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Antimicrobial activity of leaf extracts of *Justicia adhatoda* L. in comparison with vasicine

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ABSTRACT

Objective: To ascertain the antimicrobial activity of methanolic leaf extracts of *Justicia adhatoda* and vasicine against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus flavus*. **Methods:** The antimicrobial activity of the concentrated leaf extracts of *J. adhatoda* was evaluated by determination of the diameter of zone of inhibition against bacteria and fungi. $25 \mu\text{g ml}^{-1}$ concentration was used to check the antimicrobial activity of plant extracts and vasicine. Minimum inhibitory concentrations and minimum microbicidal concentrations were determined against all the pathogens. Sensitivity of the pathogens was also checked with four standard antibiotics, ciprofloxacin and ofloxacin for bacteria and nystatin and amphotericin B for fungi. **Results:** The phytochemical studies revealed the presence of alkaloids in the extracts were active against both bacteria and fungi. Studies on the minimum inhibitory concentration of the extracts on the test organisms showed that the lowest minimum inhibitory concentration and minimum microbicidal concentrations were demonstrated against *Serratia marcescens*, *Escherichia coli* and *Pseudomonas aeruginosa* and the highest minimum inhibitory concentration was exhibited against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae*. Among fungi *Aspergillus flavus* showed lowest minimum inhibitory concentration whereas *Candida albicans* and *Cryptococcus neoformans* showed highest minimum inhibitory concentration. **Conclusion:** The present study revealed that *J. adhatoda* has broad spectrum of antimicrobial activity and a potential source of antimicrobial agents that could be useful for chemotherapy and control of infectious diseases.

1. Introduction

Medicinal plants represent a rich source of antimicrobial agents. Plants are used medicinally in different countries and are a source of many potent and powerful drugs[1]. It has been established that up to 25% of the drugs prescribed in conventional medicines are allied directly or indirectly to natural substances mostly of plant origin. In recent years, pharmaceutical companies have spent a lot of time and money in developing natural products extracted from plants, to produce more cost effective remedies that are affordable to the population[2].

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties. In recent years, antimicrobial properties of medicinal plants are being increasingly reported from different parts of the

world. It is expected that plant extracts showing target sites other than those used by antibiotics will be active against drug-resistant microbial pathogens. However, very little information is available on such activity of medicinal plants [3]. Considering the vast potentiality of plants as sources for antimicrobial drugs with reference to antibacterial and antifungal agents, a systematic investigation was undertaken to screen the antibacterial and antifungal activity from *J. adhatoda*.

Justicia adhatoda L. (Family Acanthaceae) is a shrub, widespread throughout the tropical regions of Southeast Asia [4]. The name, *J. adhatoda* L. and *Adhatoda zeylanica* Medic are used synonymously. It is commonly known as Vasaka or Malabar nut. It is a perennial, evergreen and highly branched shrub (1.0 m to 2.5 m height) with unpleasant smell and bitter taste. It has opposite ascending branches with white, pink or purple flowers. It is a highly valuable Ayurvedic medicinal plant used to treat cold, cough, asthma and tuberculosis [5]. Its main action is expectorant and antispasmodic (bronchodilator) [6].

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Moreover the importance of Vasaka plant in the treatment of respiratory disorders can be understood from the ancient Indian saying, “No man suffering from phthisis needs despair as long as the Vasaka plant exists”. Thus the frequent use of *J. adhatoda* has resulted in its inclusion in the WHO manual “The Use of Traditional Medicine in Primary Health Care” which is intended for health workers in south–east Asia to keep them informed of the restorative utility of their surrounding flora [5]. The major alkaloids of the plant, vasicine and vasicinone, have been found to be biologically active and are the area under discussion of many chemical compounds and pharmacological studies. The source of the drug ‘Vasaka’ is well known in the indigenous system of medicine for its beneficial effects, particularly in bronchitis [7].

In the present study, the antimicrobial activity of methanolic extract of *J. adhatoda* was determined against Gram positive, Gram negative pathogenic bacteria and fungi along with pure vasicine and reference antibiotics using disc diffusion method and agar well diffusion method. Minimum Inhibitory Concentration and Minimum Microbicidal Concentration of methanolic extract of *J. adhatoda* were also determined.

2. Materials and methods

2.1. Collection of plant material

Fresh leaves of plants free from disease were collected from healthy *J. adhatoda* plant. The leaves were washed thoroughly 2–3 times with running water and once with sterile distilled water, then air–dried on sterile blotter under shade and placed in hot air oven at a temperature of 50°C for 4 – 5 days till the weight became constant. Plant materials were regularly examined to check any fungal growth or rotting. The dried plant materials were powdered to obtain a very fine particle size using sterile clean mortar and pestle.

2.2. Preparation of plant extracts

Twenty grams of the powder were soaked in 50 ml absolute methanol in 250 ml sterile conical flask, incubated at 37°C with shaking at 120 rpm for 30 minutes and kept for 24 hours. After 24 h, the extract was filtered rapidly through four layers of gauze. The content was then filtered with Whatman No. 1 filter paper and the residue was again treated with 50 ml of absolute alcohol and incubated as mentioned earlier. It was repeated 3 times. The pooled up filtrates were evaporated to dryness using a desiccators. The dried extract was finally reconstituted in 5 ml of absolute ethanol and estimated the total concentration of alkaloid present in it as vasicine [8]. Then packed in separate sterile glass vials as aliquots which containing $25 \mu\text{g ml}^{-1}$ of vasicine and stored at 4 °C until use. Aliquots of pure vasicine (standard) of same

concentration was also prepared and stored.

2.3. Culture and maintenance of test microorganisms for antimicrobial studies

Bacterial cultures of *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes* and fungal cultures of *Aspergillus flavus*, *Candida albicans* and *Cryptococcus neoformans* were obtained from the Microbiology Laboratory of our department. All the bacterial strains were maintained on nutrient agar (NA, Hi–Media) at 37°C and fungi were maintained on Sabouraud’s Dextrose agar (SDA, Hi–Media) at room temperature. Bacteria were inoculated in nutrient broth (NB, Hi–Media) and incubated at 37°C for 24 hours for doing the test. Mueller– Hinton Agar (MHA, Hi–Media) and Sabouraud’s Dextrose Agar (SDA) were used for testing the antibacterial and antifungal activity respectively.

2.4. Preparation of inoculums

Each 24 hour culture suspension of microorganisms was standardized to 25% transmittance at 560 nm using an ultraviolet (UV) –visible spectrophotometer for obtaining 106 colony forming units (CFU) ml^{-1} . McFarland standards are used as a reference to adjust the turbidity of microbial suspension so that the number of microorganisms will be within a given range. For the preparation of the 0.5 McFarland standard, 0.05ml of barium chloride ($\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$) (E. Merck, India) was added to 9.95 ml of 0.18M H_2SO_4 (1.0% w/v) (E. Merck, India) with constant stirring. The McFarland standard tube was tightly sealed to prevent loss by evaporation and stored for up to 6 months. To aid comparison the test and standard were compared against a white background with a contrasting black line. Fungal isolates were standardized to 106 spores ml^{-1} by using spectrophotometer at 530 nm and were adjusted to 80% to 85% transmittance.

2.5. Antimicrobial activity screening

Agar–well diffusion methods (Ahmad and Beg, 2001), and paper disc diffusion methods (Kirbey–Bour method) [9] were employed to determine the antimicrobial activities for methanolic extracts. Twenty microlitres of methanolic extracts of the leaves at concentration of $25 \mu\text{g ml}^{-1}$ and pure vasicine with $25 \mu\text{g ml}^{-1}$ concentration were used against the test microorganisms.

2.5.1. Antibacterial and antifungal screening by agar well diffusion method

Approximately 20 ml of sterile Muller–Hinton Agar (MHA) and Sabouraud’s Dextrose Agar (SDA) was poured into sterile Petri plates and allowed to set. Plates were then seeded with

0.5 ml of a 24 h old bacterial culture and using a sterile glass (L) rod made a lawn culture. SDA plates were seeded with fungal cultures. The plates were allowed to dry. For doing agar well diffusion method, wells are made on the plate with the aid of a sterile hole puncture (8.0 mm diameter). Twenty microlitres of the plant extract and vasicine were poured into the respective wells. The plates thus prepared were left at room temperature for ten minutes, allowing the diffusion of the extracts into the agar. Then the plates with bacterial culture and fungal culture plates except *Aspergillus flavus* were placed in the incubator at 37°C for 24 hours. The plates with *A. flavus* were kept at room temperature for 48–72 hours. After incubation for 24 hrs at 37°C, the plates were observed. The antibacterial activity of the plant extract and vasicine was assessed by an inhibition zone surrounding the well. The zone of inhibition was measured and expressed in millimeters.

2.5.2. Antibacterial and antifungal screening by paper disc diffusion method

Sterile Muller–Hinton Agar (MHA) and Sabouraud's Dextrose Agar (SDA) culture plates were prepared as agar well diffusion method. Sterile filter paper discs (diameter 6mm for bacteria and 13 mm for fungi) impregnated with 20 μ L of extract and vasicine at concentration of 25mg ml⁻¹ were applied over each of the culture plates previously seeded with the 0.5 McFarland and 106 CFU ml⁻¹ cultures of bacteria and 106spores ml⁻¹ of fungi respectively. Bacterial cultures and those of *Candida albicans* and *Cryptococcus neoformans* were then incubated at 37°C for 24 h while the plates with *A. flavus* cultures were incubated at room temperature (28°C – 32°C) for 48 h. Paper discs impregnated with 20 μ L of a solution of 25mg ml⁻¹ of ciprofloxacin and ofloxacin (for bacteria) (Hi Media) and nystatin and amphotericin B (for fungi) (Hi Media) as standard antimicrobials were used as positive control for comparison in both methods. Sterilized paper discs without extracts or antibiotics were used as negative control for both. Antimicrobial activity was determined by measurement of zone of inhibition around each paper disc. Overall, cultured bacteria with halos equal to or greater than 7 mm and fungi with 10 mm halos were considered susceptible to the tested extract [10]. For each organism three replicate trials were conducted.

2.6. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration of the extract was estimated for each of the test organisms in triplicates. To 0.5ml of varying concentrations of the extracts (25.0, 12.5, 6.25, 3.17, 1.58, 0.781 and 0.39 mg ml⁻¹) 2ml of nutrient broth was added and then a loopful of the test organism previously diluted, approximately to 106CFU ml⁻¹(for bacterial isolates) and 106spores ml⁻¹ (for fungal isolates), was introduced to the tubes. The procedure was repeated on the test organisms using the standard antibiotics (ciprofloxacin and ofloxacin for bacteria and nystatin and amphotericin B for fungal isolates). A tube containing nutrient broth only was seeded with the test organisms as described above to serve as control. All tubes containing cultures were then incubated at 37°C for 24 h while tubes containing *A. flavus* spore cultures were incubated for 48 h at room temperature (28°C – 32°C). After incubation the tubes were then examined for microbial growth by observing for turbidity. The MIC values were interpreted as the highest dilution of the sample, which showed no growth.

2.7. Determination of minimum microbial concentration (MMC)

To determine the MMC, for each set of test tubes in the MIC determination, a loopful of broth was collected from those tubes which did not show any growth and inoculated on sterile nutrient agar (for bacteria) and Sabouraud's dextrose agar(for fungi) by streaking. Nutrient agar and Sabouraud's agar were only streaked with the test organisms respectively to serve as control. Plates inoculated with bacteria were then incubated at 37°C for 24 hours while those inoculated with fungi were incubated at room temperature (28°C – 32°C) for 48 hours. After incubation the concentration at which no visible growth was seen and noted as the minimum bactericidal concentration.

3. Result

Phytochemical constituent present in the plant extract is alkaloid, mainly vasicine. Results of the antimicrobial

Table 1. Antibacterial activities of methanolic extract of *J. adhatoda*, pure vasicine standard, and reference antibiotic discs

| Name of organisms tested | Zone of inhibition (mm) | | | |
|---------------------------------------|---|---|--|---|
| | methanolic extract of <i>J. adhatoda</i> (25 μ g ml ⁻¹) | Pure vasicine standard (25 μ g ml ⁻¹) | Ofloxacin (25 μ g ml ⁻¹) | Cipro–floxacin (25 μ g ml ⁻¹) |
| <i>Klebsiella pneumoniae</i> | 9.5±0.2 | 10.2±0.2 | 8.8±0.0 | 10.0±0.2 |
| <i>Escherichia coli</i> | 12±0.2 | 12.5±0.2 | 9.1±0.1 | 11.0±0.0 |
| <i>Pseudomonas aeruginosa</i> | 5.0±0.2 | 6.0±0.2 | 2.0±0.0 | 8.0±0.0 |
| <i>Streptococcus pyogenes</i> | 9.0±0.4 | 9.8±0.2 | 7.0±0.1 | 8.2±0.1 |
| <i>Staphylococcus aureus</i> | 12.5±0.3 | 12.8±0.3 | 9.5±0.0 | 10.8±0.2 |
| <i>Serratia marcescens marcescens</i> | 8.9±0.25 | 8.2±0.2 | 7.8±0.1 | 9.0±0.1 |

Table 2.Antifungal activities of methanolic extract of *J. adhatoda*, pure vasicine standard, and reference antibiotic discs

| Name of organisms tested | Zone of inhibition (mm) | | | |
|--------------------------------|--|--|--|--------------------------------------|
| | methanolic extract of <i>J. adhatoda</i> (25 $\mu\text{g ml}^{-1}$) | Pure vasicine standard (25 $\mu\text{g ml}^{-1}$) | Amphotericin B (25 $\mu\text{g ml}^{-1}$) | Nystatin (25 $\mu\text{g ml}^{-1}$) |
| <i>Aspergillus flavus</i> | 9.6 \pm 0.2 | 10.5 \pm 0.2 | 12.0 \pm 0.0 | 14.0 \pm 0.2 |
| <i>Candida albicans</i> | 14.8 \pm 0.2 | 14.2 \pm 0.2 | 11.0 \pm 0.2 | 12.0 \pm 0.2 |
| <i>Cryptococcus neoformans</i> | 10.9 \pm 0.15 | 11.5 \pm 0.2 | 10.0 \pm 0.1 | 11.0 \pm 0.2 |

Table 3.Minimum inhibitory concentration (MIC) and Minimum microbicidal concentration (MMC) of methanolic extracts of *J. adhatoda* L.

| Sl.No | Name of the organisms tested | MIC ($\mu\text{g ml}^{-1}$) | MMC ($\mu\text{g ml}^{-1}$) |
|-------|--------------------------------|-------------------------------|-------------------------------|
| 1 | <i>Klebsiella pneumoniae</i> | 6.25 | 6.25 |
| 2 | <i>Escherichia coli</i> | 3.125 | 6.25 |
| 3 | <i>Pseudomonas aeruginosa</i> | Not Detected | Not Detected |
| 4 | <i>Streptococcus pyogenes</i> | 25 | 25 |
| 5 | <i>Staphylococcus aureus</i> | 12.5 | 25 |
| 6 | <i>Serratia marcescens</i> | 3.125 | 6.25 |
| 7 | <i>Aspergillus flavus</i> | 3.125 | 6.25 |
| 8 | <i>Candida albicans</i> | 12.5 | 25 |
| 9 | <i>Cryptococcus neoformans</i> | 25 | 25 |

activity of the plant extract and standard vasicine along with reference antibiotics are shown in Table 1 and 2. The results showed that the plant extract was effective against the organisms studied.

The antimicrobial activity of plant extract was variable according to various organisms. The inhibition zones ranged between 5 mm to 13.2 mm diameter.

The results obtained in the present study relieved that *J. adhatoda* extract possesses potential antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* and antifungal activity against *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus flavus*. When tested by disc diffusion method, the highest antibacterial activity of 12.5 mm in *Staphylococcus aureus* and least activity recorded in *Pseudomonas aeruginosa*, measured 5 mm.

Antifungal activity of leaf extract showed significant activity when compared with the standard vasicine and standard antibiotics. Among the three tested fungi better antifungal activity of 14.8 mm was obtained against *Candida albicans* and least activity of 9.6 mm against *Aspergillus flavus*. When compared with nystatin and amphotericin B very good antifungal activity obtained against *Candida albicans* and *Cryptococcus neoformans*.

These results were compared with standard antibiotics used, nystatin and amphotericin B and ofloxacin and ciprofloxacin. But the extract showed higher activity than the given standard antibiotic against *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Escherichia coli* and antifungal activity against *Candida albicans* and *Cryptococcus neoformans*.

Results of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) are shown in Table.5.3. The result showed that *Streptococcus pyogenes*

had the highest MIC (25 $\mu\text{g ml}^{-1}$) and MMC (25 $\mu\text{g ml}^{-1}$), while the lowest MIC of 3.125 $\mu\text{g ml}^{-1}$ was shown by *Serratia marcescens* and *Candida albicans* respectively.

4. Discussion

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the in vivo antibacterial activity assay. Many reports are available on the antiviral, antibacterial, antifungal, antihelminthic, antimolluscal and anti-inflammatory properties of plants^[1]. Some of these observations have helped in identifying the active principle responsible for such activities and in the developing drugs for the therapeutic use in human beings.

However, not many reports are available on the exploitation of antifungal or antibacterial property of plants for developing commercial formulations for applications in crop protection.

Emergence of multi drug resistance in human and animal pathogenic bacteria as well as undesirable side effects of certain antibiotics has triggered immense interest in the search for new antimicrobial drugs of plant origin. *Pseudomonas aeruginosa* was the most resistant strain of all the bacteria used in this study. In fact, Gram negative bacteria, especially *P. aeruginosa* are frequently reported to have developed multi drug resistance to many of the antibiotics. Therefore, it is not surprising to learn that *P. aeruginosa* is the least responding bacterial strain to the tested plant extract^[11].

When comparing the antimicrobial activity of the tested samples with the reference antibiotics, the inhibitory potency of tested extracts could mostly be considered as important (Table 1 and 2). This is due to the fact that medicinal plants

are natural origin, which means more safety for consumers, and are considered that they are being low risk for resistance development by pathogenic microorganisms.

The highest MIC and MMC values of *Staphylococcus aureus* is an indication that either the plant extracts are less effective on some gram positive bacteria or that the organism has the potential of developing antibiotic resistance, while the low MIC and MMC values for other bacteria is an indication of the high efficacy of the plant extracts (Table 3).

Present study revealed that the plant extracts inhibited bacterial growth but their effectiveness varied. Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. Continued further exploration of plant derived antimicrobials is needed today. Thus, the study ascertains the value of plants used in Ayurveda, which could be of considerable interest to the development of new drugs.

The demonstration of activity against both Gram-negative and Gram-positive bacteria and fungi is an indication that the plant can be a source of bioactive substances that could be of broad spectrum of activity. Thus the broad spectrum of antibacterial activity by *J. adhatoda* may help to discover new chemical classes of antibiotic substances that could serve as selective agents for infectious disease chemotherapy and control.

Conflict of interest statement

We declare that we have no conflict of interest.

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