Antibacterial and antifungal analysis of crude extracts from the leaves of *Callistemon viminalis*.

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**Abstract**

Antimicrobial resistance is a major cause of significant morbidity and mortality globally. Ethnomedicine provides avenues for identification of compounds with antimicrobial properties and potential new antibiotics. *Callistemon viminalis* is an ethnomedicinal plant used in Jamaica to treat intestinal illnesses. Methanol, hexane and aqueous extracts of *Callistemon viminalis* were tested against eight common bacteria and a single fungus of medical importance using a quantitative agar well diffusion test and tube dilution assay. All plant extracts showed antimicrobial activities against the selected microorganisms; the methanol extracts were most effective. The aqueous extract on the other hand, was very effective as a bactericidal agent against the intestinal pathogens. These results support the ethnomedicinal claim that *C. viminalis* is an effective treatment for bacterial causes of intestinal illnesses.

**Introduction**

Jamaica has a rich history in the use of diverse medicinal flora for traditional healing. These medicinal plants are generally used to treat various medical conditions including skin infections, stomachaches and respiratory conditions (Morton, 1981; Melendez, 1982; Duke, 1985). Scientific research on the antimicrobial efficacy of many of the plants used for medicinal purposes in Jamaica is lacking. The presence of active antimicrobial compounds in plants represents a useful area for development of natural products that can be used as substitutes for antibiotics resistant to pathogenic bacteria and fungi. Furthermore, they provide the foundation for the development of new antimicrobials. The study will also confirm if there is a biological basis to the claim that the ethnomedicinal plant has useful medicinal purposes (Cowan, 1999).

Native and introduced plants have been used in Jamaica for medicinal purposes for several generations (Mitchell and Ahmad, 2006). *Callistemon viminalis* is native to New South Wales, Australia (Elliot, 1982); however, since its introduction to Jamaica as a small ornamental tree referred to locally as 'bottlebrush', it has been used to prepare a hot drink locally referred to as “tea” for the treatment of gastro-enteritis, diarrhea and skin infections (Cowan, 1999). There has been a marked decrease in the development of new antimicrobial agents by major pharmaceutical companies over the past decade or so. This is cause for great concern as there has been a marked increase in the development of resistance to the existing antimicrobial agents by microbes. Despite the fact that plants represent a potential source of new antibiotics, no work has been conducted on the antimicrobial properties of *C. viminalis* grown in Jamaica.

Methicillin resistant *Staphylococcus aureus* (MRSA) has become a major focus in the medical and pharmaceutical industry. It is a common cause of hospital acquired infection and has become resistant to a wide range of antimicrobial agents making it one of the more difficult pathogens to treat with conventional antibiotics (Braun et al, 2004; Gold and Moellering, 1996). *P. aeruginosa* is also of concern as this pathogen causes infections that are difficult to treat due to multi-drug resistance (CDC NNIS, 1999). Similarly the prevalence of Shiga toxin-producing *Escherichia coli* (STEC), *Shigella sonnei* and *Salmonella enteritidis* have been alarming in hospitalized diarrheal patients. Thus the need to find alternative source of treatment for these infections is necessary (Boerlin, 1999; Paton, 1998).

This study was designed to determine the antimicrobial activity of one of Jamaica’s ethno-medicinal plants, *C. viminalis*, against some intestinal pathogens (*Escherichia coli*, *Salmonella enteritidis*, *Shigella sonnei* and *Bacillus cereus*), skin pathogens (*Staphylococcus aureus* and *Streptococcus pyogenes*), and two other commonly encountered nosocomial pathogens (*Pseudomonas aeruginosa* and *Candida albicans*).
Materials and Methods

Collection of plant materials

Leaves of *C. viminalis* were collected from the Mona Campus of The University of the West Indies, in Kingston, Jamaica. The identity of the plant was confirmed by the taxonomist in the herbarium of the Department of Life Sciences at the University. A voucher specimen [number 35337] of *C. viminalis* was preserved in the herbarium.

Selection of bacterial and fungal strains

The test bacteria used were *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteriditis*, *Shigella sonnei*, *Bacillus cereus* ATCC 6633, *Staphylococcus aureus* and *Streptococcus pyogenes* and the fungus *Candida albicans*. These organisms were chosen because they are commonly isolated pathogens from hospitalized patient with intestinal ailments, blood and skin infections. All microorganisms were obtained from the Department of Microbiology, University of the West Indies Mona, Jamaica.

Preparation of plant extract

The leaves were washed and dried at room temperature for nine days and then crushed into coarse powder using a grinder. The powder was used for the methanol, hexane and distilled water extractions at 67, 66 and 92 °C respectively; 20 g of powder was added to a thimble and then placed in a Soxhlet extractor. Heat was applied to a round bottom flask which was placed at the base of the Soxhlet extractor. The process was continued for 18 hours. The extracts were then placed on rotary evaporators at 67 and 92 °C respectively to remove the methanol/hexane and water. A sample of 0.1 g of the dried leaf extract was dissolved in 10 ml of water and used to make two fold serial dilutions, to give 10 extract concentrations (100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4 and 0.2 mg/ml). These were used as the extracts in the microbial test (Barriada-Pereira, 2003).

Media used

Muller Hinton broth was used as the primary medium for the tube dilution to determine the minimal inhibitory concentration (MIC) for each test microorganism (NCCLS, 2000). Muller Hinton agar plates were used to plate out agar for the well diffusion assay.

Preparation of Bacterial and Fungal Suspension

A sterile wire loop was used to place the test bacteria or fungi into a test tube with peptone water over an open flame. The concentration of the inoculum was 0.5 McFarland’s standards (ca. $10^8$ CFU/ml) (Baker et al, 1983).

Tube dilution Assay

The MIC values of *C. viminalis* extracts were determined using two-fold broth micro-dilution to prepare extract concentrations of 100, 50, 25, 12.5, 6.3, 3.2, 1.6, 0.8, 0.4, and 0.2 mg/ml; 1 ml of each extract was added to test tubes containing 1 ml of sterile MH media. The tubes were then inoculated with a drop of microbial suspension and incubated at 37 °C for 24 h. Amphotericin B (0.05 mg/ml) and tetracycline (0.05 mg/ml) were used as positive controls for the fungus and bacteria, respectively, except for methicillin resistant *Staphylococcus aureus* (MRSA) where vancomycin (0.05 mg/ml) was used and *Pseudomonas aeruginosa* where gentamicin (0.05 mg/ml) was used. Water was used as the negative control. The MIC value was determined macroscopically after 24 hr of incubation in comparison with the growth and sterility controls (Demarsh et al, 2001). MH plates were divided into six different sections and labeled with the different concentrations on the base of the plates; these were used to plate out the contents of each tube in the respective sections of the plates. The plates were incubated for 18-24 hr at 37 °C, after which the MBC were recorded. Six replicates were done for each extract concentration and controls against the bacteria and three replicates for the fungi (NCCLS, 2000).

Well Diffusion Assay

Antimicrobial susceptibility testing was done using the well diffusion method to detect the presence of anti-bacterial or anti-fungal activities of the plant samples (Perez et al., 1990). A sterile swab was used to evenly distribute bacterial or fungal culture over the appropriate medium as stated previously. The plates were allowed to dry for 15 minutes before use in the test. Wells were then created and a pipette was used to place 30 ul of the crude extract of *C. viminalis*.
*viminalis* into each well. The same extract was used on each plate; with a total of two plates used for each extract including two wells for the positive and negative controls. The negative and positive controls were the same as used in the tube dilution assay. The plates were incubated at 37 °C for 24 hours after which they were examined for inhibition zones. A caliper was used to measure the inhibition zones. Twelve replicates were done for each concentration of the different extracts, and each experiment was repeated six times to ensure reliability.

**Results**

The results illustrated in Tables 1 and 2, indicated that all three crude extracts from the leaves of *Callistemon viminalis* showed antibacterial and antifungal activity against both the Gram-positive (*Bacillus cereus*, *Staphylococcus aureus*, MRSA, and *Streptococcus pyogenes*), Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, and *Shigella sonnei*) and the fungus *Candida albicans*. However, the three crude extracts of *C. viminalis* were more potent antibacterial agents against the Gram-positive bacteria and fungus (MBCs of 0.8 – 6.3 mg/ml) than the Gram-negative bacteria (MBCs of 6.3 – 50 mg/ml) (Table 1). For each test microbe, the aqueous or methanol extract produced a lower MIC and MBC than the corresponding hexane extract.

**Table 1. MIC and MBC of Crude Aqueous, Hexane and Methanol leaf Extract of C. viminalis using the tube dilution assay**

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Average MIC / MBC of the Extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>12.5</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>12.5</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>6.3</td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>6.3</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>1.6</td>
</tr>
<tr>
<td>MRSA</td>
<td>1.6</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>1.6</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>3.2</td>
</tr>
</tbody>
</table>

(-) no growth observed, (+) growth observed, * MBC and n=18

The antimicrobial sensitivity of the crude extracts and their potency were assessed quantitatively by determining the MIC, MBC and zones of inhibition, respectively as given in Tables 1 and 2. In this study, the growth of MRSA was remarkably inhibited by the aqueous, methanol and hexane extracts giving an MIC of 1.6, 0.8 and 1.6 mg/ml respectively by the tube dilution method; and maximum inhibition zone as high as 31, 29 and 35 mm respectively by the well diffusion method. The methanol extract’s average zone of inhibition for MRSA [25.61± 2.11mm] was larger than that of non-methicillin resistant *S. aureus* [17.41±1.10 mm] (Fig i & ii). The growth of *P. aeruginosa* was also inhibited but to a lesser extent by the methanol extract, with MIC of 12.5 mg/ml and inhibition zones as high as 14 mm (Table 2). This is in comparison to the control Gentamicin (one of the commonly used anti-pseudomonal drugs), which produced inhibition zones of 10.1±0.15 mm.

Fig i and ii Aqueous leaf extract inhibition of growth of (i) *Staphylococcus aureus* and (ii) Methicillin Resistant *S. aureus* (MRSA) via well diffusion assay.
Table 2. Antibacterial and Antifungal Activity of Aqueous and Organic Extracts of *Callistemon viminalis* using the well diffusion method

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aqueous Extract</td>
</tr>
<tr>
<td></td>
<td>Aqueous</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>+++</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>+++</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>+</td>
</tr>
<tr>
<td><em>Salmonella enteritidis</em></td>
<td>+++</td>
</tr>
<tr>
<td><em>Shigella sonnei</em></td>
<td>+++</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>+++</td>
</tr>
<tr>
<td>MRSA</td>
<td>+++</td>
</tr>
<tr>
<td><em>Streptococcus pyogenes</em></td>
<td>+++</td>
</tr>
<tr>
<td>FUNGUS</td>
<td>++</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>+</td>
</tr>
</tbody>
</table>

(-) no activity, n=18

The diameter of the inhibition zone was scored as + (7-10mm), ++ (11-15mm) and +++ (16-20mm).

The results of the well diffusion assay corroborate the results of the tube diffusion. For the Gram-negative bacteria, (*Bacillus cereus*, *Staphylococcus aureus*, MRSA, and *Streptococcus pyogenes*) zones of inhibition were all above 15 mm for all three extracts indicating their high antibacterial potency.

For the Gram-positive bacteria *Escherichia coli*, *Salmonella enteritidis*, and *Shigella sonnei*, the aqueous extracts produced the strongest antibacterial activity with lower MBC (6.3 – 12.5 mg/ml) and larger zones of inhibition (>15 mm) than those produced by the other two extracts while for *Pseudomonas aeruginosa* the methanol extract was the strongest (MBC 12.5 mg/ml, 11-15 mm zone of inhibition) (Tables 1 and 2). The aqueous extract was more effective against the gastro-intestinal pathogens (*S. enteritidis*, *S. sonnei*) than the methanol extracts (Tables 1 and 2).

All three crude extracts were effective at inhibiting growth of the fungus *C. albicans* producing zones of inhibition of >15mm for the methanol and hexane extracts and the lowest MIC and MBC's of 1.6 and 3.2 mg/ml respectively for the methanol crude extract.

**Discussion**

Antibiotics were medical miracles during the Second World War but are now becoming impotent bacterial weaponry. This has caused an urgent need for the search of new and innovative ways to control bacterial invasions especially by multi-resistant pathogens such as *S. aureus* (MRSA) and *P. aeruginosa* (Lewis, 1995). Natural alternative treatments for bacterial infections may provide a pathway for the development of new antimicrobial agents.

This study indicated that all three extracts (aqueous, methanol and hexane extracts) of *Callistemon viminalis* were more potent against the Gram-positive (MBCs of 0.8 – 6.3 mg/ml) than the Gram-negative bacteria (MBCs of 6.3 – 50 mg/ml) (Table 1). These results are similar and consistent with reports on related plant extracts which reported inhibition zone sizes varying from 7 mm for *Pseudomonas aeruginosa* to 33 mm for *Staphylococcus aureus* while the MIC ranged from 0.1 mg/ml for *S. aureus* to over 200 mg/ml for *E. coli* (Mansouri et al 2008; Akinyemi et al, 2005;
Ndakwe et al, 2007; Mbata et al, 2006; Goyal et al, 2008; Dewanjee, 2007). In general, the Gram-negative bacteria have shown less sensitivity to plant extracts possibly as a result of their extra lipopolysaccharide and protein cell wall that provides a permeability barrier to the antibacterial agent (Adwan et al, 1998).

This study emphasizes that the medicinal plant C. viminalis, commonly used in Jamaica and other regions of the world, is active against hospital strains of MRSA, Bacillus cereus, Staphylococcus aureus, Streptococcus pyogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella enteritidis, Shigella sonnei, and Candida albicans. Especially important is its high bioactivity against MRSA, a bacterium resistant to the methicillin antibiotic. The reason for the larger zone of inhibition to MRSA compared to the non-methicillin resistant S. aureus strains used is not known. However, this finding was consistent on repeated experiments and warrants further investigation.

Inhibition zone sizes that were obtained that support the findings of the well diffusion method. The MBC values were in most cases higher than the MIC values, suggesting that the plant’s crude extracts were bactericidal at high concentrations and bacteriostatic at low concentrations. It can be deduced that C. viminalis crude extracts are strong antimicrobial agents when compared to other medicinal plants. Previous work conducted by Oyedeji et al (2009) on the composition and bioactivity of the essential oil of C. viminalis demonstrated remarkable antimicrobial activity.

Overall, the test pathogens were more sensitive to the methanol and aqueous extracts than to the hexane extract. This suggests that some of the active compounds in the crude extracts are polar and thus dissolved readily in the methanol and water while the hexane extract may have dissolved out non-polar compounds that possess less antimicrobial activity. Previous studies have noted alcohols to be reliable and consistent solvents for the extraction of antimicrobial substances from medicinal plants (Ahmad et al, 1998).

The hexane extracts may contain non-polar compounds that inhibit the growth of skin pathogens (S. aureus, S. pyogenes and the enteric B. cereus), but have less effect on the growth of intestinal pathogens (S. sonnei, S. enteritidis, and E. coli). Although compounds in the hexane extract showed little activity against intestinal pathogens and may seem insignificant, they might have novel bioactive phytochemical compounds present in low concentration. These same compounds on further purification might demonstrate increased antimicrobial activity. Thus, since activity was demonstrated in all extractions, they indicate a potential source of antimicrobial agent and should be studied further.

The potency of C. viminalis is attributed to the action of the phytochemical/ secondary compounds it contains (Balandrin et al, 1985). These secondary compounds are secreted by plants naturally, in response to environmental pressure or as a defense mechanism to animal attacks or plant diseases. Further studies will determine what compounds are active in the various extracts. However; the essential oil of C. viminalis may be a target for investigations since it has been shown to contain compounds with bioactivity (Oyedeji et al 2009). The bioactive compounds may not be limited to those already identified in the essential oils.

Secondary compounds that would normally be extracted in the polar extracts included alkaloids, flavonoids and some phenols while non-polar secondary compounds may include tannins, terpenes, and quinines. The essential oil studies by Srivastava et al, (2002) and Oyedeji et al (2009) showed that the main phytochemicals in the essential oil from C. viminalis were 1,8 cineole (61%), a-pinene (24%), and menthyl acetate (5.3%). These three compounds are all non-polar terpenes and could therefore possibly be present in the hexane extract.

In this study, water was used to dissolve the crude extracts to confirm or refute the ethnomedicinal claim that C. viminalis has medicinal properties when prepared as a hot drink. This study supports the traditional claim by native Jamaicans that a C. viminalis hot water extract may have medicinal practices through antimicrobial action. Furthermore this study also indicates that the extracts using polar solvents (water and methanol) were more effective than those using hexane as the solvent, supporting the use of “teas” to treat intestinal illnesses.

References


