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In Vitro Nitric Oxide Scavenging Activity Of Methanol Extracts Of Three Bangladeshi Medicinal Plants

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The methanol extracts of three medicinal plants named *Phyllunthus freternus, Triumfetta rhomboidae* and *Casuarina littorea* were examined for their possible regulatory effect on nitric oxide (NO) levels using sodium nitroprusside as a NO donor in vitro. Most of the extracts tested demonstrated direct scavenging of NO and exihibited significant activity and the potency of scavenging activity was in the following order: *Phyllunthus freternus* > Leaves of *Triumfetta rhomboidae* > *Casuarina littorea* > barks of *Triumfetta rhomboidae* > roots of *Triumfetta rhomboidae*. All the evaluated extracts exhibited a dose dependent NO scavenging activity. The methanolic extracts of *Phyllunthus freternus* showed the greatest NO scavenging effect of 60.80% at 200 µg/ml with IC_{50} values 48.27 µg/ml as compared to the positive control ascorbic acid where 96.27% scavenging was observed at similar concentration with IC_{50} value of 5.47 µg/ml. The maximum NO scavenging of Leaves of *Triumfetta rhomboidae,* barks of *Triumfetta rhomboidae,* roots of *Triumfetta rhomboidae* and *Casuarina littorea* were 53.94%, 50.43%, 33.23% and 54.02% with IC_{50} values 97.81 µg/ml, 196.89 µg/ml, > 200 µg/ml and 168.17 µg/ml respectively. The present results suggest that these plants might be potent and novel therapeutic agents for scavenging of NO and the regulation of pathological conditions caused by excessive generation of NO and its oxidation product.

Keyword: Nitric Oxide Scavenging Activity, Antioxidant Activity, Active Nitrogen Species, Sodium Nitroprusside.

1. INTRODUCTION: Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Nitric Oxide (NO) is used in various types of disorders like AIDS, cancer, alzheimer's and arthritis by cytotoxic effects.(Sainani et al.,1997). DNA fragmentation, neuronal cell death and cell damage occur as the toxicity of overproduction of NO. (Dawson et al.,1992). Bioorganic macromolecules (DNA or proteins) not effected directly with the presence of NO. As in aerobic conditions NO is very unstable and producing intermediates (NO_2, N_2O_4, N_3O_4) reacts with oxygen. In this reaction the stable products nitrite and nitrate will also

produce (Marcocci et aI.,1994a,b) and peroxynitrite will produce by reacting with superoxide (Wink et aI., 1991). These progenitors products are genotoxic, the deamination of guanine, cytosine and adenine is mediated primarily by the N_2O_3. In addition to that the formation of nitrosoamines and deamination of the DNA bases, recent studies indicate that the NO may also act by affecting the enzymatic activities of several thiol rich DNA repair proteins like DNA alkyl transferase, formamopyrimidine-DNA glycosalase and the DNA ligase that play vital role to meet the genetic integrity (Wink et aI., 1991). At present it is increasing evidence to suggest that NO and its derivatives produced by the activated phagocytes may have a genotoxic effect and may contribute in the multistage carcinogenesis process (Wink et aI., 1991). By antioxidant defense systems the production of these reactive species in healthy organism is approximately balanced. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals (Osawa et al., 1990; Houghton et aI., 1995). *Phyllunthus freternus* is a herb that grows up to 60 cm. Entire plant is used for the treatment of jaundice and leucorrhoea; considered astringent, deobstruent, stomachic, diuretic, febrifugal and antiseptic; used in dyspepsia, colic, diarrhea and dysentery; also employed in dropsy, gonorrhoea diseases of urinogenital system (Mohammed yusuf et al.).The plant is effectively used in deadly diabetes disease. The phytochemical studies conducted for standardization of the extract showed the presence of tannins and flavonoids as major phytoconstituents (Munish Garg1 et al.). Total phenolic contents and two major flavonoids rutin and quercetin have been isolated from alcoholic extract. *Triumfetta rhomboidae* Small, branched shrub to 3 ft tall. Fruits flowers and leaves are used in medicine as demulcent and astringent. Bark and fresh leaves are used in diarrhea and decentery. Flowers rubbed with sugar and water are given in gonorrhea to stop burning caused by urine (NRCS. 2009.). The plant was investigating experimentally the possible antitumor effect and antioxidant role of methanol extract of *Triumfetta rhomboidea* (METR) leaves against Ehrlich ascites carcinoma (EAC) bearing Swiss albino mice (Sivakumar,1Sunil Mengani et al.). *Casuarina Littorea* is an evergreen tree to 46 m tall, usually with single trunk and open, irregular crown. Bark reddish brown to gray, rough, brittle, peeling. Leaves are used in colic. Bark is astringent and useful in diarrhea and dysentery. A lotion of it is reported to be efficacious in beriberi. Powdered seeds are applied as plasters in headaches (NRCS 2009). Fresh roots of *Phyllunthus freternus* (family Euphorbiaceae) are beneficial in jaundice. Leaves and roots with rice water used as poultice on swellings, ulcers etc. Latex is also used to sores and ulcers. The plant is said to be useful in diabetes (Ghani, 2003). The fruit of *Triumfetta rhomboidea* (family Tillaceae) is believed to promote parturition. Leaves and flowers are used against leprosy. Root is diuretic; used in dysentery; hot infusion is taken to facilitate childbirth. (Ghani, 2003).The Plant *Casuarina Littorea* (Family Casuarinaceae) is used as a lotion of it is reported to be efficacious in beriberi. Powdered seeds are applied as plasters in headaches (Ghani, 2003).

Literature review reveals that scanty or no NO scavenging activity studies have been reported on those medicinal plants. Here we presented the evaluation of in vitro nitric oxide scavenging activity of methanol extracts of *Phyllunthus freternus,* Leaves of *Triumfetta rhomboidae, Casuarina littorea,* barks of *Triumfetta rhomboidae,* roots of *Triumfetta rhomboidae* carried out at Department of Pharmacy, Jahangirnagar University Bangladesh.

2. MATERIALS AND METHODS

a. Chemicals

All the chemicals used in the experiment were analytical grade. Ascorbic acid was obtained from (Merck, Germany) chem. Naphthyl ethylene diamine dihydrochloride was obtained from Sigma Chemical Co, India. Sodium nitro prusside was obtained from Loba chemie, India.

Collection and Identification of *Plant material*
Whole plant *Phyllunthus freternus,* Leaves, Barks and Roots of *Triumfetta rhomboidae* and Barks of *Casuarina littorea* were collected from Jahangirnagar University Dhaka, Bangladesh in May, 2008, and identified by the experts of National Herbarium, Bangladesh). Voucher specimens for these collections have been retained in the National Herbarium, Bangladesh. and accession no. for the identified plants *Phyllunthus freternus, Triumfetta rhomboidae* and *Casuarina littorea* are 32763, 32571 and 34997 respectively.

b. Extraction
The collected and identified plant parts were cleaned by separating the unwanted plants or plants parts. The plant parts were shade dried in open air for three weeks. The prepared plant parts were ground in a course powder with the help of a suitable grinder. The powder has to store in an airtight container under cool and dry place until further analysis required. About 200 gm of powdered materials for each plant parts were taken for extraction using Soxhlet Apparatus and Maceration method. Methanol was used as extracting solvent. The Methanol extract thus obtained was evaporated under rotavapour until dried. The concentrates were designated as crude extract of Methanol.

c. Phytochemical screening
The freshly prepared methanolic extracts of the selected plants were qualitatively tested for the presence of chemical constituents. Phytochemical screening of the extract was performed using the following reagents and chemicals: Alkaloids with Dragendorff's and Mayer's reagent, flavonoids with the use of Mg and HCI; tannins with ferric chloride and potassium dichromate solutions, steroids with sulfuric acid and saponins with ability to produce suds. Gum was tested using Molish reagents and concentrated sulfuric acid. These were identified by characteristic color changes using standard procedures (Trease et aI., 1983). By phytochemical screening the chemical constituent of plants can be detected and the isolated compounds are used for various pharmacological effects. In the present study methanolic extracts of *Phyllanthus freternus*, *Triumfetta rhomboidae* and *Casuarina littorea* screened for the presence of alkaloids, flavonoids, gums, saponins, tannins, steroids which have definite medicinal importance. Alkaloids are very common in many medicinal plants that have significant physiologic and pharmacological properties. Steroids contain widely distributed natural compounds that are used for abnormality of reproductive tract in human. Tannins have astringent and antimicrobial properties. Saponin containing plants materials are used in many parts of the world as detergents (Trease et aI., 1983). Antimicrobial properties saponin containing plants materials are used in many parts of the world as detergents. The therapeutic and other pharmacological properties of medicinal plants is directly depends upon the presence of various chemical constituents. So investigation of chemical constituents of the plant should have some direct relationship with local medicinal uses. The results of various qualitative chemical tests for the detection of chemical constituents of whole plant of *Phyllanthus freternus,* leaves, barks and roots of *Triumfetta rhomboidae* and barks of *Casuarina littorea* using their methanolic and petroleum ether extracts are shown in the following table.

3. Assay of Nitric oxide scavenging activity
Nitric oxide scavenging activity can be estimated by the use of Griess IlIosvoy reaction (Garrat, 1964).The compound sodium nitroprusside is known to decompose in aqueous solution at physiological pH (7.2) producing NO□. Under aerobic conditions, NO□ reacts with oxygen to produce stable products (nitrate and nitrite). The quantities of which can be determined using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10mM) in phosphate buffered saline was mixed with different concentrations (5 - 200µg/ml) of methanol extract of each plant were dissolved in methanol and incubated at 30^0C for 2 hours.

The same reaction mixture without the extract but the equivalent amount of ethanol served as the control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3P0_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with Naphthylethylenediamine dihydrochloride was immediately read at 550nm. Inhibition of nitrite formation by the plant extracts and the standard antioxidant ascorbic acid were calculated relative to the control. Inhibition data (percentage inhibition) were linearized against the concentrations of each extract and standard antioxidant. IC_{50} which is an inhibitory concentration of each extract required to reduce 50% of the nitric oxide formation was determined.

4. Statistical analysis

All experiments were performed thrice and the results averaged Data were expressed as mean ± SD. Linear regression analysis was used to calculate IC_{50} for each plant extract.

Table 1: Results of Phytochemical Screening

	Methanol extracts				
Test for	*Phyllanthus freternus* (Whole plant)	*Triumfetta rhomboidae*			*Casuarina littorea* (Barks)
		Leaves	Barks	Roots	
Alkaloids	+	+	+	+	+/-
Saponins	+	+	+	+	+
Flavonoids	-	-	+	-	+
Steroids	+	+	+	-	+
Tannins	+	+	+	+	+
Gums	+	+	+	+	+

'+' Indicates Presence
'-' Indicates Absence
'+/-' Indicates Presence but slightly

5. RESULTS AND DISCUSSION

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons, etc. and is involved in the regulation of various physiological processes (H. Lata et al.). Excess concentration of NO is associated with several diseases (Ialenti et al.). NO is generated in biological tissues by specific nitric oxide synthesis (NOSs), which metabolizes arginine to citralline with the formation of NO via a five electron oxidative reaction (R. Ross.1993). These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation of solutions of sodium nitroprusside in PBS at 25˚c for 2 h resulted in linear time dependent nitrite production, which is reduced by the tested methanolic extracts of the plants. NO scavenging capacity is determined by the decrease in the absorbance at 550 nm, induced by antioxidants. In order to evaluate the antioxidant potency through NO scavenging by the test samples, the change of optical density of NO was monitored. Figure shows the comparative NO scavenging activity of the extract. The results of phytochemical screening are given in Table-1. The results of NO scavenging activity of the selected plant extracts are shown as percent of NO scavenging in Table 2. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as $N0_2$, N_20_4, N_30_4, $N0_3$–. and $N0_2$ are very reactive. These compounds are responsible for altering the structural and functional behavior of many cellular components. Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25^0 C for 2 h resulted in linear time-dependent nitrite production, which is reduced by the tested methanolic extracts of *Phyllanthus freternus,* leaves, barks and roots of *Triumfetta rhomboidae* and barks of *Casuarina littorea*. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. It is to be noted that *Phyllanthus freternus* a greater inhibition comparative to other plant extracts but less than ascorbic acid which has shown 96.27% inhibition of NO. The maximum NO scavenging

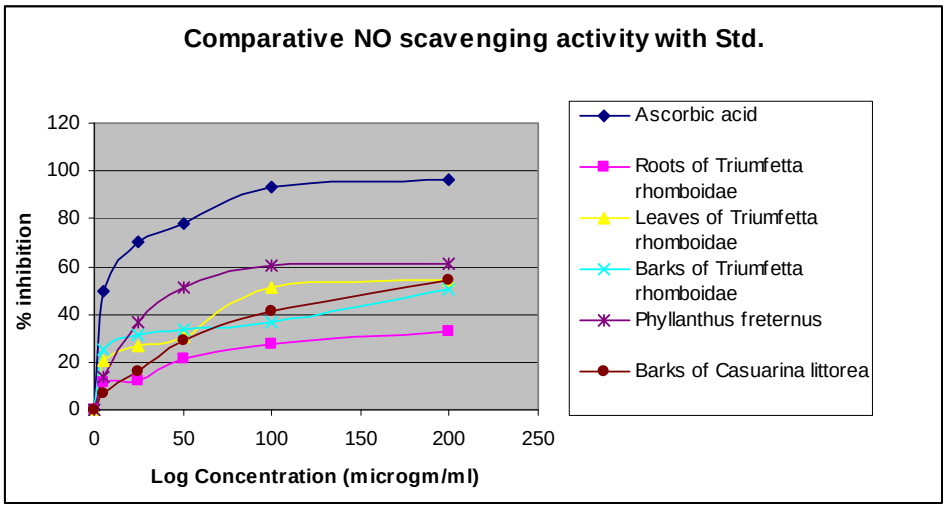
of Leaves of *Triumfetta rhomboidae*, barks of *Triumfetta rhomboidae*, roots of *Triumfetta rhomboidae* and *Casuarina littorea* were 53.94%, 50.43%, 33.23% and 54.02% with IC50 values 97.81 µg/ml, 196.89 µg/ml, > 200 µg/ml and 168.17 µg/ml respectively. preliminary phytochemical screening of the selected plant extracts, all the extract showed the presence of alkaloids, saponin, tannins and gums (Table-1). Flavonoids is present only in the barks of *Triumfetta rhomboidae* and *Casuarina littorea.* Steroids is present in all tested plant extracts except roots of *Triumfetta rhomboidae*. Phenolic compounds and flavonoids have been reported to be associated with antioxidative action in biological systems, acting as scavengers of singlet oxygen and free radicals (Rice-Evans et aI., 1997; Jorgensen et aI., 1999). The nitric oxide scavenging activity of flavonoids and phenolic compounds are known (Kim et aI., 1998; Kim et aI., 1999; Middleton et aI., 1996; Crozier et aI., 2000; Madson et aI., 2000; Jagethia et aI., 2004), we can speculate that these constituents might be responsible for the observed nitric oxide scavenging activity. The adoption of crude extracts of plants, such as infusions, for self-medication by the general public (Houghton, 1995), has arisen in the possibility that the impact of several diseases may be either. ameliorated or prevented by improving the dietary intake of natural nutrients with antioxidant properties, such as vitamin E, vitamin C, B-carotene and plant phenolics such as tannins and f1avonoids (Haslam, 1996). Our findings suggest that all of the four plants have the property to counteract the effect of NO formation due to the presence of tannins and f1avonoids and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in vivo.

6. CONCLUSION

We conclude from the above discussion that those methanolic extracts have antioxidant activity by scavenging the nitric oxide free radical. It is very much helpful for investigation of new drugs for various free radical generation diseases by identifying the compound isolation process.

Table 2: Scavenging of Nitric oxide by the methanolic extracts of selected plants.

Concentration	% of Scavenging of NO					
µg/ml	Whole plants of *Phyllunthus freternus*	Leaves of *Triumfetta rhomboidae*	Barks of *Triumfetta rhomboidae*	Roots of *Triumfetta rhomboidae*	Barks of *Casuarina littorea*	Ascorbic acid
5	13.731±0.006	20.294±0.006	25.538±0.011	11.316±0.010	6.564±0.009	49.528±0.043
25	36.816±0.018	26.896±0.023	31.083±0.009	12.448±0.010	16.409±0.014	69.974±0.014
50	51.000±0.011	30.215±0.008	33.308±0.009	21.275±0.007	29.008±0.013	77.857±0.007
100	60.166±0.015	50.924±0.013	36.929±0.016	27.575±0.008	41.418±0.010	93.361±0.019
200	60.807±0.005	53.942±0.005	50.434±0.018	33.233±0.008	54.017±0.006	96.266±0.012
IC_{50}	48.27	97.81	196.89	> 200	168.17	5.47



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