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Original article

ANTIOXIDANT POTENTIAL OF THE ETHANOL EXTRACT OF THE LEAVES OF *VITEX NEGUNDO* L.

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Abstract

The antioxidant potential of the ethanol extract of Vitex negundo L. was evaluated by using 1, 1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide, total antioxidant capacity and erythrocyte (RBC) membrane stabilization assays. The extract showed significant activities in all antioxidant assays compared to the reference antioxidant ascorbic acid in a dose dependent manner. In DPPH and hydrogen peroxide scavenging assays the IC_{50} values of the extract were found to be 178.43 µg/mL and 158.93 µg/mL, respectively. Total antioxidant activity was also increased in a dose dependent manner. The ethanol extract of V. negundo also significantly protect the lyses of red blood corpuscles by stabilizing the membrane in hypotonic medium. These results suggest that V. negundo may act as a chemopreventative agent, providing antioxidant properties and offering effective protection from free radicals.

Key Words: *Vitex negundo, Verbenaceae, Antioxidant, Lipid peroxidation, DPPH, Total antioxidant capacity, Reactive oxygen species.*

Vitex negundo L. Yapraklarının Etanollu Ekstresinin Antioksidan Aktivitesi

Vitex negundo yapraklarının etanollu ekstresinin antioksidan aktivitesi DPPH, Hidrojen peroksit, kullanılarak tayin edilmiştir, ayrıca total antioksidan kapasitesi ve eritrosit membran stabilizasyonu tayin edilmiştir. Askorbik asidin referans olarak kullanıldığı yöntemlerin hepsinde ekstre doza bağlı olarak kuvvetli aktivite göstermiştir. DPPH ve hidrojen peroksit süpürme çalışmalarında ekstrenin IC_{50} değerleri sırasıyla 178.43 µg/mL ve 158.93µg/mL olarak bulunmuştur. Total antioksidan aktivite doza bağlı olarak artmaktadır. Ayrıca V.negundo hipotonik ortamdaki membranın stabilizasyonu ile kırmızı kan hücrelerinin erimesini önlemiştir. V.negundo'nun antioksidan özellikleri ve serbest radikallerden koruma sağlaması nedeni ile kimyasal koruyucu olarak önerilebilir.

Anahtar Kelimeler: *Vitex negundo, Verbenaceae, Antioksidan, Lipit peroksidasyonu, DPPH, Total antioksidan kapasite, Reaktif oksijen türleri*

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INTRODUCTION

In the past few years, there has been growing interest in the involvement of reactive oxygen species (ROS) in several pathological situations. ROS produced *in vivo* include superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). H_2O_2 and $O_2^{\bullet -}$ can interact in the presence of certain transition metal ions to yield a highly-reactive oxidizing species, the hydroxyl radical ($^{\bullet}OH$) (1). The oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases, such as cancer, liver injury and cardiovascular disease (2). Although the body possesses such defense mechanisms, as enzymes and antioxidant nutrients, which arrest the damaging properties of ROS (3, 4), continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them, and cause irreversible oxidative damage (5). Therefore, antioxidants with free radical scavenging activities may have great relevance in the prevention and therapeutics of diseases in which oxidants or free radicals are implicated (6). In this respect, polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity (7-10). Currently, the possible toxicity of synthetic antioxidants has been criticized. It is generally assumed that frequent consumption of plant-derived phytochemicals from vegetables, fruit, tea, and herbs may contribute to shift the balance toward an adequate antioxidant status (11). Thus interest in natural antioxidant, especially of plant origin, has greatly increased in recent years (12).

Vitex negundo Linn. (Verbenaceae), a large aromatic shrub with typical five foliolate leave pattern, is found throughout Bangladesh, the greater part of India and the Himalayas (13, 14). The leaves contain a pale greenish yellow essential oil. An alkaloid, nishindine, and a glucoside, hentriacontane, sterols, β-sitosterol and its acetate derivative, stigmasterol, ascorbic acid, *p*-hydroxybenzoic acid, carotene and amino acids have also been isolated from the plant (14). Leaves and twigs contain flavonoid glycosides of wogonin, aurosin, vitexin, myricetin, and also luteolin, leucodelphinidin, leucocyanidin rhamnoside, vanillic acid and *p*-hydroxybenzoic acid (15). Leaves of *V. negundo* have been investigated for its anti-inflammatory activity in past, including its mechanism of action, first noticed non-steroidal anti-inflammatory drugs (NSAID) like activity of *V. negundo* (16). Similarly, fresh leaves of *V. negundo* have been suggested to possess anti-inflammatory and pain suppressing activities possibly mediated *via* prostaglandin (PG) synthesis inhibition, antihistaminic, membrane stabilizing and antioxidant activities (17). Leaves of *V. negundo* are known to possess various antioxidant chemical constituents like flavonoids, vitamin C and carotene (18) which may have a modulatory effect on oxidative stress or endogenous antioxidants. *In vitro* antiradical potential of the freeze-dried root extract of *V. negundo* was investigated by Munasinghe et al. (2001) determining their abilities to scavenge DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical and to inhibit hydroxyl radical-mediated damage to deoxyribose (19). This is the first report which has indicated that *V. negundo* can produce reduction of oxidative stress.

As a part of our ongoing investigation on natural antioxidants from local medicinal plants of Bangladesh (20, 21), in this paper, we reported a study of the antioxidant activity of the leaves of *Vitex negundo.* The evaluation of antioxidant power was performed *in vitro* by DPPH and H_2O_2 scavenging, total antioxidant capacity and erythrocyte membrane stabilizing assays.

MATERIALS AND METHODS

Chemicals

DPPH (1, 1-diphenyl, 2-picrylhydrazyl), TCA (trichloroacetic acid) and ferric chloride were obtained from Sigma Chemical Co. USA. Ascorbic acid was obtained from SD Fine Chem. Ltd., Biosar, India. Hydrogen peroxide (H_2O_2) was obtained from Qualigens Fine chemicals, Mumbai, India.

Plant material

Leaves of *V. negundo* were collected from Siddeswari campus, Stamford University, Bangladesh in June 2007, and identified by Professor Dr. Abdul Ghani (Stamford University, Dhaka, Bangladesh); a voucher specimen (SU-MAA-2007-1) for this collection has been retained in the Pharmacognosy Laboratory, Stamford University, Dhaka, Bangladesh.

Extraction

The shade-dried leaves were coarsely powdered and extracted with mixture of ethanol: water (7:3, v/v) by a Soxhlet apparatus at a temperature of 40-45° C. The solvent was completely removed by rotary evaporator and obtained greenish gummy exudates. This crude extract was used for further investigation for potential antioxidant properties.

Antioxidant Activity Test

DPPH radical scavenging activity

Qualitative analysis

The ethanol extract was applied on a TLC plate as a spot (100µg/ml) for chromatographic separation of the extract using the mobile phase ethanol: chloroform (95:5, v/v). It was allowed to develop the chromatogram for 30 minutes. After completion of the chromatogram the whole plate was sprayed with DPPH (0.15 % w/v) solution using an atomizer. The color changes (yellowish color development on pinkish background on the TLC plate) were noted as an indicator of the presence of antioxidant substances.

Quantitative analysis

The free radical scavenging capacity of the extract was determined using DPPH. A methanolic DPPH solution (0.15%) was mixed with serial dilutions (1 to 500 µg/ml) of *V. negundo* extract and after 10 minutes, the absorbance was read at 515 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer). Ascorbic acid was used as reference. The inhibition curve was plotted and IC_{50} values obtained by Probit analysis (22).

Scavenging of hydrogen peroxide

A modified method based on that of Ruch et al. (1989) was used to determine the ability of the extract to scavenge hydrogen peroxide (23). Hydrogen peroxide (43 mM) was prepared in phosphate buffered saline (pH 7.4). Reference (ascorbic acid) and extract solutions were prepared at concentrations of 50 to 250 mM. Aliquots of reference or extract solutions (3.4 ml) were added to 0.6 ml of hydrogen peroxide 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 as follows: (24)

% H_2O_2 Scavenging = (Absorbance of Control - Absorbance of Sample)/ Absorbance of Control x 100

Determination of total antioxidant capacity

The antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure describe by Prieto et al. (1999) (25). 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. A 0.3 ml extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95^0C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (HACH 4000 DU UV – visible spectrophotometer) against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Membrane stabilizing activity

Preparation of erythrocyte suspension: Whole blood was obtained using heparinized syringes (contained anticoagulant EDTA) from rats through cardiac puncture. The blood was washed three times with isotonic buffered solution (154 mM NaCl) in 10 mM sodium phosphate buffer (pH 7.4). The blood was centrifuged each time for 10 minutes at 3000 rpm.

Hypotonic solution-induced rat erythrocyte haemolysis:

Membrane stabilizing activity of the extract was assessed using hypotonic solution-induced rat erythrocyte haemolysis (26). The test sample consisted of stock erythrocyte (RBC) suspension (0.50 ml) mixed with 5 ml of hypotonic solution (50 mM NaCl) in 10 mM sodium phosphate buffered saline (pH 7.4) containing the extract (0.25- 2.0 mg/ml) or indomethacin (0.1 mg/ml). The control sample consisted of 0.5 ml of RBC mixed with Hypotonic -buffered saline solution alone. The mixtures were incubated for 10 min at room temperature and centrifuged for 10 min at 3000 g and the absorbance of the supernatant was measured at 540 nm. The percentage inhibition of haemolysis or membrane stabilization was calculated according to modified method described by Shinde et al. (1999) (26).

% Inhibition of haemolysis = 100 x $\{OD_1\text{-}OD_2/OD_1\}$

Where:

OD1 = Optical density of hypotonic-buffered saline solution alone

OD2 = Optical density of test sample in hypotonic solution

RESULTS AND DISCUSSION

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 (27, 28). DPPH radical scavenging activity was qualitatively assayed by using a chromatogram of *V. negundo* ethanol extract on a TLC plate and the generation of yellow color on pinkish background on TLC plate confirms the presence of antioxidant substance in the sample extract. The quantitative DPPH radical scavenging activity of *V. negundo* is given in Table 1. This activity was found to amplify with increasing concentration of the sample extract. DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 515-517 nm and also for a 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 IC_{50} value of the extract was

Table 1: Scavenging of free radical by crude ethanol extract of *V. negundo* in DPPH and H_2O_2 scavenging method.

Sample	IC_{50} (µg/mL)	
	DPPH scavenging assay	H_2O_2 scavenging assay
Ethanol extract of *V. negundo*	178.43	158.93
Ascorbic acid	34.11	135.29

178.43 µg/mL, as opposed to that of ascorbic acid (IC_{50} 34.11 µg/mL), which is a well known antioxidant.

Hydrogen peroxide itself is not very reactive, but sometimes is toxic to cells because it may give rise to hydroxyl radical in the cells (29). Dietary polyphenols have also been shown to protect mammalian and bacterial cells from cytotoxicity induced by hydrogen peroxide, especially compounds with the *o*-dihydroxy phenolic structure such as quercetin, catechin and, gallic acid and caffeic acid ester (30). Therefore, the flavonoid compounds of *V. negundo* extract along with carotene and ascorbic acid present therein may probably be involved in removing the H_2O_2. Hydrogen peroxide (H_2O_2) generates singlet oxygen ($^1O_2$) and a hydroxyl radical ($^{\cdot}OH$), which then become very powerful oxidizing agents. Not only $^1O_2$ and $HO\cdot$ but also H_2O_2 can cross membranes and may oxidize a number of compounds. While H_2O_2 itself is not that reactive (31), it can generate the highly reactive hydroxyl radical through the Fenton reaction (29). Thus, the scavenging of H_2O_2 is an important antioxidant defense mechanism (31).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH + OH^-$$

The decomposition of H_2O_2 to H_2O involves the transfer of electrons.

$$H_2O_2 + 2H^+ + 2e \longrightarrow 2H_2O$$

The scavenging of H_2O_2 by ascorbic acid and the extract of *V. negundo* after incubation for 10 min increased with increasing concentration. The extract exhibited higher H_2O_2 scavenging activity than ascorbic acid at similar concentrations. The IC_{50} values of the extract and ascorbic acid were 158.93 µg/mL and 135.29 µg/mL, respectively (Table 1). Total antioxidant capacity of the *V. negundo* extract, expressed as the number of equivalents of ascorbic acid, is shown in Figure 1. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. It is well known that the vitality of cells depends on the integrity of their membranes (32).

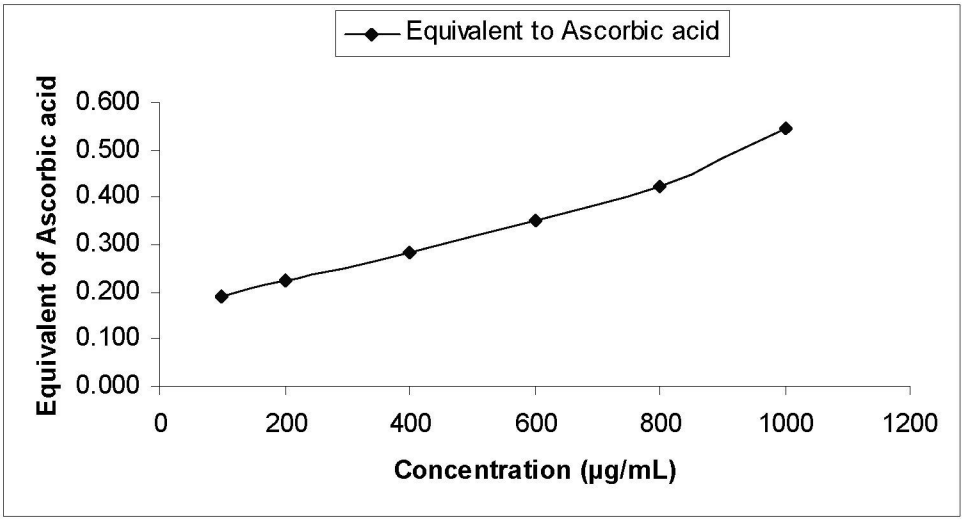

Figure 1: Total antioxidant capacity of the ethanol extract of *V. negundo.* Values are given for two consecutive experiment and expressed as mean ± SD

Exposure of red blood cell to injurious substances such as hypotonic medium and phenyl hydrazine results in lysis of its membrane accompanied by haemolysis and oxidation of haemoglobin. The haemolytic effect of hypotonic solution is related to excessive accumulation of fluid within the cell resulting in the rupturing of its membrane. Such injury to RBC membrane will further render the cell more susceptible to secondary damage through free radical-induced lipid peroxidation (32, 33). This notion is consistent with the observation that the breakdown of bio-membranes leads to the formation of free radicals, which in turn enhance cellular damage (29, 34). The progression of bone destruction seen in rheumatoid patients for example, has been shown to be due to increased free radical activity (35, 36).

Table 2: Membrane stabilizing activity of the ethanol extract of *Vitex negundo*.

Concentration	Absorbance	% Protection
Hypotonic medium 50 mM	0.350± 0.010	–
V. negundo 0.25 Mg/mL	0.270±0.001	11.23±1.51
0.5 mg/mL	0.254±0.006	16.50±1.41
1.0 mg/mL	0.202±0.004	33.54±1.23
1.5 mg/mL	0.108±0.004	62.63±2.17
2.0 mg/mL	0.047±0.003	85.54±1.71
Indomethacin (0.1 mg/mL)	0.051±0.003	85.26±0.41

Values are average of duplicate experiments and represented as mean ± standard deviation.

The extract of *V. negundo* at concentration range of 0.25-2.0 mg/mL significantly protected the rat erythrocyte membrane against lysis induced by hypotonic solution (Table 2). In contrast, indomethacin (0.10 mg/ml) offered a significant protection of the rat RBC against the damaging

effect of hypotonic solution. At a concentration of 2.0 mg/mL, the extract produced 85.54% inhibition of RBC haemolysis as compared with 85.25% produced by indomethacin. A possible explanation for the membrane stabilizing effect could be an increase in the surface/volume ratio of the cell, which could be brought about by either expansion of the membrane or shrinkage of the cell and interaction of membrane protein (37). Moreover, it has also been reported that the deformability and cell volume of erythrocyte are closely related to the intracellular content of calcium, and hence it could be speculated that the cytoprotective effect of the extract on the erythrocyte membrane might be due to the ability of the extract to alter the influx of calcium into the erythrocyte (26). Since the membrane of RBCs is similar to that of lysosome, the effect of either drugs or extracts on human RBC membranes could be extrapolated to the stabilization of lysosomal membranes. Again, free radicals are known to be one of the major causes of Parkinson disease (31), Alzheimer type dementia (38) and bone destruction in rheumatoid patients (35). It is therefore expected that compounds with membrane-stabilizing properties, should offer significant protection to cell membrane against injurious substances (34, 39). *V. negundo* can produce reduction of oxidative stress mainly by reducing lipid peroxidation, whereas it has failed to modulate endogenous antioxidant enzyme (SOD) activity. However, this finding is contradictory to the findings (observed pro-oxidant role of *V. negundo* rather than anti-radical activity) of Munasinghe et al. (19). This discrepancy might be due to a different experimental set-up in the form of different methods used, different part of the plant used and different method of extraction used in their study (40). Finally, based on the displayed antioxidant potential of the ethanol extract of *V. negundo* it can generally be concluded that the antioxidant activity may be due to the presence of flavonoids, ascorbic acid and carotene (18) but, which of these components was most responsible for the effects observed is yet to be elucidated.

As the present study has demonstrated the effectiveness of the ethanol extract of *V. negundo* as an antioxidant, determination of the natural antioxidant compounds from this plant extract may help to develop either new drug candidates or at least the template for 'lead' generation for antioxidant therapy.

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