

Original Research

## *Vitex negundo* Linn.: phytochemical composition, nutritional analysis, and antioxidant and antimicrobial activity

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**Abstract:** *Vitex negundo* (VN) is a widely used plant in folk medicine, namely for the treatment of jaundice, wounds, body ache, toothache, asthma, eye pain, and migraine. These effects have been increasingly attributed to its chemical composition. Here, we assessed the VN chemical and nutritional composition and biological activity, with particular emphasis on antioxidant and antimicrobial effects. VN methanol and hexane extracts revealed the presence of important phytochemical, such as terpenoids, polyphenol, steroids, saponins, phenolic compound and flavonoids. Total phenolic content of VN methanol extract from bark was 1082.473 mg/g GAE and that of leaves was 1382.984 mg/g GAE. The total flavonoids content in VN methanol extract from VN bark was 127.744 mg/g QE and that of leaves was 123.776 mg/g QE. VN methanol extract from bark exhibited high antioxidant effects ( $IC_{50}$  = 38.47 ppm). The content (%) of ash, moisture, crude fiber, crude protein and fat in VN leaves was, respectively, 7.86%, 18.35%, 6.52%, 9.687% and 6.19%. VN leaves methanol extract revealed antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus*, with inhibition halos being, respectively, 13 mm and 14 mm, and the MBC values were found to be 1.562 mg/mL and 6.25 mg/mL, respectively. Similarly, VN bark methanol extract led to inhibition halos of 18 mm and 15 mm for *B. subtilis* and *S. aureus*, respectively, and the MBC values were found to be 2.372 mg/mL and 0.245 mg/mL. GC-MS analysis of the VN bark methanol extract revealed that monoolein was the major compounds, with an area of 0.57%. Thus, our results encourage the potential use of VN as a medicinal product, with high protein contents, and prominent antioxidant and anti-bacterial effects.

**Key words:** Phytochemical screening; Methanolic extract; Antimicrobial activity; GC-MS analysis; FTIR analysis.

### Introduction

Our society is slowly progressing towards herbal formulations, which in practice are known to be extremely valuable against a large array of diseases (1). Nature consists of different resources of chemovar and pharmacophores and the naturally available resources provide an alternative to modern medicine in drug discovery (2). Naturally available plants in traditional medicine are the most inexpensive and easily reachable source of the dealing in the primary healthcare system (3). Medicinal plants are used to treat different diseases (4), with more than 85,000 plant species being already recognized for medical use globally, known to possess antimicrobial,

antioxidant, antidiabetic, antimalarial, anthelmintic, anticancer effects (5).

Five leave caste tree (*Vitex negundo* Linn.) belongs to the Verbenaceae family (6), comprises 75 genera and nearly 2500 species and is commonly known as Simali in Nepali, Nirgandi (Hindi), and Nirgundi (Sanskrit). It is a deciduous shrub, occur in tropical to temperate regions (up to 2200 m from east to west). The leaves have five leaflets in a palatal arrangement, which are lanceolate, 4–10 cm long. This plant is commonly found near bodies of water, recently disturbed land, grasslands, and mixed open forests Native from tropical Eastern and Southern Africa and Asia, it is also found in Afghanistan, Bangladesh, India, Bhutan, Pakistan, Myanmar,

Malaysia and Nepal, although mainly found in tropical to temperate regions, especially in South Asian country India (7). Ethnobotanically, it is believed that *Vitex negundo* (VN) is used for the treatment of jaundice (8), wounds (9), body ache (10), toothache (11), asthma (12), eye pain (13), and migraine (14). VN bark is used as verminosis and ophthalmopathy, while flowers are used for cholera and the fruit as an anthelmintic. The entire plant is used for diuretic inflammations, antipyretic and antiseptic (15-17). VN contains different class of secondary metabolites, like polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids. Its leaves consist of viridiflorol, sabiene, 4-terpineol, and gamma-terpiene (18), butanoic acid, p-hydroxy benzoic acid, oleanolic acid (19), angusid, vitamin C, nishindine, sitosterol (20). This plant also consists of vetugnoside, negundoside, 5 hydroxy-7,4'-dimethoxy flavones (21). To the polyphenolic compounds present in this plant, a high antioxidant potential has been proposed, investigated by applying various recognized *in vitro* systems (22). p-Hydroxybenzoic acid and  $\beta$ -sitosterol have been the most commonly isolated phenolic compounds from VN (23). Besides the above referred bioactive effects, it also shows anti-HIV, larvicidal (24), and mosquito repellent activity (25). In this sense, the present study aimed to characterize the phytochemical and nutritional composition of VN, and to assess their antioxidant and antimicrobial effects.

## Materials and Methods

### Plant Material

The fresh leaves and bark of VN were collected from Panchtahr district, Nepal. The taxonomic identification of the plants was done at the Central Department of Botany, Tribhuvan University, Kirtipur by judging the preserved herbariums sensibly.

### Extraction method

The collected fresh leaves and barks were washed with tap water to remove the contaminants. The leaves and barks were shade dried and grinded into the powder form and stored in a clean zipped plastic bag until further use. The phytochemicals present in the powdered leaves and bark were extracted by cold percolation method using methanol and hexane as solvents. Powdered leaves and bark (50 g) of VN were kept separately in clean and dry conical flasks. Three hundred mL solvent was added to each flask and kept for 10 days with frequent shaking. The mixtures were decanted and filtered with the help of cotton plug and thus obtained filtrates were concentrated with the help of rotary evaporator by distillation process at temperature below 70 °C. The concentrated filtrates were kept in a beaker wrapping with aluminium foil containing small pores to facilitate the evaporation of the solvent. After complete evaporation of the solvent, semisolid extracts were obtained and stored in a refrigerator (26).

### Phytochemical analysis

The phytochemicals present in the two different VN plant extracts (bark and leaves) were analyzed by following the protocol given by Ciulei (27).

### Total phenolic content

The total phenolic content (TPC) was analyzed by Folin-Ciocalteu colorimetric method based on oxidation-reduction reaction as described by Waterhouse (28). Gallic acid was used as the standard as it is less expensive and purely available than other options.

### Total flavonoid content

The total flavonoid content (TFC) was determined by aluminium chloride (AlCl<sub>3</sub>) colorimetric assay (29), using quercetin as the standard.

### Proximate analysis of nutritional value

The approximate analysis for VN nutritional composition was determined according to the protocol given by AOAC (30).

### Biological activity assessment

#### Antibacterial assay

Bacterial growth inhibition was tested by using agar well plate method and measured in the form of zone of inhibition (ZOI) or minimum bactericidal concentration (MBC) (31), a modification of the dilution method was used.

#### Antioxidant assay

The antioxidant activity of the different plant extracts was done by DPPH radical scavenging method as described by Blois (32). Data obtained will be recorded as a mean of three determinations of absorbance for each concentration, from which linear correlation coefficient (R<sup>2</sup>) value will be calculated. The regression equation is given as:

$$y = mx + c$$

Where, y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, concentration of the extract will be calculated. The inhibitor concentration against the percentage activity was plotted. Using the linear ( $y = mx + C$ ) or parabolic ( $y = ax^2 + bx + c$ ) equation on this graph for  $y = 50$  value x point becomes IC<sub>50</sub> value.

## Results

### Phytochemical assay

In phytochemical analysis of VN leaves, carbohydrates, terpenoids, steroids, saponins, phenolic compounds and flavonoids were found to be present, al-

**Table 1.** Phytochemical analysis of leaves and bark of *Vitex negundo*.

S. N.	Phytochemicals	Hexane	Methanol
1	Phenolic compounds	+	+
2	Alkaloids	-	+
3	Carbohydrates	+	+
4	Terpenoids	+	-
5	Steroids	+	-
6	Saponins	+	+
7	Tannins	-	+
8	Flavonoids	+	+

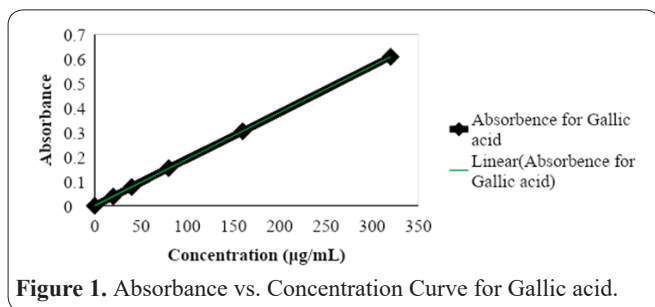


Figure 1. Absorbance vs. Concentration Curve for Gallic acid.

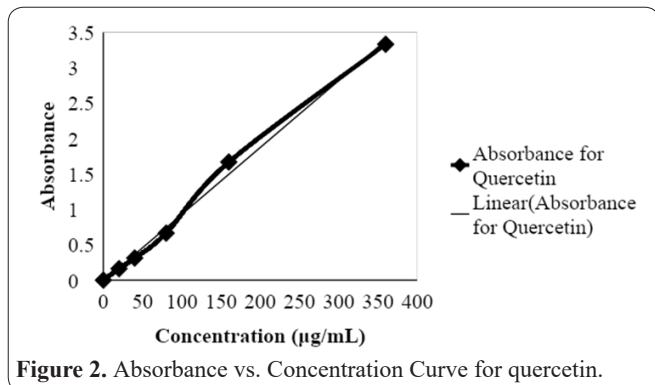


Figure 2. Absorbance vs. Concentration Curve for quercetin.

though tannins and alkaloids were absent in the hexane extract (Table 1). In the VN methanol extract, phenolic compounds, alkaloids, carbohydrates, saponins, tannins and flavonoids were present, while terpenoids and steroids were absent in VN leaves. In the phytochemical analysis of VN bark extracts, our data revealed similar contents to that found in VN leaves extracts.

**Total phenolic and flavonoid contents**

Using the calibration curve and absorbance values of VN leaves and bark methanol extracts (20, 40, 80, 160 and 320 µg/mL), the TPC was calculated as 1082.98 and 1382.89 mg/g gallic acid equivalents (GAE), respectively (Figure 1). Using the calibration curve and absorbance values of VN leaves and bark methanol extracts (20, 40, 80, 160 and 320 µg/mL), the TFC was calculated as 123.78 and 127.77 mg/g quercetin equivalents (mg/g QE) (Figure 2). The highest TPC and TFC were observed to the VN bark methanol extract (TPC=1382.89 mg/g GAE and TFC=127.77 mg/g QE).

**Antioxidant activity**

The antioxidant activity of different VN extracts were obtained by plotting % of free radical scavenging vs concentration, and IC<sub>50</sub> values of respective extracts were determined (Figure 3-6). The IC<sub>50</sub> value of different extracts was calculated and compared with the IC<sub>50</sub> value of ascorbic acid, used as positive control. In brief, the antioxidant activity is inversely proportional to the IC<sub>50</sub> values obtained, i.e. an extract/fraction/compound with small IC<sub>50</sub> values is more potent as antioxidant than those having higher IC<sub>50</sub> values.

The IC<sub>50</sub> values of VN leaves and bark methanol and hexane extracts were found as 70.93, 38.47, 50.61 and 46.66 µg/mL, respectively (Figure 7). When compared with the IC<sub>50</sub> value of the standard, ascorbic acid (24.40 µg/mL), these extracts revealed remarkable antioxidant effects. Worth of note is the IC<sub>50</sub> value obtained to VN bark methanol extract (38.47 µg/mL), which was slightly higher than that of ascorbic acid (24.40 µg/mL), being this extract the most prominent one. The observed

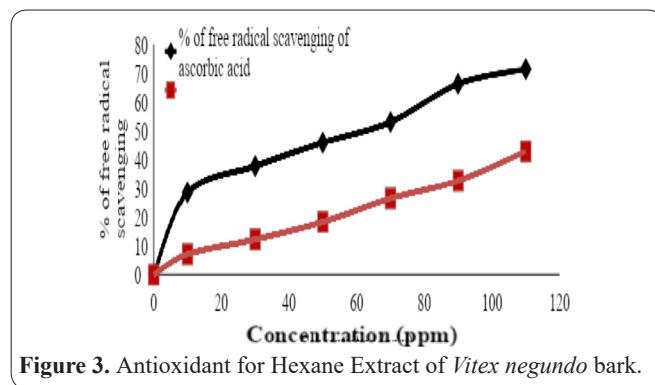


Figure 3. Antioxidant for Hexane Extract of *Vitex negundo* bark.

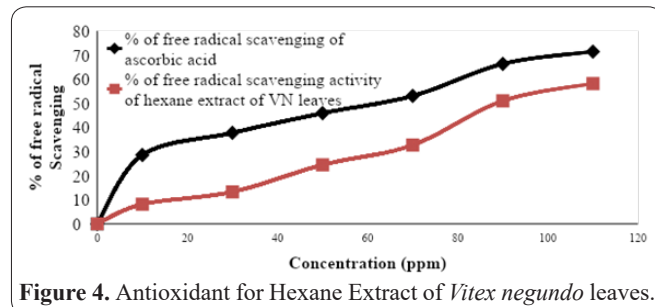


Figure 4. Antioxidant for Hexane Extract of *Vitex negundo* leaves.

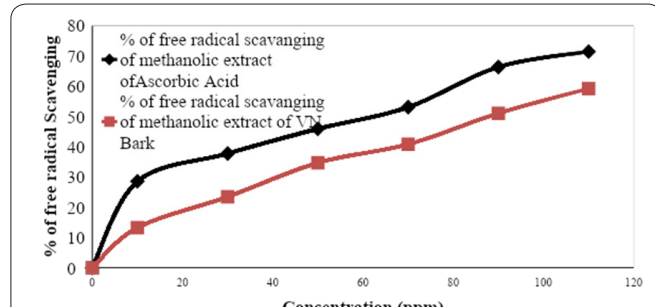


Figure 5. Antioxidant for Methanolic Extract of *Vitex negundo* bark.

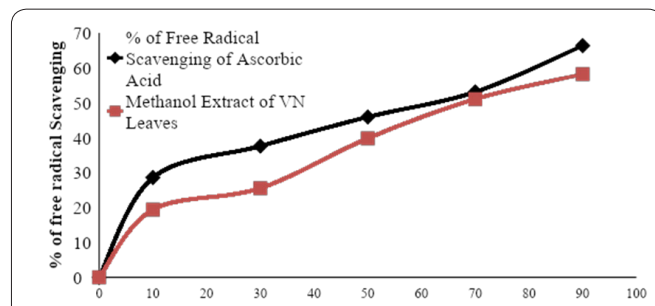


Figure 6. Antioxidant for Methanolic Extract of *Vitex negundo* leaves.

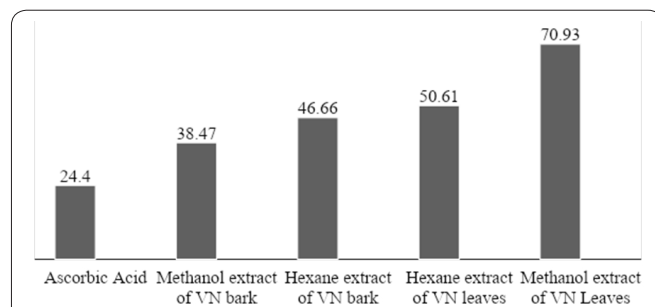


Figure 7. IC<sub>50</sub> values for ascorbic acid and different extract of *Vitex negundo*.

activity is directly correlated with both TPC and TFC obtained to VN bark methanol extract, with these compounds being increasingly correlated as determinant to the final bioactivities of extracts. Particularly to flavo-

**Table 2.** Antibacterial activity of methanolic extract of VN leaves.

S. N.	Bacteria	Reference Culture	ZOI value (mm)			MBC (mg/mL)
			Positive Control Ampicillin	Negative Control Methanol	Plant extract	
1	<i>Bacillus subtilis</i>	ATCC6051	25	0	13	1.562
9	<i>Staphylococcus aureus</i>	ATCC653P	23	0	14	6.25

**Table 3.** Antibacterial activity of methanolic extract of *Vitex negundo* bark.

S. N.	Bacteria	Reference culture	ZOI value (mm)			MBC (mg/mL)
			Positive Control Ampicillin	Negative Control Methanol	Plant extract	
1	<i>Bacillus subtilis</i>	ATCC6051	25	0	18	2.372
2	<i>Staphylococcus aureus</i>	ATCC653P	23	0	15	0.245

**Table 4.** Proximate % composition of VN bark.

S.N.	Parameters	Percentage (%)
1	Ash	7.86
2	Moisture	18.35
3	Crude Fiber	6.52
4	Crude protein	9.687
5	Fat	6.19

noids, it has been stated that they are the main responsible for antioxidant effects through scavenging free radicals or chelating effects.

### Antibacterial activity

The antimicrobial activity of VN leaves methanol extract resulted in ZOI of 13 mm and 14 mm for *Bacillus subtilis* and *Staphylococcus aureus*, respectively, while to the other microorganisms, no visible effects were observed at the tested doses. The MBC values for the two bacteria were found to be 1.56 mg/mL and 6.25 mg/mL, respectively (Table 2). Similarly, the antimicrobial activity for VN bark methanol extract resulted in ZOI of 18 mm and 15 mm for *B. subtilis* and *S. aureus*, respectively, while the other microorganism tested were found to be resistant. The MBC values for the two bacteria were found to be 2.37 mg/mL and 0.24 mg/mL, respectively (Table 3).

### Proximate analysis of *Vitex negundo*

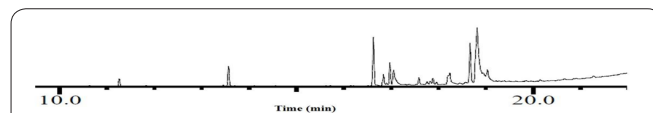
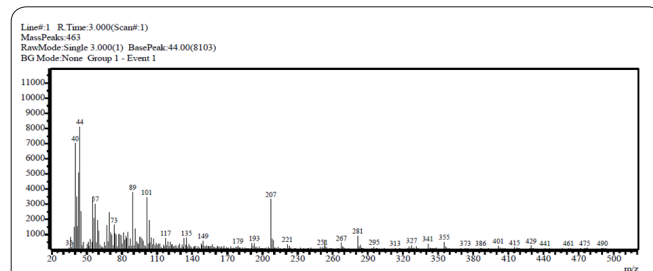
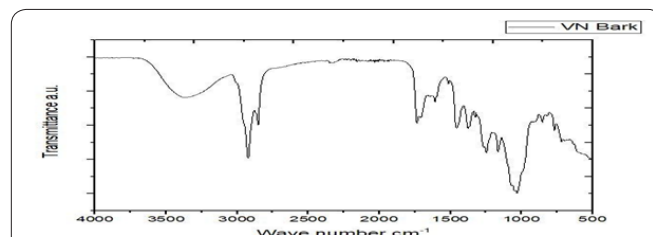
The proximate analysis is a set of methods to get data on the nutritional value of foods. In proximate analysis of VN bark chemical composition, ash, moisture, crude fiber, crude protein and fat were found to be 7.86%, 18.35%, 6.52%, 9.687% and 6.19%, respectively, clearly highlighting that VN bark contains interesting amounts of crude fiber and protein (Table 4).

### GC/MS analysis

This method was accomplished by using a standard protocol (33). From the chromatograms and mass spectra obtained to VN bark methanol extract (Figure 8 and 9), 12 major compounds are of special relevance (Table 5). Among them, caryophyllene, caryophyllene oxide, methyl palmitate, methyl octadecanoate, monoolein and ledol compounds appears to be responsible for both antioxidant and antimicrobial effects observed. From the obtained results regarding antioxidant and antibacterial effects, and considering the GC-MS data, VN bark methanol extract showed the most potent activity.

**Table 5.** List of compounds obtained from methanolic extract of VN bark.

S.N.	Name of Compounds	Area%
1	Caryophyllene (E)	0.03
2	Caryophyllene oxide	0.01
3	Viridiflorol	0.10
4	Methyl palmitate	0.14
5	Beyerene isomer	0.04
6	Longifolene	0.02
7	Kolavenol	0.03
8	Mannol	0.18
9	Phytol	0.08
10	Methyl octadecanoate	0.12
11	Monoolein	0.57
12	Ledol	0.01

**Figure 8.** Chromatogram for methanolic extract of VN bark.**Figure 9.** m/z values for the respective chromatogram of methanolic extract of VN bark.**Figure 10.** FTIR analysis for bark of *Vitex negundo*.

### FTIR analysis

The FTIR spectrum of VN bark showed -OH (alcohol), -NH (amide), -CH, -C=O (Carbonyl), -C=O (Aldehyde), -C=C (Aromatic), -C-O (Acid) and -C-O (Ether) groups obtained in 3363, 3390, 2841, 1734, 1732, 1448,

**Table 6.** FTIR analysis for methanolic extract of VN bark.

S.N.	Functional Group	Type of Vibration	Characteristics Vibration (cm <sup>-1</sup> )[15]	Observed Range (cm <sup>-1</sup> )
1	-OH (Alcohol)	Stretch	3200-3600	3363
2	-NH (Amide)	Stretch	3300-3500	3390
3	-CH	Stretch	2850-3000	2841
4	-C=O (Carbonyl)	Stretch	1670-1820	1734
5	-C=O (Aldehyde)	Stretch	1740-1720	1732
6	-C=C (Aromatic)	Stretch	1400-1600	1448
7	-C-O (Acid)	Stretch	1210-1320	1238
8	-C-O (Ether)	Stretch	1000-1300	1041

1238 and 1041 cm<sup>-1</sup>, respectively (Figure 10). Thus, in this extract, alcoholic, amide, carbonyl, aldehydic, etheric compounds were potentially present (Table 6), as shown in the GC-MS analysis, and ester compounds showed feasible biological effects.

## Discussion

In this study, methanol and hexane extracts of *Vitex negundo* leaf and bark were evaluated for their phytochemical, antioxidant using DPPH, total phenolic content, total flavonoid content, antibacterial activity, proximate analysis, GC-MS and FT-IR studies. The diverse methods used are vital due to presence of bioactive components in the plants that maybe responsible for biological activity of therapeutic plants.

The TPC and TFC are correlated to the various biological activity, anticancer activity, anti-ageing effect (34). Moreover, the high antioxidant activity witnessed by plant is due to synergistic effects of flavonoids or phenolic compounds. Studies showed that antioxidant activity of flavonoids account to be better than that of individual flavonoid (35). Thus, high antioxidant activity of *Vitex negundo* was due to presence of high amount of phenolic and flavonoid content (i.e. TPC in leaves and bark 1082.984 and 1382.894 mg/g GAE as methanol solvent and TFC in leaves and bark 123.776 and 127.774 mg/g QE as methanol solvent respectively).

The antioxidant activity of the plant depends on various factors like composition, hydrophobic or hydrophilic nature of the compounds, and quality of solvent used, type of extraction used, temperature and conditions of the test systems (36). The parameter used to measure antioxidant is IC<sub>50</sub>, it means antioxidant required to reduce the concentration of DPPH by 50% (37). A lower IC<sub>50</sub> value, higher will be the antioxidant power (38). In our research the lowest value of IC<sub>50</sub> for methanolic extract of *Vitex negundo* (38.47%) which is comparable to the standard natural antioxidant (L-ascorbic acid, IC<sub>50</sub> value 24.4%). This result was also verified by the presence of high amount of TPC and TFC in methanolic extract of *Vitex negundo*. Some research paper revealed that *Vitex negundo* contains fewer amounts of TPC and TFC content (89.71 mg/g GAE and 63.11 mg/g QE) (39).

Gram positive bacteria are less resistant to the synthetic antibiotics as compared to Gram negative bacteria (40). In this study, two Gram positive bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) were significantly inhibited their growth by using VN plant extract. *B. subtilis* is commonly found in soil, water plant and animal tissues. This bacterium can cause number of diseases

and food poisoning and *S. aureus* acts as opportunistic pathogen, can cause of skin infections, respiratory infections such as sinusitis, and food poisoning (41). In this study, VN methanolic extract of bark and leaves give you an idea about a comparable ZOI value with a reference antibiotics ampicillin and could be subjected for antiseptic purpose by analyzing its side effects. The antimicrobial activity measured by us was found comparable in range as reported by several research papers (42) and different phytochemicals like ledol, n-hexadecanoic acid phytol, caryophyllene, benzoic acid etc. were reported to inhibit bacterial activity (43). Some previous study showed that minimum bactericidal concentration is relatively higher (>5) for methanolic extract of VN bark and leaves (42).

In the proximate analysis, the ash and moisture values were found high. Similarly, crude fiber and crude protein were also found much more than expected. The higher fat value witnessed the plant contain oily substances. The obtained data was almost similar as previously reported by Nepalese researchers (43). But this result is quite different from foreign plant sample. Following AOAC standard method of analysis, leaves of VN collected in Philippines were shown to contain less amount of moisture (16.95%) as compared to sample used in Nepal (18.37%). But, ash, crude fibre, fat and proteins in Philippines were shown to contain more (8.00, 28.00, 7.00 and 13.73% respectively) (44) than sample used in Nepal. These differences may be due to climatic condition, season of sample collection, maturity of the plant selected and experimental condition.

From GC-MS analysis of *Vitex negundo*, various bioactive compounds like Caryophyllene E (terpenoids), Caryophyllene oxide (terpenoids), Viridiflorol (phenolic compound), Methyl palmitate (fatty acid), Beyerene isomer (terpenoids), Longifolene (liquid hydrocarbon), Kolavenol(diterpenoids), Mannol (diterpenoids), Phytol( Diterpene alcohol), Methyl octadecanoate, Monoolein, Ledol were elucidated with their area percentage. It is believed that these phytochemicals are accountable for various biological activities such as antioxidant, antimicrobial, anti-fungus activity, hepatoprotective activity etc. As we performed GC-MS of methanolic extract of VN bark. The compounds obtained by this process are quite similar to the compounds elucidated in some papers (VN leaves) (42, 43). The different functional groups present in the compounds of different class of secondary metabolites were confirmed by using Fourier transform infrared spectroscopy (FT-IR). Different functional groups were seen in their respective standard functional group range.

Carbohydrates, terpenoids, polyphenol, steroids, saponins, phenolic compound and flavonoids were present as secondary metabolites. The TPC and TFC value for methanolic extract of VN bark was found to be higher than methanolic extract of VN leaves i.e. in hexane and methanolic extract of bark has shown highest antioxidant activity. The antimicrobial activity of methanolic extract of VN bark was potent ZOI towards *Bacillus subtilis* and *Staphylococcus aureus* than the methanolic extract of VN leaves. From proximate analysis, Fat and crude protein were sufficiently present. From GC-MS analysis, it has been found that the phytochemicals responsible for the biological activities such as antimicrobial, antioxidant etc. were identified. From FTIR analysis, functional group of phytochemicals obtained from GC analysis were elucidated. Thus, *Vitex negundo* plant selected showed major biological activities, however, they cannot directly be referred for pharmaceutical usage. Further extensive phytochemical and pharmacological studies along with mechanism of action are crucial not only to estimate this preliminary experiment but also to characterize and isolate the unknown compounds to inaugurate their pharmacological properties.

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### Conflict of Interest

The authors declare there is no conflict of interest.

### Authors Contributions

NK supervised and contributed to the overall data curation and writing of the manuscript; CD did the experimental works and chemical preparations; NNM revised the manuscript and offline research; JSR, BS and NM helped in data curation and manuscript editing and RCB guided the execution of the experiments and editing of the article.

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