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Antioxidant and cytotoxic potential of methanol extract of *Tabernaemontana divaricata* leaves

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Abstract

The methanolic extract obtained from the leaves of *Tabernaemontana divericata* (Family: Apocynaceae) was evaluated for *in vitro* antioxidant potential by determination of total antioxidant capacity, assay of nitric oxide scavenging activity and reducing power test. The extract was also screened for its cytotoxic effect using brine shrimp lethality bioassay. The results revealed potent antioxidant property in all antioxidant assays compared to the reference antioxidant, ascorbic acid in a dose dependent manner. Further, the methanolic extract of *Tabernaemontana divericata* showed significant cytotoxic effect (LC_{50}: 3.12µg/ml) compared with positive control, Vincristine Sulphate (LC_{50}: 0.331µg/ml).

***Key Words*:** *Tabernaemontana divaricata,* Apocynaceae, Antioxidant, Cytotoxicity.

INTRODUCTION

Human beings have been utilizing plants for basic preventive and curative health care since time immemorial. It has been reported that phytochemicals, non-nutritive chemicals present in fruits and herbs may protect human from a host of diseases for their biological activities (Argal and Pathak, 2006). Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity (Newman and Cragg, 2007). Evaluation of the local flora exploited in traditional medicine for various biological activities is a necessary first step in the isolation and characterization of the active principle and further leading to drug development (Rahman *et al.*, 2011). A large number of plants have been screened as a viable source of natural antioxidants including tocopherols, vitamin C, carotenoids and phenolic compounds which are responsible for maintenance of health, to help the human body reduce oxidative damage and protection from coronary heart diseases and cancer (Yanga *et al.*, 2002). In view of these *Tabernaemontana divaricata* was studied for its potential antioxidant and cytotoxic effects.

Tabernaemontana divaricata (Bengali name - Togor; Family - Apocynaceae) is a beautiful evergreen shrub, about 54cm high, with large shiny leaves, crepe jasmine flowers, may appear sporadically all year. *T. divaricata,* garden plant in tropical countries, is a rich source of alkaloids with various pharmacological properties. It has been used in the folk medicine for anti-infection, anti-inflammation, analgesic, anti-tumour, antioxidative effect and the effect in neuronal activity (Ghani, 2003; Pratchayasakul *et al.*, 2008). In this study, the antioxidant and cytotoxic properties of the methanolic extract are being reported to validate the traditional use of the crude drug through *in vitro* evaluation.

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EXPERIMENTAL METHODS

Plant materials

Tabernaemontana divaricata was collected from Dhaka in March 2008 and was identified by Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen (DCAB accession no.: 32069) has been deposited. The leaves of the plant were first sun dried and then ground into coarse powder.

Extraction of plant materials

About 100gm of powered material was taken in a clean, flat-bottomed glass container and soaked in 500ml of 90% methanol. 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. The filtrate (methanol extract) obtained was evaporated under ceiling fan and in a water-bath until dried. It rendered a gummy concentrate of blackish color. The gummy concentrate was designated as crude extract of methanol. To get preliminary idea about the active constituents present in the plant leaves extracts different chemical tests were performed and showed the presence of alkaloid, flavonoids and tannins (Evans, 1989).

Antioxidant property

Qualitative assay

A suitably diluted stock solutions (sample solutions) were spotted on pre-coated Silica gel TLC (Thin layer chromatography) plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extract and to choose the solvent system in which stock solutions run well. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved bands was observed for 10 minutes and the color changes (yellow on purple background) were noted (Sadhu *et al.*, 2003).

Quantitative assay

Free radical scavenging activity of the methanol extract was evaluated by determination of total antioxidant capacity, assay of nitric oxide scavenging activity and reducing power test. In all methods ascorbic acid was used as standard.

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure of Prieto and colleagues (Prieto *et al.*, 1999). The assay is based on the reduction of Mo(VI)–Mo(V) by the extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695nm using a spectrophotometer (Hach, DR-4000U) against blank after cooling to room temperature. Methanol (0.3ml) in the place of extract was used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Assay of Nitric oxide scavenging activity

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 of methanolic extract of *T. divaricata* dissolved in methanol and incubated at room temperature for 150 min. The same reaction mixture without the methanol extract but the equivalent amount of methanol served as the control. After the incubation period, 0.5ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-naphthyl) ethylene-diamine-dihydrochloride was added. The absorbance of the chromophore formed was read at 546 nm (Sreejayan and Rao, 1997).

Reducing power test

The reducing power of the extract was determined according to the method of Oyaizu (Oyaizu, 1986). Different amounts of extracts (50-250mg) in 1ml of methanol were mixed with phosphate buffer (2.5ml, 0.2mol/l, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5ml, 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 (650 x g at room

Table 1: Total antioxidant capacity of methanolic extract of *Tabernaemontana divaricata*.

Materials	Concentration (µg/ml)	Equivalent to ascorbic acid
Methanol extract of *Tabernaemontana divaricata*	10	0.065±0.09
	25	0.102±013
	50	0.243±0.17
	125	0.636±0.10
	250	1.034±0.06
	500	1.991±0.12

temperature) for 10 min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Cytotoxicity study

Brine shrimp lethality bioassay (Mclaughlin, 1982; Persoone, 1988) technique was applied for the determination of cytotoxic property of methanolic extract of *T. divaricata*.

Preparation of positive control group

Vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20µg/ml from which serial dilutions were made using DMSO to get 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml, 0.625µg/ml, 0.3125µg/ml, 0.15625µg/ml, 0.078125µg/ml and 0.0390µg/ml. Then positive control solutions were added to the pre-marked vials containing ten living brine shrimp nauplii in 5ml simulated sea water to get the positive control groups.

Preparation of negative control group

100µl of DMSO was added to each of three pre-marked glass vials containing 5ml of simulated sea water and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test was considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

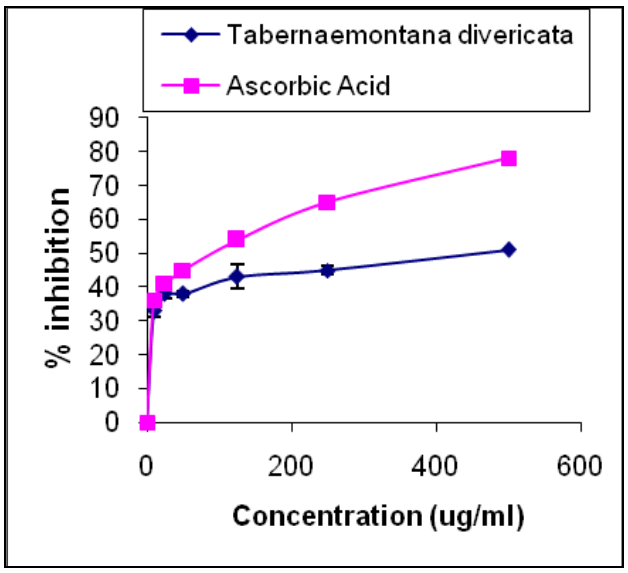

Figure 1: Nitric oxide scavenging activity of methanolic extract of *Tabernaemontana divaricata*.

RESULTS

Antioxidant property

Qualitative assay

The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands.

Quantitative assay

Total antioxidant capacity

Total antioxidant capacity exerted by the extract is concentration dependent. It was observed that the extract was likely to have the capacity of reduction of Mo (VI) to Mo (V) by the antioxidant principle and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695nm. The antioxidant activity is expressed as the number of equivalents of ascorbic acid (**Table 1**).

Nitric oxide scavenging activity

From **Figure 1**, it is observed that the extract is likely to have concentration dependent nitric oxide scavenging activity. The leaves may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions

Table 2: LC_{50} data of the test sample of *Tabernaemontana divaricata* in brine shrimp lethality bioassay.

Sample	LC_{50} (µg/ml)
Vincristine Sulphate	0.331±0.13
Methanolic extract of *Tabernaemontana Divaricata*	3.12±0.11

initiated by excess generation of NO that are detrimental to the human health. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions (Moncada *et al.*, 1991).

Reducing power

Reduction ability of the extract has been investigated from the Fe^{+++} to Fe^{++} transformation using the method followed by Oyaizu (Oyaizu, 1986). Earlier authors (Tanaka et al. 1988; Duh, 1998) have observed a direct correlation between antioxidant activity and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh, 1998) which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. **Figure 2,** demonstrates the reduction ability of *T. divaricata.*

Cytotoxic property

Following the procedure of Mayer and colleagues (Meyer *et al.*, 1982) the lethality of all the crude extracts to brine shrimp were determined on *A. salina*. The LC_{50} obtained from the best-fit line slope was found to be LC_{50} 3.12±0.11µg/ml, Vincristine sulphate as positive control **(Table 2)**. This clearly indicates the presence of potent bioactive principles in this crude extract of which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents (Meyer *et al.*, 1982).

DISCUSSION

There is a growing interest in the investigation of natural antioxidant compounds from plants, since they contain secondary metabolites with structural diversity (Joseph and Priya, 2011). In comparison with the synthetic compounds that are currently available, a good natural antioxidant will have a higher potency and lower toxicity. A number of known antioxidants as well as yet unknown antioxidants are supposedly present in plants (Haripyaree *et al.*, 2010). These antioxidants are going to do a lot of good to human health by sequestering the hazardous free radicals which are generated due to physiological errors in the cells. Hence, there is currently a strong interest in plants as pharmaceuticals, especially from edible plant parts, because these compounds play an important role, preventing free radical induced diseases such as cancer and atherosclerosis.

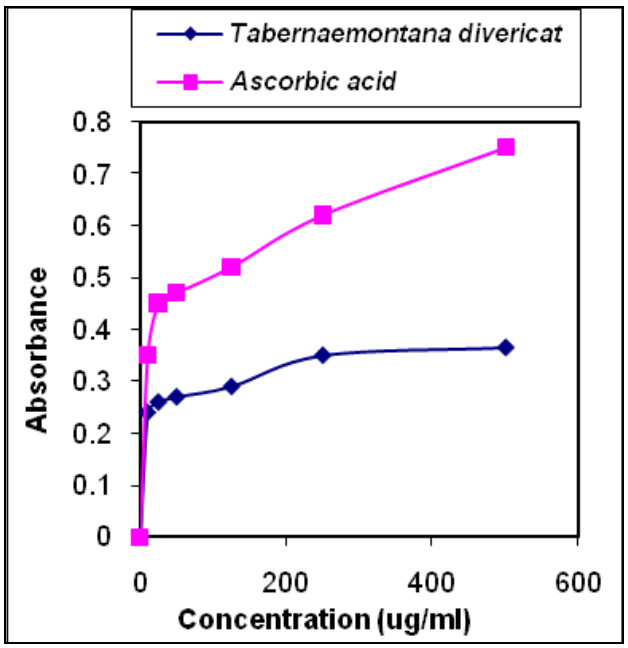

Figure 2: The reducing power of methanolic extract of *Tabernaemontana divaricata.*

There is a close association between cytotoxic properties of natural compounds with their anticancer effect (Mans *et al.*, 2005). Mounting evidence supports that the natural bioactive compound are good candidate for anticancer drugs.

CONCLUSION

Our present study demonstrates the antioxidant and cytotoxic potentialities of *T. divaricata*, which would improve our understanding to the biological role of the plant and future avenue to develop new anticancer therapeutics.

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