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Preliminary Investigation on the Extracts of the Fungal Endophytes from *Mitracarpus scaber* for Antimicrobial Activity

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Abstract
Endophytes have been shown to possess the capacity to produce novel bioactive compounds including those with antimicrobial properties. The aim of this research is to determine the antimicrobial property of the fungal endophytes isolated from *Mitracarpus scaber* (Rubiaceae) Zucc. The leaves of *M. scaber* were freed of debris under running water, sterilized, the lamina and midrib of the leaves cut into smaller fragments and then inoculated unto Sabouraud's dextrose agar (SDA) plates from where fungal endophytes grow, which were purified by repeated sub culturing and these pure fungal endophytes were then subjected to solid state fermentation using rice medium. Ethyl acetate was then used to extract the product of fermentation and extracts were concentrated by means of a rotary evaporator set at 55^0C. The concentrated extracts were then screened for antimicrobial activity against some common test organisms such as *Aspergillus niger*, *Candida albicans*, *Bacillus subtillis*, *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli*. Exactly five (5) fungal endophytes were isolated from the leaves of *M. scaber* and labeled appropriately. The endophytic fungal extracts elicited antimicrobial activities down the concentration gradient against the test isolates. This research reveals that *Mitracarpus scaber* elicits antimicrobial activities and could be a potential candidate for development into antibiotics that could be used in therapy.

Keywords: Antimicrobial activity, Extracts, Fungal endophytes, *Mitracarpus scaber*, Preliminary investigation

Introduction

Endophytic fungi also known as fungal endophytes have been shown to inhabit the tissues of plants without causing any symptoms of disease (Bacon &White, 2000). These endophytes are said to have mutualistic relationship with their host plants. The host plants provide nutrients for the endophytes, while the endophytes protect their hosts from insect attacks as well as conferring the plant with the ability to withstand a number of adverse climatic conditions (Saikkonen et al., 1998; Yang et al., 1994; Siegel et al., 1985). These endophyes could exist as bacteria or fungi; however, the more frequently isolated one is the fungal endophytes (Staniek et al., 2008). Studies have shown that most endophytic fungi could be a source of novel bioactive compounds that could find great use in medicine, agriculture and in the industries (Akpotu et al., 2017b; Deshmukh et al., 2015; Suryanayanan et al., 2009).

Medicinal plants such as *Mitracarpus scaber* (Rubiaceae) Zucc have been used from time immemorial to treat health issues in various towns in Nigeria and other sub-Saharan African countries (Abere et al., 2007). It is a perennial annual herb usually growing as high as 30cm tall or in some cases shorter. Its indigenous names in Nigeria include Obuobwa (Igbo), Gududal (Hausa) and Irawo lle (Yoruba) (Abere et al., 2007; Gbile, 1984; Hutchinson & Dalziel, 1948). The plant had found usefulness in folkloric medicine for the treatment of venereal diseases, toothaches and leprosy. It is believed that the biological activities elicited by this medicinal plant may be due to the endophytes inhabiting it (Strobel & Daisy, 2003).

This study therefore, was carried out to obtain the endophytic fungal extracts in this medicinal plant and to determine their antimicrobial potentials.

Materials and Methods

Study area

The leaves of apparently healthy *M. scaber* were harvested from a wet, slightly stony and wind prone area by the road sides on Ago-Iwoye, 25 km from Sagamu in Ogun State, South-Western Nigeria located on latitude 3.92^0N, 6.95^0E, with an annual rainfall ranges from 1250 to 2190 mm commencing from mid-March to mid-November and mean annual minimum and maximum temperatures of about 20^0C and 30^0C respectively (Meteo consult Retrieved 18th May 2021)

Plant sampling and Isolation of the fungal endophytes

The leaves were freed of debris and subsequently treated to remove ectophytic fungi and other unwanted organic matter by the methods described by earlier workers (Akpotu et al., 2017a; Arnold et al., 2000). Small sized lamina and midribs (about 1.5 cm) of the leaves were inoculated onto Sabouraud's dextrose agar (SDA) plates impregnated with chloramphenicol to inhibit the growth of contaminants of bacterial origin. The plates were made tamper proof and then incubated at 27^0C for four (4) days. The isolates were then sub-cultured to obtain pure isolates.

Fermentation of the fungal endophytes and extraction of the products of fermentation

This was carried out using the methods of Akpotu et. al. (2017a) and Onyegbule et. al. (2014) with a little modification. Briefly, the pure fungal isolates were inoculated onto rice medium in 1000 mL conical flasks. The flasks were allowed to stand undisturbed for 28 days. The products of fermentation were then extracted using 500 mL of the solvent, ethyl acetate, filtered using filter paper and the extracts were finally concentrated using rotary evaporator set at 55^0C.

Antimicrobial assay

This was carried out using a modified method of Akpotu et. al. (2017a). Briefly, the concentrated extracts were evaluated for their antimicrobial activities by challenging them with test organisms such as *Salmonella typhi*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtillis*, *Aspergillus fumigatus* and *Candida albicans* obtained from the Pharmaceutical Microbiology Laboratory of Olabisi Onabanjo University and standardized to 0.5 McFarland's standard. The solvent, Dimethyl sulphoxide (DMSO), was used to prepare a stock solution of 1 mg/mL from which it was further diluted by two-fold serial dilution into concentrations of 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL and 0.0625 mg/mL respectively. They were then dispensed into 6mm holes dug out from a freshly prepared Mueller Hinton agar (MHA) plates (for those impregnated with bacterial isolates) and Sabouraud's dextrose agar (SDA) plates (for those impregnated with fungal isolates) respectively. They were then incubated at 37^0C (bacterial isolates) and 27^0C (fungal isolates). The controls of gentamicin (for bacterial isolates) and fluconazole (for fungal isolates) were dispensed into the central holes. The DMSO was used as negative control. The entire plates were then incubated for 24 hours at 37^0C (for bacterial test organisms) and 48 hours at 27^0C (for fungal test organisms). The inhibitory zone diameters (IZDs) were finally measured and recorded.

Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 22.0 was the package employed in the data analysis by one way analysis of variance (ANOVA) and significance taken at $p<0.05$.

Results

Five (5) endophytic fungi were isolated from the leaves of *M. scaber*; two (2) from the lamina (LA1 and LA2) and three (3) from the midrib (MR1, MR3 and MR4). In Table 1, we observe that the endophytic fungal extracts from MR1 showed antimicrobial activity against the different test organisms and in some cases showing higher inhibitory zone diameters (IZD) than the standard antibiotics.

Table 1: The Inhibitory Zone Diameter of MR1 extract against the clinical isolates

Inhibition zone diameter (mm)							
Test Isolates	**MR1 Extract Concentration (mg/mL)**						
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**Gentamicin (10µg/mL)**	**DMSO**
ST	19.3	18.4	17.5	15.3	13.5	20.7	0.0
EC	20.7	18.6	17.7	16.5	15.5	17.0	0.0
SA	20.7	17.7	16.5	15.6	15.0	18.7	0.0
BS	19.5	19.4	18.6	17.5	15.0	19.0	0.0
					Fluconazole (10µg/mL)	**DMSO**	
CA	22.0	20.0	19.4	18.3	17.0	18.0	0.0
AN	21.0	19.0	16.5	15.5	15.4	18.0	0.0

ST: *Salmonella typhi*, **EC**: *Escherichia coli*, **BS**: *Bacillus subtillis*, **SA:** *Staphylococcus aureus*, **CA**: *Candida albicans*, **AN**: *Aspergillus niger*, **DMSO**: Dimethyl sulfoxide.

The MR3 fungal extracts showed varying antimicrobial property against the test organisms as shown in Table 2 with the highest IZD usually shown at 1mg/mL and this drops down the concentration gradient

Table 2: The Inhibitory zone diameter of MR3 extract against the test organisms

Inhibition zone diameter (mm)							
Test organisms	**MR3 Extract Concentration (mg/mL)**						
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**Gentamicin (10µg/mL)**	**DMSO**
ST	19.5	18.4	18.0	17.0	15.0	24.3	0.0
EC	21.5	20.2	19.2	17.0	15.0	20.0	0.0
SA	22.0	21.0	19.1	18.0	17.0	19.0	0.0
BS	20.0	18.1	17.5	15.5	15.0	18.0	0.0
					Fluconazole (10µg/mL)	**DMSO**	
CA	21.0	20.5	17.5	16.0	14.0	18.0	0.0
AN	22.0	21.0	20.0	19.0	16.0	18.0	0.0

ST: *Salmonella typhi*, **EC**: *Escherichia coli*, **BS**: *Bacillus subtillis*, **SA:** *Staphylococcus aureus*, **CA**: *Candida albicans*, **AN**: *Aspergillus niger*, **DMSO**: Dimethyl sulfoxide.

Just like in the earlier tables, in Table 3, the MR4 fungal extracts showed antimicrobial activity against the entire test organisms with their zones of inhibition decreasing down the concentration gradient

Table 3: The Inhibitory zone diameter of MR4 extract against the test organisms

Inhibition zone diameter (mm)							
Test organisms	**MR4 Extract Concentration (mg/mL)**						
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**Gentamicin (10µg/mL)**	**DMSO**
ST	21.7	19.0	15.0	13.0	15.0	18.3	0.0
EC	21.5	18.5	18.2	15.0	14.5	17.0	0.0
SA	19.0	18.0	16.5	16.0	14.4	17.0	0.0
BS	22.0	19.3	17.5	17.0	15.1	15.0	0.0
						Fluconazole (10µg/mL)	**DMSO**
CA	20.0	18.1	16.5	16.0	14.0	18.0	0.0
AN	17.0	17.0	16.0	14.5	14.0	18.0	0.0

ST: *Salmonella typhi*, **EC**: *Escherichia coli*, **BS**: *Bacillus subtillis*, **SA:** *Staphylococcus aureus*, **CA**: *Candida albicans*, **AN**: *Aspergillus niger*, **DMSO**: Dimethyl sulfoxide.

At 1mg/mL concentration, the fungal extracts from LA1 elicited higher antimicrobial activity (22.5mm) against the *E. coli* test organism than the standard antibiotic (18.0mm) as shown in Table 4.

Table 4: The Inhibitory zone diameter of LA1 extract against the test organisms

Inhibition zone diameter (mm)							
Test organisms	**LA1 Extract Concentration (mg/mL)**						
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**Gentamicin (10µg/mL)**	**DMSO**
ST	18.1	18.1	17.0	16.0	15.0	18.0	0.0
EC	22.5	20.5	18.2	17.0	17.0	18.0	0.0
SA	19.0	17.5	17.1	16.0	14.4	17.0	0.0
BS	22.0	22.0	21.5	17.1	15.0	17.0	0.0
						Fluconazole (10µg/mL)	**DMSO**
CA	21.0	20.0	20.0	18.1	17.0	18.0	0.0
AN	20.0	19.3	18.1	17.0	16.0	18.0	0.0

ST: *Salmonella typhi*, **EC**: *Escherichia coli*, **BS**: *Bacillus subtillis*, **SA:** *Staphylococcus aureus*, **CA**: *Candida albicans*, **AN**: *Aspergillus niger*, **DMSO**: Dimethyl sulfoxide.

The endophytic fungal extracts from LA2 showed varying degree of antimicrobial activity against the test isolates, with their IZDs decreasing down the concentration gradient.

Table 5: The Inhibitory zone diameter of LA2 extract against the test organisms

Inhibition zone diameter (mm)							
Test organisms	**LA2 Extract Concentration (mg/mL)**						
	1	**0.5**	**0.25**	**0.125**	**0.0625**	**Gentamicin (10µg/mL)**	**DMSO**
ST	17.5	17.0	16.0	16.0	15.0	20.0	0.0
EC	22.5	20.5	19.2	18.0	17.0	20.0	0.0
SA	21.0	20.0	19.1	19.1	17.0	19.0	0.0
BS	20.0	20.0	18.5	17.0	15.0	18.0	0.0
						Fluconazole (10µg/mL)	**DMSO**
CA	22.0	20.5	20.1	18.0	17.0	20.0	0.0
AN	21.0	19.5	19.1	18.0	16.0	19.0	0.0

ST: *Salmonella typhi*, **EC**: *Escherichia coli*, **BS**: *Bacillus subtillis*, **SA:** *Staphylococcus aureus*, **CA**: *Candida albicans*, **AN**: *Aspergillus niger*, **DMSO**: Dimethyl sulfoxide.

Discussion

Five (5) fungal endophytes were isolated from the leaves of *M. scarber*; two (2) from the lamina and three (3) from the midrib. This is in tandem with earlier researches that fungal endophytes could be isolated from the leaves of medicinal plants inoculated unto a suitable culture medium (Akpotu et al., 2017b; Radu & Kqueen, 2002; Kumaresan & Suryanarayanan, 2001). This lends further credence that fungal endophytes could actually be isolated from plants growing in a difficult ecological niche, as in the case of our sample location, where the plant was obtained by the roadside from a wet, slightly stony area and with the leaves being pitted regularly by strong winds (Arnold et al., 2000). A very important property of the fungal endophytes are their ability to produce a variety of bioactive molecules that could elicit antibacterial, antifungal, antiviral or antioxidant properties said to have the capacity to protect their host plants against pathogens thereby prolonging their existence (Tan & Zou, 2001).

All the endophytic fungal extracts showed antimicrobial property against the test isolates evaluated, whose potencies decreases down the concentration gradient (i.e. from concentrations of 1 mg/mL to 0.0625 mg/mL) (Tables 1 to 5). This is consistent with earlier studies (Akpotu et al., 2017a; Onyegbule et al., 2014; Bacon & White, 2000). The inhibitory zone diameters (IZDs) range from 13.5 mm - 22.5 mm (bacterial test organisms) and 14.0 mm – 22.0 mm (fungal test organisms) of which the highest IZD of 22.5 mm was given by the LA1 fungal extracts against *E. coli* test organism at a concentration of 1 mg/mL (Table 4) and the lowest IZD of 13.5 mm given by the MR1 fungal extracts against the *S. typhi* test organism at a concentration of 0.0625 mg/mL (Table 1). More of the fungal extracts elicited greater activity against the fungal test organisms than the bacteria test organisms and in some cases given IZDs higher than the standard antibiotics. This phenomenon has equally been observed (Selim *et al*., 2012).

Conclusion

Fungal endophytes reside asymptomatically in their host plant tissues in a relationship best described as mutualistic. Endophytic fungi are important lead for the development of novel bioactive compounds with various useful modes of action including antimicrobial. The endophytic fungal extracts evaluated against the test organisms elicited varying antimicrobial activities and in some cases giving inhibitory zone diameters greater than the standard antibiotic used in this study. Further studies would therefore be carried out using High performance Liquid Chromatography coupled to diode array detector (HPLC-DAD) to determine the actual biomolecule responsible for the antimicrobial activity elicited by these fungal extracts, isolate them and finally formulated into a suitable dosage form to further increase the antibiotics available for clinical use- human and veterinary practice.

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