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Research Article
Antibacterial Activities Investigation of *Leucas calostachys* Root Extracts

[1]Nicholas K. Kimutai, [1]Philip A. Ogutu and [2]Charles Mutai

[1]Department of Biological Sciences, Masinde Muliro University of Science and Technology, Kenya
[2]Department of Medical Laboratory Sciences, Masinde Muliro University of Science and Technolony, Kenya

Abstract

Background and Objective: Traditional healers have long used plants to treat bacterial infections caused by resistant bacteria. Hence, there is an increased interest in the ethnopharmacological approach to identify new novel compounds from plants to treat these infections. The objective of this study, therefore, was to test successive extracts and their fractions. **Materials and Methods:** The roots of *Leucas calostachys* were obtained, dried and ground. The total methanol extract was obtained and screen for antibacterial activities. Successive extracts were extracted using four different solvents hexane, dichloromethane, ethyl acetate and methanol. The bioactive solvent extracts were fractionated. Successive extracts and fractions were then screened for antibacterial activities against ten pathogenic bacteria *in vitro* using the disk diffusion method. **Results:** The results indicated the solvent extracts and fractions were active against both gram-negative and gram-positive bacteria with the lowest MIC value of 3.15 mg mL^{-1} obtained from total methanol extract against methicillin-resistant *Staphylococcus aureus*. **Conclusion:** This demonstrated that hexane extracts and fractions could be helpful in the management of resistant bacterial infections. This work is the first attempt to fractionate and test fractions of *L. calostachys*, which can be used for the development of phytomedicine.

Key words: *Leucas calostacys*, lamiaceae, successive extracts, bioactive, infections, fractions and phytomedicine

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Corresponding Author: Kimutai K. Nicholas, Department of Biological Sciences, Masinde Muliro University of Science and Technology, Kenya
Tel: +254-724-392-857

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Traditional herbal remedies and medicinal plants as primary health are widely practised in Kenya and other parts of the world. Today, as much as 80% of the people in the world depend on traditional medicine as primary health care[1]. The emergence of antibiotic-resistant microorganisms has led to the search for new potential effective plants and plant constituents against these pathogenic microorganisms, for instance, *Staphylococcus aureus*[2]. Indigenous people are a valuable asset to ethnobotanical research owing to their understanding of native plants and their applications[3]. The high cost of manufactured prescription drugs and inaccessibility to the Western medical health care system has contributed to over-reliance on traditional medicine. On the other hand, while standard healthcare facilities are accessible, traditional medicine is regarded from a cultural perspective in the treatment of various diseases as an efficient and acceptable system[4]. This necessitates the antimicrobial activities to be carried out on these plants to provide scientific proof for their use as well as their safety for humans[5]. Furthermore, further screening of plants even those that have been screened elsewhere in the world is necessary. This is because antimicrobial activities vary depending on the phytochemicals present which is affected by geographical conditions[6].

The most common approach to choosing plants for pharmacological studies is ethnopharmacology. This is because different plant components can be used to treat diarrhoea, tuberculosis, fever, bronchitis and cholera among others. These plant components have shown to act as an important source for the development of new chemotherapeutic medication that can be beneficial for the treatment of bacterial infections[7].

Leucas calostachys Oliv. locally known as "Ngechepchiat" among the Nandi community of Kenya, is a shrub of the Lamiaceae family, commonly found in the bushlands in the northern parts of Nandi county. The roots and leaves of the plant boil in water and used to treat heartburns (ulcers) and pneumonia in the county. In other parts of Kenya it is used for the treatment of diarrhoea, coughs, cold flu, abdominal pains and measles as documented by Ochwang'I *et al.*[8] The plant is also used for the management of peptic ulcers, abdominal distention, heartburn and as a synergistic plant[9]. The uses afore mention above is due to the presence of secondary metabolites it contains. Lamiaceae family is known for their triterpenoids and essential oils. Phytochemical screening of chloroform leaf extracts of *L. calostachys* has been reported to have terpenoids, alkaloids and phenols, though it is considered understudied[10]. Many essential oils have been isolated from members of this family[8,11]. These compounds can be utilized for drug development. This study will, therefore, provide a scientific basis of *L. calostachys* extracts to be use in herbal remedies as well as a guide in the isolation of the bioactive compounds from this plant. This study also investigated the antibacterial activities of the constituents from *L. calostachys*.

MATERIALS AND METHODS

Plant materials: The root barks of *Leucas calostachys* was collected in December, 2017 at (Mosoriot) northern part of the county. The geographical coordinates are 0.3189°N, 35.1668°E. It was identified at the department of biological science, University of Eldoret staff where a voucher specimen (KN/Ndi/17/05/028) was deposited. The collected plant root barks were washed using tap water, peeled off, chopped into small pieces and air-dried for 3 weeks.

Initial extraction of plant material: The identification of the active plant constituents started with the antibacterial test of crude extracts[12]. A sample of the powdered bark weighing 50 g was exhaustively extracted with methanol. The extraction was carried out in a 250 mL conical flask with 200 mL of the respective solvent added. The extracts were let to stand for 24 hrs at room temperature and filtered through Whatman No. 1 filter paper. The solvents were removed using a rotary evaporator and air-dried for 3 days. The extracts were put in sterile airtight vials weighed and kept in a desiccator at 4°C in readiness for use.

Successive extractions of plant material: Successively extractions were carried out by dissolving ground material (1 kg) in 2 L of hexane for 48 hrs. The soaked material was filtered and the crude extracts collected in a clean container. The crude extract was then concentrated using a rotary evaporator and left to dry in the open air for three days, weighed and kept in a desiccator at 4°C in readiness for use. The residue after extraction with hexane was soaked in 2 L of dichloromethane for 48 hrs, the extracts were filtered. The filtrate was concentrated and solvent recovered by distillation using a rotary evaporator. The extract was concentrated, dried for three days, weighed and kept in a desiccator. The residue after extraction with dichloromethane was dried and soaked in 2 L of ethyl acetate for 48 hrs, filtered, concentrated, dried, weighed and kept in a desiccator. The residue after extraction with ethyl acetate was soaked in 2 L of methanol for 48 hrs, filtered, concentrated, dried, weighed and kept in a desiccator. The extracts were tested for antibacterial activities.

Bioassays

Bacteria used: The bacteria used in this study included standard and clinical isolates. These were obtained from the Centre for Microbiology Research (CMR)-KEMRI and Masinde Muliro University of Science and Technology (MMUST). The Gram-positive bacteria were *Staphylococcus aureus* ATCC 25923 and clinical isolate of Methicillin-resistant *Staphylococcus aureus* (MR*SA*) and *Enterococcus faecalis* while the Gram-negative bacteria were *Escherichia coli* ATCC 25922, ESBL *Escherichia coli*, *Pseudomonas aeruginosa* ATCC 27853, *Citrobacter freundii*, *Klebsiella pneumonia*, *Salmonella typhi* and *Shigella sonnei*.

Disk diffusion assay: Antibacterial activities of the extracts and fractions were determined using the paper disk diffusion method. Positive controls were set against standard antibiotic gentamicin while negative controls were set using disk impregnated with solvent (DMSO).

Minimum inhibitory concentration (MIC): The MIC of the active plant extracts was determined using the broth microdilution method against the test microorganisms. The method is recommended by the National Committee for Clinical Laboratory Standards now Clinical Laboratory Standard Institute (CLSI) (NCCLS, 2002). The tests were performed in 96 well micro-titer plates. Plant extracts were dissolved in respective solvents and transferred into microtiter plates to make serial dilutions ranging from 10^1, 10^2, 10^3 …… 10^{10}. The final volume in each well was 100 µL. The wells were inoculated with 5 µL of microbial suspension and incubated at 37°C for 24 hrs. The MIC was recorded as the lowest extract concentration demonstrating no visible growth as compared to the control broth turbidity[13]. Wells that were not inoculated with microbial suspensions served as controls. All the assays were done in triplicates and average values computed and tabulated.

Minimum bactericidal concentration (MBC): The MBC was determined by collecting a loop full of broth from those wells which did not show any growth in MIC assay, two wells above and two wells below the MIC value and inoculated on sterile Muller-Hinton agar by streaking and incubated at 37°C for 24 hrs. The highest dilution that did not yield a colony fraction on a solid medium was considered as MBC[13].

RESULTS AND DISCUSSION

Masses of successive extractions: Hexane extract recorded a percentage yield of 0.61while methanol extracted the highest percentage of 0.91. This is due to its high polarity to extract polar and some nonpolar substances compared to other solvents used[14]. The percentage yield obtained given in Table 1 showed that increasing the polarity of the extracting solvent increases the yield except for dichloromethane which provides a lower yield compared to hexane extract, as a result, methanol yielded the highest yields.

Antibacterial activities: Methanol extracts were more active than the successive extracts and fractions with an inhibition zone of 12.00 mm and MIC of 3.15 mg mL^{-1} against *S. aureus* given in Table 2. This is because methanol extracts contain phenolic compounds such as flavonoid, flavonol and anthraquinones that are active against bacteria[15]. However, in some cases, compounds can be antagonistic to each other leading to low activities[16].

The antibacterial activities of the successive extracts showed hexane extracts were the most active of all the extracts, followed by ethyl acetate in Table 3. This probably means that hexane extract was able to extract more bioactive compounds from *L. calostachys* possibly because it is a less polar solvent. Besides, the compounds in this plant might have been more soluble in hexane than other solvents. The antimicrobial activity was screened using the agar disc diffusion method according to Clinical and Laboratory Standard Institute (CLSI)[17]. The observed variation in terms of efficacy among the successive extracts may be due to differences in the polarity of the organic solvents used during the extraction process that resulted in the differential distribution of bioactive ingredient among the extracts. This suggests that the root part of *L. calastachys* contains several antibacterial compounds of different polarities.

Table 1: Masses of successive extracts in grams and percentage yields

Extraction solvent	Mass (g)	Yield (%)
Hexane	6.07	0.61
Dichloromethane	5.01	0.50
Ethyl acetate	7.24	0.72
Methanol	9.13	0.91

Table 2: Minimum inhibitory concentration and minimum bactericidal concentration for the crude extracts in (mg mL^{-1}) against sensitive bacteria

Extract	*S. a* MIC	MBC	*P. a* MIC	MBC	MR. *S. a* MIC	MBC
Crude	3.15	66.66	Ns	Ns	12.5	33.30
Gentamicin	10.4	22.50	3.13	16.66	0.78	8.33
-ve, DMSO	-	-	-	-	-	-

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, DMSO: Dimethyl sulfoxide, MR. *S. a*: Methicillin-resistant *Staphylococcus aureus*, *S. a*: *Staphylococcus aureus*, *P. a*: *Pseudomonas aeruginosa*. Values are means of test replicates±standard deviation (n = 3), Ns: Non-significant

Table 3: Inhibition zones in millimetres of successive extracts of *Leucas calostachys*

		Average inhibition zone diameters in millimetre for each test microorganism								
Extracts	MR*S.a*	*S.a*	*P.a*	*E.c*	E.*f*	*S.s*	*K.p*	*C.f*	E.*E.c*	*S.t*
Crude	11.63	12.00	6.00	6.00	8.00	6.00	8.33	6.00	6.00	8.46
H	10.37	10.73	12.03	6.00	6.00	6.00	6.00	11.04	6.00	6.00
D	6.00	6.00	6.00	6.9	6.00	6.00	6.00	6.00	6.00	6.00
E	7.27	9.40	8.00	7.12	6.00	6.00	6.00	6.00	6.00	6.00
M	7.13	7.20	6.00	7.33	6.00	6.00	6.00	6.00	6.00	6.00
+ cont.	21.00	23.0	23.00	21.00	21.00	20.00	18.00	21.00	22.17	22.00

Values are means of test replicates±standard deviation (n = 3), H: Hexane, D: Dichloromethane, E: Ethyl acetate, M: Methanol, +ve control: Gentamicin, MR.*S. a*: Methicillin-resistant *Staphylococcus aureus*, *S. a*: *Staphylococcus aureus*, *P. a*: *Pseudomonas aeruginosa*, *E. f*: *Enterococcus faecalis*, *S. s*: *Shigella sonnei*, *K. p*: *Klebsiella pneumonia*, *E.c*: *Escherichia coli*, E. *E.c*: ESBL. *Escherichia coil S.t*: *Salmonella typhi*, *C. f*: *Citrobacter freundii*, NB: Diameter of the disk was 6mm, hence 6.0 in the results means there is no activity

Table 4: Mean inhibition zones in millimetres of fractions extracts

		Mean inhibition zone diameters in millimetre for each test bacteria							
Fractions	MR*S.a*	*S.a*	*P.a*	*E.c*	E.*f*	*K.p*	*S.s*	*C.e*	*S.t*
LCF1	7.23	7.21	6.00	6.00	6.00	6.00	6.00	6.00	6.00
LCF2	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
LCF3	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
LCF4	10.37	10.23	6.00	6.00	11.63	6.00	6.00	6.00	6.00
LCF5	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
+ cont.	21.0	23.0	23.0	21.0	21.0	18.0	20.0	21.0	22.0

Values are means of test replicates±standard deviation (n = 3), MR.*S. a*: Methicillin-resistant *Staphylococcus aureus*, *S. a*: *Staphylococcus aureus*, *P. a*: *Pseudomonas aeruginosa*, *E. f*: *Enterococcus faecalis*, *S. s*: *Shigella sonnei*, *K. p*: *Klebsiella pneumonia*, *E.c*: *Escherichia coli*, E. *E.c*: ESBL. *Escherichia coil S.t*: *Salmonella typhi*, *C. f*: *Citrobacter freundii*

All the successive extracts were active against the selected gram-positive bacteria than gram-negative bacteria with an inhibition zone of 7-11mm shown in Table 3 and Fig. 1. This could be because gram-negative bacteria have an additional outer membrane comprising of a highly hydrophilic lipopolysaccharide layer[18]. This layer restricts penetration of hydrophobic and amphipathic compounds, which encompasses many drug compounds making them less sensitive. *S. aureus* and MR *S. aureus* were the most susceptible bacteria probably because they are associated with secondary infections treated by traditional healers as well as their genetic factors[19]. However, Hexane, dichloromethane and ethyl acetate extracts were also active against *P. aeruginosa* and *E. coli* with hexane extracts having the highest zone of inhibition of 12.03 mm against *P. aeruginosa* (Table 3, Fig. 1). This strongly suggests that this solvent play a key role in the extraction of active phytochemicals[20]. In this study, hexane solvent seems to be a superior solvent for the extraction of plant antimicrobial compounds. This is in agreement with the previous studies[21].

Hexane extracts being most active were further fractionated into different fractions using column chromatography and combined according to their Rf values and tested (Table 3). Fraction LCF1 and LCF4 were active on gram-positive bacteria while the other fraction was inactive against all the selected bacteria. Besides, fraction LCF4 was also active against *S. aureus* and *E. faecalis* with an inhibition zone of 11.63 mm and 10.23 respectively, given in Table 4 and Fig. 2. The other bacteria tested *K. pneumonia*, *S. sonnei* and *E. coli* were resistant on all fraction extracts tested in Table 4 and Fig. 3. The results illustrated that the active component was eluted at fraction 4. However, the activity was lower than that of total methanol crude extracts. The plausible reason for this is the synergism of compounds present in methanol extracts (Table 2).

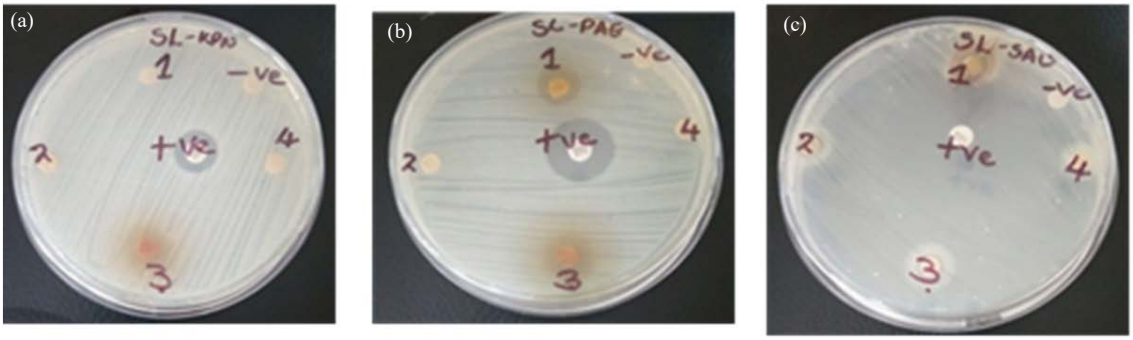

Fig. 1(a-c): Plates showing inhibition zones of successive extracts against *K. pneumonias*, *P. aeruginosa* and *S. aureus*
(a): *K. pneumonia*, (b) *P. aeruginosa* and (c) *S. aureus*

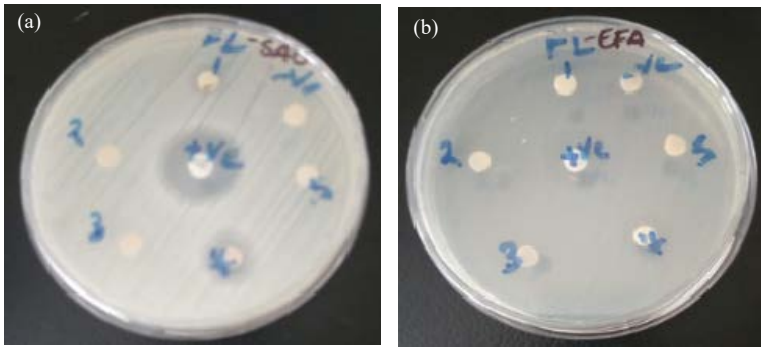

Fig. 2(a-b): Plates showing inhibition zones of fraction extracts against *S. aureus* and *E. faecalis*
(a) FL4-*S. aureus* and (b) FL5-*E. faecalis*

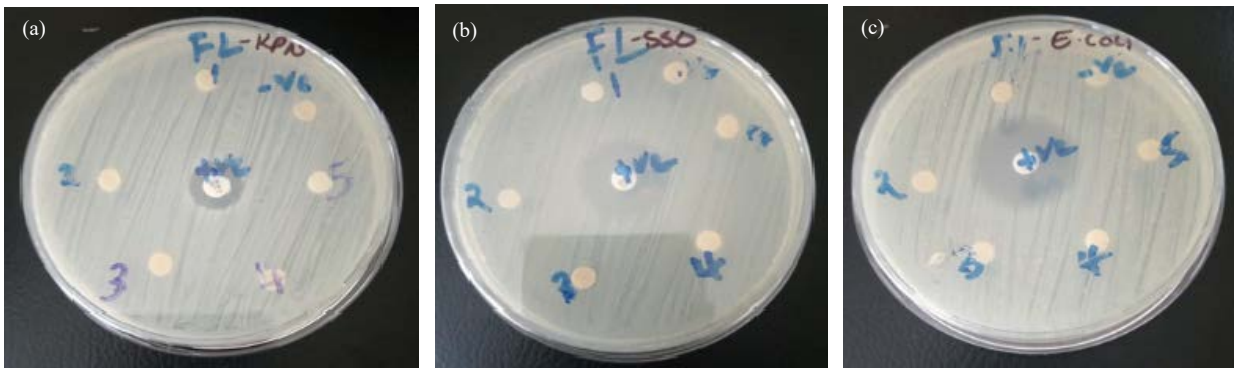

Fig. 3(a-c): Plates showing inhibition zones of fraction extracts against *K. pneumonias*, *S. sonnei* and *E. coli*
(a) FL1-*K. pneumonia*, (b) FL2-*S. sonnei* and (c) FL3-*E. coli*

CONCLUSION

Infections caused by resistant bacteria are among the most difficult to treat infections. Medicinal plants such as *L. calostachys* that are used traditionally to treat such infections are appropriate to confront this problem. Therefore, extracts and fractions of *L. calostachys* root extract obtained and tested against ten selected strains of bacteria. Methanol crude extract recorded low bacterial resistance followed by successive hexane extracts and was high with fraction extracts. These results are important for the identification of the bioactive compounds from this

plant. Methanol extract and successive hexane extract have compounds (antibacterial activity) that can be isolated and use as a basis for the development of new phytomedicine.

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SIGNIFICANCE STATEMENT

This study discovered that methanol crude extracts and successive hexane extracts have compounds that can be beneficial for the development of phytomedicine. This study will help the researchers to uncover the critical areas of isolation of compounds from *L. calostachys* root extracts that many researchers were not able to explore. Thus, a new theory of isolating bioactive compounds may be arrived at, by carrying out bio guided fraction isolation.

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