International Journal of Research Studies in Biosciences (IJRSB)
Volume 3, Issue 1, January 2015, PP 191-197
ISSN 2349-0357 (Print) & ISSN 2349-0365 (Online)
www.arcjournals.org

Phytochemical Screening, Antimicrobial and Antioxidant Activities of Selected Fungi from Mount Singai, Sarawak, Malaysia

G. M. Liew[1]*, H. Y. Khong[1], C. J. Kutoi[1]

[1]Faculty of Applied Sciences
Universiti Teknologi MARA, Kota Samarahan, Sarawak, Malaysia
**liewgm@sarawak.uitm.edu.my*

A. K. Sayok[2]

[2]Institute of Biodiversity and Environmental Conservation (IBEC),
Universiti Malaysia Sarawak
Kota Samarahan, Sarawak, Malaysia

Abstract: *Fungi play important roles in the forest ecosystem as the main decomposers, symbionts in mutualistic association with other organisms, and as parasites. Some fungi are consumed as food and some like Ganoderma are well known for its medicinal values, thus, these treasures are still waiting to be uncovered. The study area, Mount Singai in Bau District, Sarawak, Malaysia, was settled by Bisingai Bidayuh tribe for almost 300 years before they moved downhill to 14 villages some 40 years ago. A recent survey on fungi in the area discovered more than 50 species. Seven species were selected, namely Amauroderma rugosum, Earliella scabrosa, Fomitopsis dochmia, Ganoderma australe, Lentinus sajor-caju, Microporus xanthopus, and Trametes pubescens for assay of their antimicrobial activities against Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginos, Escherichia coli and Clostridium difficile using Minimum Inhibitory Concentration method. All fungi demonstrated strong inhibition towards the five bacteria while F. dochmia, however, only exhibited strong inhibition against S. pyogenes. Phytochemical screening showed that among all the fungi, only G. australe contains alkaloids as supported by its high activity in 1,2-diphenyl-2-pricrylhydrazyl radical scavenging. All fungi indicated absence of triterpene and steroid content with L. sajor-caju recorded the highest presence of saponins while A. rugosum and M. xanthopus showing the least. These findings showed that the selected fungi from Mount Singai have great potential in the development of pharmaceutical and dermatological products and thus warrant further investigation.*

Keywords: *Fungi, phytochemical screening, antimicrobial, antioxidant, Mount Singai, Sarawak, Malaysia*

1. INTRODUCTION

Fungi are microorganisms found in various forms such as yeasts and molds, as well as the more familiar mushrooms. Abundant worldwide, most fungi are inconspicuous because of the small size of their structures, and cryptic lifestyles in soil, on dead matter, and as symbionts with plants, animals or other fungi. They may become noticeable when fruiting, either as mushrooms or molds.

Being part of the forest flora, they play important roles in the forest ecosystem. Some fungi have mutualistic association with trees in the form of mycorrhizae, whereas some are parasites. Their most important role in the ecosystem is in the decomposition of organic compounds returning important nutrients to the soil and environment benefiting plants that get their sustenance from the soil's nutrients.

Many fungi are also being used by human for various purposes. They have long been used as a direct source of food such as mushrooms and truffles, as a leavening agent for bread, and in

fermentation of various food products such as wine, beer, and soy sauce to name a few. Known primarily as a culinary delight, the shiitake mushroom is also believed to help boost the immune system and restore balanced cholesterol levels. The maitake (*Grifola frondosus*) is traditionally used in Asia as an energy tonic to increase vitality. Several species of *Ganoderma* such as *G. lucidum* contain many bioactive compounds (~400) such as triterpenoids and polysaccharides. Reishi (*G. lucidum*) has been used for centuries as a vital part of traditional Chinese medicine and has been clinically shown to balance energy and glucose levels, support cardiovascular health, enhance the immune system and assist with liver detoxification. *Cordyceps sinensis*, a powerful antioxidant, has been used for centuries as a general tonic for promoting longevity, vitality, and endurance. *Agaricus blazei* are medicinal mushrooms comprised of powerful nutrients called beta-glucans and other polysaccharides that have been clinically shown to enhance the immune system. Since the 1940s, fungi have been used for the production of antibiotics. The discovery of penicillin from the fungus *Penicillium chrysogenum* was first used successfully to treat an infection caused by a bacterium in 1941. In doing so, it revolutionized treatment of disease. Many formally fatal diseases caused by bacteria became treatable, and new forms of medical intervention were possible.

A survey on fungi undertaken in late 2010 and early 2011 as part of the multidisciplinary study of Mount Singai in Bau District, Sarawak, Malaysia discovered more than 50 species of fungi. Among them, several species have some importance related to medicines and drugs. In late 2012, fungal specimens were collected and seven species were selected for phytochemical screening and assay of their antimicrobial and antioxidant activities. Reported here are the results of the preliminary studies which may have great potential and interest in the development of pharmaceutical and dermatological products.

2. MATERIALS AND METHOD

2.1. Study Area

The 550-meter Mount Singai (1^{o}29’48.86”N, 110^{o} 9’48.21”E) is a solitary mountain located in the northwestern side of Bau District, Sarawak, Malaysia. It is mainly composed of sandstone with thin soil layer and steep rocky outcrop at the northwestern aspect while the more gentle sides with thicker soil layer harbor typical mixed dipterocarps species of tropical rainforest. The south and southwestern part of the mountain area was settled by Bisingai Bidayuh, native tribe, in eight longhouses for almost 300 years before they moved downhill to 14 villages some 40 years ago. Having been abandoned, the village sites are quasi pristine albeit showing a mosaic vegetation pattern of different composition and successional stages resulting from the settlers’ occupation and activities therein especially subsistence farming of hill rice. Introduction of cash crops of rubber and fruit trees in the area had somewhat changed the vegetation composition to a more fruit tree-dominant and/or rubber tree-dominant in some sites at the foothills and nearby the old village sites. The Catholic Memorial Pilgrimage Centre established in the late 1990s at the old village site is the main conspicuous structure. This center and its proximity to the state capital, Kuching had consistently received droves of visitors, pilgrims and visitors alike, to the area.

2.2. Testing Materials

From a survey on fungi undertaken along the main trail from the hill bottom to the mountain top in late 2010 and early 2011 as part of the multidisciplinary study, more than 50 species of fungi were discovered. Additional fungal specimens were collected in late 2012 and seven species, namely *Amauroderma rugosum*, *Earliella scabrosa*, *Fomitopsis dochmia*, *Ganoderma australe*, *Lentinus sajor-caju*, *Microporus xanthopus*, and *Trametes pubescens* (Figure 1) were selected for the studies. These fungal materials were air-dried for 14 days and ground with a commercial grinder. The resultant powdered fungi were extracted with ethanol for 24 hours at room temperature. The solvent was removed by filtration and evaporated using rotavapors. The extracts were stored at 4˚C.

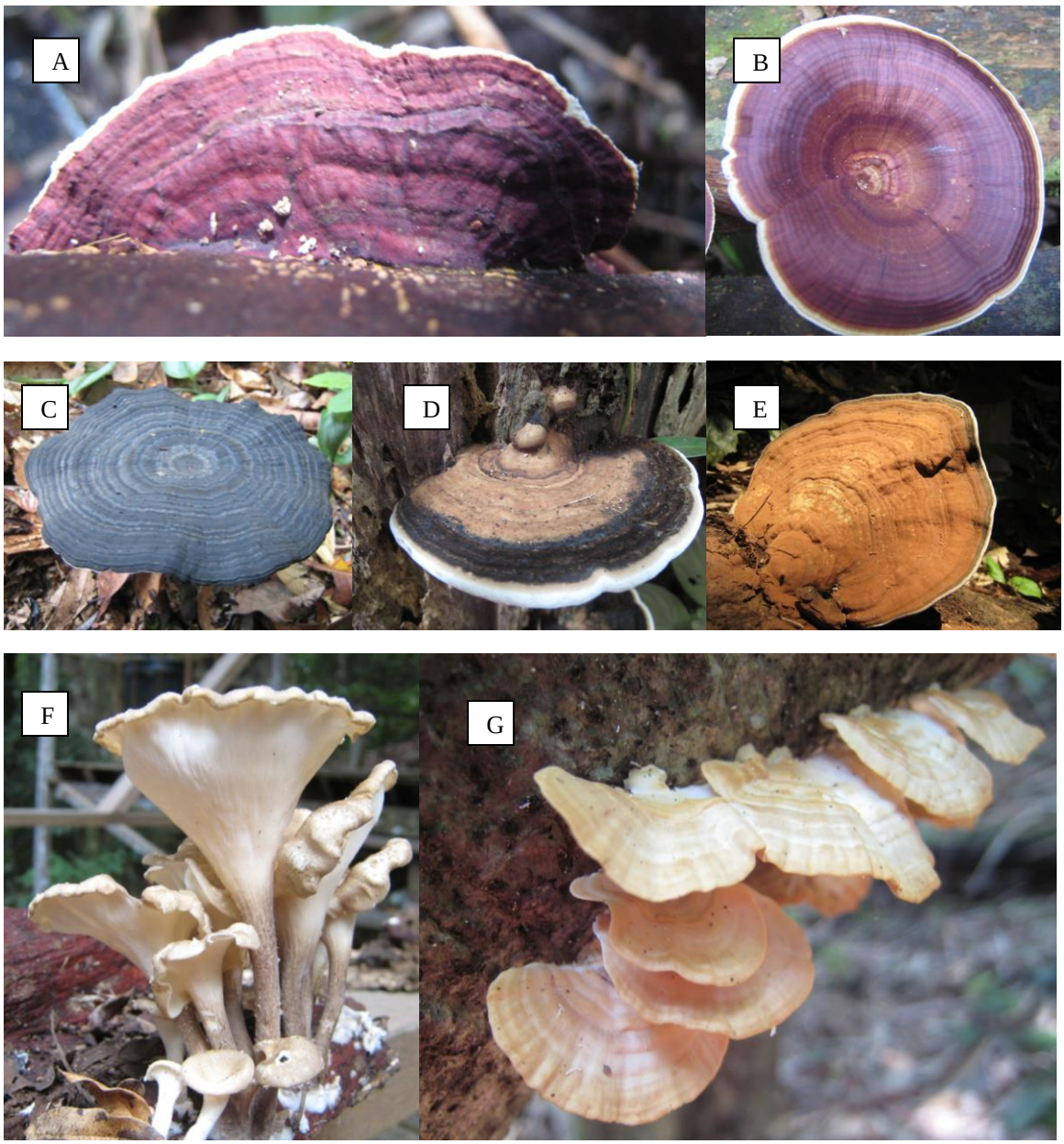

Figure1. *Fungal species. A. Earliella scabrosa B. Microporus xanthopus C. Amauroderma rugosum D. Fomitopsis dochmia E. Ganoderma australe F. Lentinus sajor-caju G. Trametes pubescens*

2.3. Phytochemical Screening

Phytochemical screening was undertaken to examine the fungal extracts for the presence of a range of chemical groups. Among those groups screened are alkaloid, triterpenes/steroids and saponins.

2.3.1. Alkaloid Test

The procedure of Culvenor and Fitzgerald (1963) as described by Said et al (1990) was used to determine the presence of alkaloids. A 2 g of crude extract was added with 10 mL of chloroform ammonia solution ($CHCl_3/NH_4OH$). This extract solution was filtered through cotton wool in a test tube. One milliliter of 2M sulphuric acid was added into the filtrate. The mixture was shaken and left for 1-2 minute until two layers were formed. The upper layer (acid aqueous) was transferred into another small test tube using a pipette dropper. The acid aqueous solution was tested with Mayer reagent. The production of a turbid solution or a white precipitate which indicated positive test for alkaloids was quantified and classified into four graduations by visual assessment. The 4+ represented the highest concentration with dense and white precipitate while 1+ for clear.

2.3.2. Triterpene/Steroid Test

The presence of triterpenes and steroids was determined by Liebermann Burchard test (Simes et al, 1959) as described by Said et al (1990). A 1 g subsample of the above powdered fungi was placed in a 50 mL conical flask with 30 mL of ethanol was added into it. The mixture was boiled for 15 minutes. The solution was then filtered and the filtrate was evaporated to dryness using a hot water bath. The dried extract was mixed with 10 mL of diethyl ether, shaken, filtered into an evaporating disc and then evaporated to dryness. The filtrate was combined and added with two drops of acetic anhydride and shaken well. After that, one drop of concentrated sulphuric acid was dripped along the wall of the disc. The formation of a bright purple, red or pink coloration which indicated the occurrence of triterpenes while blue or green colorations indicate steroids were quantified as 1+, 2+, 3+ and 4+ (lowest to highest) based on the color intensity formed.

2.3.3. Saponin Test

The presence of saponins was tested using froth test (Said et al, 1990; Simes et al, 1959). The residue that did not dissolve in diethyl ether from the previous triterpenes/steroids test was transferred into a test tube. Ten milliliters of distilled water was added and shaken vigorously for 30 seconds before allowed to set for 30 minutes. The resultant height and time of foam formation lasted was recorded. The positive saponin test was indicated by the formation of 1 cm or more stable foam. Foam lasting ½ hour, 1 hour, 1½ hours and 2 hours were given relative values of 1+, 2+, 3+ and 4+ respectively.

2.4. Antioxidant

The antioxidant activity was measured using 1,2-diphenyl-2-pricrylhydrazyl (DPPH) to evaluate free radical scavenging activity according to Blois (1958). Scavenging of DPPH represents the free radicals reducing activity of antioxidant based on the one-electron reduction. Scavenging of a stable DPPH free radical determines the antioxidant potential of the test sample (fungal powder) against injury in a biological system. Each sample of 1.0 mg mL^{-1} were prepared in triplicates, in which 200 µL of samples (1.0 mg mL^{-1}) was added to 200 µL of DPPH (1 mM in ethanolic solution) and 600 µL of absolute ethanol (AR Grade) in a 10 mL amber bottle with screw cap. The mixture was shaken and allowed to stand at room temperature for 10 minutes. Then the absorbance at 520 nm was measured with spectrophotometer (Perkin-Elmer-Lambda 35). Ascorbic acid (200 ppm) was used as a positive control. The radical scavenging activity was calculated as follows:

$$\frac{\text{(Absorbance of negative control – Absorbance of positive)}}{\text{(Absorbance of negative control – Absorbance of sample)}} \times 100$$

2.5. Minimum Inhibitory Concentration (MIC)

This was determined by broth micro dilution method using 96-well microplates against the five bacteria, namely *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginos, Escherichia coli* and *Clostridium difficile* (Gulluce et al, 2004). The nutrient broth (NB) (13 g L^{-1}, OXOID CM1) was prepared and autoclaved at 121ºC for 15 minutes. A crude extract sample of 14.4 mg was dissolved in 2 mL DMSO to prepare a concentration of 1800 µg mL^{-1} and refer as a stock solution. The inoculated microbial strain was prepared from the 24-hour broth culture suspension and was adjusted to 0.5 McFarland standard turbidity. The sterile NB was added (100 µg mL^{-1}) to wells in row B to H. The stock solution was added (100µg mL^{-1}) to row A and B. The well mixture of NB and sample at row B were transferred to each well in order to obtain a two-fold serial dilution of stock samples(1800, 900, 450, 225, 112.5, 56.25, 28.13 and 14.07 µg mL^{-1}). The prepared bacteria were added (100 µg mL^{-1}) to all wells (A to H). The plate was then covered, sealed and incubated at 37˚C for 24 hours. After which, bacterial growth was observed through turbidity and presence of pellet at the bottom of the plate.

2.6. Minimum Bactericidal Concentration

Minimum Bactericidal Concentration (MBC) was used to confirm or extend evaluating the results of MIC by determining the growth of bacteria. The solution at the most clear stage/concentration in the 96-wells plate during the MIC analysis was taken out and spread on the plate containing agar using sterilized cotton bud. The plate was sealed and incubated at 37˚C for 24 hours. The

absence of bacterial growth indicated MBC result is the same as MIC while their presence showed concentration MBC one step lower than MIC.

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening

From the seven fungi screened for the presence of alkaloid, triterpene/steroid and saponins, only one sample, namely *Ganoderma australe* contains alkaloids (Table 1).

Table1. *Phytochemical screening on selected fungi*

Fungal Species	Alkaloid	Triterpene	Steroid	Saponins
Earliella scabrosa	0	0	0	1+
Microporus xanthopus	0	0	0	<20 minutes
Amauroderma rugosum	0	0	0	<20 minutes
Fomitopsis dochmia	0	0	0	1+
Ganoderma australe	2+	0	0	2+
Lentinus sajor-caju	0	0	0	3+
Trametes pubescens	0	0	0	2+

0 = *negative*

This is expected as reported in several studies (Matos et al, 2007; Joo et al, 2008; Yuen and Gohel, 2005; Xu et al, 2011) which showed that several species of *Ganoderma* contain many bioactive compounds (~400), such as triterpenoids and polysaccharides. This positive result was supported by the high activity in DPPH radical scavenging as shown in Table 2 below. On the other hand, the absence of alkaloids in *Lentinus sajor-caju* is somewhat surprising as studied by Sharma et al (2013) in northwest India showed that genus *Lentinus* in general contain high amount of alkaloids with this species (*Lentinus sajor-caju)* was one of the top among the ten species tested. These results may due to the different geographical locations. Therefore, it is interesting to further study on their chemical profile.

All samples indicated absence of triterpene and steroid content but showed presence of saponins with *Lentinus sajor-caju* recorded the highest saponins while *Microporus xanthopus* and *Amauroderma rugosum* demonstrated weak saponins properties.

3.2. Antioxidant Activity

Seven ethanolic extracts of fungi underwent antioxidant evaluation to test its effectiveness, prevention, interception and repair mechanism against injury in a biological system. The results in Table 2 show that *Ganoderma australe* demonstrated the highest DPPH scavenging activity of 89.56% followed by *Amauroderma rugosum* a distant second with 57.16% of inhibition and considered as moderate activity. The rest showed low radical scavenging activity, ranging from 6.48% to 37.22%. Genus *Ganoderma* comprising about 80 species are polypore fungi which grow on wood and many of them found in tropical regions. Because of their extensive use in traditional Asian medicines, and their potential in bioremediation, it is an economically important genus. Herbalists consider ganoderma an adaptogen, or natural regulator, suppressing the immune system if it is overactive and boosting it if it is underactive.

Table2. *Antioxidant, DPPH scavenging activity of selected fungi*

Fungal Species	DPPH Value (%)
Earliella scabrosa	21.27
Microporus xanthopus	6.48
Amauroderma rugosum	57.16
Fomitopsis dochmia	37.22
Ganoderma australe	89.56
Lentinus sajor-caju	16.12
Trametes pubescens	14.10

Many health claims are made on the effect that ganoderma has on the immune system. These claims are based primarily on the presence of high molecular weight polysaccharides and free

radical antioxidants in ganoderma extracts. *G. lucidum* (Reishi), for example, is one of the most popular medicinal fungi in China, Japan, and the United States (http://www.healthline.com /galecontent/ganoderma). Based on these evidences, *G. australe* topping the list for antioxidant is not surprising.

3.3. Antimicrobial Assay (MIC and MBC)

All the crude fungal extracts are classified as strong inhibitors as the Minimum Inhibitory Concentration **(**MIC) values recorded were <500 µg mL^{-1} except for *Fomitopsis dochmia* (Table 3). This fungus also showed weak inhibitory activity for all bacteria except *Streptococcus pyogenes.* These bacteria are the causes of many important human diseases, ranging from mild superficial skin infections to life-threatening systemic diseases. MIC was defined as the lowest concentration that had no macroscopically visible growth. The microbial growth was indicated by the turbidity and presence of pellet at the bottom of the well and these assays were repeated twice in triplicates. These results are confirmed with the Minimum Bactericidal Concentration (MBC) test.

Table3. *Antimicrobial assay against five bacteria*

Fungal Species	CD (µg mL^{-1})	SP (µg mL^{-1})	PA (µg mL^{-1})	SA (µg mL^{-1})	EC (µg mL^{-1})
Earliella scabrosa	225	112.5	28.13	225	225
Microporus xanthopus	225	112.5	56.25	225	225
Amauroderma rugosum	225	112.5	112.5	225	225
Fomitopsis dochmia	1800	225	1800	1800	1800
Ganoderma australe	225	112.5	225	225	56.25
Lentinus sajor-caju	225	225	112.5	225	225
Trametes pubescens	112.5	112.5	225	450	225

Bacteria:

CD: *Clostridium difficile* **SP**: *Streptococcus pyogenes*

PA: *Pseudomonas aeruginos* **SA**: *Staphylococcus aureus* **EC**: *Escherichia coli*

4. CONCLUSION

All selected fungi except *Fomitopsis dochmia* demonstrated high antimicrobial activity. Thus, six fungi (*Amauroderma rugosum, Earliella scabrosa, Ganoderma australe, Lentinus sajor-caju, Microporus xanthopus,* and *Trametes pubescens* have great potential to be developed into pharmacological products. In comparison, *G. australe* has the most anti- oxidative function which may be due to its high alkaloid content. Contrary to studies elsewhere in the world, the absence of alkaloids in *L. sajor-caju* is somewhat surprising. More researches may need to be undertaken to verify this anomaly. Mount Singai contains a rich resemblance of fungi typical of tropical forest area which have socio-economic importance warranting the area to be protected and conserved.

ACKNOWLEDGEMENTS

We would like to thank Research Management Institute (RMI), Universiti Teknologi MARA (UiTM) and Ministry of Higher Education (MOHE), Malaysia for providing financial support through the FRGS [600-RMI/FRGS 5/3 (113/2012)] and funding from Shell and Ministry of Tourism Malaysia through UNIMAS for partial funding during the expedition to enable data collection. Last but not least, the cooperation from the local communities, Bisingai Bidayuh is very much appreciated.

REFERENCES

[1] Culvenor C. C. J. and Fitzgerald, J. S., A Field Methods for Alkaloids Screening of Plants, Journal of Pharmacy Science 52: 303-304 (1963).

[2] Said, I. M., Din, L. B., Samsuddin, M. W., Zakaria, Z., Yusoff, N. I., Suki, U., Manap, A., Ibrahaim, A. Z. and Latiff, A., A Phytochemical Survey of Ulu Kincin, Pahang, Malaysia, Malaysian National Journal 43: 250-266 (1990).

[3] Simes, J. J. H., Tracey, J. G., Webb, L. J. and Dunstan, W. J., An Australian Phytochemical Survey III. Saponins in Eastern Australian Flowering Plants, Commonwealth Scientific and Industrial Research Organization (CSIRO) Bulletin 281: 1-31 (1959).

[4] Blois M. S., Antioxidant Determination by the Use of Free Radical, Nature 26: 1199-1200 (1958).

[5] Gulluce, M., Ozer, H., Baris, O., Daferera, D., Sahin, F. and Polissiou, M., Chemical Composition of the Essential Oils of *Salvia aethiopis* L., Turk. J. Biol. 30: 231-233 (2004).

[6] Matos, A. J., Bezerra, R. M. and Dias, A. A., Screening of Fungal Isolates and Properties of *Ganoderma applanatum* Intended for Olive Mill Wastewater Decolourization and Dephenolizatio, Lett. Appl. Microbiol. 45 (3): 270-275 (2007). doi:10.1111/j.1472-765X.2007.02181.x. PMID17718838

[7] Joo, S. S., Ryu, I. W., Park, J. K., Yoo, Y. M., Lee, D. H., Hwang, K. W., Choi, H.T., Lim, C.J., Lee, D.I. and Kim, K., Molecular Cloning and Expression of a Laccase from *Ganoderma lucidum*, and its Antioxidative Properties, Mol. Cells 25(1): 112-118 (2008). PMID 18319622.

[8] Yuen, J. W. and Gohel, M. D., Anticancer Effects of *Ganoderma lucidum*: a Review of Scientific Evidence, Nutr Cancer 53 (1): 117 (2005). doi: 10.1207/s15327914nc5301_2. PMID 16351502.

[9] Xu, Z., Chen, X., Zhong, Z., Chen, L. and Wang, Y., *Ganoderma lucidum* Polysaccharides: Immunomodulation and Potential Anti-tumor Activities, American Journal of Chinese Medicine 39 (1): 15-27 (2011). doi: 10.1142/S0192415X11008610. PMID 21213395.

AUTHOR'S BIOGRAPHY

Gee-Moi Liew, graduated from University of Malaya with B.Sc. (Hons.) Botany and M.Sc. in mycology. Currently, she is a senior lecturer in Biology at Universiti Teknologi MARA (UiTM), Sarawak, Malaysia. Her research interest is on the tropical fungal diversity, especially the macrofungi in tropical forests.

Heng-Yen Khong serves as Associate Professor at Universiti Teknologi MARA, Sarawak since 2012. She has contributed in teaching and research for about 20 years. She has administrative experiences as laboratory coordinator, course coordinator and Head of Programme. Currently, she is the Head of RiND, Atta-ur-Rahman Institute of Natural Products Discovery Sarawak Chapter, UiTM Sarawak. Her research interest includes Commercialization of Indigenous plants; Bioassay study of plant extracts; Fractionation extraction, isolation and structural elucidation of pure compounds from natural resources for developing drug discovery and Analytical Chemistry.

Clifford Junaidi Kutoi, graduated from Universiti Teknologi MARA (UiTM), Shah Alam, Malaysia in B.Sc. (Hons) of Applied Chemistry. Currently, he is doing Master Degree in Chemistry (Natural Product).

Alexander K Sayok, from Kuching Sarawak, is a graduate in forestry with BS from Louisiana (1984) and MS (1986) and doctorate (1991) from Texas, USA. Since September 2008, he serves as a Research Fellow/Associate Professor at Institute of Biodiversity and Environmental Conservation, Universiti Malaysia Sarawak (UNIMAS), lecturing in Environmental Law and Natural Resources Management and conducts research on biodiversity in various community forests including plants in Sarawak towards effort for conservation and sustainable use, and food plants diet and health relationship.