Antioxidant, Cytotoxic, Membrane Stabilizing and Antimicrobial Activities of Bark and Seed of *Entada phaseoloides* (L.) Merr.: A Medicinal Plant from Chittagong Hill Tracts

Fahima Aktar[1], Md. Ruhul Kuddus[1], Sk. Omar Faruque[2], Farhana Rumi[3], Mohiuddin Abdul Quadir[1] and Mohammad A. Rashid[1,*]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh*

[2]*Department of Pharmacy, State University of Bangladesh, 77 Satmosjid Road, Dhanmondi, Dhaka-1207, Bangladesh*

[3]*Department of Pharmacy, Manarat International University, Mirpur-1, Dhaka-1217, Bangladesh*

Abstract: The methanolic crude extract of the bark and seed of *Entada phaseoloides* (L.) Merr. and its different organic soluble partitionates were screened for antioxidant, cytotoxic, membrane stabilizing and antimicrobial activities. The crude extract and carbon tetrachloride and aqueous soluble fractions of both bark and seed showed higher level of total phenolic content (TPC, 245.59, 240.22, 240.03 & 117.0 mg of GAE/gm of dried extract). In the DPPH assay, the crude extract of bark and its chloroform and aqueous soluble fractions demonstrated strong antioxidant property with the IC_{50} of 3.24, 1.55 and 3.6 μg/ml, respectively whereas all the fractions of seed extract revealed mild antioxidant activity. The petroleum ether soluble fraction of both seed and bark exhibited significant cytotoxicity (LC_{50} = 1.54 & 5.4 μg/ml) which confers the presence of bioactive metabolites in this plant. On the other hand, the crude extract of seed and petroleum ether soluble fraction of bark inhibited the hemolysis of RBC of rat's blood by 78.89 and 57.43%, respectively as compared to 84.44% exerted by acetyl salicylic acid (0.10 mg/ml). In antimicrobial screening, the carbon tetrachloride soluble fraction of bark showed significant antimicrobial activity against *Staphylococcus aureus* (zone of inhibition = 17.0 mm) with MIC and MBC values of 7.81 μg/ml and 125 μg/ml, respectively.

Keywords: Antimicrobial, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC).

1. INTRODUCTION

The role of plants in maintaining human health is well documented [1]. Plants are used medicinally in different countries and are source of many potent and powerful drugs [2]. The active principles of many drugs found in plants are secondary metabolites [3, 4]. Therefore, basic chemical investigation of these plant extracts is also vital for their major phytoconstituents. Most of the molecules in plants are secondary metabolites, of which at least 12,000 have been isolated and characterized [5]. *Entada phaseoloides* (L.) Merr., (Bengali name- Gila) is a medicinal plant growing in Forests of Chittagong Hill Tracts and Sylhet [7]. The plant has been used widely as a folk medicine in China for the treatment of jaundice, stomachic, constipation and hernia [8]. The seeds possess anti-inflammatory [9] and antiulcer [10] activities. Phenylacetic acid derivatives i.e. 2-hydroxy-5-butoxyphenylacetic acid and 2,5-dihydroxyphenylacetic acid methyl ester isolated from the seed of this plant demonstrated significant cytotoxicity against cultured human cancer cells [11]. Previous phytochemical investigations led to the isolation of entadamides A-C [12], saponins [13], phascoloidin [14], entadamide A-*β*-*D*-glucopyranosyl-(1→3)-*β*-*D*glucopyranoside [8], etc.

As a part of our continuing investigation of medicinal plants of Bangladesh [15, 16], we evaluated the antioxidant, cytotoxic, membrane stabilizing and antimicrobial activities of Kupchan fractions of methanolic extract of the bark and the seed of *E. phaseoloides* to verify the traditional use of the plant as potential source of antimicrobial, anticancer and antioxidant agents.

2. MATERIALS AND METHODS

2.1. Plant Materials

The bark and seed of the plant were collected from Chittagong Hill Tracts in 2010. A voucher specimen for this collection has been deposited in Bangladesh National Herbarium, Mirpur, Dhaka-1216 (Accession no. 35452). The bark was cut into small pieces and both bark and seeds were sun dried for 7 days followed by oven drying for 24 hours at 40 °C to facilitate proper grinding.

*Address corresponding to this author at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka, Dhaka-1000, Bangladesh; Tel: 880-2-9661900-73, extn- 8130, 8131, 8137; Fax: 880-2-8612069; E-mails: rashidma@univdhaka.edu or rashid_phdu@yahoo.com

ISSN: 2223-3806 / E-ISSN: 1927-5951/11

2.2. Extraction and Isolation

The powdered materials (550 g) of each of bark and seed was separately soaked in 1.0 liter of methanol in a large conical flask for 7 days with occasional shaking and stirring. The whole mixture was then filtered off through a cotton plug followed by Whatman filter paper no.1 and the filtrate thus obtained was concentrated with the help of a rotary evaporator. An aliquot (5.0 g) of the concentrated methanol extract of both samples was separately fractionated by the modified Kupchan partitioning protocol [17], which afforded pet ether (650.0 mg), carbon tetrachloride (950.0 mg), chloroform (450.0 mg) and aqueous (2.05 g) soluble materials from the crude methanolic extract of bark while the seed extract provided pet ether (450.0 mg), carbon tetrachloride (650.0 mg), chloroform (450.0 mg) and aqueous (1.8 g) soluble materials.

2.3. Total Phenolics Analysis

The total phenolic content of *E. phaseoloides* was measured by employing the method [18] involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as standard. To 0.5 ml of extract solution (2.0 mg/ml) in water, 2.5 ml of Folin-Ciocalteu reagent (diluted 10 times with water) and 2.0 ml of sodium carbonate (7.5 % w/v) solution were added. After 20 minutes of incubation at room temperature, the absorbance was measured at 760 nm using a UV-visible spectrophotometer. Total phenolics were quantified with the help of calibration curve obtained from gallic acid (0-100 µg/ml). The phenolics content of the sample was expressed as mg of GAE (gallic acid equivalent)/gm of the dried extract.

2.4. DPPH Assay

The free radical scavenging activity (antioxidant capacity) of the extracts on the stable radical 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Brand- Williams *et al.* [19]. 2.0 ml of a methanol solution of the extract at different concentration was mixed with 3.0 ml of DPPH-methanol solution (20 µg/ml). The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of *tert*-butyl-1-hydroxytoluene (BHT) and ascorbic acid (ASA) by UV spectrophotometer [20]. Inhibition of free radical DPPH in percent (I %) was calculated as follows:

$$I\% = (1 - A_{sample}/A_{blank}) \times 100$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test material) and A_{sample} is the absorbance of the sample. Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with percentage of inhibition against extractive/standard concentration.

2.5. Cytotoxicity Screening

DMSO solutions of the extractives were applied against *Artemia salina* in a one-day in *vitro* assay [21]. For the experiment, 4 mg of each of the Kupchan fractions was dissolved in DMSO and solutions of varying concentrations such as 400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.78125 µg/ml were obtained by serial dilution technique. Vincristine sulphate and DMSO were used as the positive and negative control, respectively.

2.6. Membrane Stabilizing Activity

The membrane stabilizing activity was assessed by using hypotonic solution induced hemolysis of mice erythrocyte [22]. To prepare the erythrocyte suspension whole blood was drawn with a syringe (containing anticoagulant EDTA) from mice through cardiac puncture. The blood was centrifuged and blood cells were washed three times with 154 mM NaCl solution in 10 mM sodium phosphate buffer (pH 7.4) through centrifugation for 10 min at 3000 *g*. The test sample consisting of stock erythrocyte (RBC) suspension (0.50 ml) was mixed with 5.0 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (2.0 mg/ml). The control sample consisted of 0.5 ml of RBCs mixed with hypotonic-buffered saline alone. The mixture was incubated for 10 min at room temperature, centrifuged for 10 min at 3000 *g* and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of either hemolysis or membrane stabilization was calculated using the following equation-

$$\% \text{ inhibition of hemolysis} = 100 \times (OD_1 - OD_2/OD_1)$$

where, OD_1 = optical density of hypotonic-buffered saline solution alone (control) and OD_2 = optical density of test sample in hypotonic solution.

2.7. Antimicrobial Activity

The preliminary antimicrobial activity of the extractives was determined at 400µg/disc by the disc diffusion method [23] against a number of Gram positive and Gram negative bacteria and fungi. The

bacterial and fungal strains used in this experiment were collected as pure cultures from the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR, B). Here, standard Kanamycin (30µg) disc was used as reference.

2.8. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

Minimum inhibitory concentrations (MIC) are important to monitor the activity of new antimicrobial agents [24] and are generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism [25]. The MIC was determined for crude methanolic extract (ME) and its carbon tetrachloride (CTSF), chloroform (CSF) and aqueous (AQSF) soluble fractions of the bark extract by serial dilution technique [23]. Thirteen test tubes were taken, ten of which were marked as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and the rest three were assigned as TM (only medium), TMC (Medium + extractive solution) and TMI (medium + inoculum). In all the test tubes containing the calculated amount of broth medium, the sample from the mother solution was added with serial dilution which gave solutions of varying concentration (1000 µg/ml to 0µg/ml). Lastly, the inoculum was added to the test tubes, shaken using rotamixer and incubated at 37 °C for 24 hours. The control test tube (TM) was used to ascertain the sterility of the medium. After 24 hours the test tubes were checked for the microbial growth and the clear test tubes compared to McFarland turbidity standards were marked for the MIC determination. The lowest concentration (highest dilution) of antibiotic that prevented the turbidity was considered as the minimal inhibitory concentration (MIC). At this dilution, the antibiotic is bacteriostatic. The minimum bactericidal concentration (MBC) was determined by sub-culturing the broth media into fresh agar medium from each tube in which no growth was visible. The growth of one colony indicated a 99.8% fall in viable count [25].

3. RESULTS AND DISCUSSION

The present study was undertaken to evaluate the antioxidant, cytotoxic, membrane stabilizing and antimicrobial activities of different extractives of bark and seed of *E. phaseoloides* and the results have been summarized in Figures 1 to 4 and Table **1**.

In total phenolic content analysis, among all the extractives of bark of *E. phaseoloides*, the crude extract and its carbon tetrachloride and aqueous soluble fractions showed highest phenolic content (TPC, 245.59, 240.22 and 240.03 mg of GAE/gm of extractives, respectively). On the other hand, the aqueous soluble fraction (AQSF) of seed was found to contain highest amount of phenolic content (TPC, 117.0 mg of GAE/gm of extractives) (Figure **1**).

In free radical scavenging (DPPH) assay, all fractions of bark revealed strong antioxidant activity. The chloroform soluble fraction (CSF), crude methanolic extract (ME) and carbon tetrachloride soluble partitionate (CTSF) of bark showed significant antioxidant property with IC_{50} of 1.55, 3.24 and 3.6 µg/ml, respectively, while the aqueous soluble fraction (AQSF) of seeds demonstrated moderate antioxidant property with the IC_{50} of 54.78 µg/ml in comparison to the standard *tert*-butyl-1- hydroxytoluene (BHT) with IC_{50} of 15.5 (µg/ml) (Figure **2**).

Table 1: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Test Samples of Bark of *E. phaseoloides*

	ME		CTSF		CSF		AQSF		Ciprofloxacin	
Test microorganisms	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Bacillus megaterium	-	-	15.62	250	15.62	125	31.25	500	0.31	2.5
B. cereus	31.25	250	-	-	-	-	-	-	0.31	1.25
Escherichia coli	-	-	-	-	-	-	250	1000	0.62	1.5
Sarcina lutea	62.50	500	-	-			15.62	125	1.25	5.0
Shigella dysenteriae	15.62	250	-	-	-	-	125	500	0.31	0.62
Staphylococcus aureus	-	-	7.812	125	-	-	-	-	0.62	2.5
Vibrio parahemolyticus	-	-	31.25	500			15.62	125	1.25	2.5
Candida albicans	-	-	62.50	500	-	-	-	-	0.31	0.62

Where, ME = Methanolic extract, CTSF= Carbon tetrachloride soluble fraction, CSF = Chloroform soluble fraction and AQSF = Aqueous soluble fraction of crude methanolic extract.

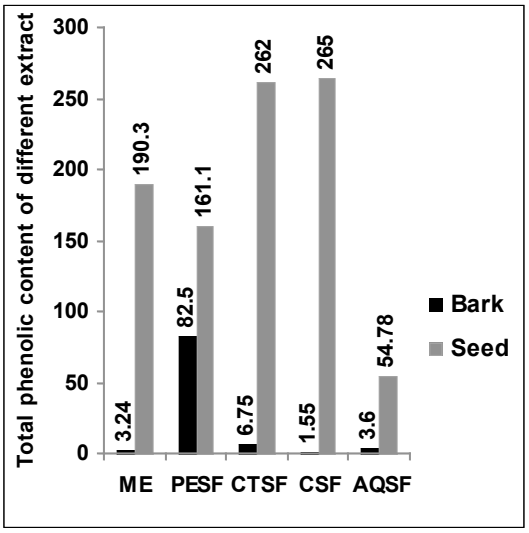

Figure 1: Total phenolic content (mg of GAE/gm of dried extract) of different extractives of bark and seed of *E. phaseoloides*.

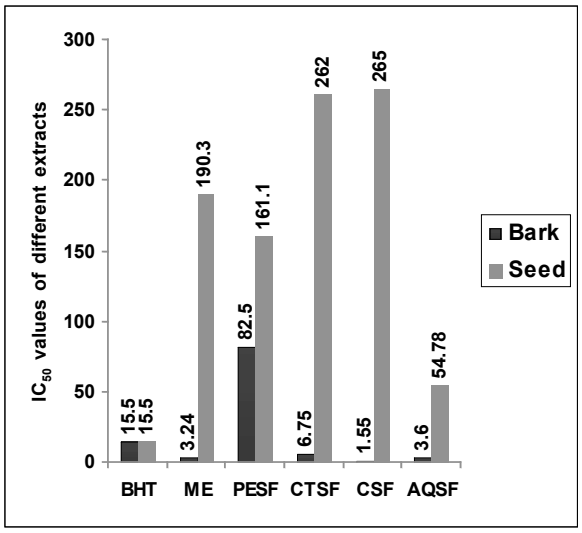

Figure 2: Free radical scavenging activity (IC_{50}) of different extractives of bark and seed of *E. phaseoloides*

In the brine shrimp lethality bioassay, the pet ether soluble fraction (PESF) of methanol extract of both the seed and bark revealed strong cytotoxicity with LC_{50} value of 1.54 and 5.4 µg/ml, respectively (Figure **3**). According to the LC_{50} values, all the extractives were toxic to shrimp nauplii.

The extractives of both bark and seed, at concentration 2.0 mg/ml, significantly protected the lysis of mice erythrocyte membrane induced by hypotonic solution, as compared to the standard acetyl salicylic acid (0.10 mg/ml). The crude methanol extract (ME) and its aqueous soluble fraction (AQSF) of seed exhibited 78.89% and 65.52% inhibition of hemolysis of RBC, respectively as compared to 84.44% produced by acetyl salicylic acid (0.10 mg/ml) (Figure **4**). The pet ether soluble extractives (PESF) of bark also revealed significant inhibition of hemolysis of RBC.

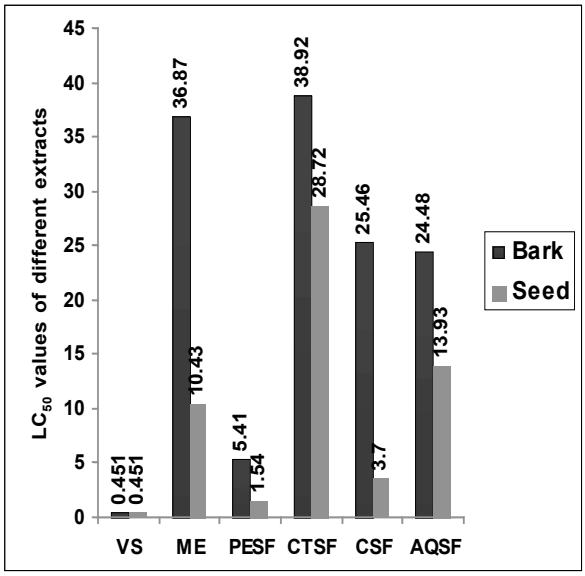

Figure 3: Cytotoxic activity (LC_{50}) of different extractives of bark and seed of *E. phaseoloides.*

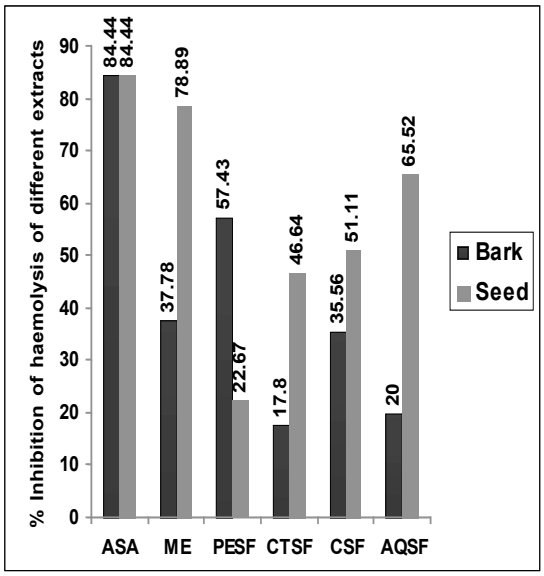

Figure 4: Membrane stablizing activity of different extractives of bark and seed of *E. phaseoloides.*

In antimicrobial investigation all the fractions of bark exhibited mild to moderate antimicrobial activity (zone of inhibition = 9.0-17.0 mm) against all the test organisms, while the fractions of seed were almost inactive. The crude methanol extract of bark exhibited mild activity against *S. dysenteriae* (zone of inhibition = 15.0 mm), *B. cereus* (13.0 mm), *S. lutea* (13.0 mm), *E. coli* (12.0 mm), *V. parahemolyticus* (12.0 mm). The

carbon tetrachloride soluble fraction of crude methanolic extract of bark also showed moderate antimicrobial activity against *Staph. aureus* (having zone of inhibition of 17.0 mm), *B. megaterium* (14.0 mm), *V. parahemolyticus* (14.0 mm), *C. albicans* (14.0 mm). Both the chloroform and aqueous soluble fraction exhibited mild activity against the test organisms.

Thus all fractions of bark were subjected to determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The carbon tetrachloride soluble fraction exerted highest inhibitory activity against *Staph. aureus* (zone of inhibition = 17.0 mm). Relatively less polar compounds revealed better antimicrobial activity as the carbon tetrachloride soluble partitionates showed significant antimicrobial activity than the aqueous and chloroform soluble partitionates of the bark extract.

The MIC value of carbon tetrachloride soluble fractions (where no bacterial growth was observed in broth media) was found to be 7.81µg/ml and the MBC value (where no bacterial growth was observed in agar media) was found to be 125µg/ml against *Staph. aureus* (Table **1**). No microbial growth was observed in the test tubes marked with TM (containing medium only) and TMC (medium + test sample, no inoculum) indicating that the medium and the test sample were not contaminated by microorganism and the total investigation was performed properly in the sterile condition. Microbial growth was observed in the test tube designated by TMI (medium + inoculums) revealing the fact that there was no problem with the subcultured microorganisms.

CONCLUSION

It can be concluded that the methanolic crude extract and its Kupchan fractions of bark and seed showed significant cytotoxic activities which suggest the presence of secondary metabolites with biological properties such as anticancer agent. In antimicrobial screening, all fractions of bark exhibited mild to moderate antimicrobial activity (zone of inhibition = 9.0-17.0 mm) against the test organisms. The carbon tetrachloride soluble fraction of bark showed significant antimicrobial activity against *Staph. aureus* (zone of inhibition = 17.0 mm) with MIC and MBC values of 7.81 µg/ml and 125 µg/ml, respectively. Thus, this plant could be subjected to extensive chromatographic separation and purification processes to isolate bioactive compounds for the discovery of novel therapeutic agents.

ACKNOWLEDGEMENT

The authors wish to thank the authority of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) for supplying test animal to perform these investigations.

REFERENCES

[1] Moermann DE. An analysis of the food plants and drug plants of native North America. J Ethnopharmacol 1996; 52: 1-22. http://dx.doi.org/10.1016/0378-8741(96)01393-1

[2] Srivastava J, Lambert J, Vietmeyer N. 1996. Medicinal plants: An expanding role in development. World Bank Technical; paper. No. 320.

[3] Ghani A. Introduction to Pharmacognosy. Ahmadu Bello University Press, Ltd. Zaria, Nigeria; 1990; p. 45-47, 187-97.

[4] Haroon AM. Effect of some macrophytes extracts on growth of Aspergillus parasiticus. Egyptain J Aqua Res 2006; 32: 301-13.

[5] Ndubuisi JA, Emeka OE, Luke, UN. Physicochemical properties of chloroform extract of water hyacinth (Eichhornia crassipes). The African J Plant Sci Biotech 2007; 1: 40-2.

[6] Roy S, Uddin MZ, Hassan MA, Rahman MM. Medico-botanical report on the Chakma community of Bangladesh. Bangladesh J Plant Taxon 2008; 15: 67-72.

[7] Xiong H, Xiao E, Zhao Y, Yang G, Mei Z. Sulfur-containing amides from Entada phaseoloides. Acta Pharmaceutica Sinica 2010; 45: 624-6.

[8] Ikegami F, Sekine T, Aburada M, Fujii Y, Komatsu Y, Murakoshi I. Synthesis of entadamide A and entadamide B isolated from Entada phaseoloides and their inhibitory effects on 5-lipoxygenase. Chem Pharm Bull (Tokyo). 1989; 37: 1932-3. http://dx.doi.org/10.1248/cpb.37.1932

[9] Ramakrishna, D., Pavan, KK., Mukkanti, K, Abedulla, KK. Antiulcer activity of the seeds of Entada phaseoloides. Pharmacologyonline 2008; 3: 93-9.

[10] Jinrui D, Leonardus BSK, Soefjan T, Kosasih P, John MP, Kinghorn AD. Phenylacetic acid derivatives and a thioamide glycoside from Entada phaseoloides. Phytochemistr, 1991; 30: 3749-52. http://dx.doi.org/10.1016/0031-9422(91)80102-7

[11] Ikegami F, Sekine T, Duangteraprecha S, Matsushita N, Matsuda N, Ruangrungsi N. Entadamide C, a sulphur-containing amide from Entada phaseoloides. Phytochemistry 1989; 28: 881-2. http://dx.doi.org/10.1016/0031-9422(89)80135-9

[12] Perumal S, Klaus B, Harinder PS. Chemical composition, protein fractionation, essential amino acid potential and antimetabolic constituents of an unconventional legume, Gila bean (Entada phaseoloides Merrill) seed kernel. J Sci Food Agric 2002; 82: 192–202. http://dx.doi.org/10.1002/jsfa.1025

[13] Barua AK, Chakrabarty M, Datta PK, Ray S. Phaseoloidin, a homogentisic acid glucoside from Entada phaseoloides. Phytochemistry 1988; 27: 3259-61. http://dx.doi.org/10.1016/0031-9422(88)80038-4

[14] Sikder MAA, Siddique AB, Hossian AKMN, Miah MK, Kaisar MA, Rashid MA. Evaluation of thrombolytic activity of four Bangladeshi medicinal plants, as a possible renewable source for thrombolytic compounds. J Pharm Nutrition Sci 2011; 1: 4-8.

[15] Kaisar MA, Rahman MS, Rahman MZ, Hasan CM, Rashid, MA. A review on phytochemicals from some medicinal plants

of Bangladesh. J Pharm Nutrition Sci 2011; 1: 87-95. http://dx.doi.org/10.6000/1927-5951.2011.01.01.14

[16] Van Wagenen BC, Larsen R, Cardellina JH, Ran dazzo D, Lidert ZC, Swithenbank C. Ulosantoin, a potent insecticide from the sponge Ulosa ruetzleri. J Org Chem 1993; 58: 335-7. http://dx.doi.org/10.1021/jo00054a013

[17] Skerget M, Kotnik P, Hadolin M, Hras A, Simonic M, Knez Z. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem 2005; 89: 191-8. http://dx.doi.org/10.1016/j.foodchem.2004.02.025

[18] Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technol 1995; 28: 25-30. http://dx.doi.org/10.1016/S0023-6438(95)80008-5

[19] Choi HY, Jhun EJ, Lim BO, Chung IM, Kyung SH, Park DK. Application of flow injection-chemilumineacence to the study of radical scavenging activity in plants. Phytother Res 2000; 14: 250-3. http://dx.doi.org/10.1002/1099-1573(200006)14:4<250::AID-PTR587>3.0.CO;2-J

[20] Meyer BN, Ferrigni NR, Putnam JE, Jacobsen JB, Nicholsand DE, Mclaughlin JL. Brine shrimp; a convenient general bioassay for active plant constituents. Planta Med 1982; 45: 31-4. http://dx.doi.org/10.1055/s-2007-971236

[21] Shinde UA, Phadke AS, Nair AM, Mungantiwar AA, Dikshit VJ, Saraf MN. Membrane stabilizing activity - a possible mechanism of action for the anti-inflammatory activity of Cedrus deodara wood oil. Fitoterapia 1999; 70: 251-7. http://dx.doi.org/10.1016/S0367-326X(99)00030-1

[22] Bauer AW, Kirby WMM, Sherries JC, Tuck M. Antibiotic susceptibility testing by a standardized disc diffusion method. J Am Clin Pathol 1966; 45: 493-6.

[23] Jennifer MA. Determination of minimum inhibitory concentrations. J Antimic Chemother 2001; 48: 5-16.

[24] Turnidge JD, Ferraro MJ, Jorgensen JH. Manual of Clinical Microbiology. 8th Ed. Washington. American Society of Clinical Microbiology; 2003; p.1103.

[25] Brumitt W, Hamilton-Miller MT, Gooding A. Importance of methodology in determining bactericidal and bacteriostatic activities of Azlocillin and ticarcillin against Pseudomonas aeruginosa. J Med Microbiol 1984; 17: 37- 44. http://dx.doi.org/10.1099/00222615-17-1-37

Received on 24-11-2011 Accepted on 02-12-2011 Published on 28-12-2011

DOI: http://dx.doi.org:10.6000:1927-5951.2011.01.02.13