ASSESSMENT OF BIOACTIVE COMPOUNDS PRODUCED BY ENDOPHYTIC FUNGUS ISOLATED FROM *SCLEROCARYA BIRREA* PLANT

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ABSTRACT

This research was aimed at the characterization of bioactive components of endophytic fungi isolated from the stem bark of Sclerocarya birrea plant using NMR, GC-MS and FT-IR. The stem of this plant was collected, prepared and screened for endophytic fungi. The isolated endophytic fungi were fermented for large scale production on potato dextrose broth and extracted with ethyl acetate. This crude extract was fractionated and purified over silica-gel column chromatography. The fractions obtained were tested for antimicrobial activity and most active isolate (SB7-FO-F_{10}) were characterized. Isolate SB7-FO-F_{10} showed antibacterial activity with zone of inhibition of 13mm, 14mm and 14mm against E. coli, Streptococcuspyogenes and Pseudomonas aeruginosa respectively. From the spectral analyses, isolate SB7-FO-F_{10} afforded a compound characterized to be 6-(5-ethoxypentyl) 1-pentyl-2-methylhex-2-enedioate.

Key words: Bioactive, Characterization, Column Chromatography, Endophytic Fungi.

INTRODUCTION

Natural products, because of their ecological safety and greater variety of chemical structures compared to synthetic compounds, are regarded as powerful, promising source of novel compounds as well as hopeful choice for innovation in both medicine and agro chemistry. Plant is the chief source of natural products, while those from microorganisms tend to be more bioactive than the synthetic ones [1, 2]. Microorganisms are underutilized sources of natural products. They evolve in biosynthesis pathway and mechanism for synthesizing a rich arsenal of complex secondary metabolites, particularly those from bacteria and fungi. Very little of them are known [3, 4].

Sclerocarya birrea is an African medicinal plant used to cure diseases and heal injuries. The larval stage of the beautiful green African moth *Argemamimosae* feeds on marula leaves [5-7]. In Nigeria and some other African countries, the stem bark, roots and leaves *of S. birrea* are used for an array of human ailments, including malaria fever, diarrhoea, dysentery, stomach ailments, headache, toothache and body pains [8]. Anticonvulsant effect of aqueous stem bark of *S. birrea* extract in mice was reported by Ojewale *et al* [9].

An endophytic fungus has been described as fungi that asymptomatically colonize healthy plant tissues, even though they may after incubation or a latency period cause disease [10]. Several endophytes secret secondary metabolites that protect plant against insect, pests, pathogenetic organism and herbivores [11]. They proved to be the promising sources of biologically active product of interest for specific medicinal applications [12]. Researches toward identification of bioactive metabolites lead to discovery of many new active compounds from different types of endophytic fungi such as podophyllotoxin, a well-known aryl tetralin lignan with potent anticancer, antiviral, antioxidant, antibacterial, immunostimulation and anti-rheumatic properties [13-19] and anti-fungal agents, oxysporidinone [20] and 6-epi-oxysporidinone [21], which were isolated from the Fusarium genus. This present research was aimed to screen, identify the potential novel antibioatic-producing endophytic fungi from the tissue of Sclerocarya birrea plant and characterize the isolated bioactive component using NMR, GC-MS and FT-IR

MATERIAL AND METHOD

Plant Sampling and Isolation of Endophytic Fungi

The plant samples were collected from Botanical and Ecological Gardens, Department of Botany, Bayero University, Kano (Nigeria). The plant surface was sterilized according to [22-24] and the sterilized segments were placed in a petri dish containing the potato dextrose agar (PDA) medium with chloramphenicol 280 $mgml^{-1}$ to suppress bacterium contamination. After an incubation of seven days, new fungal colonies were monitored and picked out. Individual fungal colonies were picked out and sub-cultured in PDA and the isolated fungi were stored at low temperature.

Identification of Fungi

The fungal isolates were prepared on slides and stained with lactophenol cotton blue reagent and examined with bright-field and phase-contrast microscope [24].

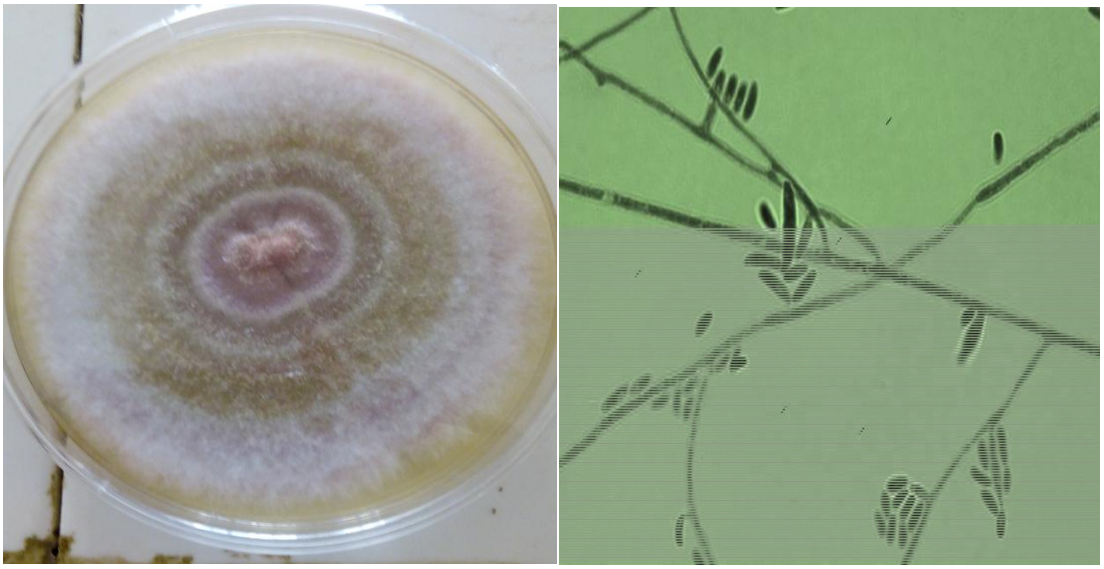

Plate 1: *Fusarium oxysporum*

Cultivation for Screening of Secondary Metabolites

About 100 mL of the potato dextrose broth was dispensed in 250 mL conical flask each and autoclave at 121 ^{0}C, 15lb for 15 min. Plug of the fungal material of about 2cm x 2cm were cut into each flask covered with bunk and kept static for about 30 days at room temperature. Flasks were regularly observed for fungal purity and similarities while variations were considered as contaminated and were discarded.

Extraction of the secondary metabolites from *Fuseriumoxysporum*

The mycelium was separated from the liquid by suction filtration using vacuum pump and the fungal mycelium was soaked in methanol prior to extraction, while the broth filtrate was extracted with ethyl acetate thrice. The broth ethyl acetate was partitioned with brine solution to remove unwanted materials. It was afterwards separated from the brine solution and dried with anhydrous $MgSO_4$ and finally concentrated at reduced pressure. The mycelia were soaked in methanol thrice for one week each and filtered by suction to afford the methanolic extracts which was concentrated at reduced pressure to yield orange-red residue. The methanolic crude extract was further partitioned between water and ethyl acetate (1:1) to yield the ethyl acetate fraction which was washed with brine solution and dried with anhydrous $MgSO_4$ and finally concentrated at reduced pressure to yield an orange-red solid 11.1g which was labeled as **SB7-FO.**

Plate 1: Mycelium of *F. oxysporum* grown on Potato Dextrose Broth

Purification of SBO-FO Ethyl Acetate Extraction

The ethyl acetate crude extract (5.0 g) was subjected to silica gel column chromatography in a glass column (121 x 2.5cm capacity), eluting with a total volume of 500 mL of hexane-chloroform gradient (100:0.00; 1:1; 2:3; 1:4; and 0.00:100), followed by chloroform: ethyl acetate, 9: 1; 4:1; 3:2; 1:1; 2:3; 1:4; 100% ethyl acetate and finally washed with acetone. About 50 mL of eluent was collected at intervals. The entire column produced 120 fractions. These fractions where pooled to give nineteen fractions which were later pooled to give three fractions.

Purification of F67 of SB7-FO

Fraction obtained from F_{67} of SB7- FO samples was further purified over silica gel column chromatography eluting total volume of 100 mL hexane (100 %), hexane: chloroform (60:40; 50:50; 40:60; 100 % chloroform), chloroform: ethyl acetate (60:40; 50:5; 40:60; 100% ethyl acetate) and finally washed with 100 % acetone. About 20 mL eluent was collected at an interval which resulted in 50 fractions. F_{29}= obtained from the main column of F_{67} (SB7-FO) sample was further purified to give eleven fractions after pooling those with the same Rf value and code with SB7-FO-F_n where n is the fraction number.

Antibacterial assay

To assess the antimicrobial activity of the endophytic fungi, five clinically isolated *Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, strains were obtained from Microbiology Department, Bayereo University, Kano. Two loop fills of the standard inoculum were steadily streaked onto the prepared nutrient agar plates and discs of different concentrations as well as amoxicillin which served as the positive control were aseptically placed and pressed firmly onto each plate using sterile forceps at about 40 mm apart. After 15 min of free diffusion time, the plates were incubated at 37 ^{o}C for 24 h. Diameters of zones of inhibition were measured using a millimeter rule and recorded to the nearest whole number.

RESULTS AND DISCUSSION

During the screening of endophytic fungi from the tissue of *S. birrea* plant, three different species of *Fusarium* were isolated. The potential novel antibiotic-producing endophytic fungi were identified for exact genus according to its morphological features and conidia as *F. oxysporum.* Based on preliminary antimicrobial study, about sixty liters of *F. oxysporum* were fermented on potato dextrose broth for a period of four months to yield 11.7 g of crude ethyl acetate extract which was later subjected to purification over silica-gel column chromatography.

Fractions from fungal strain SB7-FO showed only antibacterial activity (Table 1), even though their action against the microorganisms used was selective. SB7-FO-F_5, SB7-FO-F_{10} and SB7-FO-F_{30} were found to show potent antibacterial activity, with SB7-FO-F_{10} been more effective against *P. aeruginosa* with zone of inhibition of 14 mm at 1000µg/mL followed by 13 mm and 14 mm at 1000µg/mL for *S. pyogenes* and *E.coli* respectively. SB7-FO-F_{30} was more effective on *S. aureus* with zone of inhibition of 12 mm at 1000µg/mL followed by *S. pyogenes* with 14mm at 1000µg/mL, then *E.coli* with zone inhibition of 10 mm at 1000µg/mL and SB7-FO-F_5 was more effective against *S. pyogenes*, *S. aureus*, *K. pneumoniae* and *P. aeruginosa* with inhibition of 14 mm, 11 mm, 12 mm and 10 mm at 1000µg/mL respectively. The effect of these fractions was average when compared with the effect of the drug as control which has a maximum zone of inhibition of 26mm at 10µg/disc against *K. pneumoniae.*

The potential endophytic fungi have been identified for the exact genus based on morphological colony and spore or conidia characteristic as *F. oxysporum.* The compound isolated in this study

was characterized as a fatty acid ester 6-(5-ethoxypentyl) 1-pentyl-2-methylhex-2-enedioate and possess a potential biological activity.

Table 1: Antibacterial Activity of Column Fractions (F_{29} of F_{67}) from the Crude Extract of SB7-FO

Sample	Concentration (µg/disc)	Zone of Inhibition (mm)				
		E. coli	*S. pyogenes*	*S. aureus*	*K. pneumoniae*	*P. aeruginosa*
SB7-FO-F_5	10	08	10	07	08	08
	100	08	10	07	08	10
	1000	09	14	11	12	10
SB4-FO-F_{10}	10	08	07	08	08	09
	100	10	10	09	10	09
	1000	13	13	11	12	14
SB7-FO-F_{15}	10	NA	07	NA	NA	NA
	100	NA	07	NA	NA	NA
	1000	NA	10	NA	NA	NA
SB7-FO-F_{23}	10	NA	NA	NA	NA	NA
	100	NA	NA	NA	NA	NA
	1000	NA	NA	NA	NA	NA
SB7-FO-F_{24}	10	08	NA	NA	07	NA
	100	10	NA	NA	08	07
	1000	10	NA	NA	08	07
SB7-FO-F_{30}	10	07	07	10	07	07
	100	08	10	10	09	08
	1000	10	14	12	09	12
SB7-FO-F_{35}	10	NA	NA	NA	NA	NA
	100	NA	NA	NA	NA	NA
	1000	NA	NA	NA	NA	NA
N mgw						
SB7-FO-F_{40}	10	NA	NA	NA	NA	NA
	100	NA	NA	NA	NA	NA
	1000	NA	NA	NA	NA	NA
SB7-FO-F_{45}	10	NA	07	NA	07	NA
	100	07	NA	08	08	NA

	1000	07	NA	08	08	NA
Amoxicillin	10	25	19	26	24	26

Keys: **NA** = No Activity means zone of inhibition equal to 6mm. (+) = Zone of inhibition greater than 6mm. ***E. coli***= *Escherichia coli*,***S. pyogenes***= *streptococcus pyogenes*, ***S. aureus***= *Staphylococcusaureus*, ***P. aeruginosa***= *Pseudomonas aeruginosa*, ***K. pneumoniae***= *Klebsiella pneumoniae.*

Previous researches revealed that fatty acids and their esters exhibited strong antimicrobial activity against microorganisms, demonstrating some specificity for individual microbial species [25] and another report by Rahmani and Heydarian [26] which indicated that fatty acid ester are active metabolites in *Salicornia* species. The compound isolated in this work was found to possess potent antimicrobial activity against *S. pyogenes* and *P. aeruginosa* followed by *S.aureus* and *E.coli.* This is in agreement with finding of Chandrasekharan *et al* [27] that studied antimicrobial activity of fatty acid ester extracts of four halophytic plants, *Arthrocremumindicum, Salicornia brachiata, Suaedamaritime* and *Suaedamonoica* belonging to the family chenopodiaceae. Their composition was analyzed by GC-MS and the result was promising. Compounds of this type (fatty acid esters) were found to possess various types of biological activities as reported by Gehan *et al* [28] which stated that a similar compound, octadecanoic acid methyl ester possessed the ability to inhibit the growth of pathogenic bacteria and fungi. 10, 13-eicosadienoic acid methyl ester has been reported to possess excellent antibacterial and antifungal activities [29].

This present study is in agreement with reports of Huda [30] which reported the antimicrobial activity *F. oxysporum.* It also agrees with the finding of Abubakar *et al* [31] who reported that the stem bark of *Sclerocarya birrea* is used as traditional medicine in Kano metropolis. It is also supported by the statement of Muhammad *et al* [29] about the antimicrobial activity of some fatty acid esters isolated from *Nigella sativa* and that of Saikkonen *et al* [11] which stated that plants and their associated endophytes were found to produce the same natural compounds. This research supported few researchers [32-35] who reported the antimicrobial activities of biosynthesized compounds by plant endophytes.

CONCLUSION

The stem bark of *Sclerocarya birrea* plant was screened for endophytic fungi and four different species were isolated. Strain of two species was found to possess potent antimicrobial activity. The column chromatograph of crude extracts of these strains produced fractions that possess significant antimicrobial activity with some showing selectivity against the tested pathogens. A bioactive compound which was found to possess a significant anti antibacterial effect against the tested organisms was isolated from the crude ethyl acetate extract of *F. oxysporum* and characterized using NMR (^{1}H, ^{13}C), GC-MS and FT-IR as 6-(5-ethoxypentyl) 1-pentyl-2-methylhex-2-enedioate.

RECOMMENDATION

Further research using advance analytic techniques such as 2-D or 3-D NMR, high resolution GC-MS is recommended to elucidate and propose the exact structure of the isolated compound

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