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Screening, identification and antimicrobial activity of mycoparasitic fungus (*Aspergillus* sp.) from Philippine aglibut sweet tamarind

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Abstract

Due to the increasing resistance of pathogenic microorganisms, *Aspergillus* sp. isolated from Aglibut Sweet Tamarind's bark of Pampanga State Agricultural University was evaluated for potential mycoparasitism and antimicrobial activity. The micrograph obtained from Scanning Electron Microscopy (SEM) Analysis reveals that *Aspergillus* sp. is a potential mycoparasite; further, its identity was 99% which was confirmed through 18s rDNA of its ITS1 forward and ITS4 reverse sequences by Polymerase Chain Reaction (PCR) Amplification and Sequencing. Moreover, Thin-layer Chromatography (TLC) was used to identify the bioactive compounds of *Aspergillus* sp. The chemical groups such as Glycosidic flavonoid, Alkaloid and Anthrones were also present which can express the desired activity. Complete Randomized Design (CRD) was carried out with the following treatments; T1 (suspensions), - control (DMSO) and + control (streptomycin for bacteria: ketoconazole for fungus). Paper-disc Diffusion confirms that the suspensions of *Aspergillus* sp. have significant antimicrobial potential as shown in the zones of inhibition in *S. aureus and S. cerevisiae* but with lower activity in *E. coli*. Thus, *Aspergillus* sp. is a potential mycoparasite and source of new drugs and drug products.

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Introduction

There has been a growing concern on possible sources of antibacterial, antifungal, and/or cytotoxic products and fungi were revealed to be excellend sources of bioactive compounds for such activities (Neff, 2011). Thus, sources and activities of various fungal species are worth investigating (Hu *et al.,* 2002).

At the Pampanga State Agricultural University in the country Philippines, a well known commodity which is a subject of investigation, is the Philippine Aglibut Sweet Tamarind (AST). It is a registered variety in the National Seed Industry Council (NSIC) of the Philippines (Reyes, 2016). Tamarind (*Tamarindus indicus*), dubbed as the "brown gold" is one of the minor crops of the Philippines with great ethnomedicinal potentials and as possible sources of natural products. Of the total New Chemical Entities (NCEs) approved by the United States Food and Drug Administration (US-FDA) from 1981-1982, 50% are natural products, natural products-derived, or natural product mimics (Newton, 2003). And it is believed that the AST may be a possible source of anti-microbial natural products through mycoparasitism. Santos, Bagunu, Gracilla, Bagunu & Totaan (2016) reported the presence of fungi and bacteria in fruits of the AST fruit, but, this was managed by the application of 5KGy irradiation and this was found to be microbiologically and cytogenetically safe. So far, there is a dearth of literature on microbial studies of the Philippine AST, particularly on mycoparasitism, hence, this study.

Mycoparasitic fungi, are a group of fungal species that act as parasites to other parasites. The invaded fungi often suffer damage from the colonization, suggesting the possible *production* of anti-fungal metabolites by the mycoparasites (Loito, Woo, & Fernandez, 1998), a mechanism necessary to combat pathogenic fungi such as *E.coli, S. aureus,* and *S. cerevisiae*.

Hence, this study initially aimed to isolate and identify potential mycoparasitic fungi of Aglibut Sweet Tamarind which may have potential antimicrobial activity against *E. coli, S. aureus and S. cerevisiae* considering the bioactive components of the mycoparasites.

Materials and methodology

Collection of Samples

Samples of barks, fruits and leaves were collected from Aglibut Sweet Tamarind trees at the Pampanga State Agricultural University, Magalang, Pampanga. These were placed in sterile plastic bags and were processed immediately to reduce the risk of contamination (Yin *et al.*, 2012).

The fungi were isolated from 10 different and healthy Aglibut Sweet Tamarind trees with 10 meters apart and 10 years of age at the vicinity of Crop Science, College of Agriculture Systems and Technology, Pampanga State Agricultural University, Magalang, Pampanga. Aseptic technique was applied to ensure that the fungal strains were acquired from the tree samples.

Isolation of Potential Mycoparasitic Fungi

The barks, fruits and leaves were cut with a sterilized scalpel (Yin *et al.*, 2012). The cut portions were washed in distilled water, disinfected by soaking in 70% ethanol for 30 seconds and in 1.0% sodium hypochlorite for 10 minutes. These were then rinsed in sterilized distilled water, and dried in sterilized tissue paper (Arnold *et al.,* 2000). The samples were inoculated in Petri plates of prepared Potato Dextrose Agar (PDA) supplemented with streptomycin (0.5g/ 50mL) and incubated at room temperature (25-30°C) until fungal growths were evident. The antibiotic was used to prevent the growth of bacteria (Alwakeel, 2013).

Purification of Potential Mycoparasitic Fungi

The growing spores were removed with a sterilized inoculating loop and transferred to fresh Potato Dextrose Agar (PDA) plates. Then, the potential mycoparasites were sub-cultured and purified on another fresh Potato Dextrose Agar (PDA). The purity was assessed by examining the morphology of their colony. The media were again supplemented with streptomycin (0.5g/ 50mL) to prevent bacterial growth. Cultures were incubated in room temperature (25-30°C) for several days, always inverted and were maintained under controlled for future applications (Yin *et al.,* 2012).

Identification of Potential Mycoparasitic Fungi

Morphological Identification

Morphological identification was done in reference to the Dichotomous Key available (Sandhu *et al*., 2014).

Scanning Electron Microscopy (SEM) Analysis

Interactions of fungi were monitored and region of potential mycoparasitism was chosen and sent to the University of the Philippines Los Bańos, Laguna for Scanning Electron Microscopy (SEM) Analysis (Melo and Faull, 2004).

Molecular Identification

The potential mycoparasite was prepared in Potato Dextrose Agar (PDA) slant and sent to the University of the Philippines Diliman, Quezon City for its identification based on the 18s rDNA of the ITS1 forward and ITS4 reverse sequences prepared by the following methods: DNA Extraction, Agarose Gel Electrophoresis, PCR Amplification, Purification and Capillary Sequencing (Yin *et al.,* 2012). The sequences were aligned by viewing their chromatograms on Bioedit and were analyzed using BLAST Tool. The nucleotides were copied and were pasted for the details.

Mass Cultivation, Extraction and Identification of Bioactive Compounds from the Potential Mycoparasite

The mass cultivation, extraction and identification of possible bioactive compounds were conducted at Central Luzon State University, Science City of Muńoz, Nueva Ecija.

The fungus was grown on Potato Dextrose Broth (PDB) by putting agar block containing fungal mycelia as the inoculum in each 250mL Erlenmeyer flask containing 200mL of the medium and was incubated in room temperature (25-30°C) for 14 days (Yin, *et al*., 2012).

After two weeks, the fungus on broth was filtered through Whatman filter paper 1 to remove the mycelium mats. Then, the crude extracts were harvested. In extracting the bioactive compounds, the Cetyl Trimethylammonium Bromide (CTAB) buffer was used. The 2x buffer was incubated for about 15 minutes at 65°C in a recirculating water bath.

A 500µl of the harvested crude extracts of the fungus was transferred to each 1.5mL micro centrifuge tube. Afterward, 500µl of pre-warmed 2x CTAB buffer was added next to 50µl of 20% SDS. These were mixed thoroughly by a vortex. Each tube span is at 12,000rpm for 5 minutes, spinning down cell debris. Each tube was incubated in a water bath at 65°C for 30 minutes to 1 hour. The tubes were cooled briefly and 200µl of chloroform was added. Lastly, tubes were mixed thoroughly by a vortex and span at 10,000rpm for 30 minutes. The lower phase contains the bioactive compounds (Baltazar and Totaan, 2016).

The possible bioactive compounds were identified using the Thin-layer Chromatography with 3 replications. A 1ul of the suspensions was applied as spot on each TLC plates. Then, they were developed in different solvent systems: Chloroform; Ethyl acetate: Hexane (7:3); Ethyl acetate: Hexane (3:7); Ethyl acetate: Hexane (1:1); Hexane; Methanol; Toluene: Chloroform (9:11); Toluene: Acetone: Chloroform (40:25:35); n-Butanol: Acetic acid: water (4:1:5) upper phase; Chloroform: Acetic acid: water (50:45:5); Chloroform: Methanol (5:1). The chromatograms were then air dried and developed using freshly prepared spray reagents for specific bioactive compounds. Spray reagents were prepared as described for the constituents tested. The Antimony (III) chloride is the spray reagent for Flavonoids and Steroids prepared by a solution of 10% Antimony (III) chloride in Chloroform. Potassium ferricyanide-ferric chloride is the spray reagent for Phenols, Tannins and Flavonoids prepared by a mixed of equal volumes of 1% Potassium ferricyanide solution and 1% Ferric chloride solution. Colors were intensified by subsequent spraying of 2M Hydrochloric acid. Dragendorff's reagent: Munier Machebouef is the spray reagent for Alkaloids prepared by Solution A: 0.85g Bismuth (III) nitrate dissolved in a mixture of 10mL Acetic acid and 40mL water and Solution B: 8g Potassium iodide dissolved in 20mL water. Equal parts of Solutions A and B were mixed as the Stock Solution. A 1mL of Stock Solution was mixed with 2mL and 10mL of water to come up with the spray reagent.

Three, 5-Dinitrobenzoic acid: Kedde reagent is the spray reagent for Cardenolides prepared by Spray Reagent A: 2% Methanolic solution of three, 5-Dinitrobenzoic acid and Spray Reagent B: 5.7% Methanolic solution of Potassium hydroxide.

As to treatment, the chromatogram was sprayed lightly with Spray Reagent A with excess Spray Reagent B. Methanolic potassium hydroxide: Borntrager reagent is the spray reagent for Coumarins, Anthraquinones, Anthrones and Phenols prepared by a 5% solution of Potassium hydroxide in Methanol. As the treatment, the chromatogram was sprayed, dried and viewed under visible and UV 365 nm. Magnesium acetate in Methanol is the spray reagent for Anthraquinones prepared by a 0.5% solution of Magnesium acetate in Methanol. As the treatment, the sprayed chromatogram was heated for five minutes at about 90°C. Van-Urk-Salkowski test is the spray reagent for Indoles prepared by Solution A: 1g p-Dimethylaminobenzaldehyde dissolved in 50mL of Hydrochloric acid and 50mL of absolute alcohol and Solution B: 2.03g Hydrate ferric chloride dissolved in 500mL water and 300mL of Sulfuric acid. A one part of Solution A was mixed with three parts of solution B. As the treatment, the sprayed chromatogram was developed at 100°C for five minutes. Lastly, Vanillin-sulfuric acid is the spray reagent for Higher alcohols, Phenols, Steroids and Essential oils prepared by adding 50mL concentrated Sulfuric acid to 10mL 2% Vanillin in Ethanol mixed with cooling. As the treatment, the sprayed chromatogram was developed at 120°C until maximum color of the spot is obtained (Aguinaldo *et al.*, 2005).

Antimicrobial Activity

The antimicrobial activity screening was conducted at the Central Luzon State University, Science City of Muńoz, Nueva Ecija.

Test Microorganisms

The test bacterial strains used were *E. coli* (ATCC25922) and *S. aureus* (ATCC25923) obtained from the Biochemical, Organic and Natural Products Laboratory, Department of Chemistry, Central Luzon State University, Science City of Muńoz, Nueva Ecija. The test fungal strain used was *S. cerevisiae* (BIOTECH Acc. No. 2055) purchased from the Philippine National Collection of Microorganisms, BIOTECH, University of the Philippines Los Bańos, Laguna.

Preparation of Inoculum

The method of Aguinaldo *et al.*, (2005) was used as a guide with some modifications. A loop full of bacteria was taken from the culture slants and was transferred into two test tubes containing 10mL Nutrient Broth (NB). Then, they were incubated for 25-30°C within 24 hours. Also, a loop full of fungus was taken from the culture slant and transferred into a test tube containing 10mL Potato Dextrose Broth (PDB). Afterwards, it was incubated in 25-30°C for 48 hours.

The turbidity of the growing broth cultures was adjusted with Nutrient Broth (NB) and Potato Dextrose Broth (PDB) to obtain turbidity comparable to that of the 0.5 McFarland standards. The comparisons were done using the solution's optical density read at 600nm using UV-vis Spectrophotometer. Nutrient Broth (NB) and Potato Dextrose Broth (PDB) solutions were used as blank.

Preparation of Plates

A 20mL of autoclaved Nutrient Agar (NA) and Potato Dextrose Agar (PDA) were poured in sterilized Petri plates and were allowed to solidify for 15 minutes. The plates were incubated for 24 hours to assure that there were no contaminations. Then, 4mL of seeded agar were poured into each base agar and were allowed to solidify.

Preparation of Paper-discs

Paper-discs (Whatman filter paper 1) measuring approximately 6mm in diameter were prepared using a paper puncher. These paper-discs were placed in a petri plate and sterilized in an autoclave.

Screening of Antimicrobial Activity

The Paper-disc Diffusion Method was used for the antimicrobial activity test with replication.

Paper-discs of 6mm (Whatman filter paper 1) were soaked in the suspensions, positive controls and negative control, and dried for 15 minutes. Then, these were transferred into the Nutrient Agar (NA) and Potato Dextrose Agar (PDA) plates with seeded agar. The plates were incubated at 25-30°C within 24 hours for bacteria and 48 hours for fungus. The zones of inhibition were measured and recorded in millimeters (mm) using Vernier caliper and were interpreted according to the standard measurements.

The suspensions were concentrated using Eppendorf concentrator for 2 hours (20.3mg for bacteria: 22.4mg for fungus) and were diluted with DMSO (0.203mL for bacteria: 0.224mL for fungus). Streptomycin for bacteria (500mg/ 50mL) and ketoconazole for fungus (2500mg/5mL) were used as positive controls. Then, DMSO was used as the negative control.

Results and discussion

The potential mycoparasitic fungal isolate was initially identified through its morphological and molecular characteristics and it is an *Aspergillus* sp.

Morphological Identification and Scanning Electron Microscopy (SEM) Analysis

The colony was cottony with a pale green color that covers the plate within seven days (Plate1). Hyphae were present. Chains of spores are unbranched and emerged from phialides which radiate from a swollen vesicle. The conidiophores are coarsely roughened, often more noticeable near the vesicle. Conidia are globose and/or sub-globose.

The presumed *Aspergillus* sp. and its host fungus in the cultured medium showed typical hyphae interaction, a potential mycoparasitism shown in the micrograph (Fig. 1). As a matter of fact, fungi are often parasitic to other fungi. One way of their mycoparasitism is when the hyphae grows and/or coils along with the other hyphae.

Aspergillus sp. is under the Phylum Ascomycota which has chitin, β-glucan, and mannoproteins (glycoprotein), the main macromolecular components in its cell walls. These macromolecular components contribute to the cell wall lysis (dissolve and digest) of the host fungi and maybe with the combination of antibiotics, another mechanism of action of mycoparasitic fungi. In addition, *Aspergillus sp*. can be developed to antibiotics and beneficial immunosuppressant.

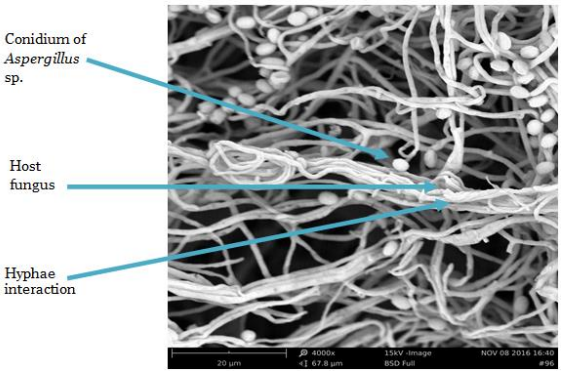


Fig. 1. SEM micrograph showing the microscopic features and potential mycoparasitism of *Aspergillus* sp.

Image obtained from the Scanning Electron Microscopy (SEM) Analysis showed ultra-structural features that support the morphological identification and positioning of the fungus into the genus *Aspergillus*, and its potential mycoparasitism.

Molecular Identification

The potential mycoparasitic fungus was identified based on the 18s rDNA of its ITS1 forward and ITS4 reverse sequences. The chromatograms on Bioedit with the aligned sequences were analyzed using BLAST Tool and revealed that the fungus showed a similarity to the genus *Aspergillus* with a 99% identity, a 96% query cover and a sequence ID: KX015944.1. This molecular level of identification clearly states that the fungus is an *Aspergillus sp*. (Fig. 2 and 3).

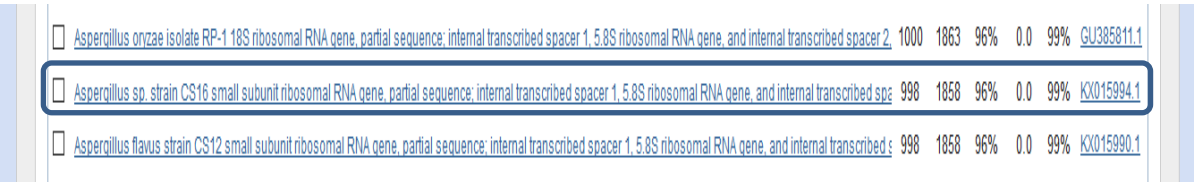


Fig. 2. Molecular identity, query cover and sequence ID of the amplified DNA sequences of *Aspergillus* sp.

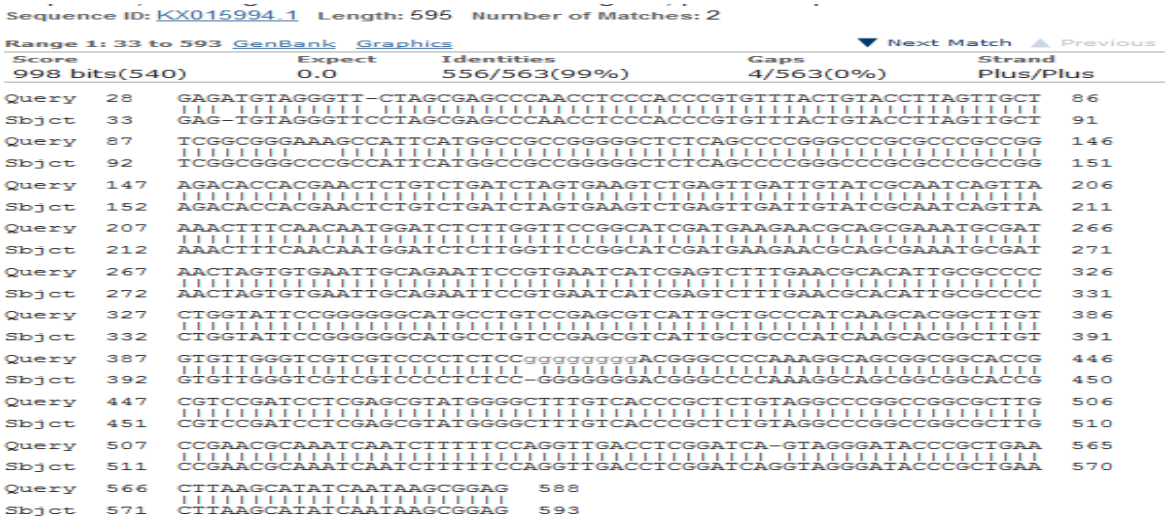


Fig. 3. DNA sequencing alignment.

To identify the bioactive compounds of the mycoparasite, TAC or Toluene: Acetone: Chloroform (40:25:35) was selected as the suitable solvent system before running the Thin-layer Chromatography (TLC). TAC was able to move effectively along the TLC plates showing well separation and less tailing. This gives a more desired result in identifying the bioactive compounds of *Aspergillus* sp. Thin Layer Chromatography (TLC) is a powerful tool in the analysis of the chemical components of extracts/suspensions. The bioactive compounds of the suspensions of *Aspergillus* sp. were identified using this method. Fig. 4, 5 and 6 showed the secondary metabolites present in the fungus. It was found that Glycosidic flavonoids with fluorescent visible spot under UV 365 nm were present, Alkaloids with a brown-orange visible spot immediately on spraying were present but not stable and Anthrones with yellow zone under UV were present. The arrows indicate the points of positive results from the suspensions of *Aspergillus* sp. on the TLC plates.

Antimicrobial Activity

The inhibitory activity of the suspensions of *Aspergillus* sp. against *E. coli*, *S. aureus* and *S. cerevisiae* was evaluated as shown in plate2, 3 and 4 with a 6mm paper-disc in a Vernier caliper as a reference (Fig.7). The bar graphs summarized the zones of inhibition caused by the bioactive compounds of *Aspergillus* sp., positive controls and negative control after 24 hours of incubation for bacteria (*E. coli* and *S. aureus*) and 48 hours of incubation for fungus (*S. cerevisiae*). The treatments showed zone of inhibitions against *E. coli*, *S. aureus* and *S. cerevisiae*, but were considered to be partially active. The results may be due to the presence of bioactive compounds in the suspensions tested by TLC. The Glycosidic flavonoids, Alkaloids and Anthrones have antimicrobial activities based on recent studies. Zones of inhibition showing less than 10 mm are considered to be inactive. 10-13 mm is partially active, 14-19 mm is active and more than 19 mm is very active.

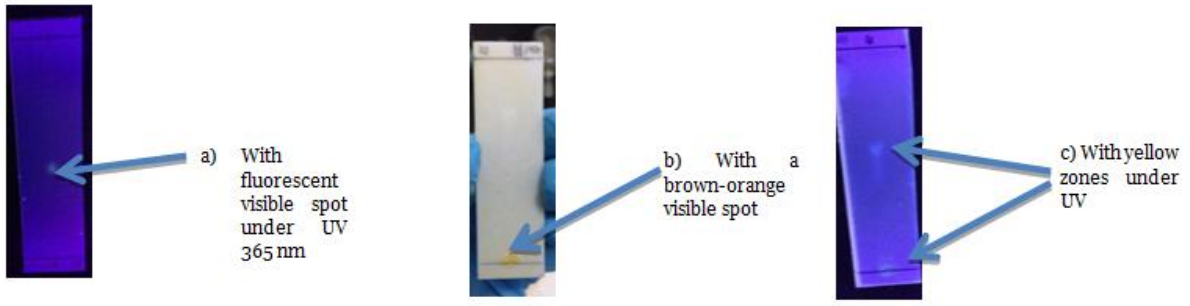


Fig. 4. Bioactive Compounds as shown in TLC plates a) glycosidic flavonoids b) alkaloids c) Anthrones.

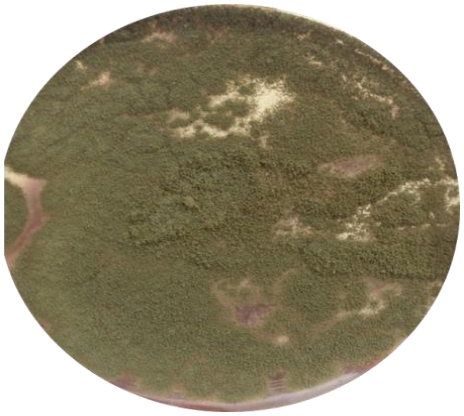
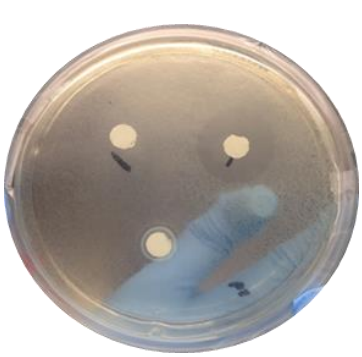


Plate 1. Macroscopic features of *Aspergillus* sp. from Aglibut Sweet Tamarind's bark.



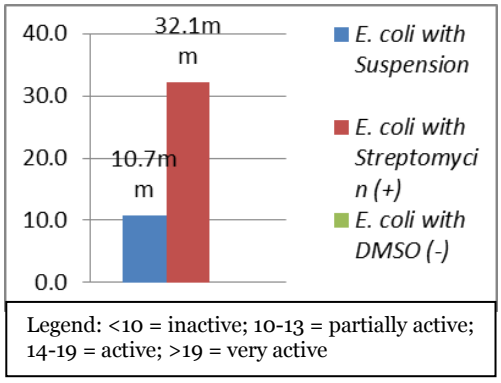
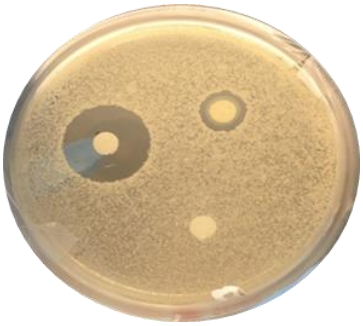


Plate 2. Suspension was partially active with 10.7mm zone of inhibition on *E. coli* (bacterium).



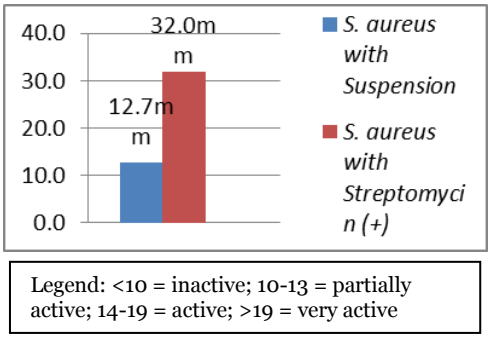


Plate 3. Suspension was partially active with 12.7 mm zone of inhibition on *S. aureus*.

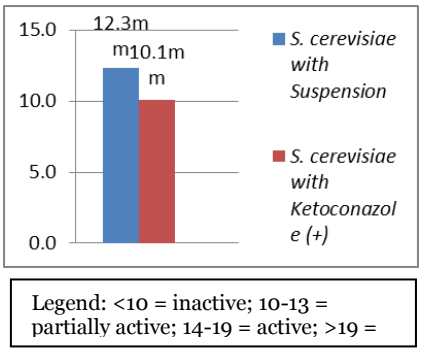


Plate 4. Suspension was partially active with 12.33mm zone of inhibition on *S. cerevisiae* (fungus).

Conclusions

Based on the findings, *Aspergillus* sp. is a potential mycoparasite isolated from Aglibut Sweet Tamarind's bark. The bioactive compounds extracted from *Aspergillus* sp. of Aglibut Sweet Tamarind's bark showed the greatest zone of inhibition on *S. aureus* and *S. cerevisiae* but with lower activity on *E. coli*. Thus, the *Aspergillus* sp. has potential antimicrobial activity. Streptomycin still showed the highest zones of inhibition on *E. coli* and *S. aureus*. While on *S. cerevisiae*, ketoconazole was not that effective though it is considered as a broad spectrum antifungal.

Recommendations

From the findings and conclusion, it is recommended that further experiments be made to demonstrate the cell wall degrading enzyme of *Aspergillus sp*. to further confirm its potential mycoparasitism. Since there is a potential antimicrobial activity, isolation of specific bioactive compound for further antimicrobial screening and Nuclear Magnetic Resonance (NMR) Spectroscopy may be conducted. Potential mycoparasites from other parts of Aglibut Sweet Tamarind may be isolated.

Acknowledgement

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