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New naphthalene derivative isolated from *Diaporthe* sp. host to *Syzygium cordatum* Hochst.ex Krauss plant

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Fungal endophytes are regarded as inexhaustible sources of pharmaceuticals and agrochemicals with profound antibacterial, anticancer or antifungal activities. *Diaporthe* sp., an endophytic fungus residing in medicinal plant *S. cordatum*, showed a good antagonism against bacterial pathogens of beans; *Pseudomonas syringae pv phaseolicola* (*Psp*) and *Xanthomonas axonopodis pv phaseoli (Xap),* with zones of inhibitions of 14.00 ± 1.15 and 17.00 ± 0.58 mm against the test organisms respectively. Large scale fermentation of *Diaporthe* sp. was performed on rice media after which ultrasonic extraction on methanol was done to yield methanol crude extract. Methanol crude extract was then partitioned between hexane and ethyl acetate to yield their respective crude extracts. Ethyl acetate fraction of *Diaporthe* sp. yielded one new naphthalene derivative compound which was accorded IUPAC name as 3-methoxy-5-methylnaphthalene-1, 7-diol after series of purifications on column chromatography as well as preparative high-performance liquid chromatography (pHPLC). Structure determination of isolated compounds was performed on 1D and 2D Nuclear Magnetic Resonance (NMR) spectroscopy experiments as well as a mass spectrometer to affirm its molecular mass. The F3 that yielded compound 1 had palpable antibacterial activities against *Psp* and *Xap,* with corresponding Minimum Inhibitory Concentration (MIC) values of 2.50 mg/ml (7.00 ± 0.00 mm) and 1.25 mg/ml (7.67 ± 0.33 mm) against the tests organisms respectively. These slight MIC values are chiefly attributed to the presence of active secondary metabolites in the fungal extracts that act against the test pathogens. This has therefore confirmed that fungal endophytes and their extractives have desirable antibacterial activities hence can be used in the formulation of agrochemicals or used as bio-control agents in crop protection especially in common beans (*Phaseolus vulgaris L*).

Key words: Endophytes, *Diaporthe* sp, bean bacterial pathogens.

INTRODUCTION

Syzygium cordatum Hochst.ex Krauss (Myrtaceae) plant is an evergreen water-loving tree, which grows to a maximum of 20 m (Maroyi, 2018). It is native to high altitudes, swampy and riverine sites (Orwa et al., 2009). Traditionally, the ripe fruits were used in brewing fermented drinks whereas, pounded stem bark was used as fish poison (Maroyi, 2018). In central Africa the plant is used as a remedy for stomach ache and as an anti-

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diarrheal agent (Sibandze et al., 2010). *S*. *cordatum* houses asymptomatic microorganisms called endophytes that resides inside the tissues of the plant and spend part or whole of its life colonizing intercellular inside the tissues of health plants (Noor et al., 2018). These endophytes can either be fungal or bacterial; of the two, fungal endophytes are the highly isolated strain (Radji et al., 2011).

Currently, fungal endophytes are the key prospects in the development of new drugs used in fighting diseases in people, animals and plants (Aly et al., 2010). Extractives from fungal endophytes have been reported to possess desirable antibacterial, antifungal, anticancer activities, hence can be used in the formulation of new agrochemicals as well as pharmaceuticals (Alvin et al., 2014; Noor et al., 2018).

In Kenya most of the farmers have adopted chemical control system in fight against these bacterial pathogens, however, lot of problems have been reported associated to the usage of these synthetic agrochemicals, these include; pesticide residue on crops, environmental pollutions due to non-biodegradability of these chemicals and that pathogens have developed resistance towards these synthetic agrochemicals (Hahn, 2014). This then has prompted a search for safer agrochemicals from natural sources, which include checking on anti-phytopathogenic of fungal isolates and their extractives against selected plant pathogens.

The main objective of this study was to isolate endophytic fungi host to the plant *S. cordatum* (water berry) and test their antibacterial activities against bean bacterial pathogens, *Pseudomonas syringae pv phaseolicola* and *Xanthomonas axonopodis pv phaseoli.* The study was justified because currently bean production is at 100,000 tons annually as opposed to the expected output of 450,000 tons annually here in Kenya (Gichangi et al., 2019). This mismatch in production and supply of common beans (*Phaseolus vulgaris L*), is partly attributed to bacterial infections; Halo blight caused by bacterium *P. syringae pv phaseolicola* and Common Bacterial Blight (CBB) caused by *bacterium X. axonopodis pv phaseoli* (Schwartz, 2011), hence the basis of this study.

MATERIALS AND METHODS

Collection of plant materials

Fresh leaves and stem bark of *S. cordatum plant were collected from Mt. Elgon forest (01*° 08ˈ00ˈˈ N 34° 35ˈ00ˈˈE or 1.13333°N, 34.583333°E), taken to the Botany Department of Egerton University for scientific identification by a taxonomist. Isolation of fungal endophytes was thereafter done within eight hours after the collection of plant materials to avoid drying up at the biotechnology lab of Egerton University.

Isolation of endophytic fungi

Endophytic fungi were isolated from internal plant tissues using the method used by Tian et al. (2017). In this method, the leaves and stem of the selected healthy plants were washed in running tap water to remove any soil or other foreign materials and blotted dry. The leaves/stems were then sectioned to approximately 1 mm by 4 mm sizes. The sectioned plant materials were then surface-sterilized for 5 min in 10 ml of 1% sodium hypochlorite followed by 10 ml 70% ethanol. Thereafter the materials were rinsed three times with sterile distilled water to wash off disinfectants. The surface-disinfected materials were then plated in Petri-plates containing Potato Dextrose Agar (PDA) amended with 200 mg/L concentration of streptomycin sulphate. The plates were sealed using parafilm and incubated at 25 ± 2°C in a light chamber. The Petri dishes were daily checked to monitor the growth of fungal endophytes from the plant materials. On the emerging of the vegetative parts of the fungi from plant segments, isolation and sub-culturing were done bringing them to pure culture by series of serial sub-culturing.

Molecular identification of fungal endophytes

DNA extraction

The endophytic fungal genomic DNA was extracted using Bio Basic EZ -10 Spin column miniprep kit according to manufacturer's instructions (Bio Basic Inc.). 60 mg of the 7-day old fungal mycelia were placed into the screw cap reaction tube. Approximately 5 to 10 of 1.4 mm Precellys ceramic beads were added to the tube and the sample was covered with 200 µl of the plant cell lysis buffer (PCB). The mixture was homogenized in a Precellys 24 homogenizer and incubated on a heating block set at 65°C for 20 min. After incubation, 30 µl of protein precipitation solution was added to the sample and incubated for 20 min on ice. The sample was centrifuged at 12000 revolutions per minute at 4°C for 2 min. A clear lysate formed as a result of centrifugation was transferred onto an EZ-10 spin column using a micropipette. The Phosphate Buffer Solution (250 µl) was added to the lysate and the samples were incubated for 4 min at room temperature with occasional mixing. The mixture was centrifuged at 12000 rpm at 4°C for 30 s and thereafter the flow-through was discarded. Awash solution (400 µl) was added to the sample and centrifuged at 12000 rpm at room temperature for 30 s and the supernatant discarded. This step was repeated twice. The samples were then centrifuged at 12000 rpm at room temperature for 1 min. The collection tubes of the EZ -10 Spin columns were replaced with clean 1.5 ml reaction tubes. About 50 µl 65°C warm elution buffer was added to the filtrate and incubated at room temperature for 2 to 3 min, after which final centrifugation at 10000 rpm was done for 2 min. The EZ-10 Spin columns were discarded and the DNA was stored at 4°C for further use.

Polymerase chain reaction (PCR) amplification

The polymerase chain reaction (PCR) was conducted by amplifying the Internal Transcribed Spacer (ITS) region of the ribosomal DNA. Polymerase Chain Reaction amplification was done in a final volume of 25 µl by mixing 2 µl of the genomic DNA with 23 µl of the master mix. The master mix was prepared by adding 0.5 µl of the forward primer ITS IF (CTTGGTCATTTAGAGGAAGTAA) into a 2 ml Eppendorf tube, followed by 0.5 µl of the reverse primer ITS4 (TCCTCCGCTTATTGATATGC), 12.5 µl of the jump start ready mix (20 mM Tris-HCL, pH 8.3, 100 mM KCL, 4 mM $MgCl_2$, 0.002% gelatin, 0.4 mM dNTPs, inert dye, stabilizers, 0.03 unit/ml Taq DNA polymerase, JumpStart ^{TM} Taq Ready Mix^{TM} and 9.5 µl of distilled water). The mixture was vortexed for 30 s. About 2 µl of distilled water was mixed with 23 µl of the master mix was used as a negative control. Amplification was done in a thermal cycler with an initial denaturation of 5 min at 94°C, 1 min for annealing at 52°C, 1

min for elongation at 72°C and a final elongation of 10 min at 72°C. The quality and quantity of the PCR products were checked on a 0.8% agarose gel with Midori green dye in 1× TAE buffer for 20 min at 100 volts and visualization was done in a UV trans-illuminator (Nippon Genetics Europe GMbH).

Sequencing

The amplified PCR products were purified according to the Bio Basic EZ-10 spin column following the manufacturer's instructions. The PCR reaction mixture was placed in 1.5 ml microfuge tubes and 5 ml of buffer B3 added. The mixture was then transferred to EZ-10 spin columns and left to stand for 2 min at room temperature and centrifuged at 10000 rpm for 1 min. The supernatant was removed and about 750 µl of wash solution was added to the column and further centrifuged at 10000 rpm for 1 min. The washing step was reaped twice. The columns were transferred into a clean 1.5 ml microfuge tubes and about 20 µl of elution buffer was added. This was incubated at room temperature for 2 min and then centrifuged at 10000 rpm for 1 min to elute the DNA. Sample preparation for sequencing was done by preparing 1:1 primer (ITS1F and ITS 4): distilled water dilution in addition to the amplified fungal endophyte DNA. Sequencing was done using an automated Illumina genome analyzer 11X DNA sequencing machine. The sequences (Appendix 1) were compared with those available in the public database GenBank by using Basic Local Alignment Search Tool (BLAST) sequence matches (https://www.ncbi.nlm.nih.gov/nucleotide/MK442578.1?report=genbank&log$=nucltop&blast_rank=1&RID=C4MDYYPG016), *Diaporthe* sp with close correlation to that of *Diaporthe anacardii*. The sequences were aligned using Clustal W software.

Antagonistic assay

Antimicrobial activity of isolated endophytic fungi against bean bacterial pathogens was done using the method explained by Arya and Sati (2011). Where; the pathogenic bacteria; *P. syringae pv phaseolicola* (gram-negative bacterium) *and X. axonopodis pv phaseoli* (gram-negative bacterium) were inoculated in 50 ml conical flasks containing nutrient agar medium and incubated at 37 ± 2°C for 24 h. After 24 h, one loop of each pathogenic culture was transferred to Erlenmeyer flasks containing distilled water and shaken until the turbidity of bacterial suspension is comparable to the turbidity of 0.5 McFarland's standard solution ($1.5 \times 10^{\wedge 8}$ CFU/ml) which was prepared by mixing 0.05 mL of 1.175% Barium chloride ($BaCl_2.2H_20$) in 9.95 mL of 1% sulphuric acid). 100 µL of each suspended bacteria ($1.5 \times 10^{\wedge 8}$ CFU/ml) were inoculated in Petri dishes containing Muller Hinton agar using a sterile micro-dispenser. The six-millimeter diameter of 7 day actively growing mycelial plugs from Potato Dextrose Agar (PDA) plates was cut using a sterile cork borer and placed on the surface of the Muller Hinton agar media. These plates were sealed with parafilm and incubated at 37 ± 2°C for 24 h for complete diffusion of antimicrobial compounds and observed for the zone of inhibitions. The zones of inhibition were measured in millimeters using a ruler scale. The experiment was performed in triplicates.

Large scale fermentation of fungal endophytes

This was done in a solid media experiments which were carried out in 21, 500 mL Erlenmeyer flasks containing 90 g of parboiled rice in 90 mL distilled water per flask, it was then autoclaved at 120°C for 40 min for each fungal strain. Agar plugs (6 mm diameter) were cut from the 7-day-old original cultures on PDA agar and placed on the surface of sterile rice media**.** One flask, without inoculum, was kept for control use. After 21 days of incubation, 200 mL of methanol was added to each flask then followed by ultrasonic extraction using ultra-sonic cleaner. The methanol was then filtered and evaporated using a Rotary evaporator under reduced pressure to yield the methanol extract. The methanol extract was then be subjected to liquid-liquid partitioning with hexane (to remove fatty acids) and ethyl acetate. The resulting organic layer was evaporated under reduced pressure to produce the hexane and ethyl acetate extracts.

Chromatographic analysis of ethyl acetate fractions

A good solvent system for running the column was determined first using a series of TLC analysis. The solvents employed were those used during extraction (hexane, ethyl acetate, and methanol). The ethyl acetate crude of SC-S(9) identified as *Diaporthe* species, was subjected to column chromatography using silica gel with mesh size 70 to 230 STM supplied by Scharlau Lab chemical supplies Limited. Crude sample was reconstituted with a little amount of distilled ethyl acetate to make a paste-like slurry. The sample was then loaded into an evenly packed silica gel column using a clean micro-pipette. A mobile phase of methanol: ethyl acetate: hexane (2: 5: 3) was used in eluding the packed column. In each case, fractions from the column were collected in glass test-tubes. All the eluted fractions were monitored through TLC analysis and using a UV lamp for spots visualization then subsequently grouped thereafter. Test-tubes with identical fractions were pooled together. SC-S (9) yielded four fractions, named F1-F4. All the fractions were screened for antibacterial activities against selected bean bacterial pathogens. Those that showed good activities were further taken for MIC analysis and further purified using preparative HPLC.

Purification of active fractions using PHPLC

Preparative High-Performance Liquid Chromatographic system (Shimadzu-UFLC prominence), fitted with an auto-sampler (Model-SIL 20AC HT) and UV-Visible detector (Model-SPD 20A) was used in the separation of compounds. Dry samples obtained from column chromatography were re-dissolved in HPLC grade methanol each to make a concentration of 20 mg/ml. The prepared solutions were centrifuged using Bio-Cote centrifuge, to enhance the sedimentation of solids that may block the column. 150 µl of centrifuged samples were then loaded onto an Auto-sampler. This separation was performed on the Kromasil reverse-phase ODS C18 5 µm column (4.6 × 250 mm). Gradient separation was performed using mobile phase A (100 % Millipore water) and mobile phase B (100% HPLC grade methanol). The separation condition was set as follows; 10% of B in A at injection time, 80 % of B in A at 30^{th} minute, 100% of B at 31^{st} to 37^{th} minute then normalized to 10% of B in A at 38^{th} minute to 45^{th} minute where the separation process was stopped, and the collected fractions removed and later concentrated under a reduced pressure in Rotary evaporator to yield pure compounds. The oven temperature was maintained at 40°C and a flow rate of 3 ml/min. Chromatographic separations was monitored at absorbance ranges of 220 to 420 nm. Both Millipore water and methanol were of analytical grade supplied by Scharlau Lab supplies limited, F3 of SC-S(9) which was identified to have desirable antibacterial activities against test organisms, yielded one compound which was recorded as compound **1**. High field 1D and 2D NMR spectroscopy and mass spectrometer were performed for Compounds **1**.

Antibacterial assay for crude extracts, fractions and pure compounds

Paper disc diffusion inhibition was used to screen for antibacterial

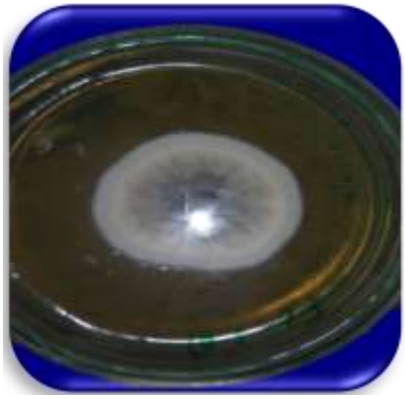

Figure 1. Sprouting *Diaporthe* sp. fungal endophyte in PDA media.

activities of crude ethyl acetate extracts, fractions from column chromatography, and the purified compounds. 100 µ of bacterial suspensions (1.5 × $10^{\wedge 8}$ CFU/ml) was homogeneously spread on sterile Mueller Hinton agar (38 g/l) in Petri dishes. The ethyl acetate fungal extracts, fractions from column chromatography, and pure compounds were prepared by dissolving them in 0.1% DMSO in distilled sterile water. The sterile paper disc was soaked in 50 mg/ml concentration of the prepared extracts then placed at the Centre of MHA plates containing the bean bacterial pathogens. A sterile disc dipped in 0.1% DMSO was used as a negative control, while standard chloramphenicol antibiotic was used as a positive control for the anti-bacterial activities. The plates were sealed using para-film and inoculated at 37 ± 2°C for 24 h, after which the zones of inhibitions were measured in millimeters using a ruler scale. This experiment was done in triplicates.

Determination of minimum inhibitory concentration (MIC)

The extracts and the pure compounds showing anti-bacterial activity in the pre-screening assay were evaluated for their Minimum inhibitory concentration (MIC) using a method explained by Balouiri et al. (2016), whereby; serial dilutions of the identified extracts and compounds with appreciable inhibition zones were dissolved using 0.1% dimethyl sulfoxide (DMSO) in distilled sterile water. The crude extracts and the purified compounds were diluted to the following serial geometric dilutions: 75, 50 and 25%. The subsequent minimum inhibition zones were used to determine the array for MIC analyses. In all cases, the cultured plates were incubated at 37 ± 2°C for 24 h. The lowest concentration able to induce inhibition zones was considered as the MIC. The experiment was performed in triplicates.

Structure elucidation

Nuclear magnetic resonance (NMR) spectroscopy: The ^{1}H, ^{13}C, DEPT, HSQC, COSY, and HMBC NMR spectra were recorded on the Bruker Advance 500 MHz NMR spectrophotometer at the Technical University of Berlin, Germany. The readings were done in Deuterated DMSO and chemical shifts assigned by comparison with the residue proton ^{1}H and carbon, ^{13}C resonance of the solvent. Tetramethyl-silane (TMS) was used as an internal standard and chemical shifts were given as δ (ppm). The off-diagonal elements were used to identify the spin-spin coupling interactions in the ^{1}H-^{1}H/COSY (Correlation spectroscopy). The proton-carbon connectivity, up to three bonds away, was identified using ^{1}H-^{13}C HMBC (Heteronuclear Multiple bond Correlation). HSQC spectrum (Heteronuclear Single Quantum Coherence) was used to determine the connectivity of hydrogen to their respective carbons atoms.

Mass spectrometry: The compound's mass spectra were recorded on FinniganTripple Stage Quadrupole Spectrometer (TSQ-70) with an electron spray ionization (ESI) method in the analysis. Thermo X Calibur Qual computer software was used in the analysis of the mass chromatograms.

Statistical analysis

Comparison of means for antibacterial activities was done using SPSS version 25.0 and the most bioactive endophytes or secondary metabolites were selected based on their antibacterial activities as shown by the sizes of inhibition zones. The difference in the mean inhibitory effect of each fungal extract was determined using one-way ANOVA; where the correlation of their antibacterial activities against positive and negative control was performed through Tukey's Honestly Significant Difference (HSD) test, at $P < 0.05$, Turkey's test). NMR data of pure compounds were analyzed using Bruker topspin 3.5 software.

RESULTS AND DISCUSSION

Isolation and identification of fungal endophytes

One fungal isolate assigned isolation code SC-S (9) was isolated from the stem bark of *S. cordatum* (Figure 1). Molecular identification was used in placing the fungal isolate to its respective taxonomical level. The molecular identification was done by using the ITS barcoding to generate the corresponding ribosomal sequences, ITS region is considered as the most conserved region in fungal endophytes hence was picked as a genomic marker in the identification of the fungal isolate (Badotti et al., 2017). The neighbor-joining approach was used in determining the phylogenetic relationship of the fungal isolate with a subsequent comparison with that from NCBI (National Centre for Biotechnology Information) and UNITES (User-friendly Nordic ITS Ectomycorrhiza) databases. The results confirmed that SC-S (9) belongs to the genus *Diaporthe* of phylum Ascomycota. However the hints obtained from BLAST search tool using ITS sequences in NCBI and UNITE suggests that the fungal isolate could not be identified to its species name, even though, a similarity index of 98.75% was recorded in a BLAST search against *Diaporthe anarcadii*; this could not be conclusively used in determining its species name. This indicates that the use of BLAST search tool could have exhibited drawbacks due to inaccurately identified sequences present in the fungal databases or that the use of the ITS region as a genomic marker has inefficiencies and limitation in identifying fungi to their respective taxonomical levels (Badotti et al., 2017). *Diaporthe* is the most encountered genera of fungal endophytes in several host plants. The genus is acknowledged to be a source of enzymes and bioactive

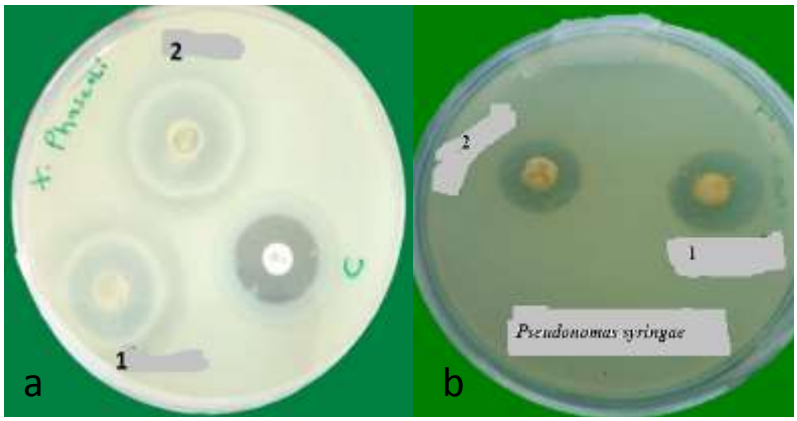

Figure 2. Dual culture assay of *Diaporthe* sp against (a) *Xap* and (b) *Psp.*

secondary metabolites having anti-bacterial, anti-cancer and anti-fungal activities (Radji et al., 2011; Gomes et al., 2013; Li et al., 2015).

As compared to this study, endophytic fungi of genus *Diaporthe* has also been isolated from plants such as acacia, *Maytenus ilicifolia, Berberis vulgaris* having substantial anti-bacterial and antifungal activities (Aly et al., 2010). Therefore, this depicts that endophytic fungi are prospective sources of anti-bacterial compounds.

Antagonistic assay

Dual culture assay was used in ascertaining the antibacterial activities of the fungal isolate against the test organism. *Diaporthe* sp showed desirable antibacterial activities against *X. axonopodis pv phaseoli* and *P. syringae pv phaseolicola* with corresponding zones of inhibition of 17.00 ± 0.58 mm and 14.00 ± 1.15mm respectively. The fungal isolate *Diaporthe* sp, showed a profound antagonism against the selected phytopathogens *Xap* and *Psp,* suggesting that the isolated fungus is not pathogenic to the host plant due to its activity to act against pathogens (Bisht et al., 2016). Fungal endophytes colonize the host plant's tissues and adapt to it, henceforth creating an antagonistic nature against pathogens (Bisht et al., 2016). Generally, their antagonistic capacity would disperse, reduce, suppress, or induce resistance against pathogens. As compared to the present study, it was clear that the fungal isolate *Diaporthe* sp suppresses the development of phytopathogens (*Psp* and *Xap*), evidently shown on the inhibitory zones in dual culture assay (Figure 2).

Fungal endophytes possessing bioactivities against pathogens plays a key role in the host plant by ensuring that the host is safe from pathogenic attacks and hence have an intimate correlation in general development and physiological activities of the host plant (Noor et al., 2018).

Extraction and purification of secondary metabolites

Diaporthe sp. yielded 2.61 g of hexane crude and 1.82 g of ethyl acetate crude after liquid-liquid portioning. Ethyl acetate on purification on column chromatography yielded four tractions named; F1 (10 mg), F2 (30.45 mg), F3 (100.18) and F4 (40.98 mg). F3 of *Diaporthe* sp. due to its antibacterial activity against the test organisms was further selected for further purification on pHPLC, where one pure compound was isolated. Methanol was used as a first-line extractant because most of the bioactive molecules are soluble in it (Kaufmann and Christen, 2002).

Minimum inhibitory concentration (MIC) determination

MIC assay against X. axonopodis pv phaseoli

Secondary metabolites present in all the extracts showed activities against bacterium *X. axonopodis pv phaseoli (*Table 1*).* F3 and F1 of SC-S (9) showed palpable MIC values of 1.25 mg/ml (7.67 ± 0.33 mm) and 1.25 mg/ml (7.00 ± 0.00 mm) against *Xap* respectively, other MIC values are shown in Table 1. The information above shows that the extracts contain a mixture of secondary metabolites that can yield lead compounds to be used in the formulation of agrochemicals to be used in anti-phytopathogenic control in beans (*P. Vulgaris L*.).

MIC assay against P. syringae pv phaseolicola

Most of the fungal extracts did not show noticeable activity against the bacterium *P. syringae pv phaseolicola*. F3 and F2 of SC-S (9) showed appreciable MIC value of 3.75 mg/ml (10.33 ± 0.33 mm) and 2.50 mg/ml (7.00 ± 0.00 mm) respectively, other MIC values

Table 1. Zones of inhibition of serially diluted fungal extractives against test organisms.

		ZoI ± SE mm		
	EA	**Dilution (%)**	***X. axonopodis***	***P. syringae***
SC-S(9) *Diaporthe* sp.	F1	100	12.00 ± 0.58 ^{b,c}	**7.00 ± 0.00** ^{b}
		75	11.00 ± 0.58 ^{b,c}	0.00 ± 0.00 ^{a}
		50	9.67 ± 0.33 ^{b}	0.00 ± 0.00 ^{a}
		25	**7.00 ± 0.00** ^{b}	0.00 ± 0.00 ^{a}
	F2	100	12.33 ± 0.88 ^{b,c}	12.00 ± 0.33 ^{c}
		75	11.00 ± 0.58 ^{b,c}	**10.33 ± 0.33** ^{b,c}
		50	9.00 ± 0.58 ^{b}	0.00 ± 0.00 ^{a}
		25	**7.00 ± 0.00** ^{b}	0.00 ± 0.00 ^{a}
	F3	100	13.33 ± 0.67 ^{c}	10.33 ± 0.88 ^{b,c}
		75	11.00 ± 0.58 ^{b,c}	7.33 ± 0.33 ^{b}
		50	9.67 ± 0.33 ^{b}	0.00 ± 0.00 ^{a}
		25	**7.33 ± 0.33** ^{b}	0.00 ± 0.00 ^{a}
	F4	100	14.33 ± 0.33 ^{c}	12.33 ± 0.33 ^{c}
		75	12.33 ± 0.33 ^{b,c}	11.00 ± 0.58 ^{b,c}
		50	9.67 ± 0.33 ^{b}	7.00 ± 0.00 ^{b}
		25	**7.67 ± 0.33** ^{b}	0.00 ± 0.00 ^{a}
		100	11.67 ± 0.88 ^{b,c}	0.00 ± 0.00 ^{a}
		75	10.00 ± 0.58 ^{b,c}	0.00 ± 0.00 ^{a}
		50	8.00 ± 0.58 ^{b}	0.00 ± 0.00 ^{a}
		25	**7.00 ± 0.00** ^{b}	0.00 ± 0.00 ^{a}

EA, ethyl acetate.

are shown in Table 1.

The values given are the mean of the three experiments ± Standard error (S.E). The highlighted values show the zones of inhibition (ZoI) regarded to give MIC values of the fungal extracts. Extracts sharing the same letter (s) within the columns are not significantly different in their anti-bacterial activities, while those with a different letter(s) are significantly different in their activities ($P<0.05$, Turkey's test). The coding, EA representing ethyl acetate crude extracts of the fungal endophytes while F (1-4) represents fractions eluted from column chromatographic analyzes. The red-colored zones of inhibition represent MIC values of extracts at different geometric dilutions. The reported antibacterial activities of the extracts at different geometric concentrations are attributed to the presence of bioactive molecules that act against them.

Kirby-Bauer disk diffusion method reveals that fractions yielded on column chromatography were all active against bacterium *X. axonopodis pv phaseoli*, with F3 and F1 of *Diaporthe* sp. showing the highest activities against *X. axonopodis pv phaseoli* with their corresponding MIC value of 1.25 and 1.25 mg/ml, respectively. The smaller MIC values depict that the extractives are resistant to the pathogens at very low concentrations. The antibacterial activities of fungal extracts against selected bean pathogens were based on their growth suppressing capacity against the test organism on MHA media as expressed by inhibitory zones.

Antibacterial results of this study, correlate with a research study done by Wanga et al. (2018). Whereby, the ethyl acetate of fungal endophyte *Fusarium solani* isolated from *Markhamia lutea* which showed considerable antibacterial activity against *X. axonopodis pv phaseoli* with a zone of inhibition of 14.00 ± 0.00 mm.

The bacterium, *P. syringae pv phaseolicola* was very resilient to most of the fungal extracts, it is only, F1 and F3 of SC-S (9) which had significant activities against this bacterium (Table 1). The results obtained were in agreement with that of Wanga et al. (2018) where most of the fungal and plant extracts showed minimal activities against bean bacterial pathogen *P. syringae pv phaseoli*

The resistivity of bacterium *P. syringae pv phaseoli* bacterium is associated with the many modes under which the bacterium can easily mutate. Secondly, it is due to the presence of an E-flux pump; an internally developed mechanism, which is substrate-specific, and functions to prevent the accumulations of antibacterial drugs within their systems, which in turn prevents them

Table 2. NMR data of compound 1.

S/N	^{13}C δ_C ppm	DEPT	HSQC δ_H ppm	HMBC
1	164.7	C	-	-
2	99.0	CH	6.59	1,3,4,9
3	166.1	C	-	-
4	103.0	CH	7.21	2,3,9,10
5	138.2	C	-	-
6	118.1	CH	6.70	8,10,11
7	152.8	C	-	-
8	101.7	CH	6.62	6,7,10
9	98.4	C	-	-
10	108.2	C	-	-
11	25.0	CH_3	2.73	5,6,10
O-CH_3	55.8	CH_3	3.90	3

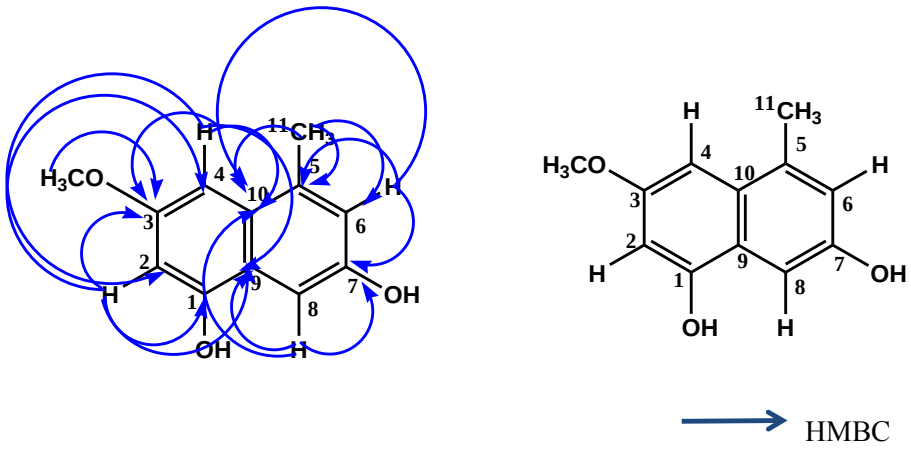

Figure 3. Structure and HMBC correlations of compound 1.

from reaching the target sites.

Characterization of secondary metabolites

In this study, **one** pure compound was isolated from *Diaporthe* sp. The pure isolate was characterized using spectroscopic techniques which include; 1D, 2D NMR experiments, and mass spectrometer.

Compound **1,** was isolated as a white solid at room temperature from endophytic fungus *Diaporthe* sp (SC-S (9) having a molecular mass of 204 and a molecular formula of $C_{12}H_{12}O_3$. It has a hydrogen deficiency index of seven, corresponding to; two fused aromatic rings and five carbon double bonds within the aromatic systems.

^1H NMR reveals that compound **1** had four aromatic protons resonating at; δ_H 6.59 (H-2), δ_H 7.21 (H-4), δ_H 6.70 (H-6) and δ_H 6.62 (H-8) corresponding to the carbon signals resonating at δ_C 99.0(C-2), δ_C 103.0 (C-4), δ_C 118.1 (C-6) and δ_C 101.7 (C-8) in the HSQC spectrum respectively. A highly de-shielded methyl proton resonating at δ_H 2.73 (H-11) corresponding to the carbon signal at δ_C 25.0 (C-11) in the HSQC spectrum. One methoxy proton resonating at δ_H 3.90 corresponds to the carbon signal at δ_C 55.8 in the HSQC spectrum.

^{13}C NMR and DEPT spectra revealed that compound **1** had a total of 12 carbon atoms of which; four were methine carbons at δ_C 99.0(C-2), δ_C 103.0 (C-4), δ_C 118.1 (C-6) and δ_C 101.7 (C-8), one methyl carbon at δ_C 25.0 (C-11), one methoxylated carbon at δ_C 55.8 and six quaternary carbon at, δ_C 164.7 (C-1), δ_C 166.1 (C-3), δ_C 138.2 (C-5), δ_C 152.8 (C-7), δ_C 98.4 (C-9) and δ_C 108.2 (C-10).

HMBC spectrum showed that proton resonating at δ_H 6.59 (H-2) correlates with carbon signals at C-1, C-3, C-4, and C-9 while that resonating at δ_H 7.21 (H-4) correlates with C-2, C-3, C-9 and C-10. In addition methoxy protons resonating at δ_H 3.90 correlates with C-3. Other HMBC correlations are shown in Table 2 and Figure 3. Data from NMR experiments gave compound

23 and IUPAC name as 3-methoxy-5-methylnaphthalene-1, 7-diol.

Information coined from Table 2 was collectively used in developing the structure for compound **1.** Compound **1**, is a Naphthalene derivative with, hydroxyl, methyl, and methoxy groups attached to it. Naphthalene is a group of volatile organic compounds that contains two fused benzene ring with a skeletal molecular formula of $C_{12}H_8$. It has been used in the production of phthalic anhydride that acts as a moth repellant, this is so because the presence of conjugated double bonds within the two fused benzene rings enhances its nucleophilicity hence regarded to be more reactive as compared to benzene (Daisy et al., 2002). Its derivatives are known to possess strong antimicrobial, insecticidal and vermicidal activities (Würthner and Stolte, 2011).

Muscodor albus an endophytic fungus isolated from *Terminalia prostrate* produces volatile organic compounds such as naphthalene having significant antimicrobial activities (Kouipou and Boyom, 2019). Other naphthalene derivatives such as 2-naphthol, 1-iodonaphthalene among others have also been isolated from endophytic fungus *M. albunus* with noticeable antimicrobial activities against series of plant pathogens (Strobel, 2011).

Endophytic fungi produce volatile organic compounds such as naphthalene and its derivatives to restrict the growth of other fungal or bacterial competitors within their environment especially in the host plant (Zhi-Lin et al., 2012; Kouipou and Boyom, 2019). It is evident therefore that compound **1** being one of these naphthalene derivatives possesses strong antimicrobial activities especially towards plant pathogens. This was in line with bioassay results obtained after screening of fungal extracts against bean bacterial pathogens *P. syringae pv phaseolicola* and *X. axonopodis pv phaseoli*, whereby, fraction 3, that yielded compound **1** had significant antibacterial activity, with corresponding zones of inhibition of 12.33 ± 0.88 and 10.33 ± 0.88 mm against *Xap* and *Psp* respectively. This is so because compound **1** has a naphthalene ring which acts as a strong nucleophile hence enhances destabilization of bacterial cell membranes, which in turn inhibits their growth. This compound is new and therefore through the present study it is reported for the first time. These results provide scientific validity and credence to the use of this plant in the treatment of bean's infections caused by some of the bacteria used in this study and highlights the usefulness of endophytic fungi from the stem bark of S*. cordatum* in the treatment of bacterial infections.

Conclusion

The study reveals that *S. cordatum* houses important endophytic fungi of genus *Diaporthe* and phylum Ascomycota. *Diaporthe* sp. isolated from the stem bark tissues of *S. cordatum* plant having an appreciable antibacterial activity against bean bacterial pathogens, P. *syringae pv phaseolicola* and *X. axonopodis pv phaseoli*; this is attributed to the mixture of secondary metabolites in it. Extractives from fungal endophyte *Diaporthe* sp*,* showed a noticeable anti-bacterial activity against *X. axonopodis pv phaseoli* but drably against *P. syringae pv phaseolicola.* One novel naphthalene derivate was isolated from the fungal extracts as a white solid at room temperature; a naphthalene derivative which was named as; 3-methoxy-5-methylnaphthalene-1, 7-diol (compound **1).** Bioactivity of this compound is associated with the presence of naphthalene ring, which enhances its nucleophilicity and presence of hydroxyl and methoxy groups attached to the main ring, which also act as ring activators, in turn enhancing its reactivity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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APPENDIX

Appendix 1. ITS consensus sequence of the isolated fungal endophyte.

Isolation code	DNA coding	Specific identity
SC-S(9)	TCCGTTGGTGAACCAGCGGAGGGATCATTGCTGGAACGCGCCCCAGGCGCACCCAG AAACCCTTTGTAAACTTATACCTTACTGTTGCCTCGGCGCAGGCCGTCCCCTATGGGG TCCCTTGGAAACAAGGAGCAGCCGGCCGGCGGCCAAGTTAACTCTGTTTTTAAACTG AAACTCTGAGTACAAAACATAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCT GGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGT GAATCATCGAATCTTTGAACGCACATTGCGCCCTCTGGTATTCCGGAGGGCATGCCT GTTCGAGCGTCATTTCAACCCTCAAGCCTGGCTTGGTGTTGGGGCACTGCCTGTAAA AGGGCAGGCCCTGAAATATAGTGGCGAGCTCGCCAGGACTCCGAGCGTAGTAGTTAA ACCCTCGCTTTGGAAGGCCTGGCGGTGCCCTGCCGTTAAACCCCAACTTCTGAAAAT TTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAA	*Diaporthe* sp.