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Screening of Phytochemical and In Vitro Antioxidant Efficacy of *Vitex negundo* L. leaf Extract

Authors
M.Janakiraman[1], K.Jeyaprakash[2]
[1]Ph.D Research Scholar, PG and Research Department of Biochemistry,
Rajah Serfoji Govt College (Autonomous), Thanjavur -613 005, Tamilnadu, India
Email: *mugavaiprofmj@gmail.com*
[2]Head, PG and Research Department of Biochemistry,
Rajah Serfoji Govt College (Autonomous), Thanjavur -613 005, Tamilnadu, India
Email: *jeypee5@gmail.com*

Abstract
Objective: *The present study was carried out to evaluate the qualitative and quantitative phytochemical and In vitro antioxidant activities of methanolic leaf extract of vitex negundo.*
Materials and Methods: *Vitex negundo leaves were extracted with three solvents like methanol,ethanol and water. Three extracts of vitex negundo were tested for different phytoconsituents and the In vitro antioxidant activity of the methanolic extract was studied by using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, ferric reducing power activity, hyderogen peroxide scavenging activity, superoxide scavenging activity and nitricoxide scavenging activity. The total Phenolic contents and total flavanoid contents were also estimated.*
Results: *The yield of phytochemicals is in the order of methanol extract > ethanol extract > water were obtained. Finally, methanolic extract was selected for investigation on the analysis of total phenol content, total flavanoid content and in vitro antioxidant activity. Total phenolic content was estimated as 163.43 mg/g of extract and total flavonoid content was estimated 86.25 mg/g of extract. Free radical scavenging activity was determined by DPPH assay (86.2.% inhibition at 1000 µg/ml concentration),ferric reducing power scavenging activity was determined (95.2% inhibition at 1000 µg/ml concentration), hydrogen peroxide scavenging activity was determined (49.2.% inhibition at 1000 µg/ml concentration),superoxide radical scavenging activity was determined (77.2 .% inhibition at 1000 µg/ml concentration) and nitricoxide radical scavenging activity was determined (98.4.% inhibition at 1000 µg/ml concentration) at highest concentration of methanol extract of vitex negundo in this study.*
Conclusion: *The present study concluded that the in vitro antioxidant activity of Vitex negundo L. was mainly due to the presence of the phenolic compounds like tannins and flavonoid compounds like quercetin.*
Key words: *Phytochemical,Vitex negundo L, In vitro antioxidant, Total phenols, Total flavonoids*

1. Introduction

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. [1].

The effects of free radicals on human beings are closely related to toxicity, disease and aging. [2].

Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS). Reactive oxygen species (ROS), include free radicals such as superoxide (O^{2-}) hydroxyl radical ($^{\cdot}OH$), peroxyl radical ($ROO^{\cdot}$) as well as non-radical species such as hydrogen peroxide (H_2O_2) [3].Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human

diseases including cancer, atherosclerosis and the aging process [4].

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 [5].

Vitex negundo Linn. (Lamiaceae) known as vellanochi in tamil and 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 [6]. 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 [7]. 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 [8]. They are also used as tonics, vermifuge, lactagogue, anti bacterial, antipyretic and anti histaminic agents [9].As the leaves of *Vitex negundo* L. possess the many medicinal properties, this study was carried out to investigate the qualitative analysis of phytochemicals,quantitative analysis of the total phenols, total flavanoid content and in vitro antioxidant properties of methanolic extract of *Vitex negundo Linn.*

2. Materials and Methods

2.1. Plant Material

The leaves of *Vitex negundo* were collected from Manapparai, near Trichy district, Tamilnadu, India. The botanical identity of the plant material was authenticated by Botanical Survey of India, Coimbatore, Tamilnadu, India and a voucher specimen of the plant material was deposited in the department under the number BSI/SRC/5/23/2014-2015/TECH/540 for further study.

2.2. Chemicals

All chemicals were procured from Ponmani Chemicals Suppliers, Tiruchirappalli, Tamilnadu, India.

2.3. Preparation of Extract

Vitex negundo leaves were dried under shade and then powdered with a mechanical grinder to obtain a coarse powder. 25gms of the *Vitex negundo* powder was transferred into different conical flask (250ml).The conical flask containing 100ml of different solvents viz. Ethanol, Methanol and water. The conical flask containing plant powder and solvent was shaked it well for 48 hours by free hand. After 3 days, the extracts were filtered using whatmann filter paper No.1 and were transferred into china dish. The supernatant was completely removed by keeping the china dish over a boiling water bath at 45°c. The obtained extracts were stored at 4°c in air tight bottle until further use.

2.4. Phytochemicals Screening

2.4.1. Qualitative Phytochemicals Screening

Methanol,ethanol and water extract of *vitex negundo* were tested for different phytoconsituents using standard procedures [10].

2.4.2. Quantitative Phytochemicals Screening

2.4.2(A).Determination of Total Phenol

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to mark in the 50ml flask and left to react for 30 min for colour development. This was measured at 505 nm [11].

2.4.2(B).Determination of Total Flavanoids

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No.42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight [12].

2.5.Determination of In vitro Antioxidant activity

2.5.1. Determination of DPPH scavenging activity

Different aliquots of 0.2 to 1 ml of sample extract solutions were taken in different test tubes. To these entire tubes methanol was added and made up to 1ml. To this 4m1 of methanolic DPPH was added and shaken well. The mixture was allowed to stand at room temperature for 20min. The control contains only methanol and DPPH. The readings were noted at 517 nm against methanolic blank. The change in absorbance of the samples was measured. Free radical scavenging activity was expressed as the inhibition percentage calculated by using the formula Percentage of antioxidant activity = [A - B/A] x 100.Where, 'A' is absorbance of control & 'B' is absorbance of sample [13].

2.5.2. Determination of Reducing Power Activity

1 ml of plant extract was mixed with phosphate buffer (2.5 ml 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml). The mixture was incubated at 50˚c for 20 minutes. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5ml) was mixed with distilled water (2.5ml) and Ferric cloride (0.5ml, 0.1%) and absorbance measured at 700nm. Increased absorbance of the reaction mixture indicates stronger reducing power. The activity was compared with ascorbic acid standard. Percentage inhibition scavenging activity was calculated by ($A_{control}$xA_{test}/$A_{control}$) x100.Where $A_{control}$ is the absorbance of the control. A_{test} is the absorbance in the presence of the sample [14].

2.5.3. Determination of Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide solution (2 mM/L) was prepared with standard phosphate buffer (pH 7.4). Different concentration of the extracts in distilled water was added to 0.6 ml of hydrogen peroxide solution. Absorbance was determined at 230 nm after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. Ascorbic acid was used as standard. Percentage inhibition of H_2O_2 radical scavenging activity was calculated by ($A_{control}$xA_{test}/$A_{control}$) x 100.Where $A_{control}$ is the absorbance of the control. A_{test} is the absorbance in the presence of the sample [15].

2.5.4. Determination of Superoxide Scavenging Activity

Superoxide anions were generated in samples that contained in 3.0ml, 0.02ml of the leaf extracts (20mg), 0.2ml of EDTA, 0.1ml of NBT, 0.05ml of riboflavin and 2.64ml of phosphate buffer. The control tubes were also set up where DMSO was added instead of the plant extracts. All the tubes were vortexed and the initial optical density was measured at 560nm in a spectrophotometer. The tubes were illuminated using a fluorescent lamp for 30 minutes. The absorbance was measured again at 560nm. The difference in absorbance before and after illumination was indicative of superoxide anion scavenging activity [16].

2.5.5. Determination of Nitricoxide Scavenging Activity

The reaction was initiated by adding 2.0ml of sodium nitroprusside, 0.5ml of PBS, 0.5ml of leaf extracts (50mg) and incubated at 25°C for 30 minutes. Griess reagent (0.5ml) was added and incubated for another 30 minutes. Control tubes were prepared without the extracts. The absorbance was read at 546nm against the reagent blank, in a spectrophotometer [17].

2.6. Statistical analysis:

The determinations were conducted in triplicate and results were expressed as mean ± SD. Statistical analysis were done by analysis of variance using ANOVA test followed by student' t- test with $P < 0.05$ as considered as significance.

3. Results

3.1. Result of Preliminary Phytochemical Analysis

Different phytochemicals like alkaloids, glycosides, saponinins, tannins, terpenoids, reducing sugars, phenolic compounds, flavanoids, protein and carbohydrates were identified and the results were given in Table.1. Our results indicates the presence of phytochemicals in the order of methanol extract > ethanol extract > water extract. The above data showed the more yield of phytochemicals in methanol extract and hence the methanolic extract was selected for the further studies like total phenolics, total flavonoids and in vitro antioxidant studies.

3.2. Results for Total Phenol and Total flavanoid contents

Total phenol content and total flavanoid content were given in Table 2. The total phenol content of *Vitex negundo* was found to have 163.45±0.13 mg/g and the total flavanoid content of *Vitex negundo* was found to have 86.25±0.11 mg/g respectively.

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

Phytochemicals	Ethanol	Methanol	Water
Tannin	++	+	++
Phlobatannins	-	-	-
Saponin	++	++	++
Flavonoids	+	++	+
Steroids	++	++	++
Terpenoids	+	++	+
Triterpenoids	+	+	++
Alkaloids	-	+	-
Carbohydrate	+	+	+
Amino acid	-	+	-
Anthroquinone	+	++	++
Polyphenol	+	++	+
Glycoside	+	+	+

Table 2.Quantitative Analysis of *Vitex negundo* extract.

Phytoconstituents	Results (mg/gm)
Total Phenols	163.45
Total flavonoids	86.25

3.3. Results of In vitro Antioxidant Activity

3.3.1. DPPH free radical scavenging activity

Table 3 and fig 1 shows the DPPH scavenging effect increased with the increasing concentrations of *Vitex negundo* leaf extract as compared to standard ascorbic acid and highest DPPH scavenging activity of *Vitex negundo* was observed as 86.2. % inhibition at 1000 μg/ml concentration which indicates the DPPH scavenging effective of *vitex negundo* as compared to ascorbic acid.

Table 3: DPPH free radical scavenging activity of *Vitex negundo* and standard ascorbic acid.

Concentration (μg/ml)	DPPH (% inhibition)	
	Vitex negundo	Ascorbic acid (standard)
200	20.5±0.02*	28.6±0.05
400	52.2±0.07*	59.4±0.01
600	68.1±0.01*	73.6±0.04
800	79.4±0.06*	86.2±0.02
1000	86.2±0.02*	94.1±0.05

All values were expressed as mean±SD (n=3). Statistically significant of *p < 0.05 compared to standard.

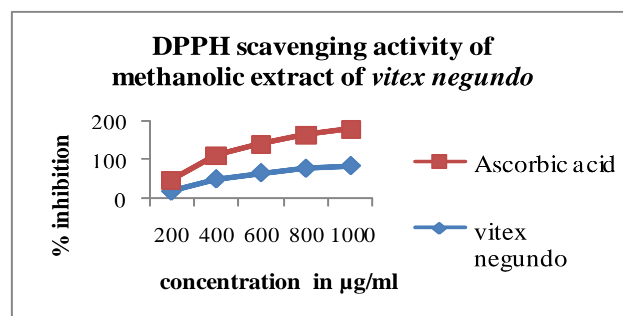

Fig 1. DPPH free radical scavenging activity of methanolic extract of *Vitex negundo* and standard ascorbic acid.

3.3.2. Ferric Reducing Power Activity

Table 4 and fig 2 shows the ferric reducing power effect increased with the increasing concentrations of *Vitex negundo* leaf extract as compared to standard ascorbic acid and highest ferric reducing power activity of *Vitex negundo* was observed as 95.2% inhibition at 1000 μg/ml concentration which indicates the reducing power effective of *Vitex negundo* as compared to ascorbic acid.

Table 4: Reducing power activity of *Vitex negundo* and standard ascorbic acid.

Concentration (µg/ml)	Reducing power activity (% inhibition)	
	Vitex negundo	Ascorbic acid (standard)
200	28.9±0.01*	32.6±0.02
400	61.2±0.03*	65.4±0.01
600	77.3±0.01*	81.2±0.03
800	88.4±0.02*	92.2±0.01
1000	95.2±0.01*	99.3±0.02

All values were expressed as mean±SD (n=3).Statistically significant of *p < 0.05 compared to standard.

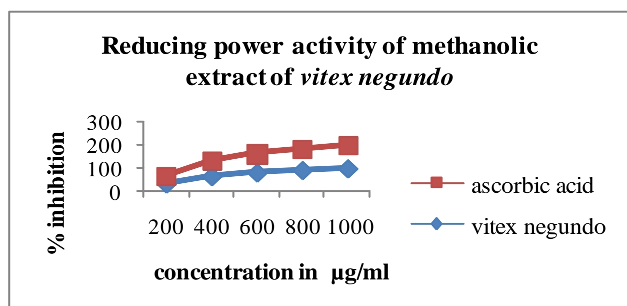

Fig 2: Reducing power activity of *Vitex negundo* and standard ascorbic acid.

3.3.3..Hydrogen Peroxide Scavenging Activity

Table 5 and fig 3 shows the hydrogen peroxide scavenging effect increased with the increasing concentrations of *Vitex negundo* leaf extract as compared to standard ascorbic acid and highest hydrogen peroxide scavenging activity of *Vitex negundo* was observed as 49.2 % inhibition at 1000 µg/ml concentration which indicates the hydrogen peroxide scavenging of *Vitex negundo* as compared to ascorbic acid.

Table 5: Hydrogen peroxide scavenging activity of *Vitex negundo* and standard ascorbic acid.

Concentration (µg/ml)	Hydrogen peroxide scavenging activity (% inhibition)	
	Vitex negundo	Ascorbic acid (standard)
200	10.2±0.01*	17.9±0.03
400	19.1±0.03*	36.2±0.01
600	28.6±0.01*	52.4±0.03
800	39.1±0.04*	71.2±0.02
1000	49.2±0.01*	87.6±0.01

All values were expressed as mean±SD (n=3). Statistically significant of *p<0.05 compared to standard.

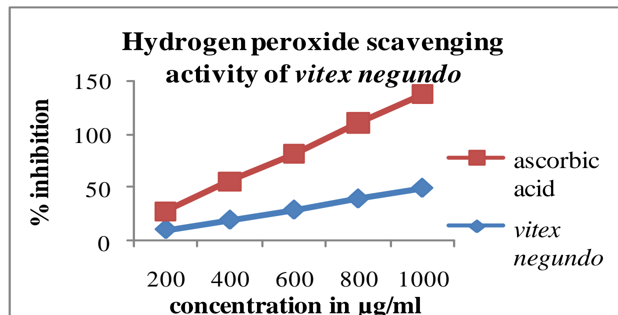

Fig 3: Hydrogen peroxide scavenging activity of methanolic extract of *Vitex negundo* and standard ascorbic acid.

3.3.4. Superoxide Scavenging Activity

Table 6 and fig 4 shows the superoxide scavenging effect increased with the increasing concentrations of *Vitex negundo* leaf extract as compared to standard ascorbic acid and highest superoxide scavenging effect of *Vitex negundo* was observed as 77.2 .% inhibition at 1000 µg/ml concentration which indicates the superoxide scavenging effective of *Vitex negundo* as compared to ascorbic acid.

Table 6: Superoxide radical scavenging activity of *Vitex negundo* and standard ascorbic acid.

Concentration (µg/ml)	Superoxide radical scavenging activity (% inhibition)	
	Vitex negundo	Ascorbic acid (standard)
200	15.2±0.02*	23.1±0.03
400	32.1±0.04*	45.4±0.01
600	43.4±0.06*	64.1±0.03
800	62.4±0.01*	84.6±0.02
1000	77.2±0.03*	97.2±0.01

All values were expressed as mean±SD(n=3).Statistically significant of *p < 0.05 compared to standard.

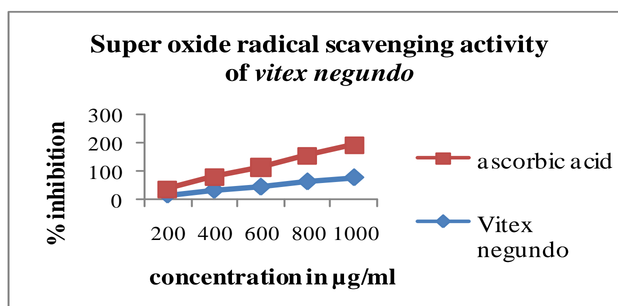

Fig 4: Superoxide radical scavenging activity of methanolic extract of *Vitex negundo* and standard ascorbic acid.

3.3.5..Nitricoxide Scavenging Activity

Table 7 and fig 5 shows the nitricoxide scavenging effect increased with the increasing concentrations of *Vitex negundo* leaf extract as compared to standard ascorbic acid and highest nitricoxide scavenging effect of *Vitex negundo* was observed as 98.4.% inhibition at 1000 µg/ml concentration which shows the nitricoxide scavenging effective of *Vitex negundo* as compared to ascorbic acid.

Table 7: Nitricoxide radical scavenging activity of *Vitex negundo* and standard ascorbic acid.

Concentration (µg/ml)	Nitric oxide radical scavenging activity (% inhibition)	
	Vitex negundo	Ascorbic acid (standard)
200	22.1±0.01*	30.1±0.01
400	39.2±0.03*	46.4±0.02
600	61.8±0.04*	68.2±0.02
800	84.2±0.01*	89.8±0.03
1000	98.4±0.06*	106.2±0.01

All values were expressed as mean± SD(n=3).Statistically significant of *p < 0.05 compared to standard

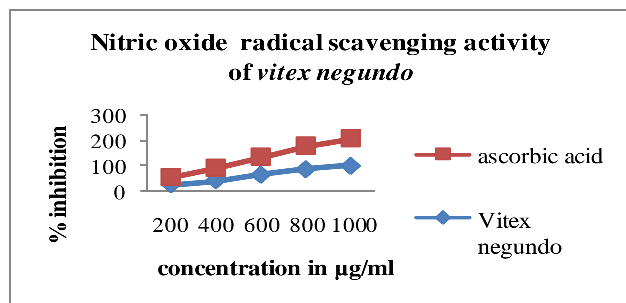

Fig 5: Nitric oxide radical scavenging activity of methanolic extract of *Vitex negundo* and standard ascorbic acid.

4. Discussion

The presence of secondary metabolites like tannins, saponin, carbohydrates, glycosides, alkaloids, flavonoids, terpenoids, steroids, polyphenols and anthraquinones of *vitex negundo* suggests that the plant might be of medicinal importance and supports the bases for some of the ethno-uses[18].Due to the presence of flavonoids and phenol suggests that the plant might have an antioxidant, anti-allergic, anti-inflammatory, antimicrobial, anticancer activity [19]. The presence of tannins shows that the plant is astringent as documented and suggests that it might have antiviral and antibacterial activities and can aid in wound healing and burns [20]. Saponins and glycoside are also very important classes of secondary metabolites as some are cardio active and used in treatment of heart conditions [21].

The phenolic and flavanoids are widely distributed secondary metabolites in plants having antioxidant activity and have wide range of biological activities as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, anti-atherosclerosis, cardiovascular protection and improvement of endothelial function, as well as inhibition of angiogenesis and cell proliferation activities [22]. This study also proved that many dietary polyphenolic and flavanoid constituents derived from *Vitex negundo* plants are more effective antioxidants than ascorbic acid and thus might contribute significantly to the protective effects in vivo [23].

The present study was also revealed the in vitro antioxidant properties of *Vitex negundo* which might be due to the presence of phenolic and flavanoid compounds in methanolic extract[24]. DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichiometrically with the number of electrons taken up [25] Such reactivity has been widely used to test the ability of compounds of plant extracts to act as free radical scavengers [26].Though the extracts showed good DPPH scavenging activity but it was less effective than standard Ascorbic acid. This activity is due to presence of phenolic and flavanoid components in the plant extracts[27].

In ferric reducing antioxidant power assay, a yellow colour of the test solution changes to various shades of green and blue is depending upon the reducing power of each compound. The presence of radicals causes the conversion of the ferricyanide complex used in this method to the ferrous form. Therefore by measuring the formation of pearls Prussian blue spectroscopically, a higher absorbance indicates a higher reducing power[28]. Reducing power of activity in the *Vitex negundo* extracts indicated that some components in the extract were electron donors that could react with the free radicals to

convert them into more stable products to terminate radical chain reaction [29].

Hydrogen peroxide is a biologically relevant, non-radical oxidizing species may be formed in tissues through oxidative processes. Hydrogen peroxide which in turn generate hydroxyl radicals (•OH) resulting in initiation and propagation of lipid peroxidation. The ability of the extracts to quench (•OH) seems to be directly related to the prevention of the lipid peroxidation and appears to be moderate scavenger of active reactive oxygen species [30]. Methanolic extract of *vitex negundo* showed good hydrogen peroxide scavenging activity but it was less effective than standard ascorbic acid[31].

Superoxide anion radical is generated by four-electron reduction of molecular oxygen into water. This radical also formed in aerobic cells due to electron leakage from the electron transport chain super oxides are generated from molecular oxygen of oxidative enzymes and as well as non-enzymatic reactions such as auto oxidation by catecholamines [32]. The studies also proved on super oxide free radical scavenging activity of methanolic leaf extract of *Vitex negundo* was noticed significant reduction of the super oxide anions[33].

Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antimicrobial and antitumor activities. [34]. Methanolic plant extracts showed more nitric oxide scavenging activity but it was less effective than standard ascorbic acid [35].

5. Conclusion

According to the results of this study, it was clearly indicated that the methanol extract of *vitex negundo* has significant in vitro antioxidant activity. *Vitex negundo* can be used as easily accessible source of natural antioxidants and as a possible food supplement industry and pharmaceutical industry. Therefore, it was suggested that further study could be performed on the isolation and characterization of the antioxidant content of the *vitex negundo.*

Conflict of Interest

The authors declare that there are no conflicts of interest. The research received no specific grant from any funding agency in the public, community, or non-for profit sectors.

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Authors Profile

Dr.K.Jeyaprakash working as Head, PG and Research Department of Biochemistry, Rajah Serfoji Govt College (Autonomous),Thanjavur, Tamilnadu, India. He has got more than 20 years of teaching experience and published more than 25 papers in national and international journals. he also guiding M.Phil and Ph.D research scholars in Bharathidasn university and Bharathiyar university, Tamilnadu, India.

Janakiraman M completed M.Sc and M.Phil Degree in Biochemistry from Bharathidasan university, Tiruchi, Tamilnadu, India. Currently, he is pursuing Ph.D in Biochemistry, PG and Research Department of Biochemistry,Rajah Serfoji Govt College(Autonomous), Thanjavur, Tamilnadu, India.