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Assessment of Biological Activity, Total Phenolic Content, and Cytotoxicity of Ethyl Acetate Extracts from an Endophytic Fungus, *Lasiodiplodia pseudotheobromae* IBRL OS-64

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ABSTRACT

The biological activities of ethyl acetate extracts of *Lasiodiplodia pseudotheobromae* IBRL OS-64 were assessed in the present study. Antioxidant activity was measured using the DPPH scavenging assay, antibacterial activity via broth microdilution, and total phenolic content using the Folin-Ciocalteu method. *In vivo* toxicity was determined using a brine shrimp lethality assay. The fraction extract exhibited the highest antioxidant activity, with an EC_{50} of 441.6 µg/ml, surpassing the crude extract's. The EEELP showed a relatively low phenolic content of 13.273 µg GAE/mg extract. Regarding antibacterial activity, both crude and fraction extracts demonstrated MIC values ranging from 62.5 to 250 µg/ml against Gram-positive bacteria, while MBC values ranged from 125 to 500 µg/ml for both extracts. The MIC values for crude and fraction extracts against Gram-negative bacteria were 250-500 µg/ml, whilst the MBC values for both crude and fraction extracts were in the range of 500–2,000 µg/ml. The findings also revealed that Gram-positive bacteria were more susceptible to both extracts (crude and fraction) than Gram-negative bacteria. The crude extract exhibited a non-cytotoxic effect with an LC_{50} value of 2054.88 µg/ml for acute exposure and a low cytotoxic effect with an LC_{50} value of 199.69 µg/ml for chronic exposure. Meanwhile, the fraction extract demonstrated a non-cytotoxic effect for both acute and chronic with an LC_{50} value of 5744.14 µg/ml and 1035.94 µg/ml, respectively. Therefore, the present study suggests that the fraction extract could be an effective pharmaceutical agent and safe for drug development due to its low toxicity.

1. Introduction

Fungal endophytes isolated from medicinal plants are a potential source of antioxidants and antibacterial with favourable cytotoxicity activities (Adeleke & Babalola 2021). Fungal endophytes of medicinal plant origin might inherit the mutagenic or genotoxic traits from their hosts due to their close relationship. According to Zhao *et al.* (2011), because of their long history of co-evolution and cordial relationship with their hosts, certain endophytes are able to develop bioactive chemicals that are similar to those coming from their hosts. Numerous earlier research works discussed the cytotoxic, antibacterial, and antioxidant properties of endophytic fungus. For example, Danagoudar *et al.* (2018) showed the cytotoxic and antioxidant activity of endophytic fungus isolated from the medicinal plant, *Tragia involucrata* L. They revealed that the endophytic fungus, *Cryptendoxyla hypophloia*

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CGJ-D2 demonstrated significant antioxidant activity with the IC_{50} value of 260.00±1.45 mg/ml, and another fungal endophyte, *P. citrinum* CGJ-C2 extract showed significant cytotoxicity against MCF-7 as well as MOLT-4 cell lines with the IC_{50} value of 1.09 and 2.38 µg/ml, respectively. On the other hand, Gunasekaran *et al.* (2017) reported the antioxidant and antibacterial activities of endophytic fungi isolated from *Mussaenda luteola*. They found that the *Alternaria* sp. (ML4) ethyl acetate extract had the highest total flavonoid and phenolic concentration (56.45±0.10 mg of RE/g and 108.65±0.12 mg of GAE/g), respectively. At a dosage of 300 µg/ml, the extract demonstrated 85.20% DPPH scavenging activity. Additionally, it demonstrated antibacterial activity against multiple test species, such as *Escherichia coli* (10 mm), *Staphylococcus aureus* (9.0 mm), and *Pseudomonas aeruginosa* (9.2 mm).

A member of the Botryosphaeriaceae family, *Lasiodiplodia pseudotheobromae* is found on a wide range of host plants, including monocotyledons, dicotyledons, gymnosperms, and angiosperms, where it exists as an endophyte, a parasite, and a saprophyte Adetunji & Oloke (2013). It has been recognized as a species that are important pathogen of various plants and can exhibit several symptoms in infected plants such as dieback, canker, and black rot (Castro-Medina *et al.* 2014; Kwon *et al.* 2017; Nogueira Junior *et al.* 2017). Nowadays, the species has been reported as endophytes with several pharmacological potentials including antioxidants, cytotoxicity, and antimicrobial activities (Lu *et al.* 2014; Taufiq & Darah 2018; Zhou *et al.* 2018). However, extensive studies regarding the antioxidant, antibacterial, and cytotoxicity potential of the species were very scarce. Therefore, the purpose of this research is to explore the cytotoxicity, antioxidant, and antibacterial activity of *L. pseudotheobromae* IBRL OS-64, an endophytic fungus that was isolated from *Ocimum sanctum* L. The results of this investigation offer important information about the biological activities of the species. Additionally, it promotes the potential use of this species as a new drug producer with antioxidant activity that is safe for human consumption.

2. Materials and Methods

2.1. Endophytic Fungus and Cultural Maintenance

The endophytic fungus, *L. pseudotheobromae* IBRL OS-64 was deposited at the Industrial Biotechnology Research Laboratory (IBRL), School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. In addition to a powdered host plant (2 g/L), the fungal culture was grown and maintained on potato dextrose agar (PDA) [brand: Merck] and incubated at 37°C for 7 days. The fungal cultures were kept at 4°C until further use. Subculturing was performed every month to ensure their purity and viability.

2.2. Maintenance of Test Bacteria

Eight test bacteria including *Streptococcus mutans* ATCC 700610, *Staphylococcus aureus* (clinical sample), Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591, *Bacillus subtilis* IBRL A3, *Yersinia enterocolitica* (clinical sample), *Klebsiella pneumoniae* ATCC 13883, *Shigella boydii* ATCC 9207 and *Escherichia coli* IBRL 0157 were deposited at the IBRL, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. All bacterial cultures were grown and maintained on nutrient agar (NA) except *S. mutans* which was grown on brain heart infusion agar (BHIA) and incubated at 37°C for 24 h before being kept at 4°C until further use. Glycerol stock was prepared, and subculturing was conducted once a month to ensure their purity and viability.

2.3. Culture Medium

In 1,000 ml of host plant water extract, 20 (g/L) yeast extract (Merck, Germany), 40 (g/L) sucrose, and 0.5 (g/L) were added to produce yeast extract sucrose (YES) broth (Jalil & Ibrahim 2022). Before that, 1,000 ml of distilled water was mixed with two grams of host plant powder, and the mixture was heated for thirty minutes. A Muslin cloth was used to filter this suspension. After using a pH meter to reduce the culture medium's pH down to 6.0, it was autoclaved for 15 min at 121°C.

2.4. Fermentation and Extraction

Two mycelial plugs of 3-d-old fungal culture were transferred into 250 ml Erlenmeyer flasks containing 100 ml YES broth. The flasks were incubated at 30°C in the dark for 16 d without agitation. Muslin cloth was used to separate the fermentative broth and fungal biomass, and filter papers (Whatman, No.1) were used to filter the mixture. Next, three extractions of the filtered broth were performed using an equivalent volume of ethyl acetate (1:1; v/v). To create ethyl acetate crude paste, the ethyl acetate crude extract was gathered, concentrated using a rotary evaporator, and allowed to dry in a fume hood (Jalil & Ibrahim 2022).

2.5. Preparation of Fraction F5

To elucidate fraction F5, the open column system was packed using the slurry packing method, as stated by Salituro & Dufresne (1998) and Jalil & Ibrahim (2022). The packing medium used was silica gel 60, and the beads had sizes ranging from 40 to 63 nm. There was a glass chromatographic column used, measuring 47.0 cm in length and 22.0 mm in diameter. About 35.0 g of silica gel and 100 ml of ethyl acetate were weighed and combined to make a pourable slurry. Following that, the glass column system was filled with three-fourths of the slurry. The column was tapped using a rubber pipe to ensure adequate packing and stop bubbles from forming during the packing process. The extract was then added after the packed column chromatography had been left to settle for a whole night. After the crude extract was diluted in methanol, it was eluted using the same solvent system that was used for column packing. Because the fractions were colourless, they were grouped by volume, with 20 ml of each fraction. Each fraction was air-dried under the fume hood chamber in order to concentrate it. Every fraction was analysed on a TLC plate, and the fractions with similar Rf values were combined.

2.6. Antioxidant Activity

With minor adjustments, the free radical scavenging properties of the ethyl acetate fungal extract of *L. pseudotheobromae* IBRL OS-64 were investigated using the methodology outlined by Duan *et al.* (2006) and Nor Afifah *et al.* (2011). DPPH stock solution was prepared by dissolving the chemical (24 mg) in 100 ml (99.5%) methanol (Merck) to achieve a final concentration of 0.16 mM. The DPPH stock solution was freshly prepared and kept in a Scotch bottle wrapped with aluminum foil at -20°C. The ethyl acetate crude/fraction extract of *L. pseudotheobromae* IBRL OS-64 and quercetin (positive control) were weighed and dissolved in 100% methanol to obtain stock samples with an initial concentration of 500.00 µg/ml. The DPPH free radical scavenging assay was performed in a flat bottom 96-well microtiter microplate. A 100 µL of the sample was added to 100 µL of 0.16 mM methanolic DPPH solution in the flat bottom 96-well microtiter microplate. The final concentration of the sample in each well was 2,000 µg/ml. Due to the colour intensity of the crude/fraction extract, 200 µL of sample blank extract without the addition of the DPPH solution was subjected to the well. For negative control, four wells were added with the mixture of dimethyl sulfoxide (DMSO) and DPPH solution, and another well consisted of a mixture of DMSO and ethanol. Besides that, standard quercetin was used as a positive control, and 2,000 µg/ml quercetin was two-fold diluted to twelve concentrations. After that, the 96-well microtiter microplate was covered with aluminium foil and left to incubate for 30 min at 40°C in the dark. Following the incubation period, the mixture's absorbance was measured at a wavelength of 517 nm. The following formula was used to determine the extract's capacity to scavenge the DPPH free radical:

$$\text{Scavenging activity on DPPH free radicals (\%)} = \frac{A_1 - A_2}{A_1} \times 100\%$$

The values of A_1 and A_2 corresponded to the absorbance of the control and sample, respectively. Additionally, using GraphPad Prism Software (GraphPad, USA), the effective concentration (EC_{50}) for the sample and quercetin (standard) was estimated. Three sets of experiments were conducted.

2.7. Total Phenolic Content (TPC)

Using certain adjustments, the total phenolic content of the crude extract was measured in 96 wells of microtiter plates using the methods outlined by Nor Afifah *et al.* (2011). By dissolving 1.0 mg of extract in 1.0 ml of DMSO, 1.0 mg/ml crude extract was created. Following that, 50 µL of the sample was added into 25 µL 10% Follin-ciocalteu reagent in the wells and it was left for 5 to 10 min under a dark condition at room temperature (30±2°C). Next, the mixture in the well was supplemented with 100 µL of distilled water and 25 µL of 2% sodium carbonate (Na_2CO_3). Gallic acid served as a positive reference point. Sixteen concentrations of the 1 mg/ml gallic acid solution were then created by diluting it. The Gallic acid mixed with the Follin-ciocalteu reagents was treated the same as for the sample extract. For negative control, 125 µL distilled water was added to 25 µL of 2% sodium carbonate (Na_2CO_3). After 30 minutes of incubation at room temperature, the sample's absorbance was measured at 760 nm using DMSO as the blank. Three duplicates of the experiment were conducted, and the findings were reported as Gallic acid equivalent (GAE) in µg GAE/mg of extract.

2.8. Antibacterial Activity

The antibacterial activity of the crude and fraction extracts was examined via MIC and MBC determination according to the broth microdilution method as

described by Taufiq & Darah (2018) using a sterile, 96-well, U-shaped microtiter microplate. The fungal extract was diluted one-fold in sterile Mueller-Hinton broth (MHB) medium, and 100 µL of the extract was added to each microtiter plate well. After that, each well received 100 µL of the test microorganisms' inocula at a final volume of 200 µL, or around 1×10^7 CFU/ml. This meant that each well's final concentration of bacterial inocula was 1×10^7 CFU/ml. The reference drug utilized was chloramphenicol. As controls, there was a bacterial inoculum and 5% ethyl acetate. 40 µL of 0.2 mg/ml p-iodonitrotetrazolium violet (INT) dissolved in 99.5% ethanol was added to each well as a growth indicator after a 24-hour incubation period at 37°C.

The MIC values were then determined to calculate the MBC of the crude extract of fungal ethyl acetate. Following a 24-hour incubation period at 37°C, the viable cells from wells that showed no microbial growth were determined using a conventional viable plate count on MHA. After a 24-hour exposure period, the lowest concentration of the extract that prevents the test bacteria from growing visibly was determined to be the MIC value. In the meantime, the lowest extract concentration that inhibits 99.9% of the bacterial growth was recognized as MBC value.

2.9. Brine Shrimp Lethality Test (BSLT)

Through an *in vivo* toxicity study, the crude extract of *L. pseudotheobromae* IBRL OS-64 was evaluated for its toxicity against brine shrimp, *Artemia salina*. The procedures outlined by Vanhaecke *et al.* (1981), McLaughlin & Rogers (1998), and Nor Afifah *et al.* (2011) were followed when conducting the brine shrimp lethality test. One gram of brine shrimp, *A. salina* eggs from Great Salt Lake (Sander brine shrimp Com Inc., USA) was added into a 2 L beaker containing 1,000 ml of 3.8% artificial seawater (38.0 g of sea salt in 1.000 ml of sterile distilled water). The beaker was placed under a light source to ensure and maintain a temperature of 30±2°C. For constant aeration, an aquarium pump was used in the hatching chamber. Stock extract for treatment with a concentration of 1,000 mg/ml was prepared by dissolving 1,000 mg of fungal crude extract in 400 µL of dimethyl sulfoxide (DMSO) followed by adding 600 µL of artificial seawater. DMSO was used as a solvent to dissolve the extract whereas the artificial seawater was used as a diluent. Then, the stock solution was further diluted to obtain a series of concentrations ranging from 625 to 10,000 µg/ml with artificial seawater. Ten 48-h-old nauplii that were previously hatched were transferred into each universal bottle containing different extract concentrations using a Pasteur pipette. For control, the nauplii were grown in a universal bottle containing artificial seawater without the extract. The nauplii in respective universal bottles were then incubated at 25°C under dark conditions. The condition of the nauplii that was exposed to different extract concentrations was observed under a dissecting microscope after 6 h of incubation for acute toxicity and 24 h for chronic toxicity. The nauplii were considered deceased if they did not show any movement. The percentage of mortality was calculated using the formula below:

$$\text{Percentage of Mortality} = \frac{N_d}{N_s} \times 100\%$$

Whereby N_d is the number of dead nauplii and N_s is the number of survived nauplii (Elmer-Rico and Micor 2007).

The linear graph Log10 of extract concentrations versus the percentage of mortality was plotted and the best-fit line was generated to obtain a linear regression equation. LC_{50} of the fungal extract for acute (6 hours) and chronic toxicity (24 hours) was calculated according to the linear regression equation, respectively.

2.10. Statistical Analysis

The experimental data were expressed as mean ± standard deviation (SD), and each experiment was carried out in triplicate (n = 3). The One-Way ANOVA was utilised to analyse the data using SPSS 15.0, and the Duncan test was employed to determine whether there were any mean differences. If ρ was less than 0.05, the findings were deemed statistically significant.

3. Results

3.1. DPPH Scavenging Activity

Figure 1 exhibits the effect of scavenging of DPPH radicals on quercetin, and fungal crude fraction extract at different concentrations, respectively. The results revealed that the crude and fraction extract exhibited a concentration-dependent antioxidant scavenging activity. It was shown that the crude and fraction extracts can scavenge approximately 70% and 86% of DPPH free radicals activity at a concentration of 2,000 µg/ml, respectively. Table 1 shows the EC_{50} for standard (quercetin) was 14.57 µg/ml, the fungal ethyl acetate crude extract was 441.60 µg/ml, whereas

the fraction F5 with the value of 208.1 µg/ml. Both extracts were significantly lower than the quercetin as a standard. It is interesting to note that the Fraction F5 had higher scavenging activity compared to crude extract with scavenging activity increased by up to 70%. The present study suggested that the purification of the extract could increase the scavenging activity of DPPH free radicals. This underscores the importance of purification in maximizing the therapeutic potential of natural extracts.

3.2. Total Phenolic Content (TPC)

Figure 2 shows the standard curve for the phenolic content of GA and the mathematical equation of the curve is y = 0.0055x + 0.11 with R2 = 0.9107. Gallic acid equivalent (GAE) per milligram (mg) extract was used to denote the unit of total phenolic content (TPC). Based on the equation, the TPC of ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 was 13.27 µg GAE/mg extract (Table 2). Meanwhile, the TPC fraction F5 was observed to be 6.41 µg GAE/mg extract. According to the present finding, the fungal extract has a low total phenolic concentration.

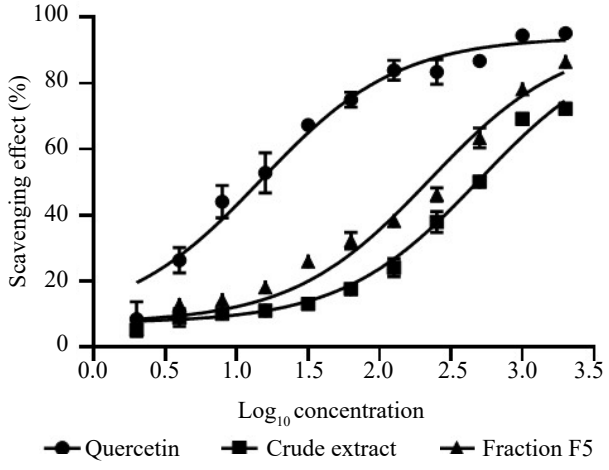

Figure 1. Effect of scavenging of DPPH radicals on quercetin, fungal crude extract, and fraction (F5) at different concentrations

Table 1. EC_{50} value of fungal crude extract, fraction F5, and quercetin

Sample	EC_{50} (µg/ml)
Fungal crude extract	441.6[a]
Fraction F5	208.1[b]
Quercetin (standard)	14.57[c]

3.3. Antibacterial Activity

The MIC and MBC values of the crude and fractional extracts against both Gram-positive and Gram-negative bacteria are displayed in Table 3. The findings showed that the MIC values for crude and fraction extracts, respectively, ranged from 62.5 to 250 µg/ml and 125 to 250 µg/ml for Gram-positive bacteria. Meanwhile, the MBC values for both crude and fraction extracts were 125–500 µg/ml. On the other hand, the MIC values for crude and fraction extracts towards Gram-negative bacteria were 250–500 µg/ml, whereas the MBC values for both crude and fraction were in the range of 500–2,000 µg/ml. The finding revealed that both types of extracts showed higher susceptibility towards Gram-positive bacteria compared to Gram-negative bacteria. The extracts (crude and fraction) exhibited bactericidal effects towards all the Gram-positive and Gram-negative bacteria except *K. pneumoniae* (fraction) which is a bacteriostatic effect since the MBC/MIC ratio was beyond four. As for MRSA ATCC 33591, the MIC and MBC values for the fraction are significantly less than the values of crude extract. The findings indicated the fractionation of the extract could

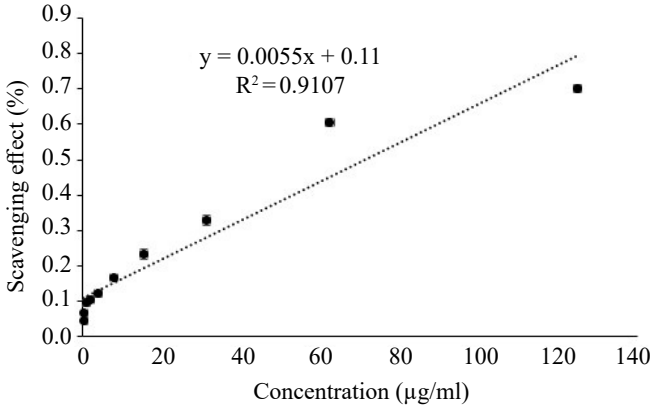

Figure 2. Standard curve of Gallic acid

Table 2. Total phenolic content of fungal crude extract and fraction F5

Sample	Total phenolic content (µg GAE/mg extract)
Fungal crude extract	13.27
Fraction F5	6.41

Table 3. *In vitro* activity of pathogenic bacteria for MIC and MBC values

Test bacteria	Crude		Fraction F5	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
Gram-positive bacteria				
S. mutans	125	125	125	125
S. aureus	250	500	250	500
MRSA ATCC 33591	250	250	125	125
B. subtilis IBRL A3	62.5	125	125	125
Gram-negative bacteria				
Y. enterocolitica	250	500	250	500
K. pneumoniae ATCC 13883	250	1,000	250	2,000
S. boydii ATCC 9207	500	2,000	500	2,000
E. coli IBRL 0157	500	2,000	500	2,000

eliminate inactive compounds that disturb the accuracy of the antibacterial effect of the extract. For *B. subtilis* IBRL A3, the MIC values of the crude extract were slightly less compared to the fraction (F5). The results revealed that the higher antibacterial activity in the crude extract might be a combination of more than one bioactive compound or a synergistic effect among the bioactive substances. A similar phenomenon was also observed on *K. pneumoniae* ATCC 13883 since the MBC values of the fraction extract were increased from 2,000 µg/ml to 1,000 µg/ml for crude extract.

3.4. Brine Shrimp Lethality Test (BSLT)

Several concentrations of crude extract were tested on laboratory-cultured brine shrimp, *A. salina* to assess the preliminary toxicity effect of the extract. The percentage of mortality of the brine shrimp exposed with potassium dichromate, fungal crude, and fraction extracts were calculated and the graphs were plotted against log10 concentration as shown in Figures 3, 4, and 5, respectively. The results revealed a dose-dependent toxicity effect for both crude and fraction extracts, wherein the percentage of mortality increased as the concentration of the extract increased. Table 4 shows the LC_{50} values for potassium dichromate, crude, and fraction extract. For acute toxicity, the LC_{50} value of potassium dichromate was 169.02 µg/ml whereas, for chronic toxicity, the LC_{50} value was 8.84 µg/ml. The results revealed that potassium dichromate was very toxic to the brine shrimp as both LC_{50} values were not exceeding 1,000 µg/ml. Meanwhile, the LC_{50} value for acute and chronic toxicities of the fungal crude extract was 2054.88 µg/ml and 199.69 µg/ml, respectively. The results demonstrated that the crude extract was non-toxic for acute toxicity but slightly toxic after 24 h exposure time (chronic toxicity). As for fraction extract, the LC_{50} value after 12 h exposure time was 5744.14 µg/ml (acute toxicity) whereas, for chronic toxicity, the value of LC_{50} was 1035.94 µg/ml. The results indicated that fraction F5 was not toxic to the brine shrimp as both LC_{50} values were exceeding 1,000 µg/ml. The present study revealed that the purification of the crude extract could change the toxicity level of the extract whereby the fraction extract demonstrated a non-toxic effect towards brine shrimp compared to the toxic effect of crude extract.

4. Discussion

The DPPH method was first introduced in the year 1958 by Marsden Blois using cysteine as a model of antioxidants and the method has undergone several modifications with the advancement of techniques and instruments (Kedare & Singh 2011). However, the main concept remains the same and many researchers have implemented this method to evaluate the potential of biological extract as an antioxidant agent due to the simplest and most rapid method approaches. According to Sultana *et al.* (2007), DPPH free radical assay is widely performed since it is a rapid and stable method to detect and determine the potential of a particular bioactive compound as a free radical scavenger. Generally, the concept of DPPH assay was the ability of the DPPH free radical (1,1-diphenyl-2pikrylhydrazine) to scavenge the hydrogen atom donated by an antioxidant compound in the biological extract to form a stable DPPH (diphenylpikrylhydrazine). During the scavenging activity, the purple color of stable DPPH was decolorized into a yellowish color which indicated the presence of an antioxidant agent in the tested biological extract (Ara & Nur 2009).

In the present study, the antioxidant activity of the fungal crude extract was significantly lower than quercetin (standard). According to Dhankhar *et al.* (2012), the lower antioxidant of crude extract may be due to the mixture of various substances accumulated in the extract and some of them might inhibit the potency of active compounds. In comparison, Kaur & Mondal (2014), reported that *O. sanctum* showed high antioxidant activity with 81.8% DPPH scavenging activity. This might be due to the presence of Rosmarinic acid in the chemical constituents of the *O. sanctum* plant which acts as a powerful antioxidant agent (Pattanayak *et al.* 2010). A similar observation

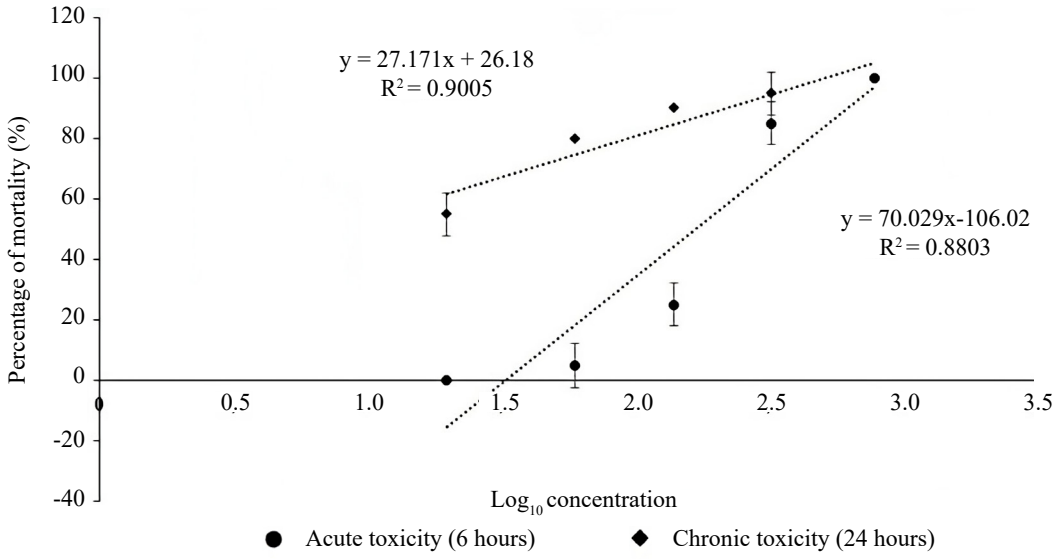

Figure 3. Toxicity of potassium dichromate on brine shrimp, Artemia salina at 6 hours (acute toxicity) and 24 hours (chronic toxicity)

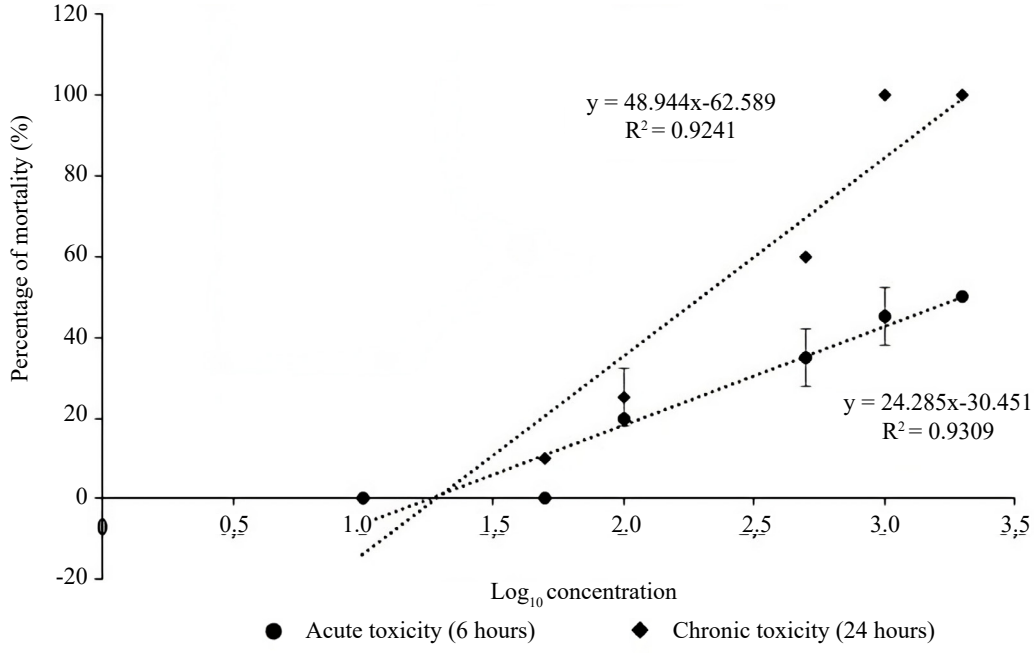

Figure 4. Toxicity of potassium dichromate on brine shrimp, *Artemia salina* at 6 hours (acute toxicity) and 24 hours (chronic toxicity)

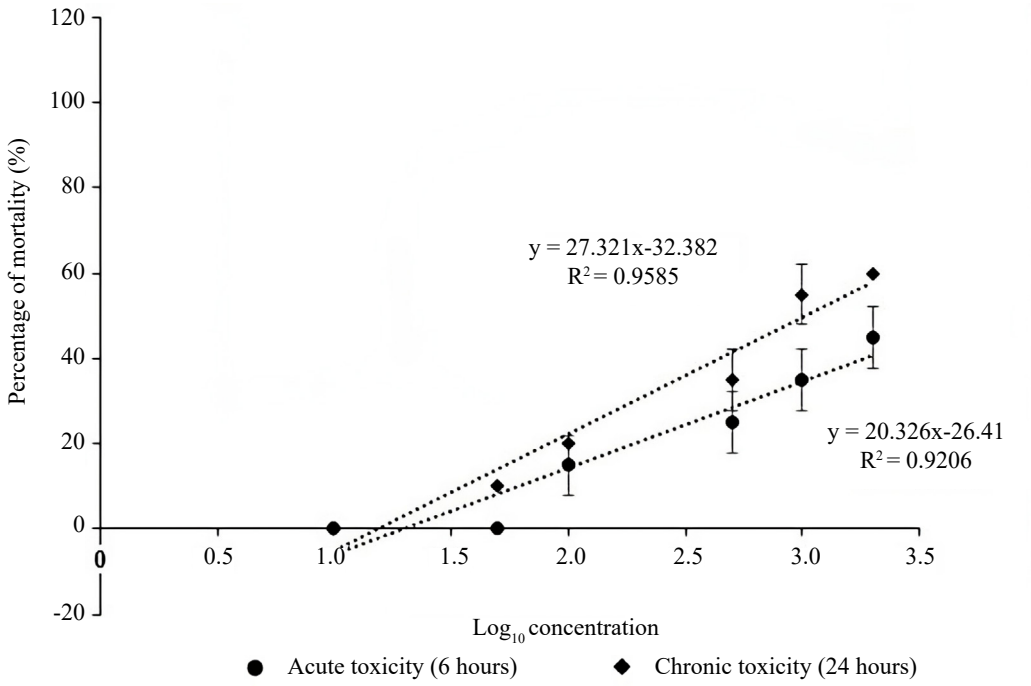

Figure 5. Toxicity of fraction 5 of the fungal extract on brine shrimp, *Artemia salina* at 6 hours (acute toxicity) and 24 hours (chronic toxicity) of exposures

Table 4. Summary of LC_{50} values for fungal crude extract and positive control

Sample	LC_{50} values (µg/ml)	
	Acute toxicity (6 hours)	Acute toxicity (24 hours)
Fungal crude extract	2054.88[a]	199.69[a]
Fraction F5	5744.14[b]	1035.94[b]
Potassium dichromate	169.02[c]	8.84[c]

was reported by Kandasamy *et al.* (2015). The present study suggests that the ability of fungal endophytes to produce phytochemical compounds (antioxidants) mimicking their host plant could be due to their close relationship. Pan *et al.* (2017) stated that the interaction between fungal endophytes and their host via chemical and physical signals could promote and enhance plant growth hormone through phytochemicals production such as antioxidants without resulting in any biotic stress to the host when they invade the host tissues. The result was consistent with the finding of Jinu *et al.* (2015) who claimed that antioxidant activity, cholestanol glucosidase produced by *L. theobromae* isolated from *Saraca asoca* showed a concentration-dependent towards scavenging activity.

However, the fungal ethyl acetate crude extract was observed to have lower EC_{50} as compared to quercetin and thus, indicated that the crude extract had lower DPPH free radicals scavenging activity. The low free radicals scavenging activity of the ethyl acetate crude extract could be related to its purity. According to Barbosa-Pereira *et al.* (2013), the extract must be purified to eliminate all undesirable and inert substances to improve its antioxidant activity. On the other hand, purification of the crude extract could remove fractions with limited antioxidant activity and thus, enable a fair level of antioxidant activity to be obtained from the original natural extracts that possess relatively small amounts of desired antioxidant compounds. Moreover, the purity of the extracts is crucial to ensure the safety and identity of antioxidant compounds to be consumed by humans such as food additives (Diaz-Reinoso *et al.* 2006). The low antioxidant activity of the crude extract also may be affected by the selection of solvent and solubility of the extract. For instance, polyphenol compounds are usually more soluble in less polarity organic solvents than water and the solubility is influenced by the polar properties of the polyphenols (Haminiuk *et al.* 2014).

The Follin-Ciocalteu assay was employed to ascertain the total phenolic content (TPC) of the fungal crude extract, with gallic acid (GA) serving as the reference. Due to its simplicity, reproducibility, and convenience, the assay was used to assess TPC (Pushpalatha *et al.* 2011). A prior investigation found

that the ethyl acetate crude extract of the endophytic fungus *L. pseudotheobromae* IBRL OS-64 contained the phytochemical component, phenol (Jalil & Ibrahim 2022). In the 1% aqueous ferric chloride test, they revealed that a spot, S7 on the TLC plate turned reddish-brown when aqueous ferric chloride was applied indicating the presence of phenol (a positive result). Their finding suggests the presence of phenol in the ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64. Since Huang *et al.* (2005) proposed that phenolics were the primary antioxidant components in the majority of endophytes, the TPC investigation was conducted. Aside from that, earlier research documented the connection between total phenolic contents and antioxidant activity. For instance, Liu *et al.* (2010) reported that the presence of phenolic compounds influenced the higher antioxidant activity of an endophytic fungus, *Xylaria* sp. isolated from *Ginkgo biloba*. Similarly, a phenolic compound isolated from a fungal endophyte, *Cephalosporin* sp. exhibited significant radical scavenging and antioxidant activities (Priya & Joseph 2011).

The present results showed that low total phenolic content resulted in low antioxidant activity of a fungal extract. According to Sultana *et al.* (2007), the antioxidant potential of any sample is linearly correlated with the total phenolic content. The occurrence of hydroxyl groups is attributed to the free radical scavenging ability of phenols (Yadav *et al.* 2014). Low total phenolic content in ethyl acetate crude extract may be due to the polarity of the organic solvent or the solubility of the extract. Methanol is usually used to extract polar and medium polar phenolic compounds such as phenolic acids and flavonoid glycosidases (Harborne 1998). Kandasamy *et al.* (2015) reported that the methanol extract of *Nigrospora* sp. and *Drechslera* sp. harboured the highest phenolic content. On the other hand, the diversity in the structure of phenolic compounds which ranged from simple to polymerized forms might affect their solubility behaviour (Babbar *et al.* 2014). In comparison, the previous study reported that *O. sanctum* leaf possesses a significant TPC with a value of 44.43 µg GAE/mg extract (Tupe *et al.* 2013).

Generally, phenolic compounds such as tannins (Bhardwaj *et al.* 2015), phenols (Shoba and Sathiavelu 2018), and flavonoids (Das *et al.* 2017) have been reported as the contributors to the antioxidant activity of various endophytic fungal extracts. The phenolic compounds are well-known antioxidant agents that serve as free radical terminators and they can scavenge free radicals, chelate metals, and inhibit lipoxygenase (Ramesha & Srinivas 2014). The phenolic compounds expressed their antioxidant activity through various mechanisms of action including the inhibition of reactive oxygen species (ROS) by reducing the chelated metal ions. Phenolics possess carboxyl and hydroxyl groups that can bind particularly copper and iron (Jung *et al.* 2003). They are believed to be inactive iron ions by chelating and suppressing the superoxide-driven Fenton reaction, which is the most important source of ROS (Rice-Evan *et al.* 1997; Arora *et al.* 1998). Besides that, phenolic antioxidant agents can interrupt lipid peroxidation by trapping lipid alkoxyl radicals. However, this activity depends on the position and number of the hydroxyl group in the molecules as well as the structure of the molecules (Milic *et al.* 1998). For instance, Arora *et al.* (2000) reported that the phenolic antioxidants alter the peroxidation kinetics by modifying the lipid packing order. They were observed to stabilize membranes by decreasing membrane fluidity and thus, hinder the diffusion of free radicals, resulting in the restriction of the peroxidation reaction. In addition to that, the phenolic compounds are also able to directly scavenge the active oxygen species molecules including hydrogen peroxide, superoxide, hydroxyl radical, and peroxyl radicals. They can stabilize active molecules by donating electrons and hydrogen atoms (Michalak 2006).

The emergence and rapid spread of antibiotic-resistant strains have become a new fear among the medical and scientific communities (Lushniak 2014; Ventola 2015). Thus, this phenomenon has urged researchers to search for new antibiotics to combat resistant strains. A natural product is known as one source of antimicrobial drugs and endophytic fungi have proven they are a promising source of bioactive compounds with pharmaceutical properties. The present study revealed that both extracts either crude or fraction from endophytic fungus exhibited significant activity against Gram-positive and Gram-negative bacteria. However, Gram-positive bacteria are more susceptible to both extracts compared to Gram-negative bacteria. Given that Gram-negative bacteria have more sophisticated cell structures than Gram-positive bacteria, including an outer membrane layer, a thin peptidoglycan layer, and periplasm, this observation may be the result of the two types of bacteria having different cell structures. The outer

membrane layer, which separates the periplasm from the external environment and acts as a selective barrier to block antibiotic compounds while allowing essential nutrients to enter the cell, is a structure that distinguishes between the two types of bacteria (Beveridge 1999). It is made up of lipopolysaccharides, proteins, and phospholipids. In addition, membrane proteins called porins bind the outer membrane by functioning as specialized channels that permit the passage of hydrophilic molecules of a particular size into the periplasm (Miller & Salama 2018). Since the MBC values are fewer than four times the MIC values, the results also showed that the extracts had a bactericidal impact on nearly all test microorganisms. As reported by Krishnan *et al.* (2010), antimicrobial substances are classified as bacteriostatic agents if the MBC/MIC ratio exceeds 4, and bactericidal agents if the ratio is less than or equal to 4. Besides that, the fraction extract demonstrated a significant antibacterial activity towards *S. mutans, B. subtilis,* and MRSA since the MIC and MBC values are the same. Similar MIC and MBC values suggested that the test bacteria were very sensitive to the antibiotic component in the extract, according to Ding *et al.* (2009). Additionally, the results showed that certain bacteria had a higher susceptibility to the fraction than the crude extract, and vice versa. According to Soltanian *et al.* (2016), the crude extract was shown to be more effective than its fraction. They proposed that this occurrence could be attributed to the combined action of many mycochemical elements. In addition, the effectiveness of natural medications was impacted by the synergistic and additive effects of many phytochemical compounds rather than a single component (Martins *et al.* 2013). Ginsburg & Deharo (2011) state that the synergistic effect is when a combination of multiple bioactive compounds can exert a greater effect or inhibition than a single compound, whereas the additive effect occurs when different bioactive compounds are combined and interact in a mixture to exhibit an effect similar to a single compound. The recent study also revealed that the fractionation of the extract can improve its antibacterial activity against test bacteria. The outcome was in line with Yimta *et al.* (2014) who reported that the fractionation method concentrated the active compound in the fractions FJ and FI and enhanced the antibacterial activity of the *T. avicennioides* extract against *P. mirabilis*.

The toxicity of the fungal crude extract was investigated using a brine shrimp lethality assay. This method is an efficient tool to access the cytotoxic effect of bioactive compounds including fungal toxins (Sarah *et al.* 2017). The brine shrimp lethality assay was selected for primary cytotoxicity assessment of ethyl acetate crude extract produced by *L. pseudotheobromae* IBRL OS-64 because it is relatively inexpensive, simple, rapid, easily mastered and the most important thing is it required only a small amount of test compound (Wu *et al.* 2013b). *Artemia* sp. was used as a test organism in the present study since it is a euryhaline species that possesses a great osmoregulation capacity which contributes to greater resistance to the toxic effects (Rao *et al.* 2007). This species is highly demanded among researchers to access cytotoxicity because of the rapid generation time, the availability of the nauplii hatched from commercial dormant eggs, small body size, ease of culture under laboratory conditions, good pieces of knowledge of its ecology/biology, and the high adaptability of various experimental conditions (Libralato *et al.* 2016). Furthermore, it has been reported that *Artemia* sp. is greatly vulnerable to toxins, especially at the early stage of its development and optimal sensibility can be achieved after 48 hours of exposure to the test materials (Carballo *et al.* 2002).

Therefore, results revealed that fungal extract was non-toxic for acute toxicity whereas, as for chronic toxicity, it was considered moderately toxic. The moderate LC_{50} value of chronic toxicity shown by the fungal extract indicated that it has potential as an anti-cancer or anti-tumor agent. Carballo *et al.* (2002) reported that the brine lethality test has a good relationship to detect antitumoral compounds in natural extracts from a pharmacological point of view. The high toxicity of the extract that resulted in the high lethality of brine shrimp demonstrated high anti-cancer potential. For instance, Sahgal *et al.* (2010) reported that the methanolic extract of *Swietenia mahogany* L. seed exhibited a moderate toxicity effect against brine shrimp after 24 hours with the LC_{50} value of 680 µg/ml and they suggested that the extract may contain cytotoxic compounds. However, further detailed study needs to be conducted to verify the potential of the extract as an anti-cancer agent. Endophytic fungi are reported to possess the ability to produce a large number of chemically different secondary metabolites including steroids, alkaloids, phenols, isocoumarins, xanthones, quinones, and terpenoids these substances are known to have antimicrobial, antifungal, antiparasitic, cytotoxic,

anticancer and antiviral activities (Frisvad *et al.* 2008; Manganyi & Ateba 2020; Salvatore *et al.* 2020; Attia *et al.* 2022a, 2022b). Khan *et al.* (2016) reported secondary metabolite, naphthoquinone isolated from an endophytic fungus, *Cladosporium* sp. showed a noticeable cytotoxicity effect in brine shrimp lethality test and at the same time exhibited antibacterial activity against some bacteria including *E. coli, S. aureus*, and *P. aeruginosa*. They also revealed that the ethyl acetate crude extract and the fractions of endophytic fungus isolated from the bark of Rauwolfia serpentina demonstrated high toxicity activity with the LC_{50} values in the range of 1.2 to 42.8 µg/ml and the toxicity increased from crude to fraction extract. On the other hand, the previous study revealed that the fraction extract (Ma10) of an endophytic fungus, *P. minioluteum* was not toxic to brine shrimp, *A. salina* with LC_{50} values of 1,480 µg/ml for acute toxicity and 1,130 µg/ml for chronic toxicity. They also claimed that the fraction Ma10 demonstrated significant in vitro antibacterial activity against the Staphylococcal strain, MRSA (Yenn *et al.* 2015).

According to Debnath & Hussain (2013), methanolic crude extracts of *Ocimum sanctum* possess an intense *in vitro* cytotoxic effect towards nauplii and suggest their potential use in traditional medicine. They also reported that *n*-hexane, ethyl acetate, and chloroform extracts exhibited a non-toxic effect with LC_{50} values of 4365, 5370, and 10,000 µg/ml, respectively. In contrast, Krishnaraju *et al.* (2006) reported that the whole plant extract of *O. sanctum* exhibited significant lethality to brine shrimp. They revealed that the extract was highly toxic to the brine shrimp with LC_{50} values of 30 µg/ml for chronic toxicity. Besides that, Audipudi *et al.* (2013) revealed that chloroform extract, ethyl acetate extract, methanol extract, and ethanolic extract of *O. sanctum* showed significant toxicity on brine shrimp with LC_{50} values of 157.24 µg/ml, 116.25 µg/ml, 198.04 µg/ml, and 468.40 µg/ml, respectively. They also suggested that the extracts might have anti-tumor potentials.

Moreover, a previous study revealed the secondary metabolites of *L. theobromae* possess potential phytotoxic, cytotoxic, and antimicrobial activities including depsidones, diketopiperazines, and lasiodiplodins (Salvatore *et al.* 2020). Depsidones are cyclic ethers, or ester-like depsides, that are produced via the polymalonate pathway and are related to diphenyl ethers. Compounds from the depsidones family have been shown to exhibit a variety of biological activities, including antiproliferative, antimalarial, cytotoxic, antibacterial, radical scavenging, antihypertensive, anti-trypanosomal, anti-malarial, anti-leishmanial, herbicidal, larvicidal, antioxidant, and antifungal properties. It has been demonstrated that the *Lasiodiplodia* species produce diketopiperazines and their derivatives which when tested on mammalian cells for cytotoxicity, demonstrated minimal action. Moreover, twelve lasiodiplodins that were extracted from an endophytic mangrove strain were tested in vitro against the human cancer lines THP1, MDA-MB-435, A549, HepG2, and HCT-116 to determine their cytotoxic properties and lasiodiplodins derivatives including hydroxyheptyl or hydroxynonyl moieties demonstrated modest levels of cytotoxicity (Salvatore *et al.* 2020). In comparison, the ethyl acetate crude extract of *L. pseudotheobromae* IBRL OS-64 isolated from *O. sanctum* leaf showed non-toxic and mild-toxic for acute and chronic toxicity, respectively. This may be due to the production of secondary metabolites by the fungus that occurred under favourable conditions with the presence of their host in the culture medium. Due to their mutualistic relationship with host plants, endophytic fungi may produce secondary metabolites with reduced toxicity to not harm and cause death to their host tissues (Strobel & Daisy 2003). However, Hardoim *et al.* (2015) suggested that the endophytes might show their pathogenicity under certain conditions. For instance, an ergot fungus, *Claviceps purpurea* can produce alkaloids that are toxic to predators under stress conditions in association with its host thus preventing its host plant from predation. The statement agreed with Owen and Hundley (2004) who claimed that the bioactive compounds produced by endophytes are relatively low toxicity towards higher organisms.

Toxicological properties of natural products such as plants need to be carried out even though they are used as food sources, but some of them may have mutagenic or genotoxic properties (Tulay & Ozlem 2007). Recently, there have been numerous studies regarding the pharmacology and toxicity of the medicinal plants used by humans since it is crucial to achieve a safe treatment with plant products (Parra *et al.* 2001). Similarly, the toxicity of endophytic fungal extract also needs to be evaluated since they may produce mycotoxins that might not be safe for human use (El-Maghraby & Shabany 2014). Moreover, fungal endophytes isolated from the medicinal plant

might inherit the mutagenic or genotoxic from their hosts due to their close relationship. Zhao *et al.* (2011) reported that some endophytes can produce similar bioactive compounds as those that originated from their hosts due to a long period of co-evolution and a friendly relationship between both of them. On the other hand, antioxidants are compounds that can delay or inhibit the oxidation of a substrate while present in minute amounts. Nutrition is the most important source of antioxidants and many of them belong to the phenol family (Fusco *et al.* 2007). Cai *et al.* (2004) recommended antioxidant sources containing various bioactive compounds from natural sources for aging prevention. Antioxidants are crucial for food preservation since they can impede the oxidation process and support health promotion provided by a variety of dietary supplements, functional food ingredients, and nutraceuticals (Shahidi & Zhong 2015). Therefore, extensive studies on antioxidant activity, pharmacology, and toxicity of fungal extract are crucial to developing antimicrobial drugs that are safe for human use.

Finally, it is possible to propose *L. pseudotheobromae* IBRL OS-64 of *O. sanctum* origin as a natural source of antibacterial and antioxidant substances. It is necessary to assess the antibacterial qualities of its extracts further and identify the elements causing the effects. Some of the extracts inhibit *Bacillus subtilis*, a significant foodborne pathogen, which suggests that the active compounds from this fungus may be used to prevent foodborne illnesses. But before any compounds are used in medicine, toxicity and safety concerns must be resolved. Additionally, further studies for the purification of the active compound from *L. pseudotheobromae* IBRL OS-64 are needed. The present study suggests that the bioactive compound of *L. pseudotheobromae* IBRL OS-64 origin could be an effective pharmaceutical agent and safe for drug and food additive development due to its low toxicity.

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