TLC Screening Profile of Secondary Metabolites and Biological Activities of Salisapilia tartarea S1YP1 Isolated from Philippine Mangroves

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Abstract: The Salisapilia species are estuarine oomycetes of the mangrove and saltmarsh ecosystem. To date, reports on the secondary metabolites and biological activities of these microorganisms are wanting. In this study, secondary metabolites in broth ethyl acetate extracts (BEAE) and mycelial ethyl acetate extracts (MEAE) of Salisapilia tartarea S1YP1 isolated from yellow senescent mangrove leaves were screened by Thin Layer Chromatography (TLC). Extracts were assayed for antioxidant, antibacterial, α-glucosidase inhibition, and cytotoxic activity. The TLC detected anthraquinones, anthrones, flavonoids, phenols, and triterpenes in both BEAE and MEAE. Coumarins were detected in BEAE but not in MEAE. Quantifying the total phenolics and total flavonoids content of the extracts in terms of gallic acid and quercetin equivalents, respectively shows that BEAE has higher total phenolic and flavonoid contents than MEAE. BEAE exhibited significant antioxidant activities through measurements of free radical scavenging activity against DPPH, hydroxyl, nitric oxide, and superoxide anion radicals as well as the ability to chelate Fe²⁺ metal ion. BEAE significantly inhibited in a dose-dependent manner α-glucosidase activity and selectively inhibited HepG2 cell proliferation. Antioxidant, α-glucosidase inhibitory, and cytotoxic activities have not been observed for MEAE. Both BEAE and MEAE do not have antibacterial activity.

Key words: Halophytophthora, mangrove oomycete, Salisapilia, secondary metabolites

1 Introduction

Estuarine and mangrove oomycetes are morphologically and a genetically diverse group of fungal-like eukaryotes of the phylum Oomycota¹. They act as decomposers of leaf litters in marine environments while some are pathogenic to crustaceans, marine nematodes, and algae²⁻⁴. Of the different taxa of estuarine oomycetes, Salisapilia is a morphology divergent group to Halophytophthora and are widely known to inhabit the mangrove and saltmarsh ecosystem⁵, ⁶. Knowledge of their biodiversity and biological activities is limited. In the Philippines, the first report of Halophytophthora species was documented in 2001 in Panay island⁷ and none yet for Salisapilia species. Of the known oomycetes and thraustochytrids, Halophytophthora, Halophytophthora for the former group, and Schizochytrium and Thraustochytrium for the latter are the taxa commonly explored for the production of poly- and monounsaturated fatty acids⁸⁻¹⁰. To date, no reports on the biological activities of secondary metabolites from congeners of Salisapilia are existing. Yet unknown, these group of microorganisms may provide a rich source of potential compounds with beneficial contributions to pharmaceutical and aquaculture industry.

Marine natural products are considered to exhibit a high and broad range of biological activities¹¹ with distinct chemical structures¹²⁻¹⁰. The unique structures are possibly attributed to the peculiarity of the marine environment²⁰. Yearly, hundreds of new and biologically active...
compounds are found in marine organisms. Because of this, their novel structures and biosynthetic pathways captivate scientists, which are reflected by the increasing number of studies about marine microorganisms.

In this study, a strain, coded as S1YP1 isolated from a fallen senescent leaf of Avicennia sp. was morphologically identified as Salispilia tartarea. The ethyl acetate extracts of S. tartarea S1YP1 were screened for the presence of general classes of secondary metabolites (i.e. alkaloids, terpenoids, phenolics). Further, extracts were investigated for antioxidant, antibacterial, α-glucosidase inhibition and cytotoxicity activity against normal human fibroblasts ATCC PCS-201-010 and cancer cell lines (i.e. HepG2 cells ATCC 8065, Kidney KDN4, Cervical [CRV] and Lung [LNG] cancer).

2 Materials and Methods

2.1 Isolation and purification of Salisapilia species

Fallen yellow to brown senescent Avicennia sp. leaves were collected from the mangrove forest in Samal Island, Davao del Norte, Philippines (N7°10’20.658”, E125°41’32.41.68’). Following the methods of Pang et al. in 2015 and Say et al. in 2017, leaves were washed thrice with 50% sterile distilled-seawater, wiped dry, and the explan strips were placed on clarified V8 juice (Campbell’s, USA) saltwater agar (V8SA) plates for the isolation and purification of Salisapilia species. Clarified V8 juice solution was prepared by mixing 3 g CaCO₃ in 300 mL distilled water and then centrifuged for 10 min at 3000 rpm. The V8SA contains clarified V8 juice, 50% sterile seawater and 20 g/L agar supplemented with 500 mg/mL Nystatin and 30 mg/mL Streptomycin and Penicillin.

2.2 Growth curve determination of S. tartarea S1YP1

Agar blocks (0.5 cm³) from plates of grown S. tartarea S1YP1 were transferred into flasks with 20 mL clarified V8S broth medium and then incubated for 21 d and harvested every two days interval. Set-ups were prepared in three replicates. Mycelia were filtered using a pre-weighed Whatman filter paper. The excess mycelial broth was washed twice with a normal saline solution (NSS). The mycelia were oven dried at 50°C for 1-3 h, or until the liquid has evaporated. The weight of mycelia per flask was recorded. The growth curve, time (h) vs. mycelial biomass (mg) was constructed.

For the biomass production, agar blocks (30 pcs, 0.5 cm³) were transferred into 10 sterilized 500-mL glass bottles containing 300 mL clarified V8S broth medium incubated for 30 d.

2.3 Screening of the secondary metabolites in extracts of S. tartarea S1YP1

Agar blocks of grown S. tartarea S1YP1 were inoculated into 40 sterilized 125-mL Erlenmeyer flasks with 20 mL of clarified V8S broth medium and incubated for 90 d at 25–30°C, and then the secondary metabolites were extracted daily. The content of the flasks was filtered, separating the filtrate (spent broth media) and residue (mycelia). The filtrate was then extracted with ethyl acetate as well as the residue. The ethyl acetate extracts from the broth (BEAE) and mycelia (MEAE) were concentrated in vacuo. The extracts were screened for the presence of secondary metabolites by thin layer chromatography (TLC) with the solvent system composition of hexane:ethyl acetate:acetone:methanol:chloromethane at 6:33:22:6:33 for MEAE, and 8:33:33:8:17 BEAE. The TLC plates were dried and visualized under a viewing cabinet that incorporates short wave (254 nm) and long wave (365 nm) UV-emitting mercury lamps, prior to chemical visualization using different spray reagents specific for secondary metabolites.

2.4 Total phenolic content

The modified Folin-Ciocalteau procedure was used to measure the total phenolic content of the extracts. The gallic acid stock solution (1 mg/mL) and working concentrations of 0, 10, 25, 50, 100, 250 and 500 μg/mL were prepared in deionized water. Folin-Ciocalteau reagent and gallic acid were obtained from Sigma-Aldrich Co (St. Louis, Mo, USA). For the modified Folin-Ciocalteau procedure the following were added in sequence into a borosilicate tube: 50 μL standard or sample, 80 μL distilled H₂O and 20 μL Folin-Ciocalteau reagent. After mixing the samples, 50 μL of 20% Na₂CO₃ was added. The sample mixtures were allowed to stand for 1 h at room temperature and were mixed after every addition of reagents. The absorbance was measured at 725 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland). The phenolic content was measured and results were expressed as mg gallic acid equivalents (GAE) /gram sample. All experiments were done in eight replicates.

2.5 Total flavonoid content

The total flavonoid content of the extracts was determined using the aluminum chloride colorimetric method. Each extract was dissolved in 1 mL of dimethyl sulfoxide (DMSO). One hundred microliters (100 μL) of this solution was mixed with 100 μL of 2% AlCl₃ methanolic solution. After incubation at room temperature for 15 min, the absorbance was measured at 430 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland). Quercetin (Sigma-Aldrich, St. Louis, Mo, USA) was used to construct a standard calibration curve. The levels of total flavonoid contents in the sample extracts were calculated from this calibration curve. The results

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were expressed as mg quercetin equivalents (QE)/gram sample. All experiments were done in eight replicates.

2.6 Antioxidant activity assays

The free radical scavenging and antioxidant activities of the extract were determined by DPPH \(^{25}\), superoxide anion \(^{26}\), hydroxyl \(^{27}\), and ferrous ion chelating activity \(^{28}\). A decrease in absorbance in the presence of the extract indicates free radical scavenging activity. Experiments of the aforementioned assays were performed in nine replicates. The standard procedure for the percentage of free radical scavenging activities was calculated using the equation:

\[
\text{% scavenging activity} = \left(\frac{A_{\text{negative control}} - A_{\text{sample}}}{A_{\text{negative control}}} \right) \times 100
\]

The 50% inhibitory concentration (IC\(_{50}\)) of free radicals was calculated from the plot of percentage inhibition against concentration.

2.6.1 DPPH free radical scavenging assay

The free radical scavenging assay was done according to the method of Clarke \textit{et al.} in 2013 \(^{29}\). Briefly, 4 mg of DPPH was dissolved in 100 mL methanol and 25 μL of the extract was added to 180 μL of this DPPH solution. The standards (quercetin and butylated hydroxytoluene (BHT)) and the negative control (no extracts or standards) were also tested in the same manner. The mixture was shaken vigorously and was incubated for 30 min in a dark room at room temperature. The absorbance was measured at 517 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland) according to the standard assay procedure for free radical scavenging activity \(^{29}\).

2.6.2 Nitric oxide assay

In this assay, the nitric oxide radical generated from sodium nitroprusside was measured \(^{30}\). Briefly, the reaction mixture (5 mL) containing sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.3), with or without the extract at different concentrations was incubated at 25°C for 3 h. The nitric oxide radical thus generated interact with oxygen to produce the nitrite ion. This was assayed at 30 min interval by mixing 1.0 mL incubation mixture with an equal amount of Griess reagent (0.2% naphthylethylenediaminedihydrochloride and 2% sulfanilamide in 5% phosphoric acid). The absorbance of the chromophore (purple azo dye) formed during the diazotization of nitrite ions with sulfanilamide and subsequent coupling with naphthylethylendiaminedihydrochloride was measured at 546 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland).

2.6.3 Superoxide anion scavenging activity assay

The superoxide anion scavenging activity was assessed according to the method of Ani \textit{et al.} in 2006 \(^{31, 32}\). In this assay, superoxide anions were generated in the samples by adding 100 μL of 0.3 mM phenazine methosulfate (PMS) to the reaction mixture (consisting of 100 μL 1.0 mM nitro blue tetrazolium (NBT), 100 μL 3.0 mM nicotinamide adenine dinucleotide (NADH) with either extract or standard (quercetin or BHT). The final volume was adjusted to 1 mL with 0.1 M phosphate buffer (pH 7.8) and incubated at room temperature for 3 min. The absorbance at 560 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland) was measured against the blank samples following the standard assay procedure for the free radical scavenging activity. All reagents used in this assay were obtained from Sigma-Aldrich Co. (St. Louis, Mo, USA).

2.6.4 Ferrous ion chelating activity

The ferrous ion chelating activity was measured according to the method of Le \textit{et al.} in 2007 \(^{28}\). The reaction mixture contains 50μL of extract or standard (EDTA), 10 μL of FeCl\(_2\) (0.6 mM in water) and 90 μL of methanol. The negative control contains all the reagents except the extract or EDTA. The mixture was shaken well and incubated at room temperature for 5 min. Ten microliters (10 μL) of ferrozine (5 mM in methanol) were added and incubated at room temperature for 10 min. The absorbance was measured at 562 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland) using methanol as blank. The chelating activity was calculated using the aforementioned equation.

2.6.5 Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was measured using the deoxyribose method \(^{33}\). Hydroxyl radicals are generated by a Fenton reaction. In the method, 35 μL of the following were added in sequence for the reaction mixture: 2.8 mM 2-deoxy-2-ribose, 10 mM phosphate buffer (pH 7.4), 25 μM ferric chloride, 100 μM EDTA, 2.8 μM hydrogen peroxide, 100 μM ascorbic acid, and various concentrations (10-1000 μM/mL) of the extracts in a final volume of 1 mL. The mixture was incubated at 37°C for 1 h. Then 350 μL of 1% thiobarbituric acid (TBA) in 50 mM sodium hydroxide and 350 μL of 2.8% trichloro acetic acid (TCA) solution were added. The color of the mixture was developed by heating at 90°C for 20 min. The same method was done to the reaction mixture without the deoxyribose for the blank. After cooling, 200 μL of the mixture was transferred to 96-well microtiter plates and the absorbance was measured at 532 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland).

2.7 Antibacterial activity using Kirby-Bauer disk diffusion assay

The antibacterial activities of the extracts were determined using the Kirby-Bauer disk diffusion assay \(^{34, 35}\), against \textit{Staphylococcus aureus} (ATCC 29213), \textit{Bacillus cereus} (USTCMS 1009), \textit{Micrococcus luteus} (USTCMS 1060), \textit{Escherichia coli} (ATCC 25922), \textit{Klebsiella pneumonia} (USTCMS 1041), \textit{Proteus mirabilis} (USTCMS 1070), and \textit{Salmonella typhimurium} (ATCC 14028), all of which were obtained from the University of Santo Tomas.
2.8 α-glucosidase inhibition assay

Inhibitory α-glucosidase activities were determined spectrophotometrically (Thermo Scientific Multiscan 60) in a 96-well microtiter plate based on p-nitrophenyl-α-D-glucopyranoside (PNPG) as a substrate following the slightly modified method described in the studies of Krakenaite and Glemza in 1983[31], Cremonesi et al. in 2003[32] and Li et al. in 2007[33]. Briefly, 20 μL of enzyme solution (0.8 U/mL α-glucosidase in 0.01 M potassium phosphate buffer (pH 6.8) containing 0.2% bovine serum albumin (BSA) and 120 μL of the test compound or the extract in 0.5% DMSO were mixed, and pre-incubated at 37°C prior to initiation of the reaction. After 15 min of pre-incubation, p-nitrophenyl α-D-glucopyranoside (PNPG) solution (20 μL) [5.0 mM PNPG in 0.1 M potassium phosphate buffer (pH 6.8)] was added and incubated together at 37°C for 15 min. After incubation, 0.2 M Na2CO3 (80 μL) in 0.1 M potassium phosphate buffer was added to the wells to stop the reaction. The amount of p-nitrophenol (PNP) released was quantified at 405 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland). The % inhibition of α-glucosidase activity was calculated using the equation:

\[
\text{% inhibition of } \alpha-\text{glucosidase activity} = \left( \frac{A_{\text{negative control}} - A_{\text{sample}}}{A_{\text{negative control}}} \right) \times 100
\]

The inhibitory concentrations at 50% inhibition (IC50) values were calculated using Graph Pad Prism version 6 (Windows Vista).

2.9 Cell viability test: MTT assay

The toxicity of the extracts was determined using the MTT method according to Mosmann in 1983[34] following some modifications by Žegura et al. in 2007[35]. The HepG2 cells (ATCC 8065), Kidney (KDN4), Cervical (CRV) and Lung (LNG) cancer and fibroblast cells (ATCC PCS-201-010) provided by the Mammalian Tissue Culture Laboratory of University of Santo Tomas, were seeded onto 96-well microplates at a density of approximately 1.0 × 104 cells/mL and were then incubated for 24 h at 37°C with 5% CO2 to facilitate the attachment of the cells. After incubation, cells were treated with 100μL of the extracts at different concentrations (31.25, 62.5, 125, 250, and 500 μg/mL) and the standard doxorubicin at different concentrations (10, 5, 2.5, 1.25, 0.625 μg/mL). These were further incubated for 24 h at 37°C with 5% CO2. After incubation, the media was removed and 20μL of MTT at 5 mg/mL PBS was added. Cells were further incubated at 37°C with 5% CO2 for 4 h after which 100 μL DMSO was added to each well. Absorbance was read at 570 nm (Multiskan Go Microplate Spectrophotometer, Thermo Fisher Scientific, Finland). The assay was performed in triplicates. The % viability of the cells was calculated using the equation:

\[
\text{% cell inhibition} = \left( 100 - \frac{A_{\text{treated}}}{A_{\text{untreated}}} \right) \times 100
\]

The inhibitory concentrations at 50% inhibition (IC50) values were calculated using Graph Pad Prism version 6 (Windows Vista).

The selectivity index was also calculated to determine the specificity of the crude extracts and the standard using the equation[36]:

\[
\text{Selectivity Index (SI)} = \frac{\text{IC}_{50} \text{ Normal Cells}}{\text{IC}_{50} \text{ Cancer Cells}}
\]

2.10 Statistical analysis

Experimental results were expressed as a mean ± standard error (SEM). Independent t-test and one-way analysis of variance (ANOVA) were used to compare two or more groups of data. Post Hoc Analysis using Tukey HSD and LSD were used to find the significant differences between groups. p values of less than 0.05 were considered significant. SPSS software version 21 was used for statistical analysis.

3 Results

3.1 S. tartarea growth curve and secondary metabolites

Similar to Halophytophthora species, S. tartarea was cultivated on clarified V8-seawater agar plates following the methods of Say et al. in 2017[37]. Like other microorganisms, S. tartarea exhibit four succeeding (Lag, Exponential, Stationary, and Decline) phases. Growth curve of S. tartarea on V8S broth was simultaneously observed based on the weight of their biomass produced until the last day of incubation (day 28). Secondary metabolites were produced during the stationary (day 21, 504th hr) phase of its growth (data not shown).

Percolation and concentration in vacuo with ethyl acetate yielded 0.08% (w/w) for the mycelia extract (MEAE) and 25% (w/v) for the broth extract (BEAE). The extracts appeared as brownish-yellow syrupy consistency with an unpleasant odor. The weight of the mycelia before extraction was 15.81 g, whereas the volume of the broth was 1.25 L, and that of the media as the control was 300 mL. The weight of the concentrated extracts for BEAE, MEAE, and media were 311, 121, and 11 mg, respectively.

3.2 TLC analysis of secondary metabolites

The TLC detected triterpenes, antherquinone, anthrone, flavonoids, and phenols in BEAE and MEAE. In addition, coumarin was also observed in BEAE but not in MEAE. To determine whether the metabolites observed in the ethyl acetate extracts were attributed to the V8 media, a TLC was also done for the media (Fig. S1). The medium contains triterpenes, phenols, and flavonoids (Table 1).
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3.3 Total phenolic and total flavonoid contents

The total phenolic content (TPC) and the total flavonoid content (TFC) of BEAE (Table 2) were found to be 94.83 ± 23.05 mg gallic acid equivalents (GAE) and 56.27 ± 19.15 mg quercetin equivalents (QE), respectively per gram of crude extract. The TPC and TFC per gram of MEAE were calculated at 56.27 ± 19.15 mg gallic acid equivalents (GAE) and 56.23 ± 1.28 mg quercetin equivalents (QE), respectively. Both TPC and TFC contents were measured using a calibration curve for gallic acid and quercetin, respectively. The BEAE showed the higher content of phenolics and flavonoid than MEAE.

3.4 Antioxidant activity of S. tartarea S1YP1 extracts

The BEAE and MEAE were tested for antioxidant activities through DPPH·, NO, O2·−, and HO· radical scavenging and Fe²⁺ chelating assays. The IC₅₀ values were calculated using the plot of inhibition percentage against the concentration of extracts. The IC₅₀ was calculated by using a Graph Pad Prism version 6 (non-linear regression). Table 3 showed the calculated IC₅₀ of the standards, BEAE, and MEAE.

BEAE showed DPPH· free radical scavenging activity in a concentration-dependent manner with an IC₅₀ of 162.88 ± 5.21 μg/mL. MEAE showed no inhibition of this free radical. The DPPH free radical scavenging assay offers the first approach of evaluating the antioxidant potential of an extract. According to Prakash et al. in 2001, this assay measures the overall antioxidant capacity of a compound or an extract, thus only the BEAE was further tested for other antioxidant activities.

Nitric Oxide (NO) production was reduced by BEAE in a concentration-dependent manner, with an IC₅₀ of 384.16 ± 36.77 μg/mL. Nitric oxide is a reactive nitrogen species (RNS), which reacts with superoxide radical (O2·−) to form the peroxynitrite which is cytotoxic. The excessive production of nitric oxide may result in tissue damage and vascular collapse.

Table 1 | Classes of secondary metabolites detected from S. tartarea S1YP1 BEAE, MEAE, and V8 media by TLC viewed under UV at 365 and 245 nm.
| Chemical constituent | BEAE | MEAE | V8 media |
|----------------------|-------|-------|----------|
| Alkaloids            | −     | −     | −        |
| Steroids             | −     | −     | −        |
| Anthraquinones       | +     | +     | −        |
| Flavonoids           | +     | +     | +        |
| Phenols              | +     | +     | +        |
| Triterpenes          | +     | +     | −        |
| Anthrones            | −     | −     | −        |
| Cardenolides         | −     | −     | −        |
| Coumarins            | −     | −     | −        |
| Indoles              | −     | −     | −        |

Table 2 | The total phenolic content (TPC) and the total flavonoid content (TFC) of S. tartarea S1YP1 BEAE and MEAE expressed as mg gallic acid equivalent (GAE) and mg quercetin equivalent (QE) per gram of extract respectively. Values are mean ± SD.

| Extract | TFC (mg QE/ g extract) | TPC (mg GAE/ g extract) |
|---------|------------------------|-------------------------|
| BEAE    | 69.57 ± 6.83           | 94.83 ± 23.05           |
| MEAE    | 56.23 ± 1.28           | 56.27 ± 19.15           |

Table 3 | A comparison of the antioxidant activities of the standards and S. tartarea S1YP1 BEAE and MEAE. Values were expressed as IC₅₀.

| Radicals | Ascorbic Acid | BHT | Quercetin | EDTA | BEAE | MEAE |
|----------|---------------|-----|-----------|------|------|------|
| DPPH     | 3.76 ± 0.35   | 154.18 ± 5.54 | 13.92 ± 0.33 | NT   | 162.88 ± 5.21b,c,d | NI   |
| NO       | 30.25 ± 1.73  | 45.57 ± 8.17  | 77.19 ± 13.52 | NT   | 384.16 ± 36.77b,c,e | NT   |
| O₂       | NT            | 237.75 ± 27.89 | 60.23 ± 32.79 | NT   | 391.06 ± 5.09b,c,e | NT   |
| OH       | NT            | 11.19 ± 1.74  | NT         | NT   | 78.37 ± 1.91b   | NT   |
| Fe       | NT            | NT            | 31.00 ± 0.84 | 541.32 ± 5.43b,d | NT   |

NI= No inhibition (IC₅₀ > 2000 μg/mL); NT = Not tested
a = significantly different to ascorbic acid; b = significantly different to BHT; c = significantly different to Quercetin; d = significantly different to EDTA; (where p < 0.05)
The superoxide ($O_2^\cdot -$) derived from dissolved oxygen by PMS-NADH detected in the coupling reaction reduces nitrobluetetrazolium. The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The BEAE exhibited superoxide radical scavenging activity with an IC$_{50}$ of 391.06 ± 5.09 μg/mL, and further inhibited HO• production in a concentration-dependent manner with an IC$_{50}$ of 78.37 ± 1.91 μg/mL.

3.5 Antibacterial activity of S. tartarea S1YP1 Extracts

The BEAE and MEAE were screened for antibacterial activity. However, both extracts at 20 μg/disc, did not inhibit any of the test bacteria.

3.6 α-glucosidase inhibition of the crude extracts of S. tartarea S1YP1

Both BEAE and MEAE were evaluated for their α-glucosidase inhibitory activity (Fig. 1) but only BEAE showed α-glucosidase inhibition with an IC$_{50}$ of 9.004 ± 0.529 μg/mL. The significant α-glucosidase inhibitory activity shown by BEAE may have implications of potential utility in controlling blood sugar levels.

3.7 Cytotoxicity of the S. tartarea S1YP1 Extracts

Both the BEAE and MEAE were tested for inhibitory activity on the growth of four cancer cell lines and normal fibroblasts. The MEAE exhibited inhibitory activities of Kidney Cancer Cells with an IC$_{50}$ of 773.2 μg/mL, Cervical Cancer Cells with an IC$_{50}$ of 56.55 μg/mL and HepG2 with an IC$_{50}$ of 185.6 μg/mL. The MEAE did not show inhibition of lung cancer cells. The BEAE exhibited stronger inhibitory activity against Kidney Cancer Cells with an IC$_{50}$ of 32.49 μg/mL, Cervical Cancer Cells with IC$_{50}$ of 254.9 μg/mL, Lung Cancer Cells with IC$_{50}$ of 59.20 μg/mL and HepG2 with IC$_{50}$ of 19.34 μg/mL (Table 4). Figure 2 shows the...
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The altered morphology of HepG2 cells treated with 31.25 μg/mL of MEAE and BEAE compared to the untreated cells and those treated with DOX (0.625 μg/mL). The altered morphology of HepG2 cells by BEAE is evidence of the cytotoxic effects of BEAE on HepG2 and explains the high and selective inhibitory effects of BEAE against HepG2 cells. The BEAE, however, exhibits low cytotoxic effects on normal human dermal fibroblasts with an IC50 of 1390.06 μg/mL which was weaker than the effects of MEAE on human normal dermal fibroblast with an IC50 of 414.33 μg/mL. Figure 3 shows the comparison of the morphology of untreated human dermal fibroblast, treated human dermal fibroblast with Doxorubicin HCl (DOX), treated human dermal fibroblast with BEAE and treated human dermal fibroblast with MEAE. The integrity of the fibroblasts’ cell membrane was still distinct after treatment with BEAE while a rupture was observed in cells treated with MEAE. This indicates that BEAE is less toxic than MEAE.

### Table 4

| Cell Line     | IC50 (μg/mL) | Selectivity Index (SI) |
|---------------|--------------|------------------------|
|               | BEAE         | MEAE                   | DOX        | BEAE         | MEAE       | DOX        |
| FibroblastsN  | 1390.06      | 414.33                 | 2.63       |
| KidneyC       | 32.49        | 773.2                  | 0.2760     | 42.78        | 0.54       | 9.53       |
| CervicalC     | 254.9        | 56.55                  | 2.59       | 5.45         | 7.33       | 1.02       |
| LungsC        | 59.20        | NI                     | 9.09       | 23.48        | NI         | 0.29       |
| HepG2C        | 19.31        | 185.6                  | 0.65       | 71.99        | 2.23       | 4.05       |

NI = No inhibition (IC50 >2000 μg/mL); N Normal cell; C Cancer cell; C+ Crude extracts; C++ Standard

4 Discussion

Most of the marine-derived microorganisms produce their secondary metabolites at 21 days of incubation which often vary from organism to organism. This was observed similarly for S. tartarea S1YP1 whose secondary metabolites were detected only after its 21-day incubation on V8S broth medium. Secondary metabolites are understood to be compounds which are not directly involved in the normal growth, development, or reproduction of an organism. Several reports have mentioned that production of secondary metabolites is influenced by fermentation parameters (i.e. culture medium, available nutrients, pH, temperature, salinity, and glucose concentrations). The synthesis of secondary metabolites begins during the sta...
tionary phase when growth slows down; which in the case of _S. tartarea_ S1YP1 reached between 21 to 28 days. Interestingly, the culture medium used may have an appreciable influence in the growth of _Salisapilia_. Profiles of their secondary metabolites may be different when the organism was grown on another medium (e.g., peptone-yeast extract-glucose in 50% salt water for fatty acid production of _Halophytophthora_ spp.)\(^{15, 16}\). The proposed one strain many compounds (OSMAC approach)\(^{17}\) may have occurred for _S. tartarea_ using the V8S broth medium but could produce different secondary compounds when cultured in PYGS. According to Bode _et al._\(^{45}\), a single organism has the ability to increase secondary metabolites when there is an alteration of cultivation parameters (i.e., media composition, aeration, and others). The V8 broth used in this study was a combination of several vegetable extracts with tomato as the main ingredient. This medium has a high pH level and often neutralized during media preparations. The V8 juice agar medium is mostly used for the efficient induction of secondary metabolites may be different when the organism at the stationary phase when growth slows down; which in the case of _S. tartarea_ S1YP1 reached between 21 to 28 days. Interestingly, the culture medium used may have an appreciable influence in the growth of _Salisapilia_. Profiles of their secondary metabolites may be different when the organism was grown on another medium (e.g., peptone-yeast extract-glucose in 50% salt water for fatty acid production of _Halophytophthora_ spp.)\(^{15, 16}\). The proposed one strain many compounds (OSMAC approach)\(^{17}\) may have occurred for _S. tartarea_ using the V8S broth medium but could produce different secondary compounds when cultured in PYGS. According to Bode _et al._\(^{45}\), a single organism has the ability to increase secondary metabolites when there is an alteration of cultivation parameters (i.e., media composition, aeration, and others). The V8 broth used in this study was a combination of several vegetable extracts with tomato as the main ingredient. This medium has a high pH level and often neutralized during media preparations. The V8 juice agar medium is mostly used for the efficient induction of secondary metabolites.

The various antioxidant activities exhibited by the extracts of _S. tartarea_ S1YP1 may be due in part to the presence of secondary metabolites. The phenolic and flavonoid contents in BEAE are higher than MEAE which explain the varied antioxidant activities in BEAE which are possibly extruded out of the mycelia.

The ability of BEAE to scavenge DPPH free radicals indicate the potential use of the secondary metabolites of _S. tartarea_ S1YP1 as protective agents against cellular damage. Free radicals damage cell structures through reaction with nucleic acids, lipids, and proteins. The extracts of _S. tartarea_ S1YP1, through its free radical scavenging activity, can preserve the integrity of the membrane and aids in the proper functioning of cells. The cell membrane is composed of a lipid bilayer which is very susceptible to oxidative damage. Free radical-mediated DNA damage has been found in various cancer tissues. Thus, it has been hypothesized that the free radical scavengers could prevent or limit the damage by free radicals\(^{26, 49}\). The prevention of DNA damage by antioxidants may also prevent cancer and tumor formation. Similarly, the binding of free radicals to proteins alters the conformation and function of proteins. The free radical scavengers thus play an important role in maintaining the integrity and functions of biomolecules of cells.

The BEAE exhibited a reduction in the nitric oxide (NO) radical which is another important chemical mediator generated by endothelial cells, macrophages, and neurons. NO can also produce Reactive Nitrogen Species (RNS) which are toxic and is involved in the regulation of various physiological processes\(^ {50}\). Excess concentration of NO is associated with several diseases.

The BEAE also exhibited superoxide radical scavenging activity. This superoxide radical is toxic as it is deployed by the immune system to kill invading microorganisms. In phagocytes, superoxide radical is produced in large quantities by the enzyme NADPH oxidase for use in oxygen-dependent killing mechanisms of invading pathogens. The superoxide radical is also deleterious when produced as a by-product of mitochondrial respiration (most notably by Complex I and Complex III), as well as of several other enzymes, for example, xanthine oxidase\(^ {51}\).

The BEAE also exhibited significant inhibition of hydroxyl radicals (\(\text{HO}^+\)). The hydroxyl radicals can occasionally be produced as a by-product of the immune activation. Macrophages and microglia most frequently generate this compound when exposed to very specific pathogens, such as certain bacteria. The hydroxyl radical can damage the macromolecules such as carbohydrates, modify the nucleic acid sequence, lipid peroxidation, and amino acids\(^ {52-54}\).

Although BEAE is not as good as EDTA as an iron-chelating agent, the dose-dependent reduction in the concentration of the metal may still be an indication of the utility of the BEAE in anti-oxidation mechanisms. The transition metal ions, especially iron, can induce lipid-peroxidation by the Fenton reaction (\(\text{H}_2\text{O}_2 + \text{Fe}^{2+} \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot\)) and can also accelerate lipids peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can perpetuate the chain reaction. Metal ion chelating capacity is significant since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. One of the most important mechanisms of action of secondary antioxidants is the chelation of prooxidant metals. Iron and other transition metals (copper, chromium, cobalt, vanadium, cadmium, arsenic, and nickel) promote oxidation by acting as catalysts of free radical reactions\(^ {55}\).

Neither BEAE nor MEAE showed antibacterial activity against any of the bacterial isolates. Although TLC indicates the presence of the general classes of triterpenes, anthraquinone, anthrone, flavonoids and phenols; however, none of these compounds has antibacterial activity. Since _Salisapilia_ species play an important role in the mangrove ecosystem, it can be inferred that these microorganisms do not produce any antimicrobial compounds as it lives symbiotically with diverse microorganisms in the mangrove ecosystem.

The \(\alpha\)-glucosidase inhibitory activity exhibited by BEAE indicate its potential use in controlling blood sugar levels in _Diabetes mellitus_. The \(\alpha\)-glucosidase is a carbohydrate-hydrolase enzyme located in the brush border of the small intestine which acts on \(\alpha, 1-4\)-bonds and which releases glucose to be absorbed and circulated into the bloodstream. Inhibitors of \(\alpha\)-glucosidase reduce the amount of glucose being absorbed into the blood preventing the rise
in blood glucose (hyperglycemia), especially after a meal. Hyperglycemia has been a classical risk factor in the development of diabetes. The significant \( \alpha \)-glucosidase inhibitory activity shown by BEAE indicates its potential as an anti-hyperglycemic agent. This inhibitory effect may be attributed to any of the secondary metabolites anthraquinone, anthrone, or triterpene present in the extract. The identity of the compound to which \( \alpha \)-glucosidase inhibitory activity is attributed to may be pursued for further characterization of the nature of the \( \alpha \)-glucosidase inhibitory activity.

Based on the criteria set by the American National Cancer Institute, an extract showing an IC\(_{50}\) < 20 \( \mu \)g/mL is considered a potent cytotoxic agent\(^{(27)}\). To be useful as an anticancer agent its selectivity index (SI = IC\(_{50}\) normal cells/IC\(_{50}\) cancer cells) should be greater than the SI of Doxorubicin\(^{(26)}\). The BEAE preferentially inhibited HepG2 cells and is considered potent (with an IC\(_{50}\) of 19.31 \( \mu \)g/mL). Its SI for HepG2 (71.99) is greater than the SI of Doxorubicin for the HepG2 (4.05). The cytotoxic and potential anticancer activity demonstrated by BEAE may be attributed to anthrone and anthraquinone\(^{(29)}\). The identification of the active compound as an anticancer agent would be an essential next step for future development in pharmaceutics.

**Conclusion**

This preliminary study on *Salisapilia* S1YP1 is the first report on the *Salisapilia* species screening of secondary metabolites and biological activities. Results suggest that BEAE exerts antioxidant activities by scavenging DPPH\(^{\cdot}\), NO, O\(_2\)\(^{\cdot}\), and HO\(^{\cdot}\) and chelating Fe\(^{2+}\) metal ion in *vitro* while BEAE inhibits \( \alpha \)-glucosidase activity *in vitro*. This, however, represents only a fraction of a deeper research on the chemical and biological profile that can be explored from these species. The activities of BEAE are not better compared to the standards used. It can be attributed to the fact that BEAE is not yet a pure compound but rather a crude extract that still contains various chemical compounds and impurities. The *Salisapilia* species as a source of chemical agents for cancer treatment are yet unexplored. BEAE has very low toxicity on normal human fibroblasts but toxic and selective to HepG2 cells. Thus, BEAE has a possible chemo-preventive property and may be a potential anti-hyperglycemic agent.

In this respect, future directions for research may rely on the ability to isolate and propagate species for the isolation of bioactive compounds and to further explore the estuarine environment for new species of this group of organisms. The unique nutrient requirement and bioactive potential of *S. tartarea* serve as an interesting basis for other related species to be explored for its pharmaceutical importance.

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**Supporting Information**

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