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In Vitro Nitric Oxide Scavenging Activity of Ethanol Leaf Extracts of Four Bangladeshi Medicinal Plants

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ABSTRACT

The ethanol leaf extracts of four medicinal plants named *Hibiscus mutabilis, Leucas aspera, Ixora coccinea* and *Polyalthia longifolia* 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 exhibited significant activity and the potency of scavenging activity was in the following order: *Leucas aspera > Ixora coccinea > Hibiscus mutabilis> Polyalthia longifolia.* All the evaluated extracts exhibited a dose-dependent NO scavenging activity. The ethanolic leaf extract of *Leucas aspera* showed the greatest NO scavenging effect of 80.50% at 320 µg/ml with IC_{50} value of 94.15 µg/ml as compared to the positive control ascorbic acid where 74.56 % scavenging was observed at similar concentration with IC_{50} value of 62.48 µg/mL. The maximum NO scavenging of *Ixora coccinea*, *Hibiscus mutabilis* and *Polyalthia longifolia* were 79.65 %, 78.60% and 70.67 % with IC_{50} values of 43.72 µg/ml, 147.64 µg/ml and 167.08 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.

Keywords: Nitric oxide scavenging activity, Antioxidant study, Active nitrogen species.

INTRODUCTION

Nitric oxide (NO) is an important chemical mediator generated by endothelial cells, macrophages, neurons and involved in the regulation of various physiological processes. Excess concentration of nitric oxide is implicated in the cytotoxic effects observed in various disorders like AIDS, cancer, alzheimer's, and arthritis (Sainani et al., 1997). Overproduction of NO can mediate toxic effects, *e.g.* DNA fragmentation, cell damage and neuronal cell death. (Dawson et al., 1992). NO does not interact with the bioorganic macromolecules such as the DNA or proteins directly. However, in the aerobic conditions, the NO molecule is very unstable and reacts with the oxygen to produce, intermediates such as NO_2, N_2O_4, N_3O_4 the stable products nitrate and nitrite (Marcocci et al., 1994a,b) and peroxynitrite when reacted with superoxide (Wink et al., 1991). These products progenitors are highly genotoxic, the deamination of guanine, cytosine and adenine is mediated primarily by the N_2O_3. In addition to 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 a critical role in the maintenance of the genetic integrity (Wink et al., 1991). There is now 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 al., 1991). The production of these reactive species in healthy organism is approximately balanced by antioxidant defense systems. Antioxidant agents of natural origin have attracted special interest because they can protect human body from free radicals (Osawa et al., 1990; Houghton et al., 1995). *Hibiscus mutabilis* (*Malvaceae*) is a large bushy shrub or small tree, about 8 ft in height. The Plant material is used in traditional medicines for their emollient in pectoral and pulmonary complaints. It is prescribed as a stimulant and leaves are applied to the swellings (Anonymous, 1959 and Kirtikar et al., 1990). A flavonone glycoside naringenin, eriodictyol, ilicyanin and chrysanthemin have been isolated from the plant (Ishikura, Nariyaki. 1973, Chauhan et al., 1979). *Leucas aspera* (Labiatae) (darkolos or dandokolos in Bangladesh) is a common aromatic herb and grows abundantly in Bangladesh and also in the wide area of South Asia. Traditionally, the decoction of the whole plant is taken orally for

analgesic-antipyretic, antirheumatic, antiinflammatory, and antibacterial treatment, *etc*., and its paste is applied topically to inflamed areas. (Ghani, 1998). Some reports have been published on the chemical constituents such as sterols, fatty acids, lactones, long-chain compounds, aliphatic ketols, and phenols (Chaudhury and Ghosh 1969, Pradhan et al., 1990, Misra et al., 1992, 1993 and1995). *Ixora coccinea L*.(Bengali name: Rangan) belongs to the family *Rubiacae*, is a common flowering shrub native to Asia including Bangladesh, Southern India, and Sri Lanka (Ghani, 2003). Leaves are given in diarrhea (Ghani, 2003). Flowers are used in the treatment of dysentery, leucorrhoea, dysmenorrhoea, hemoptysis and catarrhal bronchitis (Ghani, 2003). Roots possess stomachic and sedative properties. *I. coccinea* flowers showed chemoprotective effects on cyclophosphamide-induced toxicity by increasing the life span of treated mice (Latha and Panikkar, 1999). Ether and methanol extracts of *I. coccinea* dry leaves have antimicrobial activity (Annapurna *et al*., 2003). Flowers have cytotoxic and antitumor activity in mice (Latha and Panikkar, 1998). Aqueous leaf extract of *I. coccinea* leaves showed antinociceptive activity in mice (Ratnasooriya et al., 2005a, b). The extract of *I. coccinea* flowers contains triterpenoid, ursolic acid (Latha and Panikkar, 1999). *Polyalthia longifolia* is a tall, handsome, evergreen tree with a straight trunk and horizontal branches (Krishnamurthi A, 1969). It belongs to the family Annonaceae (Chen et al., 2000) which compromises 120 genera and more than 2000 species. It is locally known as Debdaru. The ethnopharmacological claims for Polyalthia longifolia include the use of its bark as a febrifuge. It depressed the heart, lowered blood pressure and stimulated respiration (Faizi et al*.,* 2003). The fungicidal effect of *P.* longifolia has also been reported by many workers (Shivpuri 1997, Nair and Chanda, 2006).

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 ethanol leaf extract of *Hibiscus mutabilis, Leucas aspera, Ixora coccinea and Polyalthia longifolia* carried out at Department of Pharmacy, Stamford University Bangladesh.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the experiment were analytical grade. Ascorbic acid was obtained from SD Fine chem. Ltd, Biosar, India. Naphthyl ethylene diamine dihydrochloride was obtained from Roch-light ltd, Suffolk, England. Sodium nitro prusside was obtained from Ranbaxy lab, Mohali, India.

Collection and Identification of Plant material

Leaves of *Hibiscus mutabilis, Leucas aspera, Ixora coccinea and Polyalthia longifolia* were collected from 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 *Hibiscus mutabilis, Leucas aspera, Ixora coccinea and Polyalthia longifolia* are 32540, 32536, 32549 and 32548, respectively.

Extraction

About 100 gm of powered material of each plant was taken in a clean, flat-bottomed glass container and soaked in 500 ml of 80% ethanol. The container with its contents was sealed and kept for a period of 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through Whatman filter paper (Bibby RE 200, Sterilin Ltd., UK). The filtrate (ethanolic extract) obtained for each plant was evaporated under ceiling fan and in a water- bath until dried. All of the extracts rendered concentrate of greenish black color.

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 HCl; 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 al., 1983).

Assay of Nitric oxide scavenging activity

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). The procedure is based on the method, where sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Greiss 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 (10-320 µg/mL) of ethanol extract of each plant were dissolved in methanol and incubated at 25^{O} C for 150 min. 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_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamme was read at 546 nm (Sreejayan & Rao, 1997). 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.

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: Phytochemical screening of the selected plants.

Phytochemicals	*Hibiscus mutabilis*	*Leucas aspera*	*Ixora coccinea*	*Polyalthia longifolia*
Alkaloid	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Gums	+	+	+	+
Saponins	+	+	+	+
Steroid	+	-	+	-

(+) = Positive, (-)=Negative

RESULTS AND DISCUSSION

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 NO_2, N_2O_4, N_3O_4, $NO_{3-,}$ and NO_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^{O} C for 2 h resulted in linear time-dependent nitrite production, which is reduced by the tested ethanolic extracts of *Hibiscus mutabilis, Leucas aspera, Ixora coccinea and Polyalthia longifolia*. 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 *Hibiscus mutabilis*, *Leucas aspera and Ixora coccinea* have caused a greater inhibition than ascorbic acid which has shown 74.56% inhibition of NO. The IC_{50} value of *Ixora coccinea* is 43.72 µg/ml which is lesser than that of ascorbic acid (Table- 2). In preliminary phytochemical screening of the selected plant extracts, all the extract showed the presence of flavonoids and tannins (Table-1)**.** 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 al., 1997; Jorgensen et al., 1999). The nitric oxide scavenging activity of flavonoids and phenolic compounds are known (Kim et al., 1998; Kim et al., 1999; Middleton et al., 1996; Crozier et al., 2000; Madson et al., 2000; Jagethia et al., 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 flavonoids (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 flavonoids and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in vivo.

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

Concentration µg/ml	% of scavenging of NO				
	Hibiscus mutabilis	***Leucas aspera***	***Ixora coccinea***	***Polyalthia longifolia***	***Ascorbic acid***
10	24.12 ±0.002	30.56 ±0.004	40.56 ±0.004	15.46 ± 0.003	42.43 ±0.002
20	29.10 ±0.001	38.65 ±0.001	45.69 ± 0.003	24.67 ± 0.002	43.21 ±0.001
40	35.16 ±0.003	47.50 ± 0.002	52.78 ±0.005	30.56 ± 0.003	48.79 ±0.003
80	40.45 ±0.001	54.30 ± 0.002	58.99 ±0.007	40.78 ± 0.001	55.56 ±0.001
160	50.42 ±0.002	57.60 ± 0.001	64.23 ±0.004	55.89 ± 0.002	61.34 ±0.002
320	78.6 ± 0.002	80.50 ±0.001	79.65 ±0.006	70.67 ± 0.002	74.56 ± 0.002
IC50	147.64 (0.9877*)	94.15 (0.9145*)	43.72 (0.9268*)	167.08 (0.9175*)	62.48(0.9587*)

Here n=3 and values are presented as mean ± standard deviation. *= regression coefficient.

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