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EVALUATION OF TOTAL FLAVONOLS, TOTAL PROANTHOCYANIDINS CONTENT AND THROMBOLYTIC ACTIVITY OF METHANOL EXTRACTS OF THREE BANGLADESHI PLANTS

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ABSTRACT

Premna esculenta (Roxb.), *Crotalaria pallida (*Aiton.) and *Euphorbia neriifolia* (Linn.) are three Bangladeshi plants, which examined for determination of proanthocyanidins, flavonols content and thrombolytic activity of methanol extracts. *Crotalaria pallida* showed highest content of flavonols (82.45±0.44 mg Quercetin/g) and proanthocyanidins (120.97±1.02 mg Catechin/g). It also showed highest clot lysis effect, which was 41.81±4.12%. *Premna esculenta* and *Euphorbia neriifolia* exhibit 17.27±1.57% and 34.57±1.99% compared with standard Streptokinase 75±0.09% clot lysis. They both showed well flavonols content, but proanthocyanidins content of *Euphorbia neriifolia* (17.86±0.26 mg Catechin/g) is less, where *Premna esculenta* had 106.62±1.29 mg Catechin/g.

Keywords: thrombolytic activity, *Premna esculenta* (Roxb.), *Crotalaria pallida (*Aiton.) and *Euphorbia neriifolia* (Linn.)

INTRODUCTION

Plants naturally produce thousands of phytochemicals. The flavonols and proanthocyanidins are just one of many groups of phytochemicals, which have great impact as antioxidant. Flavonols represent a class of secondary metabolites with diverse functions in plants including ultraviolet protection, pathogen defense, and interspecies communication. It is another kind of flavonoid. They are also known as modulators of signaling processes in plant and animal systems and therefore are considered to have beneficial effects as nutraceuticals. Proanthocyanidins are a diverse group of plant-derived oligomeric compounds, and are members of the flavonoid group of molecules. There is mounting evidence that proanthocyanidins and proanthocyanidin-rich foods and beverages contribute to vascular health and reduce risk of vascular outcomes [1, 2] through acting as free radical scavengers, reducing platelet aggregation and blood pressure, and improving nitric oxide homeostasis and endothelial function [3-7]. Although these vascular benefits of flavonoids are not limited to the proanthocyanidin class of flavonoids, based on mounting mechanistic and animal model data showing improved renal function and outcomes with proanthocyanidin supplementation [8-12], it appears the renoprotective benefit is limited to the specific flavonoid class of proanthocyanidins.

Premna esculenta (Roxb) (Family: Verbenaceae) is a small, branching shrub and grows in shady, wet places in primary rain forests of Bangladesh and India and have been traditionally used by tribal people of Chittagong Hill Tracts of Bangladesh in the treatment of gout, jaundice, lipoma (tumor), and edema. The leaves of the plant are applied for the treatment of arthritis and bacterial and fungal infection. [13] Although the plant has traditionally

been used in the treatment of various types of pain and inflammatory disorders (arthritis, gout, edema) in Bangladesh, no scientific data are available to validate these uses as the plant yet have not been undergone any chemical or pharmacological investigation. Therefore, the present study was undertaken to evaluate the anti-nociceptive, anti-inflammatory and CNS depressant activities of the leaf extracts of *Premna esculenta*.

Crotalaria is one of the largest genera in tropical Africa. The genus includes 690 species that are mainly situated in Africa and Madagascar [14]. Species have also been found in Bangladesh, India, USA and China. *Crotalaria pallida* Aiton. belongs to the family fabaceae. This is an erect shrub, annual or short-lived perennial herb of 1.5 m or more tall. Flowers are eaten as a vegetable in Cambodia, where the seeds are roasted and grounded for use as a sort of coffee beverage. The roots are sometimes chewed with betel nuts in Vietnam. In traditional medicine, the plant is used to treat urinary problems and fever, a poultice of the roots is applied to swelling of joints and an extract of the leaves is taken to expel intestinal worms [15].

Euphorbia neriifolia Linn. (Euphorbiaceae) grows luxuriously around the dry, rocky, hilly areas of Chittagong. It is a herb full of spine, popularly known as Monsagij, Indian spurge tree and Milk Hedge. The leaves are thick succulent, 6 to 12 inches long, ovular in shape. *E. neriifolia* leaves are used as aphrodisiac, diuretic and also used in the treatment of bronchitis, bleeding piles and in ano-rectal fistula [16]. The plant is useful in abdominal troubles, bronchitis, tumors, leucoderma, piles, inflammation, and enlargement of spleen, anemia, ulcers, fever and in chronic respiratory troubles [17].

The aim of the present studies to determine the total flavonols and total proanthocyanidins content and thrombolytic activity of methanol extracts of leaves of *Premna esculenta* , leaves of *Crotalaria pallida* and whole plants of *Euphorbia neriifolia* by established method.

METHOD AND MATERIAL

Plant collection and identification: Leaves of *Premna esculenta* , leaves of *Crotalaria pallida* and whole plants of *Euphorbia neriifolia* were collected from local village of Chittagong, Bangladesh in the month of October 2014. The plants were authenticated by Dr. Shaikh Bokhtear Uddin, Associate Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

Preparation of Extract: Each of the plant materials was dried and ground (Moulinex Blender AK-241, Moulinex, France) into powder (40-80mesh, 700 g) and soaked for 7 days with 2–3 days interval in 3.0 L of methanol at room temperature (23 ± 0.5°C). Filtrate obtained through cheesecloth and Whatman filter paper No. 1 was concentrated under reduced pressure at the temperature below 50°C using rotary evaporator (RE 200, Sterling, UK). The extracts (yield 3.8–5.2% W/W) were all placed in air tight glass tube.

Chemicals and equipment: All other chemicals and reagents were of analytical grade. Methanol and hydrochloric acid purchased from Merck (Germany). Aluminium chloride, vanillin and sodium acetate was purchased from Fluka (Fluka chemie GmbH, CH-9471 Buchs). Quercetin, Catechin was purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England). Shimatdzu Biospec 1601 UV visible spectrophotometer (Shimatdzu, Japan) was used to measure the absorbance. Lyophilized streptokinase vial (15, 00,000 IU) was purchased from Square Pharmaceuticals Ltd.

Total flavonols: Total flavonol content was determined by adopting the procedure described by Kumaran and Karunakaran.[18] The reaction mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/l) sodium acetate solution. The absorbance at 440 nm was measured after 2.5 h at 20°C. Total flavonol content was calculated as mg/g of quercetin equivalent from the calibration curve using the equation: $Y = 0.0255$ x, $R^2 = 0.9812$, where x is the absorbance and Y is the quercetin equivalent.

Determination of total proanthocyanidin: Total proanthocyanidin was determined based on the procedure of Oyedemi *et al*.[19] To 0.5 ml of 1 mg/ml of the extract solution was added 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature and the absorbance was measured at 500 nm. Total proanthocyanidin content was evaluated at a concentration of 0.1 mg/ml and expressed as catechin equivalent (mg/g) using the calibration curve equation: $Y = 0.5825$x, $R^2 = 0.9277$, where x is the absorbance and Y is the catechin equivalent.

***In Vitro* Thrombolytic activity:** This test was performed according to the method described by Prasad *et al* [20]. In the commercially available lyophilized streptokinase vial (15, 00,000 IU) 5 mL sterile distilled water was added and mixed properly. This suspension was used as a stock solution from which appropriate dilution was made. Five milliliter of venous blood was drawn from the healthy volunteers (*n*=10) without the history of oral contraceptive or anticoagulant therapy and was distributed (0.5 mL/tube) to each ten previously weighed sterile micro centrifuge tube and incubated at 37 °C for 45 min to form the clot. After the clot formation, serum was completely removed without disturbing the clot and each tube having clot was again weighed to determine the clot weight. A volume of 100 µL of ethanol extract (10 mg/mL) was added to each micro centrifuge tube containing pre weighed clot. As a positive control, 100 µL of streptokinase and as a negative control 100 µL of distilled water were separately added to the control tube numbered. All the tubes were then incubated at 37 °C for 90 min and observed for clot lysis. After incubation, fluid released was removed and tubes were again weighed to observe the difference in weight after clot disruption. Difference obtained in weight taken before and after clot lysis was expressed as percentage of clot lysis.

Statistical analysis: Antioxidant analysis was carried out in triplicates. Thrombolytic test carried out with 10 volunteer. Data were presented for antioxidant and thrombolytic test as mean ± S.E.M. Data were analyzed using one way factorial ANOVA tests using SPSS followed by Dennett's tests. Microsoft Excel 2007 (Roselle, IL, USA) was used for the statistical and graphical evaluations.

RESULTS

Total flavonols: The total flavonol content of the extracts measured as mg quercetin/g dry wt. Highest flavonol content showed in the methanol extract of *Crotalaria pallida* leaves and it was 82.45±0.44 mg /g of Quercetin equivalent and other results are shown at table 1.

Determination of total proanthocyanidin: Total proanthocyanidin were significantly higher in methanol extract of *Crotalaria pallida* which is 120.97±1.02mg/g of catechin equivalent and other results are shown at table 1

Thrombolytic activity: Addition of 100µl SK, a positive control (15,00,000 I.U.) to the clots along with 90 minutes of incubation at 37°C, showed (75± 0.09 %) clot lysis. Clots when treated with 100µl sterile distilled water (negative control) showed only negligible clot lysis (4.8±0.12%). The in vitro thrombolytic activity study revealed that *Crotalaria pallida* showed highest clot lysis effect, which was 41.81±4.12%.Statistical representation of the effective clot lysis percentage by our all herbal preparation, positive thrombolytic control (Streptokinase) and negative control (sterile distilled water) is tabulated in (Table2) and showed in Figure 1.

DISCUSSION

Phytonutrients, the chemicals that help plants defend against environmental challenges, such as damage from pests or ultraviolet light; appear to provide humans with protection as well. Mounting research shows their effectiveness in preventing and treating a range of conditions including everything from cancer and heart disease to diabetes and high blood pressure. Flavonols are a kind of flavonoid compound that works as an antioxidant. Flavonols could be one element of a dietary approach to the maintaining and improving not only of cardiovascular health, but also specifically brain health. Preliminary study suggests that a drink containing flavonols may improve some aspects of cognitive function in older people. However, larger, longer-term studies are needed before we can draw any firm conclusions on whether foods like this have any significant benefits.

It was found that proanthocyanidin intake in moderately low quantity was effective in up-regulating the antioxidant defense mechanism by attenuating lipid peroxidation and protein oxidation. It is also noticed changes in the cholinergic system indicating an increase in the acetylcholine esterase concentration with a moderate reduction in acetylcholine esterase activity. They suggest that proanthocyanidin may have a potent benefit of enhancing cognition in older rats. [21] It is believed that proanthocyanidins may offer benefits of bone health. Because when researcher extracted proanthocyanidins from an Amazonian medicinal plant (Sangre de grado; Croton palanostigma) in two preparations, prograd and zangrado (majority of short-chain oligomers). They found prograd had a promising safety profile, significant Chondroprotective and antioxidant actions, and directly inhibited MMP activity and promoted the production of the cartilage repair factor, IGF-1. [22]
Thrombolytic agents are used to disrupt already formed blood clots in clinical settings where ischemia may be fatal (acute myocardial infarction, pulmonary embolism, ischemic stroke, and arterial thrombosis).

Thrombolytic drugs dissolve blood clots by activating plasminogen which forms a cleaved product called plasmin.

CONCLUSION

The results stated above showed that all extract possessed significant flavonols and proanthocyanidins content. Except *Euphorbia neriifolia*, this has low proanthocyanidin content. *Crotalaria pallida* showed good thrombolytic activity, because it has good amount of secondary metabolite. *Premna esculenta* showed less thrombolytic activity, where *Euphorbia neriifolia* exhibit well clot lysis activity compared with streptokinase. All of these content and effect of methanol extracts of *Premna esculenta*, *Crotalaria pallida* and *Euphorbia neriifolia* evidenced that they could be a very good source of natural medicines on standard formulation.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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Table 1: Total flavonol and total proanthocyanidin content of methanol extracts of *Premna esculenta*, *Crotalaria pallida* and *Euphorbia neriifolia.*

Phytochemicals(mg/gm)	*Premna esculenta*	*Crotalaria pallida*	*Euphorbia neriifolia*
Total Flavonol (mg Quercetin/g)	**69.14±0.14^{b}**	**82.45±0.44^{a}**	**73.32±0.43^{b}**
Total Proanthocyanidin (mg Catechin/g)	**106.62±1.29^{a}**	**120.97±1.02^{b}**	**17.86±0.26^{b}**

Values are mean ± SEM, (n = 3). The different superscripted (a, b) values have significantly different ($^{a}P < 0.05$, $^{b}P < 0.001$) from the other sample in same row.

Table 2: Thrombolytic Activity of *P. esculent*, *C. pallida and E. neriifolia* compared with Streptokinase.

Treatment	% clot lysis
Water	**4.8±0.12**
P. esculenta	**17.27±1.57^{b}**
C. pallida	**41.81±4.12^{b}**
E. neriifolia	**34.57±1.99^{a}**
Streptokinase	**75±0.09^{b}**

Values are mean ± SEM (n = 20); $^{a}P < 0.05$, $^{b}P < 0.001$, Dunnett test as compared to negative control. Statistical representation of the effective clot lysis percentage by herbal preparations, positive thrombolytic control (streptokinase), and negative control (sterile distilled water) processed by paired *t*- test analysis (Dunnett test).

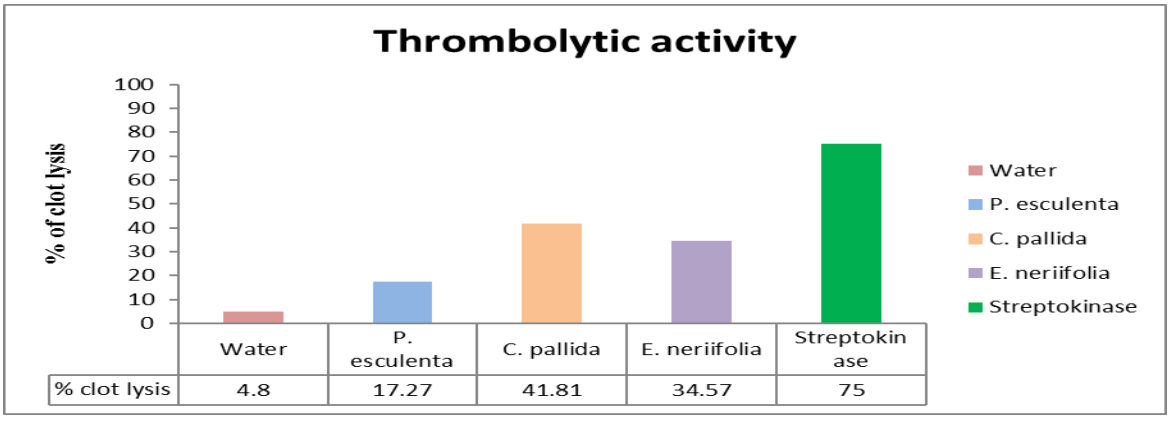

Figure 1: Thrombolytic Activity of *P. esculent, C. pallida and E. neriifolia* compared with Streptokinase.

REFERENCES

1. Mink PJ, Scrafford CG, Barraj LM, Harnack L, Hong CP. Flavonoid intake and cardiovascular disease mortality a prospective study in postmenopausal women. American Journal of Clinical Nutrition, 2007; 85: 895-909.
2. McCullough ML, Peterson JJ, Patel R, Jacques PF, Shah R. Flavonoid intake and cardiovascular disease mortality in a prospective cohort of US adults. American Journal of Clinical Nutrition, 2012; 95: 454-464.
3. Fine A. Oligomeric proanthocyanidin complexes history, structure, and phytopharmaceutical applications. Altern Med Rev, 2000; 5: 144-151.
4. Hooper L, Kroon PA, Rimm EB, Cohn JS, Harvey I. Flavonoids, flavonoid-rich foods, and cardiovascular risk: a meta-analysis of randomized controlled trials. Am J Clin Nutr, 2008; 88: 38-50.
5. Bondonno CP, Yang X, Croft KD, Considine MJ, Ward NC. Flavonoid-rich apples and nitrate-rich spinach augment nitric oxide status and improve endothelial function in healthy men and women: a randomized controlled trial. Free Radic Biol Med, 2011; 52: 95-102
6. Sano T, Oda E, Yamashita T, Naemura A, Ijiri Y. Anti-thrombotic effect of proanthocyanidin, a purified ingredient of grape seed. Thromb Res, 2005; 115: 115-121.
7. Cortes SF, Valadares YM, Oliveira AB, Lemos VS, Barbosa MPT. Mechanism of endothelium-dependent vasodilatation induced by a proanthocyanidin-rich fraction from Ouratea semis errata. Planta Med, 2002; 68: 412-415.
8. Yanarates O, Guven A, Sizlan A, Uysal B, Akgul O. Ameliorative effects of proanthocyanidin on renal ischemia/reperfusion injury. Ren Fail, 2008; 30: 931-938.
9. Nakagawa T, Yokozawa T, Satoh A, Kim HY: Attenuation of renal ischemia-reperfusion injury by proanthocyanidin-rich extract from grape seeds. J Nutr Sci Vitaminol, 2005; 51: 283-286
10. Avramovic V, Vlahovic P, Mihailovic D, Stefanovic V. Protective effect of a bioflavonoid proanthocyanidin-BP1 in glycerol-induced acute renal failure in the rat: renal stereological study. Ren Fail, 1999; 21: 627-634.
11. Stefanovic V, Savic V, Vlahovic P, Cvetkovic T, Najman S. Reversal of experimental myoglobinuric acute renal failure with bioflavonoid from seeds of grape. Ren Fail, 2000; 22: 255-266.
12. Lee YA, Kim YJ, Cho EJ, Yokozawa T. Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. J Agr Food Chem, 2007; 55: 9395-9400.
13. Uddin SN, Traditional uses of Ethnomedicinal plants of the Chittagong Hill Tracts, 1st ed, Bangladesh National Herbarium, Dhaka : 2006; 764.
14. Limoux MM, Vandyke BE, Motet AN, Tinley PM. An evaluation of molecular and anatomical characters in the genus *Crotalaria*. *S.* Afr. J. Bot, 2009; 75: 410.
15. Chong KY, Tan HTW, Corlett RT: A Checklist of the Total Vascular Plant Flora of Singapore Native, Naturalized and Cultivated Species. Raffles Museum of Biodiversity Research, National University of Singapore, Singapore: 2009; pp. 273.
16. Kirtikar KR, Basu BD: Indian Medicinal Plants. Vol. II, Dehradun, India, International Book Distributers: 1996; p. 1581.
17. Anonymous the Wealth of India, New Delhi. Raw Material, III (D-E). C.S.I.R. Publication, 1952: 226.
18. Kumaran A, Karunakaran RJ. In vitro antioxidant activities of methanol extracts of five *Phyllanthus* species from India. Food Sci Techno, 2007; 40: 344-52.
19. Oyedemi SO, Bradley G, Afolayan AJ. In vitro *and* In vivo *antioxidant activities of* aqueous extract of *Strychonos henningsii* Gilg. Afr J Pharm Pharmacology, 2010; 4:70-78.
20. Prasad S, Kashyap RS, Deopujari JY, Purohit HJ, Taori GM, Daginawala HF. Development of an *in vitro* model to study clot lysis activity of thrombolytic drugs. Thromb J 2006; 4(14): 1-4.
21. Devi A, Jolitha AB, Ishii N. Grape seed proanthocyanidin extract (GSPE) and antioxidant defense in the brain of adult rats. Med Sci Monet, 2006; 12(4):124-129.
22. Miller MJ. Chondroprotective effects of a proanthocyanidin rich Amazonian genonutrient reflects direct inhibition of matrix metalloproteinase's and upregulation of IGF-1 production by human chondrocytes. J Inflame Lond, 2007; 14: 4-16.