Studies on Analysis of Antioxidant and Enzyme Inhibitory Activity of *Vitex negundo* Linn.

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ABSTRACT

The current study was designed to investigate the leaf extracts of *Vitex negundo* Linn. for their antioxidant and enzyme inhibitory (α-amylae and urease) activity. The antioxidant capacity of the different extracts (methanol, acetone and aqueous) of this plant was evaluated by DPPH (1,1-diphenyl-2-picrylhydrazyl) and reducing power tests. The plant exhibited good DPPH radical scavenging activity and moderate reducing power potential Further, all the extracts of *V. negundo* were reported to possess good anti-alpha amylase and anti-urease activity of greater than 50% in all the solvents used at a concentration of 1 mg/mL. Thus the study provided scientific evidence to the traditional uses of this plant in the treatment of obesity, diabetes, ulcers, kidney stones etc. Therefore, the leaf extracts of this plant can be selected for further investigation to determine their therapeutic potential.

Keywords: *Vitex negundo*, leaf extracts, DPPH, reducing power, α-amylase, urease.

INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and a sufficient number of modern drugs have been derived from natural sources, many of these isolations were based on the use of these agents in traditional medicine[1]. The use of plants for treating various diseases is as old as the human species. A plant is said to be medicinal if it produces active compounds which are therapeutically more effective[2]. Plants are traditionally being used for medicinal treatment of numerous human disorders including infectious diseases caused by different microorganisms.

Medicinal plant parts are commonly rich in phenolic compounds such as flavonoids, phenols, stilbenes, tannins, coumarins, lignans, lignins etc. These chemical compounds have multiple biological effects including antimicrobial and antioxidant[3, 4]. So the medicinal plants with antioxidant potential are usually employed as an alternative source of medicine to mitigate the diseases associated with oxidative stress[5,6].

Antioxidants, also called inhibitors of oxidation, are compounds which retard or prevent the oxidation and in general prolong the life of oxidizable matter[7]. The oxidants or free radicals are species with very short half-life, high reactivity and damaging activity towards macromolecules like proteins, lipids and DNA. Antioxidant-based drug formulations are widely used for the prevention and treatment of complex diseases such as Alzheimer's disease, cancer, atherosclerosis, stroke and diabetes[8].

There is an increasing interest in natural antioxidants present in medicinal plants, which might help preventing oxidative damages. It has been established that the antioxidant effect of plant and plant-based products is mainly attributed to phenolic compounds such as flavonoids and phenolic acids[9]. Phenolic compounds from medicinal plants exhibit strong antioxidant activity and may help to protect the cells against the oxidative damage caused by free radicals[10]. Antioxidants from plants mediate oxidative damage caused by ROS (Reactive Oxygen Species), and research has focused on the role of antioxidants for the treatment and prevention of deadly diseases.

Enzyme inhibition by plant derived products has now become an important part of the modern drug discovery research. Studies in this field have already led to the discovery of wide variety of drugs useful in a number of diseases and have been used for treating certain physiological disorders. Specific inhibitors mostly interact with enzymes and block their progression towards their corresponding natural and synthetic substrates[11]. α-amylase and urease are two important enzymes that are associated with a number of clinical conditions. α-Amylase and its inhibitors are drug design targets for the treatment of certain disorders like diabetes, obesity and hyperlipaemia[12]. To overcome these detrimental effects and identify natural inhibitors of α-amylases from plant based sources is now the primary concern of scientific research. Furthermore, urease contributes to arthritis and gastric intestinal infections and ultimately the urease imbalance leads to peptic ulcers[13]. Urease inhibitors may be effective therapies for the treatment of various diseases such as ulcers, kidney stones, caused by urease-dependent pathogenic microbes. However, the commercially available urease inhibitors are of low stability and toxic which prevent their clinical use[14]. Therefore, the search for novel urease inhibitors with improved stability and low

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toxicity is necessary to improve life quality of human beings and other animals.

Vitex negundo Linn. (commonly known as Nirgundi) belonging to family Verbenaceae occurs in tropical to temperate regions (up to 2200 m from east to west) and grows gregariously in wastelands and mixed open forests. This species is grown commercially as a crop in parts of Asia, Europe, North America and West Indies. Within India, it is found throughout the greater part of India, ascending to an altitude of 1500 m in the outer Himalayas[15].

The leaves of *V. negundo* are antibacterial, antitumor, astringent, febrifuge, sedative, tonic, vermifuge and anodyne. Leaves also possess certain other medicinal properties e.g. inflammatory, analgesic, removes foetid discharges and worms from ulcers, analgesic, antihistaminic property, snake venom neutralizing capacity, hepatoprotective and CNS depressant activities Flowers are astringent, febrifuge, anti diarrhoeic and prescribed in liver complaints[16]. In view of its above mentioned useful properties, we planned to analyse *Vitex negundo* for its antioxidant and enzyme inhibitory activities (anti-alpha amylase and anti-urease).

MATERIALS AND METHODS

Collection of Plant Material
Leaves of *Vitex negundo* were plucked and collected from Devthana-Nandri area of District Sirmaur, Himachal Pradesh, India. The collected plant material was brought to the laboratory for further analysis.

Processing of Plant Material
Leaves of *V. negundo* were washed thoroughly under tap water and then with 2% Mercuric chloride. After that the leaves were cut into smaller pieces for quick drying. Cleaned leaves were shade dried for 15-20 days. The dried plant material was crushed into fine powder with the help of pestle mortar. Finally the fine powder was stored in an air tight container at room temperature.

Antioxidant Activity Test
DPPH Radical Scavenging Activity Assay
The free radical scavenging activity of plant extracts was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described by Blois[17]. Briefly, to 1 mL of different concentrations (20, 40, 60, 80 and 100 µg/mL) of plant or test extract, 1 mL of DPPH (0.1 Mm in methanol) was added. Corresponding blank sample was prepared and ascorbic acid was used as reference standard. Mixture of 1 mL methanol and 1 mL DPPH solution (without plant extract) was used as control. All the tests were carried out in triplicate and the decrease in absorbance was measured at 517 nm after 30 minutes in dark using UV-VIS spectrophotometer. The percentage of inhibition was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Where, $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of sample

Graphs were plotted against percent inhibition v/s conc. of plant extracts and standard ascorbic acid in order to find out the values of slope and y-intercepts. IC$_{50}$ value (the amount of antioxidant required to decrease the initial

DPPH concentration by 50%) for each extract and ascorbic acid was evaluated using the following equation given below:

$$IC_{50} = \frac{50 - Y - Intercept}{Slope}$$

Reducing Power Assay
The reducing power was determined according to the method described by Oyaizu[18]. Different concentrations of plant extract (20, 40, 60, 80 and 100 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K$_3$Fe(CN)$_6$] (2.5 mL, 1%). 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 minutes. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl$_3$ (0.5 mL, 0.1%) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as the standard. Phosphate buffer (pH 6.6) was used as blank solution. Higher absorbance of the reaction mixture indicated greater reductive potential. Experiment was performed in triplicates at each concentration to evaluate percent reducing power. The % reducing power (antioxidant activity) was calculated by using the formula:

$$\text{\% Reducing power} = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

Where, $A_{control}$ is the absorbance of control; A_{sample} is the absorbance of sample

Graphs were plotted against percent inhibition v/s conc. of plant extracts and standard ascorbic acid in order to find out the values of slope and y-intercepts. IC$_{50}$ value for each extract and ascorbic acid was evaluated.

Enzyme Inhibitory Activity Test
α-Amylase inhibition assay
The α-amylase inhibition activity of different plant extracts was determined by some modifications in the method proposed by Giancarlo *et al.*[19]. The starch solution (1% w/v) was obtained by boiling and stirring 1 g of potato starch in 100 mL of sodium phosphate buffer for about 30 minutes. The porcine pancreatic α-amylase enzyme (EC 3.2.1.1; purchased from Sigma Aldrich-3176) was prepared by mixing 0.01 g of α-amylase in 10 mL of sodium phosphate buffer (pH 6.9) containing 0.0006 Mm sodium chloride. The extracts were dissolved in DMSO to give concentrations from 0.2 to 1.0 mg/ mL (0.2, 0.4, 0.6, 0.8 and 1.0 mg/ mL). The colour reagent was a solution containing 0.1 g of 3,5-dinitrosalicylic acid plus 2.99 g sodium potassium tartrate in 0.16 g sodium hydroxide and phosphate buffer (10 mL). Fifty microliter of each plant extract and 150 mL of starch solution as well as 10 mL of enzyme were mixed in a 96 well plate and incubated at 37°C for 30 min. Then, 20 mL of sodium hydroxide and 20 mL of colour reagent were added and the closed plate placed into a 100°C water bath. After 20 min, the reaction mixture was removed from the water bath and allowed for cooling, thereafter α-amylase activity was determined by measuring the absorbance of the mixture at 540 nm using a UV-VIS spectrophotometer. Blank samples were used to correct the absorption of the mixture, in which the enzyme was replaced with the buffer solution. Also, a control

Table 1: Free radical (DPPH) scavenging activity (%) of the tested medicinal plants at different concentrations.

Concentration (µg/mL)	Methanol extract	Acetone extract	Aqueous extract	Ascorbic acid
20	19.05±2.00	17.00±0.00	19.40±0.75	35.24±0.50
40	29.00±0.00	29.80±2.20	26.20±1.40	50.54±0.42
60	40.00±0.00	42.10±1.30	34.50±1.70	62.35±1.20
80	54.34±1.80	54.66±1.30	48.00±0.55	74.14±0.00
100	68.72±2.44	62.00±2.00	54.70±0.45	83.26±2.20
IC_{50} (µg/mL)	72.52	75.51	89.09	41.44

Values are given as mean ± SD

Table 2: Antioxidant activity percentage (%) of tested medicinal plants by reducing power method at different concentrations.

Concentration (µg/mL)	Methanol extract	Acetone extract	Aqueous extract	Ascorbic acid
20	18.25±1.40	11.50±0.65	11.25±0.70	26.55±2.25
40	29.90±0.15	20.55±2.20	20.20±1.45	43.44±0.45
60	38.00±0.00	31.80±0.30	31.56±2.70	59.90±1.20
80	45.34±1.20	40.56±1.00	39.00±0.55	72.15±0.54
100	57.70±2.44	49.10±2.30	46.40±1.45	88.30±1.50
IC_{50} (µg/mL)	85.91	100.54	105.72	49.40

Values are given as mean ± SD

Table 3: α-Amylase inhibitory activity (%) of the tested medicinal plant extracts at different concentrations.

Concentration (µg/mL)	Methanol extract	Acetone extract	Aqueous extract	Acarbose
0.2	21.10±2.05	17.30±0.30	19.00±1.66	29.50±0.70
0.4	33.00±0.90	26.80±1.20	25.20±1.40	40.85±2.15
0.6	45.20±0.30	32.15±0.38	32.50±0.70	56.45±1.25
0.8	57.34±1.80	44.16±1.30	40.40±0.55	66.22±0.52
1.0	66.27±2.25	55.60±2.22	51.75±0.44	78.56±0.45
IC_{50} (µg/mL)	0.79	0.91	0.99	0.53

Values are given as mean ± SD

Table 4: Urease Inhibitory activity (%) of the tested plant extracts at different concentrations.

Concentration (µg/mL)	Methanol extract	Acetone extract	Aqueous extract	Thiourea
0.2	24.10±2.05	18.30±0.30	19.20±1.15	28.38±0.78
0.4	31.00±0.90	29.80±2.20	26.20±1.40	41.58±0.55
0.6	44.20±0.30	42.10±0.30	34.50±0.70	56.30±1.20
0.8	55.34±1.80	54.66±1.30	44.00±0.55	69.20±0.50
1.0	69.72±2.20	60.00±2.00	52.70±0.40	81.26±1.25
IC_{50} (µg/mL)	0.68	0.76	0.94	0.51

Values are given as mean ± SD

reaction was used, in which the plant extract was replaced with 50 mL of DMSO and the maximum enzyme activity was determined. Acarbose solution was used as a positive control in the concentration range of 0.2-1.0 mg/mL. The assay was performed in triplicate and the mean absorbance was used to calculate percentage of α-amylase inhibition. The inhibition percentage of α-amylase was assessed by the following formula:

$$\% \text{ α-Amylase Inhibition} = \left(\frac{\Delta A \text{control} - \Delta A \text{sample}}{\Delta A \text{control}}\right) \times 100$$

Where, $\Delta A_{control} = A_{test} - A_{Blank}$

$\Delta A_{sample} = A_{test} - A_{Blank}$

The concentration of the extract (inhibitor) required for 50% of enzyme inhibition (IC_{50}) for all the crude extracts was determined from corresponding dose-response curves of percentage inhibition versus inhibitor concentration and compared to acarbose, a known inhibitor of α-amylase. The % Inhibition was plotted against the concentration of a sample and a logarithmic regression curve was established to calculate the IC_{50} value for each sample which is the concentration of the given sample required to inhibit the activity of urease enzyme by 50%. Data are expressed as mean ± standard deviation (SD).

Urease inhibition assay

The enzyme inhibition was determined through catalytic effects of urease on urea by measuring change in absorbance in the absence and in the presence of inhibitor at 640 nm, using UV-VIS spectrophotometer. All the plant
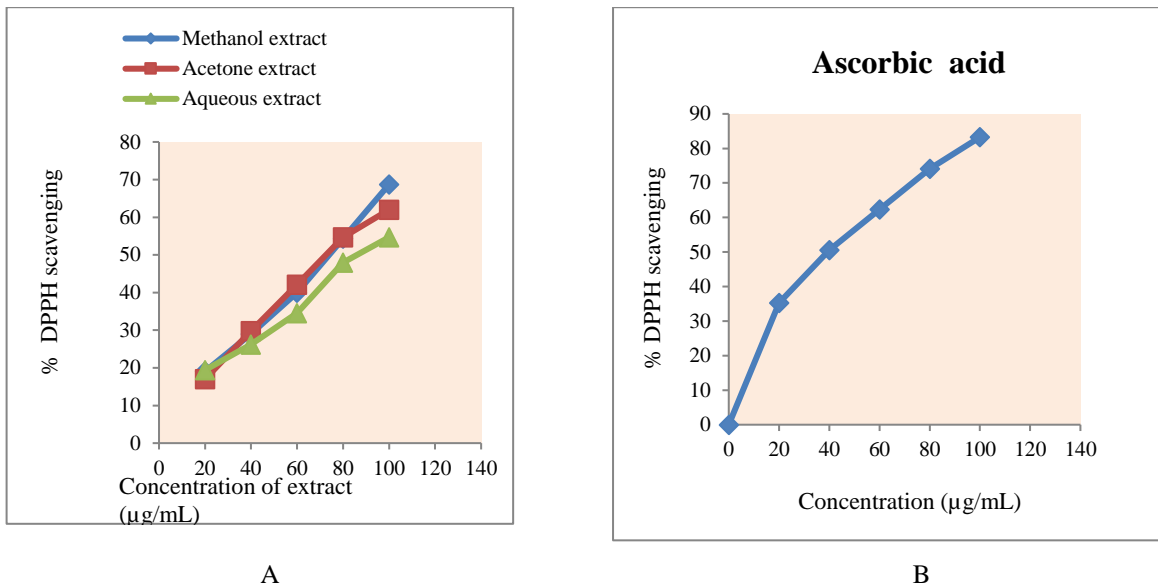
A

B

Figure 1: Percent scavenging (DPPH) activity of plant extracts at concentration range of 10-100 µg/mL (A) *V. negundo* and (B) Standard curve of Ascorbic acid.
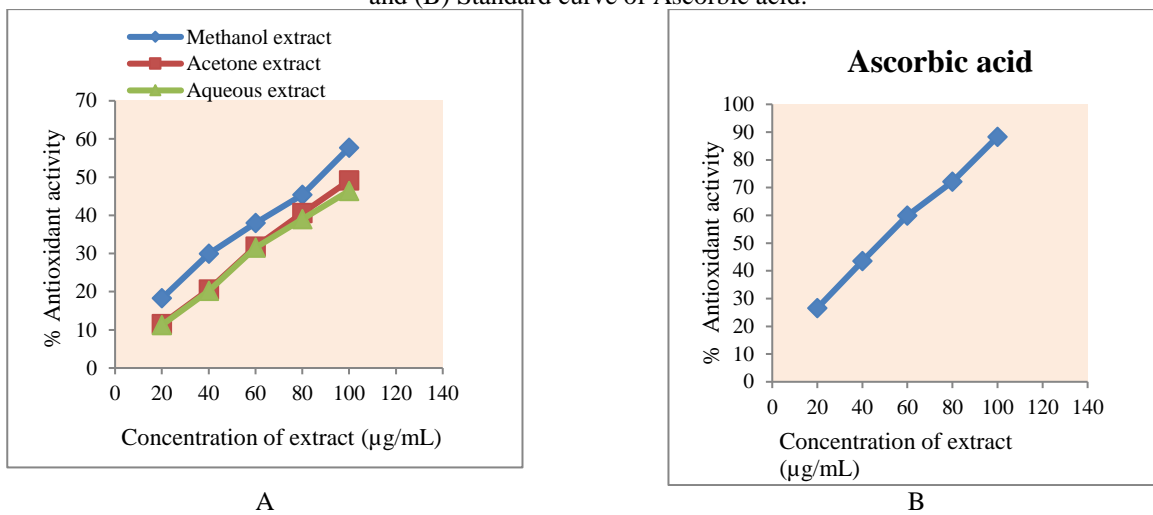

A

B

Figure 2: Antioxidant activity percentage (reducing power) of different plant extracts at concentration range of 10-100 µg/mL (A) *V. negundo*; (B) Standard curve of Ascorbic acid.
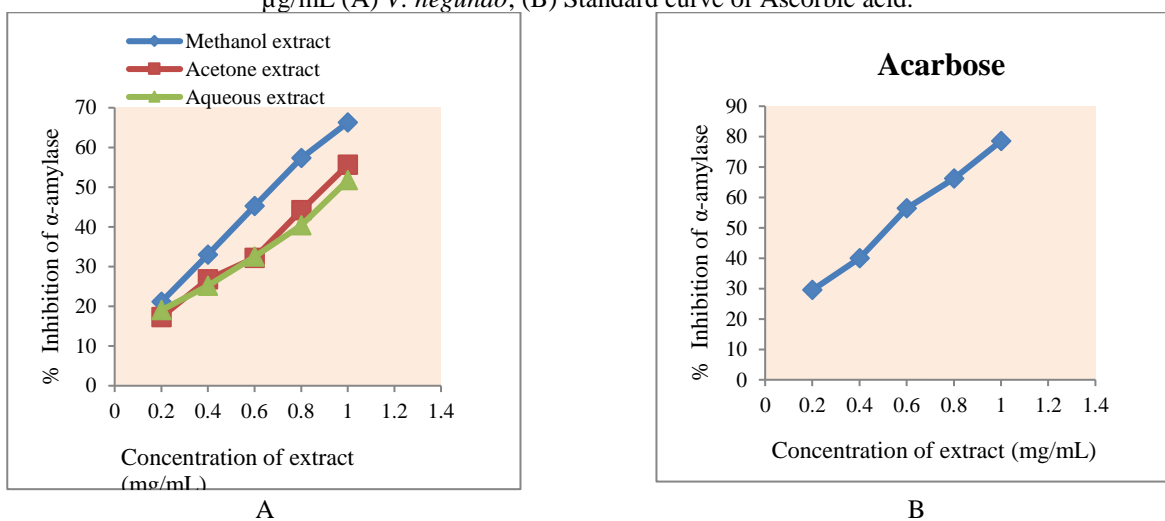

A

B

Figure 3: α-amylase inhibition profile of different extracts against porcine α-amylase at a concentration range of 0.2-1.0 mg/mL: (A) *V. negundo* (B) Standard curve of Acarbose.

extracts were tested for their urease inhibitory activity at a concentration of 1.0 mg/mL. Herbal extracts that exerted significant inhibition, were tested in a concentration range of 0.2 to 1.0 mg/mL (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL). For urease inhibition assays after addition of 10 mL of phosphate buffer to accurate weight of enzyme, sonication was performed for about 60 seconds, followed by centrifugation and absorbance of upper solution was measured at 280 nm. By using equation A= εbc, where c is concentration of solution (mol/L), b is length of the UV cell and ε represents molar absorptivity, one can calculate the concentration of initial urease solution. After proper dilution, the concentration of enzyme solution was adjusted to 2 mg/mL. Reaction mixture containing 1.2 mL of phosphate buffer solution (10 Mm potassium phosphate, 10 Mm lithium chloride and 1 Mm EDTA, pH 8.2 at 37ºC), 0.2 mL of urease enzyme solution, and 0.1mL of test compound was subjected to incubation for 5 minutes. After pre-incubation 0.5 mL (66 Mm) of urea was added to the reaction mixture and incubated for about 20 minutes. Urease activity was determined by measuring the ammonia released during the reaction by modified spectrophotometric method as described by Weatherburn[20]. Briefly, 1 mL each of phenol reagent (1% w/v sodium nitroprusside) and an alkaline reagent (1% w/v NaOH and 0.075% active chloride NaOCl) were added to each test tube. The control contained all the reagents except the sample. The increase in absorbance at 640 nm was measured after 30 minutes. The percent inhibition was determined using the formula:

% Urease Inhibition = $\left(\frac{A_c - A_s}{A_c}\right) \times 100$

Here A_s is the absorbance of the sample under study while A_c is the absorbance of the control. Each experiment was repeated thrice and average was calculated. Thiourea was used as a positive control. Data are expressed as mean ± standard deviation (SD). IC_{50} values were determined from the dose response curves.

RESULTS AND DISCUSSIONS
Antioxidant activity analysis
In the present study, extracts of *V. negundo* in three different solvents (methanol, acetone and aqueous) were tested for their free radical scavenging ability by using DPPH assay and it was observed that the plant extracts showed good potency for scavenging free radicals as shown in Table 1.1. The extracts were tested on a concentration range (20-100 µg/mL) and it was found that the activity altogether increased with increase in concentration of plant extracts (Fig. 1.1). Methanol leaf extract showed highest (68.72%) DPPH scavenging activity at a concentration of 100 µg/mL. In all cases, methanol extracts proved to be better antioxidants than the corresponding acetone and aqueous extracts. A pattern of increasing antioxidant activity with increasing polarity has been reported[21]. Our findings are in accordance with the results obtained by Tiwari and Tripathi[22] and Durairaj *et al.*[23].

Reducing power experiment is a good reflector of antioxidant activity of the plants. The reducing capacity of compounds serves as an important indicator of their potential antioxidant activity. The plant having high reducing power generally reported to carry high antioxidant potential too[24].

We investigated the reducing capacity of *V. negundo* by measuring Fe^{3+}-Fe^{2+} conversion as given in Table 1.2 and Fig. 1.2. In this experiment, Ferric ions reduced to ferrous ions with the colour of the reaction mixture changes from yellow to bluish green. Reducing power potential of extracts increased with the dose, however, plant extracts exhibited low reducing power than that of standard ascorbic acid. The methanol extract showed more reductive ability than the acetone and aqueous extracts, which was capable for neutralizing the free radicals. *V. negundo* showed 57.70%, 49.10% and 46.40% reducing power for methanol, acetone and aqueous extracts
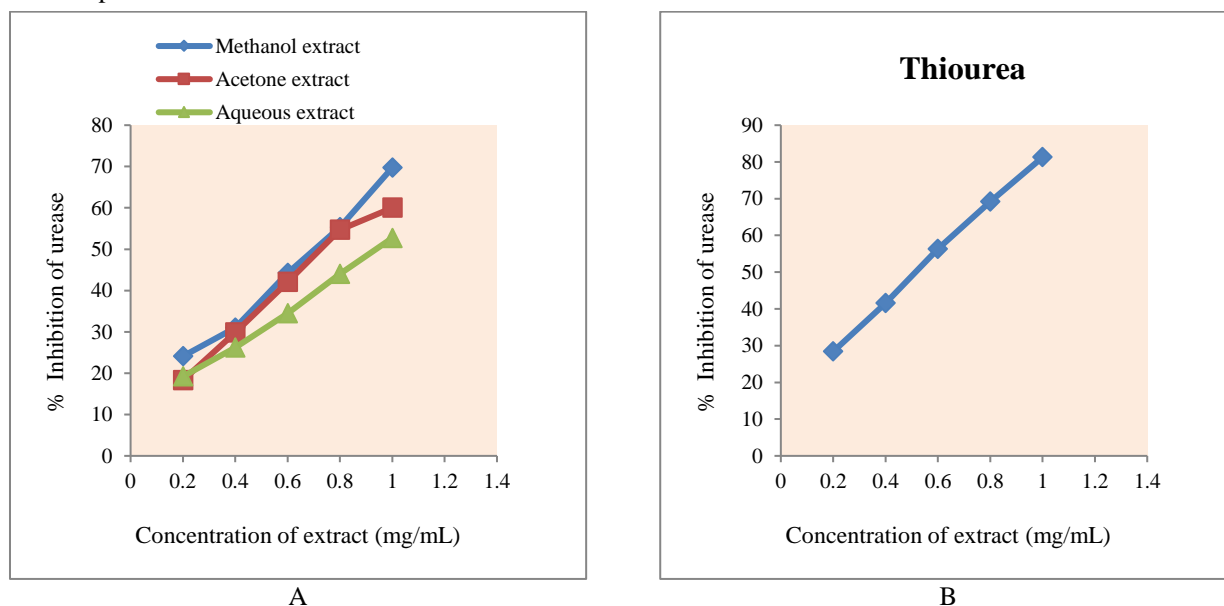

A

B

Figure 4: Urease inhibition profile of different extracts against Jack-bean urease at concentration range of 0.2-1.0 mg/mL: (A) *V. negundo* (B) Standard curve of Thiourea.

respectively at 100 µg/mL. Raghavendra *et al.*[25] and Shah *et al.*[26] have reported that *V. negundo* leaf extracts possess good reducing power which supported our findings.

Enzyme inhibitory activity analysis

In the present study, leaf extracts (methanol, acetone and aqueous) of *V. negundo* were tested for their enzyme inhibitory activity against α-amylase and urease and it was observed that the plant extracts showed ≥50% α-amylase inhibition at concentration of 1 mg/mL as shown in Table 1.3. The plant extracts showed concentration dependent inhibition of α-amylase enzyme as shown in Fig. 1.3. At a concentration of 1 mg/mL, the activity of α-amylase was 66.27±2.25, 55.60±2.22 and 51.75±0.44% for methanol, acetone and aqueous extract respectively. The inhibitory activity increased with increasing the concentration of each plant extract in the range of 0.2-1.0 mg/mL. The results further indicated that methanol extracts exhibited maximum inhibitory effects than other solvent extracts. This tends to show that the active metabolites of the different plant parts are better extracted with methanol than other solvents. As per literature survey, there is no previous report found on α-amylase inhibitory activity of this plant,

Furthermore, the urease inhibitory activities of different plant extracts were studied against jack bean urease by using phenol hypochlorite method as compiled in Table 1.4. All the extracts showed inhibition ≥50% at concentration of 1 mg/mL. All the three extracts of *V. negundo* were reported to exert good inhibitory effects on jack bean urease enzyme. Among these, methanol extract showed maximum inhibition in the range of 24.10-69.72%. However there is no literature available on urease inhibitory activity of *V. negundo*.

CONCLUSIONS

It was concluded from the above experimental observations that the plant *Vitex negundo* showed significant antioxidant and enzyme inhibitory activity at different concentrations used. Methanol leaf extract was found to be more effective followed by acetone and aqueous leaf extracts. This study suggests that the plant extracts possess potent antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases. Further investigation on the isolation and identification of antioxidant component (s) in this plant may lead to chemical entities with potential for clinical use. Current study confirmed that the leaf extracts of *V. negundo* possess anti-obesity and anti-ulcer potential through α-amylase and Jack-bean urease inhibitory activity since all the plant extracts exhibited inhibition ≥50% at concentration of 1 mg/mL. Further, this study directs future research in separating the bioactive compounds responsible for this activity.

ACKNOWLEDGEMENTS

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