Research Article

Assessment of Phytochemical Profiling and Therapeutic Potential of the Ethanolic Extract of *Vitex negundo* (L.)

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Abstract | *Vitex negundo* (L.) is a medicinal small tree of the Verbenaceae family that grows in Pakistan and India. The current study aims to identify the existence of several natural compounds (flavonoids, terpenoids, alkaloids, glycosides, and saponins) in the bark of *V. negundo* (L.) roots and stem, as well as their potential antioxidant and antibacterial properties. The crude was obtained in 95 % methanolic which was further fractionated into n-hexane (HR, HS), Chloroform (CR, CS), ethyl acetate (EtR, EtS) and butanol (BR, BS) soluble fractions by solvent-solvent extraction. In phytochemical tests of roots fractions; HR showed the presence of terpenoids, while saponins, alkaloids and flavonoids were detected in CR. EtR was enriched in terpenoids and phenols and the butanol fraction (BR) contained terpenoids and flavonoids. Similarly in stem fractions; HS contained alkaloids and phenols while terpenoids and Phenols were present CS. EtS showed the presence of flavonoids, while butanol fraction (BS) contained terpenoids and flavonoids. In Thin Layer Chromatography (TLC) examination showed, that there were several individual spots identified the existence of detected phytochemicals. Almost all the fractions showed very strong DPPH free radical inhibitory effects. Strong antioxidant activity was found for BR (IC_{50}= 62.5 µg/mL) and BS (IC_{50}= 80 µg/mL) probably due to the presence of flavonoids in butanol fractions, evident from the Fourier Transform (FT-IR) spectral analysis which gave distinct absorption of flavonoids functionalities. In antimicrobial studies, CR exhibited strongest antibacterial activity against *E. coli*, *S. flexneri* and *S. aureus* with inhibition zone of 25±0.75, 21±0.35 and 18±0.21 mm respectively. The same fraction showed very potent antifungal effect against *A. niger* and *A. flavus* with inhibition zones of 19±0.45 and 18±0.39 mm respectively. In case of stem extracts, CS exhibited strongest antibacterial activity against *E. coli*, *S. aurous* and *S. flexneri* with inhibition zone of 24±0.23, 22±0.67 and 20±0.32 mm respectively while the same fraction showed maximum inhibition of linear growth (22±0.76 and 19±0.66 mm) of *Candida albicans* and *A. niger,* respectively. We conclude that *V. negundo* (L.) has potential antimicrobial and antifungal capacities which make it a potential candidate for the development of antibiotic drugs through pharmacological formulation.

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Introduction

Medicinal plants contain phytochemicals such as flavonoids, alkaloids, terpenoids, and glycosids that are extremely effective at resisting microbiological agents. Because of their multiple beneficial characteristics, bioactive chemicals found in natural plants have ben preferred over synthetic substances (Khan *et al*., 2022). Natural products and secondary metabolites derived from living systems, primarily plants, have been proved to improve health care since ancient times. Furthermore, the success rate of modern medical technology is also dependent on medications obtained from natural resources. Natural resources' unlimited application in treating various human ailments increases their usage due to their inexpensive cost and lack of adverse effects. The World Health Organisation reports that 80% of people in poor nations rely on traditional medicines are generally economical, safe, efficient, and reliable (Khan *et al*., 2023). Natural resources, such as plants, play a crucial role in screening for unique and effective solutions drug development aims to address high costs and drug resistance (Rahman *et al*., 2023). The *V. negundo* (L.) is medicinal small tree or an erect shrub of the family Verbenaceae that grows from 2 to 8 meters in height and is used in everywhere traditional medicine, in South and Southeast Asia. *V. negundo* L. has a reddish-brown bark and five digitate leaves with, sometimes three lanceolate leaflets and their flowers are many in number mostly white and blue in colors borne in panicles 10 to 20 cm (3.9 to 7.9 in) in length (Singh *et al*., 2020). *V. negundo* is one of the important species of the genus *Vitex* that occurs in Pakistan, India, and Sri Lanka (Azhar-Ul-Haq *et al*., 2004). Therapeutic potentials of *V. negundo* are broadly divided into three categories viz; traditional medicine, folk medicine, and pharmacological evidence (Prajapati *et al*., 2004). Phytochemical studies have indicated that *V. negundo* is rich in several significant natural products like alkaloid, monoterpenes, agnuside, flavonoids, chryso-splenolvitexin, flavonoids and addition with poly-methoxy flavone (Zheng *et al*., 2009). It is also manifest from several recent studies that the quality, quantity and natures of the chemical constituents found in distinct parts of the plant (root, stem, bark, leaves, flowers, and fruit) are not the same (Akhtar *et al*., 2019). Recent studies reveal that *V. negundo* is rich in various important bioactive constituents including flavonoids, steroids, terpenes (triterpenes, diterpenes, sesquiterpenes), lignans, iridoids, and volatile oils (Gill *et al*., 2018; Koirala *et al*., 2020). Particularly, it is highly effective in treating irregular menopause, deprived lactation, bleeding disorders of the uterus and irregular menstrual periods (Sharma *et al*., 2011). The flowers are used in diarrhea, fever, and liver diseases. The fruits are used as vermifuge, and for the treatment of eye disorders and headache (Ladda and Magdum, 2012). Several studies have recognized the inflammatory properties of *V. negundo* (L.) in acute and subacute inflammation. The anti-inflammatory and pain-relieving properties of the fresh leaves of *V. negundo* (L.) are endorsed by its inhibiting potential of prostaglandin synthesis, membrane stabilization, antihistamine activity and antioxidant activity (Tandon and Gupta, 2006; Lakshmanashetty *et al*., 2010). Studies about the leaf extracts of *V. negundo* (L.) establish its antioxidant properties. A study on Freund's adjuvant induced arthritic-rats revealed that the plant extract decreases the levels of several oxidizing enzymes including and glutathione peroxidase, superoxide dismutase and catalase. The leaf extract of the plant was found rich in carotene, vitamin C and flavones which can relieve oxidative stress through decreasing the lipid peroxidation (Devi *et al*., 2007). *V. negundo* (L.) has also reported to show enzyme inhibition activity against HIV (Human Immunodeficiency Virus) type 1 reverse transcriptase, xanthine-oxidase, lipoxygenase, α-chymotrypsin, butyryl-cholinesterase and tyrosinase (Lodhi *et al*., 2008; Kannan *et al*., 2012). Leaves extract of *V. negundo* (L.) has been reported to have strong antimicrobial activity against *C. albicans, S. mutase, S. aureus, E. coli* and *K. Pneumoniae*. Moreover, its strong inhibitory activities were also reported against different fungal strains (Khatak *et al*., 2014; Koirala *et al*., 2020). *V. negundo* (L.) has high antifungal activities against the fungal strains such as A. alternate, *A. niger, C. albicans, C. lunata, T. mentagrophytes, C. neoformans, F.solani, M. canis* (Guleria and Kumar, 2006; Aswar *et al*., 2009; Mahmud *et al*., 2009). Experiments on Agrobacterium strains in a potato disc bioassay revealed that methanol extract of *V. negundo* (L.) has paramount antitumor activity in a concentration-dependent approach. In another study while using in Swiss Albino mice, it was reported that ethanolic leaf extract of *V. negundo* (L.) has potential antitumor activity against Dalton's ascitic lymphoma (Chitra *et al*., 2009). The water extract of *V. negundo* (L.) revealed a noteworthy increase in the production of rat feces and softening of stool at a dose-dependent increase level. Researchers have confirmed that anthracene

derivative is responsible for such results (Adnaik *et al*., 2008). Devani and his team in 2013 reported that the leaf extracts of *V. negundo* (L.) has inhibitory effects on the catalytic activity of α-amylase that is responsible for hydrolysis of α-1, 4-glycosidic linkages of various polysaccharides including starch, glycogen, and other oligosaccharides. Based on the findings, the researchers suggest that the plant has significant antidiabetic activity (Devani *et al*., 2013). *V. negundo* (L.) demonstrate strong insecticidal activity against several insects for instance *S. cerealella* (Angoumois grain moth), *C. maculates* (pulse beetle), *P. aperculella* (potato-tuber moth), *A. citricola* (Spirea aphid), *A. gossypii* (Jiang *et al*., 2009). The purpose of current study was to obtain crude methanolic extract and various fractions of *V. negundo* roots and stem, their phytochemical screening and to analyze antimicrobial, antifungal, antioxidant and selective enzyme inhibition activities.

Materials and Methods

Plant identification and collection

The *V. negundo* (L.) was collected from Makhay Valley, Munda, and Lower Dir in August (2019). The identification of plant was carried with help of flora of Pakistan (Munasif *et al*., 2007). The identified plant was deposited in herbarium Department of Botany at University Malakand, Dir Lower under voucher number H.UOM.BG-76.

Extraction of plant material

The bark of root and stem of *V. negundo* (L.) was washed with water thrice to remove any dust particles, and then dried under shade for one week. The barks of stem and roots were separated, chopped into small pieces, and converted to fine powder in a blender. The roots powdered material (700 g) was suspended in 95 % methanol (5 L) through cold maceration method for one week followed by filtration through neat cloth (removal of residue), then with Whatman filter paper No. 42 to remove cloudy substances. The solvent was faded away from the extract on a Rotary evaporator (Heidolph, Germany) at 40° C to obtain root crude extract (R, 120 gm). Similar treatment was carried out with the bark of stem powered material (1200 g in 10 L ethanol) which resulted in obtaining crude stem extract (S, 120 g).

Fractionation of roots and stem crude extracts

The root crude extract (R, 120 gm) was deferred in distilled water (200 mL) and the suspension was stirred constantly for four hours. This aqueous suspension was than pooled with *n*-hexane (200 mL), chloroform (200 mL), ethyl acetate (200 mL), butanol (200 mL) successively and separately in a separating funnel (20 gm/100 mL w/v ratio) to provide HR, CR, EtR and BR fractions, respectively. Similar treatment was done with the bark of stem crude extract which provided HS, CS, EtS and BS fractions respectively (Scheme 1 & 2).

Phytochemical screening tests

Primary phytochemical tests were accomplished for the crude and all the fractions by means of simple chemical testes using standard procedures as described below (Masood *et al*., 2013).

Test for alkaloids

In the current investigation, the alkaloids were identified by means of Wagner's test. Wagner's reagent was prepared by mixing 2 g of iodine with 6 g of potassium iodide and the total volume was raised to 100 ml by the addition of distilled water. The samples were prepared by taking 0.2g of the extracts in 5 ml of methanol and then 3 drops Wagner's reagent were added to the samples. The red color appeared in the bottom of test tubes shown the existence of alkaloids.

Test for phenols

Ferric chloride solution was prepared for the determination of phenols in the samples. For the preparation of ferric solution 0.2 g of the extracts and 5 mL of methanol were taken in the test tubes, then few drops of $FeCl_3$ solution were added. The appearance of bluish-black color in the test tubes confirmed the presence of phenol.

Test for saponins

For the determination of saponins content, 0.2 g of the extract was mixed with 5 mL of distilled water in a test tube. The appearance of small creamy bubbles (frothing) indicated the existence of saponins content.

Test for flavonoids

For the determination of flavonoids content 0.2 g of the extracts was added with diluted NaOH and addition of Hydrochloric acid (HCl) drops by drop. The yellowish solution of the samples turned colorless which indicated the existence of flavonoids.

Test for terpenoids

Terpenoids content were determined by chloroform/

Sulphuric acid test. The extracts (0.2 gm) were mixed with 2 ml of $CHCl_3$ (chloroform) in a test tube and then 3 ml of Sulphuric acid (H_2SO_4) was carefully added that formed a layer on its surface with a reddish-brown interface.

Thin layer chromatography (TLC)

All the fractions were monitored by normal phase pre-coated silica gel thin layer chromatography cards (20 X 20) for their constituents using simple procedures. The cards were activated, resized, spotted and dried in oven at 120˚C for one-hour prior development. Various solvents mixtures were applied as mobile phases for obtaining of chromatograms. After the development, the chromatograms were monitored visually or using UV lamp (λ_{max} 254 and 360 nm). The spots were further identified using visualization reagents for each class of the natural product by spraying the chromatogram and drying in oven. The data was recorded and the final chromatograms were showed using Chem Draw software.

DPPH free radical scavenging effect

1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging aptitudes of all the fractions were determined by the fall of reactive color between DPPH and fractions (1 mL DMSO) in line with the method of Huang *et al*. with slight modifications. Fraction solutions (0.1 mL) were mixed with 3.9 mL of DPPH solution in DMSO (0.075 mM), and the mixture was incubated in the dark at room temperature for 30 min and then centrifuged for 2 min at 2500 rpm to separate any cloudy substances. Absorbance of the samples was noted at 517 nm using UV/Visible spectrophotometer. The control was prepared by mixing 0.1 mL DMSO and 3.9 mL DPPH and then measured immediately (t= 0 min). Fractions which showed promising DPPH scavenging effect and was subsequently assayed for its IC_{50} values (Szczepaniak *et al*., 2019). The absorbance was calculated according to the following formula.

$$\% \text{ DPPH scavenging} = A\text{-}B/A \text{ X } 100 \quad \ldots.(1)$$

A= Absorbance of the mixture of DPPH solution and DMSO at t= 0 min, B= Absorbance of the mixture of DPPH solution and sample at t = 30 min.

Antimicrobial bioassays

Agar Well Diffusion Method with necessary modifications was used for the determination of antibacterial activities of the root and stem bar extracts *V. negundo* (L.). The stock solutions of the extracts were prepared in DMSO, and four bacterial strains were used for antibacterial activities which included *S. flexneri* (ATCC 14028), *B. subtilis* (ATCC 6663), *E. coli* (ATCC 15224) and *S. aureus* (ATCC 29213). A 100 µL of the solution was added to the corresponding wells containing strains. *Clarithromycin* solution (10 µg/ml) was used as a positive control and a standard. For proper and slow diffusion, the plates were kept at room temperature for 10-15 minutes followed by incubation at 37 ºC for 24 hours and finally the zones of inhibition zones were measured in millimeters (mm).

Antifungal activity

For the determination of antifungal activities, agar dilution method (Rahman *et al*., 2011) was followed while four pathogenic strains of fungi namely, *A. flavus* (ATCC 32611), *T. longifusus* (ATCC 22397), *A. niger* (clinically isolated), *C. albicans* (ATCC 2091). The stock solutions were prepared in DMSO as mentioned above and concentrations of 100 µg/mL were used for calculating the growth inhibition of tested fungi. The plates were incubated at 37 ˚C for one week and growth inhibition of fungal strains was noted by visual inspection after 7 days. Miconazole (10 µg/mL) was used as a standard positive control. Media growth was evaluated in millimetres (mm), and growth inhibition was determined by comparing it to the standard inhibition.

Fourier transformed infrared spectroscopy (FT-IR)

Cary 660 FTIR spectrometer, Agilent Technologies, USA spectrometer with ATR assembly was used to record spectra in the range of 600–4000 cm^{-1} with 256 scans per sample at 4 cm^{-1} resolutions to identify functional groups of the samples (Murad *et al*., 2019).

Results and Discussion

Phytochemical profiling

Preliminary phytochemical tests were carried out for the detection of various natural products such as flavonoids, terpenoids, alkaloids, glycosides and saponins in *V. negundo* roots and stem crude as well as fractions. The results have been illustrated in shown in the (Tables 1 and 2). The crude extract of roots (R) was enriched in saponins, terpenoids,phenols and flavonoids while alkaloids and saponins were detected in small quantities. The *n*-hexane fraction

(HR) showed presence of terpenoids and flavonoids only. Saponins, alkaloids and flavonoids were detected in chloroform fraction (CR) while ethyl acetate (EtR) showed the presence of terpenoids and phenols. The butanol fraction (BR) contained terpenoids and flavonoids in considerable amounts. Over all, the roots contained major amounts of flavonoids, terpenoids as well as promising amounts of alkaloids. The crude extract of roots (S) was enriched in saponins, terpenoids, phenols and flavonoids while alkaloids and saponins were detected in minute quantities. The *n*-hexane fraction (HS) showed presence of alkaloids and phenols. Terpenoids and Phenols, were detected in chloroform fraction (CS) while ethyl acetate (EtS) showed the presence of Flavonoids. The butanol fraction (BS) contained Terpenoids and Flavonoids in considerable amounts. Over all, the stem contained major amounts of Terpenoids, Flavonoids, phenols as well as promising amounts of alkaloids. The overall results of these phytochemical tests confirm the presence of saponins flavonoids, terpenoids and phenols in the various fractions of this plant while alkaloids were not present in considerable amounts.

Table 1: Phytochemical tests of *V. negundo* crude and various fractions from roots.

S. No	Classes	Crude	HR	CR	EtR	BR
1	Saponins	+	_	++	_	_
2	Terpenoids	++	+++	_	+++	+++
3	Alkaloids	+	_	+++	+	_
4	Flavonoids	+++	_	+++	_	+++
5	Phenols	+++	+++	_	++	_

Table 2: Phytochemical tests of *V. negundo* crude and various fractions from stem.

S. No	Classes	Crude	HS	CS	EtS	BS
	Saponins	+	+	++	_	_
	Terpenoids	++	+	+++	_	+++
	Alkaloids	+	++	++	+	_
	Flavonoids	+++	_	_	+++	+++
	Phenols	+++	++	+++	_	_

Key: -: Absent; +: Exist; ++: Exist extensively; +++: Exist very extensively.

Thin layer chromatography (TLC)

All the fractions were monitored by normal phase pre-coated silica gel thin layer chromatography cards (20 X 20) for their constituents using simple procedures. The cards were activated, resized, spotted and dried in oven at 120°C for one-hour prior development. Various solvents mixtures were applied as mobile phases for obtaining of chromatograms. After the development, the chromatograms were monitored visually or using UV lamp (λ_{max} 254 and 360 nm). The spots were further identified using visualization reagents for each class of the natural product by spraying the chromatogram and drying in oven. The data was recorded and the final chromatograms were showed using Chem Draw software.

Combine TLC of all fractions

10 mg of each extract was dissolved in methanol (1 mL) as stock sample. TLC cards (20 X 20) were activated, cut in suitable sizes and spots are applied using a small capillary. Mixture of different solvents was used as improving solvents. After development of chromatograms, each one was air dried and the spots were monitored by UV lamp followed by spraying with ceric sulphate reagent and dried in oven (110 °C) for five minutes to visualize. Majority of the spots for n-hexane fractions (HR and HS) were clearly visible in TLC-B, while TLC-C system was adopted for CR and CS extracts. TLC-D system showed good chromatogram for EtR and EtS while no system was adopted for BR and BS (Figure 1).

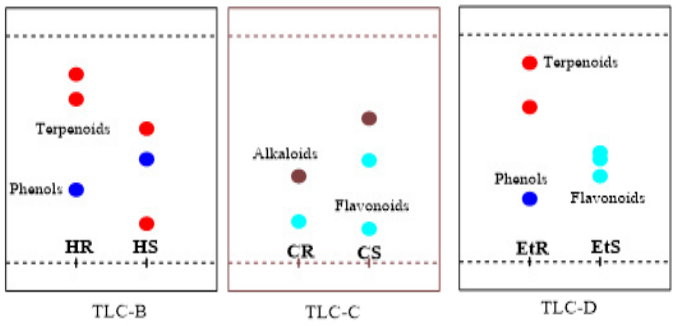

Figure 1: *TLC chromatograms developed for crude and all the fractions and visualized by detecting reagent for various classes of natural products.*

TLC for HR and HS in TLC-B solvent system

The n-hexane fractions from roots and stem labelled as (HR and HS) exhibited some spots in TLC-B solvent system was again chromatographed separately on TLC using solvent system i.e. n-hexane: EtOAc (9:1) with few drops of glacial acetic acid which resulted in few spots on TLC card. The chromatogram was further visualized with detecting reagents for phenols and terpenoids by spraying and drying on heat gun. phenols produced blue color spots while the presence of terpenoids was confirmed by spraying the plates with sulphuric acid and observing the red colors spots.

Table 3: TLC solvents systems for various fractions of *Vitex negundo* roots and stem.

S. No	TLC code	Solvent system
1	TLC-A	n-hexane: EtOAc (1:9)
2	TLC-B	n-hexane: EtOAc (9:1)
3	TLC-C	n-hexane: EtOAc (5:5)
4	TLC-D	n-hexane: EtOAc (3:7)
5	TLC-E	n-hexane: EtOAc (7:3)

Table 4: Concentration-dependent DPPH free radical scavenging effect of *V. negundo* roots fractions.

Concentration (µg/mL DMSO)	DPPH % activity of roots fractions			
	HR	CR	EtR	BR
1000	75± 0.2	90±0.23	76±0.44	97±0.12
500	72±0.3	78±0.3	56±0.3	86±0.3
250	56±0.3	66±0.3	46±0.3	72±0.3
125	33±0.3	54±0.3	23±0.3	63±0.3
62.5	22±0.3	37±0.3	--	50±0.3
31.25	--	30±0.3	--	41±0.22
IC_{50}(µg/mL)	200 µg/mL	112 µg/mL	425 µg/mL	62.5 µg/mL

Table 5: Concentration-dependent DPPH free radical scavenging effect of *V. negundo* stem fractions.

Concentration (µg/mL DMSO)	DPPH % activity of stem fractions			
	HS	CS	EtS	BS
1000	56 ± 0.2	76± 0.2	90± 0.2	94± 0.2
500	44± 0.2	65± 0.2	76± 0.2	78± 0.2
250	23± 0.2	51± 0.2	66± 0.2	70± 0.2
125	--	43± 0.2	50± 0.2	59± 0.2
62.5	--	32± 0.2	43± 0.2	48± 0.2
31.25	61± 0.26	--	33± 0.27	40± 0.26
IC_{50} µg/mL	900 µg/mL	245 µg/mL	125 µg/mL	80 µg/mL

TLC for CR and CS in TLC-C solvent system

The chloroform fractions from roots and stem labelled as (CR and CS) exhibited various spots in TLC-B solvent system (n-hexane: EtOAc (5:5)) which was visualized for the confirmation of the presence of already detected natural products. Spraying with appropriate reagents for the presence of alkaloids revealed that the CR showed a single spot (red) when sprayed with Wagner's reagent, whereas the CS showed no spot. The flavonoids were detecting by observing the both CR and CS spots in UV 256 nm. Bright colors spots showed the presence of flavonoids in both CR and CS.

TLC for EtR and EtS in TLC-D solvent system

The ethyl acetate fractions from roots and stem labelled as (EtR and EtS) exhibited various spots in TLC-D solvent system (n-hexane: EtOAc (3:7)). These spots were visualized for the confirmation of phenols, terpenoids and flavonoids. The UV visualization showed bright green spots for EtS fraction confirming the presence of flavonoids in it. Using phenol reagent, EtR showed a single spot for phenol contents while spraying with terpenoid detecting reagents, two spots in EtR showed red color indicative spots for the presence of terpenoids.

Antioxidant effects

Concentration dependent antioxidant effect of all the fractions of *V. negundo* roots and stems were determined against DPPH free radical scavenging assay. Almost all the fractions showed very strong DPPH inhibitory effect. In assessing roots fraction for antioxidant potential, strong antioxidant effect was observed for Butanol fraction (BR=97±0.12 % at 1000 µg/mL dose) which was also active at the lowest dose of 31.25 µg/mL and showing 8282±0.22 % inhibition. WhileAV3 scavenged DPPH with IC_{50} values of 120.04±0.4 µg/mL (65.4± 0.01 standard) and 125.1±0.3 µg/mL (2.0 ± 0.03 standards) against ABTS in free radical scavenging activities. The effect may be caused due to the presence of phenolic contents in plant extracts. These types of compounds could stabilize the DPPH radical in test solution or any free radical in solution by either absorbing or deactivating through bonding/coordination. Such compounds could also be able to protect membrane lipids from oxidation (lipid peroxidation) from any peroxides formed in cells. Moreover, the oxidative stress could lead to many diseases especially diabetes hence *A. violaceum* may provide some sort of remedy for problems emerging due to overflow of reactive oxygen species.

Antimicrobial activities

Antibacterial effects root barks: The all four fractions of root of *V. negundo* were tested against four human pathogenic bacteria including *Escherichia coli, Bacillus subitilis, Staphylococcus aurous, Shigella flexenari* at dose of 100 µg/mL. The crude extract of root showed a potent effect with inhibition zones of 25±0.75, 17±0.43, 18±0.21, 21±0.35mm against *E. coli, B. subitilis, S. aurous* and *S. flexenari* respectively. The chloroform fraction (CR) exhibited strongest antibacterial activity against *E. coli*, *S. flexenari and*

S. aurous with inhibition one of 25±0.75, 21±0.35and 18±0.21mm while mild activity against *B. subitilis* (17±0.43), while the rest of fraction show middle affect against the bacteria. The hexane soluble fraction (HR) remained inactive or mild against all the bacteria (Table 6). In the current study, strong inhibition was observed against the tested bacteria i.e., *E. coli, S. aurous* and *S. flexneri* is an indicative of pharmacological and potential use of this plant in various ailments especially infections which are caused by these pathogenic bacteria. The zone of inhibition and other related measurements were compared to that of reference standard (clarithromycin).

Table 6: Antimicrobial activities of *V. negundo* selected fractions from roots.

Bacteria	Zone of inhibition in mm				
	HR	CR	EtR	BR	Clarithromycin
E. coli	6±0.41	25±0.75	8±0.45	9±0.12	29±0.43
B. subtilis	4±0.32	17±0.43	7±0.66	7±0.23	21±0.53
S. aurous	--	18±0.21	5±0.44	12±0.24	24±0.76
S. flexneri	--	21±0.35	--	11±0.24	22±0.34
Fungi	**Control linear growth in mm**				**Miconazole**
A. flavus	--	18±0.39	7±0.38	14±0.22	19±0.78
A. niger	--	19±0.45	6±0.46	12±0.21	21±0.34
C. albicans	--	17±0.57	--	10±0.23	19±0.47
T. longifusus	--	14±0.68	--		17±0.36

Note: Values are mean inhibition zone (mm) ± S.D of three replicates.

Table 7: Antimicrobial and Anti-fungal activities of *V. negundo* selected fractions from stem.

Bacteria	Zone of inhibition in mm				
	HS	CS	EtS	BS	Clarithromycin
E. coli	--	24±0.23	9±0.49	20±0.34	26±0.34
B. subtilis	--	18±0.45	10±0.55	14±0.45	22±0.49
S. aurous	--	22±0.67	8±0.65	12±0.67	24±0.61
S. flexneri	--	20±0.32	6±0.34	14±0.89	22±0.57
Fungi	**Control Linear growth in mm**				**Control**
A. flavus	--	16±0.34	08±0.49	14±0.67	18±0.39
A. niger	--	19±0.66	08±0.55	12±0.23	21±0.43
C. albicans	--	22±0.76	10±0.61	12±0.19	25±0.54
T. longifusus	--	15±0.78	11±0.38	10±0.29	17±0.28

Values are mean inhibition zone (mm) ± S.D of three replicates.

Antifungal activity root barks

V. negundo chloroform fraction (CR) inhibited the growth of various fungal strains and showed to be valuable antifungal sources. The results of the antifungal activities of *V. negundo* fractions against four pathogenic fungi, *A. flavus, A. niger, C. albicans* and *T. longifusus* have been summarized in (Table 7). According to the results, the various fractionshowed maximum inhibition of linear growth; 19±0.45 and 18±0.39 against *A. niger* and A. *flavus* while 17±0.57 and 14±0.68 against *C. albicans* and *T. longifusus,* respectively. The Chloroform soluble fraction (CR) remained much active against all the tested fungi by showing very strong inhibition, especially against *A. niger* and *A. flavus* with inhibition zones of 19±0.45 18±0.39 mm (Table 6). The butanol fraction (BR) was not too much potent against *A. Flavus* (14±0.22) and *A. niger* (12±0.21) while mild active against *C. albicans* and inactive against *T. longifusus*. The hexane fraction (HR) was not potent enough. The concentrations of samples used in this assay were of 100 µg/mL each. The results show that the fraction of chloroform (CR) and ethyl acetate (EtR) confirm a conclusive remedy for fungal infections. The chloroform as well as ethyl acetate fractions is particularly useful since these constitute various phytochemicals such as saponins, terpinoids and alkaloids with potent inhibitory effects on the culture of *Aspergillus* and *Candida* species. We can hereby conclude a hopeful medicine for hair, skin and nails infections in humans by using *V. negundo* plant.

Antibacterial effects stem barks

The fractions of stem barks of *V. negundo* were tested against four diseases causing bacteria including *Escherichia coli, Staphylococcus aurous, Shigella flexenari* and *Bacillus subitilis,* at dose of 100 µg/mL. According to the data (Table 7), of fractions of *V. negundo* showed a strong effect with inhibition zones of 24±0.23, 22±0.67, 20±0.32, 18±0.455mm against *E. coli, S. aurous, S. flexenari* and *S. aurous* respectively. The chloroform fraction (CS) exhibited strongest antibacterial activity against *E. coli S. aurous* and *S. flexneri* with inhibition one of 24±0.23, 22±0.67, 20±0.32, and 18±0.45mm. Butanol fraction (BR) shows activity against *E. coli* (20±0.34) and *S. flexneri* (14±0.89), respectively. While the rest of fraction show middle affect against the bacteria. The hexane soluble fraction (HS) remained inactive or mild against all the bacteria. In conclusion, strong inhibition was observed against the tested bacteria i.e., *E. coli, S. aurous* and *S. flexneri* is a symptomatic of pharmacological and possible use of this plant in various ailments especially infections (Table 7).

Antifungal activity stem barks

V. negundo chloroform fraction (CS), butanol fraction (BS) inhibited the growth of various fungal strains at high level while the fraction of ethyl acetate (EtS) show considerable activity against the fungi. The results of the antifungal activities of *V. negundo* fractions against four pathogenic fungi, *T. longifusus A. niger, A. flavus,* and *C. albicans* a have been shortened in (Table 7). According to the results, chloroform fraction (CS) shows maximum inhibition of linear growth; 22±0.76 and 19±0.66 against *C. albicans* and A. *niger* while show minimum growth 16±0.34and 15±0.78 against *A. flavus* and *T. longifusus,* respectively. The butanol fraction (BS) remained less active against all the tested fungi by showing very less inhibition, especially against *A. niger* and *C. albicans* with inhibition zones of 12±0.23 and 12±0.19mm (Table 7). The ethyl acetate (EtS) was not too much potent against *A. niger* (08±0.55) and *A. flavus* (08±0.49). The n- hexane fraction (HR) was not potent enough. Finally, the data shows that chloroform fraction (CS) and butanol fraction (BS) show maximum potency against the fungi.

In preliminary phytochemical analysis, it was estimated that the crude extract of roots (R) contained saponins, terpenoids, phenols and flavonoids in major quantities while alkaloids and saponins were detected in minute quantities. Flavonoids were detected in chloroform fraction (CR) and butanol fraction (BR) while terpenoids in ethyl acetate (EtR). The crude extract of stem (S) was also enriched in saponins, terpenoids, phenols and flavonoids while alkaloids were present in minute quantities. Terpenoids and Phenols were present in chloroform fraction (CS) while ethyl acetate (EtS) and butanol fraction (BS) showed the presence of Flavonoids (Masood *et al*., 2013). These metabolites were further confirmed by using TLC analysis. The n-hexane fractions from roots and stem labelled as (HR and HS) showed the presence of phenols and terpenoids. Overall, two terpenoids and one phenolic compound were present HR, while two terpenoids and one phenolic compound was confirmed in HS. The chloroform fractions from roots and stem (CR and CS) showed the occurrence of flavonoids and alkaloid compounds. CR contained one flavonoid and one alkaloid while CS constituted two flavonoids and one alkaloid. The ethyl acetate fraction of root (EtR) showed a single phenolic compound while two terpenoids while the stem ethyl acetate fraction (EtS) showed the presence of three flavonoids. A total of 17 compounds were detected in all the test samples. All the fractions showed strong antioxidant effects in DPPH free radical scavenging assay. Strong antioxidant activity was found for butanol fraction of roots (BR) and stem bark (BS) fractions. The result may be initiated due to the flavonoids contents in plant extract. These compounds were also confirmed from the FTIR spectra of both BR and BS fractions (Deveoglu *et al*., 2021; Wang *et al*., 2021; Wladislaw *et al*., 1996).

These types of compounds could stabilize the DPPH radical in the solution by either absorbing or switching off through bonding. These kinds of compounds may be able to shrink the oxidation of membrane lipids (lipid peroxidation) from any peroxides produced in cells (Kumar *et al*., 2010). Moreover, oxidative pressure can cause several diseases, particularly diabetes, hence *V. negundo* (L.) delivers therapeutic effect to solve the problems due to excess of reactive oxygen species. All fractions of *V. negundo* (L.) were tested against *E. coli, B. subitilis, S. aurous,* and *S. flexenari* at 100µg/mL. The chloroform fractions of roots (CR) and stem (CS) had the strongest antibacterial activity against *E. coli, S. flexenari,* and *S. aurous,* while the other fractions were just somewhat potent. Both fractions remained effective against the tested fungus (Garcia *et al*., 2004; Kadir *et al*., 2007). These may be due to the presence of alkaloids and flavonoids. As a result, these compounds show strong cytotoxic effects when applied individually. Same results were also reported by (Khokra *et al*., 2008), that the ethanolic and ethyl acetate friction of the plant presented positive antimicrobial activity against Escherichia coli, Staphylococcus aureus, Bacillus subtilis and Pseudomonas aeruginosa bacterial strains. These findings were reliable with previous studies which defined the antimicrobial and antifungal activities of the leaves and fruits extracts of the plant. Similarly (Anbalagan *et al*., 2017) studied photochemistry and antifungal activities *of V. negundo* (L.) against pathogenic fungal strains including *C. albicans, A. niger, C. neoformans, Penicillium* spp. and presented that the plant extract showed strong antifungal activities and phytochemical analysis established the presence of alkaloids, flavonoids, and terpenoids which have strong antifungal potentials. *In vitro* and *in vivo* studies have previously established that methanol extract of *V. negundo* (L.) has potent bactericidal activity against Vibrio cholera, a key pathogenic bacterial causing cholera. Moreover, it was witnessed that the

leaves extract of *V. negundo* (L.) has the capability of protecting infant mouse models from cholera (Kamruzzaman *et al*., 2013). The compound isolated from *V. negundo* (L.) for their biological activities has strong antifungal and antibacterial activities against pathogenic bacteria and fungi (Chowdhury *et al*., 2010). Based on these findings, they reported that *V. negundo* (L.) can pose an effective therapeutic agent against life treating pathogens which make it a potential candidate for the development of antimicrobial drugs and ecofriendly bio pesticides. The anti-inflammatory effects and analgesic actives of *V. negundo* (L.) suggested that the plant has the potential to inhibit prostaglandin synthesis and antihistamine activities which can mediate pain suppressing and anti-inflammatory activities that is vigorous for wound-healing mechanisms. The antihistamine activity of the plant can be recognized to the anti-itching effect mentioned in Ayurveda medicine (Dharmasiri *et al*., 2003).

Conclusions and Recommendations

Based on our results and previous studies about *V. negundo* (L.) we conclude that *V. negundo* (L.) has potential antimicrobial and antifungal capacities which make it a potential candidate for the development of antibiotic drugs through pharmacological formulation. The compounds present in the roots and stem bark of the plant can be exploited to produce ecofriendly insecticides. Biological activities of *V. negundo* (L.) are attributed to its richness in valuable secondary metabolites such as steroids, terpenoids and phenolic compounds. Phytochemically, the crude and ethyl acetate fraction were enriched in alkaloids, terpenoids and Phenols. Both the samples successfully exhibited strong antioxidant effect in DPPH radical scavenging assays. The crude and ethyl acetate fraction showed potent antibacterial effect against all the tested bacteria at minimum dose. In antifungal assay, the crude was active in inhibiting the growth of all tested fungi while the Chloroform soluble fraction remained much potent against *A. niger* and *C. albicans*. Thus, confirming the overall cytotoxicity of plant. In enzyme inhibition assay, ethyl acetate fraction showed highest inhibition. The crude was also active in inhibiting both the enzymes *in vitro*. The study suggests a conclusive remedy for various disorders such as food poisoning and diarrhea caused by the Gram-negative bacteria such as *E. coli* and *S. flexenari* as well to treat respiratory tract infections caused by *S. aurous* (gram positive).

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Novelty Statement

This is the first study reported on the biological evaluation on *Vitex negundo,* collected from District Lower Dir, Pakistan.

Author's Contribution

Ali Hazrat and Farman Ali Khan: Gave the idea, designed and developed the methodology, wrote and reviewed the manuscript.
Muhammad Yahya, Gul Rahim and Muhammad Mukhtiar: Gave the idea and designed and developed the methodology, acquisition of the data analyzed and interpretation of data, wrote and reviewed the manuscript.
Shah Zeb Khan, Hayat Ullah and Muhammad Ajmal Khan: Acquisition of the data.

Conflict of interest
The authors have declared no conflict of interest.

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