

ANTIOXIDANT ACTIVITY OF METHANOL EXTRACTS  
OF *VITEX NEGUNDO* LINN

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**Summary**

In the present study, the comparative antioxidant potential and total phenolic content of methanol extracts of *Vitex negundo* leaf, stem and root were studied. *In-vitro* anti-lipid peroxidation activity in rat liver homogenate, nitric oxide radical, superoxide radical, peroxide radical, hydroxyl radical, DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging activity were evaluated against standard antioxidants such as  $\alpha$ -Tocopherol and Ascorbic acid. The total phenolic content and HPTLC studies were also carried out. The total phenolic content among various plant parts ranged from 283.0 to 461.0 mg gallic acid equivalent(GAE)/100 g dry sample with maximum content found in methanol extract of leaves 461.0 mg GAE/gm. The leaves showed maximum antioxidant activity with an IC<sub>50</sub> value of 0.221mg/ml for DPPH radical, 0.258 mg/ml for OH<sup>-</sup> radical, 0.642 mg/ml for anti-lipid peroxidation activity. The stem showed maximum activity with an IC<sub>50</sub> value of 0.275 mg/ml for peroxide radical, 0.290 mg/ml for superoxide radical, 0.310 mg/ml for nitric oxide radical. The results were significant as compared to standard antioxidants such as l-ascorbic acid and Tocopherol. On the basis of above results it was concluded that methanol extracts of different parts of *V. negundo* showed significant antioxidant activity. In HPTLC, the simultaneous estimation of vitexin (flavonol glycoside) and negundoside (irridoid glycoside) the marker constituent of *V. negundo* was done using the mobile phase Toluene: Ethyl acetate: Formic acid: water (2: 7: 0.8: 0.3). The negundoside was resolved at R<sub>f</sub> 0.21 and vitexin was resolved at R<sub>f</sub> 0.29 and the scanning wavelength used was 258 nm. The *V. negundo* leaves extract showed highest amount of negundoside (0.506%w/w) and vitexin (0.181%w/w). The results obtained in the current study indicate that *V. negundo* is a potential source of natural antioxidants.

**Key words:** *Vitex negundo* Linn, Verbenaceae, Free radicals, Vitexin, Negundoside.

## Introduction

*Vitex negundo* Linn. is an aromatic shrub, belonging to family Verbenaceae and distributed throughout India in warmer zones; ascending to 900 m in the North-western Himalayas. In the traditional medicine *V. negundo* is reported to be anti-rheumatic. The leaves contain irridoid glycosides like negundoside (2'-p-hydroxy benzoyl mussaenosidic acid), isomeric flavanones and flavanoids such as vitexin which is a flavonol glycoside. Dried powder of roots contains hentriacontane and stigmasterol. Two pentacyclic triterpenoids- betulinic acid and ursolic acid, have been isolated from leaves (1-3). *V. negundo* has various pharmacological activities such as anti-inflammatory, analgesic, antihistaminic (4-6), antimicrobial activity (7), hepatoprotective activity (8-10), anti-implantation activity (11), laxative activity (12), attenuates calpain activation and cataractogenesis (13), larvicidal activity (14), anti-arthritic activity (15), anti-convulsant activity (16), snake venom neutralization activity (17), oxidative stress (18) and antioxidant activity (19).

Under ordinary circumstances ROS (reactive oxygen species) such as  $O_2^-$ ,  $H_2O_2$  and  $OH^-$  generated are detoxified by the antioxidant present in the body and there is equilibrium between ROS and the antioxidant present. Though, due to ROS over production or inadequate antioxidant defense, this equilibrium is hindered favoring the ROS upsurge that culminates oxidative trauma to a variety of biomolecules including proteins, lipids, lipoproteins and DNA (20). This oxidative smash up is a decisive etiological factor implicated in numerous persistent human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases and also in the ageing process (21). Based on growing attention in free radical biology and the lack of efficient therapies for most chronic diseases such as rheumatoid arthritis, the utility of antioxidants in fortification against these diseases is warranted (22). Certain plants show antioxidant activity because of their phenolic constituents (23). The aim of present study was to evaluate the antioxidant potential of *V. negundo* using various *in vitro* models and correlation of antioxidant activity of various parts of plant extracts with presence of phenolic and other constituents.

## Methods

### Collection of plant material

Plant material of *V. negundo* was collected from plantation field been situated in a small village called Shiroor, 60 kms from Pune, in the month of October. The samples were authenticated by Agharkar Research Institute (ARI), Pune and the voucher specimen (08-123) was kept at departmental herbarium of ARI. The plant material was cleaned and dried in the shade, then powdered to 40 mesh and stored in an air tight container at 25°C.

### Chemicals

Chemicals used in this study were of analytical grade. Sodium nitroprusside, sulphanilamide, N-(1-naphthyl) ethylene diamine dihydrochloride, thiobarbituric acid, potassium nitrite, potassium ferricyanide, ascorbic acid, and ferric chloride were supplied by Sigma Chemicals. 1,1-Di-Phenyl-2-picrylHydrazyl, nitro blue tetrazolium, riboflavin and deoxyribose were purchased from Hi Media Chemicals Ltd. Mumbai, India. All reagents used for the experiment were of the analytical grade.

### **Extraction**

*V. negundo* leaves, stem and root were washed and dried at 55°C in an air dryer for 48h. Dried materials were powdered separately with a Wiley mill (Model 4276-M, Thomas Scientific, USA) to pass a 20 mesh sieve and stored in a sealed plastic bags. About 500 mg of various powders were taken in 5ml volumetric flask, mixed with 5ml of methanol and vortexed for 2 min followed by sonication (33 MHz, Roop Telesonic, India) at room temperature for 5 min. The process was repeated thrice for complete extraction. After sonication, methanolic extracts were combined and evaporated to dryness in *vacuo*. Dried extracts were obtained as 13.1 g leaves, 11.2 g stem and 12.5g root. Dried extracts were dissolved in methanol to prepare dilutions in a range of 100-500 µg/ml.

### **Phytochemical evaluation**

Preliminary phytochemical screening was carried out using standard procedures (24-25).

### **HPTLC Profile of *V. negundo***

Preparation of standard solution of negundoside (NG)

A stock solution of NG was prepared by dissolving 1 mg of accurately weighed NG in 1ml methanol. From this solution, 0.1 ml was taken and made up to 1ml with methanol in a 10 ml volumetric flask. Thus, the concentration of the stock solution of NG was 100µg/ml.

### **Calibration curve for NG**

Spots in the range of 1- 5 µl ( i.e. 100-500 ng/µl ) from the standard stock solution of NG was applied in triplicate on a TLC plate (20×10) .The distance between the spots and width of the spots was kept 6mm . The plate was developed in a solvent system of Toluene: ethyl-acetate: formic acid: water (2: 7: 0.8: 0.3 v/v) at 25 ± 2 °C temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air for 10 minutes, viewed in the UV Chamber and scanned at 258 nm. The R<sub>f</sub> values of the spots and peak areas were recorded. Calibration curves of NG were prepared by plotting peak area vs. concentration.

### **Preparation of standard solution of vitexin (VN)**

A stock solution of VN was prepared by dissolving 1mg of accurately weighed VN in 1 ml methanol. From this solution, 0.1 ml was taken and made up to 1 ml in a 10 ml volumetric flask. Thus, the concentration of standard solution of VN was 100 µg/ml.

### **Calibration curve for VN**

Spots in the range of 1-5µl (i.e. 100-500 ng) from standard solution of VN were applied in triplicate on a (20×10 TLC plate).The distance between the spots and width of each spot was kept 6mm. The plate was developed in a solvent system of Toluene: Ethyl acetate: Formic acid: water in the ratio of 2: 7: 0.8: 0.3 v/v at

25 ± 2 °C temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air for 10 minutes, viewed in the UV chamber and scanned at 258 nm. The R<sub>f</sub> values of the spots and peak areas were recorded. Calibration curves of VN were prepared by plotting peak area vs. concentration.

### **Sample application**

TLC profile of methanol extract of leaves, stem and roots was established using HPTLC. Methanol extract of the leaves, stem and roots were spotted in the range of 1- 5 µl ( i.e. 100-500 ng/µl ) on a precoated Silica-gel 60 F254 TLC plate (20×10) using Camag Linomat IV automatic sample spotter. The distance between the spots and width of the spots was kept 6mm. The plate was developed in a solvent system of Toluene: ethyl-acetate: formic acid: water (2: 7: 0.8: 0.3 v/v) at 25 ± 2 °C temperature and 40% relative humidity up to a distance of 8 cm. After development, the plate was dried in air for 10 minutes, viewed in the UV Chamber and scanned at 258 nm. The R<sub>f</sub> values of the spots and peak areas of resolved bands were recorded. Calibration curves of NG were prepared by plotting peak area vs. concentration (26-27).

### **Estimation of total phenolics**

The total phenol content (TPC) of methanol extracts of *V. negundo* was determined by the method of Folin-ciocalteu reaction using gallic acid as standard. To 100 µl of extract, add 500 µl of (50%) Folin-ciocalteu reagent followed by addition of 1ml of 20% Na<sub>2</sub>CO<sub>3</sub> solution. The mixture was incubated at room temperature for 20 min and absorbance was measured at 730 nm. The total phenol content was expressed as Gallic acid equivalents (GAE) in milligrams per gram samples (28).

### **Antioxidant assay**

#### **Determination of free radical or 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity**

The antioxidant activity, based on scavenging of stable DPPH free radical, was determined (29). Different concentrations of test sample were added to 3ml of 0.004% methanol solution of DPPH. Absorbance at 517 nm was measured after 30 min and percent inhibition activity was calculated as

$$\text{DPPH Scavenged (\%)} = (A \text{ cont.} - A \text{ test}) / (A \text{ cont.}) \times 100$$

Where, A cont. = Absorbance of control reaction

A test = Absorbance of test reaction

The antioxidant activity of the extract was expressed as IC<sub>50</sub>.

#### **Determination of nitric oxide (NO) radical scavenging activity**

Sodium nitroprusside in aqueous solution at physiological pH instinctively generates nitric oxide (NO), which intermingles with oxygen to generate nitrite ions that can be anticipated by Greiss reagent. Scavengers of NO compete with oxygen, leading to reduced production of nitrite ions. SNP (5 mM) in phosphate

buffer saline (PBS) was mixed with different concentration of (100-500 µg/ml) drug dissolved in suitable solvent and incubated at 25°C for 150 min. The above samples were reacted with Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride). The absorbance of chromophore created during diazotization of nitrite with sulphanilamide following coupling with naphthyl ethylene diamine was read at 546 nm and compared to the absorbance of standard (30-31).

#### **Superoxide anion (O<sub>2</sub><sup>-</sup>) radical scavenging activity**

The reaction mixture contains 50 mM ethylene diamine tetra acetate (EDTA), 0.1 mg/3 ml nitro blue tetrazolium (NBT), added in the order. Illuminating the reaction mixture with different concentration of sample extracts for 90 seconds started the reaction. Immediately after illumination, absorbance was measured at 590 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity (32). Ascorbic acid was used as a standard. O<sub>2</sub><sup>-</sup> radical scavenging activity was calculated by using following equation:

$$\text{O}_2^- \text{ Scavenged (\%)} = (A_0 - A_1) / (A_0) \times 100$$

Where A<sub>0</sub> was the absorbance of the control and A<sub>1</sub> was the absorbance of extract and the standard compound.

#### **Determination of hydroxyl radical (OH<sup>-</sup>) scavenging activity**

The OH<sup>-</sup> radical scavenging activity was measured according to the adapted method of Halliwell *et al.* (1987). Stock solution of EDTA (1 mM), ferric chloride (10 mM), ascorbic acid (1 mM), H<sub>2</sub>O<sub>2</sub> (10 mM) and deoxyribose (10 mM) were prepared in distilled deionized water. This assay was performed by adding 0.1 ml EDTA, 0.01 ml of FeCl<sub>3</sub>, 0.1 ml of H<sub>2</sub>O<sub>2</sub>, 0.36 ml of deoxyribose, 1.0 ml of extract (10-100 µg/ml) dissolved in distilled water, 0.33 ml of PBS (50 mM, pH 7.4) and 0.1 ml ascorbic acid in order. The reaction mixture was incubated at 37°C for 1 h. A 1.0 ml portion of incubated mixture was mixed with 1.0 ml of 10% TCA and 1.0 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop pink chromogen measured at 532 nm (33).

#### **Determination of H<sub>2</sub>O<sub>2</sub> radical scavenging activity**

A solution of H<sub>2</sub>O<sub>2</sub> was prepared in PBS (pH 7.4). H<sub>2</sub>O<sub>2</sub> concentration was determined spectrophotometrically, by measuring absorption with extinction coefficient for H<sub>2</sub>O<sub>2</sub> of 81 m<sup>-1</sup> cm<sup>-1</sup>. Extracts (100-500 µg/ml) in distilled water were added to H<sub>2</sub>O<sub>2</sub> solution (0.6 ml, 40 mM). Absorbance of H<sub>2</sub>O<sub>2</sub> at 230 nm was determined 10 min later against a blank solution containing PBS without H<sub>2</sub>O<sub>2</sub> (34).

#### **Lipid peroxidation activity**

Thoung *et al.* (2007) reported that inhibitory activity of kudingcha against mitochondrial lipid peroxidation was measured by the thiobarbituric acid reactive substance (TBARS) method. They also reported the preparation of mitochondria and measurement of lipid per oxidation was reported previously (35).

### **Statistical analysis**

Experimental results were mean  $\pm$ SEM of three parallel measurements. Analysis of variance was performed by ANOVA followed by Newmans - Keul multiple comparison test.  $p < 0.05$  was considered as significant.

## **Results & Discussion**

### **Preliminary phytochemical screening**

From the preliminary phytochemical evaluation it was proved that *V. negundo* leaves extract had alkaloids, glycosides, flavonoids, fixed oils, carbohydrates, amino-acids, terpenoids, steroids and phenols (Table 1).

### **HPTLC profile**

The HPTLC profile of methanol extract of leaves, stem and root extracts of *V. negundo* showed that leaves extract had greater concentration of negundoside (0.506% w/w) and vitexin (0.181 % w/w) when compared with the other extracts (Table 2).

### **Total phenol content**

The total phenol content was found to be maximum in leaves 461.0 mg GAE/100g (Table 3).

### **DPPH radical scavenging activity**

The free radical scavenging activity was demonstrated by DPPH and nitric oxide. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. DPPH radical was usually used as a substrate to evaluate antioxidant activity of antioxidants. The scavenging effect on the DPPH radical was greatest for leaf extract with an  $IC_{50}$  value of 0.221mg/ml at a conc. of 500  $\mu$ g/ml (Table 4).

### **Nitric oxide radical scavenging activity**

The extracts quenched NO (Nitric oxide) released by a NO donor, SNP (Sodium nitroprusside). The extracts significantly and dose dependently decreased the release of NO. The nitric oxide scavenging of *Vitex negundo* showed the methanol extract of stem to be more potent with an  $IC_{50}$  value of 0.310mg/ml at a concentration of 500  $\mu$ g/ml (Table 4).

### **Superoxide radical scavenging activity**

Drugs possessing superoxide scavenging activity decreases the reduction of NBT, which is a measure of superoxide anion scavenging activity that is indicated by reduction in absorbance at 560 nm. The results of the superoxide scavenging activity showed the stem extract to be most potent with an  $IC_{50}$  value of 0.290mg/ml (Table 4).

### **Hydroxyl radical scavenging activity**

The highly reactive  $OH^{\cdot}$  radical can cause oxidative damage to DNA, lipid and proteins. The Fenton reaction generate  $OH^{\cdot}$  radical, which degrade deoxyribose sugar of DNA using  $Fe^{2+}$  salt as an important catalytic component. The results of the hydroxyl radical scavenging activity showed the leaf extract to be potent with an  $IC_{50}$  value of 0.258mg/ml (Table 4).

**Peroxide radical scavenging activity**

H<sub>2</sub>O<sub>2</sub> radical itself not very reactive but it can sometimes be toxic to cell because it generates OH<sup>-</sup> radical in the cell. The results of the hydrogen peroxide scavenging activity showed the stem extract to be more potent with an IC<sub>50</sub> value of 0.275mg/ml (Table 4).

**Lipid peroxidation assay**

Decomposition of lipid membrane in the body leads to the formation of malondialdehyde (MDA) along with other aldehydes and enals as the end product. These react with thiobarbituric acid to form coloured complexes. Hence, these are called as the Thiobarbituric Acid Reactive Substances (TBARS). The complex of TBA-MDA is selectively detected at 532 nm using UV spectrophotometer. The leaf extract showed maximum anti-lipid peroxidation effect in liver homogenate with IC<sub>50</sub> value of 0.642 mg/ml (Table 4).

**Table1: Phytochemical analysis of methanol extracts of *V. negundo***

<b>Chemical Test</b>	<b>Leaf</b>	<b>Stem</b>	<b>Root</b>
Alkaloids	+	+	+
Terpenoids	+	+	-
Fixed oils	+	+	+
Steroid	+	-	+
Phenol	+	+	+
Saponin	+	+	-
Gums	-	-	+
Carbohydrates	+	+	+
Amino Acids	-	+	-
Glycosides	+	+	+
Resins	-	-	-
Tannins	+	+	-

(+ Present, - Absent)

**Table 2: HPTLC profile of *V. negundo* leaf, stem and roots**

Sample	Negundoside Peak area	R <sub>f</sub> value	Conc. (%w/w)	Vitexin Peak area	R <sub>f</sub> value	Conc. (%w/w)
VL	8102.4	0.21	0.506	1013.1	0.29	0.181
VS	7812.6	0.21	0.482	1005.3	0.29	0.162
VR	7462.0	0.21	0.453	973.4	0.29	0.086

**Fig 1: Peak overlay of marker compounds negundoside and vitexin**

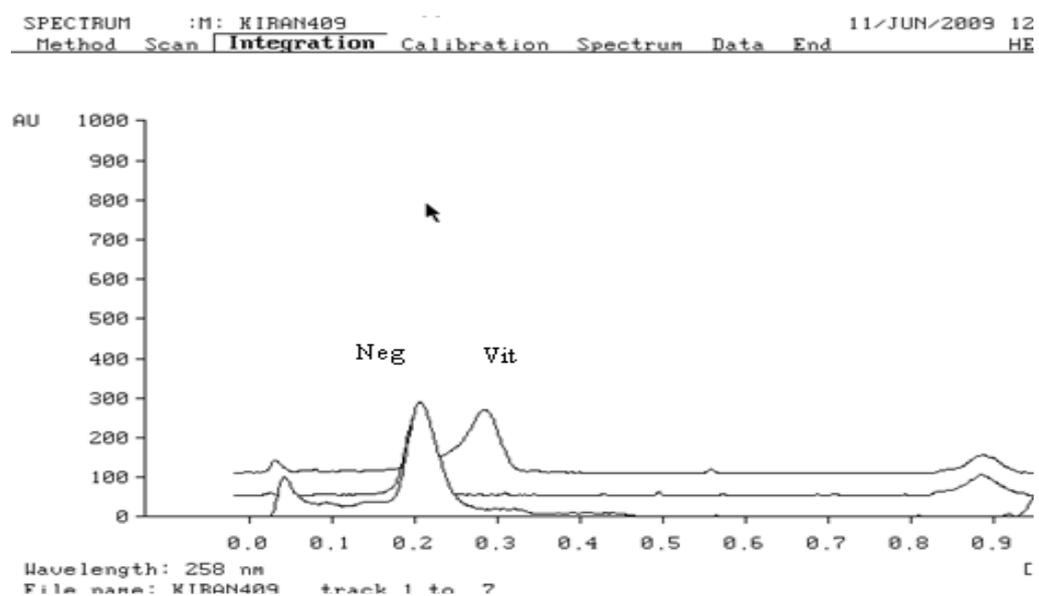


Fig 2: Spectra overlay of negundoside

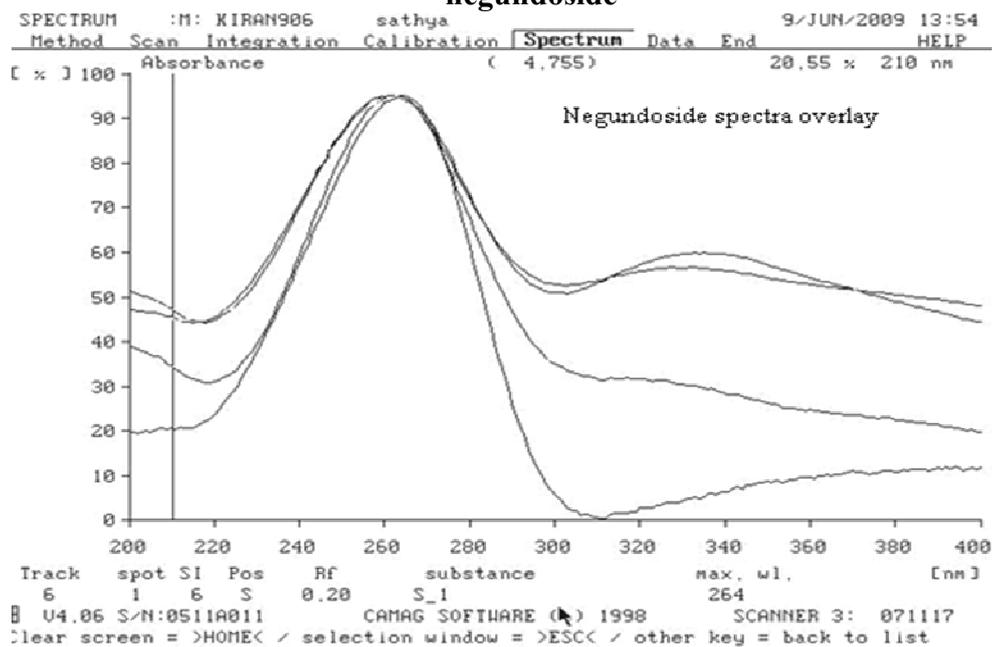
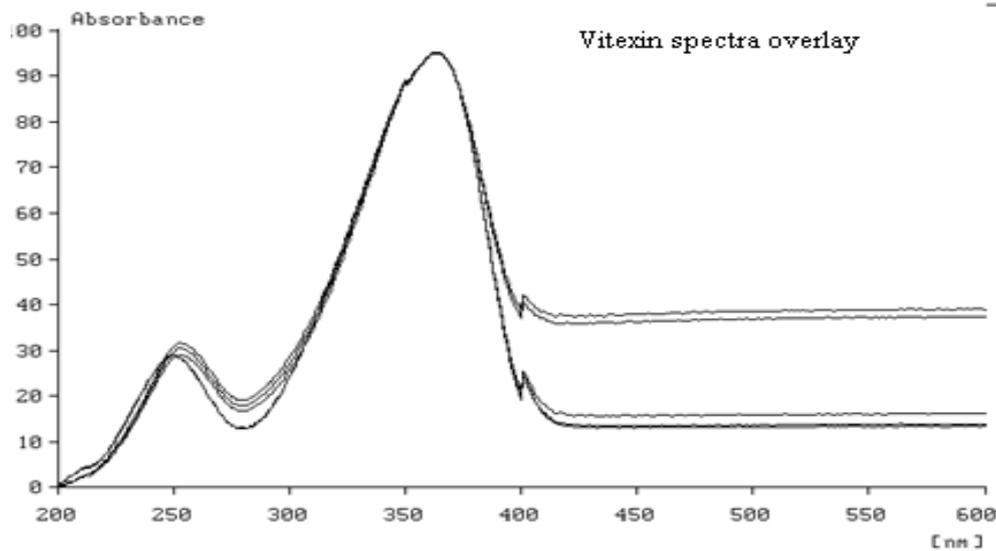


Fig 3: Spectra overlay of vitexin



**Table 3: Total phenol content in different samples of *V. negundo***

Samples	Phenol Content (mg GAE/100 g sample)
Leaves	461.0±0.001***
Stem	346.0±0.05*
Roots	283.0±0.01**

All values are expressed as mean ± SEM (n=3);

\*P<0.05 considered significant as compare to control

**Table 4: Free radical scavenging activity of methanol extracts of *V. negundo***

**IC<sub>50</sub> value (mg/ml)**

Free radical	Leaves	Stem	Roots
<b>DPPH</b>	0.221±0.05*	0.219±0.01**	0.176±0.02**
<b>H<sub>2</sub>O<sub>2</sub></b>	0.236±0.001***	0.275±0.01**	0.220±0.05*
<b>Superoxide</b>	0.218±0.05*	0.290±0.01**	0.181±0.01**
<b>Nitric Oxide</b>	0.266±0.001***	0.310±0.01**	0.205±0.01**
<b>Hydoxyl</b>	0.258±0.05*	0.217±0.05*	0.195±0.02**
<b>LPO (Liver)</b>	0.642±0.01**	0.623±0.03*	0.515±0.05*

All values are expressed as mean ± SEM (n=3);

\*P<0.05 considered significant as compared to control.

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