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INVITRO ANTIOXIDANT ACTIVITY OF *VITEX NEGUNDO* LINN BARK

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ABSTRACT

The present study shows the *Invitro* antioxidant activity of methanolic extracts of *Vitex negundo* Linn bark (VBE). The methanolic extracts of *Vitex negundo* Linn bark results shows the significant *Invitro* anti oxidant activity in a concentration dependent manner. Free radicals are fundamentals to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by body's normal usage of oxygen such as respiration and some cell mediated immune functions. The oxygen consumption inherent in cell growth leads to the generation of reactive oxygen species (ROS) (Gulcin *et al.,* 2007). Most harmful effects are produced by the reactive oxygen species (ROS) in our body, ROS are act as oxidants. Antioxidants are the substances which scavenges the oxidation process. Antioxidants are a type of complex compounds found in our diet that act as a protective shield for our body against certain disastrous diseases such as arterial and cardiac diseases, arthritis, cataracts and also premature ageing along with several chronic diseases.

Key words: Anti-oxidant, *Vitex negundo* Linn bark.

INTRODUCTION

Vitex negundo Linn is a large aromatic shrub with quadrangular, densely whitish tomentose branch lets, up to 4.5m in height, or sometimes a small slender tree, found thought the greater part of India. Bark thin, grey; leaves 3-5 foliate; leaflets lanceolate, entire or rarely leaflets smaller flower bluish purple small in penduncled cymes, forming large terminate, often compound, pyramidal panicles, drupes globose, black when ripe, 5-6nm, in diameter, invested at the base with enlarged calyx. The shrub is very common in many parts of the country and often occurs gregariously and it is usually not browsed by cattle. The shrub can be reproduced readily from cuttings and it produces the root-suckers it is useful for planting against soil-erosion. The rate of growth of the shrub is moderate; with seven rings per 2.5cm of radius giving a mean annul girth-increment of 2.3cm. It is acrid, bitter, heating, astringent, stomachic, cephalic, anthelmintic and useful in treatment of inflammations, eye diseases, spleen enlargement, bronchitis, asthma, biliousness, painful teething of children etc. It has germicidal properties. It is easily digestible and can cure morbid vata and kapha and used in arthritis, cephalgia, otalgia, inflammatory, glandular and rheumatic swellings, intestinal worms, fever, ulcers, skin diseases, nervous disorders and leprosy (Vishal R Tandon *et al.,*). But still no scientific and methodical investigation on bark of *vitex negundo* Linn reported in literature regarding its action on liver. Therefore, the present investigation has been designed to study *Invitro* anti oxidant activity of *Vitex negundo* Linn bark.

In human life oxygen is very essential, without oxygen we cannot survive. Our evolutionary ancestors developed defense mechanisms that can minimize the toxic effects of oxygen, without this protection causes the end of life. Natural defenses are imperfect; the damage of the cells caused by oxygen can be minimized by using antioxidants. A lot of research works are made in this past decades. In this study the metabolites produced by the oxygen species and with research works they have learned how to prevent the diseases caused by the reactive oxygen species. Now research works are doing for improving the antioxidant activity (Lillian, 1995).

Free radicals of different forms are constantly generated for specific metabolic requirement and quenched by an efficient antioxidant network in the body. When the generation of these species exceeds the levels of antioxidant mechanism, it leads to oxidative damage of tissues and biomolecules, eventually leading to disease conditions, especially degenerative diseases. Many plant extracts and phytochemicals have been shown to have antioxidant/freeradical scavenging properties and it has been established as one of the mechanisms of their action (Dubey *et al.,*2009).

MATERIALS AND METHODS

Plant material

The plant material consists of dried powdered bark of *Vitex negundo* Linn. Belonging to the family Verbenaceae.

Preparation of plant extract

Fresh bark of *Vitex negundo* Linn was collected from koorapalayam, Erode, TamilNadu, India. The bark was dried for one month and latter powered. This powder was then macerated with methanol for 72 h with occasional shaking. It was then filtered and the solvent was evaporated under heating mandle. The yield of methanolic extract of bark of *Vitex negundo* Linn, (VN) was 34% (w/w).

Drugs and chemicals

All the drugs and chemicals used in the study were obtained commercially and were of analytical grade.

EXPERIMENTAL PROTOCOL

1. Reducing power ability

The reducing power ability was investigated by the ferrous and ferric transformation in the presence of the plant extract. The Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. It was measured by mixing 1.0 ml extract of various concentration prepared with distilled water to 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide and incubated at 50°C for 30 min. After that 2.5 ml of trichloroacetic acid (10%) were added to the mixture and centrifuged for 10 min at 3000 g, 2.5 ml from the upper part were diluted with 2.5 ml water and shaken with 0.5 ml fresh 0.1%, ferric chloride. The absorbance was measured at 700 nm using UV-spectrophotometer. The reference solution was prepared as above, but contained water instead of the samples. Increased absorbance of the reaction mixture indicates increased reducing power. All experiments were done in triplicate using butylated hydroxyltoluene (BHT) as positive control (Yildrim *et al.,* 2001).

2. Superoxide anion scavenging activity

A reaction mixture with a final volume of 3 ml per tube was prepared with 1.4 ml of 50 mM KH_2PO_4-KOH, pH 7.4 containing 1 mM EDTA, 0.5 ml of 100 µm hypoxanthine, 0.5 ml of 100 µM NBT. The reaction was started by adding 0.066 units per tube of xanthine oxidase freshly diluted in 100 µl of phosphate buffer and 0.5 ml of test extract in saline. The xanthine oxidase was added last. The subsequent rate of NBT reduction was determined on the basis

of spectrophotometric determinations of absorbance at 560 nm. Ascorbic acid was used as standard. The results are expressed as the percentage inhibition of NBT reduction rate with respect to the reaction mixture without test compound (Guzman *et al.*, 2001).

3. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured by the ability of the extract to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (Fenton reaction) (Halliwell, 1994). The reaction mixture in a final volume of 1.0 ml contained 100 µl of 2-deoxy2-ribose (28 mM in 20 mM KH_2PO_4 buffer, pH 7.4), 500 µl of the fractions at various concentrations (50-800 µg/ml) in buffer, 200 µl of 1.04 mM EDTA and 200 µM $FeCl_3$ (1:1v/v), 100 µl of 1.0 mM hydrogen peroxide (H_2O_2) and 100 µl of 1.0 mM ascorbic acid. Test samples were kept at 37^0C for 1 h. The free radical damage imposed on the substrate, deoxyribose was measured using the thiobarbituric acid test. One ml of 1% thiobarbituric acid (TBA) and 1.0 ml 2.8% trichloroacetic acid (TCA) were added to the test tubes and were incubated at 100^0C for 20 min. After cooling, the absorbance was measured at 532 nm against a blank containing deoxyribose and buffer. Quercetin (50-800 µg/ml) was used as a positive control.

4. DPPH radical scavenging activity

The hydrogen donating ability of the extract was examined in the presence of DPPH stable radical. One millilitre of 0.3 mM DPPH ethanol solution was added to 2.5 ml of sample solution of different concentrations (10-160 µg) of extract and allowed to react at room temperature. After 30 min, the absorbance values were measured at 517 nm. Ascorbic acid was used as standard (Guzman *et al.*, 2001).

Nitric oxide radical scavenging assay

Various concentrations of the extract and sodium nitroprusside (5mM) in phosphate buffer saline (0.025 M, pH 7.4) in a final volume of 3 ml are incubated at 25^o C for 150 min. Control experiments without the test compounds but with equivalent amount of buffer is prepared in the same manner as done for the test. There after, 0.5 ml of incubation solution is removed and diluted with 0.5 ml Griess' reagent (1% sulphanilamide, 2% *O*-Phosphoric acid and 0.1% naphthyethylene diamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyethylene diamine dihydrochloride is read at 546 nm. The percentage inhibition is calculated. The experiment is done in triplicate using curcumin (50-800 µg/ml) as positive control (Sreejayan and Rao, 1997).

6. Ferrous chelating ability

The ferrous level is monitored by measuring the formation of the ferrous ion-ferrozine complex. The reaction mixture containing different concentrations of extracts (50-800µg/ml) were added to 2 mM ferrous chloride (0.1 ml) and 5 mM ferrozine (0.2 ml) to initiate the reaction and the mixture is shaken vigorously and left to stand at room temperature for 10 min. The absorbance of the solution is measured at 562 nm. The positive control are those using ascorbic acid and all tests and analysis are run in triplicate. The percentage chelating effect of Ferrozine-Fe^{2+} complex formation is calculated (Huang and Kuo, 2000).

Calculation of percentage inhibition (%I)

The concentration (µg/ml) of the extract required to scavenge the radicals was calculated by using the percentage scavenging activities at five different concentrations of the extract. Percentage inhibition (I %) was calculated using the formula,

$$I\ \% = \frac{(Ac-As) \times 100}{Ac}$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

Calculation of 50% inhibition (IC_{50})

The concentration of the extract required to scavenge 50% inhibition of radicals was calculated by using the software Graph Pad Instant.

RESULTS AND DISCUSSION

Phytochemical screening

Phytochemical screening of the bark of *vitex negundo* Linn showed the presence of steroids, triterpines, saponin glycosides, alkaloids, glycosides, tannins, and flavonoids and absence of fixed oil, gum-mucilage.

Table 1. Phytochemical screening of methanolic extract of bark *Vitex negundo* Linn

Phytochemicals	VBE
Alkaloids	+
Glycosides	+
Flavonoids	+
Saponin	+
Terpenoids	+
Tannins	+
Fixed oil	–
Gum- mucilage	–

(+) Presence of constituents
(−) absence of constituents

Table 2. Hydrogen donating ability (DPPH Assay)

Group	Concentration (µg/ml)	Absorbance at 517 nm	% inhibition	IC_{50} µg/ml
Control		0.113±0.03		
VBE	2.5 5 10 20 40	0.103 ± 0.060** 0.093 ± 0.001** 0.072 ± 0.002** 0.057 ± 0.008 0.038 ± 0.004	8.93 ± 0.82 17.77± 1.27 36.33 ± 2.34 49.55 ± 1.68 66.37± 3.70	21.47±0.21
Ascorbic acid (standard)	2.5 5 10 20 40	0.086 ± 0.0014** 0.066 ± 0.0027** 0.041 ± 0.0024** 0.024 ± 0.0040** 0.008 ± 0.0001**	26.27 ± 1.52 40.24 ± 3.78 63.50 ± 7.68 78.47± 9.42 92.66± 8.56	7.7 ± 0.31

Values are mean ± S.E.M (n=3), **P<0.01 when compared with control.

The VBE demonstrated DPPH free radical activity. The extract (VBE) reduced DPPH to yellow coloured product in concentration dependent manner. It was found that the percentage inhibition at a dose of 40 µg/ml of the VBE was almost equivalent to 10 µg/ml of ascorbic acid. The IC_{50} value of VBE was compared with the IC_{50} of standard ascorbic acid (Table 2). DPPH was reduced with the addition of VBE in concentration dependent manner and the results were statistically significant .

Table 3. Reducing power assay

Group	Concentration (µg/ml)	Absorbance at 700 nm
VBE	50	0.095 ± 0.003**
	100	0.182 ± 0.07**
	200	0.251 ± 0.08*
	400	0.431 ± 0.050
	800	0.814 ± 0.072
BHT (Standard)	50	0.262± 0.017**
	100	0.363 ± 0.082**
	200	0.427 ± 0.068**
	400	0.895 ± 0.056**
	800	1.213± 0.090 **

Values are mean ± S.E.M. (n=3), *P<0.05, **P<0.01 when compared with standard.

Table3. Shows the reductive capabilities of VBE when compared to the standard Butylated hydroxy toluene (BHT). The increase in absorbance of the reaction mixture containing the extract showed increased reducing power with increase in concentration. The reducing power increased significantly (P<0.05) with increasing amounts of the extract. However, the activity of the extract was less than the standard.

Table 4. Nitric oxide scavenging activity

Group	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC_{50} µg/ml
Control		1.186 ± 0.0017		
VBE	10	1.066 ± 0.18**	10.11 ± 1.62	55.23± 0.58
	20	0.967 ± 0.02**	18.46 ± 0.98	
	40	0.758± 0.06**	36.08 ± 2.34	
	80	0.518± 0.05	56.32 ± 5.72	
	160	0.316 ± 0.09	73.35 ± 4.66	
Curcumin (standard)	10	0.792± 0.03**	33.18 ± 2.87	25.20±0.35
	20	0.616 ± 0.05**	46.80 ± 1.64	
	40	0.408 ± 0.08**	66.14 ± 7.22	
	80	0.216 ± 0.07**	82.18 ± 4.32	
	160	0.108 ± 0.05**	91.11 ± 8.46	

Values are mean ± S.E.M (n=3), **P<0.01 when compared with control.

The scavenging of nitric oxide by VBE was concentration dependent. Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of Griess Illosvoy reaction. The VBE effectively reduced the generation of nitric oxide radicals. The IC_{50} value of extract was 55.23µg/ml which was higher than the standard curcumin 25.20µg/ml. P value of the extract was significant (P<0.01).

Table 5.Scavenging of Hydrogen peroxide

Group	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC_{50} µg/ml
Control		0.912 ± 0.043		
VBE	5	0.783 ± 0.03**	14.16 ± 1.62	31.30± 0.37
	10	0.611 ± 0.07**	33.15 ± 4.39	
	25	0.495± 0.014**	45.44 ± 6.27	
	50	0.378± 0.09**	58.55 ± 3.56	
	100	0.257 ± 0.06	71.82 ± 2.72	
α-Tocopherol (standard)	5	0.624± 0.010***	31.35 ± 3.36	9.15±0.52
	10	0.445 ± 0.04***	51.06 ± 2.32	
	25	0.265 ± 0.01***	70.93 ± 8.37	
	50	0.144 ± 0.08***	84.31 ± 4.82	
	100	0.043 ± 0.06***	95.43 ± 11.23	

Values are mean ± S.E.M (n=3), **P<0.01, ***P<0.001 when compared with control.

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. Hydrogen peroxide can cross cell membranes rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+}, and possibly Cu^{2+} ions to form

hydroxyl radical and this may be the origin of many of its toxic effects. It is therefore biologically advantageous for cells to control the amount of hydrogen peroxide that is allowed to accumulate. *Vitex negundo* Linn extract demonstrated hydrogen peroxide scavenging activity in a concentration dependent manner with an IC_{50} of 31.30 µg/ml. P value of the extract was significant (P<0.001).

Table 6. Superoxide anion radical scavenging activity

Group	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC $_{50}$ µg/ml
Control		0.139 ± 0.035		
VBE	25 50 100 200 400	0.106± 0.002** 0.087 ± 0.004** 0.067± 0.005** 0.059± 0.007** 0.038 ± 0.003	23.74 ± 3.68 37.41 ± 5.72 51.79 ± 1.67 57.55 ± 4.72 72.66 ± 9.87	93.26± 0.47
Ascorbic acid (standard)	25 50 100 200 400	0.097± 0.002*** 0.050 ± 0.004*** 0.030 ± 0.006*** 0.022 ±0.009*** 0.010 ± 0.008***	30.21 ± 2.67 64.02 ± 1.38 78.41 ± 9.67 84.17 ± 5.27 91.59 ± 10.63	45.26±0.34

Values are mean ± S.E.M. (n=3), **P<0.01, ***P<0.001 when compared with control.

The VBE was found to be a scavenger of superoxide anion generated in xanthine oxidase-NBT systems. The extract showed significant (P<0.001) superoxide inhibiting activity at a concentrations of 25-400 µg/ml. The IC_{50} of the extract was found to be 93.26µg/ml where as the IC_{50} of the standard ascorbic acid is 45.26µg/ml.

Table7. Hydroxyl radical scavenging activity

Group	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC $_{50}$ µg/ml
Control		1.057 ± 0.035		
VBE	5 10 20 40 80	0.957± 0.03** 0.837 ± 0.04** 0.731± 0.02** 0.457± 0.09** 0.278 ± 0.04	9.54 ± 0.82 16.59 ± 1.32 31.37 ± 1.98 56.78 ± 4.37 73.43 ± 5.72	38.42± 0.47
Ascorbic acid (standard)	5 10 20 40 80	0.825± 0.05*** 0.630 ± 0.02*** 0.487 ± 0.03*** 0.187 ±0.04*** 0.075 ± 0.009***	21.9 ± 1.62 39.87 ± 1.30 54.54 ± 7.45 82.72 ± 5.32 92.30 ± 9.77	18.22±0.30

Values are mean ± S.E.M. (n=3), **P<0.01, ***P<0.001 when compared with control

The hydroxyl radical was generated by Fenton – type reaction at room temperature. Scavenging of hydroxyl radical by VBE was concentration dependent. The IC_{50} value of extract was 38.42µg/ml which was higher than the standard Ascorbic acid 18.22µg/ml. The extract showed significant (P<0.01) superoxide inhibiting activity at a concentrations of 5-80 µg/ml.

Table 8. Ferrous chelating activity

Group	Concentration (µg/ml)	Absorbance at 546 nm	% inhibition	IC $_{50}$ µg/ml
Control		0.179 ± 0.0037		
VBE	10 20 40 80 160	0.140± 0.030** 0.118 ± 0.02** 0.091± 0.003** 0.063± 0.007 0.042 ± 0.006	18.50 ± 0.32 36.83 ± 3.76 48.64 ± 8.47 62.08 ± 4.39 76.63 ± 9.27	43.23± 0.24
Ascorbic acid (standard)	10 20 40 80 160	0.111± 0.08*** 0.089 ± 0.004*** 0.079 ± 0.002*** 0.039 ±0.006*** 0.018 ± 0.007***	43.28 ± 4.52 48.67 ± 2.68 60.20 ± 8.72 77.58 ± 5.38 91.85 ± 10.72	22.36±0.38

Values are mean ± S.E.M. (n=3), **P<0.01, ***P<0.001 when compared with control.

The formation of the Fe^{2+} - ferrozine complex was not completed in the presence of extract, indicating that the VBE chelates the iron. The absorbance of the VBE decreased with increase in concentration (from 10 to 160µg). The difference between VBE and the control

was statistically significant ($P<0.01$). The chelating action for the VBE was 76.63µg/ml where ascorbic acid exhibited the highest ferrous ion chelating ability (IC_{50} 43.23µg/ml).

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