BIOACTIVITY STUDIES ON CRUDE EXTRACTS OF FUNGI ISOLATED FROM *Ceriops tagal* AND SURROUNDING SOIL FROM SRI LANKAN MANGROVE ENVIRONMENT

W.C.C.N. Fernando[1], C. Ranasinghe[1*], M.M. Qader[2]

[1]Department of Chemistry, The Open University of Sri Lanka
[2] Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, USA

INTRODUCTION

The mangrove ecosystem is a unique environment which houses a large number of microbes that can tolerate harsh conditions such as high salinity, low oxygen and high temperature. Microbes found in the mangrove environment are considered as under-explored rich sources of secondary metabolites with antitumor, antibacterial, antifungal, antiviral, anticancer, antioxidant and enzyme inhibitory activities (Kohlmeyer and Kohlmeyer, 1979). This study is geared towards a preliminary investigation of the biological activities of crude extracts of three endophytic fungi from the leaves of a mangrove plant and a soil inhabiting fungus from the mangrove environment. Antioxidant activity was determined using DPPH radical scavenging assay (Kuganesan et al., 2017), while cytotoxicity was assessed by brine shrimp lethality assay (Krishnaraju et al., 2005). Some major compounds present in the biologically active fungal extracts were purified and identified by HPLC and spectroscopic methods.

MATERIALS AND METHODS

Isolation and cultivation of fungi

Healthy and disease-free leaves of the mangrove plant *Ceriops tagal* and its submerged soil were collected from Hikkaduwa (GPS: 6′ 00′ 55.0″ N 80′ 19′ 46.0″ E) in November 2019. Plant leaves were triple sterilized (Qader et al., 2017) and segments of the leaves were placed on sea water incorporated Potato Dextrose Agar (PDA) media in petri dishes and were incubated at room temperature. After 5 days, emerging fungi were serially transferred into fresh PDA media until pure cultures were isolated.

To isolate soil fungi, serial dilution method (Hasma et al., 2019) was followed. Diluted soil suspensions were spread on sea water incorporated PDA media in petri dishes and incubated at room temperature. Three endophytic fungi were isolated from the mangrove plant leaves and one fungus was isolated from mangrove soil.

Fungi emerged were sub-cultured repeatedly until pure fungi were isolated. The isolated fungi were morphologically identified. They were grown on a large scale, in sea water incorporated rice media (100 mL) in 375 mL flat bottles (x 12) and were subjected to a static fermentation for 30 days.

Extraction of fungal metabolites from fungal cultures

After the completion of the incubation period of 30 days, ethyl acetate (EtOAc) (100 mL) was added to each bottle and left for about 24 hours. The liquid was filtered off and the filtrate was evaporated to dryness under vacuum using a rotary evaporator. The crude extracts were re-dissolved in methanol (MeOH) and partitioned with *n*-hexane. Finally, both *n*-hexane and MeOH fractions were evaporated to dryness and stored at 4 °C until further use.

Preliminary screening for phytochemicals in fungal extracts

Preliminary screening tests for alkaloids (Mayer's test), phenolics (neutral ferric chloride test), steroids (Liebermann Burchard test) were carried out to identify major classes of compounds in

EtOAc extracts (Devi et al., 2012). Thin layer chromatographic (TLC) analysis was carried on precoated silica gel plates using methanol: dichloromethane (5:95) as the eluent and visualised under UV (254 and 365 nm) and by using anisaldehyde as the spray reagent.

Bioactivity studies on fungal extracts

DPPH (2,2–diphenyl−1−picrylhydrazyl) radical scavenging activity: Fungal extracts of different concentrations (500, 250, 125, 62.5, 31.25 ppm) were prepared in MeOH. DPPH reagent was prepared in MeOH (5 mg/100 mL, 2.0 mL). Aliquots of fungal extracts were mixed with an equal volume of DPPH in MeOH solution (1.5 mL). The blank for the test solution was prepared by replacing DPPH with 1.5 mL of methanol. A control was prepared by mixing 1.5 mL of DPPH with 1.5 mL of MeOH. All the samples were incubated in the dark for 10 minutes at room temperature and absorbance was measured at 517 nm wavelength. Ascorbic acid was used as the positive control. All experiments were carried out in triplicate and the mean of the readings were recorded. The percentage antioxidant activity (AOA%) was calculated as follows.

$$\% \text{ AOA} = \frac{A_C - A_S}{A_C} \text{ x } 100$$

A_C = Absorbance of the control
A_S = Absorbance of the sample

The IC_{50} values of the extracts were determined using the regression equation of the relevant AOA% vs. concentration graphs.

Brine shrimp lethality assay: Eggs of brine shrimps (*Artemia salina*) were hatched in a beaker containing artificial seawater under constant aeration and light for 48 hours. Ten active nauplii (larvae) were drawn through a pasture pipette and were exposed to different concentrations of fungal extracts (2 mL) in well plates. Wells were maintained under constant aeration and light for about 24 hours and the percentage mortality was calculated. The experiment was carried out in triplicate in a concentration series [samples were dissolved in 1.5% dimethyl sulfoxide (DMSO) and artificial seawater] and 1.5% DMSO and artificial seawater were used as the positive control.

$$\% \text{ mortality} = \frac{\text{No. of deaths of brine shrimps}}{\text{Total no. of brine shrimps}} \text{ x } 100$$

Identification of constituents of biologically active fungal extracts

Crude EtOAc extract of MP-G2 was subjected to High Pressure Liquid Chromatography (HPLC) using solvent systems consisting of acetonitrile (ACN) and water (with 0.001% HCO_2H), to isolate the major constituents in the extract. Spectroscopic analysis (^{1}HNMR and HSQC) of the isolates followed by 2D NMR dereplication tools, compounds were identified and further confirmed with the published literature data (Reher et al. 2020).

RESULTS AND DISCUSSION

Pure fungal cultures were identified by their colony characteristics and external morphology. The two endophytic fungi, MP-G1 and MP-G2 were identified as fungi belonging to the *Penicillium* genera due to the presence of branched spore producing conidiophores during maturation. MS-G3 produced solid, horn-like ascocarp fruiting bodies during maturation which is characteristic for *Xylaria*. A proper identification could not be done for MP-B1and it remains as an unidentified species.

Preliminary phytochemical screening of EtOAc extracts of MP-G1, MP-G2, MS-G3 and MP-B1 indicated the presence of phenolics by giving positive observation (dark green colouration) for the ferric chloride test. They all gave blue-green ring formation for the Liebermann Burchard test indicating the presence of steroids in them. This observation was further proved by the TLC analysis of the crude extracts. Dark blue-purple non-polar spots were observed while heating with anisaldehyde spray reagent. All four EtOAc extracts were shown to be complex mixtures of compounds on TLC ranging from low polar to mid polar metabolites. Except for MS-G3 and MP-B1, other two extracts indicated the presence of higher content of low polar compounds than high polar compounds.

DPPH assay is a simple and accurate way of measuring antioxidant activity. Antioxidant activity is proportional to the disappearance of the deep purple colour of the DPPH radical. The colour change was measured at 517 nm which is the UV absorption maxima of DPPH radical. Antioxidant activity of the MeOH fractions of the four fungi, for DPPH radical scavenging assay is shown in **Figure 1** as IC_{50} values (concentration that causes a decrease in the initial DPPH absorption by 50%), compared with ascorbic acid. A graph of concentration of the extract vs. % inhibition was plotted, and the linear regression curve was established to calculate the IC_{50} values.

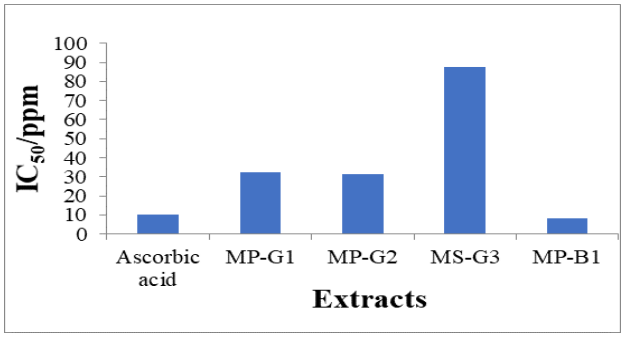

Figure 1: IC_{50} values of the MeOH fractions of the four fungi

The lower the IC_{50} value, the higher the antioxidant activity. The highest antioxidant activity was shown by MP-B1

Brine shrimp lethality assay was carried out using 24 hours old nauplii in II/III instar stage to assess their sensitivity towards the MeOH fractions of the fungal extracts. It is reported in previous studies that there is a correlation between the brine shrimp lethality assay and cytotoxicity towards antitumor activity and pesticidal activity. Therefore, this rapid, simple and inexpensive assay can be used as a prescreening test to identify anticancer, antitumor or pesticidal agents (Carballo et al., 2002).

Our results indicated that methanolic fractions of all four fungi showed cytotoxic activity against brine shrimps. Among them MP-G1 and MP-G2 showed the highest cytotoxicity with respect to the different sample concentrations used in the assay. Results obtained for the brine shrimp lethality assay are shown in **Figure 2**.

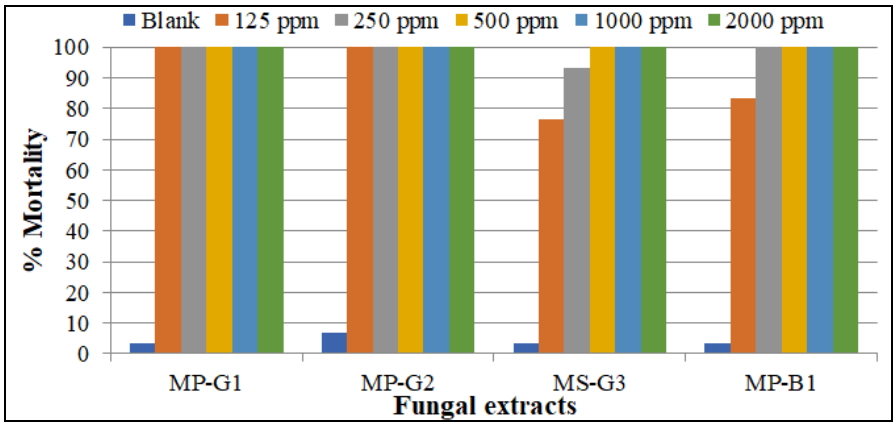

Figure 2: Percentage mortality shown by fungal extracts at different concentrations

NMR based-dereplication studies on the experimental NMR spectral data obtained for three pure compounds separated out from the EtOAc extract of MP-G2 identified as the three major fungal metabolites namely, emodin (50% ACN : water, retention time 8.9), terrain (55% ACN : water, retention time 13.2) and ergosterol (85% ACN : water, retention time 18.3). Terrein and emodin are reported to have anticancer, antitumor and cytotoxic activities. Terrein is also reported to

have antioxidant and anti-inflammatory activities (Zaehle et al., 2014). Ergosterol is the most abundant sterol found in fungal membranes which regulates the fluidity and permeability of cell membranes.

CONCLUSIONS

In the preliminary examination, the MeOH fractions of the crude EtOAC extracts of mangrove-associated endophytic fungi (two *Penicillium* species: MP-G1, MP-G2, one unidentified: MP-B1) and the soil fungus (*Xylaria* species., MS-G3) indicated the presence of phenolics, and steroids.

Biological activities of the MeOH fractions of fungal extracts assessed by DPPH radical scavenging assay and the brine shrimp lethality assay exhibited antioxidant properties and cytotoxic activity in them.

Prompted by these biological activities, an investigation on the chemical composition of the active fractions was carried out. As a result, three major fungal metabolites terrine, emodin and ergosterol were isolated and identified from the EtOAc extract of the MP-G2 fungus. The components in other extracts are yet to be identified. They could be potential sources of chemically interesting and biologically important compounds in drug discovery.

REFERENCES

Carballo, J. L., Hernández-Inda, Z. L., Pérez, P., García-Grávalos, M. D., (2002). A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. *BMC biotechnology*, 2(1), 1-5.

Devi, N. N., Prabakaran, J. J., and Wahab, F. (2012). Phytochemical analysis and enzyme analysis of endophytic fungi from *Centella asiatica. Asian Pacific Journal of Tropical Biomedicine, 2(3), S1280-S1284.*

Hasma, G. R., Poornima, M., Jeevan, P., (2019). Bioactivity of marine fungal species from seashore soil sample. *International Journal of Advanced research*, 7(6), 54-62.

Kohlmeyer, J. and Kohlmeyer, E., (1979). *Marine Mycology: The Higher Fungi*. London: Academic Press.

Krishnaraju, A.V., Rao, T.V.N., Sundararaju, D., Vanisree, M., Tsay, H.S., Subbaraju, G.V., (2005). Assessment of bioactivity of Indian medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *Journal of Applied Science and Engineering*, 3, 125−134.

Kuganesan, A., Thiripuranathar, G., Navaratne, A. N., Paranagama, P. A., (2017). Antioxidant and anti-inflammatory activities of peels, pulps and seed kernels of three common mango (*Mangifera indica* L.) varieties in Sri Lanka. *International Journal of Pharmaceutical Sciences and Research*, 8(1), 70-78.

Qader, M. M., Kumar, N. S., Jayasinghe, L., Araya, H., Fujimoto, Y. (2017). Bioactive sesquiterpenes from an endophytic fungus *Bipolaris sorokiniana* isolated from a popular medicinal plant *Costus speciosus*. *Mycology*, 8(1), 17-20.

Reher, R., Kim, H.W., Zhang, C., Mao, H.H., Wang, M., Nothias, L.F., Caraballo-Rodriguez, A.M., Glukhov, E., Teke, B., Leao, T., Alexander, K.L., Duggan, B.M., Van Everbroeck, E.L., Dorrestein, P.C., Cottrel, G.W., Gerwick, W.H. 2020. *Journal of the American Chemical Society*, *142*(9), 4114-4120

Zaehle, C., Gressler, M., Shelest, E., Geib, E., Hertweck, C., Brock, M., (2014). Terrein biosynthesis in *Aspergillus terreus* and its impact on phytotoxicity. *Chemistry & biology*, 21(6), 719-731.