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Original article

EVALUATION OF THE *in vitro* ANTI-TRYPANOSOMAL ACTIVITIES OF ETHYL ACETATE AND AQUEOUS EXTRACTS OF ENDOPHYTIC FUNGI ISOLATED FROM *Psidium guajava* LEAVES AND STEMS

[*1]Anichebe, F.N., [1]Dadah., A.J. and [1]Musa F.M.

[1]Nigerian Institute for Trypanosomiasis Research, Kaduna
[1]Department of Microbiology, Kaduna State University

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ABSTRACT

Endophytic fungi which reside in the tissues of higher plants without causing overt symptoms on the plants in which they live – are reported to produce bioactive compounds which can be used in agriculture, pharmaceuticals and the food industries. This study aim to evaluate the anti-trypanosomal activities of endophytic fungi isolated from the leaves and stems of *Psidium guajava*. Following surface sterilization, the plant parts were placed in potato dextrose agar (PDA) to initiate fungal growth. Hyphae and spores of distinct fungal growth were further sub-cultured in sabouraud dextrose broth to isolate pure fungal isolates. Each isolated fungal species was subsequently grown in a 500ml Erlenmeyer' s flask containing 100ml of liquid broth (pH 5.6) for 7 days at 28°C at 220 rpm in an incubator shaker. The respective fungal growths were filtered from the broth, air dried, weighed and stored in well labelled airtight containers. Ethyl acetate and distilled water were used for extraction, and the respective extracts were evaluated for their *in vitro* anti-trypanosomal activities. Three endophytic fungi were isolated namely *Penicillium* sp., *Aspergillus niger*, and *Mucor racemosus*. The phytochemical analyses revealed the presence of alkaloids, flavonoids, phenols, tannins, steroids, terpenoids, anthraquinone, cardiac glycoside, and phlobatannins. The *in vitro* results indicated that the extracts of the ethyl acetate and aqueous extracts of the endophytic fungi exhibited concentration dependent effect against *Trypanosoma brucei brucei* between 0.15 and 10.0 mg/ml; the median lethal concentration (LC_{50}) of the extracts showed that ethyl acetate extract of *Penicillium* sp was the most active with an LC_{50} of 0.303 mg/ml.

Keywords: Endophytic Fungi, *Psidium guajava*, *Trypanosoma brucei brucei*, Isolation

Corresponding authors email: francesnk3@gmail.com/07033760933

INTRODUCTION

African trypanosomiasis is a disease complex affecting both man and livestock. It is caused by infection with any of the several species of African trypanosomes, which are obligate heamoflagellated parasitic protozoa [1] The disease is usually transmitted through bites from infected tsetse fly; other means of transmission include needle pricks, blood transfusion, coitus, and mother-to-child transmission [2].

African trypanosomiasis is currently sub-divided into two main groups: human African trypanosomiasis (HAT) and animal African trypanosomiasis (AAT) [3]. HAT is caused by infection with either *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodensiense* [2] Trypanosome infection in human results in sleeping sickness and is usually fatal if left untreated.

African animal trypanosomiasis (AAT) is caused by infection with any one or more of the animal infective species of trypanosomes including *Trypanosoma congolense*, *T. vivax*, and *T. brucei brucei*, [4]African animal trypanosomiasis is most important in cattle but can cause serious losses in pigs, camels, goats and sheep[5]. Infection of cattle by one or more of the three African animal trypanosomes results in sub-acute, acute, or chronic disease characterized by intermittent fever, anemia, occasional diarrhea, and rapid loss of condition and often terminates in death [5]

The global disease burden due to human African trypanosomiasis is put at 60,000 case incidences with 70 million people at risk of infection [2]. In livestock, trypanosome infection has resulted in reduced livestock productivity, shortage of livestock products, and loss of resources worth 1.34 billion US dollars with about 60 million cattle at risk of infection [6]. As there are no effective vaccines against African trypanosomiasis, disease control and prevention has relied on vector control and drug treatment [6].

The chemotherapy of human and animal African trypanosomiasis has largely relied on a few repertoires of drugs, some of which are associated with several clinical limitations [7 - 10]. These shortcomings have prompted research into newer and more effective anti trypanosomal agents, usually involving the screening of large libraries of chemically synthesized compounds for potential lead moieties [11-13]. Fungi have been reported to be an excellent source of secondary metabolites [13]. Some interesting compounds produced by endophytic microbes are Cryptocin, Cryptocandin, Jesterone, Oocydin, Isopestacin, Psuedomycins and Ambuic acid [14]. The anticancer drug Taxol has been found to be expressed by certain species of fungi *Taxomyces andreanae* [15]. Our survey of literature indicated that there are only very few investigations into the anti-trypanosomal activity of secondary metabolites of fungal origin. It is therefore the objective of this study to evaluate the antitrypanosomal activity of the ethyl acetate and aqueous extracts of endophytic fungi isolated from *Psidium guajava*.

The drugs which are currently marketed by pharmaceutical companies are beset by several limitations including: non availability, high cost, resistance and cross-resistance, and especially with the drugs for human use, severe/fatal side effects, long duration of therapy, and difficulty in administration [16,17]; Thus, there is an urgent need for the development of readily available, effective, and cheaper trypanocidal agents with few or no side effects; and fungi have been reported to express bioactive secondary metabolites with uniquely diverse structural motifs that can serve as lead compounds in the development of new drugs [18,19].

MATERIALS AND METHODS

Collection of Plant Materials

Leaves and stems of Psidium guajava were collected from the kaduna state university botanical garden 10.5167° N, 7.4505° E and taken to the Department of Biological Sciences Kaduna, State University for authentication. It was then taken immediately to the laboratory for processing and sterilization.

Processing and sterilization of plant materials

The freshly collected plant samples were gently rinsed in distilled water, to remove any attached debris. The water was allowed to drain from the plant materials. After washing, the samples were cut in small pieces (3-4 cm), and surface sterilized as follows: the plant samples was treated with 70% (v/v) ethanol for 1 min, and then immersed in 10% (v/v) sodium hypochlorite for 1 min, followed by another treatment with 70% ethanol for 30 sec. Subsequently, the materials were rinsed three times (3x) in sterilized water. The plant parts were blotted on sterile blotting paper. For the control, the final sterile water rinse was plated and observed post-incubation. The absence of fungal growth indicated that the leaf surface has been sterilized [20]

Isolation and Identification of endophytic fungi

Forty grams (40g) of potatoes dextrose ager (PDA) was weighed, to which 0.04gm of streptomycin was added and mixed together. The mixture was later transferred into 1000ml conical flask and made up to one liter of double distilled water. After gently swirling of the flask to dissolve the agar, the mixture was autoclaved at 121°C for 15 min, allowed to cool to about 45°C, then poured into sterile Petri dishes and finally allowed to solidify for three days [20].

The isolation of endophytic fungi was carried out using the method described by [20]. A segment of the plant material was placed on PDA medium supplemented with antibiotic (streptomycin), with the Petri dishes sealed with paraffin and kept under dark condition (this inoculated Petri dish was referred to as the master plate). Fungal growth was observed for 2 weeks, during which growth of the fungi was been initiated [20].

Isolation from the master plate was done by transferring the hyphal tips to fresh potato dextrose ager plate (PDA) without addition of any antibiotic to obtain the pure culture which was observed after 7-10days of inoculation. The pure culture was then taken to Microbiology Laboratory, KASU, for identification [20].

Morphological Identification of Fungal Isolates

The fungi were first identified on the basis of their morphological and cultural characteristics, while molecular methods were used for confirmation.
Morphological identification of fungi was according to the method described by [20].
Cultivation of endophytic fungi
Each isolated strain of fungi was grown in Sabouraud' s broth consisting of 40g/L dextrose and 10g/L peptone. The endophyte was grown in a 500mL Erlrnmeyer' s flask containing 100mL of liquid broth (pH 5.6) for 7 days at 28°C at 220 rpm in an incubator shaker [20].

Preparation of fungal extracts

The cultures (mycelia and broth) were respectively collected at 24 hours intervals for 7 days. Mycelia was thoroughly washed

with sterile distilled water and homogenized in a cell disintegrator and subsequently extracted with aqueous and ethyl acetate by cold maceration method. The culture broth was filtered through two layers of cheesecloth, and the filtrates extracted three times with an equal volume of ethyl acetate and water. The solvents were pooled together and concentrated under reduced pressure at 35°C. The dried crude extracts were stored at -20°C until required [20].

Qualitative phytochemical screening of the extracts

The fungal extracts were screened for the presence of bio-active phytochemical constituents following the methods of [21]. Phytochemicals assayed include tannins, saponins, phenols, sterols, flavonoids, alkaloids, anthraquinones, glycosides, terpenoids and oxalates.

Test organisms: *Trypansoma brucei brucei*

The test organisms used for this study was *Trypanosoma brucei brucei*. stabilates of these trypanosome species was obtained from the cryobank maintained at the Vector and Parasitology Study Department of the Nigerian Institute for Trypanosomiasis Research, Kaduna, Nigeria. After thawing the stabilates at 37°C, trypanosomes were screened for viability by examining wet smears prepared from the stabilates in the light microscope at X400 magnification. The presence of motile trypanosomes was taken as indication of trypanosome viability [24].

Donor animals

Two Male Albino Wistar mice was utilized as donor animals. Each of these was intraperitoneally inoculated with blood suspension inoculum (0.1 ml) prepared from the stabilates. The inoculum was prepared by the addition of phosphate buffered saline-glucose (pH 7.4) to a 2ml of PBS was added to 0.2ml of blood collected from the donor mice to achieve two achieve trypanosome count of two parasite (T.brucie) per microscopic field. The mice were inoculated with 0.1 ml of the prepared blood suspension. Parasitemia was monitored in the inoculated mice two days' post inoculation to determine the establishment of active infection. Following infection with the trypanosomes, the donor mice were sacrificed when the trypanosome count was about 10^9 per milliliter of blood [2].

Determination of parasitemia

The trypanosome count in the infected mice was determined by the rapid matching method of [23]. Briefly, a drop of whole blood collected by tail snip or cardiac puncture was placed on a clean grease free glass slide and a cover glass placed over it; the blood spreads into a thin circular film. The slide was placed in the light microscope and examined at x400 magnification. The distribution of trypanosomes among the red blood cells (RBCs) was matched against the Lumsden' s chart and the approximate number of trypanosomes per milliliter of blood was estimated [22].

Collection of parasitized blood

Blood containing trypanosomes was collected into a 5 ml syringe from the donor mice by the cardiac puncture technique after chloroform anesthesia. The blood was dispensed into an ethylene diamine tetra acetate (EDTA) sample container, and gently mixed together to prevent clotting of the blood [22].

Medium and supplementation of medium RPMI 1640 medium (Caisson Laboratory, USA) was used for the *in vitro* assay. It was supplemented with 1% (w/v) glucose [22].

Reconstitution of the fungal extracts and reference drug

Solutions of the extracts and drug (Diminazene aceturate) was reconstituted in the supplemented medium. Stock solutions of the respective plant extracts was prepared by dissolving 10 mg of the extract in 1 ml of medium; subsequently, the respective stock solsutions was serially diluted to yield extracts with concentrations ranging from 1 mg/ml to 0.03125 mg/ml. The various concentrations of Diminazene aceturate was also be prepared, concentrations also ranging from 1 mg/ml to 0.03125 mg/ml [22]

In vitro **assay of the crude extract (Drug incubation survival assay):**

The Drug Incubation Survival Test (DIST) was used for this assay. 100µl of the reconstituted solutions of each of the extracts, as well as the reference drug, was separately dispensed in triplicate into wells of a 96-well microtitre plate. 30µl of the blood suspension containing *T. b. brucei* was added to each of these wells. These was gently mixed together. Control wells containing only 100µl supplemented medium and 30µl blood suspension was also included. The micro plate(s) was placed in a desiccator containing about 5% carbon dioxide and maintained at 37°C in an incubator. Wet smears were prepared from each of these wells, six (6) hours post-incubation. Each smear was examined under light microscope (X400 magnification) and the counts of motile trypanosomes was taken over three fields of view, a total of nine observations per concentration of drug/extract. Similarly, counts were also taken for smears prepared from the control wells [22]

Molecular Identification of Fungal Isolates For molecular characterization, DNA was xtracted from mycelia from pure fungal colonies. The mycelia were transferred from PDA into 250ml Erlenmeyer' s flasks containing potato-dextrose broth without shaking. After 5 days of growth at 28°C, approximately 100mg of the mycelia biomass was harvested. The genomic DNA was isolated using Qiagen DNeasy Mini Kit according to the manufacturer' s instructions.

The isolated DNA was diluted in sterile distilled water and stored at 4°C. PCR was performed using the primers ITS4 (5' -TCCTCCGCTTATTGATATGC-3') and ITS5 (5' -GGAAGTAAAGTAACAAGG-3'). The reaction was performed in a 25µL final volume containing 0.1µg of genomic DNA, 10pM of each primer, 1x *Taq* polymerase buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, and 1U of *Taq* DNA polymerase. Parameters for thermal cycle was as follows: 94°C for 3 min, 35 cycles for 30s at 94°C, 40 s at 55°C, and 35 s at 72°C and final extension of 72°C for 7 min [20]

Data Analysis

Data were expressed as means and percentages of surviving trypanosomes. One-way ANOVA was used to compare means with p values less than 0.05 ($p < 0.05$) considered as statistically significant.

RESULTS

Morphological and Cultural Identification of endophytic fungi isolated from *Psidium guajava*

On the basis of their colony characteristic, three species of endophytic fungi were isolated from the leaves and stems of *P. guajava* (Table 1.1). The first endophytic fungus (designated Sample 1) was characterized by rapidly growing colonies which matured in 3 to 5 days with growth starting as hyaline, fluffy white tufts which later changes into green shades due to conidia production. On agar plates, the colonies of Sample 1 fungus were characterized by presence of concentric rings, branched conidiopores bearing clusters of flask-shaped phialides, and smooth wall (Table 1.1). The second endophytic fungus (designated Sample 2) was a filamentous fungus that consisted of smooth colourless conidiopores and spores. On Potato dextrose agar, Sample 2 fungus grew up to 4 – 5 cm in diameter within 7 days of culture. The conidiopores were densely layered with dark brown to black coloration. The conidial heads of Sample 2 fungus appeared radiated and later split into loose columns with age (Table 1.1). Sample 3, the third endophytic fungus identified had fast growing colonies with fluffy cotton-candy like growth within 5 days of culture. The fungus had a whitish appearance when young which turned blackish-brown with age of culture. Furthermore, the fungus consisted of short and tall sporangiophores with height varying between 2 and 20 cm

Table 1. Colony characteristic of species of endophytic fungi

Isolate	Designation	Colony characteristics	Presumptive species
1	Sample 1	Rapidly growing colonies which mature in 3 to 5 days; growth started as hyaline, fluffy white tufts which later changes into green shades due to conidia production; on agar plates.	*Penicillium* sp
2	Sample 2	Filamentous fungus; consists of smooth and colorless conidiophores and spores; on PDA grows up to 4-5 cm in diameter within 7 days; conidiopores were densely layered with dark brown to black coloration; conidial heads radiate and later split into loose columns with age.	*Aspergillus* sp
3	Sample 3	Fast growing colonies with fluffy, cotton-candy like growth within 5 days; had whitish appearance when young which turned blackish-brown with age; 2- 20 cm in height consisting of short and tall sporangiophores	*Mucor* sp

Phytochemical constituents of the ethyl-acetate and aqueous extracts of endophytic fungi isolated from the leaves and stems of *P. guajava*

A total of ten phytoconstituents were assayed for in the ethyl acetate and aqueous extracts of the three fungi isolated from the surface

sterilized leaves and stems of *P. guajava*. Ethyl acetate extract of the fungi (designated Sample 1) contained nine bioactive constituents namely alkaloids, flavonoids, phenols, tannins, steroids, terpenoids, anthraquinone, cardiac glycoside, and phlobatannins, with phenols as the most abundant while saponins were absent. The aqueous extract of sample 1 fungus contained four classes of phytoconstituents namely saponins, phenols, tannins, and terpenoids, with saponins being the most abundant (Table 1.2).

The ethyl acetate extract of the fungus designated sample 2 contained all ten phytoconstituents assayed for with terpenoids being the most abundant; the aqueous extract of the same fungal species contained four of the ten constituents assayed for namely, saponins, phenols, tannins, and anthraquinones (Table 1.2).

A total of six bioactive components, namely, alkaloids, flavonoids, steroids, terpenoids, cardiac glycosides, and phlobatannins were identified in the ethyl acetate extract of the fungus designated sample 3. The aqueous extract also contained alkaloids, and flavonoids, as well as saponins, phenols, tannins, and anthraquinones (Table 1.2).

Table 1.2 Phytochemical constituents of fungal isolates.

Phytochemicals	Methods	Sample 1		Sample 2		Sample 3	
		Ethyl acetate extract	Aqueous extract	Ethyl acetate extract	Aqueous extract	Ethyl acetate extract	Aqueous extract
Alkaloids	Mayer' s test	+	-	+	-	+	+
Flavonoids	Shinoda' s test	+	-	+	-	+	+
Saponins	Frothing test	-	+	+	+	-	+
Phenols	Ferric chloride test	+	+	+	+	-	+
Tannins	Ferric chloride test	+	+	+	+	-	+
Steroids	Suphuric acid test	+	-	+	-	+	-
Terpenoids	Salkowski' s test	+	+	+	-	+	-
Anthraquinone	Bontrager' s test	+	-	+	+	-	+
Cardiac glycoside	Keller-Killani test	+	-	+	-	+	-
Phlobatannins	Hydrochloric acid test	+	-	+	-	+	-

Key: (+) = Present;
(-) = absent

In vitro anti-trypanosomal activity of the aqueous and ethyl acetate extracts of three endophytes (*Penicillium* sp, *Aspergillus niger*, and *Mucus racemosus*) isolated from *P. guajava* leaves and stems

Effect of ethyl acetate extract of *Penicillium* sp on *T. brucei in vitro*

The ethyl acetate extract of *Penicillium* sp (SE1) demonstrated significant ($p < 0.05$) anti-trypanocidal activity against *T. b. brucei*,

at varied concentrations of 10 and 5 mg/ml of the extract. No surviving trypanosomes were detected. However, between 2.5 and 0.15 mg/ml concentrations, trypanosomes were detected with the counts of the protozoa varying inversely with concentration of the extract. At 2.5 mg/ml the mean number of surviving trypanosomes was 0.17±0.41 per field. At 1.25 mg/ml it was 0.67±0.52 per field, while at 0.625 mg/ml the mean count was 7.17±2.23 per field. At 0.3125 and 0.15625 mg/ml concentration of the ethyl acetate extract, the mean counts of surviving trypanosomes were 9.67±1.03 and 14.5±2.07 per field, respectively. From the counts of surviving trypanosomes, the *in vitro* median lethal concentration 0.303 mg/ml determined for the ethyl acetate extract of *Penicillium* sp (Table 4.3).

Effect of ethyl acetate extract of *Aspergillus niger* on *T. brucei in vitro*

The ethyl acetate extract of *Aspergillus niger* (SE2) also demonstrated significant ($p < 0.05$) concentration dependent *in vitro* anti-trypanocidal activity against *T. b. brucei*: between 10 and 2.5 mg/ml concentrations of the extract, no surviving trypanosomes were detected. However, between 1.25 and 0.15 mg/ml concentrations of the extract, trypanosomes are detected: at 1.25 mg/ml concentration, the mean trypanosome count was 0.50±0.55 per field, and 16.0±2.37 per field at 0.625 mg/ml concentration, while at 0.3125 and 0.15625 mg/ml concentrations of the extract, the mean counts were 20.2±2.23 and 31.3±2.35 per field, respectively. The *in vitro*

Table 1.3: Mean *In vitro Trypanosoma* counts at varied concentration of fungal extracts/drugs after six hours' incubation

Mean Protozoa Count						
Conc. (mg/ml)	SE1	SE2	SE3	SA1	SA2	SA3
10	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	26.0 ± 5.22^{d}	27.8 ± 2.32^{c}	0.17 ± 0.41^{g}
5.0	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{f}	26.8 ± 2.32^{d}	28.7 ± 1.63^{c}	5.67 ± 1.37^{f}
2.5	0.17 ± 0.41^{e}	0.00 ± 0.00^{e}	5.50 ± 1.64^{e}	28.5 ± 1.87^{bc}	29.3 ± 2.34^{bc}	8.50 ± 1.52^{e}
1.25	0.67 ± 0.52^{e}	0.50 ± 0.55^{e}	9.50 ± 2.17^{d}	28.7 ± 2.07^{bc}	29.8 ± 1.72^{bc}	16.0 ± 2.28^{d}
0.625	7.17 ± 2.23^{d}	16.0 ± 2.37^{d}	11.7 ± 2.58^{d}	29.0 ± 1.79^{bc}	30.8 ± 3.19^{bc}	18.2 ± 1.17^{c}
0.3125	9.67 ± 1.03^{c}	20.2 ± 2.23^{c}	22.3 ± 4.08^{c}	31.7 ± 0.82^{ab}	30.2 ± 2.79^{abc}	19.2 ± 2.99^{c}
0.15625	14.5 ± 2.07^{b}	31.3 ± 3.01^{b}	28.5 ± 1.88^{b}	32.7 ± 2.07^{a}	32.3 ± 3.27^{ab}	22.2 ± 1.72^{b}
Control	33.5 ± 2.35^{a}	33.5 ± 2.35^{a}	33.5 ± 2.35^{a}	33.5 ± 2.35^{a}	33.5 ± 2.35^{a}	33.5 ± 2.35^{a}
p value	< 0.001	< 0.001	< 0.001	< 0.001	0.007	< 0.001
LC_{50}	0.303	0.531	0.932	17.14	23.01	1.533

Values are given as mean ± standard deviation. In each column, mean values with different superscripts have statistically significant difference ($p < 0.05$).

Key: SE1: ethyl acetate extract of *Penicillium* sp;
SE2: ethyl acetate extract of *Aspergillus niger*;
SE3: ethyl acetate extract of *Mucus racemosus*;
SA1: aqueous extract of *Penicillium* sp;
SA2: aqueous extract of *Aspergillus niger*;
SA3: aqueous extract of *Mucus racemosus*

median lethal concentration of the ethyl acetate extract of *Aspergillus niger* against *T. b. brucei* was 0.531 mg/ml (Table 4.3).

Effect of ethyl acetate extract of *Mucus racemosus* on *T. brucei in vitro*

The ethyl acetate extract of *Mucus racemosus* (SE3) demonstrated significant ($p < 0.05$) concentration dependent *in vitro* anti-trypanocidal activity against *T. b. brucei*: at 10 and 5 mg/ml concentrations of the extract, no surviving trypanosomes were detected. However, between 2.5 and 0.15 mg/ml concentrations, trypanosomes are detected with the counts of the protozoa varying inversely with concentration of the extract: at 2.5 mg/ml the mean number of surviving trypanosomes was 5.50±1.64 per field, at 1.25 mg/ml it was 9.50±2.17 per field, while at 0.625 mg/ml the mean count was 11.7±2.58 per field. At 0.3125 and 0.15625 mg/ml concentration of the ethyl acetate extract, the mean counts of surviving trypanosomes were 22.3±4.08 and 28.5±1.88 per field, respectively. From the counts of surviving trypanosomes, the *in vitro* median lethal concentration of the ethyl acetate extract of *Mucus racemosus* was 0.932 mg/ml (Table 4.3).

Effect of aqueous extract of *Penicillium* on *T. brucei in vitro*

The aqueous extract of *Penicillium* sp (SA1) demonstrated significant ($p < 0.05$) *in vitro* anti-trypanocidal activity against *T. b. brucei* when compared against the control; at 10 and 5 mg/ml concentrations of the aqueous extract of *Penicillium* sp., the mean counts of surviving trypanosomes were 26.0±5.22 and 26.8±2.32 per field, respectively. The counts were 28.5±1.87, 28.7±2.07, and 29.0±1.79 trypanosomes per field at 2.5, 1.25 and 0.625 mg/ml concentrations, while at 0.31 and 0.15 mg/ml, the mean counts were 31.7±0.82 and 32.7±2.07 per field. The *in vitro* median lethal concentration of the aqueous extract of *Penicillium* sp. against *T. brucei* was 17.14 mg/ml (Table 4.3).

Effect of aqueous extract of *Aspergillus niger* on *T. brucei in vitro*

The aqueous extract of *Aspergillus niger* (SA2) demonstrated significant ($p < 0.05$) *in vitro* anti-trypanocidal activity against *T. b. brucei* when compared against the control. The mean trypanosome counts were 27.8±2.32, 28.7±1.63, 29.3±2.34 and 29.8±1.72 per field at 10, 5.0, 2.5 and 1.25 mg/ml, respectively. At 0.62, 0.31 and 0.15 mg.ml concentrations the mean counts of surviving trypanosomes were 30.8±3.19, 30.2±2.79, and 32.3±3.27 per field, respectively. The *in vitro* median lethal concentration of the aqueous extract of *A. niger* against *T. brucei* was 23.01 mg.ml (Table 1.3).

Effect of aqueous extract of *Mucor racemosus* on *T. brucei in vitro*

The aqueous extract of *Mucor racemosus* (SA3) also exhibited significant ($p < 0.05$) *in vitro* anti-trypanocidal activity against *T. b. brucei* in a concentration dependent pattern. The mean trypanosome counts at 10 mg/ml was 0.17±0.41 per field, while at 5 and 2.5 mg/ml concentrations, the mean counts were 5.67±1.37 and 8.50±1.52 per field, respectively. At 1.25 and 0.625 concentrations of the aqueous extract, the mean trypanosome counts were 16.0±2.28 and 18.2±1.17 per field, while at 0.31 and 0.15 mg/ml the counts were 19.2±2.99 and 22.2±1.72 per field, respectively. The *in vitro* median lethal concentration of the aqueous extract of *M. racemosus* on *T. brucei* was 1.533 mg/ml (Table 1.3).

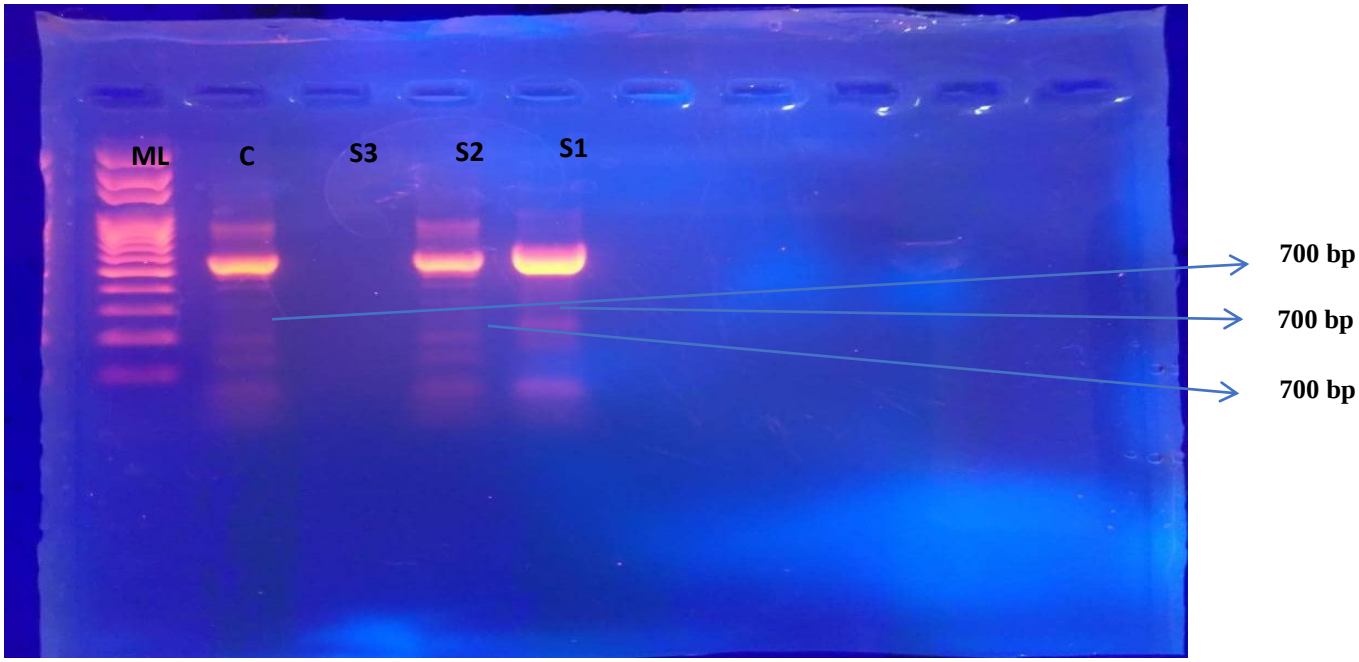

Plate I: Gel electrophoresis showing bands of PCR amplicons of DNA extracted from endophytic fungi isolated from *P. guajava* leaves and stems.

Key: ML= Molecular Ladder;
C= Control sample;
S1= Sample 1;
S2= Sample 2;
S3= Sample

DISCUSSION

The visible growth on the PDA after culturing confirm the presence of endophytic fungi in the stem and leaves of *Psidium guajava*. This corresponds to the findings of other authors who also reported the presence of endophytic fungi in *P. guajava*. Similarly, [24], also identified an endophytic fungus *Daldinia eschscholtzii* from the leaves of *P. guajava*. In another study conducted in Nkanu West Local Government Area of Enugu State, South-Eastern Nigeria, [25] identified *Fusarium sp* in the leaves of *P. guajava*. In study conducted in Cameroon, [26] isolated *Aspergillus* sp., *Botryosphaeria* sp., *Fusarium* sp., *Neoscytalidium* sp., *Xylaria* sp., *Phylosticta capitalensis*, *Cercospora apii*, *Xylaria longipes*, *Phomopsis* sp., *Phomopsis asparagi*, *Aspergillus versicolor*, *Pallidocercospora thailandica*, and *Xylaria grammica* from the leaves of *P. guajava*. Endophytic fungi are fungi that live within plant tissues, and show no symptoms of disease in their host plants [27]. According to [30], most plants harbor endophytic fungi where they facilitate nutrient assimilation, synthesis of growth promoting metabolites, insect and pest repellents, antimicrobial activities. They added that these endophytes hold enormous metabolic potential to synthesize compounds with high biological activity [28]

Other researchers have reported varying numbers of endophytic fungi in *P. guajava* and other plant materials. [24,25] each identified only one endophytic fungus in the leaves of *P. guajava*. [26], isolated 28

endophytic fungi belonging to 9 genera from the leaves of *P. guajava*. [29]in Gombe state isolated six fungal endophytes from the leaves of *P. guajava* namely *Rhizopus arrhizus, Candida albicans, Epidermophytum floccosum, Aspergillus niger, Mucor circinelloides* and *Lichtheimia corymbifera*. [30], reported the presence of *A. niger, A. fumigatus,* and *A. japonicus* in the leaves of *Pisonia grandis*. [31], isolated 11 endophytic fungi belonging to four genera (*Aspergillus, Penicillium, Fusarium,* and *Trichodema*) from the stems of *Phragmanthera capitata* collected from *Theobroma cacao*. The types and number of endophytic fungi present in plants are influenced by several factors including Factors such as species of *Psidium guaIjava*, environmental and climatic factors, age of plant and geographical factors all show that endophytic fungi present in different locations vary, please refer to continuation of the discussion.geographical location, age of plant, and environmental factors temperature, precipitation and atmospheric humidity [26]

The phytochemicals detected in the extracts of endophytic fungal isolates from *P. guajava* including alkaloids, flavonoids, saponins, phenols, tannins, steroids, terpenoids, anthraquinone, cardiac glycoside, and phlobatannins. This agreed with the reports of [32,31], who had reported the presence of secondary metabolites in extracts prepared from endophytic fungi.[32] identified flavonoids, alkaloids, phenols, saponins, steroids, tannins, terpenoids in endophytic fungi *Penicillium frequentans* isolated from *Pinus roxburghii*.[31] screened extracts of four endophytic fungi from *Phragmanthera capitata* (*Aspergillus, Penicillium, Fusarium,* and *Trichodema*) and reported the presence of flavonoids, anthraquinones, tannins, phenols, steroids, coumarins, and terpenoids, with alkaloids and saponins. In a review document, [33] reported on the ability of *Aspergillus* species to express several active secondary metabolites, such as butenolides, alkaloids, terpenoids, cytochalasins, phenalenones, ρ-terphenyls, xanthones, sterols, diphenyl ether and anthraquinones derivatives. [34] stated that these metabolites possess various biological activities, such as anti-inflammatory, anti-cancer, anti-bacterial and anti-viral activities.

[35] reported on the *in vitro* antitrypanosomal activity of two endophytic fungi isolated from rice bran. They showed that the endophytes, *Aspergillus niger* and *A. fumigatus* exhibited significant cidal properties against *Trypanosoma brucei* after six hours' incubation in graded concentrations of the fungal extracts with median inhibitory concentrations (IC_{50}) of 44.79 ± 5.32 and 49.86 ± 0.038 µg/ml, respectively. In a not too recent work, [36] noted that four novel polyketide derivatives showed significant in vitro anti-trypanosomal activity with minimum inhibitory concentration values ranging between 4.96 to 9.75 uM.

The *in vitro* results of this study indicated that the solvent extracts of the endophytic fungi possess trypanocidal activities over the range of concentration tested. Furthermore, the results showed that for each endophytic fungi, the ethyl acetate extract demonstrated significantly higher in vitro activity against trypanosomes compared to the corresponding aqueous extract. This agreed with [37] who showed that the ethyl acetate extract of *Satureja kitaibelii* was more active than corresponding aqueous extract against selected strains of bacteria and fungi.

CONCLUSION

From the findings of the study, it was deduced that the leaves and stem of Psidium guajava used in this study harboured

endophytic fungi which were amenable to both culture and isolation processes adopted; base on their morphological features, three species of endophytic fungi, namely Pencillum, Aspergillus niger, and Mucor racemous were isolated from the leaves and stems of Psidium guajava; Aqueous and ethyl acetate extract can be derived from the fungal masses of Penicillium, Aspergillus niger, and Mucor racemous that were isolated from the leaves and of Psidium guajava. The ethyl acetate extracts of Penicillium sp, Aspergillus niger, and Mucor racemous isolated from the leaves and stems of Psidium guajava possess significant in vitro activity against *Trypanosoma brucei brucei*, with the highest activity seen with the Penicillium sp extract having the least median inhibitory concentration of 0.303 mg/ml; the corresponding aqueous extracts of the three fungi demonstrated significantly lower in vitro activities against *T. brucei* and molecular characterization using ITS rRNA gene sequencing confirmed that two isolates has band size of 700bp.

Ethics Approval and Informed Consent

Ethical approval for this study was obtained from the ethical review board of the Kaduna State Ministry of Agriculture. All participants +32study and protocol for sample collection. All participants signed an informed consent form.

Authors Contribution

AFN, DAJ and MFM conceptualized the study. AFN and AJ designed the study. AFN, DAJ and MFM participated in fieldwork and data collection. AFN and MFM performed the data analysis; AFN, DAJ and MFM interpreted the data. AFN prepared the first draft of the manuscript, reviewed by AFN, DAJ and MFM. All authors contributed to the development of the final manuscript and approved its submission.

Disclosure of Conflict of Interest

None

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