Antimicrobial activity of Wedelia chinensis leaves
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ABSTRACT
This study examined the in vitro antibacterial and antifungal activities of various organic solvent extracts (petroleum ether, chloroform, ethyl acetate and methanol) of Wedelia chinensis belonging to the Asteraceae family. The extracts were tested against fifteen (both Gram positive and Gram negative) bacteria and five fungi using disc diffusion method. The susceptibility of the test microbes varied with the types of solvents used. All the extracts showed sufficient inhibitory activity to the test strains. Among the solvents, methanol showed strong antimicrobial activity. Ethyl acetate and chloroform extracts were found to have moderate effect, whereas, petroleum ether extract possessed least activity on the test bacteria and fungi. The most susceptible Gram positive bacterium was Streptococcus faecalis (26.27±0.15mm), while the most susceptible Gram negative bacterium was Pseudomonas aeruginosa (22.47±4.20mm). The Gram positive strain Streptococcus faecalis was more sensitive to the extracts (range 26.27-9.67mm) than the Gram negative strains (range 22.47-9.97mm). The significant antifungal activity was against Candida albicans (23.67±0.88mm) by the methanol extract. The antimicrobial activity of extracts was compared with the standard antimicrobials (Tetracycline for bacteria and Erythromycin for fungal isolates). The results obtained in the present study suggest that the extracts can be used in treating diseases caused by the test organisms. Hence, Wedelia chinensis can be used to discern bioactive natural products and new pharmaceutical molecules that serve in the development of new therapeutic needs.

Keywords: in vitro, disc diffusion, susceptibility and inhibitory.

INTRODUCTION
The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases (1). Bacteria for example have shown a remarkable ability to endure and adapt to their environment including the development of different mechanisms of resistance to most old and new antimicrobial agents (2). Bacterial adaptation to antibiotics has been very successful, and over the years, the increase in antibiotic resistance has generated a considerable worldwide public health problem (3). In addition, high cost and adverse side effects are commonly associated with popular synthetic antibiotics (such as hypersensitivity, allergic reactions, immunosuppression, etc) and are major burning global issues in treating infectious diseases (4). Although pharmacological industries have produced considerable number of commercial antibiotics frequently but resistance in pathogens toward these drugs too has increased at high rate and multi-drug resistant microorganisms have exacerbated the situation (5). This situation forces scientists to search for new antimicrobial substances from various sources. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (6).

Herbal medicines have been developed not only as a way to improve ancient traditional therapeutics, but also as an alternative solution for health problems. In many developing countries, 80% of the available allopathic medicines are obtained from medicinal plants (7). Plants constitute an important source of active natural products which differ widely in terms of structure and therapeutic properties. The continued investigation into the secondary plant metabolites for anti-infective agents has gained importance in recent years because of the alarming increase in resistance of pathogenic microorganisms to existing antibiotics (8). The plants are known to provide a rich source of botanical anthelmintics, antibacterials and insecticides (9).

Many plants have been used because of their antimicrobial traits, which are chiefly synthesized during secondary metabolism of the plant. Therefore, such plants should be investigated to better understand their properties, safety and efficacy (10). Researchers have shown that all different parts of the plants which include stem, root, flower, bark, leaf, etc., possess antimicrobial property (11, 12 and 13).

The screening of plant extracts as antimicrobial agents is necessary to go insight into medicinal flora and get the molecules responsible for this activity and add value to natural resources from tropical areas (14). In recent years, secondary plant metabolites (phytochemicals), previously with unknown pharmacological activities, have been extensively investigated as a source of medicinal agents (15). Thus, it is possible that phytochemicals with adequate antimicrobial efficacy will be used for the treatment of microbial infections (16). The aims of the policy of World Health Organization (WHO) on medicinal plant materials include ascertaining their safety, efficacy and specifications (17). This study is therefore aimed at assessing the effect of the leaves of Wedelia chinensis on the selected bacterial and fungal strains.

Wedelia chinensis is a perennial herb of family Asteraceae, commonly known as “Pilahamagara” or “Bhringraj” in Hindi, Wedelia in Chinese and “Manjalkarisalanganni” in Tamil (18). Traditionally, the plants are used in child birth and in the treatment of bites and stings, fever and infection. The leaves are used in the treatment of kidney dysfunction, cold, wounds and amenorrhea (19). The leaves are also used for dyeing hair and for promoting their growth. The tonic of the leaves is used in cough and cephagalia. Decoction of the leaves is used in menorrhagia and skin diseases (20, 21). The plant has also found its use in inflammations, helminthic diseases and liver disorders (22). The decoction of the plant was extremely used by the tribes in Kolli Hills of Namakkal District, Tamilnadu, India to reduce mental tension and also to induce sleep and the plant affects CNS (23). The plant has been used as astringent, bitter, acrid, anti-inflammatory and cardiotonic, and treatment of wounds, seminal weakness and viral-hepatitis (18, 24). The plant is scientifically reported to possess antioxident property which indicates its usefulness in reducing anxiety and stress in emotional conditions (25).

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MATERIALS AND METHODS
Collection and identification of plant
The leaves of Wedelia chinesis (Family Asteraceae) were collected from Coimbatore District, Tamilnadu, India. The plant was authenticated by Dr. V. Balasubramaniam, Associate Professor, Department of Botany, Kongunadu Arts and Science College, Coimbatore and voucher specimen was prepared and deposited at the Herbaria of Kongunadu Arts and Science College, Coimbatore.

Preparation of Extracts
For extraction about 50g of the shade dried and powdered leaf material was taken. The powdered material was transferred into 250 ml quick fit flask and extracted in the soxhlet extractor for 48 hours (26) using organic solvents namely petroleum ether, chloroform, ethyl acetate and methanol separately according to the increasing polarity of the solvents. The extracts were filtered over Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure to pasty mass (27) for further studies.

Test Microorganisms
The bacterial and fungal strains were obtained from the Department of Mycology, University of Madras, Chennai. The bacterial strains and the fungal strains were maintained in nutrient agar slants and potato dextrose agar slants respectively. Before use, each bacterial and fungal culture was diluted 1:100 with fresh sterile nutrient broth (28).

Gram positive bacteria:
Streptococcus faecalis, Streptococcus pyogenes, Enterococcus faecalis, Bacillus subtilis, Bacillus thuringiensis and Staphylococcus aureus.

Gram negative bacteria:
Serratia marcescens, Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis, Salmonella paratyphi, Salmonella paratyphi A, Salmonella paratyphi B, Pseudomonas aeruginosa and Escherichia coli.

Fungi:
Candida albicans Aspergillus niger, Aspergillus flavus, Curvularia lunata and Trichophyton mentagrophytes.

Disc diffusion method
The antimicrobial activity for different extracts of Wedelia chinesis was determined by the disc diffusion method (29). Both gram positive, gram negative bacterial strains and fungi were used for the test. A survey of the published literature shows that there are a number of different methods used for the assessment of antimicrobial activity; however, there is no one method that is used by all researchers and no inclusive study to determine which one is the best method for in vitro assay (30). Majority of the researchers uses one of the three following methods for the assessment of antimicrobial activity: Disc diffusion, agar dilution, and broth dilution/ microdilution method. The disc diffusion method (also known the zone of inhibition method) (29) is probably the most widely used of all methods used for testing antibacterial and antifungal activity (30). Several researchers have used the method to identify the antibacterial and antifungal activities of the plant extracts (31), compounds isolated from plants (32), and also to find out the antimicrobial resistant strains of microorganisms (33). It is important to note that the disc diffusion method demonstrated activity in vitro does not always translate to activity in vivo (30).

Principle
Disc diffusion method provides a simple and reliable test in routine clinical bacteriology in order to find out the effect of a particular substance on a specific bacterium. This method consists of impregnating small circular discs of standard filter paper with given amount of a chosen concentration of substance. The discs are placed on plates of culture medium previously spread with a bacterial inoculum to be tested. After incubation the degree of sensitivity is determined by measuring the inhibition zone produced by the diffusion of the antibiotic substances from the discs into the surrounding medium.

Procedure
Preparation of Discs
Circular paper discs of 6 mm diameter were prepared from Whatman No.1 filter paper and sterilized in an autoclave. From the plant extract, 100mg of crude extract was dissolved in the respective solvent and was loaded onto the filter paper discs to get 100mg/disc concentration for overnight and allowed to dry at room temperature in laminar air flow chamber (34, 35 and 36).

Experiment
Nutrient agar medium and Potato dextrose agar medium were prepared and the petriplates and the media were sterilized for 20 minutes at 120°C in an autoclave and approximately 20 ml of each of the medium was poured into the sterile petriplates under aseptic condition and allowed to get solidify for 15-20 minutes. It was cooled at room temperature. After cooling, the bacterial culture and fungal culture were taken (24 h old) and using an inoculation needle, the culture was applied on the surface of the respective agar medium in the form of parallel streaks using cotton swabs. The prepared discs containing the test material (leaf extract) were impregnated on the agar medium with framed forceps and gently pressed down to ensure complete contact of the disc with the agar surface uniformly seeded with the test microorganisms and the plates were incubated at 37°C for 24 h. Control plates without the plant extract i.e. a standard antibiotic Tetracycline (10µg/disc) as positive control and a negative control with only the solvent used for extraction (37, 16) were also maintained for reference (38) to determine the sensitivity of the tested microbial strains (39). There was a gradual change in concentration in the media surrounding discs. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out three times and the mean of the reading is required to ensure the reliability of the result. All tests were performed in triplicate (40).

Statistical analysis
The data were reported as Mean ± Standard deviation (n=3). For determining the statistical significance, standard error, mean and analysis of variance (ANOVA) at 5% level significance was employed. All the data were subjected to Duncans Multiple Range Test (DMRT) done by using the SPSS version 2007 WINSAT software. The P values < 0.05 were considered as significant (41).

RESULTS
Antimicrobial activity of the various solvent extracts (petroleum ether, chloroform, ethyl acetate and methanol) of leaves of Wedelia chinesis has been evaluated in vitro against fifteen bacterial and five fungal species. The results are presented in Tables 1 and 2. All the extracts used showed activities against both the bacterial and fungal isolates tested in the study. The spectrum of activity observed in the present study may be an indicative of the presence of broad spectrum antimicrobial compounds in the extracts.

Antibacterial activity
Among the extracts tested, the methanol extract was found to be more effective on the Gram positive bacterium Streptococcus faecalis with the zone of inhibition (26.27±0.15mm) followed by Enterococcus faecalis (25.27±0.15mm) (Table 1). Streptococcus pyogenes and Staphylococcus aureus were equally inhibited by the methanol extract. Moderate inhibitory zone of methanol extract was against the Gram negative bacteria Pseudomonas aeruginosa (22.47±4.20mm), Escherichia coli (21.51±0.23mm) and Salmonella paratyphi B (20.4±0.21mm) respectively. The ethyl acetate extract showed moderate activity against Streptococcus faecalis with 19.67±0.88mm as zone of inhibition. The gram negative bac-
Antibacterial activity of *Wedelia chinensis* leaves

<table>
<thead>
<tr>
<th>S.No</th>
<th>Bacterial organisms</th>
<th>Zone of inhibition (mm)</th>
<th>Control (Tetracycline)</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Methanol</th>
</tr>
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<tr>
<td>1</td>
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<td>Enterococcus faecalis</td>
<td>19.3±0.17</td>
<td>10.13±0.15</td>
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<td>25.27±0.15</td>
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<td>Bacillus subtilis</td>
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<td>6</td>
<td>Staphylococcus aureus</td>
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<td>7</td>
<td>Serratia marcescens</td>
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<td>8</td>
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<td>9</td>
<td>Proteus vulgaris</td>
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<td>Pseudomonas aeruginosa</td>
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<td>8.37±0.15</td>
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<td>16.4±0.23</td>
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<td>15</td>
<td>Escherichia coli</td>
<td>25.52±0.10</td>
<td>9.03±0.24</td>
<td>12.47±0.22</td>
<td>16.53±0.20</td>
<td>21.51±0.23</td>
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</tbody>
</table>

Values are expressed as Mean ± Standard Error of 3 replicates. Means within a column followed by a common letter are not significantly different at 5% level by DMRT.

Antifungal activity of *Wedelia chinensis* leaves

<table>
<thead>
<tr>
<th>S.No</th>
<th>Fungal organisms (Zone of inhibition in mm)</th>
<th>Control (Erythromycin)</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Candida albicans</td>
<td>18.33±0.33</td>
<td>8.67±0.33</td>
<td>12±0.57</td>
<td>17±1.15</td>
<td>23.67±0.88</td>
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<td>2</td>
<td>Aspergillus niger</td>
<td>21.33±0.88</td>
<td>10.67±0.33</td>
<td>13.67±0.88</td>
<td>18.67±0.88</td>
<td>19.67±0.88</td>
</tr>
<tr>
<td>3</td>
<td>Aspergillus flavus</td>
<td>22±1.15</td>
<td>9±0.57</td>
<td>11.6±0.88</td>
<td>15.33±0.88</td>
<td>19.33±0.88</td>
</tr>
<tr>
<td>4</td>
<td>Curvularia lunata</td>
<td>17±1.15</td>
<td>9.67±0.33</td>
<td>12.66±1.20</td>
<td>13±0.57</td>
<td>20.67±1.76</td>
</tr>
<tr>
<td>5</td>
<td>Trichophyton mentagrophytes</td>
<td>16.5±1.23</td>
<td>10.33±0.27</td>
<td>12.13±0.17</td>
<td>14.46±0.29</td>
<td>18.6±0.30</td>
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</table>

Values are expressed as Mean ± Standard Error of 3 replicates. Means within a column followed by a common letter are not significantly different at 5% level by DMRT.

DISCUSSION

Plants have been a veritable source of drugs. However, man tends to ignore the importance of herbal medicine. Medicinal plants have formed the basis of health care throughout the world since the earliest days of humanity and are still widely used and have considerable importance in international trade (42). The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these biactive compounds of plants are alkaloids, flavonoids, tannins and phenolic compounds (43).

The antimicrobial activities of *Wedelia chinensis* extracts were tested against fifteen bacterial and five fungal strains. The study showed that extracts from *Wedelia chinensis* have promising antibacterial and antifungal activities and this is probably why the plant is widely used in traditional medicine. Among the extracts, the methanol extract was found to have a more potent inhibitory effect followed by ethyl acetate while chloroform extract had moderate effect and petroleum ether extract showed least activity. Several investigations had reported that plants contain antimicrobial substances (44, 45, 46, 47 and 48). The results of the present study agree essentially with the reports of these previous workers.

The activity of the plant against both Gram positive and Gram negative bacteria and some fungal strains may be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins in the plant. Generally Gram negative bacteria are more resistant than Gram positive bacteria (49, 50 and 51). Gram-positive bacterial strains were more susceptible to the extracts when compared to gram negative bacteria. This
may be attributed to the fact that these two groups differ in their structure of the cell wall components. The ability of tannin compounds to cause the bacterial colonies to disintegrate, probably results from their interference with the bacterial cell wall (52). Majority of plant extracts have been reported to be more active against Gram-positive bacteria than the Gram-negative bacteria strains (53, 54 and 55).

Generally, the methanol extract was more active than other extracts. This may be attributed to the presence of soluble phenolic and polyphenolic compounds (56). Methanol extract was previously reported inhibitory to B. subtilis, P. aeruginosa, and S. aureus but not E. coli (57). Recent research activities on antibacterial activities of crude extracts have implicated the methanol extract for being more active than the other solvents extracts (58, 50, 54 and 55). It will seem likely that the solvent is suitable for the extraction of antibacterial compounds from crude extracts.

The antimicrobial properties exhibited by the extracts may be associated with the presence of tannins, saponins, cardiac glycosides and alkaloids found in the plant extracts. A large number of flavonoids have been reported to possess antimicrobial properties (59, 60, 61 and 62). Several studies have shown that tannins, saponins, flavonoids and phenolic compounds possess antimicrobial activities (63, 64). It is known that due to the presence of several phytochemical constituents like alkaloids, flavonoids, glycosides, saponins, tannins, steroids etc., the plant extract generally show antibacterial property (65). The antibacterial activity of phytochemicals such as terpenoids (66), saponin (67), tannin (68) and cardiology (69) isolated from plant materials have been reported. Sharma et al., (70) have observed that the leaves extract containing mainly flavonoids, tannins and alkaloids showed significant activity against microbes causing candidosis of mouth, vagina and alimentary tract. Previous Phytochemical studies of some fungicidal plants have shown the presence of tannins, alkaloids, flavonoids and saponins (71, 72, 73, 74, 75, 76, 77 and 78).

The antimicrobial activity of medicinal plants and drugs varies in their inhibitory effect, depending on the concentration of crude extracts or synthetic drug, size of inoculums, temperature, nature of organism, and rate of diffusion (79). The extracts were found to be active against Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus flavus and Trichophyton mentagrophytes. All these pathogens are known to cause several diseases and infections in humans. For instance, Staphylococcus aureus and Pseudomonas aeruginosa are most common pathogens causing serious infections (80); and Escherichia coli is an opportunist pathogen at the site of cut wound. Staphylococcus aureus and Pseudomonas aeruginosa are most common pathogens which infect the skin (81, 82). Staphylococcus aureus express surface proteins that promote attachment to host proteins that form part of the extra cellular matrix on epithelial and endothelial cell surfaces as well as being a component of blood clots (83, 84).

Bacteria of the genus Klebsiella and Enterobacter spp. are among the major causes of noso-comial infections. They often give rise to urinary and respiratory tract infections, and next to Escherichia coli, they are the most common cause of Gram-negative bac-teremia (85). Enterobacter and Klebsiella spp. are frequently described in resistant nosocomial infections (86, 87). Escherichia coli and Staphylococcus aureus are two of the most important pathogens causing serious infections (88) and can require antimicrobial resistance to prevent disease treatment effect (89). Candida species have been reported to interfere with human reproductive system leading to several disorders (90, 91 and 92). The fungal pathogen Candida albicans causes chronic infection of lungs, ear, and bones and Aspergillus flavus causes infection of joint, skin and central nervous system (93).

CONCLUSION
Based on these results, it can be concluded that plant extracts have a great potential antimicrobial compounds against microorganisms that can be used in treatment of infectious diseases caused by a range of resistant microorganisms. The study provides support for the use of these plants in the management of infectious diseases. The findings can form the basis for further studies to prepare an optimize preparation of the herbal extract to further evaluate them against a wide range of microbial strains.

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