

Antibacterial, Cytotoxic and Antioxidant Potential of *Vitex Negundo* Var. *Negundo* and *Vitex Negundo* Var. *Purpurascens* – A Comparative Study

Prashith Kekuda TR¹, Manasa M¹, Poornima G¹, Abhipsa V¹, Rekha C¹, Shivaleela P. Upashe² and Raghavendra HL^{2*}

¹ Department of Microbiology, S.R.N.M.N College of Applied Sciences, N.E.S Campus, Balraj Urs Road, Shivamogga-577201, Karnataka, India

² College of Medical and Health Sciences, Wollega University, Post Box No: 395, Nekemte, Ethiopia

Abstract

The present study was carried out to estimate the content of total phenolics, flavonoids, negundoside and agnuside and to determine antibacterial, cytotoxic and antioxidant efficacy of methanol extract of two varieties of *Vitex negundo* namely *V. negundo* var. *negundo* (Vnvn) and *V. negundo* var. *purpurascens* (Vnvp). Total phenolic and flavonoid content of leaf extracts were estimated by Folin-Ciocalteu reagent and Aluminium chloride colorimetric estimation method respectively. Negundoside and agnuside content were estimated using HPLC. Antibacterial activity of both the extracts was tested by Agar well diffusion assay. Cytotoxicity of extract was evaluated by Brine shrimp lethality bioassay. Antioxidant activity of extracts was determined by DPPH free radical scavenging and Ferric reducing assay. The content of total phenolics and flavonoid were higher in extract of Vnvp. Negundoside and agnuside contents were high in Vnvn and Vnvp respectively. Leaf extract of Vnvp was shown to display marked antibacterial, cytotoxic and antioxidant activity when compared with extract of Vnvn. Both the varieties of *V. negundo* showed marked antibacterial, cytotoxic and antioxidant activity. The extract of Vnvp displayed marked bioactivities than that of extract of Vnvn. The presence of high phenolic and flavonoid content could be attributed to the marked bioactivities of Vnvp.

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***Corresponding Author:**

Raghavendra HL

E-mail:
 Ragh_u_hl@rediffmail.com

INTRODUCTION

The genus *Vitex* belongs to the family Verbenaceae and comprises of large shrubs or small trees. *Vitex negundo* Linn is most commonly distributed on roadsides and the banks of streams and is called Lakki gida in Kannada. It is a large, silvery-tomentose shrub or small tree with bluish purple flowers in terminal panicles with short cymose branches. Leaflets are 3-5 in number, middle one slightly longer petiolulate, entire, lanceolate, acuminate, 12x2cm and membranous. Bark is thin and grey. Wood is greyish white and hard. Corolla is bilabiate and bluish purple in color. Fruit is drupe and is globular, 1 cm long and 0.3cm across with persistent calyx. Flowering occurs in January to February. Fruit setting occurs in May to July (Gamble, 1993). It is a very common aromatic plant and is used in medicine.

V. negundo is shown to possess a wide array of biological activities such as antimalarial (Nguyen-Pouplin *et al.*, 2007), anthelmintic (Merekar *et al.*, 2011), wound healing (Roosewelt *et al.*, 2011), antipyretic (RaamaMurthy *et al.*, 2010), anti-inflammatory (Dharmasiri *et al.*, 2003), analgesic (Dharmasiri *et al.*, 2003), antioxidant (Raghavendra *et al.*, 2010a), antifungal (Mahmud *et al.*, 2009), antibacterial (Devi *et al.*, 2008), hepatoprotective (Tendon *et al.*, 2008), anti-microfilarial (Sahare *et al.*, 2008), mosquito repellent (Hebbalkar *et al.*, 1992), insecticidal (Chowdhury *et al.*, 2009a), cytotoxic (Saluja *et al.*, 2010), anxiolytic (Adnaik *et al.*, 2009), Snake venom neutralizing (Alami and Gomes, 2003), antiandrogenic (Bhargava, 1989), immunostimulatory (Singh *et al.*, 2005) and CNS depressant activity (Gupta *et al.*, 1999).

There are two varieties in *V. negundo* namely *V. negundo* var. *negundo* and *V. negundo* var. *purpurascens*. In *V. negundo* var. *negundo* (locally called bili lakki), the lower surface of the leaflets is grey-pubescent and style is white. However, the lower surface is purple in *V. negundo* var. *purpurascens* (locally called kari lakki). It also differs from *V. negundo* var. *negundo* in having deep purple corolla and purple stamina filaments and style (Manilal and Sivarajan, 1982). In this study, we have estimated the content of total phenolics, flavonoids, agnuside and negundoside content of methanolic extract of leaf of both varieties of *V. negundo* and compared antibacterial, antioxidant and cytotoxic potential of extracts of leaves of both the varieties.

MATERIALS AND METHODS

Collection and Identification of Plant Materials

The plant materials were collected in the month of May 2011 from a place called Maavinakoppa, Hosanagara (Taluk), Shivamogga (District), Karnataka and authenticated by Prof. K.G. Bhat, Udupi. The voucher specimens (SRNMN/MB/Vnvn-01 and SRNMN/MB/Vnvp-01) were deposited in the department herbaria for future reference. The leaves were separated, washed well under water, shade dried and powdered mechanically using a blender.

Extraction

The powdered leaf materials (100g) were extracted using methanol solvent in Soxhlet apparatus. The methanol extract was filtered through 4-fold muslin cloth followed by Whatmann No. 1 and concentrated in vacuum under reduced pressure and dried in the desiccator (Kekuda *et al.*, 2012a). The extracts of *V. negundo* var. *negundo* (Vnvn) and *V. negundo* var. *purpurascens* (Vnvp) were stored in refrigerator in amber colored bottles till use.

Antibacterial Activity

The efficacy of extracts of Vnvn and Vnvp to inhibit bacteria was tested against three Gram negative bacteria namely *Escherichia coli* NCIM-2685, *Pseudomonas aeruginosa* NCIM-2242 and *Vibrio cholerae* MTCC-3905 and three Gram positive bacteria namely *Bacillus subtilis* NCIM-2699, *Streptococcus pyogenes* NCIM-2608 and *Staphylococcus aureus* NCIM-2079 by Agar well diffusion assay. The test bacteria were aseptically inoculated into sterile Nutrient broth (HiMedia, Mumbai) tubes and incubated for 24 hours at 37°C. The broth cultures were aseptically swabbed on the sterile Nutrient agar (HiMedia, Mumbai) plates uniformly. With the help of a sterile cork borer, wells of 6mm diameter were punched in the inoculated plates and 100 µl of extract (25mg/ml of 10%

dimethyl sulfoxide [DMSO]), standard (Streptomycin, 1mg/ml of sterile distilled water) and DMSO (10%) was added into the respectively labeled wells. The plates were kept in room temperature for an hour and then incubated at 37°C for 24 hours aerobically. The presence of zones of inhibition around the wells was observed and interpreted as an indication of antibacterial activity (Kekuda *et al.*, 2012a).

Cytotoxic Activity

The cytotoxic potential of extract of Vnvn and Vnvp was determined by Brine shrimp lethality bioassay. Briefly, eggs of brine shrimp *Artemia salina* were hatched in a container filled with air-bubbled artificial sea water which was prepared using 10 g of a commercial salt mixture (GEX Inc., Osaka, Japan) and 500 ml of distilled water. After 36-48 hours, the phototropic shrimps were collected and used for bioassay. To the vials containing different concentrations of extracts in sea water (1, 10, 25, 50 and 100 µg/ml), 25 shrimps were added and the vials were incubated at 25°C and the surviving shrimps were counted after 24 hours. The LC₅₀ values of extracts greater than 1000 µg/ml were considered inactive (non-toxic). Potassium dichromate was used as reference standard (Kekuda *et al.*, 2012a).

Antioxidant Activity

DPPH Free Radical Scavenging Assay

The ability of extract of Vnvn and Vnvp to scavenge free radicals was screened by DPPH free radical scavenging assay (Rekha *et al.*, 2012). 2ml of various concentrations, namely 2.5, 5, 10, 25, 50 and 100 µg/ml of methanol, of extracts and ascorbic acid (standard) were mixed with 2ml of DPPH solution (0.002% in methanol) and the tubes were incubated in dark at room temperature for 30 minutes. The absorbance was measured at 517nm in UV-Visible spectrophotometer. The absorbance of DPPH control was also noted. The scavenging activity (%) of each concentration of extracts and standard was calculated using the formula: $(A_0 - A_1)/A_0 \times 100$, where A₀ is absorbance of control and A₁ is absorbance of test (extract/standard). The concentration of extract required to inhibit 50% of free radicals (Inhibitory concentration, IC₅₀) was calculated for each extract.

Ferric Reducing Assay

The reducing potential of extract of Vnvn and Vnvp and tannic acid (standard) was determined by Ferric reducing assay (Rekha *et al.*, 2012). Briefly, different concentrations of extracts and standard (2.5, 5, 10, 25, 50 and 100 µg/ml of methanol) in 1ml of methanol were mixed with 2.5ml of phosphate buffer (pH 6.6), 2.5ml of potassium ferricyanide (1%) and incubated at 50 °C for 20

minutes in water bath. Afterwards, 2.5ml of trichloroacetic acid (10%) was added to each tube followed by addition of 0.5ml of ferric chloride (0.1%). The absorbance was measured at 700nm after 10 minutes. An increase in the absorbance with increase in concentration of extracts/standard indicated increasing reducing power.

Estimation of Total Phenolic Content

The Total phenolic content of extract of Vnvn and Vnvp were estimated by Folin-Ciocalteu reagent (FCR) method employed by Rekha *et al.* (2012) with minor modifications. A dilute concentration of extract (0.5 ml) was mixed with 0.5 ml diluted Folin-Ciocalteu reagent (1:1) and 2 ml of sodium carbonate (7%). The mixtures were allowed to stand for 30 minutes and the absorbance was measured at 765nm in UV-Visible spectrophotometer. A standard curve was plotted using different concentrations of Gallic acid (standard, 0-1000µg/ml). The concentration of total phenolic compounds was determined as µg Gallic acid equivalents (GAE) from the graph.

Estimation of Total Flavonoid Content

The amount of flavonoids present in the extracts of Vnvn and Vnvp was estimated by Aluminium chloride colorimetric method (Zhishen *et al.*, 1999). A dilute concentration of extract (0.5ml) was mixed with 0.5ml of methanol, 4ml of water, 0.3ml of NaNO₂ (5%) and incubated for 5 minutes at room temperature. After incubation, 0.3ml of AlCl₃ (10%) was added and again incubated at room temperature for 6 minutes. Later, 2ml of 1M NaOH and 2.4ml of distilled water were added and the absorbance was measured at 510nm using UV-Visible spectrophotometer. A calibration curve was constructed using different concentrations of Catechin (0-120µg/ml) and the flavonoid content was expressed as µg Catechin equivalents (CE) from the graph.

HPLC Analysis for Negundoside and Agnuside

The HPLC profile of negundoside and agnuside was performed by employing Shimadzu HPLC system. In order to prepare the plant samples, 500mg of each of Vnvn and Vnvp was weighed, added to 40-50 ml of HPLC Methanol, refluxed under water bath for 10 minutes, cooled, sonicated for 15 minutes and the volume was made up to 100ml with methanol. The flask was shaken well, filtered through 0.45 micron membrane filter paper and used for injection. Standards of negundoside and agnuside were prepared by dissolving each standard in HPLC methanol to get 0.2mg/ml concentration.

Negundoside and Agnuside were separated, identified and determined in leaf extract of Vnvn and Vnvp by Shimadzu HPLC consisting of a model

LC2010CHT series with UV detector. C18 (Phenomenex Luna), 2.5µ (100x 3.0 mm) reverse phase was used. Column temperature was maintained at 30°C and flow rate was set to 1.5 mL/min. 20 µL of the sample and standard was injected automatically (injected in duplicate to calculate average area). The two solvents used to make the gradient were (A) Dissolve 0.136 g of anhydrous potassium dihydrogen orthophosphate in 900 ml of HPLC grade water and add 0.5 ml of orthophosphoric acid. Make up to 1000 ml with water, filter through 0.45 µ membrane and degas in a sonicator for 3 min (B) acetonitrile. The solvent gradient as follows: 0.01-10 min 95 % of A and 5 % of B; 10-20 min 85 % A and 15 % of B; 20-25 min 75 % of A and 25 % of B; 25-30 min 85 % of A and 15 % of B; 30-35 min 95 % of A and 5 % of B. The wavelength was set to 254 nm to detect the eluent. Sample was quantified by comparing the retention time/peak areas with those of standards obtained from Sigma (St Louis, MO, USA).

Statistical Analysis

All data were expressed as Mean±Standard deviation (SD) of the number of experiments (n =3). The IC₅₀ and LC₅₀ values were calculated by Origin 6.0 software.

RESULTS

Antibacterial Activity of Vnvn and Vnvp Extract

Table 1 shows the result of antibacterial activity of methanolic extract of Vnvn and Vnvp. The extracts were effective in inhibiting all test bacteria. Gram positive bacteria were shown to be susceptible to extracts to high extent when compared with Gram negative bacteria. Among Gram negative bacteria, *P. aeruginosa* was highly susceptible to extracts followed by *E. coli* and *V. cholerae*. In case of Gram positive bacteria, high susceptibility was recorded in case of *S. aureus* followed by *S. pyogenes* and *B. subtilis*. Inhibition caused by reference antibiotic was higher than that of extracts. DMSO did not cause inhibition of any test bacteria.

Table 1: Antibacterial activity of Vnvn and Vnvp extract.

Test Bacteria	Zone of inhibition (in cm)		
	Vnvp	Vnvn	Standard
<i>E. coli</i>	1.9±0.05	1.5±0.05	2.9±0.20
<i>P. aeruginosa</i>	2.3±0.10	1.9±0.10	3.2±0.20
<i>V. cholerae</i>	1.8±0.05	1.3±0.10	2.9±0.10
<i>S. pyogenes</i>	2.5±0.20	2.1±0.05	4.3±0.05
<i>B. subtilis</i>	2.5±0.10	2.0±0.20	4.0±0.20
<i>S. aureus</i>	2.6±0.20	2.3±0.20	4.5±0.30

Brine Shrimp Lethality of Vnvn and Vnvp Extract

The result of cytotoxic potential of methanol extract of Vnvn and Vnvp in terms of mortality of

brine shrimps (%) is presented in Figure 1. The degree of lethality was directly proportional to the concentration of the extracts. The percentage mortality of shrimps was recorded higher in case of Vnvp (LC_{50} 25.14 μ g/ml) than that of Vnvn (LC_{50} 46.12 μ g/ml). Extract of Vnvp showed more lethality when compared with the reference control i.e., potassium dichromate (LC_{50} 32.77 μ g/ml). Highest mortality (100%) was observed at concentration 100 μ g/ml of both the extracts.

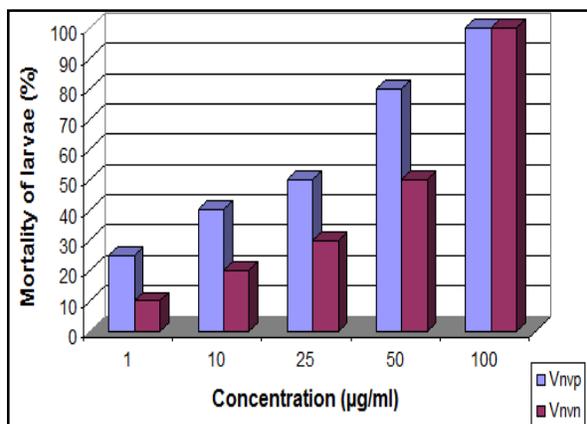


Figure 1: Brine shrimp lethality of extract of Vnvn and Vnvp.

DPPH Radical Scavenging Activity of Vnvn and Vnvp Extract

The radical scavenging efficacy of extract of Vnvn and Vnvp was tested on the basis of scavenging of DPPH free radicals. The extracts have shown a dose dependent scavenging of DPPH radicals. Among extracts, scavenging activity was highest in case of Vnvp (IC_{50} 2.69 μ g/ml) than Vnvn (IC_{50} 10.66 μ g/ml). At concentrations 100 μ g/ml, both the extracts exhibited >90% scavenging of DPPH and the scavenging effect was higher than that of ascorbic acid. Ascorbic acid scavenged radicals more efficiently (IC_{50} 2.15 μ g/ml) than extract of Vnvn and Vnvp (Figure 2).

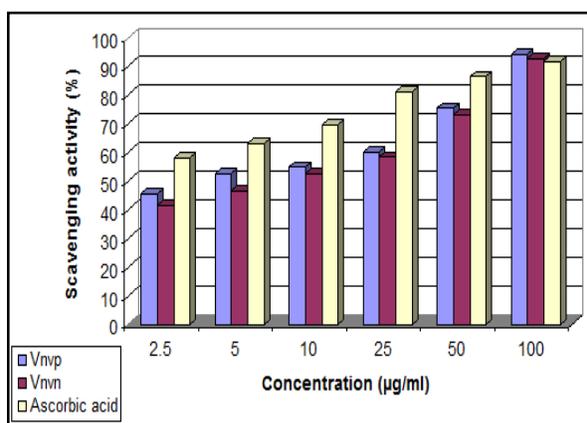


Figure 2: DPPH free radical scavenging activity of extract of Vnvn and Vnvp.

Ferric Reducing Activity of Vnvn and Vnvp Extract

The reductive capability of extract of Vnvn and Vnvp was determined by ferric reducing assay. The absorbance of reaction mixtures containing various concentrations of leaf extracts increased with increase in their concentrations and is suggestive of reducing power. Among extracts, methanol extract of Vnvp showed more reducing potential than extract of Vnvn. The reducing powers of extracts were lesser than that of tannic acid (Figure 3).

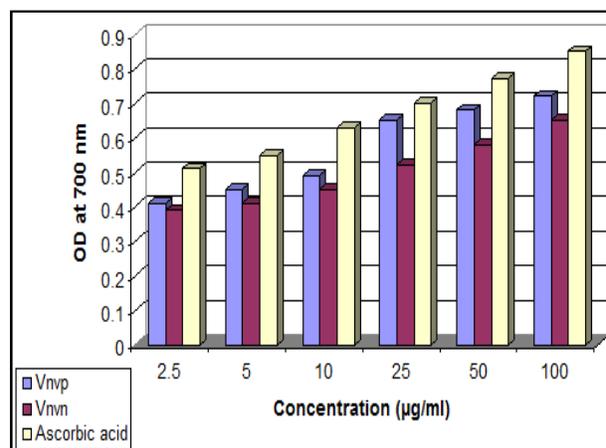


Figure 3: Ferric reducing activity of extract of Vnvn and Vnvp.

Total Phenolics and Flavonoids Content in the Vnvn and Vnvp Extract

The content of total phenolics in the methanol extract of leaf of Vnvn and Vnvp was estimated by FCR method and the result is presented as μ g GAE/mg of dry extract. It was found that extract of Vnvp contained high phenolic content (285.69 μ g GAE/mg) than that of extract of Vnvn (259.05 μ g GAE/mg). The flavonoid content was estimated by aluminium chloride colorimetric estimation method and the content of total flavonoids was estimated in terms of μ g CE/mg of dry extract. The leaf extract of Vnvp contained higher flavonoid content (30.35 μ g CE/mg) than leaf extract of Vnvn (26.86 μ g CE/mg).

Qualitative and Quantitative Estimation of Negundoside and Agnuside in Vnvn and Vnvp

The content (% w/w) of negundoside and agnuside present in the leaf extract of Vnvn and Vnvp was estimated by HPLC technique (Figure 4-7). Leaf extract of Vnvn contained high negundoside (3.5%) than extract of Vnvp (2.6%). However, the content of agnuside was highest in leaf extract of Vnvp (16.2%) when compared to leaf extract of Vnvn (3.6%).

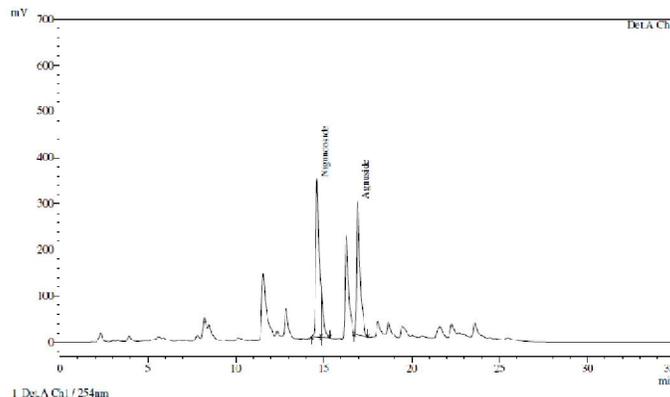


Figure 4: HPLC chromatogram of methanol extract of Vvvn.

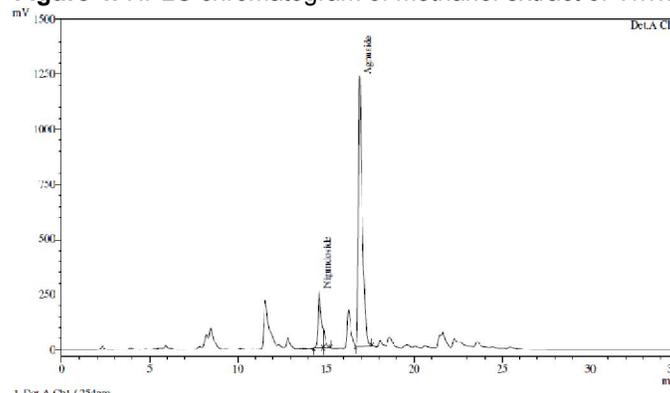


Figure 5: HPLC chromatogram of methanol extract of Vvvn

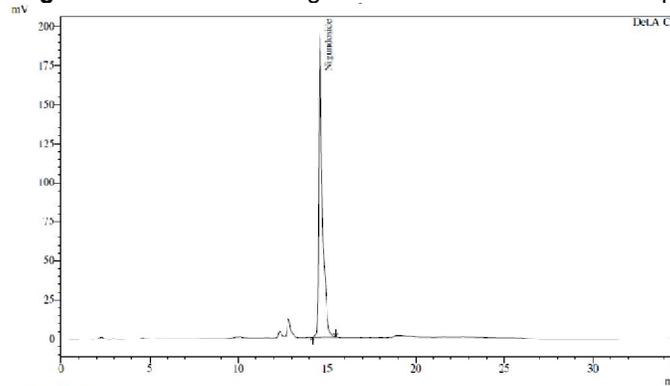


Figure 6: HPLC chromatogram of Negundoside (standard).

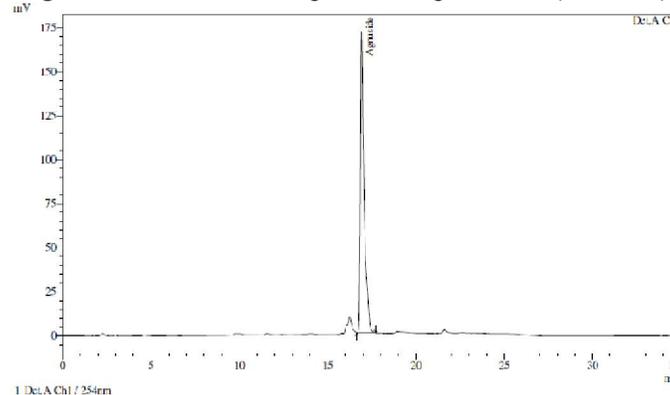


Figure 7: HPLC chromatogram of Agnuside (standard).

DISCUSSION

Throughout history, infectious diseases caused by bacteria, fungi, protozoa, helminths and viruses have been an important cause of disability and death. The discovery of antibiotics during the 20th century and their use revolutionized the field of medicine. However, the ability of bacterial pathogens to adapt and to overcome the challenges of antibiotics has made the situation worst and the disease treatment difficult. Methicillin-resistant *Staphylococcus aureus*, multidrug-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* spp., multidrug-resistant *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, multidrug-resistant TB are among the drug-resistant bacteria of today. The emergence of infections being caused by these antibiotic-resistant pathogens is a problem growing day by day and it has become a major health issue worldwide (Yoshikawa *et al.*, 2002; Lister *et al.*, 2009; Davies and Davies, 2010). The problems such as emergence of antibiotic resistant strains, high cost of antibiotics and the possible side effects associated with the use of antibiotics stimulated search for alternative for disease therapy (Gonzalez *et al.*, 1994; Weckesser *et al.*, 2007; Vadlapudi, 2012; Mahdavi *et al.*, 2012; Dahiya and Purkayastha, 2012; Martins *et al.*, 2013).

Ever since the existence of human being, plants have been exploited for several purposes including medicinal purposes. Plants are the primary source of biologically active phytochemicals present in conventional medicaments. Medicinal systems *viz.*, Ayurveda, Unani and Sidda employ the use of these plants for treatment of diseases. Ethnobotanical studies highlight the relationships between various cultures and the traditional use of plants. Several ethnic groups all over the world employ a number of plant species for treatment of various ailments ranging from mild infections to fatal infections. Often, these studies are of importance and provide essential information for development of scientific research to justify the therapeutic potential of plants (Kumar *et al.*, 2006; Martins *et al.*, 2013). Plants and their components have been investigated for antimicrobial activity. Plants have shown to possess inhibitory activity against various types of microorganisms including drug resistant microorganisms (Gonzalez *et al.*, 1994; Jayasinghe *et al.*, 2002; Weckesser *et al.*, 2007; Vadlapudi, 2012; Mahdavi *et al.*, 2012; Kumar *et al.*, 2006; Dahiya and Purkayastha, 2012; Martins *et al.*, 2013). In the present study, extract of Vnvn and Vnvp displayed marked inhibition of test bacteria. Leaf extract of Vnvp showed higher inhibition of test bacteria when compared with the inhibition

produced by leaf extract of Vnvn. Presence of high phenolic and flavonoid content could be attributed to the marked inhibitory activity of extract of Vnvp. The crude methanol extract and petroleum ether and carbon tetrachloride fractions of methanol extract of leaf of *V. negundo* showed antimicrobial activity (Chowdhury *et al.*, 2009b). Chowdhury *et al.* (2010) showed antibacterial activity of two compounds isolated from leaves of *V. negundo*. The bark extract of *V. negundo* exhibited antimicrobial activity (Khan *et al.*, 2013).

Brine shrimp lethality bioassay is an *in vivo* lethality assay that employs a simple zoologic organism as a convenient monitor for screening, discovering and monitoring various bioactivities of natural compounds. This test is very useful in determining various biological activities such as cytotoxic, phototoxic, pesticidal, trypanocidal, enzyme inhibition, and ion regulation activities. The assay can also be extrapolated for cell-line toxicity and antitumor activity. The method is rapid as it utilizes only 24 hours, inexpensive and needs no special equipment. It is even simple in that it does not require aseptic conditions to perform. The assay employs large number of organisms for validation and a relatively small amount of sample. This bioassay has been employed to determine cytotoxic activity of plant extracts (Krishnaraju *et al.*, 2006; Kekuda *et al.*, 2010a; Raghavendra *et al.*, 2010b; Kekuda *et al.*, 2012a). In our study, the methanol extract of Vnvn and Vnvp displayed cytotoxic activity as evidenced by the dose dependent mortality of brine shrimp larvae. Among extracts, higher cytotoxicity was observed in case of Vnvp extract than that of extract of Vnvn. High mortality of shrimps caused by extract of Vnvp could be ascribed to the presence of high phenolic and flavonoid content. The crude methanol extract and petroleum ether and carbon tetrachloride fractions of methanol extract of leaf of *V. negundo* were shown to exhibit cytotoxic activity (Chowdhury *et al.*, 2009b). In another study, Chowdhury *et al.* (2010) showed cytotoxic activity in terms of brine shrimp mortality of two compounds isolated from leaves of *V. negundo*. Crude methanol extract and solvent fractions of bark of *V. negundo* were shown to exhibit marked cytotoxic effect in terms of mortality of brine shrimp larvae (Khan *et al.*, 2013).

DPPH is a stable, nitrogen centred, organic free radical with maximum absorption at 517nm in alcoholic solution. The radical accepts an electron or hydrogen atom and becomes a stable diamagnetic molecule. The assay of DPPH free radical scavenging is widely used to determine radical scavenging potential of various types of substances or compounds including plant extracts (Chung *et al.*, 2006; Kaviarasan *et al.*, 2007;

Vinayaka *et al.*, 2009; Kekuda *et al.*, 2010b; Rekha *et al.*, 2012; Junaid *et al.*, 2013). In this study, we employed DPPH free radical scavenging assay for determining the radical scavenging ability of leaf extract of both *Vitex negundo* varieties. In the presence of a compound/substance capable of donating an hydrogen atom, the free radical nature of DPPH is lost and its color (purple) changes to yellow (diphenylpicrylhydrazine). The solution loses color stoichiometrically depending on the number of electrons taken up (Poornima *et al.*, 2012; Kekuda *et al.*, 2012b). The decrease in absorption of DPPH reagent in the presence of varying concentrations of leaf extracts has been monitored at 517nm. Leaf extract of Vnvp displayed stronger scavenging efficacy. It can be noticed that the extracts at high concentrations showed significant decrease in the absorption of DPPH radicals when compared with reference standard. Although the scavenging abilities of extracts were lesser than that of ascorbic acid, it was evident that the extracts showed hydrogen donating ability and therefore the extracts could serve as free radical scavengers, acting possibly as primary antioxidants (Chung *et al.*, 2006).

Ferric reducing assay is a simple assay used to measure direct reduction of $\text{Fe}^{+3}(\text{CN})_6$ to $\text{Fe}^{+2}(\text{CN})_6$. The assay is used to evaluate the reducing power of several types of samples including plant extracts. It is done by measuring the absorbance resulting from the formation of PerI's Prussian blue complex on addition of excess of ferric ions (Fe^{+3}). The reducing ability of a compounds/extract is indicated by an increase in absorbance at 700nm on increase in concentration. The reducing capacity of antioxidants is generally associated with the presence of reductones. In this assay, the presence of reductants (antioxidants) in the samples would result in the reduction of Fe^{+3} to Fe^{+2} by donating an electron (Chung *et al.*, 2006; Kekuda *et al.*, 2010b; Gulcin *et al.*, 2011; Poornima *et al.*, 2012; Rekha *et al.*, 2012 and Junaid *et al.*, 2013). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Hsu *et al.*, 2006). In the present study, it was observed that the reducing powers of extract of Vnvn and Vnvp increased with the increase of their concentrations. The higher reducing potential of Vnvp could be due to high phenolic and flavonoid contents present in it. It is evident from the study that the extracts possess reductive potential and could serve as electron donors, terminating the radical chain reactions (Chung *et al.*, 2006).

Phenolic compounds including flavonoids are widely distributed in plant kingdom. They are occurring in vegetables, fruits and medicinal plants. These compounds are known to possess different

antioxidant capacities, which can be ascribed to a broad range of pharmacological activity. Phenolic contents of plants have been extensively studied for their contribution to antioxidant activity of plants. There are many reports which correlate the total phenolic content of plants and their antioxidant activity (Tilak *et al.*, 2004; Coruh *et al.*, 2007; Rekha *et al.*, 2012; Poornima *et al.*, 2012). In the present study, we have estimated total phenolic content of leaf extracts of Vnvn and Vnvp by FCR method. In this study, the total phenolic content was found higher in the leaf extract of Vnvp when compared to Vnvn. FCR method is an oldest and widely used colorimetric method used for estimating total phenolic content of a variety of substances including plant extracts. Phenolic compounds reacts with FCR under basic conditions only (adjusted by sodium carbonate solution to pH 10) to form blue complex which has maximum absorption near 750nm. Despite the undefined chemical nature of FCR, the total phenolic assay by FCR is simple, convenient and reproducible. Thus FCR method has become a routine assay in studying the phenolic antioxidants (Chung *et al.*, 2006; Coruh *et al.*, 2007; Kekuda *et al.*, 2011; Rekha *et al.*, 2012; Poornima *et al.*, 2012; Junaid *et al.*, 2013).

In our study, we have estimated total flavonoids present in the leaf extract of Vnvn and Vnvp by Aluminium chloride colorimetric estimation method. Extract of Vnvp was found to contain high flavonoid content when compared with the extract of Vnvn. Flavonoids are polyphenolic compounds forming majority of plant secondary metabolites. These compounds are known to possess marked health promoting effects including antioxidant activity (Chua *et al.*, 2011). Aluminium chloride colorimetric estimation is widely used to quantify total flavonoid content of plant extracts (Zhishen *et al.*, 1999; Penarrieta *et al.*, 2007; Rohman *et al.*, 2010; Kekuda *et al.*, 2012b; Pavithra *et al.*, 2013a; Pavithra *et al.*, 2013b). Total flavonoid contents can be estimated by reaction with sodium nitrite, followed by the development of colored flavonoid-aluminium complex formation using aluminium chloride in alkaline condition which can be monitored spectrophotometrically at maximum wavelength of 510 nm (Rohman *et al.*, 2010).

Negundoside and agnuside are the two iridoid glycosides present in *V. negundo*. In this study, we have estimated the content of negundoside and agnuside by HPLC method. It was observed that the leaf extracts of Vnvn and Vnvp contained these two glycosides in different proportions. The negundoside and agnuside content was highest in Vnvn and Vnvp respectively. These glycosides have been reported to possess some biological activities. Negundoside and agnuside, isolated from leaves of

V. negundo of Nepal origin displayed marked inhibition of gram positive and gram negative bacteria. Agnuside was more inhibitory to bacteria than negundoside (Gautam *et al.*, 2008). Both negundoside and agnuside have been shown to possess osteogenic activity (Kumar *et al.*, 2010). Negundoside was found to protect human liver cells against CCl₄ toxicity (Tasduq *et al.*, 2008). Agnuside demonstrated to produce significant reduction in both alternate and classically-mediated hemolysis at a dose of 1:10 (Tigno and Francisco, 1993). Agnuside showed significant anti-arthritis activity in the polyarthritis test in rats. It also showed inhibition of vascular permeability and leukocyte migration *in vivo* (Pandey *et al.*, 2012).

CONCLUSION

In the present study, methanol extract of both the varieties of *V. negundo* have shown to possess marked antibacterial, cytotoxic and antioxidant activity. Extract of Vnvp exhibited higher bioactivities than that of Vnvn. The high phenolic and flavonoid content in the extract could be ascribed to the marked bioactivities of Vnvp. The content of negundoside and agnuside might have also played an important role in the bioactivities observed.

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REFERENCES

- Adnaik, R.S., Pai, P.T., Sapakal, V.D., Naikwade, N.S., Magdum, C.S. (2009). Anxiolytic activity of *Vitex negundo* Linn. in experimental models of anxiety in mice. *International Journal of Green Pharmacy* 3(3): 243-247.
- Alam, M.I., Gomes, A. (2003). Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Embllica officinalis*) root extracts. *Journal of Ethnopharmacology* 86: 75-80.
- Bhargava, S.K. (1989). Antiandrogenic effects of a flavonoidrich fraction of *Vitex negundo* seeds: a histological and biochemical study in dogs. *Journal of Ethnopharmacology* 27(3): 327-339.
- Chowdhury, N.Y., Islam, W., Khalequzzaman, M. (2009a). Insecticidal activities of stem bark extracts from *Vitex negundo* L. against *Tribolium castaneum* (Herbst). *Journal of Bio-Science* 17: 63-70.
- Chowdhury, J.A., Islam, M.S., Asifuzzaman, S.K., Islam, M.K. (2009b). Antibacterial and cytotoxic activity screening of leaf extracts of *Vitex negundo* (Fam: Verbenaceae). *Journal of Pharmaceutical Sciences and Research* 1(4): 103-108.
- Chowdhury, N.Y., Islam, W., Khalequzzaman, M. (2010). Biological activities of isolated compounds from *Vitex negundo* leaf. *Journal of Bio-Science* 18: 53-59.
- Chua, L.S., Latiff, N.A., Lee, S.Y., Lee, C.T., Sarmidi, M.R., Aziz, R.A. (2011). Flavonoids and phenolic acids from *Labisia pumila* (Kacip Fatimah). *Food Chemistry* 127: 1186-1192.
- Chung, Y., Chien, C., Teng, K., Chou, S. (2006). Antioxidative and mutagenic properties of *Zanthoxylum ailanthoides* Sieb & zucc. *Food Chemistry* 97: 418-425.
- Coruh, N., Celep, A.G.S., Oztgokce, F., Iscan, M. (2007). Antioxidant capacities of *Gundelia tournefortii* L. extracts and inhibition on glutathione-S-transferase activity. *Food Chemistry* 100: 1249-1253.
- Dahiya, P., Purkayastha, S. (2012). Phytochemical screening and antimicrobial activity of some medicinal plants against multi-drug resistant bacteria from clinical isolates. *Indian Journal of Pharmaceutical Sciences* 74(5): 443-450.
- Davies, J., Davies, D. (2010). Origins and evolutions of antibiotic resistance. *Microbiology and Molecular Biology Reviews* 74(3): 417-433.
- Devi, R.P., Kokilavani, R., Gnanapoongotha, S. (2008). Antimicrobial activity of the various leaf extracts of *Vitex negundo* Linn. *Ancient Science of Life* 28(2): 15-18.
- Dharmasiri, M.G., Jayakody, J.R.A.C., Galhena, G., Liyanage, S.S.P., Ratnasooriya, W.D. (2003). Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *Journal of Ethnopharmacology* 87(2-3): 199-206.
- Gamble, J.S. (1993). Flora of Presidency of Madras. Volume II. Bishen Singh Mahendra Pal Singh, Dehra Dun, pp 1101-1102.
- Gautam, L.N., Shrestha, S.L., Wagle, P., Tamrakar, B.M. (2008). Chemical constituents from *Vitex negundo* (Linn.) of Nepalese origin. *Scientific World* 6(6): 27-32.
- Gonzalez, A.G., Moujir, L., Bazzocchi, I.L., Correa, M.D., Gupta, M.P. (1994). Screening of antimicrobial and cytotoxic activities of Panamanian plants. *Phytomedicine* 1(2): 149-153.
- Gulcin, I., Topal, F., Sarikaya, S.B.O., Bursal, E., Bilsel, G., Goren, A.C. (2011). Polyphenol contents and antioxidant properties of Medlar (*Mespilus germanica* L.). *Records of Natural Products* 5(3): 158-175.
- Gupta, M., Mazumder, U.K., Bhawal, S.R. (1999). CNS activity of *Vitex negundo* Linn. in mice. *Indian Journal of Experimental Biology* 37(2): 143-146.
- Hebbalkar, D.S., Hebbalkar, G.D., Sharma, R.N., Joshi, V.S., Bhat, V.S. (1992). Mosquito repellent activity of oils from *Vitex negundo* Linn. leaves. *Indian Journal of Medical Research* 95: 200-203.

- Hsu, B., Coupar, I.M., Ng, K. (2006). Antioxidant activity of hot water extract from the fruit of the Doum palm, *Hyphaene thebaica*. *Food Chemistry* 98: 317-328.
- Jayasinghe, U.L.B., Jayasooriya, C.P., Bandara, B.M.R., Ekanayake, S.P., Merlini, L., Assante, G. (2002). Antimicrobial activity of some Sri Lankan Rubiaceae and Meliaceae. *Fitoterapia* 73: 424-427.
- Junaid, S., Rakesh, K.N., Dileep, N., Poornima, G., Kekuda, P.T.R., Mukunda, S. (2013). Total phenolic content and antioxidant activity of seed extract of *Lagerstroemia speciosa* L. *Chemical Science Transactions* 2(1): 75-80.
- Kaviarasan, S., Naik, G.H., Gangabthagirathi, R., Anuradha, C.V., Priyadarshini, K.I. (2007). *In vitro* studies on antiradical and antioxidant activities of fenugreek (*Trigonella foenum graecum*) seeds. *Food Chemistry* 103: 31-37.
- Kekuda, P.T.R., Raghavendra, H.L., Surabhi, K.S., Preethi, H.R., Swarnalatha, S.P. (2010a). Cytotoxic activity of methanol extract of *Abrus pulchellus* Wall (Fabaceae) leaves. *Biomedicine* 30(3): 377-379.
- Kekuda, P.T.R., Shobha, K.S., Onkarappa, R. (2010b). Studies on antioxidant and anthelmintic activity of two *Streptomyces* species isolated from Western Ghat soil of Agumbe, Karnataka. *Journal of Pharmacy Research* 3(1): 26-29.
- Kekuda, P.T.R., Vinayaka, K.S., Swathi, D., Suchitha, Y., Venugopal, T.M., Mallikarjun, N. (2011). Mineral composition, total phenol content and antioxidant activity of a macrolichen *Everniastrum cirrhatum* (Fr.) Hale (Parmeliaceae). *E-Journal of Chemistry* 8(4): 1886-1894.
- Kekuda, P.T.R., Raghavendra, H.L., Swathi, D., Venugopal, T.M., Vinayaka, K.S. (2012a). Antifungal and cytotoxic activity of *Everniastrum cirrhatum* (Fr.) Hale. *Chiang Mai Journal of Science* 39(1): 76-83.
- Kekuda, P.T.R., Rakesh, K.N., Dileep, N., Junaid, S., Pavithra, G.M., Gunaga, S.S., Megha, V.H., Raghavendra, H.L. (2012b). Antimicrobial and antioxidant activity of *Anaphalis lawii* (Hook.f.) Gamble. *Science Technology and Arts Research Journal* 1(3): 8-16.
- Khan, M.S.S., Syeed, S.H., Uddin, M.H., Akter, L., Ullah, M.A., Jahan, S., Rashid, M.H. (2013). Screening and evaluation of antioxidant, antimicrobial, cytotoxic, thrombolytic and membrane stabilizing properties of the methanolic extract and solvent-solvent partitioning effect of *Vitex negundo* Bark. *Asian Pacific Journal of Tropical Disease* 3(5): 393-400.
- Krishnaraju, A.V., Rao, T.V.N., Sundararaju, D., Vanisree, M., Tsay, H., Subbaraju, G.V. (2006). Biological screening of medicinal plants collected from Eastern Ghats of India using *Artemia salina* (Brine Shrimp Test). *International Journal of Applied Science and Engineering* 4(2): 115-125.
- Kumar, M., Rawat, P., Dixit, P., Mishra, D., Gautam, A.K., Pandey, R., Singh, D., Chattopadhyay, N., Maurya, R. (2010). Anti-osteoporotic constituents from Indian medicinal plants. *Phytotherapy* 17(13): 993-999.
- Kumar, P.V., Chauhan, N.S., Padh, H., Rajani, M. (2006). Search for antibacterial and antifungal agents from selected Indian medicinal plants. *Journal of Ethnopharmacology* 107: 182-188.
- Lister, P.D., Wolter, D.J., Hanson, N.D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: Clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clinical Microbiology Reviews* 22(4): 582-610.
- Mahdavi, B., Yaacob, W.A., Din, L.B., Nazlina, I. (2012). Antimicrobial activity of consecutive extracts of *Etligeria brevilabrum*. *Sains Malaysiana* 41(10): 1233-1237.
- Mahmud, S., Shareef, H., Farrukh, U., Kamil, A., Rizwani, G.H. (2009). Antifungal activities of *Vitex negundo* Linn. *Pakistan Journal of Botany* 41(4): 1941-1943.
- Manilal, K.S., Sivarajan, V.V. (1982). Flora of Calicut. Bishen Singh Mahendra Pal Singh, DehraDun, pp 229.
- Martins, S., Amorim, E.L.C., Sobrinho, T.J.S.P., Saraiva, A.M., Pisciotano, M.N.C., Aguilari, C.N., Teixeira, J.A., Mussatto, S.I. (2013). Antibacterial activity of crude methanolic extract and fractions obtained from *Larrea tridentata* leaves. *Industrial Crops and Products* 41: 306-311.
- Merekar, A.N., Pattan, S.R., Parjane, S.K., Nirmal, S.A., Patel, D.S., Shitre, M.R. (2011). Synergistic anthelmintic activity of rhizomes of *Acorus calamus* and roots of *Vitex negundo*. *Pharmacologyonline* 3: 209-212.
- Nguyen-Pouplin, J., Tran, G., Tran, G., Phan, T.A., Dolecek, C., Farrar, J., Tran, T.H., Caron, P., Bodo, B., Grellier, P. (2007). Antimalarial and cytotoxic activities of ethnopharmacologically selected medicinal plants from South Vietnam. *Journal of Ethnopharmacology* 109: 417-427.
- Pandey, A., Bani, S., Satti, N.K., Gupta, B.D., Suri, K.A. (2012). Anti-arthritis activity of agnuside mediated through the down-regulation of inflammatory mediators and cytokines. *Inflammation Research* 2012; 61(4): 293-304.
- Pavithra, G.M., Naik, A.S., Siddiqua, S., Vinayaka, K.S., Kekuda, P.T.R., Mukunda, S. (2013b). Antioxidant and antibacterial activity of flowers of *Calycopteris floribunda* (Roxb.) Poiret, *Humboldtia brunonis* Wall and *Kydia calycina* Roxb. *International Journal of Drug Development and Research* 5(2): 301-310.
- Pavithra, G.M., Vinayaka, K.S., Rakesh, K.N., Junaid, S., Dileep, N., Kekuda, P.T.R., Siddiqua, S., Naik, A.S. (2013a). Antimicrobial and antioxidant activities of a macrolichen *Usnea pictoides* G. Awasthi (Parmeliaceae). *Journal of Applied Pharmaceutical Science* 3(8): 154-160.
- Penarrieta, J.M., Alvarado, J.A., Bergenstahl, B., Akesson, B. (2007). Spectrophotometric methods for the measurement of total phenolic compounds and total flavonoids in foods. *Revista Boliviana De Quimica* 24(1): 5-9.

- Poornima, G., Kekuda, P.T.R., Vinayaka, K.S. (2012). Antioxidant efficacy of *Olea dioica* Roxb (Oleaceae) leaves. *Biomedicine* 32(4): 506-510.
- RaamaMurthy, J., Venkataraman, S., Meera, R., Deshmukh, K.S., Chidambaranathan, N., Devi, P. (2010). Phytochemical investigation and Antipyretic activity of leaf extract of *Vitex negundo* Linn. *International Journal of PharmTech Research* 2(2): 1068-1073.
- Raghavendra, H.L., Vijayananda, B.N., Hiremath, M.G., Kumar, V. (2010a). *In vitro* Antioxidant activity of *Vitex negundo* L. leaf extracts. *Chiang Mai Journal of Sciences* 37(3): 489-497.
- Raghavendra, H.L., Kekuda, P.T.R., Valleesha, N.C., Sudharshan, S.J., Chinmaya, A. (2010b). Screening for cytotoxic activity of methanol extract of *Putranjiva roxburghii* Wall (Euphorbiaceae) seeds. *Pharmacognosy Journal* 2(10): 335-337.
- Rekha, C., Poornima, G., Manasa, M., Abhipsa, V., Devi, P.J., Kumar, V.H.T., Kekuda, T.R.P. (2012). Ascorbic acid, Total phenol content and antioxidant activity of fresh juices of four ripe and unripe citrus fruits. *Chemical Science Transactions* 1(2): 303-310.
- Rohman, A., Riyanto, S., Yuniarti, N., Saputra, W.R., Utami, R., Mulatsih, W. (2010). Antioxidant activity, total phenolic, and total flavanoid of extracts and fractions of red fruit (*Pandanus conoideus* Lam). *International Food Research Journal* 17: 97-106.
- Roosevelt, C., Vincent, S., Sujith, K., Darwin, R.C. (2011). Wound healing activity of methanolic extract of *Vitex negundo* leaves in albino Wistar rats. *Journal of Pharmacy Research* 4(8): 2553-2555.
- Sahare, K.N., Anandhraman, V., Meshram, V.G., Meshram, S.U., Reddy, M.V., Tumane, P.M., Goswami, K. (2008). Anti-microfilarial activity of methanolic extract of *Vitex negundo* and *Aegle marmelos* and their phytochemical analysis. *Indian Journal of Experimental Biology* 46(2): 128-131.
- Saluja, M.S., Sangameswaran, B., Sharma, A. (2010). Cytotoxic activity of *Vitex negundo* against Ehrlich Ascites Carcinoma (EAC) in mice. *International Journal of PharmTech Research* 2(2): 1369-1375.
- Singh, D.D., Chitra, G., Singh, I.P., Bhutani, K.K. (2005). Immunostimulatory compounds from *Vitex negundo*. *Indian Journal of Chemistry* 44B: 1288-1290.
- Tasduq, S.A., Kaiser, P.J., Gupta, B.D., Gupta, V.K., Johri, R.K. (2008). Negundoside, an iridoid glycoside from leaves of *Vitex negundo*, protects human liver cells against calcium-mediated toxicity induced by carbon tetrachloride. *World Journal of Gastroenterology* 14(23): 3693-3709.
- Tendon, V.R., Khajuria, V., Kapoor, B., Kour, D., Gupta, S. (2008). Hepatoprotective activity of *Vitex negundo* leaf extract against anti-tubercular drugs induced hepatotoxicity. *Fitoterapia* 79(7-8): 533-538.
- Tigno, X.T., Francisco, A.D. (1993). The comparative effects of purified fractions of *Vitex negundo* L. (lagundi) and crude extracts of *Cassia alata* L. (akapulko) and *Artemisia vulgaris* L. (damongmaria) on inflammatory processes *in vitro*. *NRCP Research Journal* 3(2): 39-54.
- Tilak, J.C., Adhikari, S., Devasagayam, T.P.A. (2004). Antioxidant properties of *Plumbago zeylanica*, and Indian medicinal plant and its active ingredient, plumbagin. *Redox Report* 9(4): 220-227.
- Vadlapudi, V. (2012). *In vitro* antimicrobial activity of plant extracts of *Avicennia alba* against some important pathogens. *Asian Pacific Journal of Tropical Disease* 2(S1): S408-S411.
- Vinayaka, K.S., Swamalatha, S.P., Preethi, H.R., Surabhi, K.S., Kekuda, P.T.R., Sudharshan, S.J. (2009). Studies on *in vitro* antioxidant, antibacterial and insecticidal Activity of methanolic extract of *Abrus pulchellus* Wall (Fabaceae). *African Journal of Basic and Applied Sciences* 1(5-6): 110-116.
- Weckesser, S., Engel, K., Simon-Haarhaus, B., Wittmer, A., Pelz, K., Schempp, C.M. (2007). Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. *Phytomedicine* 14(7-8): 508-516.
- Yoshikawa, T.T. (2002). Antimicrobial resistance and aging: Beginning of the end of the antibiotic era? *JAGS* 50(7): S226-S229.
- Zhishen, J., Mengcheng, T., Janming, W. (1999). The determination of flavonoids contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry* 64: 555-559.