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Antibacterial activity of *Ventilago madrasapatana* methanol leaves extract against Uropathogens

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ABSTRACT

One of the reasons diseases are re-emerging is due to multidrug-resistant strains of bacteria in the environment because of overuse of antibiotics. The conventional antibiotics are losing their efficacy against the strains that they used to work against. These prevailing conditions push us towards the pursuit of finding new antimicrobial agents. They might prove to be the solution for the problem under consideration. In this study, we investigated the antimicrobial activity of *Ventilago madrasapatana* leaf extracts against a panel of pathogenic microorganisms. The leaves were extracted using methanol and the antimicrobial activity was evaluated using agar disc diffusion method. Our results demonstrated significant antimicrobial potential for the methanol extract, with pronounced inhibition against both gram-positive and gram-negative bacteria, including *Bacillus subtilis, Enterococcus faecalis, Klebsiella pneumoniae and Pseudomonas aeruginosa.* This assay suggests their potential application as therapeutic agents. Moreover, the phytochemical analysis indicated the presence of bioactive compounds, such as alkaloids, flavonoids, and phenolic compounds, which may contribute to their antimicrobial efficacy. These findings highlight *Ventilago madrasapatana* leaf extracts as promising sources of natural antimicrobial agents that could be developed into novel therapeutics to combat AMR and enhance public health. However, further research is needed to elucidate the underlying mechanisms of action and evaluate their safety and efficacy in clinical settings.

1. Introduction

Exploitation of antibiotics has led to the emergence and spread of multidrug-resistant microorganisms, rendering many conventional antibiotics ineffective against once-treatable infections. Consequently, the World Health Organisation (WHO) has emphasised the urgent need to discover new, effective, and sustainable alternatives to combat the rising tide of antimicrobial resistance. In recent years, due to the emergence of multi-drug resistant bacterial strains, there has been a growing interest in exploring natural sources for potential antimicrobial agents.

Medicinal plants have been used for treatment of infectious diseases and majority of emerging infectious events are caused by bacteria.[1] This can be associated with evolution of drug resistant strains.[2] Herbal medicines play an important role in Ayurveda, Homeopathy and Naturopathy.[3] Currently, efforts are being made to propose new drugs to multidrug resistant bacterial strains.[4] Such treatments have determined undesirable side effects and side effects such as diarrhoea, nausea, abnormal taste, dyspepsia, abnormal pain and headache.[5]

Among the botanical species, *Ventilago madrasapatana* seems to be a promising candidate for its purported antimicrobial properties. *Ventilago madrasapatana*, a member of the Rhamnaceae family, is a deciduous

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climbing shrub predominantly found in the tropical and subtropical regions of India, Sri Lanka, and Southeast Asia.[6] Its medicinal significance can be traced back to traditional knowledge where it is used for healing practices in indigenous communities, where *Ventilago madrasapatana* leaves were used to treat various ailments, including skin infections, wounds, respiratory disorders, and gastrointestinal disturbances.[7] The whole plant is used for the treatment of asthma, jaundice, anorexia, splenic disorders and piles.[8] It is also used for testing anti-inflammatory, anti-cancer activity[9] and anti-diabetic activity.[10] The medicinal compounds of *Ventilago madrasapatana* rupture the membrane of the bacteria, assist in enzyme activity inhibition and bacterial biofilm formation.

However, despite the promising findings, a comprehensive and systematic evaluation of the antimicrobial potential of *Ventilago madrasapatana* leaves remains incomplete, with several aspects requiring further elucidation. The existing research on Ventilago madrasapatana's antimicrobial properties often lacks standardisation in extraction methods, bioassays and reporting of results, leading to variability and challenges in comparing the outcomes of different studies. Therefore, a thorough review of the available literature is crucial to gain a comprehensive understanding of Ventilago madrasapatana's antimicrobial activity and to identify potential areas for further research and development. In pursuit of this goal, we have conducted an extensive literature analysis, encompassing a broad spectrum of scientific databases, journals, and publications, to identify relevant studies pertaining to the antimicrobial properties of Ventilago madrasapatana.

2. Materials and Methods

2.1. Sample collection

The plant materials chosen as samples of leaves of *Ventilago madrasapatana* were collected during the spring season (June to August) from Villupuram district of Tamil Nadu, India.The fresh samples were collected and rinsed in water to remove dust and any undesirable deposits on leaves. The leaves were then placed on towels to absorb excess water.

2.2. Preparation of extracts

The methanol extraction fluid (200 mL) was used to perform effective extraction. Soxhlet apparatus was used for the same for 8 hours to obtain soluble fraction. The extracted liquid was subjected to evaporation using rotary evaporator. This was done to separate the semi-solid extracts from the extraction liquid. The semi-solid extract was stored in freezer over-night. It was then freeze dried for hours at 60°C in vaccum. The extracts were then stored in air tight containers at 4°C. The dried extracts were exposed to UV rays (200-400 nm) for 24 h and to ensure any contamination they were streaked on agar plates to check if they produced colonies.[11]

3. Qualitative Analysis of Phytochemicals in the Plant Leaves Extract

The qualitative phytochemical analysis of the crude extract obtained from the leaves of *Ventilago madrasapatana* was conducted to assess the presence or absence of secondary metabolites, including alkaloids, flavonoids, saponins, tannins, cardiac glycosides, and others.[12] Standard procedures were employed for these screenings.

3.1. Tests for Alkaloids

1. *Dragendorff's test:* 50 mg of extract was dissolved in ml of 1% hydrochloric acid and filtered. Dragendorff's reagent was parallely prepared. While the filtrate from the reaction was totally collected, 2-3 drops from the filtrate were withdrawn and treated with equal amount of Dragendorff's reagent. Formation of orange-red precipitate would indicate the presence of alkaloids.
2. *Mayer's reagent test:* 1 ml of filtrate was considered and was added with a few drops of Mayer's reagent. Formation of yellowish white precipitate would indicate the presence of alkaloids.[13]

3.2. Test for flavonoids

Methanol was used as the solvent to dissolve the plant extract. For better interaction, a mildly heated piece of magnesium ribbon was added to the mixture followed by addition of concentrated hydrochloric acid. The change of colour from orange, pink, red to purple would indicate the presence of flavonoids.[14]

3.3. Test for carbohydrates

The plant extract was considered and approximately 2 ml was withdrawn from the stock extract. It was added with few drops of Molisch reagent and 1ml of concentrated sulphuric acid. Formation of red coloured ring at the interface would indicate the presence of carbohydrates.

3.4. Test for tannins

Around 300 mg of the pre prepared plant extract was considered and dissolved in about 10 ml of distilled water. This mixture was then subjected to filtration 2 ml of the resultant extract was considered in a new test tube. 1% alcoholic ferric chloride solution was prepared simultaneously as the filtration reaction proceeds. Once both the reactions are complete, prepared ferric chloride solution was added to the resultant extract. Occurrence of blue green precipitate would indicate the presence of tannins.

3.5. Test for saponins

For analysing the presence of tannins, the stock plant extract is considered and 1 gram of the extract was withdrawn. Vigorous shaking of the considered extract dissolved in ample amount of distilled water was done. Persistent foam for about ten minutes would confirm the presence of saponins.

3.6. Test for glycosides

A small amount of plant extract was considered and was added to a test tube containing glacial acetic acid and ferric chloride. This mixture is then added with concentrated sulphuric acid. Formation of brown ring at the interface would indicate the presence of glycosides.

3.7. Test for terpenoids

From the stock extract, around 500 mg of the extract was considered and dissolved in ethanol. In a new test tube, approximately equal amount of acetic anhydride was taken and was added with the ethanol-extract mixture. This was followed by addition of concentrated sulphuric acid. The colour change from pink to violet would indicate the presence terpenoids.[15]

3.8. Test for steroids

Acetic acid-Chloroform solution was prepared and was added with 100mg of the extract. For better reaction efficiency, the reactants were placed in 0°C and them was added with concentrated sulphuric acid. Formation of reddish brown ring would indicate the presence of steroids. Alternatively, a violet ring formation may also indicate the presence of steroids.[12]

3.9. Test for diterpenoids

A new test tube was considered and 1% of copper acetate solution was prepared. It was then added with the plant extract. The colour of the solution would change to emerald green colour and that would indicate the presence of diterpenoids.

3.10. Test for protein

Ninhydrin solution was considered and prepared in a fresh test tube. It was added with the plant extract and tap mixed for better reaction. It was subjected to mild heating to enhance the reaction efficiency. Formation of blue colour might indicate the presence of protein.

4. Quantitative Analysis of Phytochemicals in the Plant Leaves Extract

1. *Estimation of total phenolic content:* Upon analysis, Folin-Ciocalteu method was considered to estimate total phenol content. 0.5N Folin-Ciocalteau reagent was first prepared. For the reaction, 0.5ml of extract was considered as the sample. It was added to 0.1ml of Folin-Ciocalteu reagent. The reaction mixture was incubated at 37ºC and left alone for 5 minutes. Followed by this, 2.5 ml of saturated sodium carbonate was drip added into the reaction mixture before further incubation for 30 more minutes. The solution was then analysed at 760nm using spectrophotometer.[16]
2. *Estimation of flavonoids:* A fresh test tube and 0.5ml of the extract was considered. Aluminium chloride of known concentration and 0.5ml of 120Mm Potassium acetate were added by the sides into the previously considered test tube. The tube was incubated at room temperature for about half an hour. The resultant solution post reaction was analysed at 417nm while distilled water was used as blank and Quercetin was used as standard.[17]
3. *Estimation of alkaloid:* The extract was considered and 5 mg of it was withdrawn and was added with dimethyl sulphoxide. It was tap mixed until the contents of the test tube seemed to have dissolved. Once, the contents seemed to have dissolved, the test tube was added with a few drops of 2N hydrochloric acid. Upon filtering the solution, the filtrate was transferred to a separating funnel. Pre prepared Bromocresol green solution(5ml) and PBS(0.5ml) were added to the reaction mixture. The mixture was subjected to shaking with different volumes of chloroform from 1ml to 4ml. Post, vigorous shaking and the solution was collected in a 10 ml volumetric flask and diluted to the volume with chloroform. The resultant solution was analysed at 417nm with suitable standards. The total alkaloid content was expressed as mg of AE/g of extract.
4. *Thin layer chromatography:* In thin layer chromatography, the extract considered till now was dissolved in acetone and was homogeneously applied on a plate that was pre coated with silica gel with a couple of capillary tubes. The plates upon applying were rested on a solvent system that contained Methanol: Chloroform: Hexane in 2:2:1 ratio. Upon completion, the plates were air dried and analysed at 254nm and 356nm. The Rf values were carefully noted for further analysis.

5. Preparation and Standardisation of Bacterial Suspensions

To prepare the microorganism's suspension, standard and clinically isolated bacterial strains were cultured on nutrient broth. A full loop of each bacterial strain was inoculated into separate tubes of nutrient broth and incubated at 37°C for 24 hours. The growth in the broth was observed, and then sub-cultured on nutrient agar plates for another 24

hours. Once the growth was confirmed as the correct species under a light microscope, individual colonies were sub-cultured onto nutrient agar slopes and incubated at 37°C until sufficient growth occurred. To obtain the bacterial suspension, 1ml of the bacterial culture was diluted with 9ml of sterile saline using a pipette and gently shaken to achieve a suspension approximately (10-10 CFU/ml). The prepared suspension was stored in the refrigerator at 4°C until further use.[18]

6. Agar Disk Diffusion Method

In this test, a number of tiny, sterile disks, each measuring 6 mm in diameter, are placed on the surface of an agar plate that has already been inoculated with a standard quantity of the organism to be tested. Each disk is topped with a defined concentration of an antimicrobial agent. The disks are positioned on the plate at evenly spaced intervals using a disk dispenser or sterile forceps. The antimicrobial chemicals diffuse into the surrounding media and come into contact with the proliferating organisms when the disks are firmly in contact with the agar. For 18 to 24 hours, the plates are incubated at 35°C.[19] Following incubation, the plates are inspected for the presence of bacterial growth-inhibiting zones surrounding the antimicrobial disks. The rate at which extract components diffuse into the medium, how susceptible an organism is to extract components, how many organisms are put on the plate, and how quickly they grow are only a few of the variables that affect the extent of the zone of inhibition. Prior standardization of the test is critical. The organism is said to have "intermediate" susceptibility to a specific medicine if it cannot be categorized as either sensitive or resistant.

6.1. Statistical analysis

Arithmetic analysis of the data was performed while the standard errors were compared with control as the readings of control (distilled water) experiments in the *in-vitro* antibacterial studies of those plants were zero.

7. Results

The yield of methanol extract of obtained by Soxhlet extraction was 12.23%. Qualitative analysis for the presence of different phytochemicals in *V. madrasapatana* was performed and the results obtained are presented in Table 1 below. Notably, elevated levels of flavonoids were seen in the sample. Quantitative analysis for phenol, flavonoids and alkaloids were performed in *V. madrasapatana* and the results obtained are presented in Table 2 below. It shows the presence of maximum amount of phenol (34.03±0.34) followed by flavanoids (29.87±0.2) and alkaloids (14.91±0.31). The analysis also confirms the presence of various bioactive compounds present in methanol extracts. The sample was then subjected to thin layer chromatography and the results are shown (Figure 1). It showed the presence of different bands when exposed to light of different wavelengths 254nm, 366nm and day light.

The sample was subsequently tested for antibacterial properties with four species against antibiotic controls using agar disc diffusion method and the results are presented below. Notably, the zone of inhibition is wider for gram negative bacteria compared to gram positive bacteria.

8. Qualitative Analysis of Phytochemicals in the Plant Leaves Extract of Ventilago madraspatana Table 1.

Table 1: Preliminary phytochemical screening of Methanol solvent extracts of *Ventilago madrasapatana* leaves

S.No	Test	Methanol
1.	Alkaloids	++
2.	Flavonoids	++
3.	Tannins	++
4.	Protein	-
5.	Saponin	-
6.	Glycosides	+
7.	Terpenoids	+
8.	Phenols	++
9.	Steroid	+
10	Diterpenoids	+

Table 2: Total phenol, flavonoid, Alkaloid content of methanol leaves extract of Ventilago madraspatana

Solvents	Total Phenol(mg of GAE/g of extract)	Flavanoids(mg of QE/g of extract)	Alkaloids(mg of AE/g of extract)
Methanol	34.03±0.34	29.87±0.2	14.91±0.31

Mean±S.D

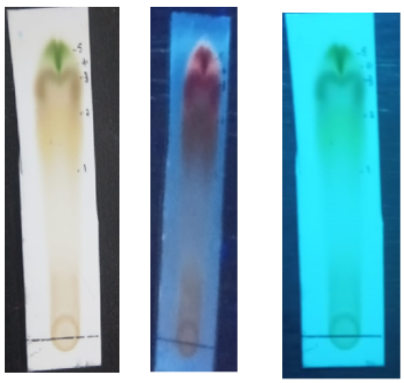

Figure 1: Thin layer chromatography of methanol leaves extract of *Ventilago madraspatana*

Table 3: Methanol extract

Type of bacteria	Name of the bacterial strains	Zone of inhibition (mm)			Antibiotic control
		25 (μg/ml)	50 (μg/ml)	100 (μg/ml)	Gentamicin (mm)
Gram positive bacteria	*Bacillus subtilis*	11.8 ± 0.26	12.01 ± 0.19	13.8 ± 0.31	14.5 ± 0.06
	Enterococcus faecalis	11.2 ± 0.16	12.5 ± 0.17	15 ± 0.14	14.8 ± 0.05
Gram negative bacteria	*Pseudomonas aeruginosa*	12.8 ± 0.25	13.4 ± 0.29	15.8± 0.21	14.6 ± 0.14
	Klebsiella pneumoniae	12.2 ± 0.24	13.5 ± 0.31	14.5 ± 0.28	14.8 ± 0.07

9. Antibacterial Activity of *Ventilago Madrasapatana* Leaf ExtractsTable 3.

9.1. Zone of inhibition concentration (mm) for various concentration of Methanol extract of Ventilago madraspatana leaves

Figure 2: Inhibition zone of antibacterial activity of methanol extract gram positive bacteria

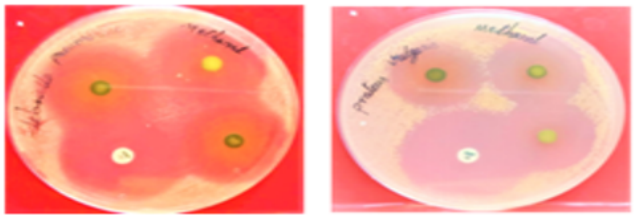

Figure 3: Gram negative bacteria

10. Discussion

The results indicate that the methanol leaves extracts of Ventilago madraspatana showed antibacterial activities toward the gram-positive bacteria (*Bacillus subtilis and Enterococcus faecalis*) as well as gram-negative bacteria (*Pseudomonas aeruginosa and Klebsiella pneumoniae*). The methanol leaves extract seems to be more effective against gram negative bacterial strains. The highest antimicrobial activity was recorded against *Klebsiella pneumonia, Pseudomonas aeruginosa* followed by *Enterococcus faecalis, Bacillus subtilis* and. The phytochemical analysis of the plant extracts reveals the presence of alkaloids, flavonoids, tannins, glycosides, terpenoids, phenols, steroids and diterpenoids that singly or in combinations may be responsible for the antimicrobial activity. The quantitative analysis indicates phenols are the major components of the extract. The demonstration of antimicrobial activity against both gram-positive and gram-negative bacteria may be indicative of the presence of broad spectrum antibiotic compounds. The phytochemicals present are secondary metabolites of plants that serve as defence mechanisms against predation by many microorganisms, insects and other herbivores.

The anti bacterial activity of Ventilago madraspatana can be majorly accounted for its flavonoids contents. Notably, elevated levels of flavonoids were seen in all stages of the extracts and prove to be consistently retained in the sample post analysis. Flavonoids are known to exert antibacterial activity via damaging the cytoplasmic membrane, inhibiting the energy metabolism of bacteria. This would in turn inhibit the synthesis of nucleic acids needed for replication. Their antibacterial activity can also be accounted to the flavonoids–membrane interaction that is related to their chemical structure, particularly the number and positions of methoxyl and hydroxyl groups. This makes flavonoids of *Ventilago madraspatama* as the primary player in inhibiting bacterial growth.

In conclusion, Ventilago madraspatana can be seen as a potential source for preparation of naturally extracted biologically active antibacterial drugs. While there are new strains emerging everyday with antibiotic resistance, the hunt for the naturally occurring bioactive compounds continues so that a long term solution to combat AMR is attained in the future.

11. Source of Funding

None.

12. Conflict of Interest

None.

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