International Journal of Modern Science and Technology, 2018;3(9):196-202.
ISSN: 2456-0235.
www.ijmst.co

Research Article

Extraction, purification and analysis of biological activity of anthocyanin like compound extracted from *Punica granatum* (Pomegranate) peel

R. Jayalakshmi, D. Yuvaraj*, J. Iyyappan, R. Gnanasekaran

Department of Biotechnology, Vel Tech High Tech Dr. Rangarajan Dr. Sakunthala Engineering College, Avadi, Chennai - 600062.

*Corresponding author's e-mail: yuvarajdinakarkumar@gmail.com

Abstract

Anthocyanins are natural plant pigments that have numerous beneficial effect for plant as well as for humans. Anthocyanin protects plants from UV radiation, infection, injury and stress. Health benefits of anthocyanins are innumerable. The aim of this work is to extract anthocyanin like compound from *Punica granatum* (pomegranate) peel, which is discarded as waste after the consumption of fruit. Both dried peels and fresh peels of pomegranate were used in this study. The idea is to obtain something valuable from waste, which can be used in a beneficial way. Anthocyanins were extracted from peel by extraction with methanol and by using soxhlet apparatus. Quantification of anthocyanins were done by pH differential method. Antioxidant assays such as DPPH assay and ABTS assay were carried out. Antimicrobial studies with bacteria and fungi were also carried out by agar well diffusion method and a good zone of inhibition was obtained, which shows that pomegranate peel is rich in antimicrobial activity. Antioxidant and antimicrobial properties were greater for dried peel in comparison with fresh peels. The extracted anthocyanins can be used as natural colorant, this may be good replacement for chemical colorant. Anthocyanin based colorants are approved for food use all over the world, however there are some regulatory differences among countries.

Keywords: Anthocyanins; Antioxidant; Antimicrobial; HPLC; Food colorants.

Introduction

Anthocyanins are widespread in plant kingdom and are observed mostly in fruits, flowers, leaves and cereal grains. Cyanidinn, delphinidin, peonidin, petunidin, malvidin and pelargonidin are the naturally occurring anthocyanins in plants [1,2]. Anthocyanins belong to group of compounds called flavonoids [3,4]. Anthocyanins are affected by structural modifications with hydroxyl, methoxyl, glycosyl especially acyl group by environment factors such as pH, temperature [5]. Stability of anthocyanins also depends on their chemical structure, concentration, oxygen, light, enzymes, metal ions and various storage conditions [1].

Anthocyanins act as antioxidants or free scavengers that prevents oxidative stress [6,7]. Evaluating antioxidant capacity of extract is helping in the areas of nutrition and food technology [8]. Anthocyanins in plants plays important role such as pigmentation, growth, reproduction, radical scavenging, signaling, defense from pathogens and parasitic attack. It is also produced in response to invading fungus, stress, injury, infection and UV irradiation [9]. Anthocyanins are used as traditional medicines in humans because of their ability to treat diseases such as hypertension, pyrexia, liver disorder, dysentery, diarrhea, urinary problem and common cold [3].They are good in fighting against degenerative diseases such as cancer, cardiovascular disease, diabetes, alzheimers's disease and ageing [10,11]. Anthocyanins are well known to reduce serum cholesterol, triglyceride, High density lipoprotein, enhance low density lipoprotein, inhibit atherosclerosis, regulate blood fat and prevent high blood pressure [12].

Anthocyanins have anti-inflammatory, antimutagenic and cancer chemo-preventive ability. In addition to that it has protective effect against gastric damage, collagen degradation, hepatic damage and increase cognitive performance [13, 14] Anthocyanin content is high in black rice [15] Acetylated procyanidin is the commonly found anthocyanin in colored rice

Received: 02.09.2018; Received after Revision: 11.09.2018; Accepted: 11.09.2018; Published: 24.09.2018

which possess antioxidant properties [7]. Sources of anthocyanins are berries, jabuticaba, rose, blueberries, raspberries, grapes, red-cabbage, raddish, apple,Tulips, orchids, mulberries. Anthocyanins being harmless make them to be used as natural water soluble colorant [16].

Materials and methods

Plant material

The studies were performed using the peels of *Punica granatum* (pomegranate) peel which were commercially grown in India. Fruits were procured from local market in Chennai. Fruits were manually peeled and employed for further extraction process.

Sample preparation

Two kinds of samples were used in this study. One sample was freshly peeled, homogenized and kept for extraction process while another sample was dried at 40°C in hot air oven, the dried sample was later powdered and then used for subsequent extraction process.

Extraction methods

Extraction using methanol

The extraction was carried according standard procedures [17] with slight modifications. 100 g of the manually separated peels were extracted with 400 ml methanol. The extraction mixture was well decanted and filtered on filter paper and the filtrate was then evaporated at 40 °C in the dark obtaining a yellow brown residue from peels (about 10 g) that were stored at -18°C.

Extraction in soxhlet apparatus

100 g of the powdered peels were extracted with 400 ml of methanol in a Soxhlet extractor in the dark. The obtained solution was evaporated at 40°C in the dark obtaining a yellow brown residue that was stored at ⁻18°C.

Quantification of anthocyanin like compound

Monomeric anthocyanin pigments undergo color change with a change in pH. The difference in the absorbance of the pigments at 520 nm is proportional to the concentration of the pigment. This method is accurate and allows rapid measurements of total anthocyanins. The total anthocyanin content (TAC) was determined by the pH-differential method [18]. 10 mg/ml extract dissolved in DMSO and 210 µl of this extract was diluted in 2 separate solutions, one of pH 1.0(0.1 M HCl, 25 mM KCl) and other of pH 4.5 (0.4 M CH_3COONa) and final volume was made upto 1ml. The absorbance of the mixtures was then measured at 535 and 700 nm against methanol as blank. The value $(Abs_{535} - Abs_{700})_{pH1.0} - (Abs_{535} - Abs_{700})_{pH4.5}$ corresponds to the absorbance due to the anthocyanins. Calculation of the anthocyanins concentration was based on a cyanidin-3-O glucoside molar extinction coefficient of 25,965 $M^{-1}\times cm^{-1}$ and a molecular mass of 449.2 g/mol. Results were expressed as micromoles cyanidin-3-O-glucoside equivalents (CGE) per gram of dry extract [17].

$$C(mg/l) = \frac{A\times MW\times DF\times 1000}{\varepsilon \times l}$$

Where MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, l is the path length calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 and ε= 26,900

Purification of anthocyanin like compound

The extracts contained some impure compounds such as free sugars and aliphatic acids which were removed using gel filtration column chromatography. In the study [19] they used sepadex LH-20 column for the purification of anthocyanin like compound. But in our study we used Q sepharose instead of sephadex LH-20 to check efficiency. These columns adsorb bioactive compounds like anthocyanins. Then, the adsorbed anthocyanin like compound was eluted using methanol/water/ 1M HCl in the ratio of 50:50:01.

Antioxidant assays

DPPH assay

The scavenging activity was determined by the method described by Okonogi [20]. 10 mg/ml extract was diluted in methanol. Then 100 µl from previously diluted extract was again diluted with methanol and final volume was made upto 1ml. Test tubes with varying concentrations of extract (10, 20, 30, 40 and 50µg) was taken and the final volume was made upto 1 ml using methanol. 3 ml of DPPH solution with absorbance 1 at 517 nm was added to each test tube and incubated in dark for 15 min. Absorbance value was taken at 517 nm. The free radical scavenging activity (RSA) as determined by the decolouration of the DPPH solution was calculated according to the formula:

Radical Scavenging Ability (%)= [(1– A_{test})/A_{blank} x 100]
Where A_{test} is the absorbance of the reaction mixture, and A_{blank} is the absorbance of the blank test. Assays were carried out for both fresh and dried samples.

ABTS Assay

The scavenging activity was determined by the method described by Re [21]. 10 mg/ml extract was diluted in methanol. Then 100 µl from previously diluted extract was again diluted with methanol and final volume was made upto 1ml.Test tubes with varying concentrations of extract (10, 20, 30, 40, and 50 µg) were taken and the final volume was made upto 1ml using methanol.3ml of ABTS solution with absorbance 1 was added to each test tubes and incubated in dark for 30 min. Absorbance value was taken at 734 nm. Methanol was used as blank. The scavenging rate of ABTS was calculated on the basis of following formula:
Radical Scavenging Ability (%) = [1– (A_{test}/A_{blank}) x 100]where A_{test} is the absorbance of the reaction mixture, and A_{blank} is the absorbance of the blank test. Assays were carried out for both fresh and dried samples.

Antimicrobial assay

Antibacterial assay

The antibacterial activity of extract was evaluated by agar well diffusion method using nutrient agar, for the assay. The bacteria available in our laboratory were used for this study, among them 3 bacteria were gram positive (*Bacillus cereus Staphylococcus aureus, Streptococcus mutans,) and* 3 were gram negative *(E. coli, Pseudomonas aeruginosa, Salmonella typhi).* The microorganism was activated by inoculating a loopful of the strain in the nutrient broth (25 ml) and incubated at room temperature on a rotary shaker. The culture was then swabbed using sterile cotton swabs. For the agar well diffusion, well was made in the plates. The test compound 10mg was diluted in 1ml DMSO and then varying concentrations of extract (100, 200, 300, 400 and 500 µg) was introduced into the well and all the plates were incubated at 37 °C for 24 h.

Antifungal assay

The antifungal activity of extract was evaluated by agar well diffusion method using potato dextrose agar, for the assay. The microorganisms available in our laboratory were used in this study, they are *Aspergillus flavus, Aspergillus niger and Candida albicans* species. The microorganism was activated by inoculating a loopful of the strain in the potato dextrose broth (25 ml) and incubated at room temperature on a rotary shaker. The culture was then swabbed using sterile cotton swabs. Then 0.2 ml of inoculum was inoculated into the potato dextrose agar media using swab method. For the agar well diffusion, well was made in the plates. The test compound 10mg was diluted in 1ml DMSO and then varying concentrations of extract (100, 200, 300, 400 and 500 µg) was introduced into the well and all the plates were incubated at 37 °C for 24-48h.

Results and discussions

Extraction of anthocyanins

The aim of this study is to extract anthocyanins from fresh and dried *Punica granatum* (pomegranate) peel. Methanol was used as solvent for extraction of anthocyanins from fresh sample and for dried sample extraction of anthocyanins was carried out in soxhlet apparatus.According to the results obtained, extraction using dried sample gave higher yield of anthocyanins.

Quantifications of anthocyanins

pH differential method was utilized to determine total anthocyanin content of pomegrante peel. Anthocyanin undergoes reversible color change with a change in pH. Samples were diluted in aqueous solution at pH 1.0 and 4.5 buffers and absorbance measurements were recorded at 535 nm and 700nm. Monomeric anthocyanin pigments is responsible for the difference in absorbance between the two buffer solutions .The total anthocyanin content (TAC) of the pomegranate peel was found to be 0.069 (mg/L) for fresh sample and 0.207 (mg/L) for dried sample, which is very low when compared to the total anthocyanin content of grapes which ranges from 25.56 to 460 mg/L [22] (Table 1).

Table 1. Quantification of anthocyanins

Method	Samples	Yield before purification, mg/L	Yield after purification, mg/L
Methanol Soxhlet apparatus	Fresh	0.069	0.24
	Dried	0.207	0.46

Antioxidant assays

Pomegranate peel has high range of antioxidant and antimicrobial properties. Antioxidant properties of extract was accessed by two assays(DPPH assay, ABTS assay). This Studies shows that dried sample contain comparitively high range of antioxidant properties than fresh sample.IC 50 values for fresh samples are 49µg/ml for DPPH assay,14 µg/ml for ABTS assay. IC50 values for dried samples are 13 µg/ml for DPPH assay,14µg/ml for ABTS assay. These values are more or less similar to the studies carried to determine the antioxidant capacity of flowers and roots of *Pyrostegia venusta* [23] (Fig. 1 and 2)

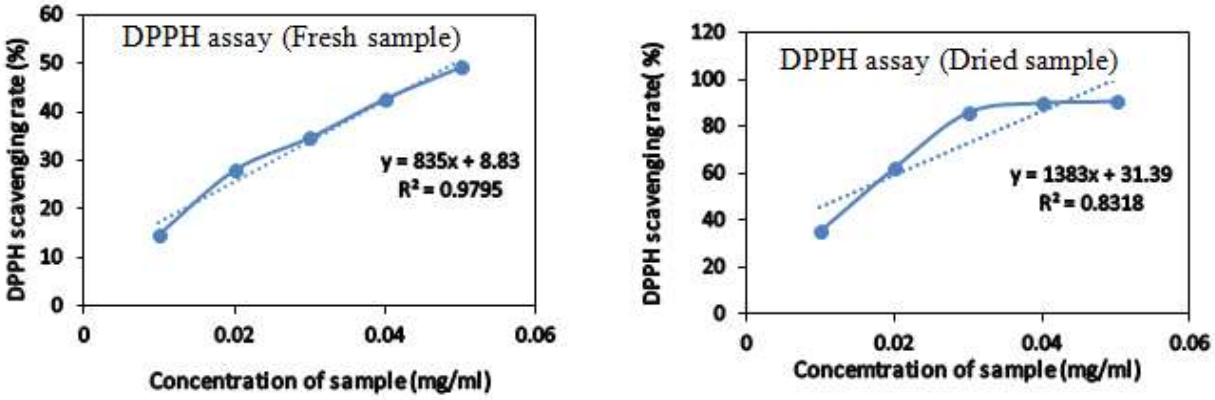


Fig. 1. DPPH assay for fresh and dried sample

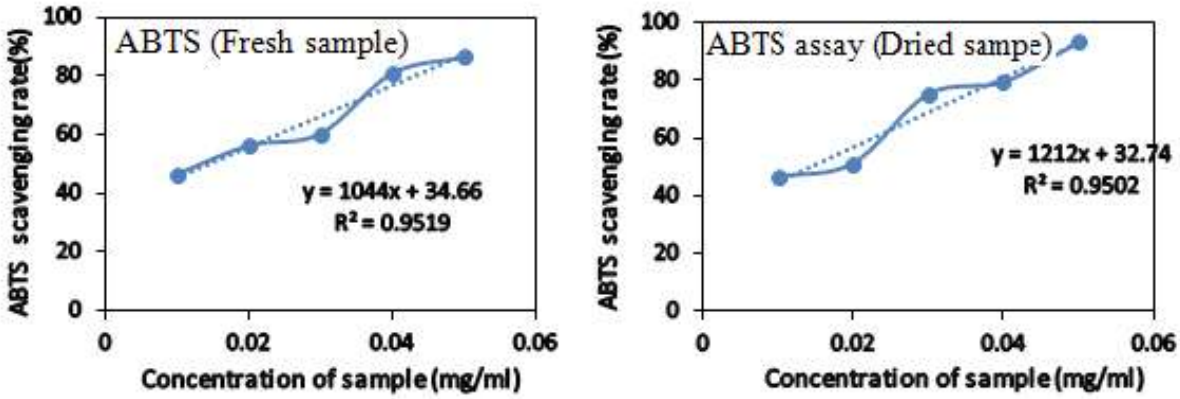


Fig. 2. ABTS assay for fresh and dried sample

Antimicrobial activity

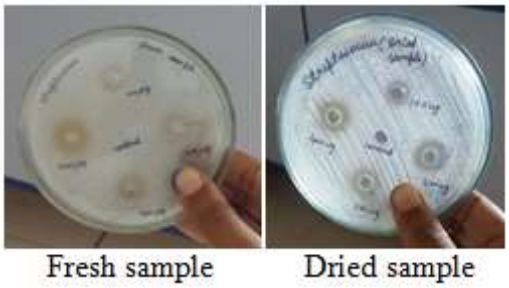
Antibacterial assay

Antimicrobial activity of investigated dried and fresh samples is almost same. From the results obtained, clear zone of inhibition could be seen even at least concentration of 100µg. Minimum inhibitory concentration were obtained for extract ranging from 0.1 mg/ml to 0.5 mg/ml. The extract has inhibition effect on both gram positive and gram negative bacteria. The diameter of the zone of inhibition was measured and is tabulate below (Table 2) (Fig. 3 to 8)

Table 2. Zone of inhibition in selected bacterial species (cm)

Species	Zone of inhibition (cm)							
	Fresh samples, µg				Dried samples, µg			
	100	200	300	400	100	200	300	400
Staphylococcus	1.3	1.5	1.6	1.9	1.2	1.7	1.8	2
Streptococcus	1.1	1.4	1.5	1.7	1.1	1.3	1.4	1.4
Bacillus	1.3	1.7	1.9	2	1.3	1.7	1.8	1.9
E.coli	1.1	1.2	1.5	1.7	1.1	1.5	1.6	1.7
Pseudomonas	1.1	1.5	1.6	1.7	1.4	1.6	1.9	2
Salmonella	1.1	1.4	1.5	1.7	1.1	1.5	1.6	1.7

Fig. 3. Zone of inhibition against *Staphylococcus aureus*



Fig, 4. Zone of inhibition against *Streptococcus mutans*

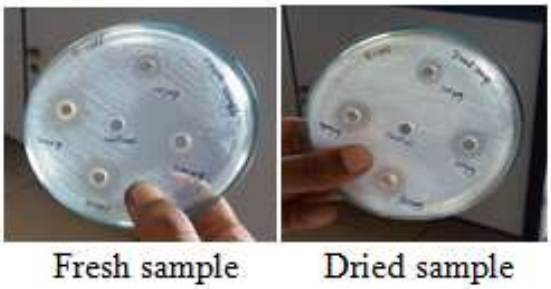


Fig. 5. Zone of inhibition against *Bacillus cereus*

Fig. 6. Zone of inhibition against *E. coli*

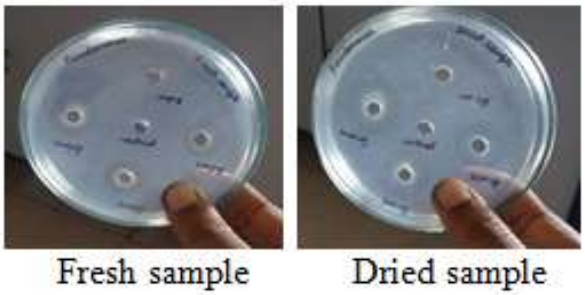


Fig. 7. Zone of inhibition of *Pseudomonas aeruginosa*

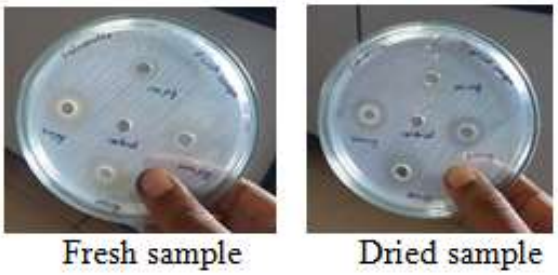


Fig. 8. Zone of inhibition of *Salmonella typhi*

Antifungal assay

The extract is capable of inhibiting fungal species also. Antifungal activity of investigated dried and fresh samples are almost same. From the results obtained, clear zone of inhibition could be seen even at least concentration of 100µg. Minimum inhibitory concentration were obtained for extract ranging from 0.1 mg/ml to 0.5 mg/ml. The diameter of the zone of inhibition was measured and is tabulate below (Table 3) (Fig. 9 to 11).

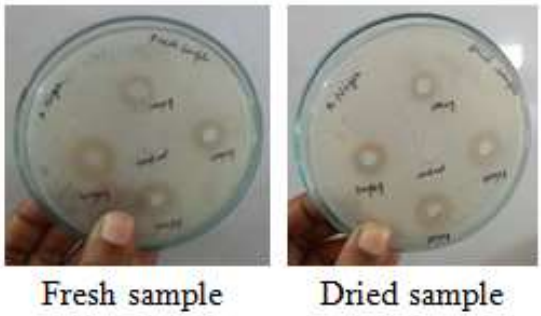


Fig. 9. Zone of inhibition of *Aspergillus niger*

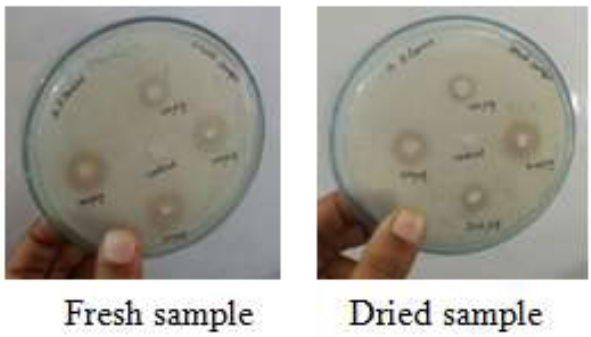


Fig. 10. Zone of inhibition of *Aspergillus flavus*

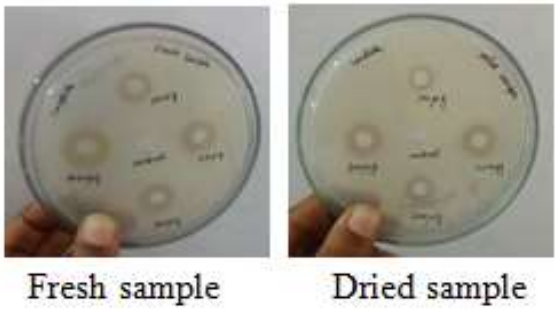


Fig. 11. Zone of inhibition of *Candida*

Table 3. Zone of inhibition in selected fungal species (cm)

Species	Zone of inhibition (cm)							
	Fresh samples, µg				Dried samples, µg			
	100	200	300	400	100	200	300	400
A. niger	1.1	1.3	1.5	1.5	1.2	1.4	1.5	1.6
A. flavus	1.1	1.2	1.4	1.5	1.1	1.4	1.5	1.6
Candida	1.1	1.1	1.4	1.5	1.2	1.4	1.5	1.7

Conclusions

Anthocyanin like compound were identified in *Punica granatum* (pomegrnate) peel, and were extracted. Methods used for extraction are dissused in this study. This study states that pomegranate peel exhibit strong antioxidant and antimicrobial activity and the results are also given for these assays. Therefore pomegranate peel instead of being waste can be used in a beneficial way. This study is a new approach to extract anthocyanin like compound from *Punica granatum* peel. The anthocyanin like compound extracted has a wide range of applications, they can be used in food industries ,cosmetics and pharmaceuticals. The extracted anthocyanin like compound will be subject for GCMS and HPLC anysis for further studies.

Conflicts of interest

The authors declare no conflict of interest.

References

[1] Sun LL, Gao W, Zhang MM, Li C, Wang AG, Su YL, Ji TF. Composition and Antioxidant Activity of the Anthocyanins of the Fruit of Berberis heteropoda Schrenk. Molecules 2014;19:19078-96.

[2] Ge Q, Ma X. Composition and antioxidant activity of anthocyanins isolated from Yunnan edible rose (Anning). Food Science and Human Wellness 2013;2:68-74.

[3] Shipp J, Abdel-Aal EM. 2010. Food Applications and Physiological Effects of Anthocyanins as Functional Food Ingredients. The Open Food Science Journal 2010;4:7-22.

[4] Kayesh E, Shangguan L, Korir NK, Sun X, Bilkish N, Zhang Y, Han J, Song C, Cheng ZM, Fang J. Fruit skin color and the role of anthocyanin. Acta Physiol Plant 2013;35:2879-90.

[5] Vankar PS, Srivastava J. Evaluation of Anthocyanin Content in Red and Blue Flowers. International Journal of Food Engineering 2010;6:4.

[6] Brito A, Areche A, Sepúlveda B, Kennelly EJ, Simirgiotis MJ. Anthocyanin Characterization, Total Phenolic Quantification and Antioxidant Features of Some Chilean Edible Berry Extracts. Molecules 2014;19:10936-55.

[7] Sarkis JR, Jaeschke DP, Tessaro IC, Marczak LDF. Effects of ohmic and conventional heating on anthocyanin degradation during the processing of blueberry pulp. LWT-Food Science and Technology 2013;51:79-85.

[8] Lima AJB, Corrêa AD, Saczk AA, Martins MP, Castilho RO. Anthocyanins, pigment stability and antioxidant activity in Jabuticaba. Revista Brasileira de Fruticultura 2011;33:877-87.

[9] Nisco MD, Manfra M, Bolognese A, Sofo A, Scopa A, Tenore GC, Pagano F, Milite C, Russo MT. Nutraceutical properties and polyphenolic profile of berry skin and wine of Vitis vinifera L. (cv. Aglianico). Food Chem 2013;140:623-29.

[10] Fawole OA, Makunga NP, Opara UL. Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. BMC Complementary and Alternative Medicine 2012;12:200.

[11] Liu GL, Guo HH, Sun YM. Optimization of the Extraction of Anthocyanins from the Fruit Skin of Rhodomyrtus tomentosa (Ait.) Hassk and Identification of Anthocyanins in the Extract Using High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (HPLC-ESI-MS). Int J Mol Sci 2012;13:6292-302.

[12] Gao Z. Extraction, separation and purification of blueberry anthocyanin using ethyl alcohol. Kem Ind 2017;66:655-59.

[13] Veggi PC, Santos DT, M. Angela A. Meireles. 2011. Anthocyanin extraction from Jabuticaba (Myrciaria cauliflora) skins by different techniques: economic

evaluation. Procedia Food Science 2011;1:1725-31.

[14] Chen H, Yu W, Chen G, Meng S, Xiang Z, He N. Antinociceptive and Antibacterial Properties of Anthocyanins and Flavonols from Fruits of Black and Non-Black Mulberries. Molecules 2018,23:1.

[15] Pengkumsri N, Chaiyasut C, Saenjum C, Sirilun S, Peerajan S, Suwannalert P, Sirisattha S, Sivamaruthi PS. Physicochemical and antioxidative properties of black, brown and red rice varieties of northern Thailand. Food Science and Technology 2015;35(2):331-38 .

[16] Castaneda-Ovando A, Pacheco-Hernandez ML, Paez-Hernandez ME, Rodriguez JA, Galan-Vidal CA. Chemical studies of anthocyanins: A review. Food Chem 2009;113:859-71.

[17] Masci A, Coccia A, Lendaro E, Mosca L, Paolicelli P, Cesa S. Evaluation of different extraction methods from pomegranate whole fruit or peels and the antioxidant and antiproliferative activity of the polyphenolic fraction. Food Chem 2016;202:59-69.

[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: 1269-78.

[19] Yang Y, Yuan X, Xu Y, Yu Z. Purification of Anthocyanins from Extracts of Red Raspberry Using Macroporous Resin, International Journal of Food Properties 2015;18:1046-58.

[20] Okonogi S, Duangrat C, Anuchpreeda S, tachakittirungrod S, Chowwanapoonpohn S. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. Food Chem 2007;103(3):839-46.

[21] Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 1999;26(9-10):1231-37.

[22] Burin VM, Falcão LD, Gonzaga LV, Fett R, Rosier JP, Bordignon-Luiz MT. Colour, phenolic content and antioxidant activity of grape juice. Food science and Technology 2010;30:1027-32.

[23] Roy P, Amdekar S, Kumar A, Singh V. Preliminary study of the antioxidant properties of flowers and roots of *Pyrostegia venusta* (Ker Gawl) *Miers.* BMC Complementary and Alternative Medicine 2011;11:69.

****

