International Journal of Advanced Research in Biological Sciences
www.ijarbs.com

Research Article

Analysis the antioxidant activity of the isolated bicyclo [2.2.1] hept-5-ene-2-carbonitrile compound from the medicinal plant – *Vitex negundo* (Linn.)

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Abstract

Vitex negundo Linn. (VN), belonging to family Verbenaceae, is an aromatic shrub distributed throughout India. In the ayurvedic system of medicine it is used as a drug of choice to manage pain, inflammation and other related diseases. It contains many polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids. *V. negundo* was investigated by employing various established *in vitro* systems antioxidant, the bicyclo [2.2.1] hept-5-ene-2-carbonitrile such as DPPH 2,2-Diphenyl-1-Picrylhydrazyl, Superoxide radical scavenging, Hydroxyl radical scavenging activity, Nitric oxide radical scavenging, Hydrogen peroxide scavenging activity and Iron reducing power assay. The antioxidant activity shows the highest inhibition of DPPH activity (95% in 120µg/mL), superoxide scavenging activity in (69.76% in 500 µg/mL), Hydroxyl radical scavenging activity in the bicyclo [2.2.1] hept-5-ene-2-carbonitrile of high activity in (67.09% in 500µg/mL), Nitric oxide radical scavenging more effective in (52.06% in 500 µg/mL), Hydrogen peroxide scavenging activity high activity in (75% in 120µg/mL) and Iron reducing power assay inhibited (75% in 120µg/mL) and it was concluded that the bicyclo [2.2.1] hept-5-ene-2-carbonitrile from the leaves of *Vitex negundo* of possess the significant antioxidant activity.

Keywords: *Vitex negundo* Linn, ayurvedic system, polyphenolic compounds , *in vitro* systems, *in vitro* systems

Introduction

Herbal medicines have been the basis of treatment and cure for various diseases and physiological conditions in traditional methods practiced such as Ayurveda, Unani and Siddha. Several plant species are used by many ethnic groups for the treatment of various ailments ranging from minor infections to dysentery, skin diseases, asthma, malaria and a horde of other indications (Dahanukar *et al*., 2000). The recent studies have investigated that the antioxidant effect of plant products is mainly attributed to phenolic compounds such as flavonoids, phenolic acids and tannins etc. (Nagavani 2010 and Cartea *et al*., 2010).

Antioxidants are the compounds with ability to neutralize free radicals, therefore prevent free radical mediated oxidative damage in cell. Antioxidant neutralize the free radicals by interfering with the oxidation process by reacting with free radicals, chelating activity, catalytic

activity and oxygen scavenging activity (Shahidi 1992 and Buyukokuroglu *et al*., 2001). Recently there has been a growing interest in the study of traditional plants for pharmaceutical applications because of its low toxicity and economic viability. In past, various plant phytochemicals *viz*; phenolic compounds, flavonoids and tannins reported to possess significant antioxidant activity against a wide variety of free radicals (Koleckar *et* al., 2008, Kirmizibekmez *et al*., 2009 and Choudhary 2011).
The focus of this study was to evaluate the *vitex negundo* plant Compound of bicyclo [2.2.1] hept-5-ene-2-carbonitrile DPPH radical scavenging activity, Superoxide anion scavenging activity, Hydroxyl redical scavenging activity, Nitric oxide scavenging activity, Hydrogen peroxide scavenging activity and iron reducing power assay by *in vitro* methods.

Materials and Methods

Plant Materials

Healthy and well grown leaves of selected plant (*Vitex negundo*) were collected from the area of Kolli Hills, Namakkal district, Tamil Nadu, India. The leaves were immediately brought to the laboratory using separate polythene bags. First they were washed with tap water, then surface sterilized in 10 per cent sodium hypochlorite solution to prevent the contamination of any microbes, then rinsed with sterile distilled water and air dried in shade at room temperature. The plant voucher specimen were (BSI/SRC/5/23/2013-2014/Tech/492) deposited and authenticated by Dr. G.V.S. Murthy, Scientist 'F' and Head Botanical Survey of India, Coimbatore, Tamilnadu, India.

Preparation of plant extracts

The leaves of the plants were air dried at room temperature for 10 days then powdered using a mixer grinder. The powdered leaves (100 g) were extracted in a Soxhlet apparatus for 72 h with methanol (Vogel 1978). The extracts were pooled and the solvent was evaporated using a rotary evaporator under reduced pressure at 40^0C. The crude extracts thus obtained were kept at 4^0C until further assay.

Identification of Antimicrobial Compound

The compound was identified by spectral studies like, IR, 1H NMR, 13C NMR and mass spectrum. The compound identified Bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

Chemicals

Nitro blue tetrazolium (NBT), sodium nitroprusside (SNP), trichloro acetic acid (TCA), 1,1-diphenyl-2-picrylhydrazyl (DPPH), potassium hexa cyano ferrate [$K_3Fe(CN)_6$] and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined by the method of (Shimada *et al.* 1992). Briefly, a 2 mL aliquot of DPPH methanol solution (25 mg/mL) was added to 0.5 mL sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517 nm in a spectrophotometer. Lower absorbance of the reaction mixture indicated higher free-radical scavenging activity. The scavenging activity of sample was expressed as 50% effective concentration (IC_{50}), which represented the concentration of sample having 50% of DPPH radical scavenging effect

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$$\text{Radical scavenging activity (\%)} = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

where A control is the absorbance of the control (ascorbic acid) and A sample is the absorbance of reaction mixture (in the presence of sample). All the tests were run in triplicates (n = 3) and the average values were calculated.

Superoxide anion scavenging activity assay

The scavenging activity of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile towards superoxide anion radicals was measured by the method of (Liu *et al.*

1997). Superoxide anions were generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 mL of Tris-HCl buffer (100 mmol, pH 7.4) containing 0.75 mL of NBT (300 μmol) solution, 0.75 mL of NADH (936 μmol) solution and 0.3 mL of different concentrations of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile. The reaction was initiated by adding 0.75 mL of PMS (120 μmol) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0\text{-}A_1)/A_0 \times 100]$$

where A_0 was the absorbance of the control (blank, without bicyclo [2.2.1] hept-5-ene-2-carbonitrile) and A_1 was the absorbance in the presence of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

Hydroxyl radical scavenging activity assay

The scavenging activity for hydroxyl radicals was measured with Fenton reaction (Yu *et al*., 2004). Reaction mixture contained 60 μL of 1 mmol of $FeCl_2$, 90 μL of 1 mmol of 1, 10-phenanthroline, 2.4 mL of 0.2 mol of phosphate buffer (pH 7.8), 150 μL of 0.17 mol of H_2O_2 and 1.5 mL of bicyclo [2.2.1] hept-5-ene-2-carbonitrile at various concentrations. Adding H_2O_2 started the reaction. After incubation at room temperature for 5 min, the absorbance of the mixture at 560 nm was measured with a spectrophotometer. The hydroxyl radicals scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0\text{-}A_1)/A_0 \times 100]$$

where A_0 was the absorbance of the control (blank, without bicyclo [2.2.1] hept-5-ene-2-carbonitrile) and A_1 was the absorbance in the presence of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

Nitric oxide scavenging activity assay

Nitric oxide radical scavenging activity was determined according to the method reported by (Garrat 1964). 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 the Griess Illosvoy reaction. 2 mL of 10 mM sodium nitroprusside in 0.5 mL phosphate buffer saline (pH 7.4) was mixed with 0.5 mL of bicyclo [2.2.1] hept-5-ene-2-carbonitrile at various concentrations and the mixture incubated at 25°C for 150 min. From the incubated mixture 0.5 mL was taken out and added into 1.0 mL of sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. Finally, 1.0 mL of naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min. The absorbance at 540 nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = [(A_0\text{-}A_1)/A_0 \times 100]$$

where A_0 was the absorbance of the control (blank, without bicyclo [2.2.1] hept-5-ene-2-carbonitrile) and A_1 was the absorbance in the presence of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

Hydrogen peroxide scavenging activity assay

Hydrogen peroxide scavenging activity of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile was estimated by replacement titration (Zhang, 2000). Aliquot of 1.0 mL of 0.1 mmol of H_2O_2 and 1.0 mL of various concentrations of bicyclo [2.2.1] hept-5-ene-2-carbonitrile were mixed, followed by 2 drops of 3% ammonium molybdate, 10 mL of 2 mol of H_2SO_4 and 7.0 mL of 1.8 mol KI. The mixed solution was titrated with 5.09 mmol of NaS_2O_3 until yellow color disappeared. Percentage of scavenging of hydrogen peroxide was calculated as:

$$\% \text{ Inhibition} = [(V_0 - V_1)/V_0 \times 100]$$

where V_0 was volume of NaS_2O_3 solution used to titrate the control sample in the presence of

hydrogen peroxide (without bicyclo [2.2.1] hept-5-ene-2-carbonitrile), V_1 was the volume of NaS_2O_3 solution used in the presence of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

Iron reducing power assay

The Fe^{3+} reducing power of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile was determined by the method of (Oyaizu 1986) with slight modifications. The bicyclo [2.2.1] hept-5-ene-2-carbonitrile (0.75 mL) at various concentrations was mixed with 0.75 mL of phosphate buffer (0.2 mol, pH 6.6) and 0.75 mL of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50^{o}C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of trichloro acetic acid (TCA) solution (10%) and then centrifuged at 3000rpm for 10 min. 1.5 mL of the supernatant was mixed with 1.5 mL of distilled water and 0.1 mL of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Results and Discussion

In the present study, three triplicate commonly used antioxidant evaluation methods such as DPPH radical scavenging activity, Superoxide anion scavenging activity assay, Hydroxyl radical scavenging activity assay, Nitric oxide scavenging activity assay, Hydrogen peroxide scavenging activity assay and Iron reducing power assay chosen to determine the antioxidant potential of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile of *Vitex negundo*.
The antioxidant activity of bicyclo [2.2.1] hept-5-ene-2-carbonitrile is shown in **Fig. 1.** The bicyclo [2.2.1] hept-5-ene-2-carbonitrile exhibited a significant dose dependent inhibition of DPPH activity in high in 95% in 120µg/ml followed by 80.59, 67.79% in different concentration. The superoxide anion radical scavenging activity of bicyclo [2.2.1] hept-5-ene-2-carbonitrile was assayed by the PMS-NADH system.

The superoxide scavenging activity of bicyclo [2.2.1] hept-5-ene-2-carbonitrile was increased markedly with the increase of concentrations 69.76% in 500 µg/mL followed by 61.76 and 52.86% in different concentration. Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction in 67.09% in 500µg/mL followed by 59.82, 51.09 and 40.18 in different concentrations. The bicyclo [2.2.1] hept-5-ene-2-carbonitrile exhibited concentration dependent scavenging activities against hydroxyl radicals generated in a Fenton reaction system and bicyclo [2.2.1] hept-5-ene-2-carbonitrile is also moderately inhibited nitric oxide in dose dependent manner the values of 52.06% in 500 µg/mL followed by 46.68, 32.12% in different concentration in 400, 300, 200 mg/mL **Fig: 2.**

bicyclo [2.2.1] hept-5-ene-2-carbonitrile demonstrated hydrogen peroxide scavenging activity in a concentration dependent and depicts the reductive effect of 75% in 120µg/mL followed by 62.59, 48.79% in different concentration 100, 80 µg/mL of bicyclo [2.2.1] hept-5-ene-2-carbonitrile. Similar to the antioxidant activity, the iron reducing power of 70% in 120 µg/mL followed by 55.59, 47.79 and 39% in different concentration of 100, 80, 60 µg/mL of bicyclo [2.2.1] hept-5-ene-2-carbonitrile increased with increasing dosage **Fig. 3.** Free radical assay is one of the most widely used methods and has become routine in establishing the antioxidant activity of herbal extracts and photochemical Hydrogen donating ability is an index of primary antioxidants. DPPH is known to abstract labile hydrogen and the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Sing *et al*., 2008).

Antioxidant effects of plant extracts are mainly due to the presence of phenolic compounds such as flavonoids, phenolic acids, tannins and phenolic diterpenes (Chew *et. al.*, 2009). They are safe to be consumed by human and animal (Preethi *et. al.*, 2010).The activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Seladji *et al*., 2014).

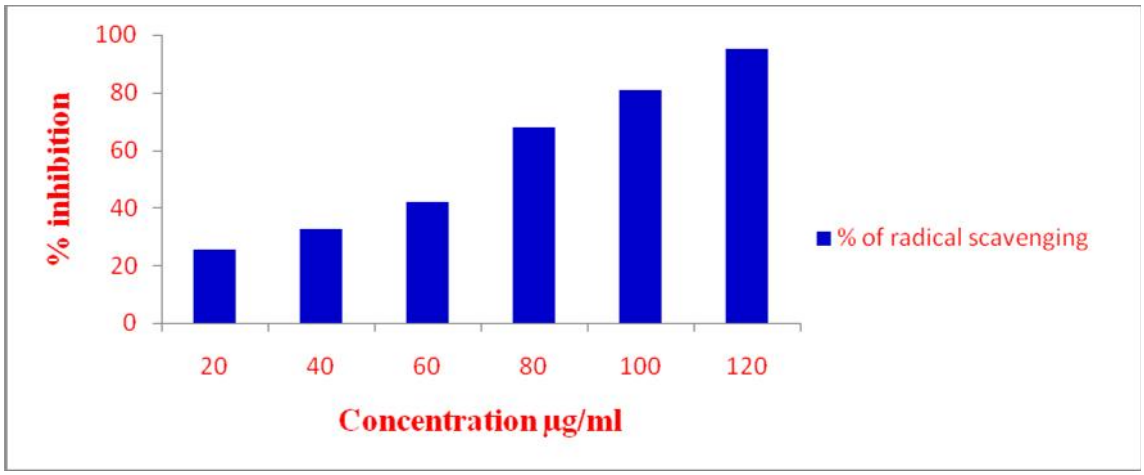

Fig: 1. DPPH radical scavenging activity of bicyclo [2.2.1] hept-5-ene-2-carbonitrile

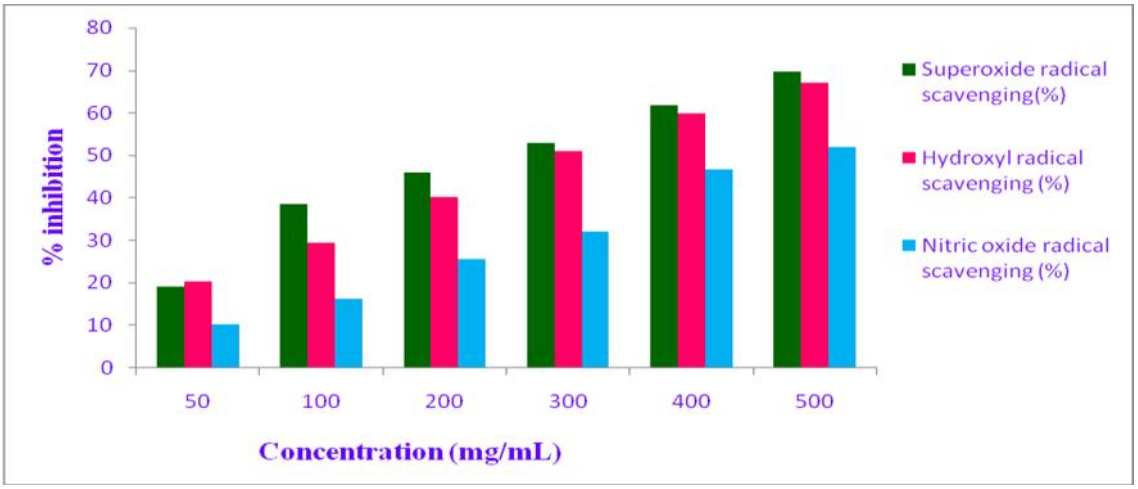

Fig: 2. Different radicals scavenging Activity of bicyclo [2.2.1] hept-5-ene-2-carbonitrile

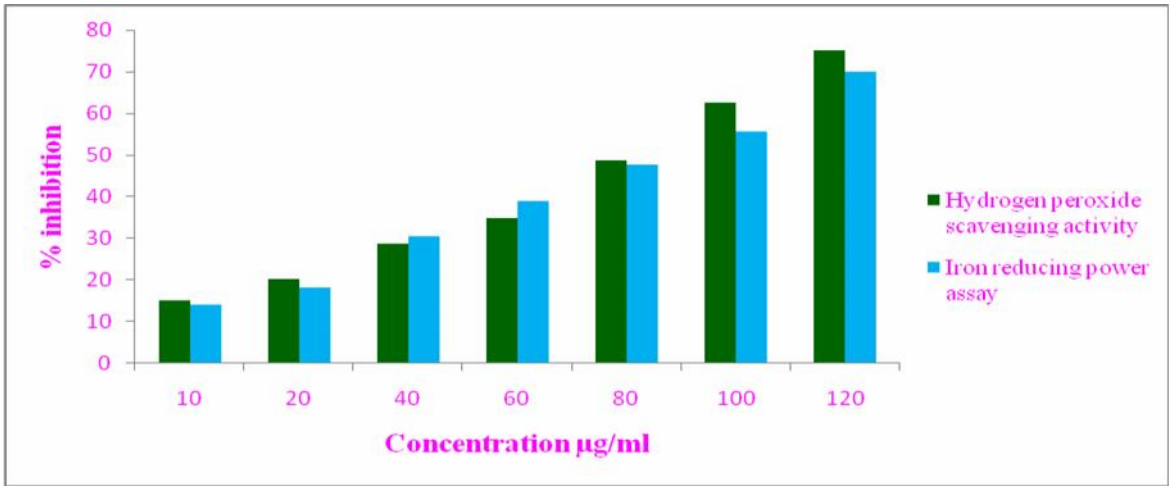

Fig: 3. Hydrogen peroxide scavenging activity and Iron reducing power assay

Conclusion

On basis of the results obtained in the present study, it was concluded that the compound of leaves in *Vitex negundo* of this species possess the bicyclo [2.2.1] hept-5-ene-2-carbonitrile significant antioxidant activity. We can say that bicyclo [2.2.1] hept-5-ene-2-carbonitrile is a better solvent for the full exploitation of the therapeutic potentials of *Vitex negundo* since it exhibited higher antioxidant properties of the compound. Further suggested to carry out the hepatoprotective and immune modulator activity of the bicyclo [2.2.1] hept-5-ene-2-carbonitrile.

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