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Contributed Paper

In vitro Antioxidant Activity of *Vitex negundo* L. Leaf Extracts

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ABSTRACT

In the present study, *Vitex negundo* L. leaf extracts were screened for their phytochemical composition and antioxidant activity. The yield of phytochemicals is in the order of ethanol extract > methanol extract > benzene extract > chloroform extract > hexane extract > ethyl acetate extract > petroleum extract. Finally we have selected ethanol extract for the antioxidant activity. Total phenolic content was estimated as gallic acid equivalents (249.96 ± 8.34 mg/g of extract) and total flavonoids estimated as catechin equivalents (166.67 ± 9.14 mg/g of extract). Free radical scavenging activity was determined by DPPH assay (79.82 ± 2.99% at 500 µg/ml concentration). Total antioxidant capacity of the extract (214.81 ± 4.07 mg ascorbic acid equivalent/g) was determined. Ferrous chelating ability was found to be 84.15 ± 1.65% at 500 µg/ml concentration and reductive ability of the extract was found to be 0.918 ± 0.035 absorbance unit at 600 µg/ml concentration. The antioxidant activity of *Vitex negundo* L. is mainly due to the presence of the phenolic compounds like, flavonoids and flavonols.

Key words: *Vitex negundo* L, DPPH, Reducing power, Phenols, Antioxidant capacity, Flavonoids.

1. INTRODUCTION

Reactive oxygen species (ROS), include free radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), peroxyl radical ($ROO^{\bullet}$) as well as non-radical species such as hydrogen peroxide (H_2O_2) [1]. *In vivo*, such species are securely coupled at their site of generation or are detoxified by endogenous antioxidative defenses, so as to preserve optimal cellular function. In pathological conditions, however, the detoxifying mechanisms are often inadequate as excessive quantities of ROS are generated. This resulting pro-oxidant shift, a process known as oxidative stress can result in the degradation of cellular components viz., DNA, carbohydrates, polyunsaturated lipids and proteins or precipitate enzyme inactivation, irreversible cellular dysfunction and ultimately cell death, if the pro-oxidant-antioxidant balance is not restored. Furthermore, ROS play a cardinal role in the aetiology of numerous diseases [2]. In recent years, there is an increasing interest

in finding antioxidant phytochemicals, because they can inhibit the propagation of free radical reactions, protect the human body from diseases [3].

Polyphenols constitute a large group of naturally occurring substances in the plant kingdom, which include the flavonoids. The plant phenolics are commonly present in fruits, vegetables, leaves, nuts, seeds, barks, roots and in other plant parts. These substances have considerable interest in the field of food chemistry, pharmacy and medicine due to a wide range of favorable biological effects including antioxidant properties. The antioxidant property of phenolics is mainly due to their redox properties. They act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers and metal chelators [4].

Vitex negundo Linn., (Verbenaceae) known as Nirgundi in Hindi, grows gregariously in wastelands and is also planted as a hedge-plant. It is an erect, 2–5 m in height, slender tree with quadrangular branchlets distributed throughout India. The leaves have five leaflets in a palmately arrangement, which are lanceolate, 4–10 cm long, hairy beneath and pointed at both ends. The leaf extract of *Vitex negundo* are generally used as a grain preservating material to protect the pulses against insects [5]. Although all parts of *Vitex negundo* are used as medicine in the indigenous system of medicine, the leaves are the most potent for medicinal use. It is used for treatment of eye-disease, toothache, inflammation, leucoderma, enlargement of the spleen, skin-ulcers, in catarrhal fever, rheumatoid arthritis, gonorrhoea, and bronchitis. They are also used as tonics, vermifuge, lactagogue, emmenagogue, antibacterial, antipyretic and antihistaminic agents. Oil prepared with it, is applied to sinuses and scrofulous sores. Its extract has also shown anticancer activity against Ehrlich ascites tumour cells [6]. Lagundi tablets were prepared from leaves of *Vitex negundo*, commercially marketed as *Ascof*® is indicator for the relief of mild to moderate bronchile asthma and cough [7]. It contains many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids. Among the chemical constituents, it has several flavonoids such as casticin, orientin, isoorientin, luteolin, lutecin-7-O-glucoside, corymbosin, gardenins A and B, 3-Odesmethylartemetin, 5-O-desmethylnobiletin and 3′,4′,5,5′,6,7,8-heptamethyoxyflavone. An antioxidant property of the extract has been reported [6]. Eventhough many reports available on the medicinal importance of *Vitex negundo*, so for its phytochemical screening and antioxidant potential has not been studied.

In the present study, shade dried leaf powder was extracted with organic solvents with different polarities viz., ethanol, methanol, chloroform, petroleum ether, ethyl acetate, benzene and hexane for qualitative phytochemical screening. Antioxidant activity was examined for ethanol extract using different antioxidant assays such as total antioxidant activity, free radical scavenging, metal chelating activity, reducing ability. Furthermore, the total phenolic and flavonoid contents were also measured in ethanolic extract and their correlation with the antioxidant activities were ascertained.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Chemicals, such as ascorbic acid, gallic acid, 1,1 Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA) and catechin were procured from Sigma Chemical Co.(St Louis, MO, USA), ferrous chloride, ferrozine and Folin-Ciocalteu reagent were from Spectrochem PVT. Ltd., (Mumbai, India). All other chemicals unless and otherwise mentioned were obtained from Himedia Laboratories and Sisco Research Laboratories

PVT. Ltd. (Mumbai, India).

2.2 Plant Material and Preparation of Extract

Fresh leaves of *Vitex negundo* were collected from the forests of Malenadu region of Karnataka state (Western Ghats), India and authenticated by the experts of Department of Botany, Sahyadri Science College, Shimoga. The leaves were shade dried and milled to get a course powder. Each 5 gms of dry powder was extracted with organic solvents with different polarities *viz*., ethanol, methanol, petroleum ether, ethyl acetate, chloroform, benzene and hexane using soxhlet apparatus, for at least 10-15 cycles. The extracts were then filtered and concentrated to a dry mass [8].

2.3 Qualitative Phytochemical Screening

Preliminary phytochemical screening for the various secondary metabolites in different extracts was qualitatively tested using standard procedures [9]. Presence of alkaloids was tested by using Mayer's, Wagner's and Hager's reagents. Iridoids were determined using Trim-Hill reagent. 5 % ferric chloride solution and aqueous basic lead solution (25%) were used to identify the presence of flavonoids and flavonols respectively in different extracts as described in the literature.

2.4 The Total Phenolic Content

The total phenolics content in the extract was determined with the Folin-Ciocalteu's reagent (FCR) [10]. In brief, 0.5 ml of extract was mixed with 2.5 ml FCR (diluted 1:10 v/v) followed by 2 ml of Na_2CO_3 (7.5 % v/v) solution. The tubes were vortexed and allowed to stand for 90 min at room temperature. Absorbance of sample was measured against a blank at 750 nm using a spectrophotometer (Shimadzu UV-1609, Japan). A calibration curve was constructed, using gallic acid as standard and total phenolic content of the extract was expressed in terms of milligrams of gallic acid (mg GAE) per gram of dry weight.

2.5 Determination of Total Flavonoid Content

Total flavonoid content of the ethanolic extract was determined according to modified method of Zhishen *et al*, [11]. Briefly, 1 ml of test sample and 4 ml of water were added to a volumetric flask (10 ml volume). 5 min after adding 0.3 ml of 5 % $NaNO_2$, 0.3 ml of 10 % $AlCl_3$. $6H_2O$ were added. After 6 min incubation at room temperature, 2 ml of 1 M NaOH was added to the reaction mixture. Immediately the final volume was made up to 10 ml with distilled water. The absorbance was measured at 510 nm against a blank spectrophotometrically (Shimadzu UV-1609, Japan). Calibration curve was constructed with catechin and results were expressed as catechin equivalents (mg catechin/g dried extract).

2.6 DPPH Radical Scavenging Activity

The free radical scavenging activity of the ethanolic extract was determined according to the method of Braca *et al*, [12]. Briefly, 0.1 ml of extract and standard compound BHA (butylated hydroxyanisole) of variable concentration (100-500 µg/ml) was added to 3 ml of 0.004% DPPH (in ethanol). The samples were incubated for 30 min in the dark and the absorbance of the reaction mixture was measured at 517 nm spectrophotometrically (Shimadzu UV-1609, Japan). The standard compound BHA was used for comparison. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The percent of DPPH radical scavenging activity was calculated as $[(Ac-Ae)/Ac]\times100$, where Ac is the absorbance of the control and Ae is the absorbance of the extract/standard.

2.7 Determination of Antioxidant Capacity by Phosphomolybdenum Method

The antioxidant activity of the ethanolic extract was determined by the phosphomolybdenum method as described by Prieto *et al*, [13]. Briefly, 0.3 ml of extract was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95 °C for 90 min and cooled to room temperature. Finally, absorbance was measured at 695 nm using a spectrophotometer (Shimadzu, UV-1609, Japan) against blank. Methanol (0.3 ml) in place of extract was used as the blank. The total antioxidant capacity was expressed as the number of equivalents of ascorbic acid (AAE).

2.8 Chelating Action on Ferrous Ion

Ferrous ion-chelating activity was determined according to the method of Dinis *et al,* [14]. The Fe^{+2} was monitored by measuring the formation of ferrous iron-ferrozine complex. Briefly, 3 ml of *Vitex negundo* extract/standard EDTA (100-500 µg/ml) and 2 mM $FeCl_2$ (0.05 ml) were added. The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and incubated for 10 mins at room temperature. Absorbance of the reaction mixture was measured at 562 nm spectrophotometrically. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated from the formula, [(Ac-Ae)/Ac] ×100, where Ac is absorbance of the control and Ae is absorbance of the extract/standard.

2.9 Total Reductive Ability

The reductive ability was determined according to the method of Oyaizu [15]. In brief, different concentrations (100-600 µg/ml) of the extract and the standard compound (BHA) in 1ml of ethanol were combined with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1 % potassium ferricyanide [$K_3Fe(CN)_6$]. Reaction mixture was incubated at 50°C for 20 min and cooled to room temperature. 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged (650 rpm at room temperature) for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. Absorbance of the reaction mixture was measured at 700 nm in a spectrophotometer (Shimadzu UV-1609, Japan). Increased absorbance of the reaction mixture indicates the increase in reduction capability.

2.10 Statistical Analysis

All experiments were repeated at least three times. The data were recorded as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1 Qualitative Phytochemical Screening

The preliminary phytochemical screening indicates the presence of alkaloids, iridoids, phenolic acids, flavonols and flavonoids in the extracts (Table 1). The presence of phytochemicals are represented as (+) and absence was represented as (-) depending upon the color intensity of the reaction products. Our results indicate the presence of phytochemicals in the order of, ethanol extract > methanol extract > benzene extract > chloroform extract > hexane extract > ethyl acetate extract > petroleum ether extract. The above data shows the more yield of phytochemicals in ethanol extract and hence the ethanolic extract was selected for the further studies like, total phenolics, total flavonoids, DPPH radical scavenging activity, total antioxidant capacity determination and reducing power assay.

3.2 Total Phenolic Content

Phenolic compounds are commonly found in both edible and inedible plants and

Table 1. Qualitative analysis of the phytochemicals of *Vitex negundo* L. leaf extracts. Symbol (+) indicates presence and (-) indicates absence of phytochemicals.

Tests	Ethanol	Methanol	Petroleum ether	Chloroform	Benzene	Ethyl acetate	Hexane
Mayer's test (Alkaloids)	+	+	-	+	+	-	-
Wagner's test (Alkaloids)	+	-	-	-	+	+	+
Hager's test (Alkaloids)	+	+	-	+	+	-	-
Trim-Hill test (Iridoids)	+	+	-	-	-	-	-
Ferric chlorideTest (Flavonoids)	+	+	+	+	-	+	+
Lead acetate test (Flavonols)	+	-	+	+	+	+	+

plant parts. They have been reported to have multiple biological effects, including antioxidant activity. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides [16]. Furthermore, phenolic extracts of plant materials have been shown to neutralize free radicals in various model systems [17]. These phenolic compounds may contribute directly to the antioxidative action. It has been suggested that up to 1.0 g polyphenolic compounds (from a diet rich in fruits and vegetables) ingested daily have inhibitory effects on mutagenesis and carcinogenesis in humans [18]. The content of phenolic compounds (mg/100g DW) in ethanolic extract, determined from regression equation of calibration curve ($y = 0.0248x + 0.0069$, $R^2 = 0.9989$) and expressed in gallic acid equivalents (GAE). The total phenolics content of *Vitex negundo* was found to be 249.96 ± 8.34 GAE/g dry weight of extract.

3.3 Total Flavonoid Content

Flavonoids comprise the most widespread and diverse group of polyphenolic plant secondary metabolites. These compounds play an important role in biological and chemical activities including free radical scavenging properties [19]. Flavonoids are potent antioxidants *in vitro*, and therefore one of the main interests in the compounds has involved in the protection against cardiovascular disease. Antioxidation is however, one of the many mechanisms through which flavonoids could exert their actions [20]. The content of flavonoid compounds (mg/100g DW) in ethanolic extract, determined from regression equation of calibration curve ($y = 0.0022x + 0.005$, $R^2 = 0.9996$) and expressed in catechin equivalents. The total flavonoid content in the ethanolic extract was found to be 166.67 ± 9.14 mg catechin/ g of plant extract.

3.4 DPPH Scavenging Activity

A concentration-dependent assay was carried out with the ethanolic extract and the results are presented in Figure 1. The percentage of inhibition was in the order: BHA (93.48 ± 0.88%) > *Vitex negundo* extract (69.82 ± 2.99%) at a concentration of 500 µg/ml. The scavenging effect was increased

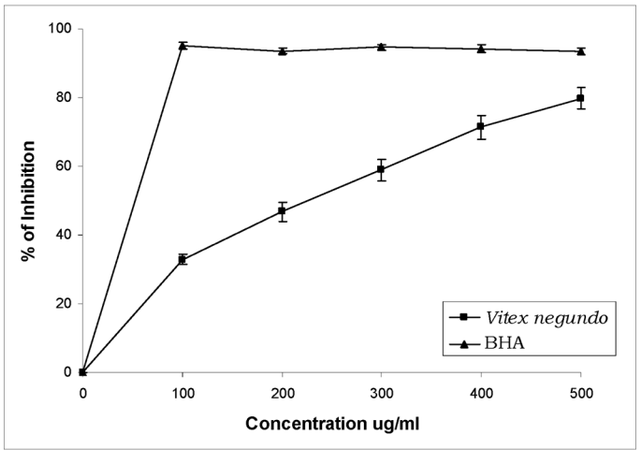

Figure 1. DPPH radical scavenging activity of ethanolic extract of *Vitex negundo*. BHA used as reference standard.

with increasing concentration. These results provide a direct comparison of the antioxidant activity with BHA. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen-donating ability. Radicals formed in the presence of oxygen ($ROO^{\bullet}$, $HO^{\bullet}$, $RO^{\bullet}$, $O_2^{\bullet -}$) are highly reactive species greatly differing in their lifetimes and chemical properties. Hence, their direct detection and evaluation is difficult. Stable radical species ($DPPH^{\bullet}$, $ABTS^{\bullet +}$) are often used for the evaluation of general radical scavenging abilities of antioxidants [21].

3.5 Total Antioxidant Capacity

Total antioxidant capacity of ethanolic extract was determined from regression equation of calibration curve ($y = 0.0121x - 0.068$, $R^2 = 0.9952$) and expressed in ascorbic acid equivalents. The total antioxidant capacity of ethanolic extract was found to be 214.81 ± 4.07 mg/g of plant extract (expressed as ascorbic acid equivalents). 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. The assay is successfully used to quantify vitamin E in seeds, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extracts [13].

3.6 Ferrous Ion-Chelating Activity

The chelating effects (%) of the ethanolic extract and standard EDTA on ferrous ion are presented in Figure 2. EDTA is a known metal ion chelator; and therefore the chelating effect of *Vitex negund*o was compared with it. The percentage of metal chelating capacity of *Vitex negund*o extract and positive control EDTA was in the order of; EDTA (99.43 ± 1.34 %) > *Vitex negundo* (84.15 ± 1.65 %) at 500 µg/ml concentration. The production of highly ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals is also catalysed by free iron through Haber–Weiss reaction ($O_2^{-\bullet} + H_2O_2 \longrightarrow O_2 + OH^- + OH^{\bullet}$) [22]. The transition metal, iron is capable of generating free radicals from peroxides by Fenton reaction and may be implicated in human cardiovascular disease [23]. Thus, the ability to chelate transition metals is considered to be an important antioxidant mode of action. It was reported

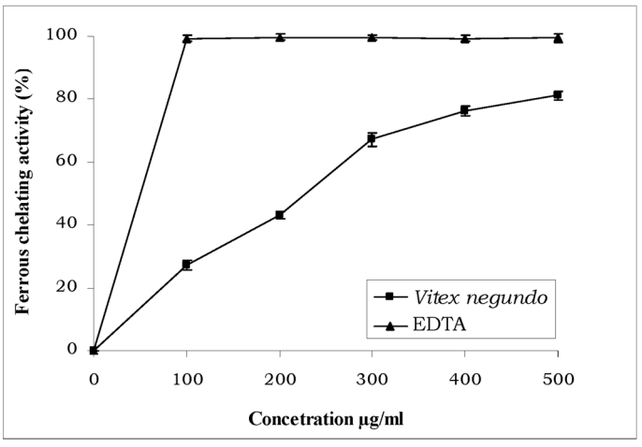

Figure 2. Chelating effects of *Vitex negundo* extract at different concentrations on Fe^{2+}. EDTA used as positive control.

that chelating agents, which form σ-bonds with a metal ion are effective as secondary antioxidants because they reduce the redox potential thereby stabilising the oxidised form of the metal ion [24]. Furthermore, it has been reported that non-flavonoid polyphenolics can reduce iron and then form Fe^{2+} polyphenol complexes that are inert [25].

3.7 Total Reductive Capability

For the measurements of the reductive ability, it has been investigated from the Fe^{3+}-Fe^{2+} transformation in the presence of extract samples using the method described by Oyaizu [15]. Figure 3. shows reducing power of ethanolic extract and standard BHA. The reducing power of ethanolic extract and standard compound BHA exhibited the following order: BHA (2.398±0.067) > *Vitex negundo* ethanolic extract (0.918±0.035) absorbance units at 600 µg/ml. The reducing properties are generally associated with the

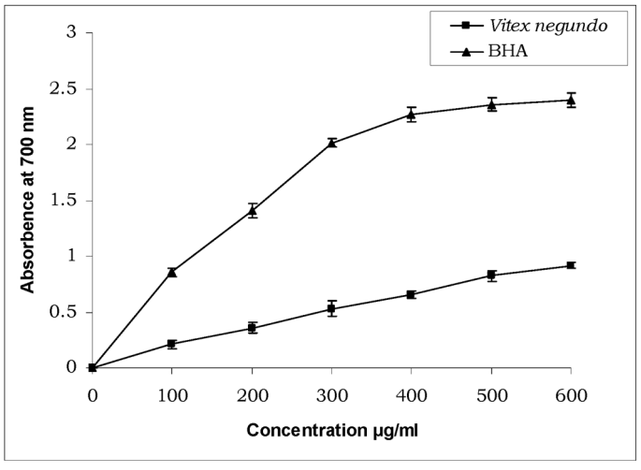

Figure 3. Reducing power of the *Vitex negundo* extract at different concentrations. BHA used as reference standard.

presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain or by donating a hydrogen atom [26].

4. CONCLUSION

The data obtained from the present study suggest the presence of phytochemicals in the ethanolic extract of *Vitex negundo* L. Total phenols and flavonoids content were also very rich in ethanolic extract. This extract contain an effective antioxidant fraction that has been confirmed by different *in vitro* assays including DPPH radical scavenging activity, ferrous chelating, reducing power assay and total antioxidant capacity. The free radical scavenging ability probably is one of the mechanisms by which herbal medicines exhibit higher antioxidant activity. Further studies are warranted for the isolation and identification of individual phenolic compounds and also *in vivo* studies are needed for understanding their mechanism of action to exploit as potent antioxidants for therapeutic applications.

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