

## ***In vitro* antioxidant property of the extract of *Excoecaria agallocha* (Euphorbiaceae)**

<sup>1</sup>Nusrat Subhan, <sup>2</sup>M. Ashraful Alam, <sup>3</sup>Firoj Ahmed, <sup>4</sup>M. Abdul Awal, <sup>5</sup>Luffun Nahar, <sup>\*5</sup>Satyajit D. Sarker

<sup>1</sup> Department of Pharmacy, Northern University, <sup>2</sup> Department of Pharmacy, Stamford University, Dhaka, <sup>3</sup> Pharmacy Discipline, Khulna University, Khulna, <sup>4</sup> Department of Pharmacology, Bangladesh Agricultural University, Mymensingh, Bangladesh, <sup>5</sup> School of Biomedical Sciences, University of Ulster, Cromore Road, Coleraine BT52 1SA, Co. Londonderry, Northern Ireland, UK

Received 18 Feb 2008; Revised 11 March 2008; Accepted 27 May 2008

### **ABSTRACT**

**Background and objectives:** *Excoecaria agallocha* L. (Family: Euphorbiaceae) is a Bangladeshi medicinal plant found predominantly in the tidal forests and swamps of the Sundarbans and other coastal areas in Bangladesh. As part of our on-going phytochemical and bioactivity studies on medicinal plants from Bangladeshi flora, the *in vitro* antioxidant property on the bark of this plant was evaluated.

**Methods:** The hydroalcohol extract of the dried and ground bark of *E. agallocha* was assessed for antioxidant activity using a series of well-established assays including the 2,2-diphenyl-1-picrylhydrazyl (DPPH), the lipid peroxidation by thiobarbituric acid (TBA), the reducing power, the nitric oxide (NO.) and the hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) scavenging assays.

**Results:** In the DPPH, the NO and the H<sub>2</sub>O<sub>2</sub> scavenging assays, the extract of *E. agallocha* displayed significant antioxidant activities with the IC<sub>50</sub> values of 179.16, 120.24 and 134.29 µg/ml, respectively. The reducing power of the extract increased dose-dependently, and the extract reduced the most Fe<sup>3+</sup> ions to the extent less than butylated hydroxy toluene (BHT). In the lipid peroxidation assay, the extract showed significant inhibition of peroxidation effect at all concentrations, with an IC<sub>50</sub> value of 189.27 µg/ml.

**Conclusion:** Since reactive oxygen species are important contributors to serious ailments such as atherosclerosis, alcoholic liver cirrhosis and cancer, the antioxidant property of the extract of *E. agallocha* as observed in the present study might be useful for the development of newer and more potent antioxidants.

**Keywords:** *Excoecaria agallocha*, Euphorbiaceae, antioxidant, lipid peroxidation, DPPH, reactive oxygen species

### **INTRODUCTION**

*Excoecaria agallocha* L. (Euphorbiaceae) is a small mangrove tree found extensively in the tidal forests and swamps of the Sundarbans and other coastal areas of Bangladesh (1). This plant is also well-distributed in a number of other countries of temperate and tropical Asia, Australasia and Southwestern Pacific (2). This plant has traditionally been used to treat sores and stings from marine creatures, and ulcers, as a purgative and an emetic, and the smoke of its bark has been used to treat leprosy (1). The bark oil has also been found effective against rheumatism, leprosy and paralysis (1). However, the milky sap of this

tree can cause temporary blindness if it enters the eyes. The sap can also cause skin blisters and irritation. Clinical trials carried out on this plant have shown its potential anti-HIV, anticancer, antibacterial and antiviral properties (3). Previous phytochemical investigations on this species revealed the presence of diterpenoids (4-10), triterpenoids (11), flavonoid (12) and phorbol esters (13). As part of our on-going phytochemical and bioactivity studies on medicinal plants from Bangladeshi flora (14-28), we now report assessment of *in vitro* antioxidant property of the bark of *E. agallocha*.

## MATERIALS AND METHODS

### *Plant material*

The bark of *Excoecaria agallocha* L. was collected during October 2003 from the Sundarbans of Karomjol, Dacope region. The plant was identified at Bangladesh National Herbarium where a voucher specimen was deposited (Accession No. -30209).

### *Extraction*

The bark of *E. agallocha* (400 g) was dried at room temperature, ground and Soxhlet-extracted with 80% aqueous ethanol. The solvent was completely removed by rotary evaporator to obtain 16.3 g of reddish gummy exudates.

### *Qualitative DPPH radical scavenging activity*

Qualitative assay was performed by the method of Sadhu et al., (29). Test samples were developed on a TLC plate (CHCl<sub>3</sub>: MeOH, 4:1) and sprayed with 0.004% w/v DPPH solution in MeOH using an atomizer. The positive activity was detected by the discolored (pale yellow) spots on a reddish purple background.

### *Quantitative DPPH radical scavenging activity*

A methanolic solution of DPPH (0.15%) was mixed with serial dilutions (1 µg to 500 µg) of *E. agallocha* extracts and after 30 min, the absorbance was recorded at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as a positive control. The inhibition curve was plotted and the IC<sub>50</sub> values were determined.

### *Nitric oxide radical inhibition assay*

Nitric oxide radical scavenging can be estimated by the use of Griess Illosvoy reaction (30). In this study, the Griess-Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-naphthylamine (5%). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and *E. agallocha* extract (10 µg to 160 µg) or standard solution (BHT, 0.5 ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25°C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. Ascorbic acid and BHT were used as positive controls.

### *Lipid peroxidation assay*

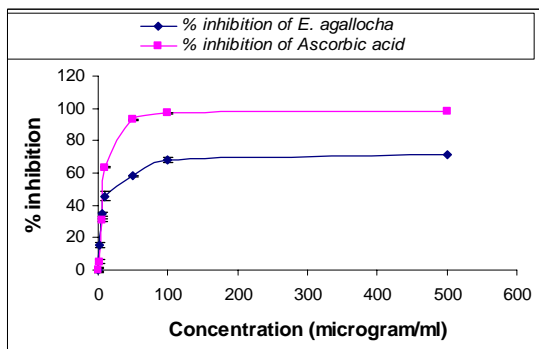
The degree of lipid peroxidation was assessed by estimation the thiobarbituric acid-reactive substances (TBARS) (31-32). Briefly, different concentrations of extracts (50–250 µg/ml) were added to the 10% liver homogenate. Lipid peroxidation was initiated by addition of 100 µl of 15mM FeSO<sub>4</sub> solution to 3 ml of liver homogenate (final concentration was 0.5 mM). After 30 min, 100 µl of this reaction mixture was taken in a tube containing 1.5 ml of 0.67% TBA in 50% acetic acid. Samples were incubated at 37°C for 1 hrs, and then lipid peroxidation was measured using the reaction with thiobarbituric acid (TBA). The absorbance of the organic layer was measured at 532 nm. All reactions were carried out in duplicates. Vitamin E was used as the positive control. The percentage of inhibition of lipid peroxidation was calculated, by the formula: Inhibition (%) = (control – test) X 100/ control.

### *Scavenging of hydrogen peroxide*

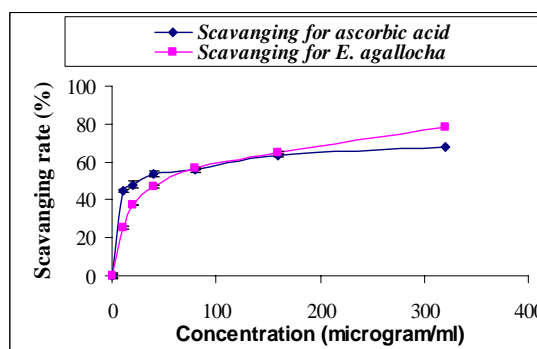
The ability of the extracts to scavenge hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was determined by the reported method (33). Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Ascorbic acid as positive control and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of standard or extract solutions (3.4 mL) were added to 0.6 mL of H<sub>2</sub>O<sub>2</sub> solution. The reaction mixture was incubated at room temperature for 10 min, and the absorbance was determined at 230 nm. The percentage of scavenging was calculated (34) as follows: % H<sub>2</sub>O<sub>2</sub> Scavenging = 100 x (Absorbance of Control- Absorbance of Sample)/ Absorbance of Control

### *Reducing power*

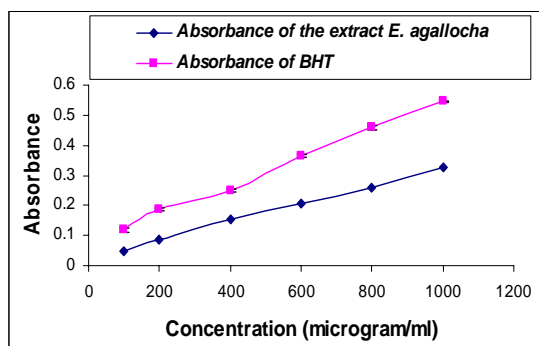
The reducing power of *E. agallocha* was determined by the method described previously (35). Different concentration of *E. agallocha* extract (100 µg – 1000 µg) in 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K<sub>3</sub>Fe(CN)<sub>6</sub>] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl<sub>3</sub> (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Butylated hydroxy toluene (BHT) was used as a positive control.



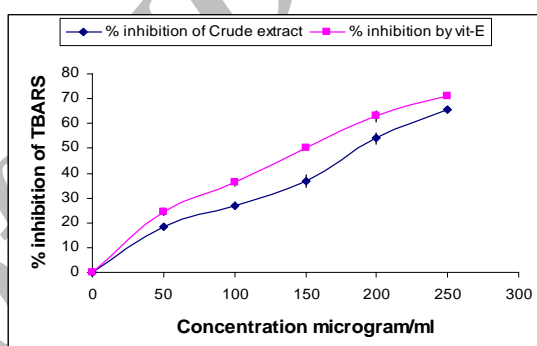
**Figure 1.** The DPPH scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean $\pm$  standard deviation).



**Figure 2.** Nitric oxide (NO $\cdot$ ) scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean $\pm$  standard deviation).



**Figure 3.** Reducing power of *E. agallocha* (values are the average of triplicate experiments and represented as mean $\pm$  standard deviation).



**Figure 4.** Effect of *E. agallocha* on TBARS on Liver homogenates of Swiss albino mice (values are the average of triplicate experiments and represented as mean $\pm$  standard deviation).

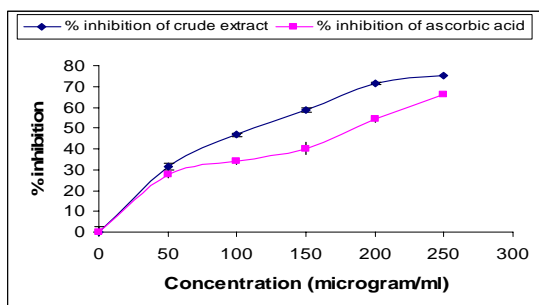
## RESULTS AND DISCUSSION

The DPPH antioxidant assay is based on the ability of 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to be decolorized in the presence of antioxidants (36). The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The TLC-based qualitative DPPH spray revealed the presence of significant antioxidant activity in the alcoholic extract of *E. agallocha* indicated by the presence of a yellowish spot on the reddish purple background of the TLC plate. In the quantitative assay the extract exhibited a notable dose dependent inhibition of the DPPH activity, with a 50% inhibition (IC<sub>50</sub>) at a concentration of 179.16  $\mu$ g/ml while the IC<sub>50</sub> value of the positive control, ascorbic acid, was found 55.91  $\mu$ g/ml (Figure 1). Nitric oxide (NO $\cdot$ ) and superoxide anion (O<sub>2</sub> $\cdot^-$ ) cause ischemic renal injury separately and these radicals work together to bring about further

damages. The toxicity and damage caused by NO $\cdot$  and O<sub>2</sub> $\cdot^-$  is multiplied as they react to produce reactive peroxynitrite (ONOO $\cdot$ ), which leads to serious toxic reactions with biomolecules (37).

Suppression of released NO $\cdot$  may be partially attributed to direct NO $\cdot$  scavenging, as the extract of *E. agallocha* decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. The scavenging of nitric oxide (NO $\cdot$ ) by the extract was increased in a dose-dependent manner as illustrated in Figure 2. At a concentration of 120.24  $\mu$ g/ml of the extract, 50% of NO was scavenged. This IC<sub>50</sub> value of extract was comparable to that of the vitamin C (IC<sub>50</sub> 107.78  $\mu$ g/ml) as positive control.

The reducing ability of a compound generally depends on the presence of reductants, which exhibit antioxidant activity by breaking the free radical chain through donation of a hydrogen atom (37). Figure 3 shows the reducing ability of the plant extract compared to that of BHT. The reducing power of the extract increased dose-dependently. However, the extract reduced the most of Fe<sup>3+</sup> ions to the extent less than BHT.

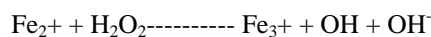


**Figure 5.** H<sub>2</sub>O<sub>2</sub> scavenging activity of *E. agallocha* (values are the average of triplicate experiments and represented as mean ± standard deviation)

During lipid peroxidation, low molecular weight end products, generally malonaldehyde, are formed by oxidation of polyunsaturated fatty acids that may react with two molecules of thiobarbituric acid to give a pinkish red chromogen (37). In the Lipid peroxidation assay, the activity of *E. agallocha* extract against non-enzymatic lipid peroxidation in rat liver microsomes was evaluated (Figure 4). Addition of Fe<sup>2+</sup>/ascorbate to the liver microsomes caused an increase in lipid peroxidation. The extract showed inhibition of peroxidation effect at all concentrations, with an IC<sub>50</sub> value of 189.27 µg/ml. The IC<sub>50</sub> value of the vitamin E as positive control was 158.26 µg/ml.

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generates a singlet

oxygen (<sup>1</sup>O<sub>2</sub>) and a hydroxyl radical (HO·), which then become powerful oxidizing agents. Not only <sup>1</sup>O<sub>2</sub> and HO· but also H<sub>2</sub>O<sub>2</sub> can cross membranes and may oxidize a number of compounds. While H<sub>2</sub>O<sub>2</sub> itself is not that reactive, it can generate the highly reactive hydroxyl radicals (HO·) through the Fenton reaction (37). Thus, the scavenging of H<sub>2</sub>O<sub>2</sub> is an important antioxidant defense mechanism.



The decomposition of H<sub>2</sub>O<sub>2</sub> to water involves the transfer of electrons.



In the H<sub>2</sub>O<sub>2</sub> scavenging assay, both ascorbic acid as positive control and the extract dose-dependently scavenged H<sub>2</sub>O<sub>2</sub>. The extract showed more potent activity with an IC<sub>50</sub> value of 134.29 g/ml opposed to 179.21 g/ml for the ascorbic acid as positive control (Figure 5).

## CONCLUSION

Since reactive oxygen species are important contributors to various serious ailments, the observed antioxidant property of the extract of *E. agallocha* in the present study might be useful for the development of newer and more potent natural antioxidants.

## REFERENCES

- Ghani, A. Medicinal Plants of Bangladesh, 2<sup>nd</sup> ed., The Asiatic Society of Bangladesh. pp. 228-229; 2003.
- GRIN Taxonomy Database, USDA, ARS, National Genetic Resources Program. Germplasm Resources Information Network (GRIN) [Online Database]. National Germplasm Resources Laboratory, Beltsville, Maryland. Available online at: <http://www.arsgrin.gov/cgi-bin/npgs/html/taxon.pl?410154>; 2008.
- Peter, K. L. N., Sivasothi, N. A Guide to the Mangroves of Singapore I: The Ecosystem and Plant Diversity, Singapore Science Centre. pp.111-112; 1999.
- Ji-Dong, W., Wen, Z., Zhen-Yu, L., Wen-Sheng, X., Yue-Wei, G., Krohn, K. Elucidation of excogallochaols A-D, four unusual diterpenoids from the Chinese mangrove *Excoecaria agallocha*. *Phytochemistry*, 2007; 68: 2426-2431.
- Li, X., Lei, J., Zheng, Y. N., Isabel, S., Lin, W. H. New ent-isopimarane diterpene from mangrove *Excoecaria agallocha* L. *Chemical Research in Chinese Universities*, 2007; 23: 541-543.
- Wang, J. D., Li, Z. Y., Xiang, W. S., Guo, Y. W. Further new secoatisane diterpenoids from the Chinese mangrove *Excoecaria agallocha* L., *Helvetica Chimica Acta*, 2006; 89: 1367-1372.
- Kang, J., Chen, R. Y., Yu, D. O. A new isopimarane-type diterpene and a new natural atisane-type diterpene from *Excoecaria agallocha*. *Journal of Asian Natural Products Research*, 2005; 7: 729-734.
- Wang, J. D., Li, Z. Y., Guo, Y. W. Secoatisane- and isopimarane-type diterpenoids from the Chinese mangrove *Excoecaria agallocha* L. *Helvetica Chimica Acta*, 2005; 88: 979-985.
- Wang, J. D., Guo, Y. W. Agallochaols A and B, two new diterpenes from the Chinese mangrove *Excoecaria agallocha* L., *Helvetica Chimica Acta*, 2005; 87: 2829-2833.
- ISI Database. URL: <http://wok.mimas.ac.uk/>; 2008.
- Zou, J. H., Dai, J., Chen, X., Yuan, J. Q. Pentacyclic triterpenoids from leaves of *Excoecaria agallocha*, *Chemical & Pharmaceutical Bulletin*, 2006; 54: 920-921.

12. Konishi, T., Yamazoe, K., Kanzato, M., Konoshima, T., Fujiwara, Y. Three diterpenoids (Excoecarins V1-V3) and a flavanone glycoside from the fresh stem of *Excoecaria agallocha*, Chemical & Pharmaceutical Bulletin, 2003; 51: 1142-1146.
13. Ericson, K. L., Beutler, J. A., Cardellina, J. H., McMahon, J. B., Newman, J. D., Boyd, M. R. A novel phorbol ester from *Excoecaria agallocha*, Journal of Natural Products, 1995; 58:769-772.
14. Ali, H. A., Chowdhury, A. K. A., Rahman, A. K. M., Borkowski, T., Nahar, L., Sarker, S. D. Pachypodol, a Flavonol from the Leaves of *Calycopteris floribunda*, Inhibits the Growth of CaCo 2 Colon Cancer Cell Line *in vitro*, Phytotherapy Research (in press), 2008.
15. Datta. B. K., Nahar. L., Rahman. M. M., Gray. A. I., Auzi. A. A., Sarker. S. D. Polygosomic acid, a new cadinane sesquiterpene, from *Polygonum viscosum* inhibits the growth of drug-resistant *Escherichia coli* and *Staphylococcus aureus* (MRSA) *in vitro*. Journal of Natural Medicine, 2007; 61: 391-396.
16. Saha, A., Masud, M. A., Bachar, S. C., Kundu, J. K., Nahar, L., Datta, B. K., Sarker, S. D. Analgesic and anti-inflammatory activities of the extracts of *Phyllanthus reticulatus* in mice model. Pharmaceutical Biology, 2007; 45: 355-359.
17. Uddin, S. J., Shilpi, J. A., Rouf, R., Ferdous, M. M., Nahar, L., Sarker, S. D. Neuropharmacological properties of *Xylocarpus molucensis*. Fitoterapia, 2007; 78: 107-111.
18. Uddin, S. J., Shilpi, J. A., Byres, M., Middleton, M., Shoeb, M., Nahar, L., Sarker, S. D. Swarnalin and *cis*-swarnalin, two new tetrahydrofuran derivatives with free radical scavenging activity, from the aerial parts of *Cuscuta reflexa*. Natural Products Research, 2007; 21: 663-668.
19. Uddin, S. J., Nahar, L., Shilpi, J. A., Shoeb, M., Borkowski, T., Gibbons, S., Middleton, M., Byres, M., Sarker, S. D. Gedunin, a limonoid from *Xylocarpus granatum* inhibits the growth of CaCo2 colon cancer cell line *in vitro*. Phytotherapy Research, 2007; 21: 757-761.
20. Alam, M. A., Ghani, A., Subhan, M., Rahman, M. M., Haque, M. S., Majumder, M. M., Majumder, M. E. H., Akter, R. A., Nahar, L., Sarker S. D. (2008) Antioxidant and membrane stabilizing properties of the flowering tops of *Anthosephalus cadamba*, Natural Product Communications 3: 65-70.
21. Uddin, S.J., Shilpi, J. A., Rahman, M. T., Ferdous, M.M., Rouf, R., Sarker, S. D. Assessment of neuropharmacological effects of *Pandanus foetidus* (Pandaceae) in mice. Die Pharmazie, 2006; 61: 262-264.
22. Rouf, R., Uddin, S. J., Shilpi, J. A., Rahman, M. T., Ferdous, M. M., Sarker, S. D. Anti-diarrhoeal effects of *Diospyros peregrina* in the castor oil-induced diarrhoea model in mice. ARS Pharmaceutica, 2006; 47: 81-89.
23. Uddin, S. J., Shilpi, J. A., Rouf, R., Ferdous, M. M., Nahar, L., Sarker, S. D. Antinociceptive activity of some Bangladeshi medicinal plant extracts. Oriental Pharmacy and Experimental Medicine, 2006; 6: 96-101.
24. Uddin, S. J., Shilpi, J. A., Alam, S. M. S., Alamgir, M., Rahman, M. T., Sarker, S. D. Antidiarrhoeal activity of the methanol extract of the barks of *Xylocarpus molucensis* in castor oil and magnesium sulphate-induced diarrhoea models in mice. J. Ethnopharmacology, 2005; 101: 139-143.
25. Rahman, M. M., Sarker, S. D., Byres, M., Gray, A. I. New salicylic acid and isoflavone derivatives from *Flemingia paniculata*. J. Nat. Prod., 2004; 67: 402-406.
26. Datta, B. K., Datta, S. K., Chowdhury, M. M., Khan, T. H., Kundu, J. K., Rashid, M. A., Nahar, L., Sarker, S. D. Analgesic, anti-inflammatory and CNS depressant activities of sesquiterpenes and a flavonoid glycoside from *Polygonum viscosum*. Die Pharmazie, 2004; 59: 222-225.
27. Haque, M. E., Haque, M., Rahman, M. M., Rahman, M. M., Khondkar, P., Wahed, M. I. I., Mossadik, M. A., Gray, A. I., Sarker, S. D. *E*-Octadec-8-en-5-ynoic acid from the roots of *Capparis zeylanica*. Fitoterapia, 2004; 75: 130-133.
28. Uddin, S. J., Shilpi, J. A., Delazar, A., Nahar, L., Sarker, S. D. Free radical scavenging activity of some Bangladeshi plant extracts. Oriental Pharmacy and Experimental Medicine, 2004; 4: 185-193.
29. Sadhu, S. K., Okuyama, E., Fujimoto, H., Ishibashi, M. Separation of *Leucas aspera*, a medicinal plant of Bangladesh, guided by prostaglandin inhibitory and antioxidant activities. Chem. Pharm. Bull., 2003; 51: 595-598.
30. Garrat, D. C. The Quantitative analysis of Drugs. Chapman and Hall Ltd, Japan, Vol 3, pp 456-458, 1964.
31. Okhawa, H., Ohishi, W., Yagi, K. Assay formulation lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem, 1979; 95:351-353.
32. Tripathi, Y. B., Sharma, M. Comparison of the antioxidant action of the alcoholic extract of *Rubia cordifolia* with Rubiadin. Indian Biochem Biophys, 1998; 35: 313-320.

33. Ruch, R. J., Cheng, S. J., Klaunig, J. E. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis*, 1989; 10: 1003-1008.
34. Oktay, M., Gülçin, I., Küfrevioğlu, I. Determination of *in vitro* antioxidant activity of fennel (*Foeniculum vulgare*) seed extracts. *Lebensm.-Wiss.U-Technology*, 2003; 36: 263- 271.
35. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. *Japanese Journal of Nutrition*, 1986; 44: 307-315.
36. Kumarasamy, Y., Byres, M., Cox, P. J., Jaspars, M., Nahar, L., Sarker, S. D. Screening seeds of some Scottish plants for free-radical scavenging activity, *Phytotherapy Research*, 2007; 21: 615-621.

Archive of SID