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Antimicrobial activity of crude extract of endophytic fungi isolated from leaves of *Kigelia africana* and *Phyllanthus niruri*

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ABSTRACT

One of the main challenges that hinder the achievement of the third-millennium sustainable development goal is the emergence of infectious agents that are resistant to existing antimicrobials. These challenges entail a search for novel bioactive compounds like exploring the untapped potential of medicinal plants and their associated endophytes. The present study aimed to investigate the antimicrobial activities of crude extracts of endophytic fungi isolated from two medicinal plants: *Kigelia africana* and *Phyllanthus niruri*. Isolates of endophytic fungi were subjected to initial screening for antimicrobial activity and the isolates that exhibited antimicrobial activity based on the zone of inhibition were genotyped by Sanger sequencing for identification. Then, antimicrobial activities of crude extracts of *Nigrospora sphaerica*, *Meyerozyma guilliermondii*, *Alternaria alternata*, and *Phyllosticta capitalensis* were evaluated by the disc diffusion method against *Escherichia coli* (Gram-negative), *Salmonella typhi* (Gram-negative), and *Staphylococcus aureus* (Gram-positive). There was high variability (8.5 -23.1 mm) of the zone of inhibition among isolates. The highest zone of inhibition was that of *M. guilliermondii* ethyl acetate crude extract against *S. aureus*. Furthermore, the lowest range (9.9 >MIC>5.0) of minimum inhibition concentration (MIC,) in mg/ml was found in n-hexane crude extract of *P. capitalensis* against *S. typhi*. However, E. *coli* was resistant to all crude extracts. Results of the present study corroborated the influence of extracting solvent on the effectiveness of crude extract of isolates against pathogen microorganisms. Also, findings demonstrated the potential of endophytes from medicinal plants for medical applications, and therefore further investigation may lead to the discovery of novel bioactive compounds potent to resistant infectious agents.

Key words: Medicinal plants; crude extracts; endophytes; ethyl acetate; n-hexane; minimum inhibition concentration

Introduction

The third-millennium sustainable development goal is to achieve good health and well-being (Harris, 2001; Kamalam, 2017; Mio et al., 2020). However, one of the challenges that hinder the achievement of this goal is the emergence of antibiotic-resistant infectious agents (Ab Rahman & Abd Aziz, 2020; Jaiyesimi, 2016; Xue et al., 2018). Antibiotic resistance is one of the top ten global public health threats (Essack et al., 2017). According to the Centre for Disease Control and Prevention (CDC), in the USA, antimicrobial resistance has been estimated to cause 2 million infections, and more than 20,000 deaths (Dadgostar, 2019; Shrestha et al., 2018). Also, antimicrobial resistance has very high financial implications; For example, in the USA, the cost associated with antimicrobial resistance is estimated to be 55 billion dollars per year (Davies et al., 2020). Furthermore, the burden of antimicrobial resistance is even huge in developing countries due to poor health facilities and lagging economies (Essack et al., 2017; Perovic et al., 2018). In Sub-Saharan Africa, about 90% of Gram-negative bacteria were found to be resistant to antibiotics chloramphenicol and cephalosporins (Leopold et al., 2014; Singh-Moodley et al., 2018). This information suggests searching for alternative drug structures, maybe through harnessing the potential of secondary metabolites from medicinal plants and their endophytes.

For thousands of years, medicinal plants have been used for the treatment of various diseases based on traditional herbal knowledge (Bamola et al., 2018; Harborne, 1993;

Polat et al., 2013). Medicinal plants like *Sapindus saponaria* and *Camptotheca acuminata* have been used for the treatment of wounds and coughing for thousands of years in Brazil and China (García et al., 2012; Santos et al., 2019). Also, in Africa, the use of medicinal plants is common practice among poor rural communities for the treatment of infection and non-infectious diseases (Aziz et al., 2017; Mbuni et al., 2020; Oladele & Adewunmi, 2008). For example, the Maasai community is one of the tribes that rely on medicinal plants for the treatment of disease due to a pastoral lifestyle (Kimondo et al., 2015; Nankaya et al., 2020). Specifically, taken as an example, *Phyllanthus niruri* has been used for the treatment of urinary tract stones, ulcers, dysentery, and liver diseases among the Haya and Sukuma communities in Tanzania. Also, *Kigelia africana* and *Phyllanthus nummulariifolius* have been used for many years in different parts of Africa including Tanzania (Nondo et al., 2016). In Tanzania, *Kigelia africana* has been used for the treatment of skin ailments, pneumonia, diabetes and malaria in Zaramo, Maasai and Chagga communities. However, the direct use of medicinal plants has environmental implications taking into consideration the effect of climate change (Catarino et al., 2019; Ncube et al., 2012). Interestingly, it has been demonstrated that medicinal plants harbor endophytes that produce secondary metabolites having a more or less similar effect as secondary metabolites of the host medicinal plant (Abba et al., 2015; Ali et al., 2019; Vieira et al., 2014)(Refs). Therefore, the isolation of endophytes from medicinal plants and allowing them to produce secondary metabolites is an interesting alternative source of new drugs, which is environmentally friendly.

Endophytes are microorganisms (bacteria or fungi or actinomycetes) that dwell inside the living plant tissues for the whole or at least part of their life cycle without causing any apparent disease symptoms in the host plant (Christina et al., 2013). The name for these endosymbiont microorganisms was coined by De Bary in 1884 (Murray, 1884), and in 1904, the first endophytic fungus was isolated from the *Lolium persicum* plant, and identified by Freeman (Tugume et al., 2016). Endophytes can be isolated from various plant parts including the seeds, stems, barks, and leaves (Gómez & Luiz, 2018). Upon entering plant tissues, endophytes reside in the intercellular spaces or the vascular system forming symbiotic associations with their hosts and hence stimulating plant growth, improving the recycling of nutrients, increasing resistance to diseases, and improving the plants' ability to withstand environmental stresses (Sudha et al., 2016). Also, researchers have shown that endophytic fungi can produce natural products with antimicrobial, antidiabetic, antiviral, anticancer and antioxidant properties. These include alkaloids, steroids, quinones, terpenoids, flavonoids, phenols and peptides (Zhang et al., 2006). Despite this well-documented information about the potential of endophytes as a sustainable alternative source of antimicrobial bioactive secondary metabolites, more remain to harness this potential from medicinal plants, particularly in Tanzania. Therefore, this study aimed to evaluate the antimicrobial activity of crude extract of endophytic fungi isolated from leaves of *Kigelia africana* and *Phyllanthus niruri* collected from Dar es Salaam Tanzania.

Materials and Methods

Plant samples and identification

Leaves from two medicinal plants with no visual disease symptoms were collected from the University of Dar es Salaam conserved forest. The two medicinal plants were *Phyllanthus niruri (Mzalia nyuma in Swahili)* and *Kigelia africana (Mwegea in Swahili)* and were identified by Botanists in the Department of Botany of the University of Dar es Salaam. Samples were kept in plastic bags and taken to the Microbiology Laboratory in the Department of Molecular Biology and Biotechnology of the University of Dar es Salaam for further processing on the same day.

Plant leaves surface sterilization

The collected samples (leaves), were subjected to surface sterilization as previously described (Burgdorf et al., 2014; Saldierna Guzmán et al., 2020). Briefly, first, plant leaves were washed vigorously with running tap water to remove all debris and soil contaminants, and it was followed by washing with sterile distilled water. Second, surface microbial contaminants were removed by dipping leaves in 70% ethanol for two minutes followed by rinsing in sterile distilled water. This was again followed by another dipping in sodium hypochlorite solution for about two minutes. Third, final surface sterilization was done by rising leaves pre-dipped in sodium hypochlorite solution with sterile distilled water, which was then followed by final dipping in 70% ethanol for 30 seconds. To test for the efficiency of surface sterilization, water from the last washing step was inoculated on Petri dishes containing potato dextrose agar (PDA; containing potato (200 g/L), dextrose (20 g/L), and agar (15 g/L), pH 6.0).

Isolation, purification, and initial screening of endophytic fungi

After sterilization of the plant materials, under aseptic conditions, the plant leaves were cut into small pieces of about one centimeter square segments to expose the interior surface of the leaves to the nutrient media. Five segments of leaves were placed in Petri dishes containing PDA media supplemented with ampicillin 25µg/ml. Petri dishes were then sealed with parafilm to prevent any contamination and

were incubated at 30°C for 5 days. Pure isolates were obtained by aseptically transferring hyphal tips into fresh PDA Petri dishes supplemented with ampicillin (25µg/ml) using a sterile blade. Plates were then sealed with parafilm and incubated at 30°C for 5 days.

Before mass cultivation, isolates were subjected to initial screening as previously described by Santos et al., (2015). Briefly, an antimicrobial assay using a solid medium that permits a rapid selection of bioactive extracts was used. Endophytes were cultivated as described above, and disks were cut from the PDA plate (about 6 mm diameter) and transferred to the surface of Petri dishes containing Müller-Hinton agar (MHA) previously spread with selected bacteria (*E. coli*, *S. typhi* and *S. aureus)*. Then, Petri dishes were incubated at 37°C for 24 hours to allow bacterial growth. Two isolates for each medicinal plant that demonstrated the highest inhibition were selected for further processing and analysis.

Molecular identification of endophytic fungi

Genomic DNA was extracted using a Quick-DNA Fungal/Bacterial Miniprep kit (Zymogen; Zymo Research Corp) and manufacturer instructions were followed. Briefly, the mycelia were transferred from Potato Dextrose Agar (PDA) to 250 mL Erlenmeyer's flasks containing Malt Extract Broth (MEB). The flasks with mycelia were kept for five days for massive growth at 30°C on a mechanical shaker. From the incubated flasks, 200 mg of mycelia were harvested and placed in 2 ml micro-centrifuge tubes for genomic DNA extraction and were followed by centrifugation. Genomic DNA was extracted using DNA Easy mini kit (Zymogen), and manufacturer instructions were followed. Quality and quantity of DNA were evaluated by gel electrophoresis: 0.8% (w/v) agarose, 1x (w/v) TBE buffer, and 75 V for 30 min. Polymerase chain reaction (PCR) was then conducted as described by Borgmann et al., (2008) to amplify the internal transcribed spacer (ITS) using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al., 1990) under the following conditions: initial denaturation at 94°C for 3 min; denaturation at 95°C for 10 secs; annealing at 56°C for 45 secs and extension at 72°C for 1 min 45 sec and the process lasted a total of 35 cycles with a final extension at 72°C for 5 min. The quality and quantity of PCR products were evaluated as described above with the exception that 80 V for 30 min and 2% agarose were used.

Sanger sequencing of amplicons and Bioinformatics analysis

Amplified DNA fragments were sent to Inqaba biotech (South Africa) for purification and Sanger sequencing. Raw DNA sequences were received electronically and subjected to trimming and generation of consensus sequences by using CLC Genomics workbench v.3.0.8 (QIAGEN). Data were further analyzed using the basic alignment search tool (BLAST) of the NCBI GenBank database. Based on similarity scores and E-values, sequences were selected and aligned using a multiple alignment algorithm and the phylogenetic trees were constructed using MEGAX.

Mass cultivation of endophytes and extraction of crude extracts

Three hundred milliliters of pre-autoclaved malt extract broth (MEB) were distributed in eight 500 mL conical flasks. Then flasks were inoculated with mycelial culture plugs of about 2 cm for each isolate. Flasks were incubated on a stationary phase for three weeks at 27°C and were observed periodically for any possible contamination. Following incubation, a double-layered muslin cloth was used as a filter to separate filtrate from the mycelial mat to obtain clear broth. Mycelia-free culture filtrates were then added with an equal volume of ethyl acetate (EA) and n-hexane solvents in separating funnels, and shaken gently after every two hours for a day. The mixtures were left overnight to allow clear separation of the two layers. Ethyl acetate/n-Hexane layers were collected and then left in a cold room for solvent evaporation and harvesting crude extracts.

Antimicrobial assay

Fungal crude extracts were screened for their antibacterial activity in duplicates using the agar diffusion method (Dhar et al., 1968; Hayhoe & Palombo, 2013) against selected pathogenic bacteria: *E. coli* (Gram-negative), *S. typhi* (Gram-negative) and *S. aureus* (Gram-positive). Crude extracts were initially dissolved with 150µL of dimethylsulfoxide (DMSO), and 30µL of the crude extracts of various concentrations (Table 2) of each isolate was transferred to disk and placed on plates spread with selected bacteria as described above. Also, 30µL of streptomycin (30µg/ml) and DMSO had similar treatments to serve as positive and negative controls, respectively. Then, plates were incubated at 37°C for 24 hours and followed by recording inhibition zones.

Minimum inhibition concentrations (MIC) of the crude extracts of ethyl acetate and n-hexane were determined by the disk diffusion method against Gram-positive (*S. aureus*) and Gram-negative (*S. typhi*) bacteria. Inoculum for the assays was prepared in 0.5 McFarland standards (approximately 1.5 × 108 CFU/mL). Two-fold serial dilution was performed for each crude extract. Each dilution was tested for antimicrobial activity by the disc diffusion method as described above. Minimum inhibition concentration in mg/ml was reported as a range between the last concentration that showed inhibition and the immediate subsequent concentration that did not show inhibition.

Table 1. *In vitro screening of antimicrobial activity of isolated endophytic fungi against the selected test microorganisms by disc diffusion method on nutrient agar (NA) media, incubated at 37°C for 23 h.*

Test organism	EA		n-H		EA		n-H		Control
	A.alternata	*P.capitalensis*	*A.alternata*	*P.capitalensis*	*N.sphaerica*	*M.guilliermondii*	*N.sphaerica*	*M.guilliermondii*	Streptomycin
E.coli	-	-	-	-	-	-	-	-	17
S. aureus	8.5	-	-	8.5	10.6	23.1	-	10.0	32
S. typhi	19.5	-	-	18.5	11.0	11.0	-	-	22.5

Numbers stand for zone of inhibition (ZOI) in mm; EA = crude extract by Ethyl acetate; n-H =crude extract by normal Hexane; - = absence of a zone of inhibition.

Results and Discussion

One of the challenges in improving the health and well-being of people is the emergence of infectious agents that are resistant to existing antimicrobials (Ab Rahman & Abd Aziz, 2020; Jaiyesimi, 2016; Xue et al., 2018). Based on the plethora of information regarding the relationship between medicinal plants and endophytes, it was hypothesized that endophytes residing in *K. Africana* and *P. niruri* found in Tanzania may produce secondary metabolites with antimicrobial activities. Therefore, in the present study endophytic fungi from the same medicinal plants were isolated, characterized, and tested for antimicrobial activities against selected pathogenic microorganisms. Indeed, results from this study demonstrated that some endophytes from *K. africana* and *P. niruri* have antimicrobial activities as summarized in Table 1.

Morphological appearance and molecular confirmation of endophytes

Initially, endophytes were isolated, purified, and characterized by both molecular and macroscopic approaches. Morphological characterization was based on color, texture, shape, edges of mycelia, and elevation from the agar surface. Some of the morphological appearances of isolated endophytes are depicted in Figures 1 and 2.

Accession numbers (Table 2) of isolates were obtained following submission of sequences in GenBank.

The phylogenetic relationship between isolates reported in the present study and others, which were obtained in database was established as depicted in Fig. 3-6.

Based on morphological appearance, in the present study, a total of 12 endophytes were isolated. This is a relatively lower number of isolates as compared to other studies. The study involved the isolation of endophytes from leaves only, and this may explain why relatively few isolates were obtained. The choice of leaves for isolation of endophytes was based on local knowledge that leaves from medicinal plants (*K. Africana and P.niruri*) are used for medical applications.

Figure 1. *Morphological appearance of Nigrospora sphaerica (MZ570966) isolated from P. niruri found in Dar es Salaam, Tanzania.*

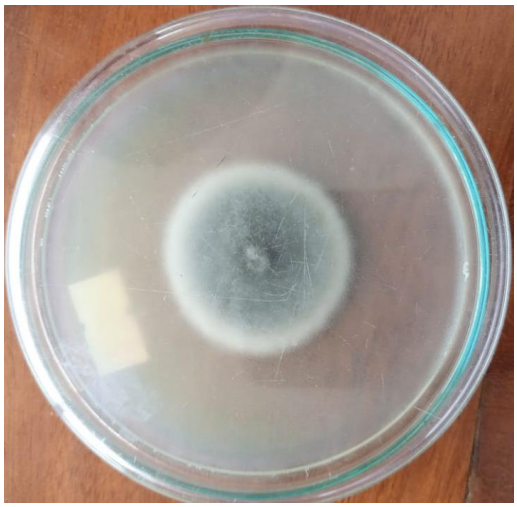

Figure 2. *Morphological appearance of A. alternata (MZ595006) isolated from K. africana found in Dar es Salaam, Tanzania.*

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Table 2. *Weight and concentration of crude extracts of endophytic fungi isolated from K. africana and P. niruri.*

K. africana	*A. alternata* (MZ595006)	EA	49.7	331.33
		n-H	20.9	139.33
	P. capitalensis (MZ595007)	EA	19.5	130.00
		n-H	11.9	79.33
P. niruri	*N. sphaerica* (MZ570966)	EA	100.6	670.67
		n-H	74.9	499.33
	M. guilliermondii (MZ570967)	EA	38.1	254.00
		n-H	98.2	654.67

EA = Ethyl acetate; n-H =normal Hexane

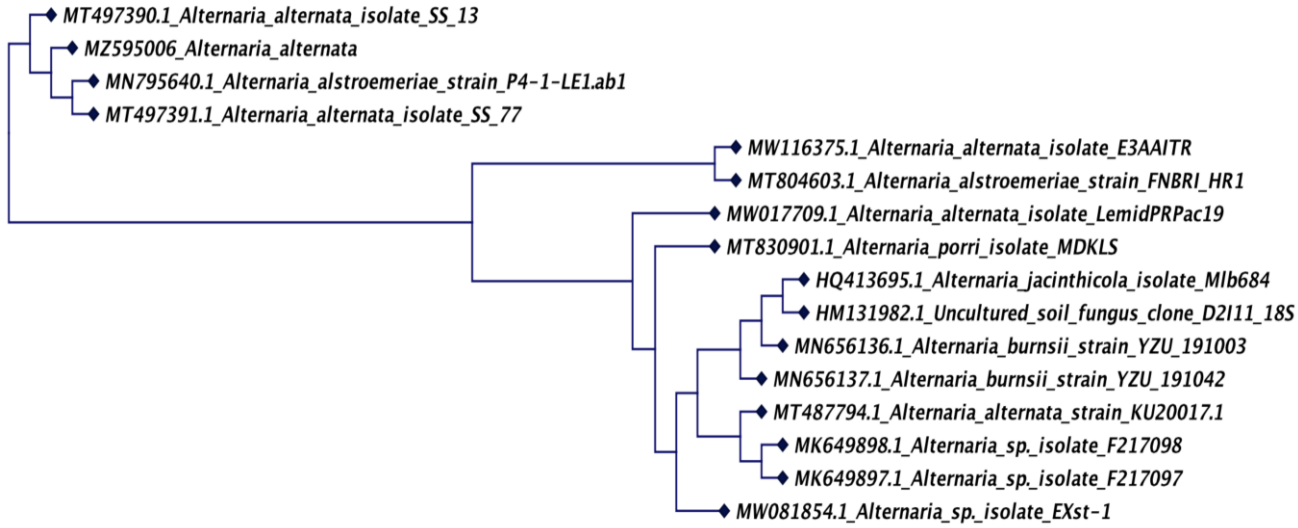

Figure 3. *A UPGM phylogenetic tree depicting relationship between A. alternata (MZ595006) and other fungi obtained from GenBank. Branches depicted on the tree had confidence values of ≥80% based on 1000 replications of Felsenstein's bootstrap method.*

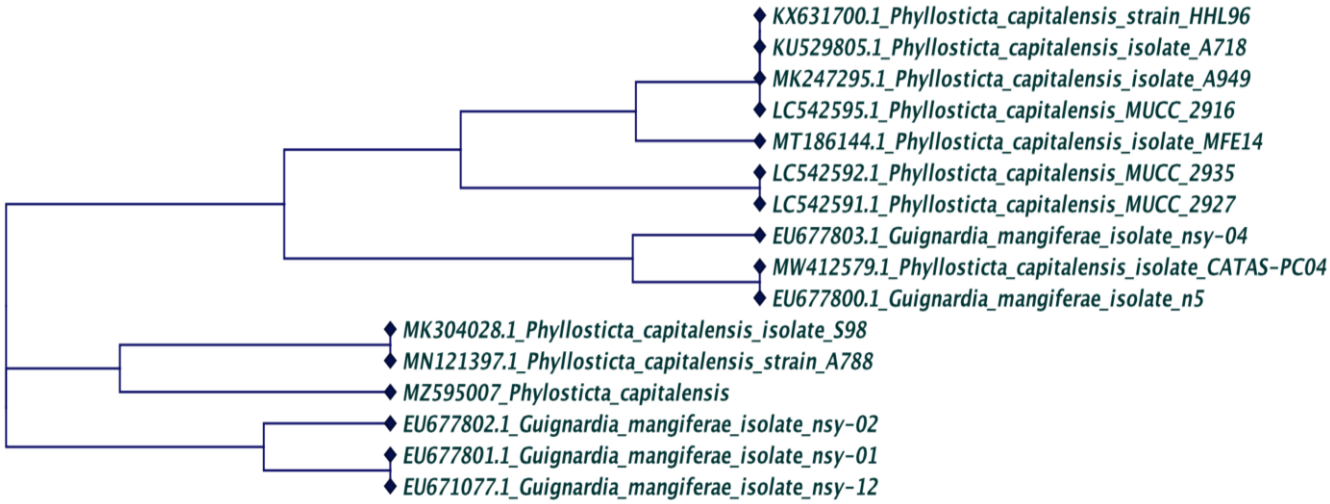

Figure 4. *A UPGM phylogenetic tree depicting relationship betwee P. capitalensis (MZ595007) and other fungi obtained from GenBank. Branches depicted on the tree had confidence values of ≥80% based on 1000 replications of Felsenstein's bootstrap method.*

Table 3. *The range of MIC (mg/ml) determined for the EA and n-H crude extracts of endophytic fungi against selected pathogenic microorganisms by the disc diffusion method performed at 37°C for 24 h.*

Test organism	EA *A.alternata*	n-H *P.capitalensis*	EA *N. sphaerica*	n-H *M.guilliermondii*	*M. guilliermondii*
S. aureus	331.3>MIC>165.7	79.3>MIC>39.7	83.8>MIC>41.9	127.0>MIC>63.5	327.3>MIC>163.7
S. typhi	41.4>MIC>20.7	9.9>MIC>5.0	335.3>MIC>167.7	127.0>MIC>63.5	163.7>MIC>81.8

Numbers are crude extracts concentration in mg/ml; MIC = minimum inhibition concentration EA = crude extract of endophytic fungi by Ethyl acetate; n-H = crude extract of endophytic fungi by normal Hexane

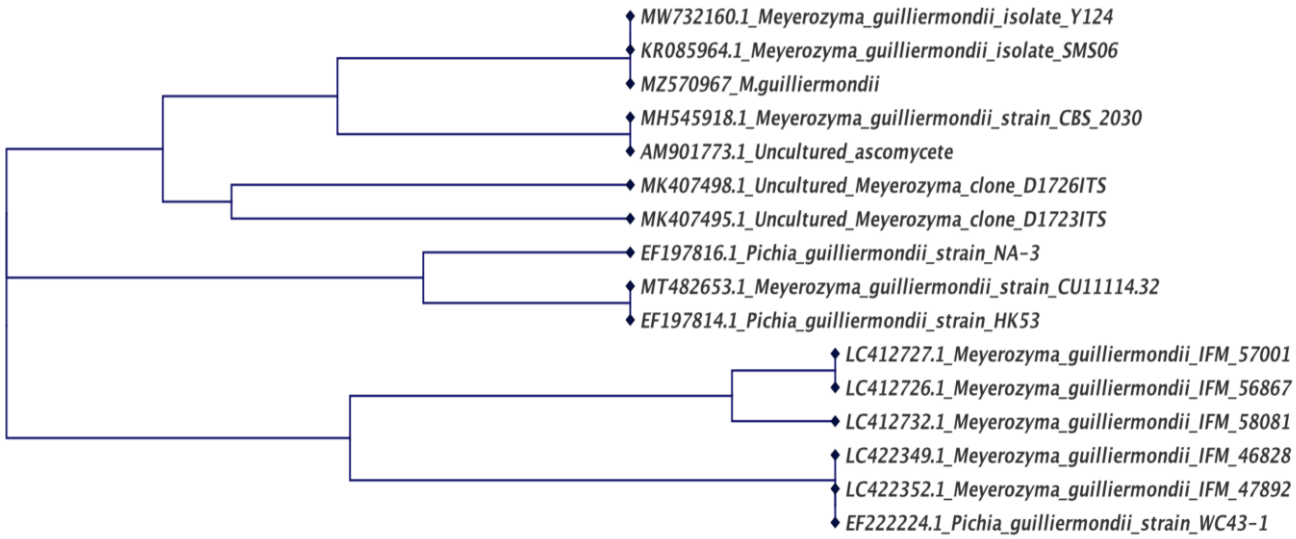

Figure 5. *A UPGM phylogenetic tree depicting relationship between M. guilliermondii (MZ570967) and other fungi obtained from GenBank. Branches depicted on the tree had confidence values of ≥80% based on 1000 replications of Felsenstein's bootstrap method.*

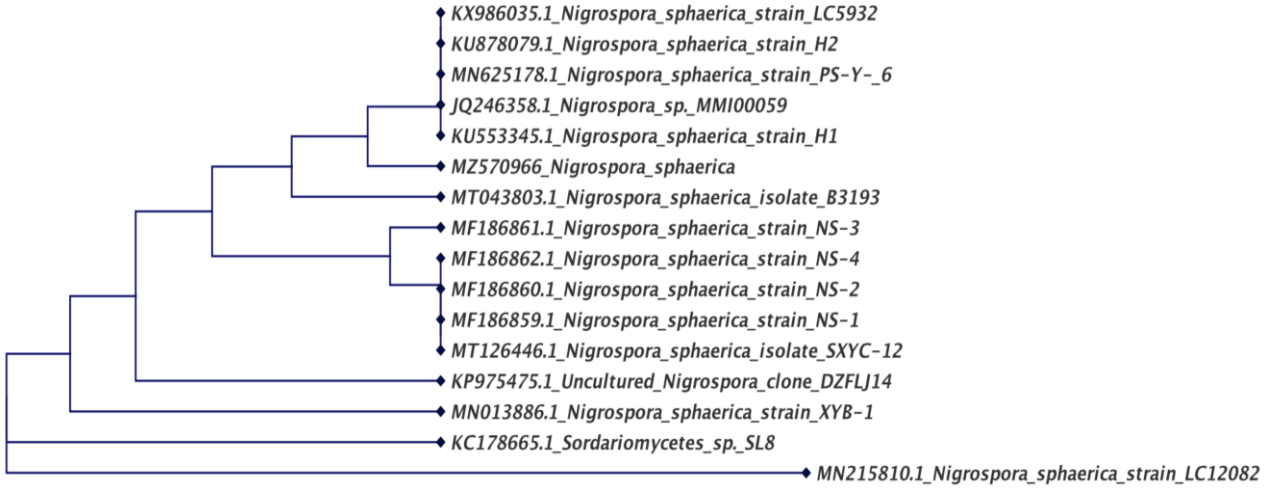

Figure 6. *A UPGM phylogenetic tree depicting the relationship between N. sphaerica (MZ570966) and other fungi obtained from GenBank. Branches depicted on the tree had confidence values of ≥80% based on 1000 replications of Felsenstein's bootstrap method.*

Following an initial screening, two isolates that showed the highest inhibition for each medicinal plant were selected for further analysis. *A. Alternate* and *P. capitalensis* were selected from *K. Africana*; whereas, *N. sphaerica* and *M. guilliermondii* were selected from *P. niruri*.

To the best of our knowledge, this is the first study to have been isolate *N. sphaerica* and *M. guilliermondii* from the leaves of *P. niruri;* However, the same isolates have been isolated and reported in other studies elsewhere (Huang et al., 2021; Ismail & Abd Razak, 2020; Valsalan & Mathew, 2020; Wu et al., 2018; Zhu et al., 2018). Taken as specific examples, Ayob et al., (2017) isolated *N. sphaerica* from *Heliocarpus americanus* in Brazil, and Pârvu et al., (2019) isolated *M. guilliermondii* from *Allium sativum* in Malaysia. Similarly, although *A. alternata* and *P. capitalensis* have been reported in other studies (Xu et al., 2021; Wang et al., 2021; Fan et al., 2020; Millar et al., 2003; Rodrigues et al., 2019; Sudharshana et al., 2019; Zhao et al., 2020), this is the first report of *A. alternata*, and *P. capitalensis* to be isolated from *K. Africana*. For example, Xu et al., (2021), isolated *P. capitalensis* from hypocotyls of the Chinese mangrove *Bruguiera sexangula*.

Endophytic fungi crude extracts

Although there was high variability of crude extract concentrations among isolates, generally, the relationship between a type of extracting solvent and the weight of crude extracts was observed as well (Table 2). The range of weight of crude extracts using ethyl acetate (EA) as solvent was between 130mg/ml and 670mg/ml for *P. capitalensis* and *N. sphaerica*, respectively; whereas, the range of weight of crude extracts using n-Hexane (n-H) as solvent was between 79.33mg/ml and 654.67mg/ml for *P.capitalensis* and *M. guilliermondii*, respectively. Regardless of the type of extracting solvent used, *P. capitalensis* generated the lowest weight of crude extracts. Also, ethyl acetate generated a higher mass of crude extracts in all isolates except for *M. guilliermondii,* where the n-H crude extract was higher (654.67mg/ml compared to 254mg/ml of EA). Variation in a mass of crude extracts may be explained by the composition of secondary metabolites generated by endophytic fungi during mass cultivation, and a type of extracting solvent used. It is well known that n-Hexane and ethyl acetate differ in polarity. The latter is mild polar and the former is non-polar (Asmah et al., 2020; Asmilia et al., 2020; Jiyane et al., 2018), suggesting that isolates were able to produce polar and non-polar secondary metabolites at different levels (Table 2).

Antimicrobial activity analysis

Assessment of antimicrobial activity of the crude extracts was by disc diffusion method, and results of a zone of inhibition (ZOI) and minimum inhibition concentration (MIC) are depicted in Table 1 and Table 3, respectively. Overall, there was high variability in the antimicrobial activity of crude extracts, which is corroborated by a wide range (8.5-23mm) of ZOI. The highest antimicrobial activity against *S. aureus* was EA crude extract of *M. guilliermondii*. For *S. typhi*, the highest antimicrobial activity was found in EA crude extract of *A. alternata*, although when compared to antimicrobial activity depicted by n-H crude extract is not statistically different ($P>0.005$).

Extracting solvent influenced the antimicrobial activity of isolates because some isolates had antimicrobial activity in one solvent and not the other. For example, *P. capitalensis* did not show antimicrobial activity when EA is used as a solvent; however, n-Hexane crude extract of *P. capitalensis* had antimicrobial activity against *S. aureus and S. typhii* (Table 1), suggesting that non-polar secondary metabolites generated by *P. capitalensis* may have antimicrobial activity. It was not a surprise that *E.coli* was resistant to all crude extracts because this has been a common trend in most reports. It is well known that Gram-negative bacteria possess lipopolysaccharide protein and peptidoglycan layer that makes their cell wall more complex as compared to Gram-positive bacteria (Dik et al., 2018), and this may explain why *E.coli* was relatively resistant to all crude extracts reported in the present study. *eight and concentration of crude extracts of endophytic fungi isolated from K. africana and P. niruri.*

The MIC values of the tested pathogens *S. aureus* and *S. typhi* ranged from 5.0 mg/mL to 335.3 mg/mL.

Conclusion

In the present study, endophytic fungi isolates (*Nigrospora sphaerica*, *Meyerozyma guilliermondii*, *Alternaria alternata*, and *Phyllosticta capitalensis)* from leaves of medicinal plants (*K. africana* and *P. niruri*) were isolated, characterized and tested for antimicrobial activities against selected pathogenic microorganism (*E. coli* (Gram-negative), *S. typhi* (Gram-negative), and *S. aureus* (Gram-positive)). Results of the present study corroborate the influence of extracting solvent on the effectiveness of crude extract of isolates against pathogen microorganisms that have antimicrobial activities. Findings demonstrated the potential of endophytes from medicinal plants for medical applications, and therefore further investigation may lead to the discovery of novel bioactive compounds potent to resistant infectious agents.

Declaration of Competing Interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Ab Rahman NH, Abd Aziz SN. 2020. Challenges in Implementing Inclusive Development Concept in Sustainable Development Goals 2030. J Undang-undang dan Masyarakat, 26:15–25.

Abba C, Eze P, Okoye F, Eboka C. 2015. Cytotoxicity and antimicrobial activities of secondary metabolites produced by endophytic fungi isolated from *Gongronema latifolia* and *Loranthus micrantus*. Planta Med, 81(16). DOI:10.1055/s-0035-1565482.

Ali EAM, Abdel-Rahman TMA, Ebrahim HA.2019. Cytotoxicity and antimicrobial activity of alkaloids extracted from *Catharanthus roseus* associated endophytic fungi. Malaysian J Microbiol, 15(1): 16-23. DOI:10.21161/mjm.115617.

Asmah N, Suniarti DF, Margono A, Mas'ud ZA, Bachtiar EW. 2020. Identification of active compounds in ethyl acetate, chloroform, and N-hexane extracts from peels of Citrus aurantifolia from Maribaya, West Java, Indonesia. J Adv Pharm Technol Res., 11(3): 107–12.

Asmilia N, Fahrimal Y, Abrar M, Rinidar R. 2020. Chemical Compounds of Malacca Leaf (*Phyllanthus emblica*) after Triple Extraction with N-Hexane, Ethyl Acetate, and Ethanol. The Sci World J, ID 2739056 https://doi.org/10.1155/2020/2739056.

Ayob FW, Simarani K, Zainal Abidin N, Mohamad J. 2017. First report on a novel *Nigrospora sphaerica* isolated from Catharanthus roseus plant with anticarcinogenic properties. Microb Biotechnol, 10(4): 926–32.

Aziz MA, Khan AH, Adnan M, Izatullah I. 2017. Traditional uses of medicinal plants reported by the indigenous communities and local herbal practitioners of Bajaur Agency, Federally Administrated Tribal Areas, Pakistan. J Ethnopharmacol, 198: 268–281. doi: 10.1016/j.jep.2017.01.024.

Bamola N, Verma P, Negi CA.2918. Review on Some Traditional Medicinal Plants. Int J of Life-Sci Sci Res, 4(1).

Borgmann S, Gruber H, Beyser K, Abel R, Reber A, Lindauer A, et al. 2008. Gel electrophoresis of polymerase chain reaction (PCR) products improves GenoType MRSA Direct PCR analysis in clinical practice. J Hosp Infect, 68(3): 276–277.

Burgdorf RJ, Laing MD, Morris CD, Jamal-Ally SF. 2014. A procedure to evaluate the efficiency of surface sterilization methods in culture-independent fungal endophyte studies. Braz J Microbiol, 45(3): 977–983.

Catarino S, Duarte MC, Costa E, Carrero PG, Romeiras MM. 2019. Conservation and sustainable use of the medicinal *Leguminosae* plants from Angola. PeerJ, doi: 10.7717/peerj.6736. eCollection

Christina A, Christapher V, Bhore S. 2013. Endophytic bacteria as a source of novel antibiotics: An overview. Pharmacog Rev, 7(13):11–16 doi: 10.4103/0973-7847.112833.

Dadgostar P. 2019. Antimicrobial Resistance: Implications and Costs. Infect Drug Resist, 12: 3903–3910. doi: 10.2147/IDR.S234610.

Davies H, Russell J, Varghese A, Mealing S, Holmes H, Woods B. 2020. PSU3 A Framework for Evaluating the Health and Cost Implications of Antimicrobial Resistance in the Context of Surgical Procedures. Value Health, 23: 738–738.

Dhar ML, Dhar MM, Dhawan BN, Mehrotra BN, Ray C. 1968. Screening of Indian plants for biological activity: I. Indian J Exp Biol, 6(4): 232–247.

Dik DA, Fisher JF, Mobashery S. 2018. Cell-Wall Recycling of the Gram-Negative Bacteria and the Nexus to Antibiotic Resistance. Chem Rev, 2018;118(12): 5952–5984.

Essack SY, Desta AT, Abotsi RE, Agoba EE. 2017. Antimicrobial resistance in the WHO African region: current status and roadmap for action. J Public Health, 39(1): 8–13. https://doi.org/10.1093/pubmed/fdw015.

Fan M, Chen X, Luo X, Zhang H, Liu Y, Zhang Y, et al. 2020. Diversity of endophytic fungi from the leaves of *Vaccinium dunalianum*. Lett Appl Microbiol, 71(5): 479–89.

García A, Rhoden SA, Rubin Filho CJ, Nakamura CV, Pamphile JA.2012. Diversity of foliar endophytic fungi from the medicinal plant *Sapindus saponaria* L. and their localization by scanning electron microscopy. Biol Res, 45(2): 139–48. doi: 10.4067/S0716-97602012000200006.

Gómez OC, Luiz JHH. 2018. Endophytic fungi isolated from medicinal plants: future prospects of bioactive natural products from Tabebuia/Handroanthus endophytes. Appl Microbiol Biotechnol, 102(21): 9105-9119. doi: 10.1007/s00253-018-9344-3.

Harborne JB. 1993. Glossary of Indian Medicinal Plants with Active Principles. Phytochem, 33(5): 1279.

Harris JM. 2001. A survey of Sustainable Development – Social and Economic Dimensions. London: Island Press, Environ Manag and Health, 12(5):529–31.

Hayhoe EJ, Palombo EA. 2013. Screening for antibacterial, antifungal, and anti quorum sensing activity. Methods Mol Biol, 1055: 219–25.

Huang Y, Sun C, Guan X, Lian S, Li B, Wang C. 2021. Biocontrol efficiency of *Meyerozyma guilliermondii* Y-1 against apple postharvest decay caused by *Botryosphaeria dothidea* and the possible mechanisms of action. Int J Food Microbiol, 2; 338:108957.

Ismail SI, Abd Razak NF. 2020. First Report of Nigrospora sphaerica causing leaf spot on watermelon (*Citrullus lanatus* L.) in Malaysia. Plant Dis https://doi.org/10.1094/PDIS-07-20-1491-PDN.

Jaiyesimi R.2016. The Challenge of Implementing the Sustainable Development Goals in Africa: The Way Forward. Afr J Reprod Health, 20(3): 13–18.

Jiyane PC, Tumba K, Musonge P. 2018. Optimisation of Croton gratissimus Oil Extraction by n-Hexane and Ethyl Acetate Using Response Surface Methodology. J Oleo Sci, 67(4): 369–377.

Kamalam DS. 2017. Transforming Our World: The 2030 Agenda for Sustainable Development. Pond J Nurs, 11(2): 42–49.

Kimondo J, Miaron J, Mutai P, Njogu P. 2015. Ethnobotanical survey of food and medicinal plants of the Ilkisonko Maasai community in Kenya. J Ethnopharmacol, 175:463–469 doi: 10.1016/j.jep.2015.10.013.

Leopold SJ, van Leth F, Tarekegn H, Schultsz C.2014. Antimicrobial drug resistance among clinically relevant bacterial isolates in sub-Saharan Africa: a systematic review. J Antimicrob Chemother, 69(9): 2337-53. doi: 10.1093/jac/dku176.

Mbuni YM, Wang S, Mwangi BN, Mbari NJ, Musili PM, Walter NO, et al. 2020. Medicinal Plants and Their Traditional Uses in Local Communities around Cherangani Hills, Western Kenya. Plants, 9(3): 331. https://doi.org/10.3390/plants9030331.

Millar BC, Xu J, Walker MJ, Boyd NAM, McMullan R, Moore JE. 2003. Isolation of *Alternaria alternata* from an emollient cream: implications for public health. Mycopathologia, 156(4): 273–277.

Mio C, Panfilo S, Blundo B. 2020. Sustainable development goals and the strategic role of business: A systematic literature review. Business strategy Environ., 29(8): 3220–3245.

Murray G.1884. Vergleichende Morphologie und Biologie der Pilze, Mycetozoen und Bacterien. Annals and magazine of natural history, 14(83): 363–366.

Nankaya J, Nampushi J, Petenya S, Balslev H. 2020. Ethnomedicinal plants of the Loita Maasai of Kenya. Environ Dev Sustain, 22(3): 2569–2589 https://doi.org/10.1007/s10668-019-00311-w.

Ncube B, Finnie JF, Van Staden J. 2012. Quality from the field: The impact of environmental factors as quality determinants in

medicinal plants. S Afr J Botan., 82:11–20. https://doi.org/10.1016/j.sajb.2012.05.009.

Nondo R, Erasto P, Moshi M, Zacharia A, Masimba P, Kidukuli A. 2016. In vivo antimalarial activity of extracts of Tanzanian medicinal plants used for the treatment of malaria. J Adv Pharm Technol Res, 7(2): 59-63 doi: 10.4103/2231-4040.179748.

Oladele A, Adewunmi C. 2008. Medicinal plants used in the management of malaria among the traditional medicine practitioners (TMPs) in South-Western Nigeria. Afric J Infect Dis, 2(1): 51.

Pârvu M, Moț CA, Pârvu AE, Mircea C, Stoeber L, Roșca-Casian O, et al. 2019. *Allium sativum* Extract Chemical Composition, Antioxidant Activity and Antifungal Effect against *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* Causing Onychomycosis. Molecules, 31:24(21).

Perovic O, Ondoa P, Laxminarayan R, Ihekweazu C, Varma JK, Park BJ, et al. 2018. Africa Centres for Disease Control and Prevention's framework for antimicrobial resistance control in Africa. Afr J Lab Med, 7(2):1–4.

Polat R, Cakilcioglu U, Satıl F. 2013. Traditional uses of medicinal plants in Solhan (Bingöl—Turkey). J Ethnopharmacol, 148(3): 951–963. doi: 10.1016/j.jep.2013.05.050.

Rodrigues CM, Takita MA, Silva NV, Ribeiro-Alves M, Machado MA. 2019. Comparative genome analysis of *Phyllosticta citricarpa* and *Phyllosticta capitalensis*, two fungi species that share the same host. BMC Genomics, 20(1): 554.

Saldierna Guzmán JP, Nguyen K, Hart SC. 2020. Simple methods to remove microbes from leaf surfaces. J Basic Microbiol, 60(8): 730–734.

Santos CM dos, Ribeiro ADS, Garcia A, Polli AD, Polonio JC, Azevedo JL, et al. 2019. Enzymatic and Antagonist Activity of Endophytic Fungi from *Sapindus saponaria* L. (*Sapindaceae*). Acta Biol Colomb, 24(2): 322–330.

Santos IP, Silva LC, Silva MV, Araújo JM, Cavalcanti MD, Lima VL. 2015. Antibacterial activity of endophytic fungi from leaves of *Indigofera suffruticosa* Miller (Fabaceae). Frontiers in Microbiol, 7;6: 350. https://doi.org/10.3389/fmicb.2015.00350.

Shrestha P, Cooper BS, Coast J, Oppong R, Thuy NDT, Phodha T, et al. 2018. Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. Antimicrob Resist Infect Control, 7: 98. doi: 10.1186/s13756-018-0384-3.

Singh-Moodley A, Perovic O, Ismail H. 2018. An overview of antimicrobial resistance surveillance among healthcare-associated pathogens in South Africa. Afr J Lab Med, 7(2): 1–6.

Sudha V, Govindaraj R, Baskar K, Al-Dhabi NA, Duraipandiyan V. 2016. Biological properties of Endophytic Fungi. Braz. Arch. Biol. Technol, https://doi.org/10.1590/1678-4324-2016150436.

Sudharshana TN, Venkatesh HN, Nayana B, Manjunath K, Mohana DC. 2019. Anti-microbial and anti-mycotoxigenic activities of endophytic *Alternaria alternata* isolated from *Catharanthus roseus* (L.) G. Don.: molecular characterisation and bioactive compound isolation. Mycology, 10(1): 40–48.

Tugume P, Kakudidi EK, Buyinza M, Namaalwa J, Kamatenesi M, Mucunguzi P, et al. 2016. Ethnobotanical survey of medicinal plant species used by communities around Mabira Central Forest Reserve, Uganda. J Ethnobiol Ethnomed, 12(5): 5-13.

Valsalan R, Mathew D. 2020. Draft genome of *Meyerozyma guilliermondii* strain vka1: a yeast strain with composting potential. J Genet Eng Biotechnol, 18(1): 54.

Vieira MLA, Johann S, Hughes FM, Rosa CA, Rosa LH.2014. The diversity and antimicrobial activity of endophytic fungi associated with medicinal plant B*accharis trimera (Asteraceae*) from the Brazilian savannah. Canad J Microbiol, 60(12): 847–856. doi: 10.1139/cjm-2014-0449.

Wang JT, Ma ZH, Wang GK, Xu FQ, Yu Y, Wang G., et al. 2021. Chemical constituents from plant endophytic fungus *Alternaria alternata*. Nat Prod Res, *35*(7): 1199–1206 https://doi.org/10.1080/14786419.2019.1639699

Wu Z, Xie Z, Wu M, Li X, Li W, Ding W, et al. 2018. New Antimicrobial Cyclopentenones from *Nigrospora sphaerica* ZMT05, a Fungus Derived from Oxya chinensis Thunber. J Agric Food Chem, 30;66(21): 5368–5372.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In PCR protocols a guide to methods and applications, Academic Press, San Diego, pp. 315–322.

Xu Z, Xiong B, Xu J. 2021. Chemical investigation of secondary metabolites produced by mangrove endophytic fungus *Phyllosticta Capitalensis*. Nat Prod Res, 35(9): 1561–1565. https://doi.org/10.1080/14786419.2019.1656624

Xue L, Weng L, Yu H.2018. Addressing policy challenges in implementing Sustainable Development Goals through an adaptive governance approach: A view from transitional China. Sustainable Dev, 26(2): 150–158.

Zhang HW, Song YC, Tan RX. 2006 Biology and chemistry of endophytes. Nat Prod Rep, 23(5): 753–771.

Zhao N, Yang J, Meng Q, Fang X, Zhang W, Li L, et al. 2020. First Report of Alternaria alternata Causing Leaf Spot on *Avena nuda* in Zhangbei, China. Plant Dis, 15(1). https://doi.org/10.1094/PDIS-03-20-0639-PDN.

Zhu X, Chen J, Zhu S, He Y, Ding W, Li C. 2018. Two new compounds from *Nigrospora sphaerica* ZMT05, a fungus derivated from *Oxya chinensis* Thunber. Nat Prod Res, 32(20): 2375–2381.