ISOLATION AND PARTIAL CHARACTERIZATION OF ANTHOCYANIN IN *SYZYGIUM POLEYCEPHALOIDES* MERR C. B. ROB.(IGOT)FRUIT EXTRACT BY THIN LAYER CHROMATOGRAPHY

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ABSTRACT

This research was an experimental study which involved analytical chemical analysis with instrumentation. The biologically active chemical compound of anthocyanin in fruit extract of Syzygium poleycephaloides Merr C.B Rob or igot had been isolated and partially characterized by Thin-Layer Chromatography.A qualitative procedure of chemical precipitation was performed isolating the precipitates of anthocyanin. The reaction of fruit extract yield a gelatinous deep red colored precipitates showing a positive result in alkali (HCl) and metal (Mg). This procedure detected the presence of one particular type of anthocyanin of the fruit extract of igot, the cyanidin. In thin-layer chromatography, fruit extract (fraction extract) of Syzygium poleycephaloides Merr C.B Rob (igot) ran side by side with the fruit extract of grapes as reference sample both showed a chromatogram of anthocyanin. Syzygium poleycephaloides Merr C.B Rob (igot) exhibited a sharp color of pink to rich reddish-pink isolated by the extracting solvent system of hydrochloric acid, acetic acid, and water (19:39.6:414 v/v) mixture. In three series of treatment, the above-mentioned solvent system showed the retardation factor (Rf) values of 0.86, 0.80, and 0.77 respectively. The other two sets of solution of extracting solvent used in this research also confirmed the presence of anthocyanin constituent. The resulting value of retardation factor was 0.72, 0.68, 0.67, 0.55, and 0.5 with a color tone of the anthocyanin chromatogram from pale pink to blue. Theoretically, every substance has unique chemical properties or identity, thus in that sense these observed values of retardation factor were coined to a specific type of anthocyanin.

INTRODUCTION

Nature is an excellent source of configuration with high stereochemical diversity having a fascinating bio-chemical activities and medicinal properties.

Research had produced authentic evidence for an immeasurable range of health benefits arising from the consumption of fruits and vegetables. In undertakings to identify the active health-promoting constituents, many researchers have concentrated on the properties of the flavonoid, a large class of phenolic compound that is abundant in most food. Most prominent among the flavonoids are the anthocyanin-plant colorants responsible for the red, purple, and blue hues in many fruits, vegetables, cereal grains, and flowers. Many pigmented fruit extracts commonly used in folk medicines have various positive therapeutic effects.

The researcher is interested in investigating the pigment of the fruit of this organic species locally known as *igot* has scientific name of *Syzygium polycephaloides* Merr C. B. Rob. The ripe black colored skinny fruit was used to isolate anthocyanin and characterize its properties.

METHODOLOGY

This study is an experimental type of research conducted at the College of Science Bio-Physical Laboratory, University of Eastern Philippines, Catarman, Northern Samar.

For sample preparation, the procedure started with the extraction of anthocyanin (pigment) of *Syzygium poleycephaloides* Merr C.B Rob (igot) fruit. The fresh plant material, 150g was treated with sufficient 95% ethyl alcohol soaked for 24-48 hours. Extract was filtered using a Buchner funnel with gentle suction. Filtrate was concentrated in the fume hood below 50°C to about 20 ml. The concentrated stock of fruit extract was stored in a cool temperature (0 to -5°C). Extraction was done in three series.

Anthocyanin Test (Wilstatter "Cyanidin" Test)

From the stock of plant extract, 10 grams was brought to incipient dryness over steam bath, then cooled off at room temperature. The reduced extract was then defatted by taking the residue with 9 ml hexane and water (2:1). Hexane was discarded. Portion of the plant extract was treated with 0.5 ml concentrated hydrochloric acid. Three to 4 pieces of magnesium turnings were added and within 10 minutes, change of color and its abundance was observed and compared to the untreated sample. Coloration occurred upon dilution of equal volume of water. A milliliter of octyl alcohol was added and the treated fruit extract was allowed to stand until a colored layer appeared.

Thin-Layer Chromatography

The coating material was prepared using 12.5 g of Silica gel G mix with 50 ml distilled water in 150 ml flask used as stationary phase for this experiment. The TLC glass plate used was 7.5cm x 16 cm.

The solvent systems which served as mobile phase by volume were the following: (S1)

toluene : chloroform : acetone(20 :12.5 :17 v/v), (S2) HCL : formic : water (19.0 :39.6:41.4 v/v), (S3) hexane :2-butane : diethyl ether(34 :7 :6 v/v)

For sample preparation, a steam bath concentrated plant extract dried up to incipient dryness then into cold. The residue fractionated with just enough diethyl ether and dissolved with a few drops of methyl alcohol. Same procedure followed to the reference sample (dark purple grapes).

For sample application, the test sample of fruit extract together with the reference sample pipetted and spotted side-by-side using capillary tube 1 cm from the lower edge of the coated glass plates of silica gel in 2 cm apart and then air-dried.

Development of chromatograms carried out through spotted plates placed in the tightly covered equilibrated chambers and the extracting solvents was allowed to migrate up to 30 minutes. The plates were removed then and air-dried. The developed chromatograms were exposed in iodine vapor allowing them for few minutes to react. Iodine spray is a mixture of iodine solution and 1.0 percent methanol.

For rf value measurement, the distance migrated by an isolated igot extract compared with the reference sample was obtained from the ratio of the distance traveled by the compound over the distance traveled by the solvent front. The distance of the new spots was measured using the unit of length from the point of origin towards the positioned new spots. The color of the spots was identified through the response of eye visualization.

The Rf value of the sample was computed as follow:

$$Rf = \frac{distance\ migrated\ by\ solute}{distance\ migrated\ by\ the\ solvent}$$

For chromatogram documentation, tracing papers were used to plot the identified spots from the point of origin up to the point marked by the solvents. Chromatograph characteristics were visible and traced, such as the distance traveled by the solute and solvent, color, shape (or chromatogram density), and the color reaction in iodine vapor.

Statistical treatment determined the test of difference for the retardation factor (Rf) value of anthocyanin between fraction extract of *igot* and grapes in three different solvent systems. After the laboratory analysis, the results were tabulated and treated statistically. The null hypothesis was tested and answered by F-test, analysis of variance (ANOVA).

RESULTS AND DISCUSSIONS

This chapter presents the properties of anthocyanin isolated from the fruit extract Kof *Syzygium poleycephalios Merr C.B Rob* locally known as *igot*. The qualitative analysis of Wilstatter test detected the presence and abundance of cyanidin molecule, a common type of anthocyanin. The test obtained an A+++ to A++ results (in heavy to moderate amount) with deep red colored gelatinous precipitates treated in the solutions of

HCl, magnesium and hexane-alcohol mixture.

Thin Layer Chromatography displayed the rich color of anthocyanin and the retardation factor of the fruit extract. The chromatogram revealed that the fruit extracts of *Syzygium poleyceophaloides Merr. C.B Rob* contained the constituent of anthocyanin similar to the reference sample of grapes. As shown in the plate, the density of the spot was large. The fruit extract of *igot* contained not only anthocyanin, it also held other unidentified constituents displaying spots of different colors when it oxidized in iodine vapor. The different solvent systems showed the color of spots (chromatogram), reddish pink, pink, pale pink, and pale bluish pink, that easily disappeared, while pale brown-yellow, brown, and red-brown appeared when exposed to iodine vapor.

The developed chromatogram in this research recorded an ultimate rf value of 0.67 to 0.86 in the extracting solvent of hydrochloric acid, acetic acid and water mixture. An rf value of 0.86 indicated that anthocyanin was 86 percent as fast as the extracting solvent migrated. Referring to the literature, this rate of migration most likely belonged to diglycosylated type of anthocyanin. An rf below 0.62 was slower than the diglycosylated anthocyanin and probably belonged to another type of anthocyanin.

Characteristics and Abundance of Anthocyanin in *Igot* Fruit Extract

Number of observation of *Igot* fruit extract	Abundance and Color Description		
	Trial 1	Trial 2	Trial 3
untreated	red pink	red pink	red pink
1	A+++ (deep red)	A++ (deep red)	A++ (deep red)
2	A+++ (deep red)	A++ (deep red)	A++ (deep red)
3	A+++ (deep red)	A++ (beep red)	A++ (beep red)
4	A+++ (deep red)	A+++ (deep red)	A++ (deep red)
5	A+++ (deep red)	A++ (deep red)	A++ (deep red)

Table 1. Wilstatter "Cyanidin" test

Table 1 showed the result of the reaction of the fruit extract of *Syzygium poleycephaliodes Merr C.B Rob* in cyanidin test. The test obtained an A+++ to A++ results indicating a heavy to moderate amount, with deep red colored gelatinous precipitates treated in the solutions of HCL, magnesium and hexane-alcohol mixture. The test detected the composition of cyanidin molecule, a common type of anthocyanin.

Table 2. Color Reaction of Anthocyanin in Extracting Solvents Viewed in Daylight and Iodine Vapor.

Solvent System: S1: Toluene:Chloroform:acetone (20:12.5:17 v/v) S2: hydrochloric acid: acetic acid:water(19:39.6:41.4 v/v) S3: Hexane:2-butanol:diethyl ether(34:7:6 v/v)			
Adsorbent plate: silica gel 250			
Visualization: Daylight and iodine vapor			
Fraction Extract in diethyl ether	light visible	iodine vapor visible	Interpretation
Igot	reddish pink Pink Bluish pink brown(trace) deep red brown	reddish pink pink bluish pink light brown (trace) light brown	anthocyanin anthocyanin anthocyanin flavonol flavonol
Grape	pink Pale pink Bluish pink(trace) Brown	pink pale pink bluish pink(trace) yellowish brown	anthocynin anthocyanin anthocyanin flavonol

Table 2 showed the rich color hue of chromatogram of the fruit extract of *Syzygium poleycephaloides* Merr C.B Rob (igot) in extracting solvents after

it was viewed in daylight and iodine vapor. The solvent systems stripped the constituent of the fruit extract showing the color of spots of reddish pink, pink, pale pink, and pale bluish pink, that easily disappeared, while pale brown-yellow, brown, and red-brown appeared when exposed to iodine vapor. The rich color tone ranged from pink, reddish pink to bluish pink appearing in *igot* fruit extract was quite similar with grapes (reference sample) from pale pink to bluish pink in trace amount.

The fruit extract contained not only anthocyanin, but also unidentified constituents indicated by other colors when it oxidized in iodine vapor. Evidently spots were closely alike to both test samples.

Retardation Factor (rf) Value of Fraction Extract of *Igot* and Grapes

Table 3. Reading of Retardation Factor of Anthocyanin Chromatogram *Igot* fruit extract versus Grape

Retardation Factor (Rf) Value						
Trial	Diethyl ether fraction extract of igot fruit			Diethyl ether fraction of extract grape fruit		
	S1	S2	S3	S1	S2	S3
1	0.67	0.86	0.50	0.50	0.72	0.09
2	0.078	0.80	0.078	0.08	0.55	0.08
3	0.04	0.77	0.04	0.04	0.68	0.45
Mean	0.262	0.81	0.206	0.206	0.65	0.208

Table 3 showed the retardation factor (rf) value of chromatogram (anthocyanin) in the fraction extract of *igot* versus grapes obtained from the ratio of the distance of migration between chromatogram and solvent. Based on the mean score of rf value under three solvent systems, the property of similarity of anthocyanin present in *igot* was closely identical to grape as indicated.

Test of Difference for the Retardation Factor (rf) Value of Anthocyanin Between Fraction Extract of *Igot* and Grapes in Three Different Solvent Systems.

Table 4. Analysis of Variance (ANOVA) for the Significant Difference of the Rf Value of Anthocyanin.

Source of variation	Df	Sum of Square	Mean Square	F-value @ a=0.05 Computed tabular	Interpretation
Between Group	2	0.716	0.143	2.75 3.11	Not Significant
Within Group	12	0.619	0.052		
Total(n-1)	17	0.38153			

Table 4 showed the F-test analyzed the difference of the rf value of anthocyanin contained in fraction extract of *igot* and grape fruit run by side in three different solvent systems. The computed F- value of 2.75 was lesser than the F-tabular value of 3.11 at 0.05 significant level at degrees of freedom 5 and 12. Therefore, there is no significant difference in rf value between the fraction extract of *igot* and in grapes run in three different solvent systems. The research null hypothesis was accepted.

CONCLUSIONS

Based on the findings drawn in this research, the following conclusions were presented:

The extract from the fruit of *Syzygium poleycephaloides* Merr. C. Rob. contains a bio-chemically active substance, in which the experimental work of this research was identified as anthocyanin with rich color of deep red of considerable amount.

The consistent value of retardation factor of chromatogram in solvent systems in this study identifies the rich composition of anthocyanin (probably a diglycosalyted) present in Syzygium *poleycephaloides* Merr. C. Rob. (igot) fruit extract exhibiting a color tone from pink , reddish pink to bluish pink. An rf value of 0.72, 0.68, 0.67, 0.55, and 0.5 in this study with a color tone of the anthocyanin chromatogram from pale pink

to blue were coined theoretically to a specific type of anthocyanin.

Statistically, anthocyanin in *igot* fruit has no significant difference to the grape fruit in term of its properties of rf value.

REFERENCES

Books

Austin, G.T. Shreven's Chemical Process Industries, 5th ed., McGraw-Hill Book Company, 1984

Broto, A.S. Statistics Made Simple, Manila Philippines, Melbros Printing Center, 2003.

Browns, William. Useful Plants of the Philippines, Melbourne Sand & Mc. Dugall, 1949.

Boyd Robert and Morrison Robert. Organic Chemistry, Philippines: Allyn and Bacon, 1986.

Chang, R., General Chemistry: The Essential Concepts, 4th New York International Edition, McGraw-Hill Companies, Inc., 2006.

Dunn and Prescott.Industrial Microbiology, 3rdedition NY: McGraw-Hill, 1958

Fritz, James and Shenk, George.Quantitative Analytical Chemistry. 5th Edition JMC Press Inc., 1987

Guevara, B.Q., editor, A Guidebook to Plant Screening: Phytochemical and Biological. Revised Edition, Research Center for the Natural Sciences, Manila, Philippines: University of Santo Tomas Publishing House, 2005.

Jackman, R.L and Smith, J.L. Anthocyanins and Batalains. In: Hendry G.A.F., Houngton J.D. editors. Natural Food Colorants. 2nd ed. London: Blackie Academic & Professional, 1996.

Lewis Richard and Sax Irving N. Hawley' Condensed Chemical Dictionary, Revised Eleventh Edition. New York: Van NostrandKeinhold Company Inc., 1987.

Mathews, Christopher et al. Biochemistry 3rd Edition, Pearson Education Ltd, Singapore, 2002.

Masterton, W. et al. Chemical principles, 6th Edition, New York: Saunders College Publishing, 1985.

Masterton, William and Hurley Cecile N. Chemistry: Principles and Reactions, 3rd Edition. New York: Saunders College Puliblishing, 1997.

Ragann S. Manual of Analysis of Fruits and vegetables Products.New Delhi: Tata McGraw-Hill PublishinG Ltd.,

Rajvaiya, N., Markandey, D.K. Environmental Analysis and Instrumentation. New Delhi: A.P.H Publishing Corp. 2005.

Skoog, Douglas et al. Fundamentals of Analytical Chemistry, 8th Edition.Thompson Books/Cole, 2004.

Wolsey, W.C. et al. Chemical Principles in the Laboratory. 11th Edition, Minnesota: Thompson Books/Cole, 2002.

Journals

Bartel and Matsuda: Science Vol 299, 2000

Banerjee, A. et al. "In Vitro Study of Antioxidant Activity of *Syzygiumcumini* Fruit." Food Chemistry 90, 2005.

De Costa, C. et al. "Analysis of Anthocyanins in Foods by Liquid Chromatography -Mass Spectrophotometry and Capillary Electrophorosis." Journal of Chromatography A. 881, 2000

De Guzman, Enriquito D., Guide to the Philippine Flora and Fauna URMC-MNR & UP, Philippines, Vol.3, 1986.

Delgado-Vargas, F. and Paredes-Lopez, O. (Eds): Natural Colorants for Food and Nutraceutical Uses. CRC Press, 2003.

EEC Regulation No.000/90, Official Journal, No.3.10.90, 1990.

Garcia, Faustino, "Distribution of Insulin- Like Principle in Different Plants and Its Therapeutic Application To A Few Cases of Diabetes Mellitus." The Philippine Journal of Science, 76, 3, 1994.

Gross P.M, "Scientists Zero in on Health Benefits of Berry Pigments," Natural Products Information Center. Retrieved on 2007-07-27.

Kamei H, Kojima T, Hasegawa M, et al. "Suppression of Tumor cell growth by anthocyanin in vitro." Cancer Invest. 13 (6), 1995.

Knekt, P. et al. "Flavonoid Intake and Risk of Chronic Disease." American Journal of Clinical Nutrition, 1 76 (3) 2002.

Kong, J.M. et al. "Analysis and Biological Activities of Anthocyanins." _*Phytochemistry*, vol. 64 (5): 2003

Lauro, G.J. and Francis, F.J. (Eds) "Natural Food Colours, Science and Technology." IFT Basic Symposium Series 14, Marcel Dekker, 2000.

Nohynek, LJ. et al. "Berry Phenolics: Antimicrobial Properties and Mechanism of Action Against Severe Human Pathogens." Nutrition and Cancer, 54 (1) 2002

Revilla, J. et al. "Identification of Anthocyanin Derivatives in Grapes skin extract, and Red Wines by Liquid Chromatography with Diode array and Mass Spectrometric detection." Journal of Chromatography A 847, 1999

University of Eastern Philippines Annual Report, 2003-2006.

Wada L. and Ou B. "Antioxidant Activity and Phenolic Content of Oregon Caneberries." J. Agric Food Chem., vol. 50, 2002.

Wolfgang, Johan von Geothe "Color Mixing Rules from Theory of Colors". Germany

Wu, X. et al. "Characterization of Anthocyanins and Proanthocyanins in some Cultivars of Ribes, Aronia and Sambucus and their Antioxidant

Capacity."Agric Food Chem., vol. 52, 2004.

Unpublished Studies

Chacon, Naya O. The Phytochemical, Microbiological and Pharmacological Screening of the Alcoholic Extract from the Bark of Lipote (*Syzygiumpoleycephaloides*) C.B. Rob (Family Myrtaceae), 1995

Chang, Ma. Te.Identification and Quantitative Determination of the Components present in Flavonoid Pigments of *ImpatientsBalsamia L. VarTriflora* (kamantigi) Flowers, 1990

Gamotin, Rosemarie L. Optimization of Processing Condition On the Stability of Anthocyanin in Yam (*DioscoreaAlaln Linn*), 2006

Lopes, Allan G. Phytochemical Screening of the Roots, Stem and Leaves of Amoreco (*AndropogmAciculatus Retz*) By Thin-Layer Chromatography, 1997

Tibayan, Chona G. Phytochemical and Microbial Screening of the Lipote (*Syzygiumpoleycephaloides*) Leaf Extract. An Experiment, 1998

Online Sources

http://Ipi.oregonstate.edu/f-woo/flavonoid.html/Antioxidantactivitiesof Flavonoid

http://www.nature.com/journal Oenology: Red wine procyanidins and vascular health (abstract)

http://www.metabolome.JP/Arita Laboratory, Department of Computer Biology. Tokyo

http://www.vitaminsupplements.org/antioxidant.Php/ phytochemicals

http://eng.ege.edu.tr/resolution

http://www.guardian.co.uk/Science

http://geocites.com/mipemen/suarshtml/SugarandAlcohol.

http://www.ac.nz/news and event html/True Colors May Shine Through with EIT's Red Wine Research

http://www.pactically.com.edible.nsf/page/Measuring alcohol Content.

http://www.winemaking.jackeller.net/problems.asp/Wine Making

http://www.meriam-webster.com/dictionary/absorptivity

http://www.oznet.ksu.edu/swe/module2/characterization-htm Robert Boyd and Rob

http://www.measurementandtechnology.com

http://www.intowine.com/health.html Brad Prescott.Health and Wine.