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ESSENTIAL OILS OF AZADIRACHTA INDICA AND VITEX NEGUNDO LEAVES EVALUATION FOR PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT ACTIVITY AND ANTIMICROBIAL ACTIVITY

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ABSTRACT

A wide range of medicinal and aromatic plants (MAPs) have been explored for their essential oils in the past few decades. Essential oils are complex volatile compounds, synthesized naturally in different plant parts during the process of secondary metabolism. They have great potential in the field of biomedicine as they effectively destroy several bacterial, fungal and viral pathogens. The presence of different types of aldehydes, phenolics, terpenes and other antimicrobial compounds means that the essential oils are effective as antioxidants as well as against a diverse range of pathogens. The goals of the study were to determine and compare the phytochemical screening, antioxidant and antimicrobial activities of the essential oils of *Azadirachta indica* and *Vitex negundo*. The phytochemical screening of the two plants showed presence of alkaloids, carbohydrates, glycosides, phenolic and tannin compounds, flavonoids, saponins, lipids and terpenoids. About the antioxidant potential, *Azadirachta indica* showed a higher ability to scavenge free radicals as compared to *Vitex negundo*, which was investigated by 2, 2- diphenyl- 1- picrylhydrazyl (DPPH) radical scavenging assay. Regarding the antimicrobial activity, *Vitex negundo* showed a better ability to inhibit the growth of pathogenic micro-organisms as compared to *Azadirachta indica*, which was investigated by the Agar diffusion method (Kirby-Bauer method, Bauer et. al.; 1966).

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INTRODUCTION

Essential oils are volatile, natural, complex compounds characterized by a strong odour and are formed by aromatic plants as secondary metabolites. They are usually obtained by steam or hydro-distillation first developed in the middle ages by Arabs. Known for their antiseptic, i.e. bactericidal, viricidal and fungicidal, and medicinal properties and their fragrance, they are used in embalmment, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthetic remedies. Up to the present day, these characteristics have not changed much except that more is now known about some of their mechanisms of action, particularly at the antimicrobial level.

In nature, essential oils play an important role in the protection of plants as antibacterial, antiviral, antifungal, insecticides and also against herbivores by reducing their appetite for such plants. They may also attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others [1].

Essential oils are extracted from various aromatic plants generally localized in temperate to warm countries like Mediterranean and tropical countries where they represent an important part of traditional pharmacopoeia. They are liquid, volatile, limpid and rarely coloured, lipid soluble and soluble in organic solvents with a generally lower density than that of water. They can be synthesized by all plant organs, i.e. buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark, and are stored in secretory cells, cavities, canals, epidermis cells or glandular trichomes.

Essential oils can be prepared from various plant sources by several processes like steam distillation, hydro distillation, enzymatic hydrolysis, enfleurage, maceration, expression, solvent extraction and fluid extraction. Most of the

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commercialized essential oils are chemo-typed by gas chromatography and mass spectrometry analysis [2].

Essential oils have been largely employed for their properties already observed in nature, i.e. for their antibacterial, antifungal, and insecticidal activities. At present, approximately 3000 essential oils are known, 300 of which are commercially important especially for the pharmaceutical, agronomic, food, sanitary, cosmetic and perfume industries. Essential oils or some of their components are used in perfumes and make-up products, in sanitary products, in dentistry, in agriculture, as food preservers and additives, and as natural remedies. Moreover, essential oils are used in massages as mixtures with vegetal oil or in baths but most frequently in aromatherapy.

Owing to the new attraction for natural products like essential oils, despite their wide use and being familiar to us as fragrances, it is important to develop a better understanding of their mode of biological action on new application in human health, agriculture and the environment. Some of them constitute effective alternatives or complements to synthetic compounds of the chemical industry, without showing the same secondary effects [3].

Thus, we can say that essential oils from plants have a number of significant properties and they can be therefore be exploited and used for the betterment of human life.

MATERIAL AND METHODS

Plant material collection

Fresh leaves of *Azadirachta indica* were collected from Vartak College, Vasai (W) while fresh leaves of *Vitex negundo* were collected from Umela village, Naigaon (W) and authentication was done by the taxonomist from Botany department.

Preparation of powder

The leaves of *Azadirachta indica* and *Vitex negundo* were washed under running tap water and dried by keeping in a hot air oven at 60°C-65°C for 3 days. The dried leaves were then homogenized using a mortar and pestle into a fine powder.

Extraction of essential oils

10 g powder of *Azadirachta indica* leaves/ *Vitex negundo* leaves were taken and extracted with 200 ml of distilled water using the Soxhlet apparatus for 5 hours at 60°C-80°C. The extracts obtained are a mixture of oil and water. The water is separated by using a rotary evaporator i.e. the extracts are concentrated to about 5-10 ml, after which essential oils are obtained. For further analysis, the solvent was completely removed from the extracts by using the rotary evaporator; the extracts were then dried at 45°C in an incubator following which they were stored in the refrigerator at 4°C, along with the essential oils.

Phytochemical analysis

Test for alkaloids

For detection of alkaloids, 500 mg of extract was dissolved in 20 ml of HCl, filtered and the following tests were performed [4].

A. 2 ml filtrate + 1 ml of Mayer's reagent by the side of the tube. A white or creamy precipitate indicates positive test.
B. 2 ml filtrate + 2 ml of Wagner's reagent by the side of the tube. A reddish-bron precipitate indicates positive test.

Test for carbohydrates

400 mg of extract was dissolved in 20 ml of distilled water, filtered and the following tests were performed [4].

A. 1 ml filtrate + 1 ml each of Fehling's solution A and B, keep in boiling water bath for few minutes. A red precipitate indicates presence of sugars.
B. 1 ml filtrate + 1 ml Benedict's reagent, keep in boiling water bath for 2 minutes. A coloured precipitate indicates presence of sugars.

Test for glycosides

500 mg of extract was dissolved in 20 ml of concentrated HCl, filtered and following test were performed [4].

A. 2 ml filtrate + 3 ml chloroform and shake, the chloroform layer was separated and 10% ammonium solution was added to it. Pink colour indicates the presence of anthraquinone glycosides.
B. 2 ml filtrate + 1 ml glacial acetic acid + 2 drops of 2% $FeCl_3$ solution, this mixture was then poured into another test tube containing 1ml of concentrated H_2SO_4. A brown ring at the interphase indicates presence of cardiac glycosides.

Test for proteins

100 mg of extract was dissolved in 10 ml of distilled water, filtered and the following tests were performed [4].

A. 2 ml of filtrate + 2 ml Millon's reagent. A white precipitate indicates presence of proteins.
B. 2 ml filtrate + 1ml $CuSO_4$ solution + 1 ml of 1% NaOH solution. A violet colour indicates presence of proteins.

Test for phenolic and tannin compounds

100 mg of extract was dissolved in 10 ml of distilled water, filtered and the following tests were performed [4].

A. 2 ml filtrate + 2 drops of 5% $FeCl_3$ solution. A dark green precipitate indicates presence of phenolic and tannin compounds.
B. 2 ml filtrate + 0.5 ml lead acetate solution. A yellow precipitate indicates presence of phenolic and tannin compounds.

Test for flavonoids

100 mg of extract was dissolved in 10 ml of distilled water, filtered and the following test was performed [4].
2 ml filtrate + 3 ml 2% NaOH solution. Yellow colour which becomes colourless on addition of dilute H_2SO_4 indicates presence of flavanoids.

Test for saponins

Extract + 4 ml distilled water, shake vigorously. Formation of foam indicates presence of saponins [4].

Test for lipids

100 mg of extract was dissolved in 10 ml of distilled water, filtered and the following test was performed.
1 ml filtrate + 1 ml distilled water + few drops of Sudan red indicator, swirl and allow to settle. Formation of red colour solution indicates presence lipids [5].

Test for terpenoids

Extract + 0.4 ml chloroform + 0.6 ml concentrated H_2SO_4 to form a layer. Formation of reddish brown colouration at the interface indicates presence of terpenoids [6].

Antioxidant activity

DPPH assay

The DPPH scavenging activity was performed using a solution of 0.1 mM DPPH in methanol solution and 1 ml solution was added in 3 ml of test samples of each dry extract having concentrations as 1, 10, 25, 50, 75, 100 µg/ml in methanol and kept in darkness. Thirty minutes later, the absorbance was measured at 517 nm. A blank was prepared by adding 1 ml methanol to each of the test samples. A negative control was also prepared by adding 1 ml DPPH reagent in 3 ml of methanol. Ascorbic acid at concentrations 5, 10, 15, 25, 50, 75, 100µg/ml was used as standard. Lower the absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

$$\text{DPPH Scavenged (\%)} = \frac{\text{A control} - \text{A test}}{\text{A control}} \times 100$$

Where 'A control' is the absorbance of the control reaction and 'A test' is the absorbance in presence of the sample or extracts. The antioxidant activity of the different extracts was expressed in % DPPH radical scavenged. The results for each tube were duplicated and the average (mean) was calculated[7].

Antimicrobial Susceptibility Test (AST)

With the help of sterile cotton swabs, the overnight cultures were then swabbed onto the sterile petri dishes containing the growth media and kept for drying. The agar diffusion method (Kirby-Bauer method, Bauer et. al.; 1966) was used to check the antimicrobial activity of the extracts. After drying, flame sterilized cork borer made of stainless steel (8 mm diameter) was used to prepare 4 cups in the medium of each petri dish. The wells were loaded with 100 µl of varying concentrations of the extract i.e. 10, 20, 30, 50 mg/ml. In a similar manner, a standard plate for bacteria by using the antibiotic streptomycin and for yeast by using the antibiotic fluconazole was performed. All plates were kept at room temperature for effective diffusion of the extracts and the standard antibiotics. Later the bacteria inoculated plates were incubated at 37°C for 24 hours while the yeast plates were kept at room temperature. Each plate was carried out in duplicates. The diameter of zone of inhibition was measured and recorded [8].

RESULTS

Phytochemical analysis

The phytochemical characteristics of *Azadirachta indica* and *Vitex negundo* leaves were tested and are summarized in the table given below. The results revealed the presence of medically active compounds in both the plants studied. From the table 1, it could be seen that alkaloids, carbohydrates, glycosides (anthraquinone and cardiac), phenolic and tannin compounds, flavonoids, saponins and lipids were present in both the plants. *Vitex negundo* also showed the presence of terpenoid compounds while *Azadirachta indica* showed absence of such compounds. However, proteins were found to be absent in the leaves of both the plants.

Table 1 Phytochemical constituents of *Azadirachta indica* and *Vitex negundo*

Phytochemicals	*Azadirachta indica*	*Vitex negundo*
Alkaloids	+	+
Carbohydrates	+	+
Glycosides		
a. Anthraquinone	+	+
b. Cardiac	+	+
Proteins	-	-
Phenolics and tannins	+	+
Flavonoids	+	+
Saponins	+	+
Lipids	+	+
Terpenoids	-	+

Keys:
\+ → Presence of compounds
\- → Absence of compounds

Antioxidant activity by DPPH assay

Azadirachta indica extract

OD of control = 0.151
Wavelength = 517 nm

Table 2 Calculation of antioxidant activity for *Azadirachta indica*

Concentration (µg/ml)	OD of Test	% Radical scavenging
1	0.145	37.086
10	0.128	51.655
25	0.116	61.589
50	0.098	76.158
75	0.093	82.119
100	0.090	86.754

Vitex negundo extract

OD of control = 0.151
Wavelength = 517 nm

Table 3 Calculation of antioxidant activity for *Vitex negundo*

Concentration (µg/ml)	OD of Test	% Radical scavenging
1	0.144	33.112
10	0.140	39.072
25	0.120	54.966
50	0.114	62.913
75	0.108	68.874
100	0.104	74.834

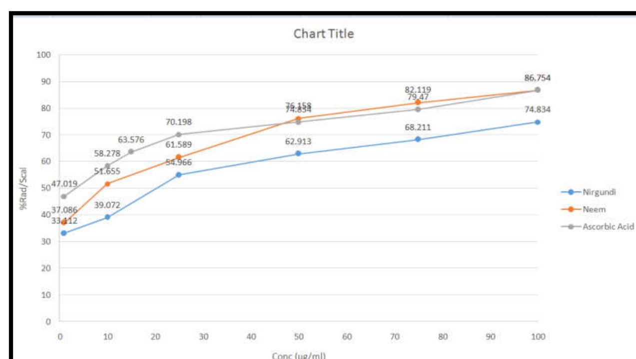
Standard Ascorbic Acid

OD of control = 0.151
Wavelength = 517 nm

Table 4 Calculation of antioxidant activity for Standard ascorbic acid

Concentration (µg/ml)	OD of Test	% Radical scavenging
1	0.109	47.019
10	0.098	58.278
15	0.093	63.576
25	0.087	70.198
50	0.082	74.834
75	0.078	79.470
100	0.072	86.754

The effect of antioxidant on DPPH is believed to be due to their hydrogen-donating ability. The DPPH assay helps measure the antioxidant activity of the plant extracts. The DPPH radical scavenging activity of *Azadirachta indica* and *Vitex negundo* leaf extracts were compared with a standard ascorbic acid solution. At concentrations of 50µg/ml, 75µg/ml and 100µg/ml, the scavenging activity of *Azadirachta indica* leaf extracts were found to be greater than that of the standard ascorbic acid. The radical scavenging activity of *Vitex negundo* leaf extracts were found to be lower than that of both the standard ascorbic acid as well as *Azadirachta indica* extracts at the different concentrations tested. Although the DPPH radical scavenging activities of the extracts were lower than that of standard ascorbic acid, it was evident that the extracts showed proton-donating ability and this could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.



Graph 1 Line graph showing the antioxidant (DPPH radical scavenging activity) of *Azadirachta indica*, *Vitex negundo* and Standard Ascorbic acid

Antimicrobial susceptibility test (AST)

Azadirachta indica

Table 5 Zone of inhibition produced by *Azadirachta indica*

Concentration (mg/ml)	Diameter of zone of inhibition (mm)				
	Streptococcus pyogenes	Staphylococcus aureus	Escherichia coli	Klebsiella pneumoniae	Candida albicans
10	-	-	-	-	-
20	-	-	-	-	13
30	-	-	14	15	15
50	15	13	16	18	19

Vitex negundo

Table 6 Zone of inhibition produced by *Vitex negundo*

Concentration (mg/ml)	Diameter of zone of inhibition (mm)				
	Streptococcus pyogenes	Staphylococcus aureus	Escherichia coli	Klebsiella pneumoniae	Candida albicans
10	-	-	-	-	-
20	13	-	13	14	12
30	14	13	14	17	14
50	18	14	18	20	20

Standard antibiotics

Streptomycin (Antibacterial)

Table 7 Zone of inhibition produced by Standard Streptomycin

Concentration (mg/ml)	Diameter of zone of inhibition (mm)			
	Streptococcus pyogenes	Staphylococcus aureus	Escherichia coli	Klebsiella pneumoniae
10	22	18	24	28
20	25	22	26	30
30	27	24	28	32
50	29	26	31	34

Fluconazole (Antifungal)

Table 8: Zone of inhibition produced by Standard Fluconazole

Concentration (mg/ml)	Diameter of zone of inhibition (mm) *Candida albicans*
10	-
20	-
30	-
50	-

The zones of inhibition (mm) exhibited by the plant extracts are given in the table 6 and 7. In case of *Azadirachta indica* extract, zone of inhibition was observed from the concentration of 20 mg/ml for *Candida albicans*, 30 mg/ml for *Escherichia coli* and *Klebsiella pneumonia* and 50 mg/ml for *Streptococcus pyogenes* and *Staphylococcus aureus*. In case of *Vitex negundo* extract, zone of inhibition was observed from the concentration of 20 mg/ml for *Candida albicans, Klebsiella pneumonia, Escherichia coli* and *Streptococcus pyogenes*, and from 30 mg/ml for *Staphylococcus aureus*. In case of the Standard antibiotic Streptomycin (antibacterial), zone of inhibitions were observed from the concentration of 10 mg/ml for all the bacterial organisms. However, the Standard antibiotic Fluconazole (antifungal), it did not show zone of inhibition at any of the 4 given concentrations for the yeast *Candida albicans*.

Several researchers have investigated the efficiency of plant extracts and their effective compounds as antimicrobial agents. Some researchers have suggested that the antimicrobial components of plant extracts (terpenoids, alkaloids, phenolic compounds) interact with the enzymes and proteins of the microbial cell membrane causing its disruption to disperse a flux of protons towards cell exterior which induces death or may inhibit enzymes necessary for amino acids biosynthesis [9]. Other researchers attributed the inhibitory effect of these plant extracts which enable them to react with proteins of microbial cell membrane and mitochondria disturbing their structures and changing their permeability [10].

DISCUSSION

Phytochemical analysis and Antimicrobial activity of Azadirachta indica and Vitex negundo

Plant has long been a very important source of drug and many plants have been screened if they contain compounds with therapeutic activity. Therefore, it is vital to evaluate the antimicrobial activity of both *Azadirachta indica* and *Vitex negundo*. In this study, the antimicrobial activity of the leaves of *Azadirachta indica* and *Vitex negundo* was evaluated by the Agar diffusion method (Kirby-Bauer method). The

microorganisms chosen to be studied were Gram positive (*Streptococcus pyogenes, Staphylococcus aureus*), Gram negative (*Escherichia coli, Klebsiella pneumonia*) and a yeast (*Candida albicans*). These organisms were chosen to be studied as they are important pathogens and also due to rapidly developed antibiotic resistance as antibiotic use increases.

A variety of herbs and herbal extracts as well as medicinal plant extracts contain different phytochemicals with biological action that can be of valuable therapeutic index. Much of the protective effect of herbal and medicinal plants has been attributed by phytochemicals, which are the non-nutrient compounds. Alkaloids, flavonoids, glycosides and phenols have been reported to exert multiple biological effects like anti-inflammatory, anti-allergic, antioxidant, anti-diabetic, anti-viral and anti-cancer, antimicrobial activities etc. *Azadirachta indica* showed the presence of alkaloids, carbohydrates, glycosides, phenolic and tannin compounds, flavonoids, saponins and lipids. Earlier workers [11], studied that phytochemical analysis of *Azadiracta indica* leaves by using different solvent such as Petroleum ether, chloroform, methanol show the presence of triterpenes, glycosides and fatty acids. Other phytochemicals studied in this analysis were absent in all extract of leaves. Antibacterial activity of *Azadiracta indica* was analyzed by previous workers showed that the chloroform extract of leaves possess significant activity, than petroleum ether and methanol extracts. Early studies proved ethanol as the most efficient solvent for extracting broad spectrum of antibacterial compounds from plants. Ethanolic extract of *Azadiracta indica* whole plant shows presence of flavonoids and tannins only. Similarly the extract of Azadiracta indica is active against *E.coli* followed by Staphylococcus aureus [12]. Earlier observation also showed the antifungal and antibacterial activity of *Azadiracta indica* [13]. *Vitex negundo*, in addition to these compounds, showed the presence of terpenoid compounds as well. Antibacterial activity of the *Vitex negundo* while plant of hexane, alcoholic and aqueous extracts against *B.subtilis, E.coli, Proteus vulgaris, S.typhimurium, P.aeruginosa* and *S.aureus* had no activity [14]. Antibacterial activity of ethanol extracts of *Vitex negundo* leaf using agar dilution method against four bacteria *B.subtilis, S.epidermidis*, *E.coli* and *P.aeruginosa* [15]. Further, studied the antibacterial activity of *Vitex negundo* on bark and leaf of petroleum ether, chloroform, methanol and aqueous extracts against *B.subtilis, S.aureus, S.epidermidis, S.typhimurium, P.aeruginosa, V.cholerae*, and *V.alginolyteus* had little activity but inhibition was measured including disc and cup that measures 6mm indicates low activity moreover less concentration of extract was taken which does not give accuracy of results.

Antioxidant activity of Azadirachta indica and Vitex negundo

To evaluate the scavenging effect of the essential oils in this study, DPPH reduction was investigated against a positive control (Standard Ascorbic Acid). The more antioxidants occurred in the oils, the more DPPH reduction will occur. High reduction of DPPH is related to the high scavenging activity performed by particular sample. At a higher concentration, these essential oils may exhibit more significant free radical scavenging activity. *Azadirachta indica* and *Vitex negundo* showed considerable antioxidant activity with the former showing a higher activity.

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