Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 3, 2015

Original Article

# EXTRACTION, STABILITY AND SEPARATION OF ANTHOCYANINS OF *IXORA COCCINEA* LINN

**NITAL PATIL*, AJIT DATAR**

**Guru Nanak Institute for Research and Development, G. N. Khalsa College, Matunga, Mumbai, Maharashtra, India, 400019**
**Email: nitss99@gmail.com**

***Received: 13 Dec 2014 Revised and Accepted: 25 Dec 2014***

**ABSTRACT**

**Objective:** In the present study, a new, simple and an efficient microwave oven extraction method was developed for maximum yield of colorant from *Ixora coccinea* flowers. The preliminary stability study of colorant was carried out to provide reliable data for application of the plant.

**Methods:** The comparison of cold extraction and microwave oven extraction method was carried out. The crude anthocyanin content was estimated by using pH differential method. The stability of color extract was evaluated for the period of one month by using UV- Visible spectroscopy. An attempt was made to separate color components of the extract by developing HPTLC method.

**Results:** The yield of microwave oven extraction of colorant is found to be 13.26% under the optimized conditions set at the temperature of 70˚C, 50 minutes time, 0.1% HCl concentration in solvent and 1:60 ratio of sample to solvent. The result indicated that the anthocyanin content is 704.73 mg/100 g to 662.79 mg/100g in fresh material. The study demonstrated that the pinkish red extract of the flowers is stable under low pH values (≤4) and unstable under alkaline conditions. It is also found to be sensitive to high temperature and light. The HPTLC profile of color showed the presence of pink and violet color major components at $R_f$ values 0.17, 0.33, 0.37, 0.56, 0.67 and 0.83.

**Conclusion:** The experimental work on *Ixora coccinea* provides enough information to tap its potential as a colorant in acidic foods or cosmetics.

**Keywords:** *Ixora coccinea*, Anthocyanins, Microwave oven extraction, Stability, HPTLC.

## INTRODUCTION

*Ixora coccinea* Linn is one of the candidates of floral anthocyanins. It belongs to the Rubiaceae family, a large glabrous shrub growing throughout forest lands and also cultivated plant in the garden [1]. It is a native to Asia and commonly known as Jungle of geranium or flame of the woods or vetchi in Ayurveda [2].

It has different flower color; namely dark pink, white, yellow and orange. Flowers are numerous and found to grow in clusters. They are bright red petaled flowers with yellow or light pink at the centre, odorous, in sessile, corymbiform, dense flowered cymes [2]. *Ixora coccinea* (IC) is grown in many countries essentially for medicinal and ornamental purpose. Depending on the disease, the flowers, leaves, roots or stems may be used to treat various ailments in Indian traditional system of medicine. Phytochemical studies indicated that the plant contains important phytochemical such as lupeol, ursolic acid, oleanolic acid, sitosterol, rutin, lecocyanadin, anthocyanins, proanthocyanidins, glycosides of kaempferol and quercetin. Flowers are reported to contain rutin, leucocyanidins glycoside, cyanidin-3-rutinoside and delphinidin monoglycoside [3, 4].

The essential oil of IC flowers is composed of 54 components; resulting 99.97% of the total components detected. [2, 5]. Nayak *et al.* reported the wound healing activity of alcoholic extract of IC flower by using dead space wound model in rats [2]. Methanolic extract of flower has antioxidant activity [6, 7]. The antitumor activity of IC flower has been studied in comparison to intraperitoneally transplanted Dalton's lymphoma [8]. The active fraction from *I. coccinea* flowers have significantly prolonged the life span of cisplatin treated mice and maintained their blood urea nitrogen levels in the near normal range, indicating its chemo protective effects [9]. Nagaraj *et al.* reported the synthesis of gold nanoparticles in aqueous medium using flower extracts of IC as a reducing and stabilizing agent [10].

Anthocyanins from IC flowers have drawn attention for their potential as natural colorant source [11, 12]. Many studies have shown that anthocyanins are not only nontoxic and non mutagenic, but have positive therapeutic activities [13-17]. The interest in anthocyanins considerably increased when synthetic food colorants, particularly red ones, began to be questioned as additives for possessing adverse health effects.

Due to their attractive colors, water solubility, along with positive therapeutic effects; IC flowers should be considered as potential substitute for synthetic colorants. To our knowledge, report or physiochemical studies of anthocyanins of IC flower is scarce. The stability of anthocyanins from IC flower has not been studies yet. To provide some reliable data for its application in food or cosmetics the effect of pH, temperature, light on the stability of crude anthocyanins was studies. In this studied, an attempt was made to develop the microwave oven extraction method over conventional cold extraction method. Our approach also involved the development of HPTLC method for separation of color or pigment extracted from both the methods.

## MATERIAL AND METHODS

### Materials

Fresh dark red color IC flowers were collected from Palghar district, Maharashtra, India. Herbarium of the plant was authenticated from BSI having voucher no GNKC - 6. All chemicals used in the study were purchased from Merck Co. and were of analytical grade.

### Equipments and apparatus

Multifunctional microwave oven (Sharp carousel) model number R-880B having 0 -1200W range and emission frequency 2450 MHz, UV- visible spectrophotometer (Shimadzu, 1650), HPTLC Linomat applicator- V and scanner III (Camag), twin trough chamber (Camag), pH meter (Lab India Pico+).

### Extraction methods

The anthocyanins of IC were extracted by conventional cold extraction method. However, to obtain maximum yield of pigment in less time, an efficient method of microwave oven extraction (MWE) has been developed.

1) Cold extraction method: Fresh flowers of IC were cut into small pieces and 1g of them was extracted using 0.1% acidified ethanol (HCl in ethanol) v/v in a conical flask. The flask was kept on the shaker at room temperature for 50 minutes. (The time was optimized.)

2) MWE and optimization of extraction conditions: There are many parameters which affect the microwave extraction efficiency, such as

particle size of the sample, liquid to solid ratio, extraction temperature, microwave power, extraction time. Based on preliminary experimental results, appropriate ranges of sample to solvent ratio, extraction temperature, acid concentration, extraction time were selected. The variables of each parameter are indicated in Table 1. After extraction, every sample was filtered through whatman paper no. 41 and centrifuged at 10000 rpm for 15 min. The percent yield was calculated for every extraction method.

**Table 1: Variables for Microwave oven extraction**

| Independent variables | Ranges |
|---|---|
| Ratio of sample: Solvent (g/ml) | 10, 20, 40, 60, 80, 100 |
| Acid concentration % (V/V) | 0.05, 0.1, 0.2, 0.3, 0.4 |
| Temperature (˚C) | 70 Constant |
| Time (min) | 10, 20, 30, 40, 50, 60, 70 |

**Total anthocyanin content**

Cyanidin -3-rutinoside is a pigment in *I. coccinea* flower, found to be a major pigment [3]. Total monomeric anthocyanin was estimated by a pH differential method [18]. Two dilutions of IC flower extract obtained by optimized MWE were prepared each one with potassium chloride (KCl) buffer (pH1.0) and the other with sodium acetate ($CH_3COONa.3H_2O$) buffer (pH4.5). Absorbance was measured simultaneously at 510 nm and 700 nm after 30 min of incubation at room temperature. The content of total anthocyanins was expressed in mg of cyanidin-3- rutinoside equivalents using a molar extinction coefficient (ε) of cyanidin-3- rutinoside of 7000L mol-1 cm-1 [19] and molar weight (MW) of 595.2 g mol-1 as follows:

$$C\ (mg/100g\ of\ fresh\ product) = \frac{\Delta A \times MW \times DF \times V \times 100}{\varepsilon \times 1 \times m}$$

Where $\Delta A = (A_{510\ nm} - A_{700\ nm})\ pH1.0 - (A_{510\ nm} - A_{700\ nm})\ pH4.5$; DF = dilution factor; 1 = path length in cm; V = volume of stock / extract in ml; m = weight of sample in g.

**Determination of stability**

The MWE was found to be efficient extraction method over cold extraction method. Therefore, the crude anthocyanins from IC flower were extracted using optimized microwave oven extraction method (as mentioned above). A fresh material of 1 g was extracted using 60 ml of acidified ethanol in microwave oven at 70˚C for 50 min. The solusion was filtered. A scan from 400 to 800 nm was performed in order to generate the characteristic absorption spectra of the sample using UV-visible spectrophotometer. The sample was prepared initially at pH = 1.0 (original pH of the extract) with absorbance reading of 0.8 at the wavelength of maximum absorption in the visible region. The sample was prepared at six different pH values (2, 4, 6, 8, 10, and 12) using 0.1N NaOH. The effect of pH on colorant stability was performed immediately after preparing the extract at different pH values. UV –visible spectra were recorded between 200-700 nm on UV–visible spectrophotometer.

In order to study the effect of temperature on the stability of IC anthocyanins, diluted samples inside capped glass vials, covered with aluminum foil, sealed with paraffin were placed at room temperature (~25˚C), 4˚C, 15˚C, and 40˚C for a period of 1 month. To study the degradation of anthocyanins under the influence of light, sample solution containing glass tubes were exposed to natural light outdoor (sunlight), natural light indoor and dark room. At appropriate time intervals, the samples were taken out to measure the absorbance at its $\lambda_{max}$ -536 nm.

**HPTLC analysis**

Sample preparation: The color extract of IC flower obtained from MWE and cold extraction methods were evaporated on water bath at 40˚C and reconstituted in acidified ethanol to make 2500 0 ppm solution. Samples were filtered through 0.45µ filter.

Chromatography of anthocyanins extracts was performed on 10 cm x 5 cm Aluminium backed Silica gel $F_{254}$ TLC plate. Samples were applied to the plate by means of Linomat –V applicator (Camag, Switzarland) equipped with a 100µl syringe. The band length was 8 mm and the application volume was 5µl. The plate was developed with BFW {n-butanol: formic acid: water (6:0.85:2 v/v/v)} as mobile phase in saturated glass Camag twin trough chamber. The saturation time was 10 min. The migration distance was approximately 80 mm and migration time ≈ 50 minutes. The separated zones were examined under visible light and evaluation was performed densitometrically with a TLC scanner –III and Win CATS software (V-1.2.3). The scanner was operated at 536 nm. The slit length and width were 6 mm and 0.45 mm respectively.

**RESULTS AND DISCUSSION**

**Optimization of MWE**

The effect of different solvent proportion on the colorant yield is shown in Figure 1A. It showed that the yield of colorant was greatly influenced by the volume of the solvent. The yield of color was affected by HCl acid concentration (0.05%, 0.1, 0.2%, 0.3%, and 0.4% respectively) is shown in Figure 1B.

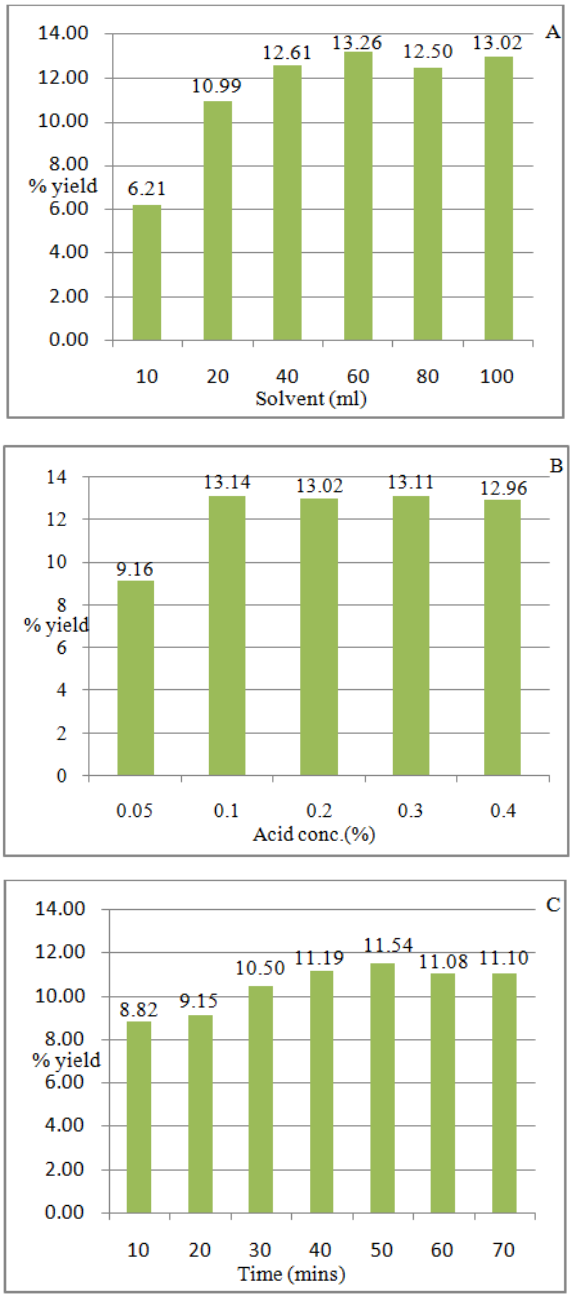



**Fig. 1: A- Effect of solvent proportion, B- Effect of acid concentration, C- Effect of time on the yield of colorant**

The temperature was kept constant at 70°C (as there was no option available) considering microwave oven model and solvent chosen. When other three factors (ratio of solvent to sample, acid concentration and extraction temperature) were fixed at 60 ml per gram, 0.1% and 70°C respectively, the result indicated that the yield of total colorant increased with the increase of microwave extraction time in the beginning of extraction. The yield reached its maximum at 11.54% in 50 minutes during microwave extraction process. When extraction time exceeds more than 50 minutes, the yield of colorant did not increase with the increase of extraction time. Hence, extraction time to be optimized is 50 minutes. The influence of extraction time on the yield of total colorant is shown in fig 1C.

**Comparison of microwave extraction (MWE) with cold extraction method**

As shown in Table 2, the optimized MWE gave yield of 13.26% of crude anthocyanins extract which was higher than of cold extraction (5.18%). The MWE has an advantage of reducing extraction time, saving energy and increased yield as compared to cold extraction.

These results indicated that the MWE method is more suitable for extraction of anthocyanins from IC flowers than cold extraction method.

**Table 2: Comparison of microwave extraction and traditional cold extraction**

| Extraction method | Cold extraction | Microwave oven |
|---|---|---|
| Ratio solid/liquid (g/ml) | 60 | 60 |
| Acid concentration (% v/v) | 0.1 | 0.1 |
| Time (min.) | 50 | 50 |
| Yield of color (%) | 5.18 | 13.26 |

**Anthocyanin content**

Anthocyanin pigments undergo reversible structural transformations with a change in pH manifested by different absorbance spectra. The oxonium form predominates at pH 1.0 while the hemiketal (colorless) form at pH 4.5. The pH-differential method is based on this reaction and allows accurate and rapid measurements of the total amount of anthocyanins, even in the presence of polymerized degraded pigment and other interfering compounds [19]. The concentration of total monomeric anthocyanin in *Ixora* flower extracts ranged 704.73 mg/100 g to 662.79 mg/100g of fresh flower.

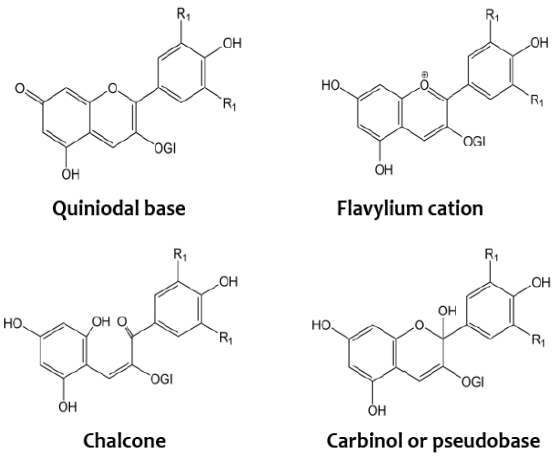



**Fig. 2: Four equilibrium forms of anthocyanins**

**Measurement of color and stability**

The characteristic pinkish red color manifested by the IC flower extract is a consequence of the presence of compounds derived from anthocyanins group [20]. The absorption spectra generated as shown in Figure 3A attributed the similar pattern for both the extract obtained from cold and MWE methods confirms that the pigment was not degraded by microwave oven extraction process. A visible spectrum of IC flower extract in acidified ethanol (pH=1.0) exhibited the wavelength of maximum absorption at 536 nm. This value is found to be close with cyanidin (λ=535 nm) [20].

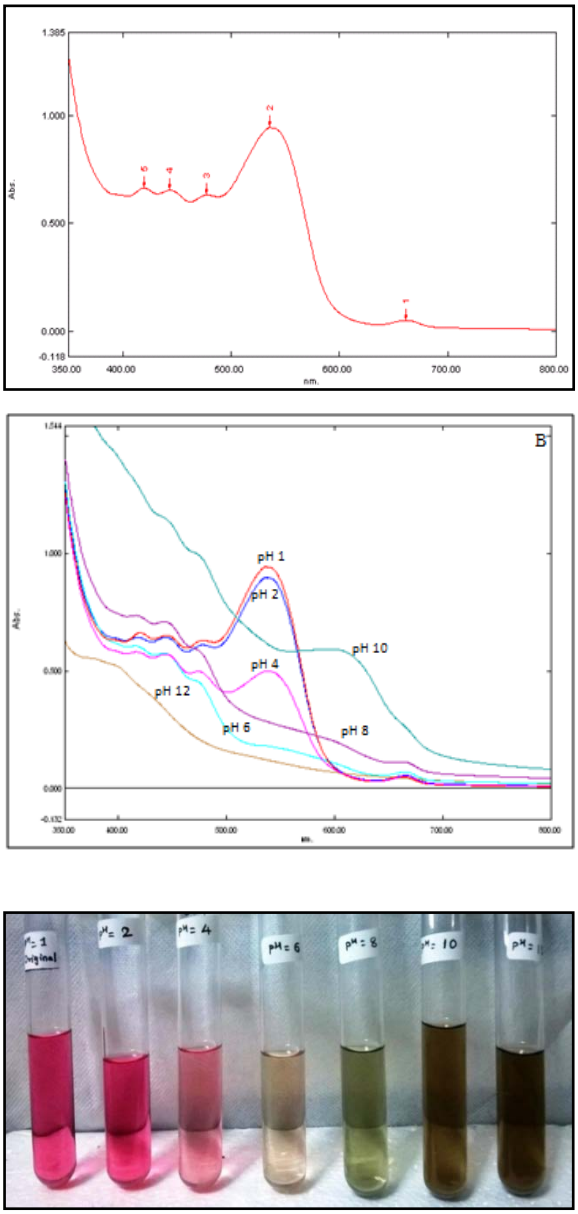



**Fig. 3: A- Visible spectra of *I. coccinea* in acidified Ethanolic extract B- Spectrum charts at different pH, C- Pictures of colorants at pH range 1.0 to 12.0**

**Effect of pH**

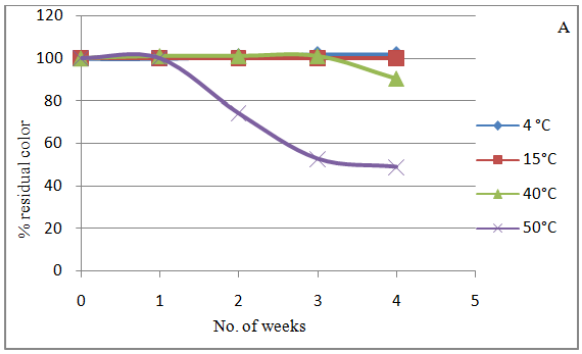
The spectral pattern of IC flower extract showed that the absorbance decrease was not significant when pH ≤ 4.0 but anthocyanins degraded significantly after pH ≥ 4.0. This demonstrated that the IC anthocyanins are stable under low pH values (≤4) and unstable under alkaline conditions. Anthocyanins are easily susceptible to pH changes due to the ionic nature of anthocyanin. Anthocyanins exist as four equilibrium forms (Figure 2), viz. the quinonoidal base, the flavylium cation, the carbinol (pseudobase) and the chalcone. Under low pH, the anthocyanin exists primarily in the form of flavylium cation

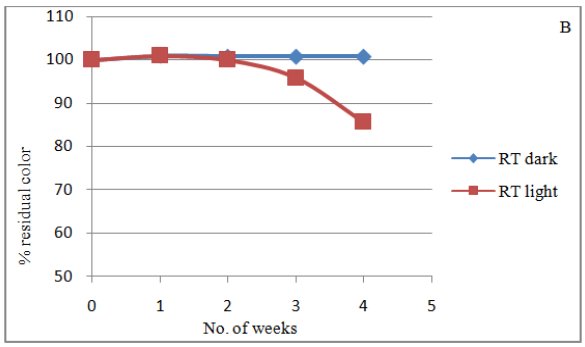
in red. As the pH is raised (>5), a rapid loss of proton occurred to form quinoidal base that tend to become blue or violet. In addition, the increase in pH causes the hydration of the flavylium cation to form a carbinol (pseudobase) or chalcone which are colorless [21]. The study of the effect of pH on the stability of anthocyanins indicated that they were susceptible to pH change. This is consistent with the result of most published studies on the stability of anthocyanins [22, 23].

**Effect of temperature**

Thermal stability of red extract of IC flower was studied at 4°C, 15°C, 40°C and 50°C. As shown in Figure 4A at low temperature anthocyanin pigment was not degraded over the period of 30 days. The pigment concentration found to be 100% residual color at 4°C and 15°C. However, the pigment degraded significantly after exposure to higher temperature 40°C and 50°C. The percent of residual color were found to be 90.4 % and 48.9 % after 30days when exposed to 40°C and 50°C respectively. The significance of the absorbance decrease was not obvious in dark condition.

There was a little difference in the absorbance of anthocyanins in the sample exposed to light condition indoor which showed 85.6 % residual color after 30 days. (Fig. 4B) As presented in Figure 4C, sunlight greatly affects the stability of anthocyanins. The color was degraded quickly and only 32.0 % remained after 10 hrs. Hence direct sunlight should be avoided in processing, storage, and usage of anthocyanins. They are best stored at dark.



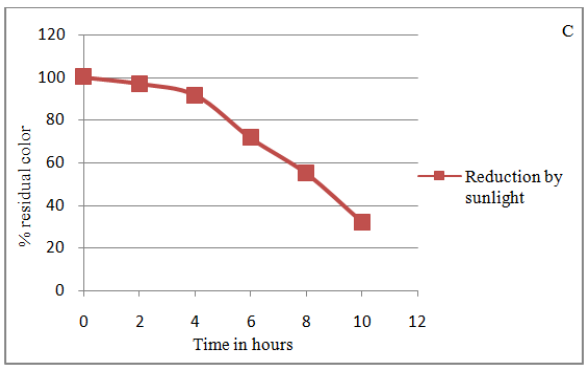




**Fig. 4: A-Effect of temperature, B- Effect of light and dark conditions, and C- Effect of sunlight**

**HPTLC**

HPTLC profile was developed to separate anthocyanins component(s) of IC flower extract. The red color extracts showed presence of pink and violet color major components at $R_f$ values 0.17, 0.33, 0.37, 0.56, 0.67, 0.83 as indicated in Table 3A and B. The chromatographic fingerprint profile of cold extract of IC flower was found to be similar to that of the microwave oven extracted extract which further confirms efficient extraction of anthocyanin components by MWE method fig. 5.

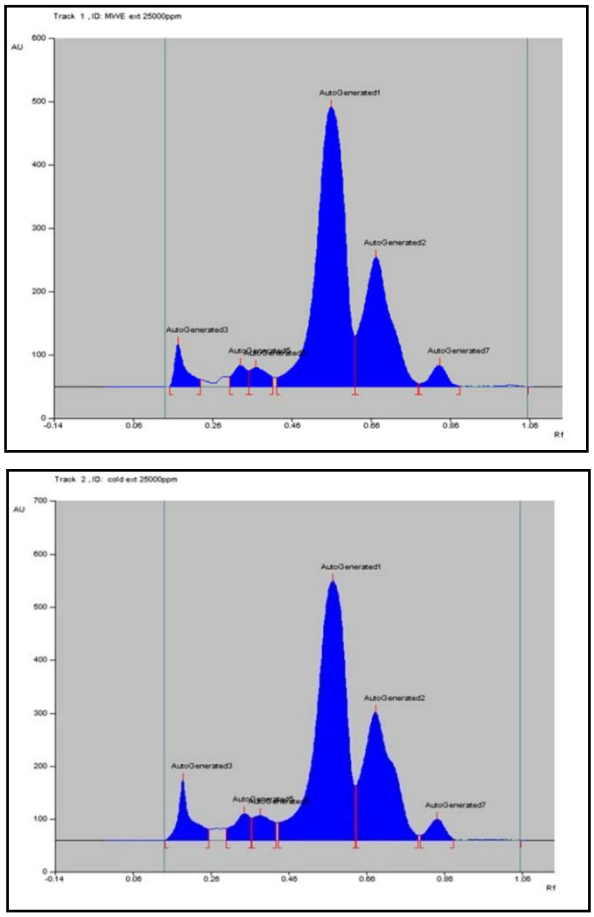


**Fig. 5: (A) HPTLC chromatograms of *I. coccinea* extract obtained from microwave oven extraction (i) and cold extraction method (ii) at 536 nm**


**Fig. 5: (B) TLC plate of *I. coccinea* extract obtained from microwave oven extraction (i) and cold extraction method (ii) in visible light**

**Table 3: (A) Peak List and $R_f$ values of Chromatogram of *I. coccinea* extract obtained by microwave oven extraction method at 536 nm**

| Peak no. | $R_f$ | Area | % Area |
|---|---|---|---|
| 1 | 0.17 | 1534.1 | 3.69 |
| 2 | 0.33 | 938.6 | 2.26 |
| 3 | 0.37 | 1071.4 | 2.58 |
| 4 | 0.56 | 25347.2 | 61.03 |
| 5 | 0.67 | 11507.0 | 27.71 |
| 6 | 0.83 | 1135.7 | 2.73 |

**Table 3: (B) Peak List and $R_f$ values of Chromatogram of *I. coccinea* extract obtained by cold extraction method at 536 nm**

| Peak no. | $R_f$ | Area | % Area |
|---|---|---|---|
| 1 | 0.19 | 2955.3 | 5.63 |
| 2 | 0.34 | 1741.6 | 3.32 |
| 3 | 0.38 | 1883.0 | 3.59 |
| 4 | 0.57 | 29851.6 | 56.87 |
| 5 | 0.68 | 14691.2 | 27.99 |
| 6 | 0.84 | 1370.4 | 2.61 |

## CONCLUSION

An optimized MWE method for IC anthocyanin extract has been developed. This is the first report on MWE of anthocyanins from IC flower which results in 13.26% yield under the optimized conditions set at temperature of 70˚C, 50 minutes time, 0.1% HCl concentration in solvent, and 1:60 ratio of sample to solvent. MWE can save time, energy and provide higher extraction yield. *Ixora coccinea* flowers have rich anthocyanin content (704.73 mg/100 g to 662.79 mg/100g) in fresh material. The crude anthocyanins extract from IC flower was stable at low pH ≤ 4 and sensitive to alkaline condition, high temperature and light. It can be considered as potential colorant in acidic foods or cosmetics. Further in vivo study need to be carried out to prove its safety. An attempt was made to separate color components of IC flowers by developing HPTLC method for the first time. HPTLC is a very simple, rapid and economical method, and it can be used for the separation of different pigments from the plants. Further research is going on to isolate and characterize the monomeric anthocyanins from IC flowers.

## ACKNOWLEDGMENT

The authors wish to express sincere thanks to G. N. Khalsa College and the Director - Dr. R. T. Sane of Guru Nanak Institute for Research and Development department for providing facilities and support. We are also thankful to Manjusha Nigudkar for her valuable help.

## CONFLICT OF INTERESTS

Declared None

## REFERENCES

1. Tondon N, Sharma P. Quality standards of Indian medicinal plants. ICMR 2013;11:242-52.
2. Elumalai A, Eswaraiah C, Venkatesh Y, Shiva Kumar B, Narendar C. Phytochemical and pharmacological profile of *Ixora coccinea* Linn. Int J Pharm Life Sci 2012;3 Suppl 3:1563-7.
3. Kharat AR, Nambiar VV, Tarkasband YS, Pujari RR. A review on phytochemical and pharmacological activity of genus *ixora*. Int J Res Pharm Chem 2013;3 Suppl 3:628-35.
4. Baliga MS, Kurian PJ, *Ixora coccinea* Linn. Traditional uses, phytochemistry and pharmacology. Chin J Integr Med 2012;18 Suppl 1:72-9.
5. Sumathy H, Sangeetha J, Vijayalakshmi K. Chromatographic fingerprint analysis of *ixora coccinea* methanolic flower extract. Int J Pharm Sci Drug Res 2011;3 Suppl 4:327-30.
6. Saha MR, Alam Md A, Akter R, Jahangir R. *In vitro* free radical scavenging activity of *Ixora coccinea* L. Bangladesh J Pharmacol 2008;3:90-6.
7. Rohini S, Shalini M, Narayanaswamy N, Balakrishnan KP. Application of natural products in cosmetics: a study of *ixora coccinea* extracts for their antityrosinase and antioxidant activities. Int J Res Cosmet Sci 2012;2 Suppl 1:1-7.
8. Latha PG, Panikkar KR. Cytotoxic and antitumor principles from *Ixora coccinea* flowers. Cancer let 1998;130(1, Suppl 2):197-202.
9. Latha PG, Panikkar KR. Chemoprotective effect of *Ixora coccinea* L. flowers on cisplatin induced toxicity in mice. Phytother Res 2001;15 Suppl 4:364–6.
10. Nagaraj B, Krishnamurthy N, Liny P, Divya T, Dinesh R. Biosynthesis of gold nanoparticles of *Ixora coccinea* flower extract and their Antimicrobial activities. Int J Pharm Biol Sci 2011;2 Suppl 4:557-65.
11. Deshpande R, Chaturvedi A. Methanol extracts of anthocyanin pigment and their suitability on fibers. J Environ Res Dev 2011;5 Suppl 3:648-51.
12. Deshpande A, Jadge D, Dhawale S, Patrakar R, Gadgul A. Flower extract of *ixora coccinea* as a natural indicator in acid base titration. J Pharm Res 2010;3 Suppl 10:2512-3.
13. Vemuri M, Kelley DS, Erickson KL. Health effects of foods rich in polyphenols. In: De Meester F, Watson RR editors. Wild-type food in health promotion and disease prevention. New Jersey, USA: Humana Press; 2008. p. 393–412.
14. Jiao Z, Liu J, Wang S. Antioxidant activities of total pigment extract from blackberries. Food Technol Biotechnol 2005;43:97–102.
15. Hale SB, Galli RL, Meterko V, Carey A, Bielinski DF, McGhie T, *et al*. Dietary supplementation with fruit polyphenolics ameliorates age-related deficits in behavior and neuronal markers of inflammation and oxidativestress. AGE 2005;27:49–57.
16. Hassimotto NM, Genovese MI, Lajolo FM. Absorption and metabolism of cyanidin-3-glucoside and cyanidin-3-rutinoside extracted from wild mulberry (*Morus nigra* L.) in rats. Nutr Res 2008;28:198–207.
17. Yao Y, Vieira A. Protective activities of Vaccinium antioxidants with potential relevance to mitochondrial dysfunction and neurotoxicity, Neurotoxicol 2007;28:93–100.
18. Lee J, Durst RW, Wrolstad RE. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants and wines by the pH differential method: collaborative study. J AOAC Int 2005;88 Suppl 5:1269-78.
19. Stoica R, Senin RM, Ion RM. Ethanol concentration effect on the extraction of phenolic compounds from *Ribes nigrum* assessed by spectrophotometric and HPLC-DAD methods. Rev Chim (Bucharest) 2013;64 Suppl 6:620-4.
20. Haborne JB. Phytochemical methods a guide to modern techniques of plant analysis, Phenolic compounds. 3rd ed. Springer Science & Business Media; 1998. p. 69.
21. Cavalcanti RN, Santos DT, Meireles MA. Non-thermal stabilization mechanisms of anthocyanins in model and food systems—An overview. Food Res Int 2011;44:499–509.
22. Kirca A, Ozkan M, Cemeroghlu B. Effects of temperature, solid content and pH on the stability of black carrot anthocyanins. Food Chem 2007;101(1):212–8.
23. Janna OA, Khairul AK, Maziah M. Anthocyanin stability studies in *Tibouchina semidecandra* L. Malaysian J Biochem Mol Biol 2006;13:22-6.