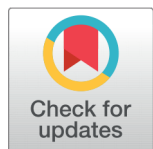


Antimicrobial activity of n-hexane and ethyl acetate extracts from *Candida tropicalis* and *Phyllosticta capitalensis* fungal endophytes

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Received 27-03-2022

Revised 07-05-2022

Accepted 07-05-2022

Published 14-05-2022

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DOI <https://doi.org/10.47419/bjbabs.v3i02.118>

Pages: 109-121

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ABSTRACT

Background ad objective: Among the notable achievements of the twentieth century was the discovery and identification of new drugs from plants against microbial infections. However, the discovery of novel drugs since then is inadequate due to emergence of resistant microbes. In an effort to discover novel drugs, the study aimed to investigate the antimicrobial activity of crude extracts from endophytic fungi isolated from *Cnidoscopus aconitifolius* and *Ocimum suave*.

Methods: Following morphological characterization and initial screening for antimicrobial activity, isolates that had higher inhibition were genotypes by Sanger sequencing. Two isolates (*Candida tropicalis* from *O. suave* and *Phyllosticta capitalensis* from *C. aconitifolius*) were tested for antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*.

Results: Overall, the range of crude extract concentration was from 152 mg/mL to 1353 mg/mL, and that of a zone of inhibition was from 7 to 21 mm. The lowest minimum inhibition concentration ($19 > MIC > 9.5$) was observed in *Phyllosticta* spp. extract against *S. aureus*.

Conclusions: Findings of the present study have shown that endophytes isolated from medicinal plants can generate secondary metabolites with therapeutic applications. Therefore, further investigations are warranted to decipher the content and structure of bioactive compounds that may be associated with the antimicrobial activity of crude extracts.

Keywords antimicrobial activity, *Cnidoscopus aconitifolius*, endophytic fungi, medicinal plants, *Ocimum suave*

INTRODUCTION

Medicinal plants are plants that have therapeutics properties.^{1,2} For example, *Echinacea purpurea*, which is found in the United States, Canada, and Europe, has been used traditionally for wound healing and to treat respiratory infections caused by bacteria.³ Also, *Angelica*

sinensis, cultivated mainly in China, Japan, and Korea, is historically used to relieve pain and treat female irregular menstruation.⁴ Furthermore, medicinal plants are widely used by local people in rural areas of Africa, particularly in Tanzania. For example, *Cnidoscolus aconitifolius* is commonly used to treat gastrointestinal infections such as diarrhoea and respiratory diseases among Zaramo and Maasai societies located in Dar es Salaam and Arusha, respectively.

Ocimum suave is commonly used to treat gastrointestinal infections such as diarrhoea and respiratory infections such as pneumonia in Tumbatu and Kizimkazi societies found in Zanzibar, Tanzania. Also, the plants have been reported to be used to treat various diseases in different parts of the World. For example, *Ocimum suave* has been used to relieve pain, fever, inflammation, and other diseases in Ethiopian people.⁵ Other related species of *Ocimum* have been reported to be used as analgesic and antipyretic in Southern Asia.⁶ Moreover, *Cnidoscolus aconitifolius* popular in South East Mexico is used to treat gastrointestinal disorders and inflammatory diseases.⁷ In Brazil, *Cnidoscolus aconitifolius* is used as analgesic, anti-inflammatory, antibiotic, and anti-diuretic herb.⁸

Most interestingly, endophytes residing in medicinal plants have been demonstrated to produce bioactive secondary metabolites that are more or less similar to the host plant.⁹ Endophytic fungi are micro-fungi that inhabit plant tissues without causing infection to the host plant.¹⁰ For example, *Pestalotiopsis fici* is the endophytic fungi found in healthy branches of *Camellia sinensis*,¹¹ and likewise, *Penicillium janthinellum* live in symbiotic association with *Solanum lycopersicum* plant without causing any known symptom¹². Furthermore, it is well known that endophytic fungi produce bioactive secondary metabolites, which are essential for the survival of the host plant.¹³ For example, endophytes isolated from *Tropaeolum majus* L and *Isatis indigotica* medicinal plants have been demonstrated to produce secondary metabolites such as polyketide, nonribosomal peptides, alkaloids, and terpenes.^{12,14} Enough evidence has shown a close relation between bioactive secondary metabolites generated by medicinal plants and bioactive secondary metabolites generated by endophytes isolated from the same plants.^{11,15} Bioactive secondary metabolites from medicinal plants and endophytes isolated from the same plant have been shown to have more or less similar antimicrobial activity.⁹ However, despite the potential of endophytes as an alternative source of bioactive compounds, little has been done to harness these potentials, particularly in tropics and subtropic areas like Tanzania. Therefore, based on this reality, this study was aimed to investigate the antimicrobial activity of crude extracts of endophytic fungi isolated from *Cnidoscolus aconitifolius* and *Ocimum suave* of Dar es Salaam (Tanzania).

MATERIALS AND METHODS

Plant samples collection and identification

In April 2021, healthy-looking *Cnidoscolus aconitifolius* and *Ocimum suave* leaves were collected from Chanika and University of Dar es Salaam (UDSM) forests in Dar es Salaam

Tanzania, respectively. A botanist in the Botany Department of UDSM initially authenticated the plants. Immediately following collection, plants materials were processed in the Molecular Biology and Biotechnology Laboratory at UDSM.

Processing of plant materials and isolation of endophytic fungi

Surface sterilization of plant samples

Collected samples of *Cnidoscolas aconitifolius* and *Ocimum suave* were first washed by running tap water to remove soil debris, followed by distilled water. The samples were then sterilized. Next, the surface sterilization was performed to eliminate epiphytic microorganisms by immersion in 70% ethanol for 1 minute, dipping in distilled water for 30 seconds, immersion in sodium hypochlorite (0.4%) for 2 minutes, dipping in distilled water for 30 seconds, immersion in 70% ethanol for 1 minute and finally tested by inoculating the last washing water into the potato dextrose agar (PDA) media (potato infusion 200.00 g/L, dextrose 20.00 g/L and 15.00 g/L of agar).

Isolation of endophytic fungi

After surface sterilization, leaves were cut into square cube pieces of about 1cm by using a sterile razor blade. Thereafter, 4 segments were placed in Petri dishes containing PDA supplemented with Ampicillin (25 mg/mL). Following inoculation, plates were incubated at 27°C for 4 days. Plates were observed frequently to check for the growth of mycelia. Thereafter, the fungi purification was done by cutting the square cubes of a hypha of growing fungi on agar using a sterile blade and transferred to separate PDA Petri-dishes supplemented with 25 mg/mL Ampicillin. The plates were incubated for 4 days at 27°C to obtain pure isolate.

Characterizations of endophytic fungi isolates

Initially, pure isolates were identified morphologically based on colour, texture, shape, edges of hyphae, and topography from the agar surface. Before mass cultivation and further analysis, isolates were subjected to initial screening as previously described by Santos et al.¹⁶ Briefly, an antimicrobial assay was used using a solid medium (Muller-Hinton agar, MHA) that permits a rapid selection of bioactive microorganisms. Endophytes were cultivated as described above, and discs were cut from the PDA plate (about 6 mm diameter) and transferred to the surface of Petri dishes previously inoculated with bacteria in MHA. Then, Petri dishes were incubated at 37°C for 24 h to allow bacterial growth. Two isolates, one for each medicinal plant with the highest inhibition, were selected for further processing and analysis.

Molecular characterization was performed on the two selected isolates and was genotyped by Sanger sequencing. First, fungal genomic DNA is extracted following procedures described in Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research). The quality and quantity of genomic DNA were evaluated by running on 0.8% (w/v) of agarose gel in 1% (w/v) Tris Boric EDTA (TBE) for 30 minutes, and the running buffer was 1% (w/v) TBE. After electrophoresis, the gel was visualized in a gel documentation instrument (Ernst Pharmacia, Beijing, China). By using ITS 1 and ITS 4 primers, Polymerase Chain Reaction (PCR) was conducted under the following conditions: Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds, elongation at 72°C for 1 minute, and final extension at 72°C for 7 minutes. The quality and quantity of PCR products were evaluated as described above, except that the agarose concentration was 1.5% (w/v).

PCR products sequencing and bioinformatics analysis

Samples were Sanger sequenced by INQABA BIOTECH Company in South Africa using the same primers, ITS1 and ITS4 that were used during PCR amplification. Raw DNA sequences were received electronically and were initially trimmed, and using CLC Genomic Workbench consensus sequences were generated. Then, sequences were subjected to BLAST search on NCBI for identification. Finally, other closely related sequences reported from different parts of the World obtained from GenBank were aligned and used to construct a phylogenetic tree to depict genetic relatedness.

Mass cultivation and extraction of crude extract

Crude extract of the endophytic fungi was extracted from separate PDA plates containing the pure endophyte isolate culture from different parts of the *Cnidoscolas aconitifolius* and *Ocimum suave* plant. Briefly, about 4 square cubes of mycelia agar of each endophytic fungi were aseptically cut from the actively grown pure culture using a sterile blade and forceps. After that, inoculated into 500 mL Erlenmeyer Flasks containing 300 mL of the autoclaved Multi Extract Broth (MEB) media. It was then put on the shaker at 150 rpm for three weeks as described by Kumar et al.¹⁷ The crude extract produced from each MEB fungal culture was obtained by separating the mycelia from broth through filtering using a filter funnel. The extracts were extracted twice with an equal volume of ethyl acetate (200 mL), and another was extracted with an equal amount of n-hexane (200 mL), then evaporated in the cold room for 4 days. The crude extract residuals obtained were weighed and dissolved in 10% dimethyl sulfoxide (DMSO), then stored at 4°C as a stock solution for antimicrobial tests.

Preparation for test organisms

Gram-positive bacteria strains of *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli* were obtained from the Microbiology Laboratory in the Department of Molecular Biology and Biotechnology at the University of Dar es Salaam. The strains were sub-cultured on nutrient agar and incubated at 37 °C for 24 hours. Then pure culture of bacteria was inoculated using inoculating loop following re-suspension in sterile saline water, and cell turbidity was assessed by comparing with 0.5 McFarland standards 1.5×10^8 (CFU/mL).

Screening for antimicrobial activity

Analysis for antimicrobial activities of each extract was determined using Kirby Bauer's disc diffusion method with some modification as detailed in Silva et al.¹⁸ The prepared inoculum was spread aseptically using nutrient agar (NA) media of 28g/1000 mL. About 25 µL/disc solvent extract was loaded to each sterile disc of 6 mm, allowed to dry, and then placed on the inoculated media. Ciprofloxacin was used as a positive control, whereas Dimethyl Sulfoxide (DMSO) 10% was used as a negative control. Petri-dishes were incubated at 37 °C for 24 hours. The antimicrobial activity of crude extracts was evaluated based on the diameter of the zone of inhibition and measured in millimetres (mm).

Minimum inhibition concentration (MIC) test

The MIC test was carried out using the microdilution method. Serial dilution of crude extracts using 10% DMSO was conducted on 96 well microplates. Following initial screening of antimicrobial activity, two isolates were selected. The test was conducted by using the two isolates. Ciprofloxacin was used as a positive control, and 10% DMSO was used as a negative control.

Crude extracts chemical composition screening

Chemical composition of crude extracts was qualitatively examined by using standard procedures.^{20,21} Crude extracts chemical constituents (saponins, comarins, alkaloids, anthraquinones, tannins, flavonoids, sterols, and triterpenes) were determined.

Data analysis

The t-test was performed using the R software (version 3.3.3; The R Foundation for Statistical Computing) to evaluate whether there is a difference in antimicrobial activity of crude extracts of isolates. Significance differences were taken at $p < 0.05$ levels.

RESULTS AND DISCUSSION

Characterization and confirmation of endophytes

There was high variability in the morphological appearance of fungal colonies based on colour, texture, shape, edges of mycelia, and topography from the agar surface. Some of the morphological appearances of isolated endophytes are depicted in Figure 1.

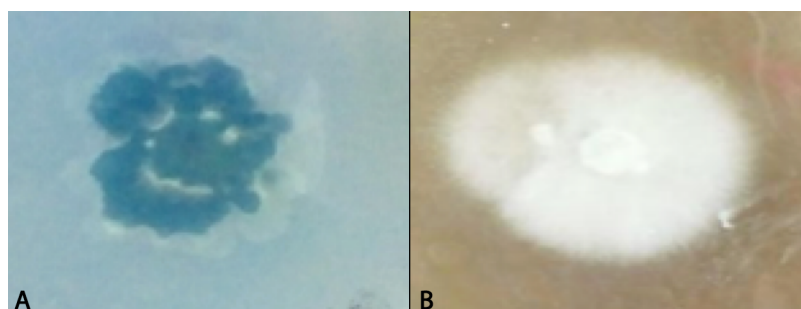


Figure 1 Morphological appearance of *Phyllosticta* sp. (MZ577137) isolated from *Cnidoscolas aconitifolius* (A); Morphological appearance of *C. tropicalis* (MZ577576) isolated from *Ocimum suave* (B).

As described in the previous section (methodology), molecular identification was performed for two isolates from each medicinal plant with the highest inhibition zone during initial screening (data not shown). In addition, phylogenetic trees (Figure 2) were generated depicting evolutionary relationships with other reported strains. Furthermore, accession numbers (Table 1) of isolates were obtained following submission in Genbank.

In the present study, two endophytic fungi isolates (*C. tropicalis* and *P. capitalensis*) were selected for the antimicrobial test based on the initial screening. This is the first report to communicate on *C. tropicalis* and *P. capitalensis* from *O. suave* and *C. aconitifolius*, respectively; however, the two endophytes have been isolated from other plant species and environments. Rocha et al.¹⁹ isolated *C. tropicalis* from comfrey leaves (*S. officinale* L) and tested for antagonism/inhibitory activity against *S. sclerotium*. Also, *C. tropicalis* have been reported to have biotechnological application. This has been corroborated by the isolation of *C. tropicalis* from high salt concentration from Amazon forest.²⁰ Therefore, the isolation of *C. tropicalis* from *O. suave* leaves is not a surprise, although the strain is known to be highly pathogenic, just second after *C. albicans*. Conversely, *P. capitalensis* is a well-established endophyte, which has been reported in several plant species and types.²¹

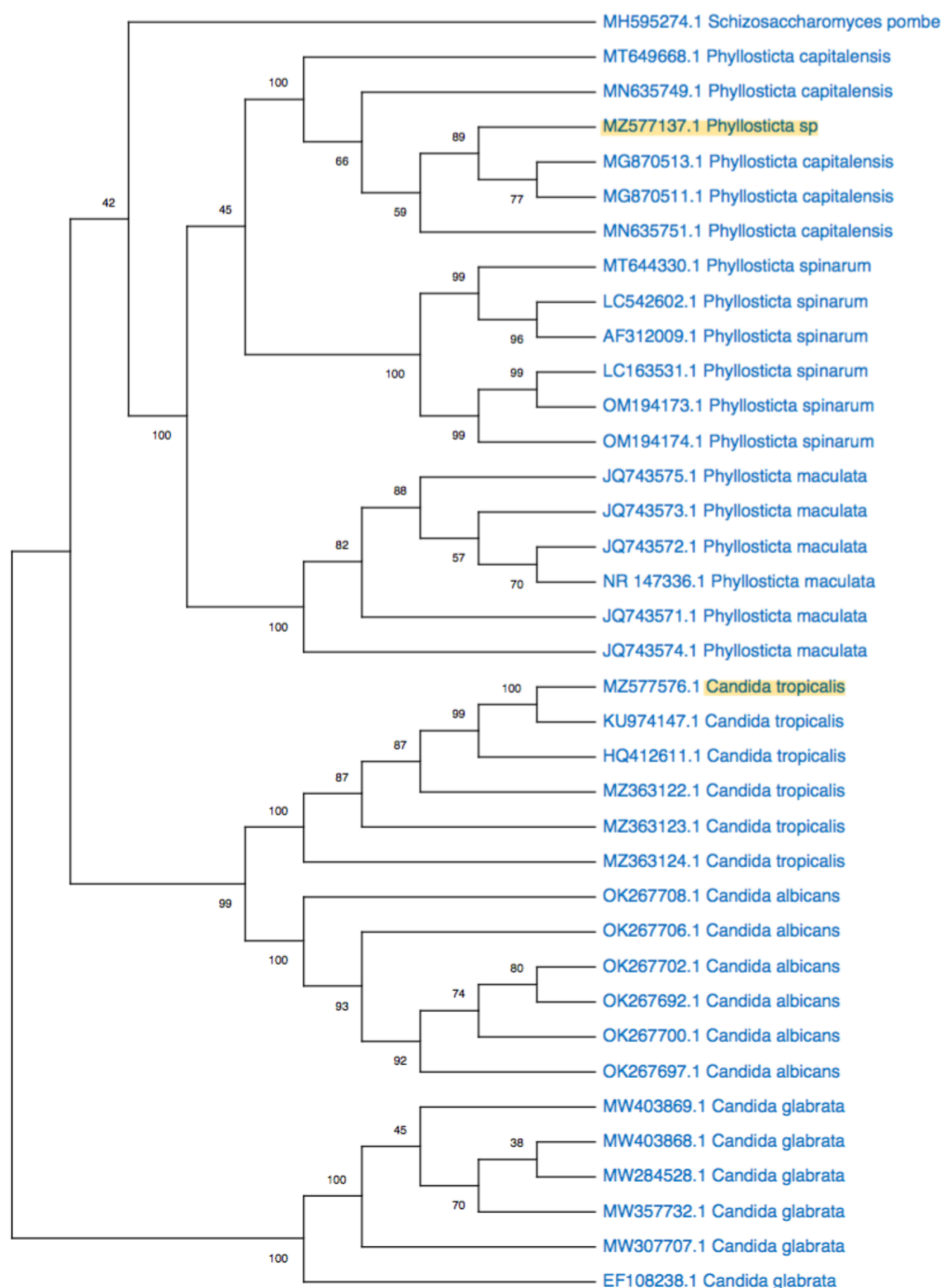


Figure 2 A Neighbor joining phylogenetic tree depicting the relationship of *C. tropicalis* and *P. capitalensis* (highlighted in yellow) with other related fungi obtained from GenBank. The MH595274.1 *Schizosaccharomyces pombe* was used as an out-group. Numbers depicted on the branches represent values based on 1000 replications of Felsenstein's bootstrap method.

Table 1 Weight and concentration of crude extracts of endophytic fungi isolated from *O. suave* and *C. aconitifolius*.

Plant name	Isolated endophytic fungi	Solvent	Weight (mg)	Concentration (mg/mL)
<i>O. suave</i>	<i>C. tropicalis</i> (MZ577576)	EA	203.00	1353.00
		n-H	35.40	236.00
<i>C. aconitifolius</i>	<i>Phyllosticta</i> spp. (MZ577137)	EA	36.10	200.66
		n-H	22.80	152.00

Weight and concentration of crude extract. EA= ethyl acetate; n-H =normal hexane.

Antimicrobial activity of endophytic fungi crude extracts

Since the discovery of most drugs and drug structures during the twentieth century,^{20,21} there has been non-equivalent speed between the discovery of novel drug structures and the emergence and re-emergence of new infectious agents.^{22,23} For example, penicillin, which was originally effective against Gram-positive organisms such as *S. aureus* later became resistant because of bacterial acquisition of resistant genes to the drug.^{24,25} These existing challenges entail searching for alternative and novel drug structures from potent medicinal plants and their associated endophytes. Enough evidence has been shown a close relation between bioactive secondary metabolites generated by medicinal plants and bioactive secondary metabolites generated by endophytes isolated from the same plants.^{9,15} Based on this information, the present study hypothesized that endophytic fungi isolated from *C. aconitifolius* and *O. suave* could generate bioactive secondary metabolites with antimicrobial activity because local rural communities have long used the plants for the treatment of various infectious agents.⁴⁻⁶ Therefore, endophytic fungi were isolated from *O. suave* and *C. aconitifolius* in the present study and tested for antimicrobial activities against *S. aureus* (Gram-positive) and *E. coli* (Gram-negative). Results have supported the hypothesis because crude extracts of isolated endophytes exhibited antimicrobial activity, as summarized in Table 2.

Table 2 Antimicrobial activity of isolated endophytic fungi against the selected test microorganisms.

Test organism	<i>C. tropicalis</i>		<i>P. Capitalensis</i>		Positive control
	EA	n-H	EA	n-H	Ciprofloxacin
<i>E. coli</i>	21	10	8	8	20
<i>S. aureus</i>	–	–	7	9	15

Numbers indicate the diameter of zone of inhibition (DZI) in mm. EA= extract of ethyl acetate; n-H= extract of normal hexane; (–) = absence of a zone of inhibition.

The finding observed from this study may be corroborated by well-established evidence, which suggests the two plants (*O. suave* and *C. aconitifolius*) where the endophytes (*C. tropicalis* and *P. capitalensis*) were isolated are validated medicinal plants.^{26,27} The medicinal plant, *O. suave*, has been demonstrated to have antioxidant properties due to the high con-

tent of flavonoids and polyphenols secondary metabolites, which are said to have cytoprotection effect.²⁸ Also, essential oil from *O. suave* was demonstrated to have antimicrobial activity against pathogens.²⁷ On the other hand, crude extract of *C. aconitifolius* has been shown to have an anti-inflammatory effect due to the high content of flavonoids. Therefore, it may be that these medicinal plants' antioxidant, anti-inflammatory, and antimicrobial activity are acquired from endophytes, including *P. capitalensis* and *C. tropicalis*, as reported in this study.

Table 3 Range of MIC determined for the crude extracts of endophytic fungi against selected pathogenic microorganisms.

Test organism	<i>C. tropicalis</i>		<i>P. capitalensis</i>	
	EA	n-H	EA	n-H
<i>E. coli</i>	338>MIC>169	118>MIC>59	100.33>MIC>50.165	39>MIC>19
<i>S. aureus</i>	–	–	50.165>MIC>25.082	19>MIC>9.5

(EA) = ethyl acetate; (n-H) = normal hexane; (–) = absence of a zone of inhibition.

There was high variability in MIC among different extracts against test organisms (Table 3). The range of MIC was 169 mg/mL EA extract of *C. tropicalis* against *E. coli* to 9.5 mg/mL n-H extract of *P. capitalensis* against *S. aureus*. Generally, from a microbiology point of view, Gram-negative microorganisms are relatively less susceptible to most antimicrobial agents than Gram-positive microorganisms.^{29,30} The lowest minimum inhibitory concentration (MIC) was the n-Hexane crude extract of *P. capitalensis* against *S. aureus*, a Gram-positive bacteria. It was challenging to compare the present observation with other studies since this is the first report on the antimicrobial activity of crude extracts of *C. tropicalis* and *P. capitalensis* isolated from *O. suave* and *C. aconitifolius*, respectively.

Preliminary chemical screening of crude extracts

Qualitative analytical data is presented in Table 4 showing chemical composition of n-hexane and ethyl acetate crude extracts from *C. tropicalis* and *P. capitalensis*. Results demonstrated presence of sterols, terpenes, tannins, comarins and saponins. The abundance in chemical composition was slightly variable between extracts. The variability in chemical composition may be explained by type of solvent that was used and endophytic fungi involved (*C. tropicalis* or *P. capitalensis*). However, alkaloids, flavonoids and anthraquinones were not found.

CONCLUSIONS

The isolation of two endophytic fungi, *C. tropicalis* and *P. capitalensis*, from *O. suave* and *C. aconitifolius*, respectively, was done first in the present study. In addition, the endo-

Table 4 Preliminary chemical screening of n-hexane and ethyl acetate crude extracts of *C. tropicalis* and *P. capitalensis*.

Metabolite	<i>C. tropicalis</i>		<i>P. capitalensis</i>	
	EA	n-H	EA	n-H
Alkaloids	—	+	—	—
Anthraquinones	—	—	—	—
Comarins	+++	+++	++	+++
Flavonoids	—	—	—	—
Saponins	++	+++	+++	++
Sterols	++	+++	+++	+++
Tannins	++	++	+++	+
Triterpenes	++	+++	+++	++

EA= ethyl acetate; n-H= normal hexane; (+)= trace; (++)= moderate; (+++)= high; (—)= absent.

phytes exhibited appreciable antimicrobial activity against *S. aureus* and *E. coli*. The results warrant further investigation to decipher bioactive secondary metabolites associated with antimicrobial activity from *C. tropicalis* and that of *P. capitalensis*.

ACKNOWLEDGMENT

The authors are grateful to Placida Kabelege and Ahmadi Ally for their help in the sample and data collection. Also, in the present study no external source of fund was obtained.

DECLARATIONS

Authors' contributions

Conceptualization: FNM. Data curation, formal analysis, investigation, methodology, project administration, resources, writing-original draft: FNM & MM. Funding acquisition, supervision: N/A. Writing-review & editing: MM & FNM.

Ethical approval and consent to participate

The research did not involve use of Humans or Animals during the experiment and therefore ethical approval was not though.

Conflict of interest

The authors declare no conflict of interest.

Funding resources

No external fund was received.

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