content was found among various pH. The pH of 3 was observed to yield maximum anthocyanin content for fresh leaves. Further, there was a significant difference (P≤0.01) between fresh and dried leaves upon varying the pH.

Table 1 Effects of different solvents on the quantity of extracted crude anthocyanin (mg C3G/g) content from moringa (*Moringa oleifera)* leaves

Solvents	Total monomeric anthocyanin content (mg C3G/g)[$#]		t test value
	Fresh leaves (FL)	Dry leaves (DL)	
S1	0.117 [a] ±0.0004	0.083 [a] ±0.0006	44.44**
S2	0.136 [b] ±0.0037	0.106 [b] ±0.0035	10.21**
S3	0.142 [c] ±0.0002	0.114 [c] ±0.0006	35.78**
S4	0.163 [d] ±0.0004	0.139 [d] ±0.0012	18.85**
S5	0.197 [e] ±0.0004	0.161 [e] ±0.0009	36.00**
S6	0.217 [f] ±0.0008	0.184 [f] ±0.0002	40.86**
S7	0.227 [g] ±0.0008	0.190 [g] ±0.0007	36.60**
S8	0.242 [h] ±0.0007	0.215 [h] ±0.0010	22.66**
S9	0.284 [i] ±0.0008	0.252 [i] ±0.0004	38.44**
S10	0.310 [j] ±0.0006	0.272 [j] ±0.0005	52.29**
S11	0.319 [k] ±0.0004	0.279 [k] ±0.0004	66.06**
S12	0.341 [l] ±0.0003	0.300 [l] ±0.0003	94.34**

[$]Average of six trials.

Total anthocyanin content.

** highly significant (P ≤ 0.01)

Small case superscripts represent significant differences between treatments.

S1 – Aqueous; S2 – Aqueous + 1% acetic acid; S3 – Aqueous+ 1% citric acid;

S4 – Aqueous +0.1% HCl; S5 – 50% Ethanol; S6 – 50% Ethanol + 1% acetic acid;

S7 – 50% Ethanol + 1% citric acid; S8 – 50% Ethanol + 0.1% HCl;

S9 – 50% Methanol; S10 – 50% Methanol + 1% acetic acid;

S11 – 50% Methanol + 1% citric acid; S12 – 50% Methanol + 0.1% HCl

Table 2. Effects of varying temperatures on anthocyanin[#] (mg C3G/g) yield using solvent ethanol acidified with 1% citric acid

Treatments	Temperature (ºC)	Anthocyanin content (mg/g) [$#]		t - test
		FL	DL	
T1	40	0.229[a] ±0.0004	0.199 [a] ±0.0003	41.94**
T2	50	0.334[c] ±0.0001	0.205[c]±0.00023	58.86**
T3	60	0.342[e] ±0.0001	0.211[d]±0.0001	83.08**
T4	70	0.337[d] ±0.0004	0.206[c]±0.0001	126.58**
T5	80	0.331[b] ±0.0002	0.202 [b]±0.0002[c]	88.12**

[$]Average of six trials.
Total anthocyanin content.
Small case superscript represents significant differences between treatments.
** highly significant ($P \leq 0.01$)

Table 3. Effects of steeping times on anthocyanin[#] (mg C3G/g) yield using solvent ethanol acidified with 1% citric acid at optimized temperature of 60 ºC

Treatments	Time (m)	Anthocyanin content (mg/g) [$#]		t - test
		(FL)	(DL)	
t1	60	0.345[b] ±0.0003	0.213[b]±0.0002[a]	116.91**
t2	90	0.352[e] ±0.0006	0.215[c]±0.0008[d]	129.80**
t3	120	0.359[f] ±0.0005	0.226[e]±0.0007[e]	185.20**
t4	150	0.349[d] ±0.0004	0.219[d]±0.0005[f]	134.34**
t5	180	0.347[c] ±0.0001	0.213[b]±0.0004[c]	119.43**
t6	210	0.341[a]±0.0003	0.209[a]±0.0004[b]	96.96**

[$]Average of six trials.
Total anthocyanin content.
Small case superscript represents significant differences between treatments
**highly significant ($P \leq 0.01$)

Table 4. Effects of varying pH on anthocyanin# yield (mg C3G/g) using selected solvent ethanol acidified with 1% citric acid at optimized temperature of 60°C and steeping time of 120m

Treatments	pH	Anthocyanin content (mg C3G/g) $^{\$\#}$		t - test
		(FL)	(DL)	
P1	1	0.363^{e} ±0.0004	0.229 ^{e}±0.0001	92.90**
P2	3	0.359 ^{d} ±0.0003	0.226 ^{d}±0.0004	266.17**
P3	5	0.312 ^{b} ±0.0013	0.205 ^{b}±0.0001	230.11**
P4	7	0.335 ^{c} ±0.0002	0.218^{c}±0.0009	229.45**
P5	9	0.302^{a} ±0.0004	0.192 ^{a}±0.0002	90.29**

$^{\$}$Average of six trials.
Total anthocyanin content.
Small case superscript represents significant differences between treatments.
** highly significant (P≤0.01)

DISCUSSION

Crude anthocyanin (mg C3G/g) extraction from moringa (*Moringa oleifera)* leaves using various solvents

Results on total anthocyanin from moringa extracted using twelve different solvents are shown in Table 1. Better anthocyanin yield was obtained when extracted using methanol acidified solvents followed by ethanol acidified solvents. Acidified methanol gave higher yield because methanol has high dielectric constant which enables extraction of more polar polyphenolic compounds compared to ethanol (Haminiuk *et al.,* 2014). Fresh leaves of moringa characterised to have maximum yield. Khoo *et al*. (2017) reported that ethanol is a safer extraction solvent for isolation of anthocyanin and the use of weak acid like citric acid is advisable for extraction of anthocyanins. Hence, considering hazardous effects of methanol and HCl on human health, ethanol solvent may be preferred for food use. Based on this, fresh leaves extracted with ethanol acidified with citric acid was selected as safe solvent and their anthocyanin content was 0.227±0.0008 (mg C3G/g). The values were in agreement with values of Omede (2016) who reported that moringa leaves had maximum anthocyanin content of 0.348±0.004 mg C3G/g despite the various extraction conditions.

Crude anthocyanin extraction (mg C3G/g) from moringa (*Moringa oleifera)* leaves using selected solvent at different temperatures

The effect of different temperatures, from 40 to 80°C, on extraction quantity of anthocyanin in fresh and dry leaves of *Moringa oleifera* was studied. Study showed that, the selected solvent (ethanol acidified with 1% citric acid) at temperature of 60ºC yielded maximum anthocyanin of 0.342±0.0001 (mg C3G/g) in fresh and 0.211±0.0001 (mg C3G/g) in dry leaves of moringa. Comparatively, fresh leaves gave the higher yield than the dry leaves. The results also showed that the increase in temperature from 40 to 60ºC gradually

increased the anthocyanin yield whereas the yield started declining beyond 60ºC. This finding correlated with the finding of Nayal and Babarb (2017) who also suggested that optimum extraction temperature for anthocyanin was 60ºC. The reason behind the lesser stability of anthocyanin is that, at higher temperatures, phenolic compounds like anthocyanins present in the crude extract degraded enzymatically by polyphenol oxidase enzyme (Luo *et al*., 2017). The results also correlated well with the findings of Khoo *et al*. (2017) who reported that the mild heat treatment up to 60ºC can be provided to prevent the oxidation of anthocyanin.

Crude anthocyanin extraction (mg C3G/g) from moringa (*Moringa oleifera)* leaves using selected solvent and temperature at varying steeping times

The present study was carried out at optimum extraction temperature of 60ºC using selected solvent (ethanol acidified with 1% citric acid) on different steeping times. The best duration for extraction was predicted from the Table 3. Comparatively, the results of treatments show that longer drying time of 210 m reduced the total anthocyanin yield with mean and SE values of 0.341±0.0003 (mg C3G/g) whereas at 120 m, the anthocyanin yield was higher with the mean and SE values of 0.359±0.0005 (mg C3G/g). The results were in accordance with the results of Nayal and Babarb (2017) who indicated that two hours of extraction at 60ºC showed better content of anthocyanins. The anthocyanin yield was found to be higher in fresh leaves compared to dry leaves.

Effect of varying pH on total anthocyanin content of moringa leaves

As observed with the results in Table 4, pH had a great influence on the anthocyanin yield. At more acidic pH (1, 3) colour of anthocyanin was bright and showed higher absorbance. But when the pH was increased, the colour changed slightly to light colour which resulted in less absorbance. The results of the study were in accordance with the results of Wahyuningsih *et al*. (2017) who also stated that anthocyanin was more stable in acidic pH and degrades at alkaline pH. Our study shows that the stability of flavylium ion was responsible for stability at acidic condition and colour degrades at increasing pH condition (Khoo *et al*., 2017).

CONCLUSION

This study was conducted to optimize various parameters (solvent, temperature, time and pH) for finding best and effective extraction condition for obtaining maximum yield of anthocyanins in moringa fresh and dry leaves. From the investigation, it was found that methanol with 0.1% HCl gave maximum yield of anthocyanin compared to other test solvents. However methanol being a toxic solvent, its adverse effects on human health should be taken into consideration in recommending for pharmaceutical and food purposes. From our results, maximum anthocyanins in Moringa fresh and dry leaves could be extracted using ethanol acidified with 1% citric acid at 60ºC for 2 h at pH of 3.

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