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Bioprospecting of endophytes isolated from selected Zimbabwean medicinal plants

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ABSTRACT

Native medicinal plants have been traditionally used to treat various bacterial infections, fungal infections, cancers, viral infections and cardiovascular diseases. Endophytes residing inside medicinal plants are capable of producing therapeutically important bioactive compounds. The study aimed to evaluate the antimicrobial activity of endophytic crude extracts against clinical isolates of Staphylococcus aureus and Escherichia coli. Acetone, methanol and ethyl acetate were used to extract the endophytic compounds. Acetone, methanol and ethyl acetate extracts from 24 endophytes were evaluated for antimicrobial efficacy against E. coli and S. aureus by agar well diffusion assay. Amplification of 16 S rRNA gene was used to confirm that the endophytes were bacteria. Whereas, M13 RAPDs were used to differentiate the endophytic bacteria, respectively. The antagonistic test was used to evaluate the interspecies interaction of endophytes in a single Petri dish. Qualitative thin-layer chromatography was used to identify the compounds present in the ethyl acetate crude extract. The 16 S rRNA PCR amplicons confirmed that nine of the most prolific endophytes are bacteria. M13 RAPDs analysis indicated that the endophytic bacteria are not the same. Endophytic acetone extracts from sample 1, 3, 9 and 17 exhibited the most potent antimicrobial efficacy against E. coli and S. aureus. Terpenoids, flavonoids and stilbenes were observed by the thin-layer chromatography charring profile. In this study, the endophytic crude extracts proved to possess compounds with antimicrobial activity against clinically important pathogens.

Keywords: *Antimicrobial activity, E. coli, S. aureus, endophytic extracts; susceptibility testing, zone of inhibition*

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1. INTRODUCTION

Antimicrobial resistance (AMR) prevalence has reached an alarming level globally and thus, poses a severe threat to environmental, animal and human health (Rather *et al.* 2017; Ribeiro *et al.* 2019). It compromises the successful prevention and treatment of infections caused by bacteria, parasites, viruses and fungi. For instance, infections which are caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are characterised by high mortality and high morbidity in humans (Wang *et al.* 2018). Zimbabwe is currently facing growing antimicrobial resistance to (human immunodeficiency virus) HIV, sexually transmitted infections, meningitis, urinary tract infections, tuberculosis, diarrhoea and malaria (Ministry of Health and Child Care *et al.* 2017). Mashe *et al.* (2019) reported that the resistance to ciprofloxacin by *Salmonella enteric (S. enteric)* in Zimbabwe, increased by 17.8% between 2014 and 2017, while resistance to cephalosporin by *E. coli* increased by 14.6% between 2012 and 2017 (Mhondoro *et al.* 2019). The control/treatment of disease treatment hinges on the availability of effective antibiotics. Thus, there is a need to bio prospect and advance conventional approaches to mitigate antimicrobial resistance. (Evrilia *et al.* 2019; Abdulsahib *et al.* 2020; Ren-Zhang *et al.* 2020; Aloqbi 2020)

Historically, Africa is considered to be the cradle of *Homo sapiens* and is an invaluable sanctuary of possibly the oldest and most diversified healthcare systems which are yet to be fully utilised (Nankaya *et al.* 2020; Traoré *et al.* 2020; Zouaoui *et al.* 2020). Approximately, 50% of the African population uses ethnomedicinal remedies for the treatment of various diseases (Shoko 2018), while, 80% of the southern hemisphere population still relies on the traditional approach to medicine based on herbal drugs (Agrawal *et al.* 2017). The Traditional Medicine Strategy (2014-2023) of the World Health Organization (WHO) supports the incorporation of traditional medicine into modern healthcare practices and strategies (Packer *et al.* 2019).

Every living plant constitute of vast and diverse niches of endophytic fungi, bacteria and actinomycetes (Fouda *et al.* 2015). Therefore, the term endophyte was coined to describe microbes which reside asymptomatically intra or intercellular in plant tissues (Lubna *et al.* 2018; Rosenblueth and Martínez-romero 2006). The genetic flexibility and adaptability of endophytes have enabled the production of a wide array of valuable secondary metabolites in response to the natural stimuli (Chadha *et al.* 2015). A study carried out in Tamil Nadu, India by Palanichamy *et al.* (Palanichamy *et al.* 2018) revealed the ability of endophytes to yield bioactive metabolites which exhibited broad-spectrum potency against *Escherichia coli (E. coli)*, *Staphylococcus aureus* (*S. aureus*), *Corynebacterium diphtheriae*, *Salmonella typhi* and *Proteus mirabilis*. Extracts from the fungal isolate MGTMMP031 which was obtained from *Vitex negundo* plant exhibited potency against both Gram-

negative and Gram-positive pathogens. In another investigation carried out in Karnataka, India of antimicrobial activity of endophytic extracts obtained from *Ocimum* species (*Tulsi*), isolates P14T1 and P13T5 were potent against *Candida albicans* and *Pseudomonas aeruginosa*. The extracts obtained from the isolates (P14T1 and P13T5) exhibited 22 mm and 21 mm zones of inhibition, respectively (Pavithra *et al.* 2012).

Environmental conditions and the genetics of trees over a geographic range influence the occurrence of endophytes harbouring a tree. The interaction between host and endophyte determine the health and productiveness of trees by overcoming the abiotic and biotic challenges. Thus, the species and strains of endophytes found in a tree from different locations are different. Therefore, it is imperative to identify and isolate endophytes found in indigenous Zimbabwe medicinal trees. The endophytic samples in this study were isolated from Musekesa (*Piliostigma thonningii*), Mupane (*Colophospermum mopane*), Muzhozho (*Vernonia amygdalina*), Mugan'acha (*Lannea discolour),* Mubvamaropa (*Pterocarpus angolensis*), Mutamba *(Strychnos pungens*), Mukute (*Syzygium. guineense*) and Marula (*Sclerocarya birrea*). Only a few species of endophytes have been isolated from native Zimbabwean medicinal plants have been studied. Thus, the broad aim of this study was to evaluate the potential of the under-explored new endophytes isolated from native Zimbabwean medicinal plants to produce bioactive compounds.

2. SUBJECTS AND METHODS:

Sample collection

The study was conducted at the University of Zimbabwe which is located at latitude 17°47'01.3"S and longitude 31°02'54.9"E, at an altitude of 1483 m above sea level (masl). The site has a warm temperate climate with an average annual temperature range of 9 °C - 22 °C and an average annual rainfall of 831 mm received mainly during the summer months from November to March. The endophytic samples were isolated from trees that are native to Zimbabwe, viz Musekesa (*P. thonningii*), Mupane (*C. mopane*), Muzhozho (*V. amygdalina*), Mugan'acha (*L. discolour), P. angolensis,* Mutamba *(S. pungens*), Mukute (*S. guineense*) and Marula (*S. birrea*). Sterile plant segments from *P. thonningii, C. mopane, V. amygdalina, L. discolour, P. angolensis, S. pungens, S. guineense* and *S. birrea* were implanted on the surface of potato dextrose agar (PDA) (Biolab®) and nutrient agar (Biolab®) in Petri dishes before being incubated at room temperature in the dark. Growth of the endophytic microorganisms was monitored. After the growing period, the endophytes were characterised according to morphology and spores. The endophytes were stored in liquid cultures under room temperature (Takarova 2018).

Isolation and Maintenance of endophytes

The liquid cultures were collected and cultivated on PDA and nutrient agar. The Petri dishes were incubated under room temperature in the dark and were monitored daily for 4-7 weeks to observe the growth of endophytic growth. The endophytes were allowed to grow for 4-7 weeks. The grown endophytes were macroscopically evaluated. The differentiated endophytes were isolated by streaking method onto PDA and (Malt extract agar) MEA until pure cultures were obtained. The 23 pure cultures obtained were numbered 1 to 23. For long term preservation, the agar blocks impregnated with endophytic colonies were immersed in 15% (v/v) glycerol and stored at -80 ℃.

Genomic DNA extraction

An aliquot 500 µl of the bacterial overnight culture was used for DNA extraction. The genomic DNA was extracted by adding 500 µl of lysis buffer into 2 ml tubes containing the fungal or bacterial samples. Each tube was mixed by inversion, and the mixture was incubated at room temperature for 10 minutes. The tubes containing the samples were then centrifuged at 10000 revolutions per minute (rpm) for 60 seconds. The supernatants were transferred into sterile 1.5 ml tubes and then centrifuged at 10 000 rpm for 1 minute. The supernatants were transferred to fresh sterile tubes, and equal volumes of isopropanol were added to each tube and the tubes mixed by inversion. The microfuge tubes were then centrifuged at 10000 rpm for 2 minutes to pellet the genomic DNA. The pellets formed were washed with 300 µl of 70% ethanol and centrifuged at 10 000 rpm for 3 minutes, and the supernatants were poured off. The pellets were dried using the speed vac. The pellets were resuspended in 50 µl of T.E. buffer (10 mM Tris-HCL, 0.1 mM EDTA, pH 8.0). The integrity and size of the endophytic genomic DNA were evaluated on 0.8% agarose gel containing 2 µl Ethidium bromide (10 mg/ml) using electrophoresis at 150 volts and 400 Amps for 30 minutes. Ultraviolet light was used to visualise the agarose gel, and a picture was taken.

16S rRNA Gene Amplification

For genotypic identification, the extracted total genomic DNA was used to amplify bacterial 16S rRNA gene regions using the universal forward primer (*27F*: AGATTTGATCCTGGCT) and universal reverse primer (*1492R*: CGGTACCTTGTTGTTAC). The PCR reaction was carried out in a 25 µl of a Master reaction mixture comprising of 5 µl of 5X PCR buffer, 2.5 µl of mM dNTPs, 1.5 µl of 25 mM $MgCL_2$, 1 µl of the forward primer and 1 µl reverse primer, 3 µl of Kirk-house *Taq* DNA polymerase, 10 µl of PCR water and 1 µl of DNA. The PCR amplification was carried out in an (Applied Biosystems model 2720 thermal cycler) using the following conditions: 1 initiation denaturation cycle at 94 ℃ for 3 minutes, 35 denaturation cycles at 94 ℃ for 30 seconds, 35 primer annealing cycles at 55 ℃ for 30 seconds, 35 extension cycles at 72 ℃ for 90 seconds, one elongation cycle at 72 ℃ for 5 minutes and the reaction is held indefinitely at 4 ℃. The PCR products were analysed on 1% agarose gel containing 2 µl Ethidium bromide (10 mg/ml) by electrophoresis at 150 volts and 400 Amps for 30 minutes. U.V light was used to visualise the agarose gel, and a picture was taken.

M13 RAPDs

Generic markers (RAPDs) were used to differentiate between the endophytic compounds that have been identified as 16 S rRNA positive. The total extracted genomic DNA was used to amplify the RAPD using the M13 forward primer (5'-(TGTAAAACGACGGCCAGT)-3'). The PCR reaction was carried out in a 25 µl Master reaction mixture which consisted of 2.5 µl 10X BD PCR buffer, 2.5 µl dNTPs, 1 µl of M13 forward primer,

2.5 µl of $MgCl_2$, 0.25 µl of Firepol *Taq* DNA polymerase, 1 µl of DNA template, 15.25 µl of PCR water. The conditions used for the PCR amplification were as follows: 1 initiation denaturation cycle at 94 ℃ for 1 minute, 40 denaturation cycles at 94 ℃ for 1 second, 40 primer annealing cycles at 38 ℃ for 1 minute, 35 extension cycles at 72 ℃ for 2 minutes, one elongation cycle at 72 ℃ for 10 minutes and the reaction is held indefinitely at 4 ℃. The PCR products were evaluated on 1% agarose gel containing 2 µl Ethidium bromide (10 mg/ml) by electrophoresis at 150 volts and 400 Amps for 30 minutes. U.V light was used to visualise the agarose gel, and a picture was taken. A dendrogram to compare the relatedness of the isolates was constructed by dendroUPGMA online tool (Garcia-Vallve *et al.* 1999).

Antagonist test

The *in-vitro* antagonistic reaction of endophyte-endophyte was tested using the antagonistic assay modified (Takarova 2018). PDA media was used for testing the antagonistic potential of endophytic isolates. The PDA media was autoclaved at 121 ℃ for 15 minutes at 15 psi pressure. After autoclaving, the sterile molten PDA media was cooled to approximately 45 ℃ and then poured into a sterile petri dish to a final depth of 4 mm. The petri dish was divided into three segments, and on each segment, an endophytic plug was inoculated onto the PDA media. The culture was incubated at room temperature and observed daily for growth behaviour.

Production of secondary metabolites

The Secondary metabolites production was induced using fermentation in malt extract broth and Luria Bertani Broth (L.B) (Sigma-Aldrich®). The fermentation broth was prepared by inoculating 2 ml of an overnight liquid culture of bacterial endophyte into 20 ml Malt Extract Broth (MEB) (Sigma-Aldrich®). For fungal endophytes, the fermentation broth was prepared by inoculating MEB with 7-15 mm mycelium scrapped from PDA surface. The endophytic liquid cultures were then fermented at room temperature for 14 days.

Test-bacteria strains

The antibacterial efficacy of endophytic crude ethyl acetate, methanol and acetone extracts was assessed against *Escherichia coli* (American Type Culture Collection (ATCC) 35218) (Gram-negative bacteria) and *Staphylococcus aureus* (ATCC 25923) (Gram-positive bacteria). The microorganisms were obtained from the Department of Biochemistry, University of Zimbabwe (17°47'01.3"S 31°02'54.9"E). An aliquot of 500 µl of each stock solution of bacteria strains was inoculated into 5 ml L.B. broth under sterile conditions and incubated at 37 ℃. After 24 hours of incubation, the bacterial inoculum was diluted with sterile broth to 10^8 CFU/ml for antimicrobial.

Determination of antibacterial susceptibility

The endophytic crude (ethyl acetate, acetone and methanol) extracts were examined for antimicrobial efficacy against *Staphylococcus aureus* and *Escherichia coli* using the agar well diffusion method (Takarova 2018). The sterilised L.B. molten agar was cooled to approximately 45 ℃ and subsequently inoculated with the test strains to make up a final concentration of 10^6 CFU/ml. The inoculated molten agar was dispensed into 90 mm diameter Petri dishes. Sterile tips with a diameter of 5 mm were utilised to punch wells into the solidified agar. A volume of 10 µl, 35 µl and 50 µl of reconstituted endophytic extracts was loaded into the agar well for antibacterial efficacy testing. The antibiotic ampicillin was used as positive controls, while 2.5% of the DMSO solution was used as a negative control. The assay plates were incubated at 37 ℃. The antibacterial activity was determined by measuring the zones of inhibition after 24 hours.

Qualitative analysis of endophytic extracts using TLC chromatography

The solvents were used as received from the supplier without further purification unless otherwise stated. Analytical thin-layer chromatography fingerprints of ethyl acetate extracts were carried out on aluminium sheets precoated with silica gel 60 (Sigma T-6770®), 10 µl of each extract was loaded as a band on the TLC analytical plates. The eluent solvent system which was used include., dichloromethane: ethyl acetate (20:80 DCM: E.A. polar). The eluted TLC plates were dried, and the separated chromatograms were visualised using ultraviolet light (254 and 365 nm wavelengths, U.V light lamp) and the bands were marked using a pencil. For optimal colour development, the TLC plates were treated with vanillin sulphuric acid spray reagent (0.1 g vanillin, 28 mL methanol, 1 mL sulphuric acid) and then was heated in an oven at 110 ℃. The elution of the metabolites, along with selected solvents, was measured, and the Rf value was calculated.

3. RESULTS:

Molecular characterisation

Genomic DNA of endophytic samples that exhibited potent antimicrobial activity were successfully extracted. Approximately 10 000 bp bands showed genomic DNA in lane 1 to 9 (Figure 1). Amplification of the 16 S rRNA gene was performed to evaluate if the endophytes are bacteria. Endophytic samples 1, 3, 9, 10, 12, 15, 18, 17 and 19 were found to be bacteria as illustrated by the 1500 bp amplicons on a 1 % ethidium bromide-stained TAE agarose gel (Figure 2). The band in lane 2 is faint which might be due to small quantities of template genomic DNA. RAPDs analysis using M13 forward primer showed that the endophytic bacteria are different as illustrated by the distribution of bands (Figure 3) and the dendrogram (Figure 4). Samples 12 and 3 are closely related and originate from the same ancestral bacteria. Also, sample 12 and 3 are distantly related to all samples as illustrated by the length of the branch. Sample 17 is distantly related to sample 1,3, 9, 10, 15, 18 and 19. Sample 1 and 18 have the same ancestral origin, while sample 10 and 19 have the same ancestral origin.

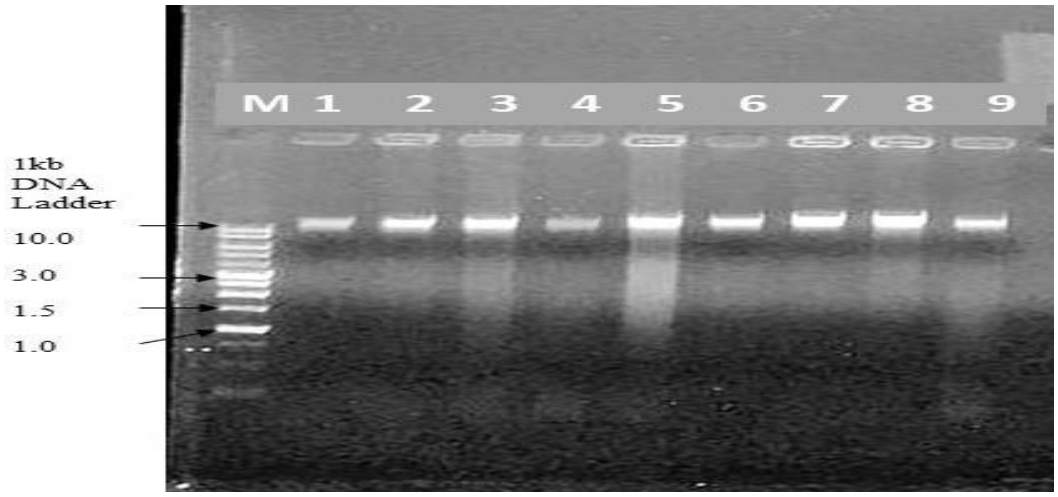

Figure 1. Ethidium bromide-stained 0.8 % TAE agarose gel showing genomic DNA of endophytes visualised using U.V. light. Lane M, Molecular weight marker (1 kb DNA ladder). Lane 1 to 9, sample 1, 3, 9, 10, 12, 15, 18, 17 and 19. Note 10.0 represents 10 000 bp.

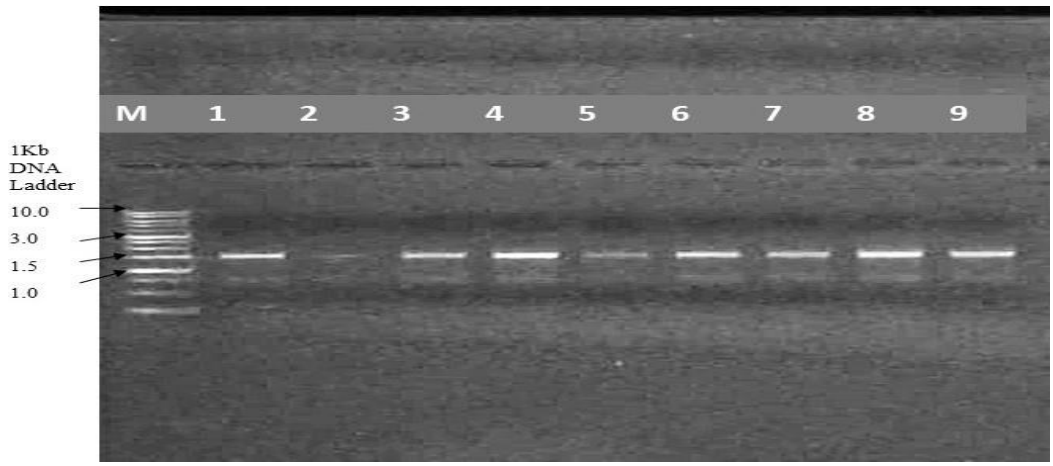

Figure 2. Ethidium bromide-stained 1 % TAE agarose gel showing polymerase chain reaction products from endophytic samples obtained by using 27F and 1492R primers to amplify approximately 1500 bp of 16 S rRNA gene. Lane M; Molecular weight marker (1 kb DNA ladder). Lane 1 to 9; 1, 3, 9, 10, 12, 15, 18, 17 and 19. Lane 2 the band is faint. Note 10.0 represents 10 000 bp.

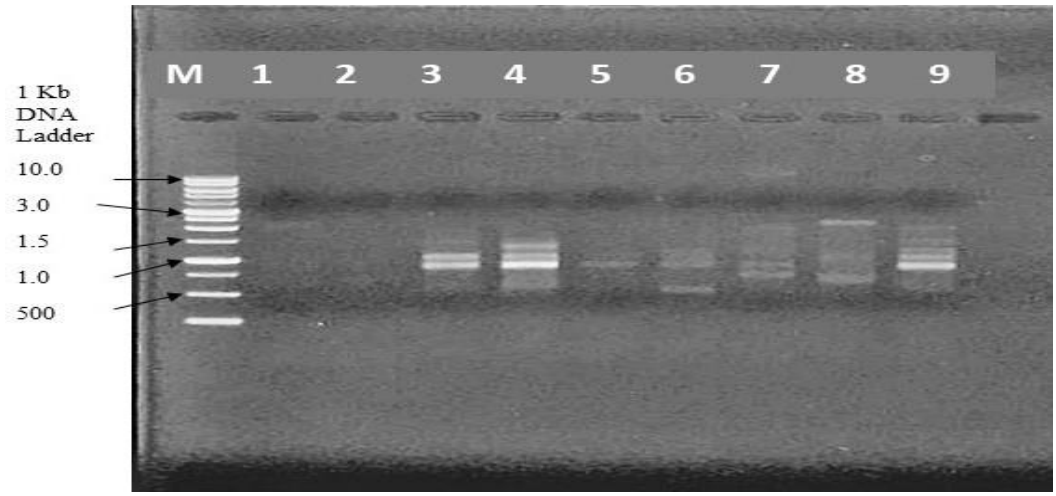

Figure 3. Ethidium bromide-stained 1 % TAE agarose gel showing M13 RAPDs amplicons visualised using the U.V. light. Lane M represents the molecular weight marker while 1 to 9 represents sample 1, 3, 9, 10, 12, 15, 18, 17 and 19. Note 10.0 represents 10 000 bp, 500 represents 500 bp.

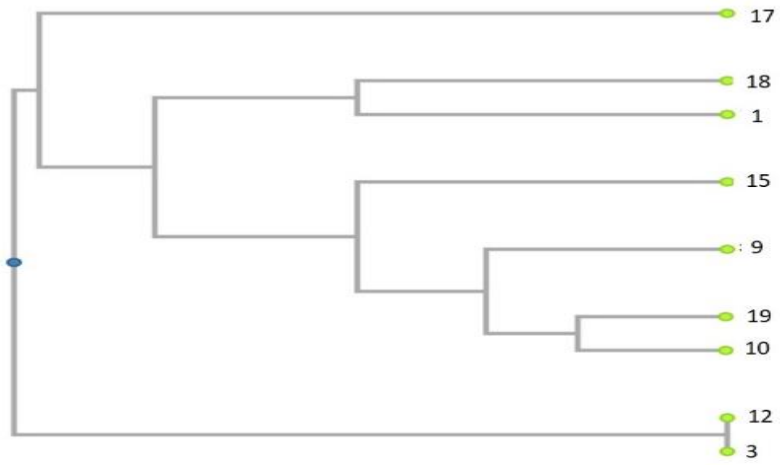

Figure 4. Dendrogram of nine endophytic bacterial isolates.

Antagonist assay

The endophytic cultures were isolated until pure isolates were obtained. The pure isolates were cultivated on PDA and nutrient agar. The pure isolates were then tested for antagonist assay. Endophytic isolates 3, 22 and 6 aggressively proliferated towards the other endophytic isolates. The proliferation stopped before physical contact with the biomass of endophytic isolates 1, 5, 8, 14, 15 and 12 signified by a zone of inhibition (Figure 5 a-h). The endophytic isolate 5 exhibited the largest zone of inhibition (Figure 5 d). The colour of the biomass of 6 changed from yellowish to slightly reddish when it approached other endophytic isolates biomass signifying interspecies communication (Figure 5 b).

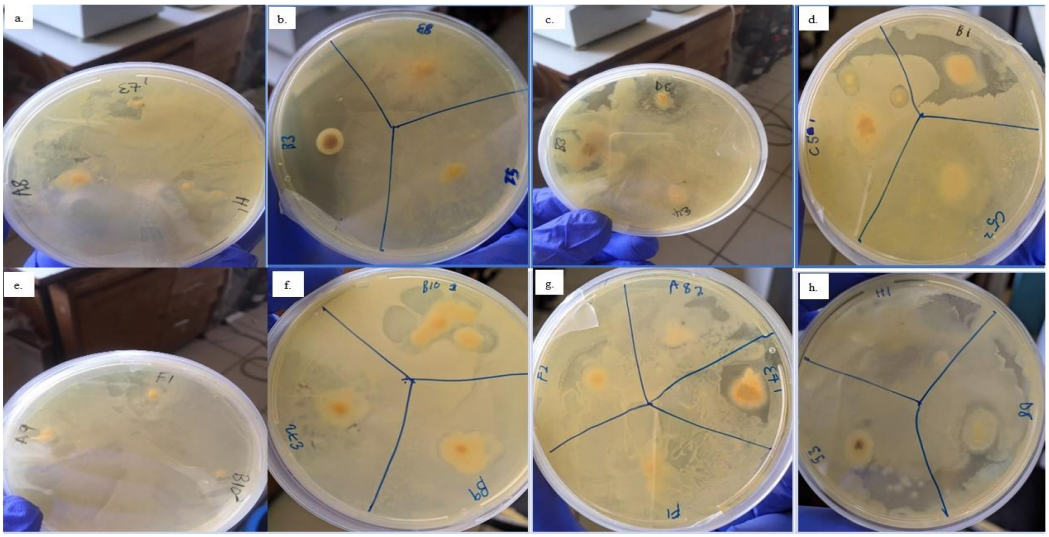

Figure 5. Interaction of different endophytic isolates observed antagonistic culture assay. (a-h) zones of inhibition exhibited by endophytic isolates. Sample number 1, 3, 5, 6, 8, 14, 15, 12 and 22 represent endophytic isolate A8, A9, B1, B3, B10[1], E7[1], E7[2], D8 and H1 respectively.

Antimicrobial efficacy of endophytic crude extracts

Evaluation of endophytic bioactive secondary metabolites as targets for discovery of novel drugs and as an alternative route of combating AMR, we investigated the antimicrobial efficacy of endophytic extracts against two bacterial species which are *S. aureus* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria) (Table 1, Table S1, Table S2). Three different volumes were used in an attempt to evaluate the relatedness of dosage, solubility and antimicrobial efficacy of the endophytic compounds. An aliquot of 50 µl from 100 µl of endophytic extracts showed incredible antimicrobial efficacy. Broadly, the zones of inhibition diameter ranged from 0-35 mm (Table 1, Table S1, Table S2). The extracts from endophytic samples 1, 3, 4 and 20 showed the strong antimicrobial potency against both *S. aureus* and *E. coli*. The endophytic extracts of samples 4, 6, 7, 8, 15, 16, 18, 19 and 23 exhibited moderate antimicrobial activity against *E. coli* only, while extracts of endophytic samples 14, 16 and 22 showed antimicrobial activity against *S. aureus* only. The extracts of endophytic samples 5, 6, 12,15, 19 and 23 exhibited moderate antimicrobial potency against *E. coli*, while 2, 12, 14, 15, 22 and 23 showed moderate antimicrobial potency against *S. aureus* (Table 1, Table S1, Table S2).

The clarity of the zones of inhibition elucidates the minimal concentrations of the endophytic crude extracts which exhibit potency against *S. aureus* and *E. coli.* The results from the agar well diffusion assay for antimicrobial effect shows that utilising acetone as the extracting solvent presents higher efficacy against *S. aureus* and *E. coli*. Notably, endophytic crude extracts from sample 1, 3, 9, 17, 18 and 19 which were extracted by acetone showed relatively the highest antimicrobial potency against *S. aureus* and *E. coli* (Table 1). Endophytic extract from sample 1 obtained from ethyl acetate as the extractant exhibited strong potency against *S. aureus* and *E. coli* while extracts from sample 8 and 23 showed potent activity against *E. coli* only (Table S1).

Acetone and ethyl acetate crude extract from sample 1 exhibited the highest antimicrobial potency against *S. aureus* and *E. coli, respectively*. The acetone extracts of endophytic sample 1 and 3 solvent exhibited the highest potency against both test organisms *S. aureus* and *E. coli*. The endophytic sample 1 showed 35 mm and 29 mm zones of inhibition against *S. aureus* and *E. coli,* respectively. While, endophytic sample 3 exhibited 23 mm and 34 mm zones of inhibition against *S. aureus* and *E. coli,* respectively (Table 1). When methanol we used as the extracting solvent, endophytic samples 3, 4 and 20 exhibited strong potency against *E. coli* and *S. aureus* (Table S2). The effects of the negative control Dimethylsulfoxide (DMSO) 2.5 % and positive control Ampicillin 10 µg/ml we observed. The findings revealed different zones of growth inhibition in positive controls. The positive control zones of inhibition for *S. aureus* and *E. coli* were 20 and 17, respectively. There was no zone of inhibition observed by DMSO as a negative control (Table S3). We selected endophytic samples 1, 3, 9 and 17, which showed strong antimicrobial potency against either *S. aureus* or *E. coli* for GC/MS spectrometry assays.

Table 1. Qualitative assessment of endophytic crude extracts' antimicrobial efficacy expressed in the diameter of the zone of inhibition (based on the translucency of zones of inhibition).

Sample. no	Endophyte isolate	Origin	Solvent used for extraction	Zone of inhibition (mm)					
				E. coli ATCC 35218			S. *aureus* ATCC 25923		
				10 µl	**35 µl**	**50 µl**	**10 µl**	**35 µl**	**50 µl**
1	A8[1]	*P. thonningii*	Acetone	9	24	29	13	27	35
2	A8[2]	*P. thonningii*	Acetone	0	6	8	6	15	20
3	A9[1]	*P. thonningii*	Acetone	15	25	34	12	17	23
4	A9[2]	*P. thonningii*	Acetone	0	9	21	0	0	5
5	B1	*C. mopane*	Acetone	0	7	18	0	0	10
6	B3	*C. mopane*	Acetone	7	14	20	0	0	0
7	B6	*C. mopane*	Acetone	14	15	25	0	0	0
8	B10[1]	*C. mopane*	Acetone	0	0	8	0	10	12
9	B10[2]	*C. mopane*	Acetone	0	10	17	20	26	30
10	C5[1]	*V. amygdalina*	Acetone	3	12	12	15	23	31
11	C5[2]	*V. amygdalina*	Acetone	2	8	15	0	0	0
12	D8	*L. discolour*	Acetone	11	17	22	6	13	21
13	E4	*P. angolensis*	Acetone	0	2	11	0	0	0
14	E7[1]	*P. angolensis*	Acetone	0	0	5	0	10	21
15	E7[2]	*P. angolensis*	Acetone	0	11	18	5	19	22
16	E8[1]	*P. angolensis*	Acetone	0	10	15	0	0	0
17	E8[2]	*P. angolensis*	Acetone	6	24	27	13	13	15
18	F1	*S. pungens*	Acetone	10	20	27	0	10	15
19	F2	*S. pungens*	Acetone	6	14	25	4	7	15
20	G1	*S. birrea*	Acetone	0	7	14	3	9	10
21	G3	*S. birrea*	Acetone	0	0	7	0	0	10
22	H1	*S. guineense*	Acetone	0	0	0	14	18	22
23	H3	*S. guineense*	Acetone	14	14	17	5	12	17
	Strong crude extract's antibacterial potency (25-35 mm)						Moderate crude extract's antibacterial potency (15-24 mm).		
	Low susceptibility of either *E. coli* or *S. aureus* to the respective endophytic crude extract (10-0 mm)						No inhibition of *E. coli* and *S. aureus* (0 mm)		

Table S1. Qualitative assessment of endophytic crude extracts' antimicrobial efficacy expressed in the diameter of the zone of inhibition (based on the translucency of zones of inhibition).

Sample. no	Endophyte isolate	Origin	Solvent used for extraction	Zone of inhibition (mm)	
				E. coli ATCC 35218	S. *aureus* ATCC 25923

				10µl	**35 µl**	**50 µl**	**10 µl**	**35 µl**	**50 µl**
1	A8[1]	*P. thonningii*	Ethyl acetate	14	17	25	6	12	20
2	A8[2]	*P. thonningii*	Ethyl acetate	7	7	13	0	8	10
3	A9[1]	*P. thonningii*	Ethyl acetate	0	2	11	0	2	10
4	A9[2]	*P. thonningii*	Ethyl acetate	0	0	0	0	3	8
5	B1	*C. mopane*	Ethyl acetate	0	0	5	0	3	5
6	B3	*C. mopane*	Ethyl acetate	0	5	7	0	7	10
7	B6	*C. mopane*	Ethyl acetate	0	1	3	0	0	0
8	B10[1]	*C. mopane*	Ethyl acetate	7	15	25	0	0	0
9	B10[2]	*C. mopane*	Ethyl acetate	0	0	0	0	0	0
10	C5[1]	*V. amygdalina*	Ethyl acetate	0	0	0	0	0	0
11	C5[2]	*V. amygdalina*	Ethyl acetate	0	0	0	0	0	0
12	D8	*L. discolour*	Ethyl acetate	2	10	16	0	0	13
13	E4	*P. angolensis*	Ethyl acetate	0	0	9	0	7	12
14	E7[1]	*P. angolensis*	Ethyl acetate	0	0	0	0	0	9
15	E7[2]	*P. angolensis*	Ethyl acetate	3	5	15	0	0	0
16	E8[1]	*P. angolensis*	Ethyl acetate	0	8	11	6	13	15
17	E8[2]	*P. angolensis*	Ethyl acetate	0	3	7	0	11	14
18	F1	*S. pungens*	Ethyl acetate	0	6	6	0	0	0
19	F2	*S. pungens*	Ethyl acetate	3	9	0	0	0	0
20	G1	*S. birrea*	Ethyl acetate	0	0	0	0	5	8
21	G3	*S. birrea*	Ethyl acetate	0	0	0	0	0	0
22	H1	*S. guineense*	Ethyl acetate	0	0	0	0	10	10
23	H3	*S. guineense*	Ethyl acetate	18	23	25	0	0	0
	Strong crude extract's antibacterial potency (25-35 mm)						Moderate crude extract's antibacterial potency (15-24 mm)		
	Low susceptibility of either *E. coli* or *S. aureus* to the respective endophytic crude extract (10-0 mm)						No inhibition of *E. coli* and *S. aureus* (0 mm)		

Table S2. Qualitative assessment of endophytic crude extracts' antimicrobial efficacy (based on the translucency of zones of inhibition and expressed in the diameter of the zone of inhibition).

Sample. no	Endophyte isolate	Origin	Solvent used for extraction	Zone of inhibition (mm)					
				E. coli ATCC 35218			S. *aureus* ATCC 25923		
				10 µl	**35 µl**	**50 µl**	**10 µl**	**35 µl**	**50 µl**
1	A8[1]	*P. thonningii*	Methanol	4	12	21	0	8	15
2	A8[2]	*P. thonningii*	Methanol	0	0	0	0	0	0
3	A9[1]	*P. thonningii*	Methanol	5	16	20	9	18	25
4	A9[2]	*P. thonningii*	Methanol	8	14	22	4	17	26
5	B1	*C. mopane*	Methanol	6	16	20	7	13	20
6	B3	*C. mopane*	Methanol	2	9	15	0	0	0
7	B6	*C. mopane*	Methanol	0	8	15	0	6	15
8	B10[1]	*C. mopane*	Methanol	8	13	15	0	0	0
9	B10[2]	*C. mopane*	Methanol	0	5	10	0	3	12
10	C5[1]	*V. amygdalina*	Methanol	0	0	0	0	0	0
11	C5[2]	*V. amygdalina*	Methanol	0	0	0	0	0	0
12	D8	*L. discolour*	Methanol	0	0	0	0	0	0
13	E4	*P. angolensis*	Methanol	0	0	0	0	0	0
14	E7[1]	*P. angolensis*	Methanol	0	0	0	0	0	0
15	E7[2]	*P. angolensis*	Methanol	8	16	20	13	15	20
16	E8[1]	*P. angolensis*	Methanol	0	5	7	0	3	7

17	E8[2]	*P. angolensis*	Methanol	0	0	0	0	10	14
18	F1	*S. pungens*	Methanol	0	0	0	0	0	0
19	F2	*S. pungens*	Methanol	10	20	23	0	0	0
20	G1	*S. birrea*	Methanol	6	14	23	9	20	25
21	G3	*S. birrea*	Methanol	0	0	0	0	0	0
22	H1	*S. guineense*	Methanol	0	6	9	0	0	0
23	H3	*S. guineense*	Methanol	0	0	8	0	2	8
	Strong crude extract's antibacterial potency (25-35 mm)						Moderate crude extract's antibacterial potency (15-24 mm)		
	Low susceptibility of either *E. coli* or *S. aureus* to the respective endophytic crude extract (10-0 mm)						No inhibition of *E. coli* and *S. aureus* (0 mm)		

Table S3. Determination of negative (2.5% DMSO) and positive control (Ampicillin 10 µg/ml) on test bacteria.

Bacteria	Positive control (Ampicillin 10 µg/ml) ZOI (mm)	Negative control (2.5% DMSO) ZOI (mm)
E. coli	17	0
S. aureus	20	0

Characterisation of endophytic extracts

The screening of the endophytic ethyl acetate extracts in this study using Thin-layer chromatography showed different chemical components as depicted by the different colours (a, b, c, d, e). The solvent system separated the compounds, DCM/EA 20:80 dichloromethane: ethyl-acetate. Most of the separated compounds may be non-polar compounds. The retention factor (Rf) of the chromatograms was obtained by dividing the distance travelled by the compound by the solvent distance. The profile of ethyl acetate samples 2, 5, 6, 7 and 11 after derivatisation with vanillin sulphuric acid methanol spray reagent contained a yellow pigment observed at (Rf value) (Figure S1 a and Figure S1 f). According to the charring profile of all the metabolites after derivatising with vanillin sulphuric acid methanol spray reagent indicated the presence of terpenoids (blueish purple) Rf value 6.5, flavonoids (yellow) Rf value 0.06, reddish (stilbenes) Rf value 0.31 (Figure S1 a and Figure S1 f).

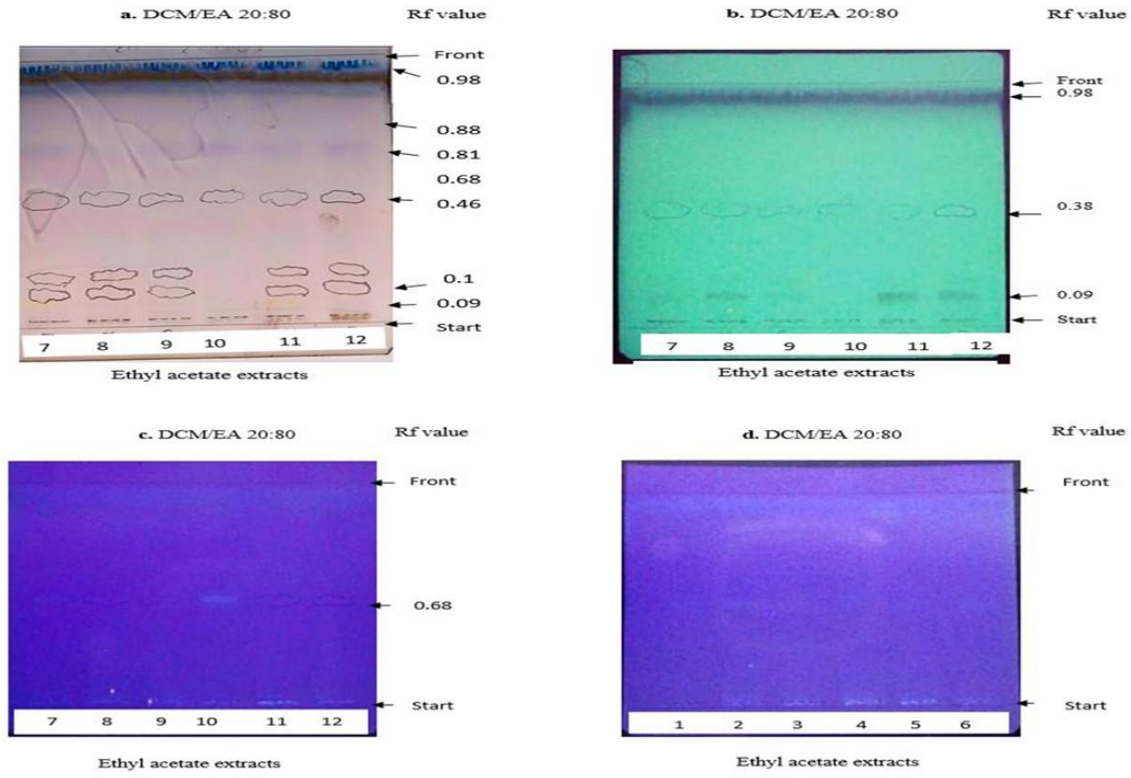

Figure 6. Thin layer chromatogram of the endophytic ethyl acetate extracts. a) after derivatisation with Vanillin-Sulphuric acid methanol reagent; b) UV 254 nm. The pencil circles are compounds bands visualised at 254/365 nm U.V. wavelength. (c, d) Thin layer chromatogram of the endophytic ethyl acetate extracts visualised at UV 365 nm.

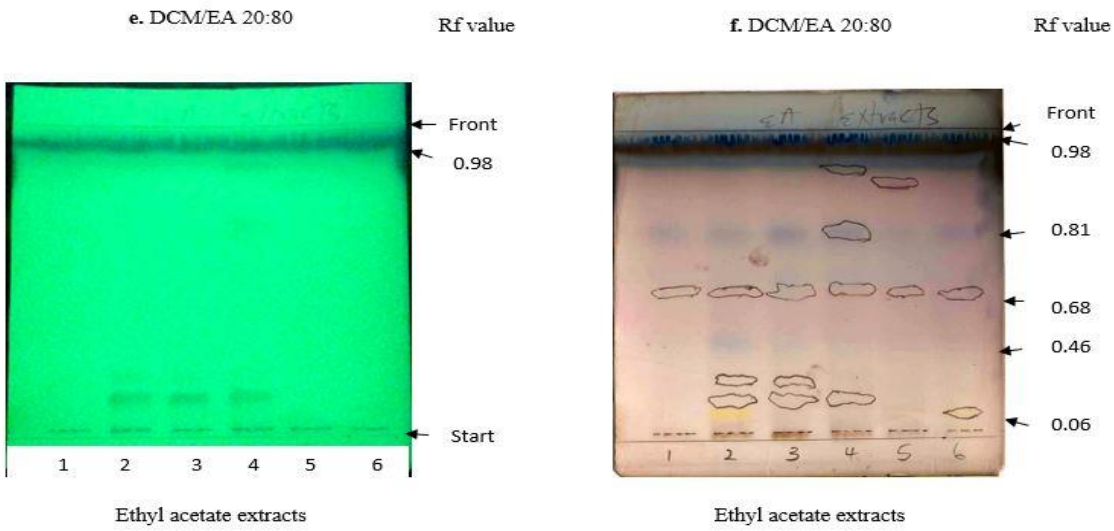

Figure 7. (e, f) Thin layer chromatogram of the endophytic ethyl acetate extracts. e) UV 254 nm; f) after spraying with Vanillin-Sulphuric acid methanol reagent. The pencil circles are compounds bands visualised at 254/365 nm U.V. wavelength.

4. DISCUSSION:

Molecular characterisation endophytes

Molecular analysis of the 9 most prolific endophytes showed that the endophytes were bacteria, and they were not the same (Figure 2, Figure 3, Figure 4). The diversity of the bacterial endophytes is because the endophytes were isolated from different hosts (trees). Also, the distribution of endophytic community is tissue-specific, implying that a single branch can harbour different types of endophytes. Noteworthy, only the RAPD sequences are easily detected because not all amplified sequences are polymorphic.

Our findings are consistent with other reports that have demonstrated that endophytic bacteria are prolific producers of important bioactive secondary metabolites (Eyre *et al.* 2019; Nguyen *et al.* 2019; Photolo *et al.* 2020). The 16 S rRNA gene is highly conserved within all bacteria genome and is a housekeeping genetic marker which is commonly used for identification of bacteria (Zin *et al.* 2020). External factors also determine the composition of microbiomes inside plants. These significant factors include weather, nutrient availability, plant species, location, soil composition and water (Eyre *et al.* 2019).

Antagonistic activity

The preliminary antagonistic screening of endophyte-endophyte in this study indicated the ability of endophytes to secrete bioactive secondary metabolites with antimicrobial properties as illustrated by the clear zones of inhibition (Figure 5). Endophytic samples 14 and 5 remarkably repelled other endophytes in the antagonistic screening assay. In contrast, *E. coli* and *S. aureus* exhibited moderate susceptibility to the extracts of endophytic sample 14 (0-21mm zone of inhibition) and sample 5 (0-20 mm inhibition). Undoubtedly, the interaction of endophytic samples in the same Petri dish as samples 14 and 5 present (Figure 5) evidence that microbial interaction promotes the production of more unique ligands that inhibit or accelerates particular functions of the cells. These findings are consistent with numerous literature which illustrate that endophytes are legitimate sources of bioactive secondary metabolites (Nuankeaw *et al.* 2020; Pelo *et al.* 2020; Taghinasab and Jabaji 2020; Wu *et al.* 2019; Yasser *et al.* 2019). The co-cultivation approach promotes interspecies interaction and has been effectively utilised in the activation of silent biosynthetic gene clusters (BGCs); thus, stimulates and improves the production of bioactive secondary metabolites. Co-cultivation of *Acremoniun pilosum F47* and *Pleosporales sp. F46* by (Wang *et al.* 2020) resulted in the discovery of new zinniol analogues (pleoniols A-C). The interaction of microorganisms is critical in the activation of the production of metabolites by fungi and bacteria (Belknap *et al.* 2020; McLean *et al.* 2019; Nguyen *et al.* 2019).

Notably, the availability of nutrition and interaction of microorganisms influence the global regulatory systems in microorganisms. Global regulators are known to indirectly or directly determine the expression of (BGCs) biosynthetic gene clusters by controlling the expression levels of cluster situated regulators (CSRs) (Belknap *et al.* 2020; Nguyen *et al.* 2020). The concentration of many valuable secondary metabolites is controlled by the tight cross-regulation of the transcription and translation of other BGCs in the genome by some CSRs. It is therefore imperative to observe and understand how the endophytic isolates empirically react to other microbes outside the plant environment.

Antimicrobial efficacy of endophytic extracts

Relative *in vitro* examination of the endophytic crude extracts showed that acetone extracts were substantially more potent than methanol and ethyl acetate extracts against the test microbes *S. aureus* and *E. coli*. This finding supports the notion that acetone is the best solvent for extracting a wide range of phyto-related (endophytic) compounds with different polarities (Famuyide *et al.* 2019a, 2019b).

In this study, the antimicrobial efficacy varied, but most of the extracts expressed strong, broad range potency. It is noteworthy that the variation in the antimicrobial efficacy by

the endophytic extracts from the same tree source in this study confirms that distinct, unprecedented and usually very complicated chemical configurations which result from the addition of different side-chain groups are secreted as secondary metabolites by endophytes. Some antimicrobial compounds exhibit stronger potency against Gram-positive bacteria than towards Gram-negative bacteria, while some antibiotics have broad-spectrum activity against both Gram-negative bacteria and Gram-positive bacteria. Thus, the antimicrobial efficacy of different bioactive metabolites is not equal in regards to bioactivity range (Torres *et al.* 2019).

The distinction in the potency of endophytic (ethyl acetate, methanol and acetone) crude extracts from different endophytic tree sources are consistent with previous studies that have illustrated the diversity in bioactive metabolites produced by different strains and genera of endophytes, hence, different antimicrobial properties (Atri *et al.* 2020; Barra-Bucarei *et al.* 2020; Islam *et al.* 2020; Photolo *et al.* 2020). A vast gene pool, building blocks and modification steps drive variations in enzymatic mechanisms which are responsible for the diverse structures of the metabolite classes (Berdy 2005).

Characterisation of metabolites secreted by endophytes

The qualitative TLC profiling of the endophytic (ethyl acetate) extracts in this research were based on the quenching intensity/colour of the extracts, and it remarkably showed the presence of numerous compounds. The charring profile of the compounds after derivatisation with vanillin sulphuric acid methanol spray reagent showed the presence of terpenoids (blueish purple), flavonoids (yellow), stilbenes (red) (Figure 7 f). Flavonoids appear (yellow, pinkish or orange) whereas stilbenes appear (bright red to dark pink colour) whilst terpenoids produce a bluish-purple colour after spraying with vanillin (Taganna *et al.* 2011). The numerous endophytic compounds gave different Rf values in dichloromethane: ethyl acetate solvent system (Figure 6 and Figure 7). The Rf values determined the polarity of the endophytic chemicals, and this also aids in the selection of an appropriate solvent system for separation of pure compounds by column chromatography (Tijjani 2018).

Terpenoids have been reported to exhibit broad-spectrum antimicrobial efficacy (Sanchez and Demain 2011; Tijjani 2018). Terpenoids non-specifically interact with bio-membranes; thus, leading to an increase in membrane fluidity, unrestrained efflux of metabolites and ions, modulation of membrane proteins and receptors ultimately resulting in cell leakage and eventually apoptosis (Wink 2010).

Flavonoids control biological systems by inhibiting numerous enzymes which include lipase, hydrolase, aldose reductase, xanthine oxidase, alkaline phosphatase, cyclooxygenase, hyaluronidase, aryluslphatse, cAMP phosphodiesterase, Ca +2 ATPase and several kinases (Aboody and Mickymaray 2020). Flavonoids extracted from *Nodulisporium* sp. an endophytic fungus which resides in Juniperus cadre have been demonstrated to exhibit antimicrobial activity (Zaferanloo *et al.* 2013).

Plants and microorganisms produce low-molecular-weight phytoalexins which are responsible for protection against infection (Filippis *et al.* 2019). A stilbene was shown to exhibit potent *in-vitro* inhibitory activity against vancomycin-resistant Enterococcus (Tan *et al.* 2020). The presence of the above mentioned phyto-related compounds in endophytic extracts might be responsible for the antimicrobial effect. The broad-spectrum antimicrobial efficacy of the extracts might be due to the synergistic effect of the flavonoids, terpenoids and stilbenes. The bioactive compounds identified in this research may be responsible for the pharmacological properties of the host medicinal plants *P. thonningii*, *C. mopane*, *P. angolensis*, *V. amygdalina*, *S. pungens*, *S. birrea* and *S. guineense*.

5. CONCLUSION:

The endophytes from the *P. thonningii*, *C. mopane*, *P. angolensis*, *V. amygdalina*, *S. pungens*, *S. birrea* and *S. guineense* are easily cultured under laboratory conditions for the synthesis of bioactive secondary metabolites through fermentation. Thus, it will protect and preserve medicinal plants from excessive harvesting, which consequently affects environmental biodiversity. Molecular characterisation of the endophytes enabled us to identify 9 prolific bacterial endophytes. RAPDs evaluation has shown that the endophytic bacteria are not the same microorganisms. However, there is a need to sequence the 16 S rRNA gene to have the exact identity of the bacterial endophytes.

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