Life Science Informatics Publications

Research Journal of Life Sciences, Bioinformatics, Pharmaceutical and Chemical Sciences

Journal Home page http://www.rjlbpcs.com/

RJLBPCS

ISSN 2454-6348

Original Research Article **DOI: 10.26479/2019.0501.12**

EVALUATION OF ANTIOXIDANT ACTIVITY, ANTIBACTERIAL POTENTIAL AND PHYTOPHARMACEUTICALS PROPERTIES OF ETHANOL EXTRACT OF LEAVES OF *VITEX NIGUNDO*

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ABSTRACT: *Vitex negundo* L. (Verbenaceae) commonly known as nirgundi, a small aromatic shrub, very popular plant which plays a great importance to the health of individual and communities with several pharmacological activities. The present study was designed to evaluate the Pharmacognostical parameters of Nirgundi leaf part followed by anatomical studies with macroscopic and microscopic examination. Physicochemical parameters such as ash values and extractive values were determined. The Nirgundi leaves were subjected to successive extraction processes by Soxhlet extraction method using 50% ethanol as solvent. Preliminary phytochemical analysis, antioxidant and antibacterial activity were also undertaken against nirgundi leaf extract. Results for Anatomical studies confirm the presence of lamina, midrib, xylem, phloem and stomata etc. that provide information about its correct identity and evaluation. Ash and Extractive values for dried Nirgundi leaf powder was under acceptance limit. Phytochemical profiling revealed significant presence of alkaloids, flavonoids, phenols, glycosides and tannins followed by presence of higher antioxidant activity, percentage inhibition activity was observed ranged from 54.9-74.54% for leaf extract. Ethanolic leaf extract also demonstrated antibacterial activity by showing highest zone of inhibition of 10mm at 1mg/ml of highest concentration against *K. pneumonia* and *E. coli* followed by 8 mm of inhibition at same concentration against *B. cereus* and *S. aureus*. Thus these findings suggest the presence of several pharmacognostic profiles for Nirgundi plant with excellent potential use in new medicinal drug discovery. However more clinical trials, molecular and chromatographic fingerprint studies need to be done to confirm the results obtained in the given study.

KEYWORDS: Ethanol, Soxhlet extraction, Nirgundi leaves, Anatomy, Bioactive compounds, DPPH activity and Zone of inhibition.

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1.INTRODUCTION

Medicinal plants, a traditional system of medicine, emphasized the use of modern medicines in the form of various formulations for the treatment of various infectious diseases caused by bacteria, fungi and viruses followed by autoimmune diseases. These medicines holds a great promise as an easily available source as effective medicinal agents to cure a wide range of ailments among the people without causing any side effects particularly in tropical developing countries like India. India, exhibits a rich source of herbal medicinal plants traditionally used by many Indians regularly from ancient time as spices, home-made remedies and so on. Ayurveda, an ancient system of Indian medicine has been recommending large number of medicinal plants drugs for medicinal use since time immortal. In spite synthetic drug chemistry field and antibiotics has reached a great advancements, but still medicinal plants continue to be one of the major biological constituents for drugs for treating various diseases of mankind. Through Clinical and pharmaceutical reports which have evidenced the status of medicinal plants by identifying the role of bioactive compounds present in them by evaluating their mode of action in human and animal systems [1]. *Vitexnegundo* L. (Verbenaceae), also known as nirgundi, Sambhalu, Mewri (Blue flowered plant) a large aromatic shrub abundantly found in all parts of Maharashtra, sub-Himalayan tract, plains of North India to the peninsular India. Young branches are covered with white tomentum. Leaves are opposite, 3-5 foliolates, acuminate tip, entire margin. The flowers are small bluish purple in color. Fruits are ovoid drupes, 1 cm long black when ripe [2]. Globally distributed in countries like tropical Eastern and Southern Africa and Asia. It also found in Afghanistan, Bangladesh, Bhutan, Cambodia, China, India, Indonesia, Japan, Korea, Kenya, Madagascar, Malaysia, Mozambique, Myanmar, Nepal, Pakistan, the Philippines, Sri Lanka, Taiwan, Tanzania, Thailand and Vietnam. Fresh berries, seeds, leaf, roots, bark & flowers of *V. negundo*are used in ayurvedic medication. *V. negundo*Linn has several Pharmacological activities such as anti-inflammatory, anti-arthritic: Control pain and inflammation useful in dispersing swellings of the joints from acute rheumatism, antifungal, antioxidant, antibacterial, antipyretic, antihistaminic, analgesic, insecticidal, ovicidal, growth inhibition and morphogenetic agents, antigenotoxic, CNS depressant activity and antifertility effects were also reported from the leaves of *V. negundo* [3]. Bioactivecompounds includes Carbohydrates, sterols, C-glycosides, flavanoids, polyphenolic compounds, terpenoids, glycosidic iridoids and alkaloids, casticin, essential oil, benzoic acid, vitamin c, flavones; 3β-Acetoxyolean-12-en-27-oic acid, 2α,

3α-dihydroxyleana-5, 12-dien-28-oic acid; 2β, 3α-diacetoxyolean-5, 12-dien-28-oic acid and 2α, 3β-diacetoxy-18-hydroxyolean-5, 12-dien-28-oic acid, Phenol, dulcitol, nigundosides, nishindaside, B-sitosterol, camphene, orientin, arteemetin, onoterpens, anguside, eurostoside and aucubin as the main chemical components [4-9].The aim of this present study is to determine the antioxidant activity and other beneficial pharmacological profile of ethanolic extract *V. negundo* leaves.

2. MATERIALS AND METHODS

Collection and Authentification of Plant material

The authenticated leaves of *V. negundo* were collected, purchased and identified from Vindhya Herbal Testing and Research Institute, Minor Forest Produce Processing & Research Centre (MFP-PARC), Bhopal, Madhya Pradesh India. A sample specimen with Laboratory reference no.VHTRL19121802ER has been deposited in the herbarium for future reference. Leaves of *V. negundo* were shade dried and coarsely powdered and used for further studies.

Chemicals, Media and Reagents

All biochemical and media used in this study were purchased from Himedia, Mumbai, India, Sigma Chemical Company, Bangalore, India and other chemicals and solvents of analytical grade were from MP Biochemicals and Merck Chemicals.

Morphological, Macroscopic and Microscopic examination

As per World Health Organization (WHO) it is recommended to do macroscopic and microscopic examination of a medicinal plant as first step of identity and purity before going for other further level experiments [10]. Fresh and dried leaves were used for morphological information and microscopically features were studied from the midrib of the leaf. Different sections were treated with potassium hydroxide (5%) and chloral hydrate (20%) for the removal of chlorophyll. Dried powdered leaves wereused for powder microscopy study. Transverse sections (TS) of fresh leaves were prepared with the help of sharp blade followed by safranin staining to identify lignified tissues. Images were taken by inverted fluorescence microscope (Leica DM 3000).

Physicochemical analysis

Total Ash

The 2 gm powdered ground drug was incinerated in a silica crucible at 450ºC temperature until free from carbon and weighed to get the total ash content [11].

Acid insoluble ash

The ash was boiled with 25 ml dilute hydrochloric acid for few minutes and filtered through an ash lessfilter paper, washed with hot water and heated at a temperature not exceeding 450º C until constant weight attained [11].

Water soluble extractive value

The 5gm of powdered ground drug with 100ml of water was treated in a stoppered flask with frequent shaking during first 6 hours and allowed to stand for 18 hours an filtered after 24 hours.

25ml of the filtrate was evaporated to dryness intared flat dish and weighed. The percentage of water-soluble extractive value was calculated [11].

Alcohol soluble extractive value

Proceed as directed for the determination of Water soluble extractive, using Alcohol instead of water [11].

Preparation of extract

About 100 gm of powdered leaf was extracted with ethanolic (50%) in a Soxhlet apparatus for 10-24hrs. The extract was filtered through Whatmann filter paper No. 41 in a pre weighed beaker and filtrate obtained was evaporated to dryness on a water bath maintained at 65°C. After evaporation the beaker was cooled at roomtemperature. After cooling, the weight of the beaker containing the residue was determined. The dried extracts were suspended in DMSO for further experimental use.

Phytochemical Investigation

Preliminary Phytochemical analysis was carried out for ethanolic leaf extract of *V. negundo* as per standard methods. Extracts obtained were performed to test the presence of alkaloids by using Mayer's test and Dragondroff's reagent, tannins by $FeCl_3$ solution and Gelatin solution, saponins by foam test, flavanoids byLead acetate and Shinoda test, glycosidal sugars by Keller-Killiani test, carbohydrates by Molish test, terpenoids, Steroids by Liebermann's test,phenols by ferric chloride test and proteins by Biuret test [12, 13].

Identification of Antioxidant activity using DPPH method

The Antioxidant activity of ethanolic leaf extract of *V. negundo* was analyzed by using DPPH (Diphenylpicrylhydrazyl) assay according to the procedure described by Blios (1958) [14, 15]. DPPH solution was prepared in 0.1mm methanol. 1-2 ml of solution was added to 3 ml of extract at different concentration of 200-1000µg/ml followed by incubation for 45 min at room temperature. After incubation absorbance was taken at 517nm against the corresponding blank solution. Ascorbic acid was taken as reference. Percentage inhibition of DPPH free radical was calculated using the following equation:

DPPH scavenged (%) activity = Absorbance of control- Absorbance of test/Absorbance of control x 100

IC_{50} values were also calculated. IC_{50} is the concentration which shows 50% inhibition.

Anti-bacterial analysis

Antibacterial analysis was carried out using the ethanolic leaf extract of *V. negundo* to assess the antibacterial activity. Antibacterial activity was analysed against *Bacillus cereus*, *Klebsiella pneumonia*, *Escherichia coli*and *Staphylococcus aureus*.The extract prepared as in the standard procedure. 1mg of dried extract wasweighed and suspended in 1ml of dimethyl sulphoxide (DMSO) to make a stock solution of 1mg/ml dose. The bacterial strain obtained from stock cultures and maintained in the Microbiology Laboratory, MFP-PARC, Bhopal, Madhya Pradesh India.

Antibacterial activity was determined by disc diffusion method to test the inhibitory zone formed by leaf extract. The sterile discs were taken and impregnated with the different concentration of leaf extract then placed on nutrient agar media plates containing bacterial cultures and incubated at 37°C for 24 hrs. After incubation, the antibacterial activity of ethanolic leaf extracts against the bacterial strains was assessed by measuring diameter of the inhibition zone formed [16].

3. RESULTS AND DISCUSSION

Morphological, Macroscopic examination

Green colored in dried condition with herbaceous odour and bitter taste. Leaves palmately compound, petiole 2.5-3.8 cm long; mostly trifoliate, occasionally pentafoliate; in trifoliate leaf, leaflet lanceolate or narrowly lanceolate, middle leaflet 5-10 cm long and 1.6 -3.2 cm broad, with 1- 1.3 cm long petiole, remaining two sub-sessile; in pentafoliate leaf inner three leaflets have petiole and remaining two sub-sessile; surface glabrous above and tomentose beneath; texture, leathery [17].

Microscopic Examination

The microscopic studies revealed the presence of xylem, phloem, lamina, stomata and trichomes etc. Lamina shows single layered epidermis having mostly unicellular hairs, bi and multicellular and glandular trichomes beingrare (Figure 2); hypodermis 1-3 layered interrupted at places by 4-8 palisade layers containing chlorophyll; a large number of veins enclosed by bundle sheath traverse mesophyll; stomata present only on the ventral surface (Figure 3), covered densely with trichomes; vein-islet and vein termination number of leaf are 23-25 and 5-7 respectively [17]. The TS of leaf was shown in Figure 1-4. Present study results were compared from anatomical Studies reported by Silvyet al., 2014 on *Vitex Leucoxylon* and *V. Negundo* [18].

Physicochemical analysis

Physical constituents of powdered drug like ash value and extractive values were obtained according to standard testing protocol. Results obtained presented in Table 1.

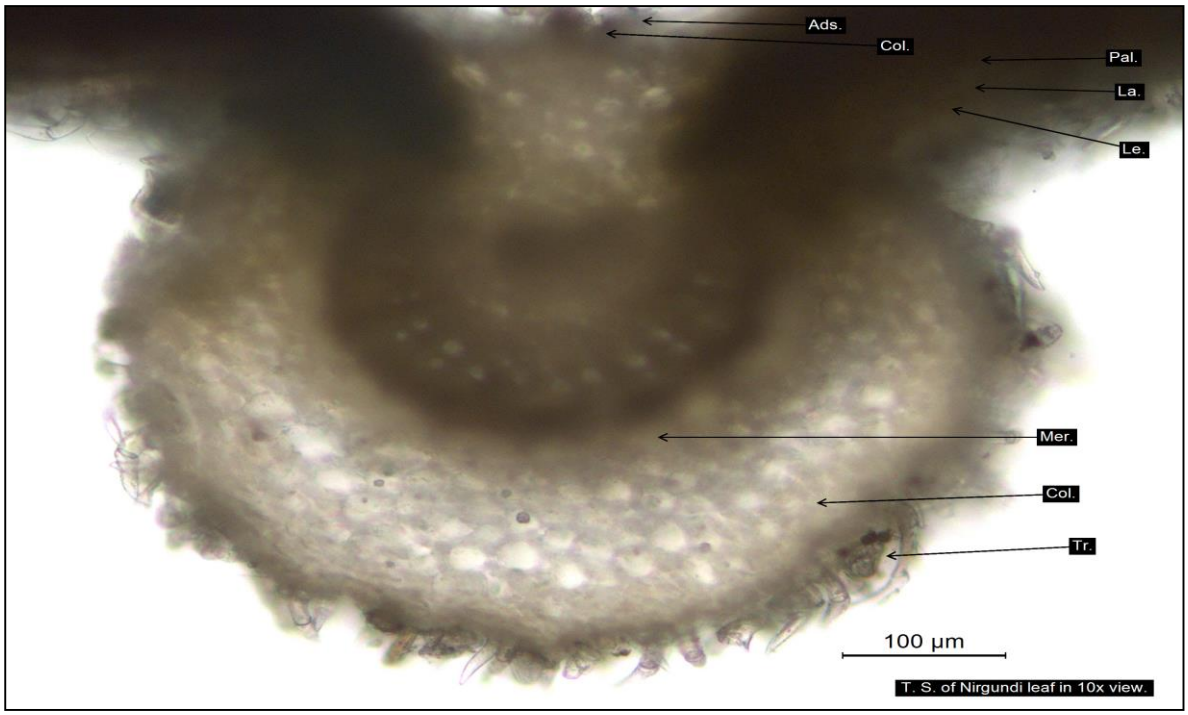

Figure1. (a): Anatomy of TS Nirgundi leaf passing through midrib (10x view); adaxial side (ads), collenchymatous tissue (col), palisade (pal), lamina (la), lower epidermis (le), meristele (mer), trichome (tr)

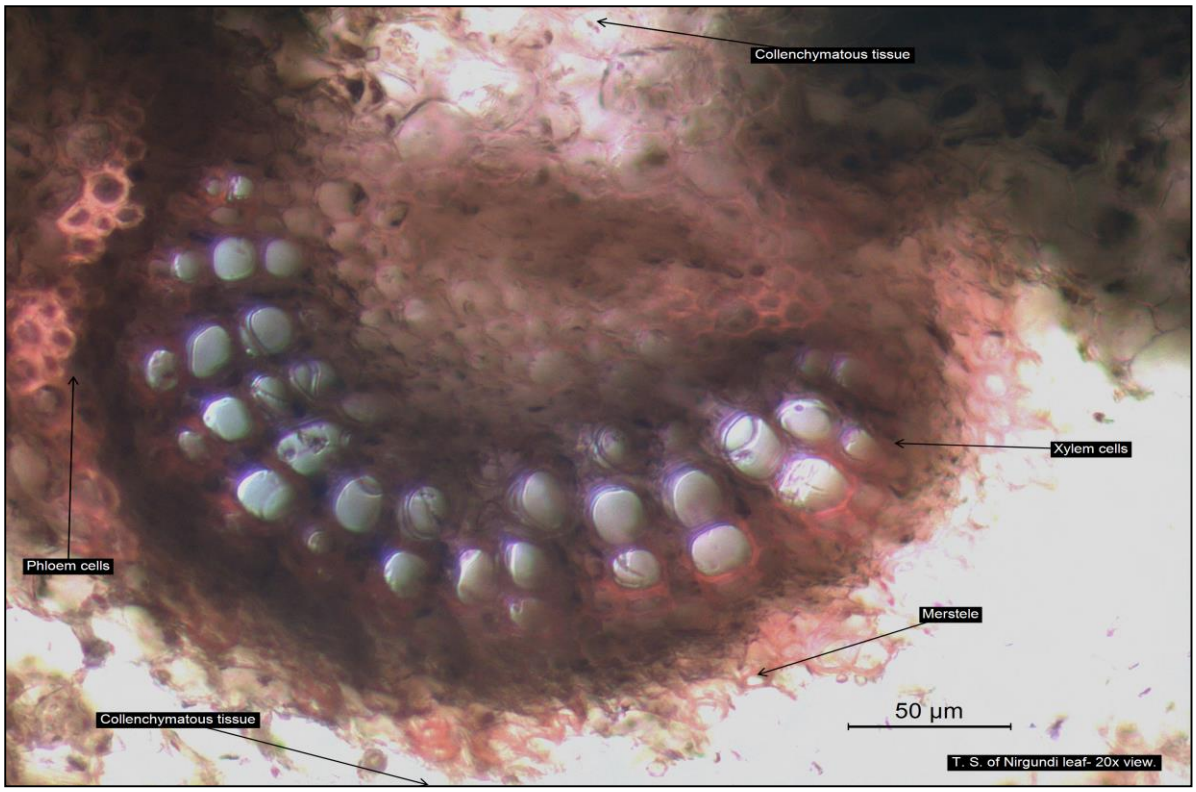

Figure 1. (b): Fine T.S. of Nirgundi leaf 20x view with Phloem and Xylem cells

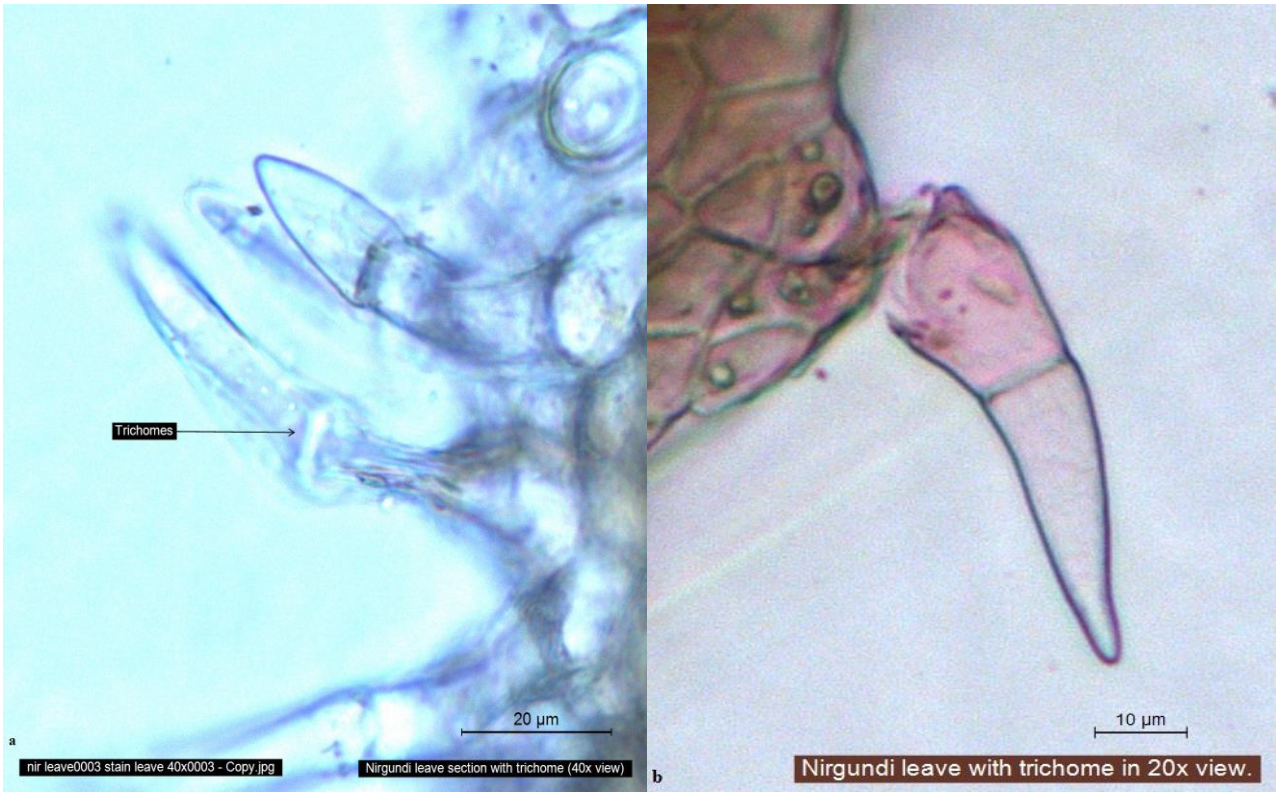

Figure 2: Nirgundi leaf section with covering trichome (a) 40x view; (b) single trichome in 20x view

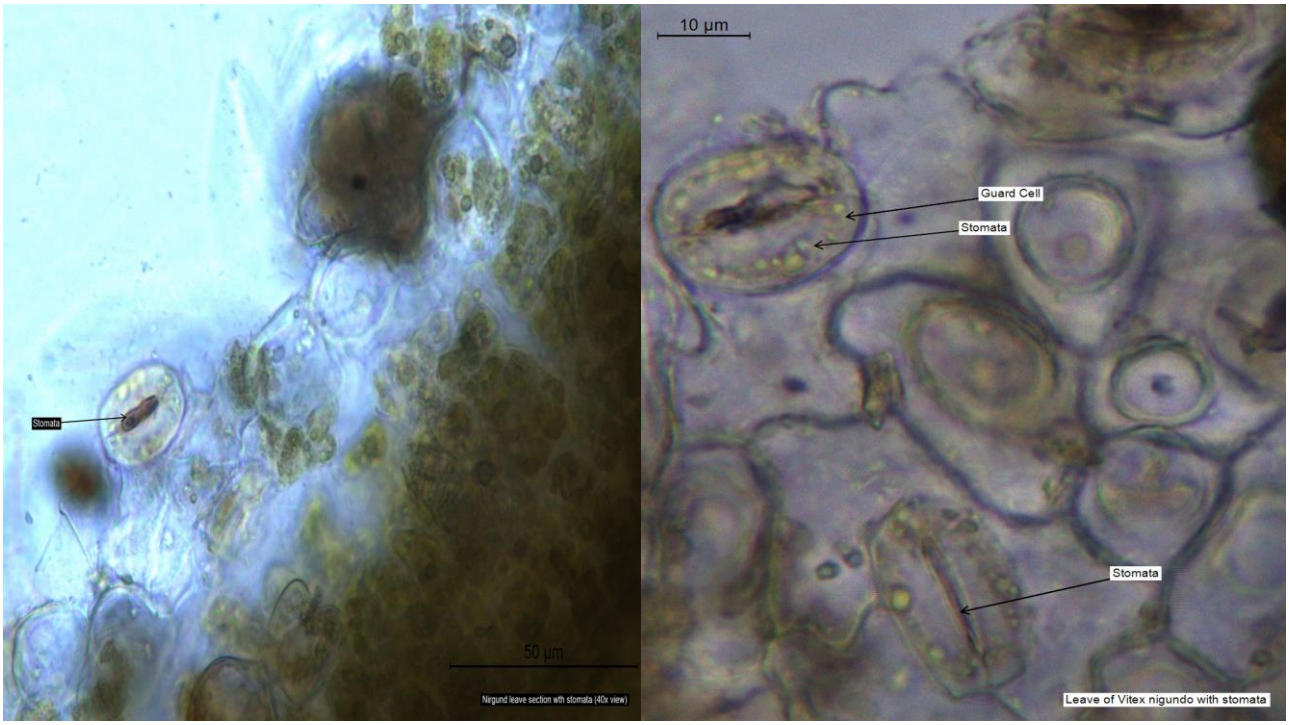

Figure 3: Nirgundi leaf lower epidermis in surface view showing stomata and guard cells on 40x view

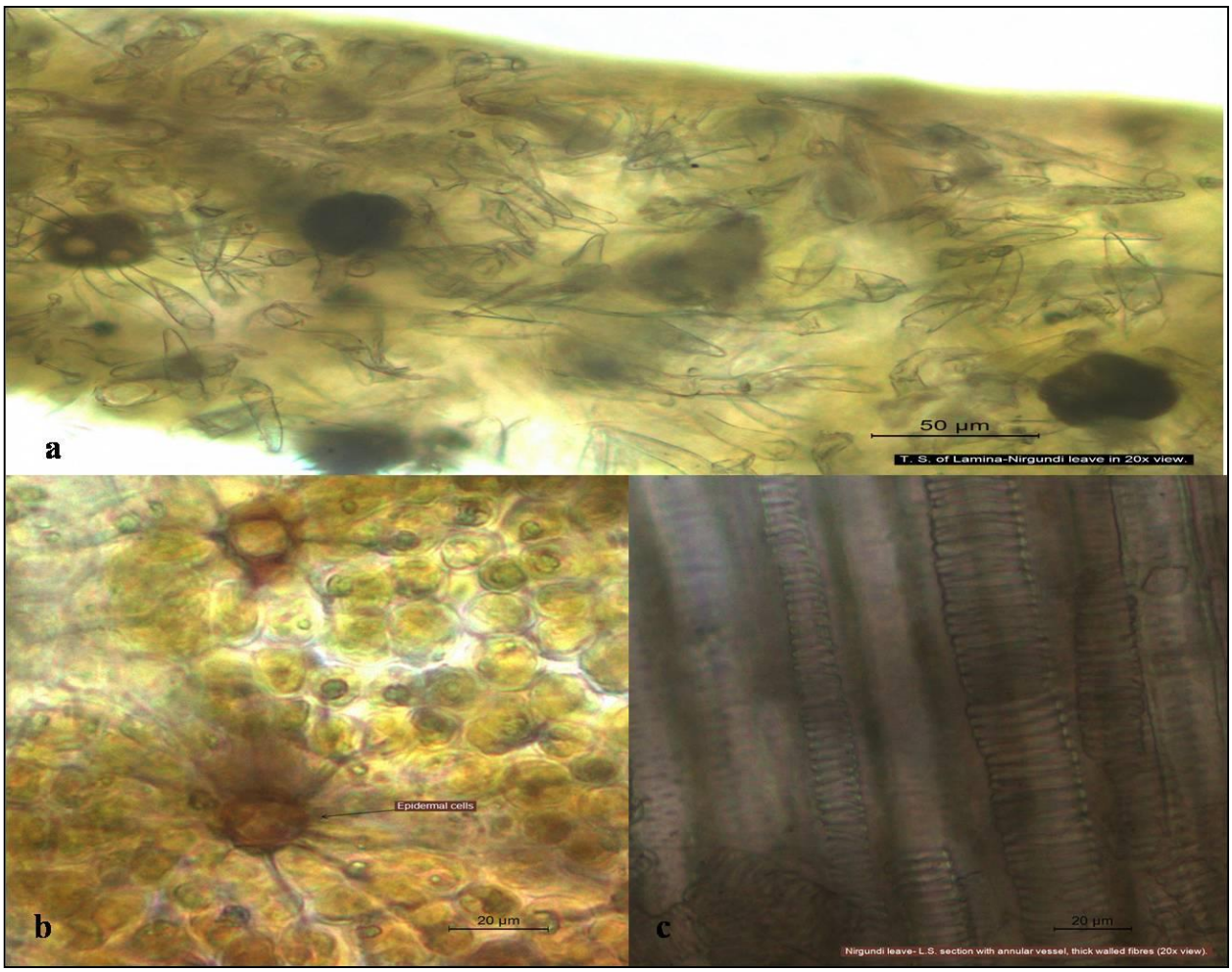

Figure 4: (a) T.S. of Nirgundi leaf with lamina in 20x view; **(b)** Epidermal cells; **(c)** Longitudinal Section (L.S.) cut section of annular vessel, thick walled fibers in 20x view

Table 1: Ash and Extractive values for dried Nirgundi leaf powder

S. No.	Test parameters	Results for Nirgundi leaf powder (%w/w)	Specification and acceptance limit (%w/w)
1	Total Ash Content	0.32%	Not more than 1%
2	Acid Insoluble Ash	0.29%	Not more than 0.2%
3	Alcohol Soluble Extractive	9.17%	Not less than 5%
4	Water Soluble Extractive	12.45%	Not less than 9%

Phytochemical Investigation

Qualitative estimation of ethanolic leaf extract of *V. negundo* through soxhlet extraction was subjected to various chemical tests to find out chemical constituents present in them. From our findings we observed positive results for Alkaloids, Tannins, Flavonoids, Phenols, Saponins and Glycosides shown in Table 2.These secondary metabolites majorly occur in complex mixtures that differ among plant organs and different development stages [19, 20].

Table 2: Phytochemical analysis of ethanolic Nirgundi leaf extract

S. No.	Phytochemical Content	Nirgundi leaf Extract
1.	**Alkaloids**	
	a) Mayer`s test	+ve
	b) Dragondroff's reagent	-ve
	c) Wagner's test	+ve
2.	**Carbohydrate test**	
	a) Molish test	-ve
3.	**Tannins**	
	a) $FeCl_3$ solution	+ve
	b) Gelatin solution	+ve
4.	**Flavonoids**	
	a) Lead acetate	+ve
	b) Shinoda test	+ve
5.	**Steroids**	
	a) Liebermann's test	-ve
6.	**Protein test**	
	a) Biuret test	-ve
7.	**Glycoside test**	
	a) Keller-Killiani test	+ve
8.	**Phenols**	+ve
9.	**Saponins**	
	a) Foam test	+ve

(+) Present; (-) Absent

Antioxidant activity

DPPH radical scavenging activity of different concentration ranging from 200-1000µg/ml was compared between leaf extract and standard ascorbic acid. The percentage inhibition activity for both samples ranged from 54.9-74.54% and 86.90-90.18% for nirgundi leaf extract and ascorbic acid respectively as shown in Table 3.

Table 3:DPPH scavenged activity of Ethanolic Nirgundi leaf extract

S. No.	Concentration (µg/ml)	Absorbance (517nm)		% Inhibition	
		Standard	Leaf extract	Standard	Leaf extract
1	200	0.028	0.121	89.81	56
2	400	0.030	0.110	89.09	60
3	600	0.027	0.070	90.18	74.54
4	800	0.025	0.096	90.90	65.09
5	1000	0.036	0.124	86.90	54.90

IC_{50} value was found to be 61.18 µg/ml nirgundi leaf extract (R^2= 0.003) where as IC_{50} value for ascorbic acid 20.03 µg/ml (R^2= 0.171) summarized in Table 4.

Table 4: IC 50 values for Standard and Ethanolic Nirgundi leaf extract

S.No.	Sample	IC 50 Value (µg/ml)
1.	Ascorbic Acid (Standard)	20.03
2.	Nirgundi	61.18

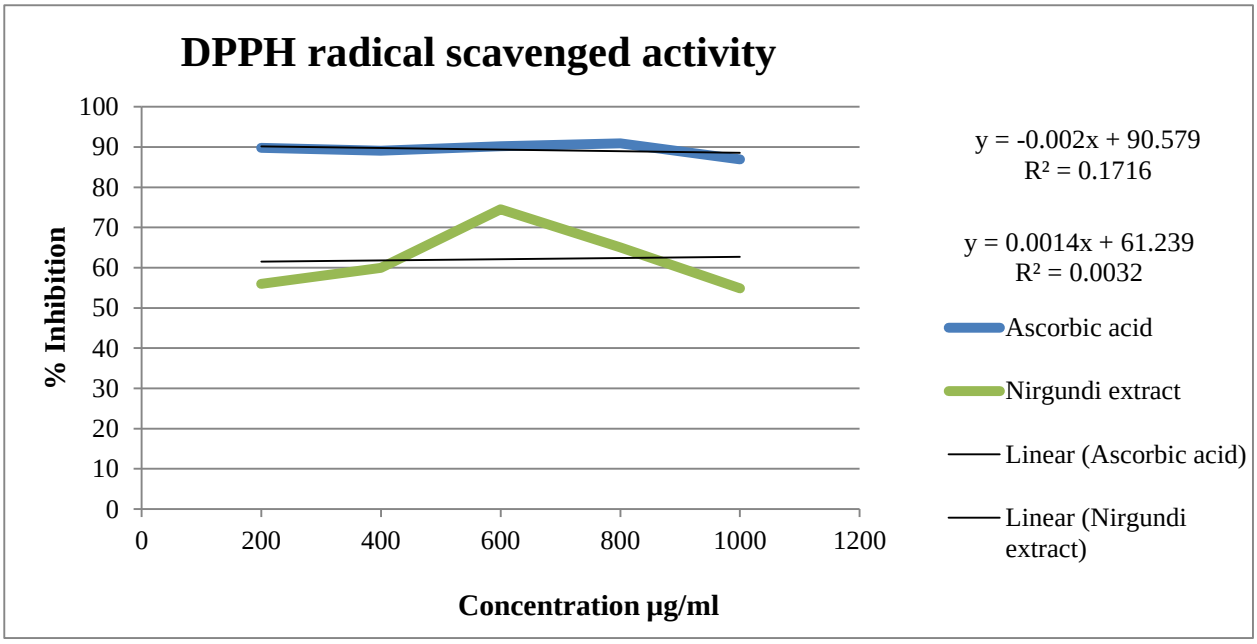

Figure 5: DPPH radical scavenging activity of Nirgundi leaf extract and ascorbic acid. Data arepresented as the percentage of DPPH radical scavenging, Data are presented as Mean± SD.

Results obtained through this activity highlight the higher percentage of DPPH. This activity depends on the reduction of purple DPPH (a nitrogen based free radical) to yellow colored diphenyl picryl hydrazine [21]. The reduction of DPPH by the extracts is carried out by transfer of hydrogen atom or e- and phenol compounds and also depends or varies on solvent type used for the plant extraction along with method, temperature, condition and plant parts to be used. Preliminary

phytochemical estimation reveals the presence of phenols which also supports antioxidant activity. The percentage of free radical activity was plotted against the corresponding antioxidant substance concentration presented in Figure 5.The results were compared to phytochemical analysis and pharmacological studies which have been reviewed and well documented on *V. negundo* [22-26].

Anti-bacterial analysis

Antibacterial activity was done using 50% ethanolic *V. negundo* leaf extract by disc diffusion method at different concentration against four bacterial strains showed varied level of zone of inhibition ranged from 1 mm-10mm. Highest zone of inhibition 10mm was observed at 1mg/ml of highest concentration against *K. pneumonia* and *E. coli* followed by 8 mm of inhibition at same concentration against *B. cereus* and *S. aureus* respectively. Next highest inhibition zone of 8 mm was recorded at 0.8mg/ml of concentration against bacterial isolates *K. pneumonia*and *S. aureus* respectively. On the other hand, minimum inhibition of ranged from 0-2 mm was observed at 0.4-0.2mg/ml of concentration against bacterial isolates used in the study. Rest of the results is tabulated in Table 5.

Table 5: Antibaterial activity shown by ethanolic extract leaf of *V. negundo*

S.No.	Pathogens	Zone of Inhibition at different concentration (mm)				
		0.2mg/ml	0.4mg/ml	0.6mg/ml	0.8mg/ml	1mg/ml
1	*B. cereus*	0	0	1	4	8
2	*K. pneumonia*	2	2	7	8	10
3	*E. coli*	0	2	5	9	10
4	*S. aureus*	2	3	5	8	8

In the present study, nirgundi leaf 50% ethanolic extract showed excellent antibacterial activity with highest zone of inhibition against all four pathogens at 0.8-1 mg/ml of concentration that may be due to presence of bioactive compounds present in the plant, on the basis of positive results obtained for phytochemicals like flavonoids, alkaloids, tannins and phenols could be responsible for the inhibitory effect of these pathogens. Present study results were compared to the already study done by Merlin Rose and Cathrine, 2011 against the bacterial pathogens such as *Salmonella paratyphi*, *K. pneumoniae*, *Vibrio cholera*, *Streptococcus mutans* and *E. coli* which were found to be susceptible in ethanol leaf extracts of *V. negundo* [27]. Ethanol and methanol leaves extracts were found to be active inhibiting agents against both gram-positive and gram-negative bacteria. Whereas, petroleum ether and chloroform extracts had better antibacterial activity against all gram-positive bacteria [28, 29].

4. CONCLUSION

V. negundo have been using in traditional medicine for several purposes as ayurvedic formulation. The present study was conducted to evaluate the pharmacognostic profile of *V. negundo* leaf part followed by phytochemical, antioxidant and antibacterial activities. Our study results revealed the presence of certain phytoconstituents like Alkaloids, Tannins, Flavonoids, Phenols, Saponins and Glycosides which makes it effective and showed good antioxidant and antibacterial activities. The bioactive compound vitexin which is a flavanoid majorly responsible for anti-cancer activity. *V. negundo* can be used as antibiotic and antioxidant agent. Further parameters like sensitive techniques like chromatography analysis for particular extraction, purification of bioactive compound followed by clinical trial on both vivo and vitro model are need to be conducted. The results obtained in this study could serve as diagnostic parameters for proper identification along with preparation of a monograph on *Vitex negundo* Linn.

ACKNOWLEDGEMENT

The authors are grateful to the Department of Microbiology, AIIMS Bhopal for their continuous support and encouragement. Also like to thanks the Vindhya Herbal Testing & Research Laboratory, (MFP-PARC), Bhopal, MP, India for laboratory support and equipment facilities. Financial support provided by the University Grants Commission (UGC), New Delhi under the Post Doctoral fellowship scheme for Women candidates for the year 2016-17 is also gratefully acknowledged.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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