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Antimicrobial Effects of Leaves of *Eucalyptus camaldulensis* on Some Microbial Pathogens

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Authors' contributions

This work was carried out in collaboration between all authors. Authors AC and AIO designed the study and wrote the protocol. All authors managed the analyses of the study and literature searches. Authors AC and MN drafted the first manuscript. Author GAO drafted the second manuscript. Authors AC and GAO read and reviewed the first and second drafts of the manuscript. All authors approved the final manuscript.

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ABSTRACT

Background: Plants are important sources of medicinal materials and have been in use since ancient times. Although numerous plants have been explored for their medicinal properties, there still remains much to be studied.
Aim: To evaluate the phytochemical constituents of *Eucalyptus camaldulensis* extracted using ethanol, methanol and petroleum ether and the antimicrobial activity of the leaf extracts.
Place and Duration of Study: The study was conducted in Jos, Plateau State, Nigeria over a period of 18 months.
Methodology: Methanol, ethanol and petroleum ether extracts of *E. camaldulensis* leaves were obtained and phytochemical constituents were determined following standard procedures. The antimicrobial effect of the extracts obtained was tested against three bacterial species including *Bacillus subtilis, Escherichia coli*, *Pseudomonas aeruginosa* and two fungi namely *Penicillium expansum* and *Candida albicans* using the agar well diffusion method, at concentrations of 400,

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200, 100 and 50 mg/ml. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also determined.
Results: The phytochemical analysis of the leaf extracts revealed that they contained alkaloids, tannins, flavonoids, saponins, carbohydrates, steroids and cardiac glycosides. All the test organisms (both bacteria and fungi) were inhibited by each of the extracts at concentrations of 400, 200, 100 and 50 mg/ml with variations. The highest concentration (400 mg/ml) of ethanol extract showed the highest inhibition for *C. albicans* (27 mm) and *B. subtilis* (27 mm), while those of methanol and petroleum ether were most effective against *C. albicans* (27.7 mm) and *B. subtilis* (27 mm). The MIC ranged between 50 mg/ml and 200 mg/ml, while MBC and MFC ranged between 100 mg/ml and 400 mg/ml for all extracts. The inhibitory effects of the plant extracts were comparable to that of commercially available antibiotics used as controls.
Conclusion: The leaf extract of *E. camaldulensis* could be a better option in the treatment of infections caused by test organisms studied if properly processed and harnessed.

Keywords: Antimicrobial; phytochemical; Eucalyptus camaldulensis; agar well diffusion; inhibition.

1. INTRODUCTION

Plants have been known from the inception of time to be an important source of medicine, with such plants commonly referred to as “medicinal plants”. The plant kingdom being diverse, consists of a variety of plants which are of value in the treatment of various infections and diseases [1,2]. While many of these plants have been discovered overtime, a lot more are yet to be discovered [3].

Medicinal plants as a group are believed to consist of approximately 800 species, and thus account for 50% of all higher flowering plants. These are further divided into 386 families and 2,200 genera respectively. Traditional or herbal medicine is said to be an age long practice which has developed overtime, with man being able to distinguish plants which are useful as medicine and those which are not [4].

The importance or usefulness of plants as medicine cannot be overemphasized. However, complications have been known to arise from indiscriminate use of medicinal plants [5]. Similarly, deaths have been reported from mistaking poisonous plants for medicinal plants as a result of scanty knowledge about the plants in question. Furthermore, medicinal plants collected in the wild may be contaminated by other species or plant part through misidentification, accidental contamination or internal adulteration, all of which may have unsafe consequences [5]. There is therefore, need for research on these plants and to experimentally analyse and evaluate them so that findings are properly documented, so as to aid appreciation and conservation of the plants. This will also help in promoting the pharmaceutical industry and preserving valuable information that could be lost to non-documentation. The chemical evaluation of herbal medicine has made it possible to transform traditional medicine from an almost invisible trade into modern industrial enterprises capable of making significant contribution to both healthcare delivery and the economic growth in developing countries.

The antimicrobial properties of medicinal plants are reported to be as a result of the production of certain secondary metabolites which vary from plant to plant [6] and these could be determined through phytochemical analysis. Many chemical compounds with medicinal value have been isolated from plants and could be further classified based on the organisms which they are active against.

The study plant *Eucalyptus camaldulensis*, though found in many parts of the world is native to Australia. The tree which can grow up to 45 meters tall has smooth bark, ranging in colour from white and gray to red-brown and with a dense crown of leaves. The base of the bole can also be covered with rough reddish brown bark [7]. The juvenile and adult leaves are stalked with a broad base tapering to the tip with the adult leaf having a dull blue–green colour. The leaf also contains several oil-producing glands in its un-veined areas [8]. Due to its natural adaptation to both temperate and tropical climate, river red gum is the most widely planted species in arid and semi-arid region around the world, and primarily in timber plantations [9].

E. camaldulensis has been historically reported in folklores to be an anaesthetic, antiseptic, astringent folk remedy for cold, colic, cough,

diarrhoea, dysentery, haemorrhage, laryngalgia and laryngitis. However, there has not been much work done on the efficacy of the plant in this part of the country. It is estimated that 80% of the world population presently rely on herbal medicine for some aspect of primary healthcare [10]. Also considering the high cost and side effects associated with synthetic drugs in the developing world, it is necessary to find cheaper and safer alternatives of treating infections. Thus, this study is aimed at evaluating the phytochemical constituents of *E. camaldulensis* extracted using ethanol, methanol and petroleum ether and the antimicrobial activity of the leaf extracts.

2. MATERIALS AND METHODS

2.1 Plant Identification and Collection

Eucalyptus camaldulensis leaves were collected from Sheda Science and Technology complex Abuja, Nigeria and authenticated at the biotechnology advanced laboratory. The leaves were placed in clean polythene bags and taken to the laboratory for analysis.

2.2 Preparation of Leaf Extracts

The modified method of [11] was used to obtain the plant extracts. The leaves were dried under shade for two weeks after which they were pulverized using a laboratory blender to obtain a powder. A hundred grams (100 g) of the powdered leaves was measured into a 500 ml conical flask to which 300 ml of 70% ethanol was added and covered with foil paper. It was incubated for 48 hours in a shaker incubator to ensure that a homogenous solution was obtained. The solution was filtered afterwards and the filtrate collected. A hundred millilitres (100 ml) of ethanol was further added to the residue and allowed to stand for 24 hours, after which it was filtered and the chaff discarded. The filtrates were pooled together and evaporated in a water bath at 40℃ to obtain the crude extract with a percentage yield of 18%. The extract was appropriately labelled and stored in a refrigerator at 4℃. The same process was carried out to obtain the crude extracts using methanol and petroleum ether with their percentage yield recorded as 16.5% and 10.6 respectively. Subsequently, to produce different concentrations of the various extracts for antimicrobial sensitivity testing, four grams (4 g) of the respective extract was dissolved in 10 ml of sterile distilled water in a test tube to obtain a stock solution of 400 mg/ml. Serial dilution was carried out to obtain 200 mg/ml, 100 mg/ml and 50 mg/ml in each case. These were kept in a refrigerator at 4℃.

2.3 Phytochemical Analysis

Phytochemical screening of the extracts was done according to standard procedures. The tests included those for alkaloids, saponins, tannins and flavonoids, glycosides and carbohydrates [12-13]. Other tests included Borntrager's test for anthraquinones, Salkowski test for steroidal ring [14], and Kellar Killiani test for cardiac glycosides.

2.4 Collection and Standardization of Microorganisms

Test organisms were clinical isolates of *Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Penicillium expansum* and *Candida albicans*, obtained from the Medical Microbiology laboratory of the Jos University Teaching Hospital (JUTH), Plateau state, Nigeria. A modified method of [15] was employed for the standardization of the test microorganisms. A loopful of the pure isolates was dispensed from broth culture into 20 ml of sterile nutrient broth in McCartney bottles which were then incubated at 37℃ for 24 hours to standardize the culture to 10^{-6}cfu/ml (McFarland standard). A loopful of the standardized culture was used for sensitivity testing.

2.5 Antimicrobial Sensitivity Test

The sensitivity of the test organisms to the various extracts was determined using the agar well diffusion method as described by [16]. Nutrient agar plates were prepared in triplicates for each of the test organisms and labelled appropriately. A volume of 0.1 ml of each standardized (McFarland standard) test organism was inoculated into separate plates using the spread plate method. A sterile cork borer of 3 mm in diameter was used to bore 5 equidistant holes on the surface of the plate with one at the centre. One-tenth of a millilitre (0.1 ml) of each extract concentration was introduced into the four peripheral holes while the hole in the middle had 0.1 ml of gentamycin (40 mg/ml) to serve as control. After leaving the plates for an hour to allow for diffusion of the extracts through the medium, they were incubated at 37℃ for 24

hours. For each triplicate culture plate, the inhibition zone around each well was measured and the mean value obtained and recorded in millimetres (mm). The same procedure was repeated for the antifungal sensitivity test using Sabouraud Dextrose Agar (SDA) medium and Itraconazole (40 mg/ml) as control.

2.6 Minimum Inhibitory Concentration (MIC)

The MIC was determined according to a procedure described by [16]. Double serial dilution of the extract was performed to obtain concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml and 6.25 mg/ml respectively. Nutrient broth was inoculated with a standardized culture of each test organism in McCartney bottles and incubated for 7 hours at 37℃. The broth was introduced into test tubes containing different concentrations of the leaf extracts and examined for turbidity after 24 hours of incubation at 37℃. The lowest concentration at which microbial growth was not observed, was recorded as the MIC for the respective extracts.

2.7 Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC)

The Minimum bactericidal concentration and Minimum fungicidal concentration were determined by sub-culturing the contents of the test tubes that showed no visible growth on nutrient agar and sabouraud dextrose agar respectively. These were incubated for 24 hours at 37℃, after which the MBC and MFC was recorded as the lowest concentration at which no further microbial colony growth was observed.

3. RESULTS AND DISCUSSION

The phytochemical analysis of the ethanol, methanol and petroleum ether leaf extracts of *E. camaldulensis* showed that flavonoids, saponins, tannins, carbohydrates and cardiac glycosides were present in all the three extracts (Table 1). This is in consonance with earlier works by researchers [17-19]. Alkaloids were absent only in the methanol extract. Anthraquinone was found to be absent in all of the extracts. Saponins, tannins, flavonoids and steroids were higher in the ethanol extracts than in methanol and petroleum ether extracts, while saponins, tannins, flavonoids, carbohydrates and cardiac glycosides were least in petroleum ether extracts. Steroids were also high in methanol extract, with a moderate presence of saponins, tannins, flavonoids, carbohydrates and cardiac glycosides. These differences could be due to varying extraction ability of the solvents used for the extraction of the bioactive components in the plant's leaf.

The antimicrobial sensitivity testing of *E. camaldulensis* leaf extracts indicated that all the three extracts (ethanol, methanol and petroleum ether) had inhibitory effects on all the pathogenic microorganisms tested. The plant extracts showed a dose-dependent inhibition of the microorganisms. The highest concentration of 400 mg/ml of the three extracts had the highest inhibition and the lowest concentration of 50 mg/ml had the lowest inhibition on the pathogens. *C. albicans* and *B. subtilis* were the most sensitive to ethanol extract at 400 mg/ml concentration, with an inhibition zone of 27 mm (Fig. 1). This was followed by *Escherichia coli* with inhibition zone of 26.5 mm. *P. expansum* had an inhibition zone of 25 mm, while *P. aeruginosa* recorded the lowest inhibition zone of 24 mm at the highest concentration (400 mg/ml). The inhibitory effect of ethanol extract at all concentrations tested, was most pronounced against *B. subtilis* and *C. albicans* and least against *P. aeruginosa* and *P. expansum*. For the methanol extract, the highest inhibition zone of 25 mm was recorded against *E. coli*, at 400 mg/ml concentration, followed by *Penicillium expansum* with an inhibition zone of 23.5 mm (Fig. 2). At all the concentrations tested, *C. albicans* was more susceptible to methanol extract than *P. expansum*. Similarly, *E. coli* was most inhibited by the methanol extract among the bacterial organisms tested. On the whole however, *E. coli* and *C. albicans* were most sensitive to inhibition by methanol extract than the other isolates tested. The petroleum ether extract showed highest inhibitory activity at 400 mg/ml against *Bacillus subtilis* with inhibition zone of 27 mm. *E. coli* and *P. expansum* had an inhibition zone of 26 mm respectively, followed by *P. aeruginosa* with inhibition zone of 23 mm and *C. albicans* which recorded the lowest inhibition zone of 20 mm at 400 mg/ml concentration (Fig. 3).

Generally, it was observed that *B. subtilis* and *C. albicans* were most susceptible to more of the plant extracts than the other organisms. *E. coli* showed highest susceptibility to methanol extract, while *P. aeruginosa* could be said to have displayed the highest resistance to all three

extracts of *E. camaldulensis*. The inhibition zones recorded suggest that ethanol extract was most inhibitory to the test organisms than methanol and petroleum ether. However, all the extracts could be said to have competed favourably with the standard drugs (gentamycin 40 mg/ml and itraconazone 40 mg/ml) used. At a concentration of 400 mg/ml, the extracts showed a higher antimicrobial activity against the test organisms than the control drugs used. At concentrations of 100 mg/ml to 400 mg/ml, ethanol extract exhibited more inhibitory activity against the fungal test organisms than itraconazole (Fig. 1).

Table 1. Phytochemical constituents of the leaf extracts of *E. camaldulensis*

Secondary metabolites	Ethanol	Methanol	Petroleum ether
Alkaloids	++	-	++
Saponins	+++	++	+
Tannins	+++	++	+
Flavonoids	+++	++	+
Steroid	+++	+++	+++
Anthraquinones	-	-	-
Carbohydrates	++	++	+
Cardiac glycosides	++	++	+

+ = Present in trace; ++ = Moderate; +++ = High; - = Absent

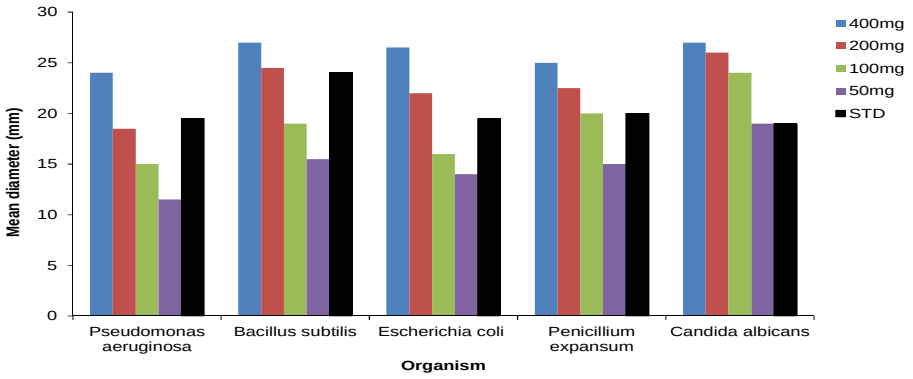

Fig. 1. Effect of different concentrations of ethanol leaf extract of *E. camaldulensis* on test organisms

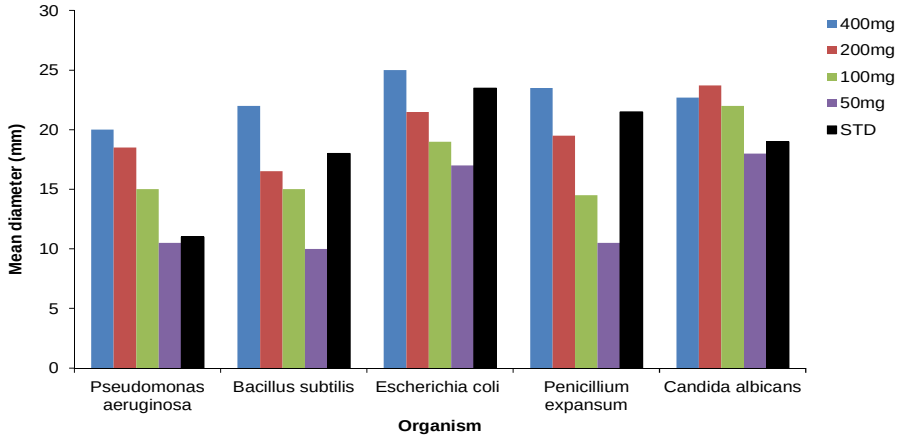

Fig. 2. Effect of different concentrations of methanol leaf extract of *E. camaldulensis* on test organisms

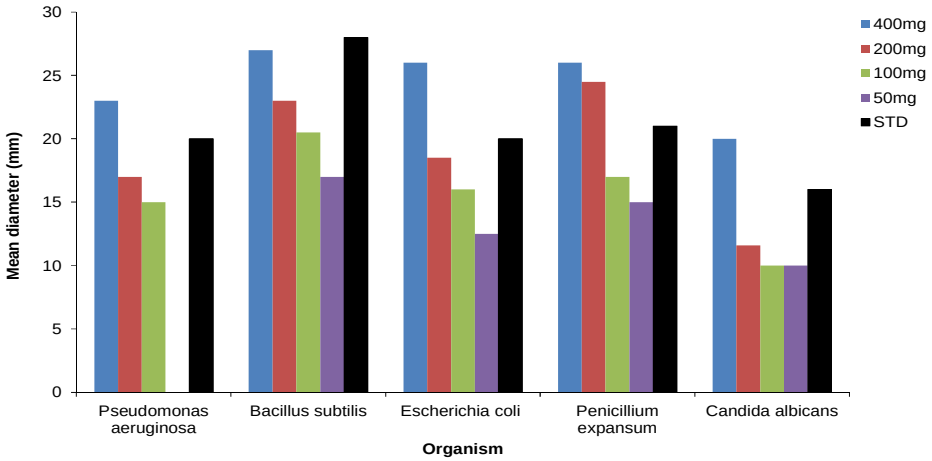

Fig. 3. Effect of different concentrations of petroleum ether leaf extract of *E. camaldulensis* on test organisms

Table 2. Minimum inhibitory concentration (mg/ml) of ethanol, methanol and petroleum ether extracts of *E. camaldulensis* on test organisms

Test organism	Minimum inhibitory concentration (mg/ml)		
	Ethanol	Methanol	Petroleum ether
P. aeruginosa	100	100	100
B. subtilis	200	100	50
E. coli	100	200	50
P. expansum	50	100	50
C. albicans	50	50	200

Table 3. Minimum bactericidal/fungicidal concentration of leave extracts on test organisms

Test organism	Minimum bactericidal and fungicidal concentration (mg/ml)		
	Ethanol	Methanol	Petroleum ether
P. aeruginosa	-	-	200
B. subtilis	400	400	200
E. coli	200	100	100
P. expansum	50	400	400
C. albicans	50	200	200

= No bactericidal activity at all concentrations used

The antimicrobial activity of the leaf extracts of *Eucalyptus camaldulensis* can be attributed to the action of the phytochemical compounds the plant contains. The antimicrobial properties of plants have been documented [20,21] and are due to the presence of alkaloids, tannins and saponins, all of which were present in the respective leaf extracts. The presence of these compounds in the family of Myrtaceae to which *E. camaldulensis* belongs have been reported [17]. The reported antiseptic properties of glycosides [22] also present in the leaf extracts could have further contributed to the antimicrobial properties as observed in this study. Many plants contain non-toxic glycosides that can release phenolics that are toxic to microbial pathogens when hydrolysed [23]. The presence of alkaloids, saponins, tannins, flavonoids and steroids suggests high toxicity and medicinal property and must have contributed to the plant's antimicrobial activity. Tannins present in the cells of plants are known to denature proteins [24] and have been reported as potent inhibitors of many hydrolytic enzymes. The proteolytic macerating enzymes used by plant pathogens are an example. The action of tannins is non-specific and the mechanism has been related to their ability to bind proteins, which results in the inhibition of cell protein synthesis. Tannins possess astringent and

homeostatic properties which have informed their wide use as topical application on sprains, bruises, and superficial wounds and infections [25]. Antifungal property of saponins has also been reported [23,26]. It is however inferred that these various bioactive components may have acted either singly or in combination to inhibit the test organisms. This aspect was not investigated in this study.

The minimum inhibitory concentration (MIC) of all extracts ranged between 50 to 200 mg/ml for all the test organisms (Table 2). Petroleum ether extract was most effective against *B. subtilis* and *E. coli* with an MIC of 50 mg/ml respectively and weakest against *C. albicans* with an MIC of 200 mg/ml. The three extracts (ethanol, methanol and petroleum ether) showed equal activity against *P. aeruginosa* (MIC = 100 mg/ml). Ethanol and methanol extracts exhibited stronger inhibition against the fungal organisms than the bacterial organisms tested. On the contrary however, the MIC of petroleum ether was lower for the bacterial organisms than the fungal organisms tested. This observation remains to be elucidated.

The minimum bactericidal concentration (MBC) and the minimum fungicidal concentration (MFC) ranged between 100 mg/ml to above 400 mg/ml in all the extracts (Table 3). The MBC of ethanol and methanol extracts for *P. aeruginosa* was observed to be above 400 mg/ml as both failed to exhibit convincing bactericidal activity at 400 mg/ml against the bacterium. Again, ethanol extract exhibited more fungicidal activity than methanol and petroleum ether extracts (MFC = 50 mg/ml respectively), while petroleum ether extract (MBC<400 mg/ml) exhibited a higher bactericidal activity than both ethanol and methanol extracts (MBC between 100 mg/ml to >400 mg/ml). *E. coli* and *C. albicans* where the most sensitive to all the extracts as observed from their lower MBC and MFC respectively. This level of sensitivity shown by *E. coli* and *C. albicans* could promote the use of the leaves of *E. camaldulensis* as treatment for sore throat, laryngitis, pharyngitis and other diseases caused by these two pathogens. The observed effectiveness of *E. camaldulensis* extracts when compared to some standard antibiotics, points to the importance of *E. camaldulensis* as a potential source of antimicrobials that would be cheaper and yet safe alternatives for treatment of microbial infections. Furthermore, the test organisms used in the study were a combination of Gram positive and negative organisms. The results which show all of these organisms to be sensitive to the extracts could further give credence to the efficacy of the plant extracts in treating a wide range of infections as the extracts are observed to be broad spectrum in activity.

4. CONCLUSION

The importance of plants in the treatment of infections has been established and documented from various researches. The want of information on the antimicrobial properties of *E. camaldulensis* in this part of the world necessitated this study. Consequently the antimicrobial effect of *E. camaldulensis* leaf extracts has been determined along with the possible active constituents to which the effects can be attributed. Although important knowledge gaps have been filled by this study, the need for further research to fully evaluate the active ingredients and their modes of action could be an important step in further promoting the development and use of *E. camaldulensis* leaves in the treatment of microbial infections and ailments.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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