ANTIFUNGAL ACTIVITY OF ETHANOLIC EXTRACT OF *Syzygium polyanthum* (WIGHT) WALP. LEAVES EXTRACT AGAINST SEVERAL TYPES OF FILAMENTOUS FUNGI AND *Candida* SPECIES

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Abstract. This study aimed to determine the antifungal activity of *Syzygium polyanthum* leaves extract against opportunistic filamentous fungi and pathogenic *Candida* species using the standard method of Clinical and Laboratory Standard Institute (CLSI). All fungi were susceptible to *S. polyanthum* leaves extract with minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) ranging between 0.63 - 1.25 mg/mL and 1.25 - 5.0 mg/mL, respectively. Moreover, the extract of *S. polyanthum* leaves inhibited more than 90% conidia germination of tested filamentous fungi. Killing time of *S. polyanthum* leaves extract on *C. albicans*, *C. glabrata*, *C. parapsilosis* and *C. krusei* was fast acting where it showed 3 $\log_{10}$ CFU/mL reduction at 4× MIC in 4 h. Morphology changes on treated strains were viewed (changing cell wall thickness, rupture, and leakage of the cell's cytoplasm). Maximum cell constituents release was observed at 4× MIC for 72 h incubation for *A. niger* with an absorbance 0.381 whereas 0.435 for *C. albicans* after 4 h incubation at 4× MIC. Therefore, results suggest that *S. polyanthum* leaves extract had antifungal activity and might be able to develop as a natural antifungal agent.

Keywords: *Syzygium polyanthum* leaves, antifungal, filamentous fungi, *Candida* species, susceptibility test.

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Introduction

Fungi consist of unicellular yeast, filamentous and multicellular moulds are widely distributed in mother of Earth [1]. *Aspergillus*, *Candida*, *Penicillium*, and *Fusarium* are the most popular and normally seen fungi to cause food spoilage [2]. For example, *Aspergillus flavus* and *Aspergillus fumigatus* release their mycotoxin (aflatoxin and fumitoxins), and produces the off-flavour and allergenic substances to cause uncountable post-harvest losses [3, 4]. On the other hand, *Candida* species are also mainly associated with rotting vegetation on plants or food. *Candida* is a heterogeneous genus containing about a quarter of all yeast species [5]. Food contaminated by fungi are resulting food poisoning and foodborne illness. Clinical symptoms include stomach upset, diarrhea, abdominal cramp, fever, dehydration, and vomiting [6]. In the current industries, synthetic antimicrobial agents such as sodium nitrite and sodium benzoates have been extensively used to prevent the growth and proliferation of foodborne pathogens or food spoilage microbes [7]. However, the continuous usage of artificial preservatives led to microbial strain, which can resist a plethora of commercial antimicrobial agents and become a public health concern [8]. Therefore, developments of natural preservatives derived from plant sources instead of chemical preservatives are gaining more attention nowadays.

Syzygium polyanthum (Wight) Walp. is categorized under the family of Myrtaceae, In Malaysia and Indonesia, *S. polyanthum* also known as *serai kayu*, *meselangan*, *salam*, *gowok*, *manting* and *kastolam* [8-9]. *S. polyanthum* leaves have been used traditionally as medicine or therapeutic agents, including effective against diabetes, diarrheal, gastritis, inflammation, hyperuricemia, hypertension, ulcer, and skin diseases [10]. Furthermore, *S. polyanthum* leaves was believed to possess antimicrobial activity against several food spoilage fungi, including *Aspergillus* spp, *Euroticum* spp. and *Penicillium* spp. [11]. Therefore, this study aimed to determine the antifungal activity of *S. polyanthum* leaves extract against several fungi strains.

Materials & Methods

Preparation of extract. Dried *S. polyanthum* leaves were purchased from Herbal Market Bandung, Indonesia, identified, and deposited in the Institute of Bioscience (IBS), Universiti Putra Malaysia. *S. polyanthum* leaves (100 g) were dried, milled and soaked with 400 mL absolute ethanol for 7 days at room temperature. Final crude extract was diluted and standardized into 10 mg/mL or 1%. For prior use, the stock solution was chilled below 4 °C condition [12].

Filamentous fungi strains, growth conditions and inoculum preparation. The filamentous fungi species, *Aspergillus flavus* ATCC 22546, *Aspergillus niger* ATCC 9029, *Rhizopus oryzae* ATCC 22580, and *Rhizopus oligosporus* ATCC 22959 were used in this study. Inoculum suspensions of filamentous fungi were prepared by the method of National Committee on Clinical Laboratory Standard (NCCLS) [13]. Briefly, fungi were grown on Potato dextrose agar (PDA) at 35 °C for 7 days. The conidia or sporangiospore and hyphae fragments mixture was collected and transferred into a sterile microcentrifuge tube. Optical density (OD) of conidial suspensions were read and adjusted between 80 to 82% transmittance for *Aspergillus* spp., and 68 to 70% transmittance for *Rhizopus* spp. These suspensions were diluted in sterile distilled water to obtain inoculum suspension was 10^4 CFU/mL.

***Candida* strains, growth conditions and inoculum preparation.** *Candida albicans* ATCC 10231, *Candida glabrata* ATCC 2001, *Candida krusei* ATCC 32196 and *Candida parapsilosis* ATCC 22019 were cultured and maintained on Sabouraud dextrose agar (SDA) for 48 h at 35 ºC. A standardized inoculum for each isolate was 5×10^6 CFU/mL.

Minimum inhibition concentration (MIC) and minimum fungicidal concentration (MFC). Broth microdilution method was applied in the evaluation of MICs and MFCs using a sterile 96-well microtiter plates. Each microdilution well contained 100 µL of two-fold diluted concentration of antifungal agent and 100 µL of inoculated broth medium (moulds: 10^4 CFU/mL; yeast: 5×10^6 CFU/mL). Serial dilution was performed from the highest extract concentration (5.00 mg/mL) to lowest extract concentration (0.01 mg/mL). All the culture plates were incubated at 35 °C for 72 h for all filamentous fungi and 48 h for *Candida* spp. The lowest concentration of antifungal agent to inhibit the microbial growth will recognised as MICs [14]. Then, all MFCs were determined by subculturing each suspension well and deposited onto PDA/SDA plates.

Inhibition conidial germination assay. Inhibition conidial germination assay was performed in a standard MOPS-buffered RPMI 1640 medium. The assay was carried out by using quantitative assay. Inoculum suspension used in this assay was adjusted to 5×10^4 CFU/mL. Then, the prepared inoculum was diluted in 1:10 in MOPS-buffer to provide a final inoculum concentration of 5×10^3 CFU/mL. Final extract concentrations were 0 × MIC, 0.5 × MIC, MIC, 2 × MIC and 4 × MIC for each tested filamentous fungus. Fungal cultures (1 mL) were incubated at 35°C for 24 h. Conidia numbers was then determined by plating on PDA and the percentage of germination inhibition was calculated.

Time kill curve assay. Time kill curve assay is a method to calculate time required to achieve inhibition on microbial growth of tested strains at different concentration of sample extract. The protocol was referred to Souza et al. [15]. *S. polyanthum* leaves extract was diluted using Sabouraud dextrose broth (SDB) medium containing inoculum (~10^6 CFU/mL) to obtain final concentrations (0× MIC, 0.5× MIC, 1× MIC, 2× MIC, and 4× MIC values) for each tested strain. The fungal suspension (0.1 mL) was serially diluted into phosphate-buffered saline (PBS; 1%) at different exposure times and then plated onto SDA. The medium plates were incubated at 35 ºC for 24 to 48 h.

Scanning electron microscope (SEM). Fresh prepared pure culture (24 h) of *A. niger* and *C. albicans* were treated with *S. polyanthum* leaves extract at the tested MIC value. Centrifuged pellets (5000 × g for 10 min) were harvested and then fixed 2.5% (v/v) glutaraldehyde for 4 to 6 h at 4 °C. Pellets were washed 3 times with 0.1 M of sodium cacodylate buffer and each washing time was 10 min. The pellets were then post-fixed with 1% osmium tetroxide buffer for 2 h at 4 °C, washed again for 3 times, and each washing was 10 min. Pellets were then dehydrated for 15 mins each, using series of acetone concentrations (35, 50, 75, and 95%). Lastly, the pellets were dehydrated 3 times using 100% acetone and each dehydrated time was 15 min. Cell suspension was transferred to the specimen basket and dried in the critical dryer for 30 min. The specimen was mounted on a stub and coated the gold on sputter. Morphologies of *A. niger* and *C. albicans* were viewed using SEM instrument.

Cell constituent release assay. Cell constituents release was determined for the fungal suspension of *A. niger* and *C. albicans* treated with *S. polyanthum* leaves extract [16]. Cell suspension of *A. niger* and *C. albicans* were centrifuged at 3000 × g for 20 min, and washed and suspended three times in 0.1% PBS (pH 7.0). The suspensions were undergone incubation at 35 ºC inside the incubator shaker (2000 rpm) for 24 h, 48 h and 72 h for *A. niger* and 30 min, 1, 2 and 4 h for *C. albicans*, at different extract concentrations (0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC). Then, the samples were centrifuged at 13400 × g for 15 min. Supernatant was collected to measure the absorbance of suspension at 260 nm by using the UV-Vis spectrophotometer.

Results and Discussion

MIC and MFC. Table 1 summarises the MICs and MFCs of *S. polyanthum* leaves extract against all filamentous fungi and *Candida* spp. MICs ranges between 0.63 to 1.25 mg/mL. *C. krusei*, *C. glabrata*, and *C. parapsilosis* were the most susceptible to *S. polyanthum* leaves extract compared to other strains (MIC: 0.63 mg/mL). MFCs for all tested strains were displayed between 0.63 to 5.0 mg/mL.

Table 1. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of *S. polyanthum* leaves extract against tested strains.

Strains	MIC (mg/mL)	MFC (mg/mL)
A. flavus ATCC 22546	1.25	5.0
A. niger ATCC 9029	1.25	5.0
R. oligosporus ATCC 22959	1.25	5.0
R. oryzae ATCC 22580	1.25	5.0
C. albicans ATCC 10231	1.25	1.25
C. glabrata ATCC 2001	0.63	0.63
C. krusei ATCC 32196	0.63	1.25
C. parapsilosis ATCC 22019	0.63	0.63

Lawsonia inersis extract (6 mg/mL) was able to inhibit *A. niger* [17]. Results indicated that *S. polyanthum* leaves extract could better antifungal effect towards A. niger than *L. inersis*. Suraj et al. [18] reported that MIC value of ethanolic extract of *Calotropis procera* was 25 mg/mL against *R. oryzae*, whereas MIC value for petroleum ether and benzene extract of *C. procera* were at 35 and 20 mg/mL, respectively. Again, *S. polyanthum* had performed a better antifungal effect against *R. oryzae* than *C. procera* extract. This presence of antimicrobial compounds (bergenin and quercetin) in *S. polyanthum* leaves extract might contribute to the activity of filamentous fungi [19-20]. No previous studies have reported for *S. polyanthum* leaves extract against non-pathogenic *C. albicans* spp. Based on Prabhakar et al. [21], *Syzygium jambolanum*, *Cassia siamea* and *Caulerpa calpelliformis* ethanol extracts had antifungal activity against *C. krusei*, *C. albicans*, *C. parapsilosis* and *C. glabrata*, at 100 mg/mL whereas *Sargassum wightii* display antifungal activity at 10 mg/mL. Hence, *S. polyanthum* had better antifungal activity than S. jambolanum, C. siamea and C. calpelliformis, while having similar activity as *S. wightii*. A previous study had isolated the main antimicrobial compounds (flavonoid, alkaloid, triterpenes, and phenolic acid) from *S. polyanthum* leaves extract to perform antifungal activity against *Candida* spp. [22].

Inhibition conidial germination assay. Based on quantitative analysis, using 4× MIC for 72 h, the percentage of conidia germinations was reduced to 0% for A. flavus and Rh using quantitative analysis. *oryzae*. Meanwhile, the germination of *Rh. oligosporus* and *A. niger* were not fully inhibited and reduced to 1% and 13%, respectively (Figures 1(a) to (d)).

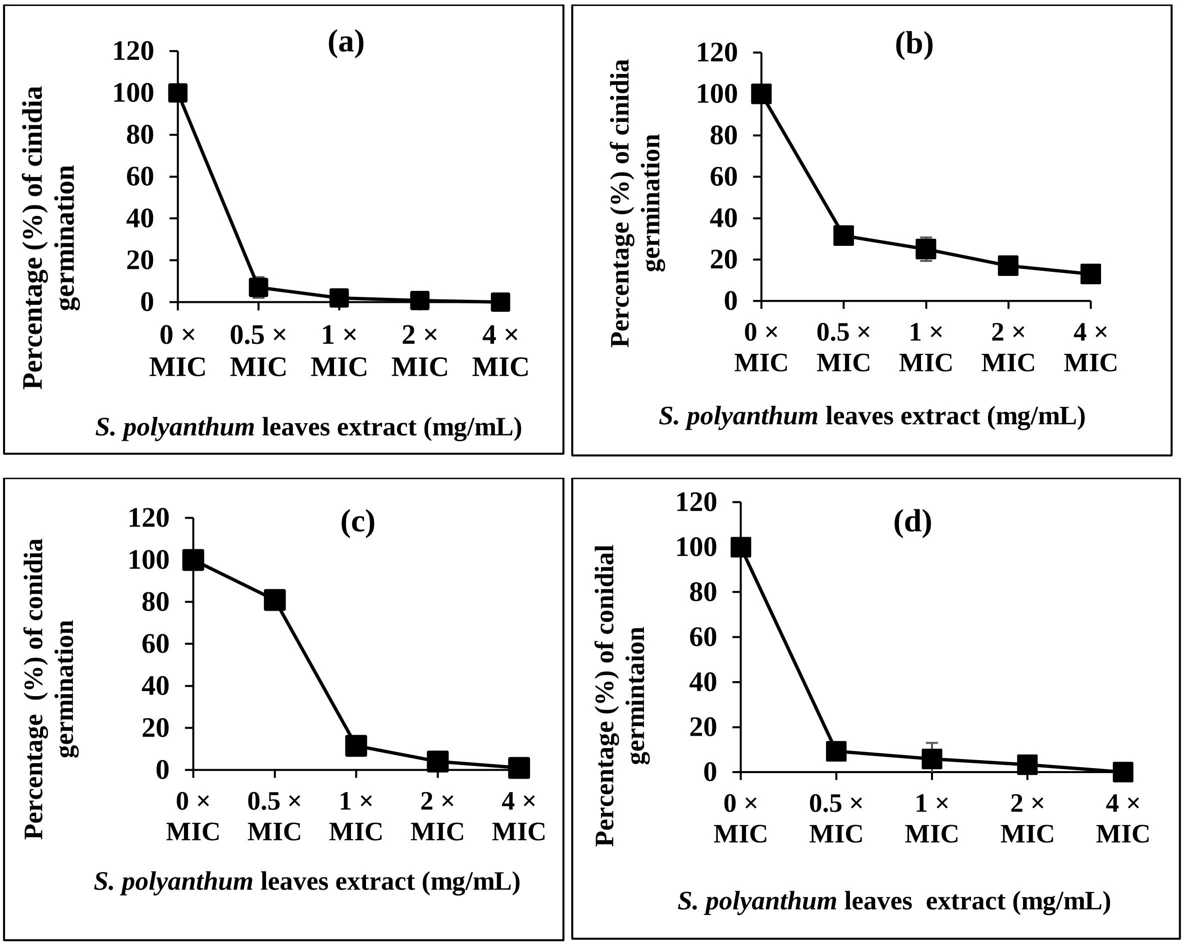

Figure 1. Effect of *S. polyanthum* leaves extract against conidia germination of filamentous fungi (a) *A. flavus*, (b) *A. niger*, (c) *R. oligosporus* and (d) *R. oryzae* at concentration of 0× MIC, 0.5× MIC, 1× MIC, 2× MIC and 4× MIC), respectively.

Generally, conidia germination of filamentous fungi was decreased with increasing extract concentration. Conidia germination of *A. flavus* and *R. oryzae* were fully inhibited. Meanwhile, conidia germination of *R. oligosporus* and *A. niger* were reduced to 1% and 13% at 4× MIC, respectively. According to Begum et al. [23], *A. indica* leaf extract was reduced conidia germination of *Rhizopus* spp. until 72% after treated 5% concentration whereas *Zingiber officinale* rhizome extract showed only 1% reduction at same concentration. *S. polyanthum* leaves extract possessed better antifungal activity than *A. indica* and *Z. officinale* extract against *Rhizopus* spp. Findings proved that the inhibitory effect against fungi spore germination might be contributed by some of the phytochemical compounds in plants.

Time kill curve assay. *C. glabrata*, *C. albicans*, and *C. parapsilosis* were able to kill at 4× MIC within 4 h, 2× MIC in 2 h, 2× MIC, and 4× MIC at 4 h incubation, respectively. The population of *C. krusei* did not kill completely; however, it was reduced to less than 3 Log_{10} CFU/mL. The killing of the *Candida* spp. immediately indicates that this extract affected irreversible damage to the structure of the test organisms when they come in contact with the extract [24]. On the other hand, according to de Toledo et al. [25], *Cympobogon citratus* had a killing effect against *C. albicans* at concentration of 0.63 mg/mL at 4 h incubation time.

Besides that, *C. glabrata* and *C. parapsilosis* can be entirely killed by *C. nardus* at 1 mg/mL concentration at 24 h exposure time. Abilities of *C. nardus* and *S. polyanthum* leaves extract to inhibit fungi spp. might due to the presence of various active compounds in the extract. The compounds were responsible for killing effect activity. Some active compounds can make microbial membranes more permeable to cause cell leaking [26].

Time kill activity of *S. polyanthum* leaves extract against *Candida* species was represented in Figures 2(a) to (d). The killing endpoints for *C. albicans* were reached after 4 h of incubation time at 4× MIC, while *C. glabrata* had been killed within 2 h at 4× MIC. The population of *C. parapsilosis* was reached the endpoint of 4 h incubation after exposed to 2 and 4× MIC. However, the population *C. krusei* reduced below 3 Log_{10} CFU/mL at 4× MIC of extract concentration for 4 h. These data demonstrated fungi inhibition by leaves extract was highly dependent on *S. polyanthum* leaves extract tested concentration and time of incubation.

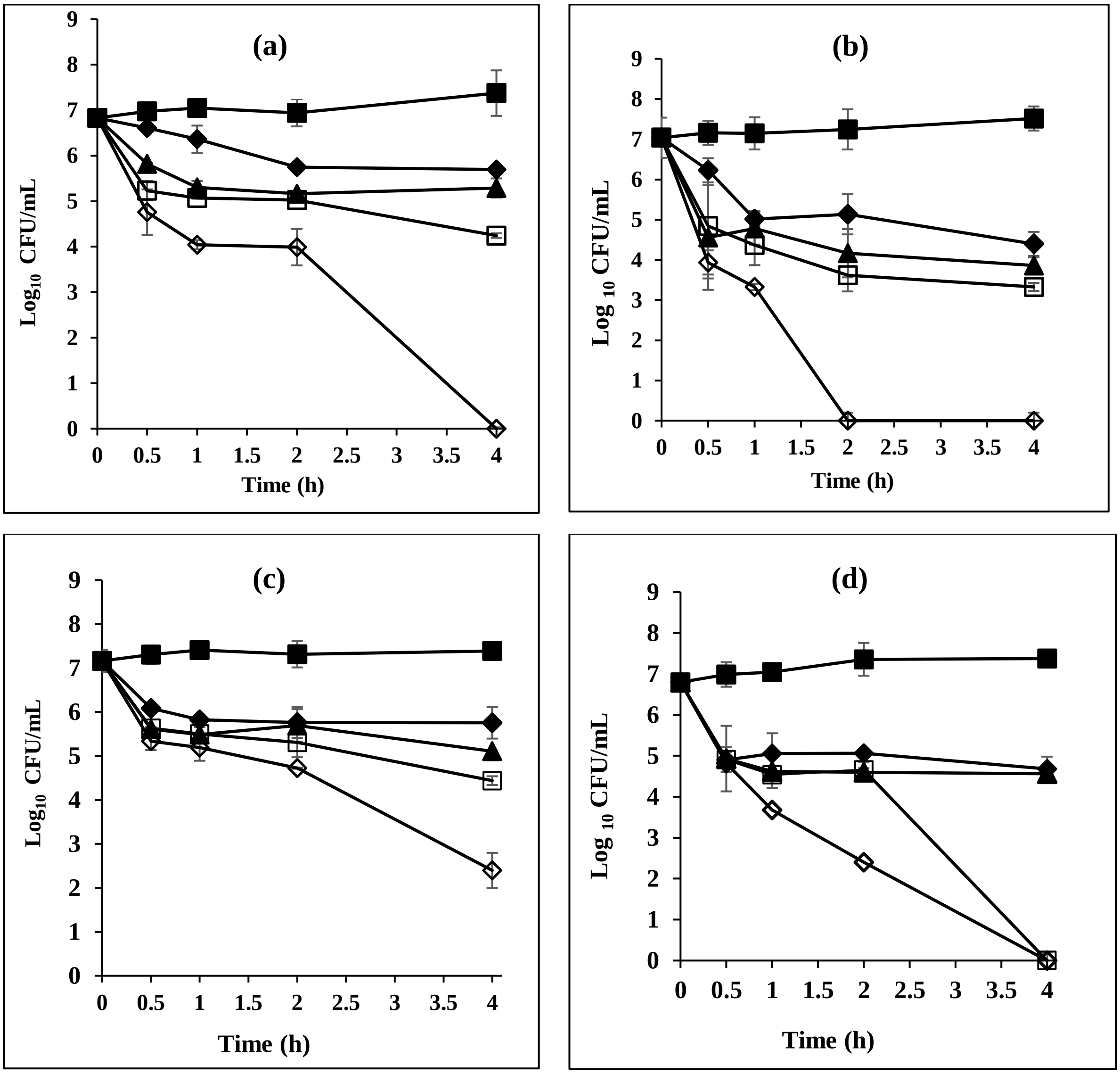

Figure 2. Time-kill curve plots for *Candida species*: (a) *C. albicans*, (b) *C. glabrata*, (c) *C. krusei* and (d) *C. parapsilopsis*, following exposure to *S. polyanthum* leaves extract at (■) 0× MIC, (◆) 0.5× MIC, (▲) 1× MIC, (□) 2× MIC and (◊) 4× MIC as respectively.

Microscopic examination using SEM revealed morphology changes in the hyphae of *A. niger* including irregular branching and loss of linearity whereas spores, showing distorted and perforated structure as shown in Figures 3(a) to (d).

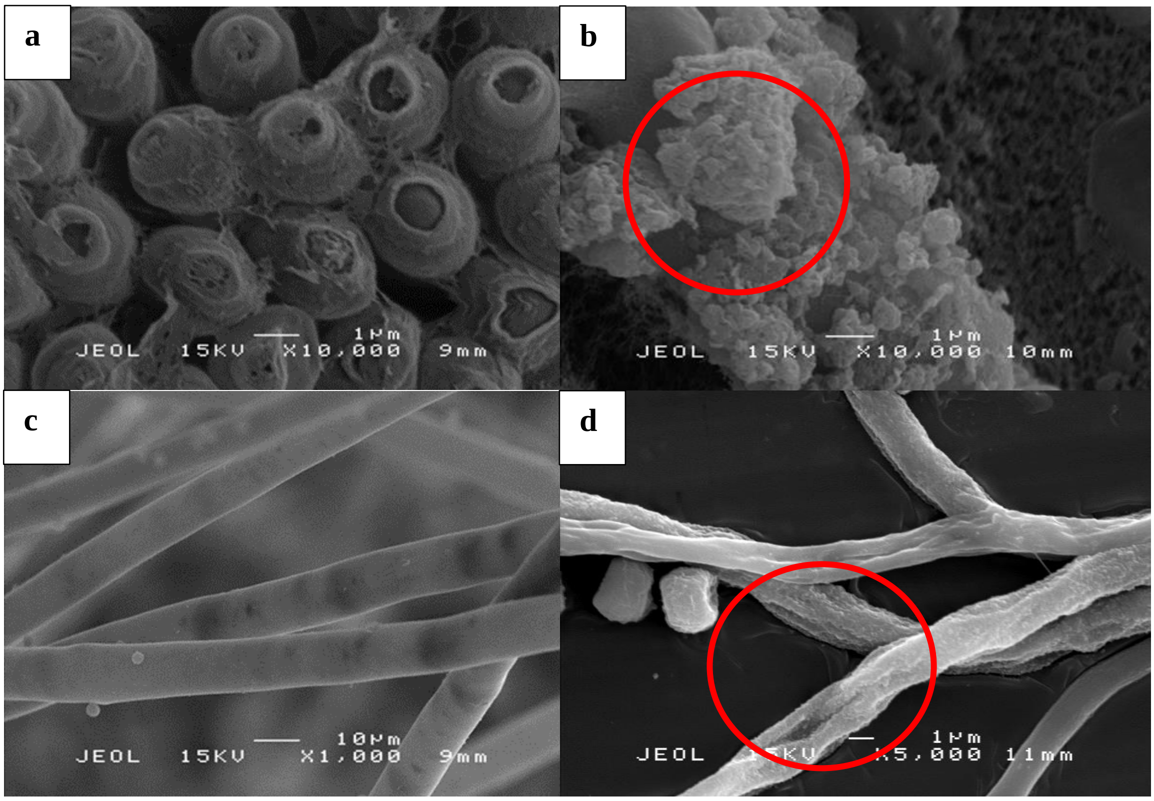

Figure 3. Morphology changes of *A. niger* spores and hyphae before (a) and (c) and after treated (b) and (d) with *S. polyanthum* leaves extract at MIC value. Spore (a) Control, (b) Treatment and Hyphae (c) Control and (d) Treatment.

Figure 4(b) showed the changes in *C. albicans* cell, including the leakage of its cytoplasm, while for control showed the intact cell (Figure 4(a)).

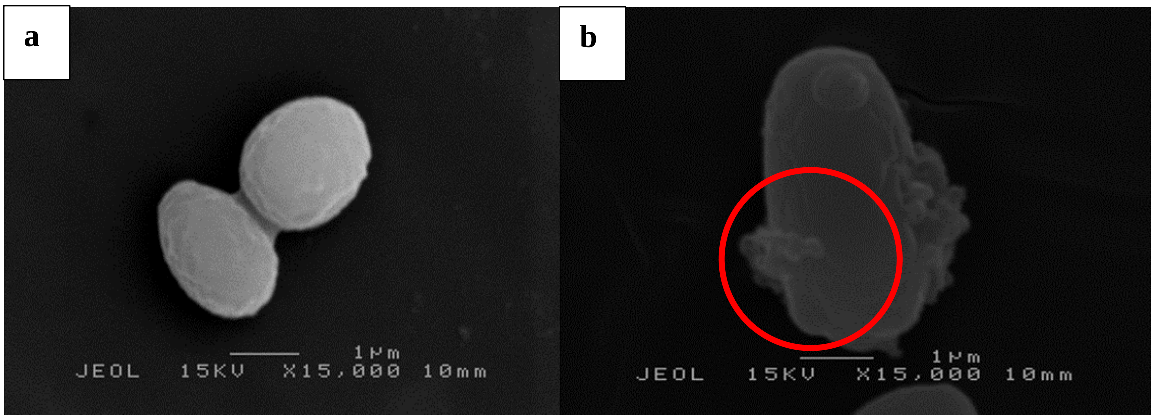

Figure 4. Morphology changes of *C. albicans* before and after treated with *S. polyanthum* leaves extract at MIC value. (a) Control and (b) Treatment.

Untreated *A. niger* (control) showed normal morphology with smooth, spherical, intact spore shape and straight, linearly hypha with smooth external surfaces and rounded apices.

Treatment extract had altered the morphology of *A. niger*, where spore showing distorted and perforated structure while the treated hyphae become irregular branching, loss of linearity, and distorted cell wall. A similar observation also declared by [27]. On the treated *C. albicans* membrane cells, there was some dissolution of cytoplasmic contents due to the loss of electron density. The cell wall was ruptured and leakage. In contrast, untreated *C. albicans* retained their cell morphological features. Same condition reported by Jackson et al. [28], where membrane disposition in *C. albicans* had been observed after being treated with *Euphorbia hirta* leaf extract at 3.125 mg/mL for 36 h. The leaves extract has lipophilicity properties to disrupt fungal cell membrane functions such as expansion, increased fluidity and permeability, respiration, and cell leakage [29]. Long periods of extract exposure to cells cause irreversible damage to cell structure [30]. This result indicates that the fungicidal mechanism of leaves extract against *A. niger* and *C. albicans* was happened by penetrating the fungal membrane to disrupt further and inhibit the cell growth.

Cell constituents release assay. Figures 5(a) and (b) showed the cell constituents release assay results for *A. niger* and *C. albicans* following exposure with *S. polyanthum* leaves extract for 72 h and 4 h, respectively.

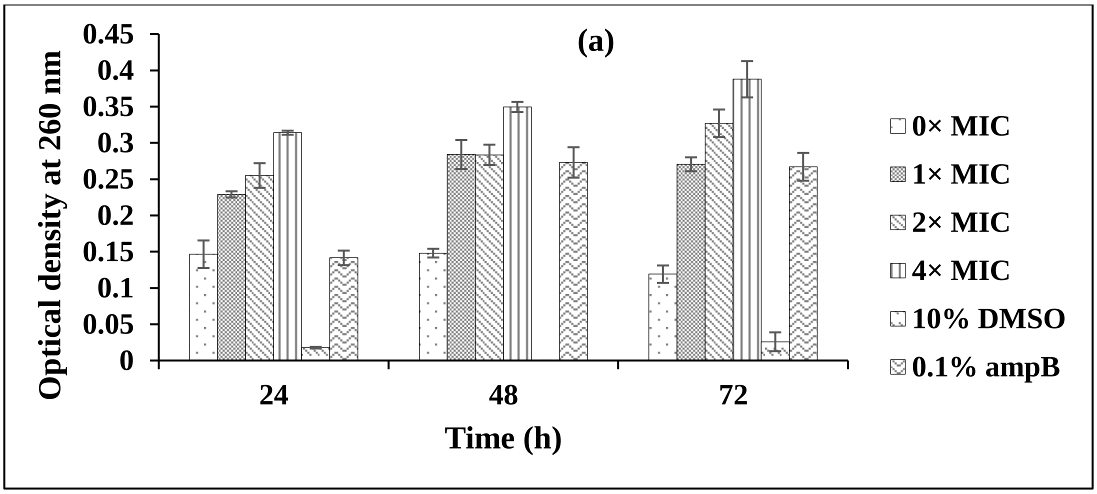

Figure 5(a). Cell constituent release analysis of *A. niger* following exposure with *S. polyanthum* leaves extract at concentration from 0× MIC to 4× MIC

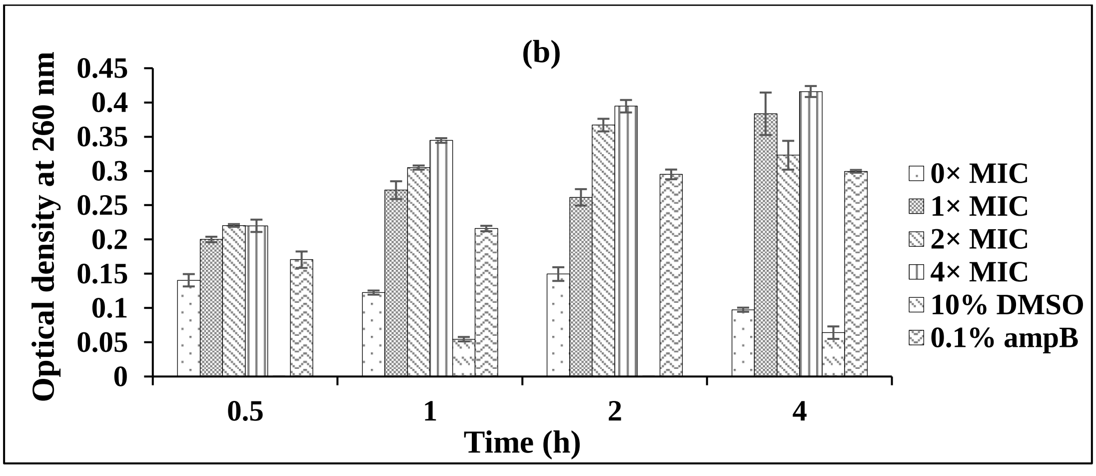

Figure 5(b). Cell constituent release analysis of *C. albicans* following exposure with *S. polyanthum* leaves extract at concentration of from 0× MIC to 4× MIC.

Finding above shows the cell constituents release increased visibly when the applied concentration of leaves extract increased and longer incubation time compared to the control. The highest release of cell constituents release was obtained during the extract treatment on *A. niger* at 4× MIC for 72 h incubation, showing an absorbance of 0.381 while for *C. albicans* showed the highest absorbance of 0.435 at 4× MIC after 4 h incubation.

Conclusion

In conclusion, the research findings were proved *S. polyanthum* leaves extract might contain the essential phytochemical compounds to perform its antimicrobial activity of the leaves extract against filamentous fungi and *Candida* species. Therefore, *S. polyanthum* leaves extract might had potential to be promoted for further study in the evaluation of natural antimicrobial compounds to be applied in future food industries.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards

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