

CHAPTER SIX

6. GENERAL DISCUSSION AND CONCLUSION

In this study, two locally grown *Ganoderma* species obtained from a farm in Semenyih were tentatively identified as *G. lucidum* and *G. tsugae* based on the morphology of basidiospore and cultural characteristics. Basidiospores of *G. tsugae* were 'rough' walled and had broad inter-wall pillars. Isolates of *G. tsugae* do not produce chlamydospores or primordia in culture and had an average growth rate of 6.8 mm/day at the optimal temperature range of 33-37°C.

In contrast, the basidiospores of *G. lucidum* appeared 'smooth' walled, characterized by narrow, numerous inter-wall pillars. Isolates of *G. lucidum* produce chlamydospores and primordia in culture after five weeks and had a higher average growth rate of 11.8 mm/day at a lower optimal temperature range between 28-33°C as compared to *G. tsugae*. However, both produced polyphenol oxidase as extracellular enzyme.

Since *G. tsugae* was the main species cultivated in the farm, it was used for further study. The fruit body chips of *G. tsugae* were ground and extracted with hexane and methanol separately. These extracts were subjected to phytochemical studies. After repeated column chromatography of the crude extracts, four compounds were isolated and identified using various spectroscopic methods.

Terpenoids were found to be present in the highest content, followed by alkaloids, saponins and flavonoids. Chemically guided fractionation based on TLC patterns of terpenoids allows four compounds (GM 1.1, GM 2.2, GM 3.6.5 and GM 4.3) to be isolated from the hexane crude extract. These compounds were identified as stellerol (GM 3.6.5), ergosterol (GM 4.3), di-(2-ethylhexyl) phthalate (DEHP) and a new brominated ergosta-type sterol (GM 1.1)

This is the first report that DEHP is being isolated from a mushroom. DEHP is the most widely used plasticizer (comprising 50% of all phthalate ester plasticizers) that softens resins. DEHP exists widely in the environment and have been reported in wide variety of substances (oil, soil, plants, and animals) (IPCS, 1992). Most occurrences have anthropogenic origins but some could be of natural origins (Mathur, 1974a, b)

The anthropogenic origins of DEHP may be postulated coming from the plastic bag during pasteurization of the substrate. The *Ganoderma* spp. belonging to the basidiomycetes has long known to bioaccumulate substances from their natural environment (Hudson, 1986). Hence, it is possible that DEHP dissociated from the plastic, bound onto the substrate and bioaccumulated by the *G. tsugae* during the course of cultivation. However, since no further investigations were carried out on the substrate or other batches of *Ganoderma tsugae*, the source of contamination of DEHP is still not conclusive.

The other three compounds, ergosterol, stellasterol and **compound 1** are found to be sterols. Sterols are of key importance in maintaining the integrity of eukaryotic membranes. For the majority of the fungi, ergosterol is the key membrane sterol. (Griffin, 1994).

In this study, ergosterol was found to be the main sterol in the fruit body of *G. tsugae*, with 0.025% (w/w), while stellasterol was observed as a subcomponent with 0.004% (w/w). This finding is not in accordance to Kac et al. (1984) which reported stellasterol as the main sterol, while ergosterol as a subcomponent in *G. lucidum*. The concentrations of steroids may be different depending on the stage and species of *Ganoderma* as well as the environment in which it is grown (Jong and Birmingham, 1992; Mizuno, *et al.*, 1995).

To our best knowledge, the new brominated egosta-type structure, **compound 1**, is the first to be isolated from *Ganoderma* species. The presence of bromine in the mushroom may be also of anthropogenic or natural origins. Further study need to be carried out to confirm its origin. However, initial investigation of the fresh substrate using wave dispersion spectrum (WDS) indicated the presence of low level of bromine.

The source of bromine may be also postulated coming from the substrate. Fumigation of rubber wood with methyl bromine in sawmill has been a practice in many countries (Tanaka *et al.*, 1991). In Malaysia, it is not certain whether this fumigation of logs is being carried out. If this is the case, biosorption of bromine residue from the substrate is possible. The bromine may then somehow get incorporated into the sterol synthesis pathway and produce **compound 1**.

Although many of the lanostane type (C_{27} lucidenic acid and C_{30} ganoderic acid) structures described earlier were not isolated, the sterols obtained in this study are very related with these structures. As shown in Figure 6.1, the lanostane is a precursor for the synthesis of ergosterol. The production of ergosterol is a multistep process from lanosterol that requires removal of both methyls at C-4 and the C-14 methyl (Figure 6.1) (Griffin, 1994).

The lanostane type lucidenic and ganoderic acids are usually present in the more polar methanol extract. Due to time constraint, the methanol fractions were not further purified.

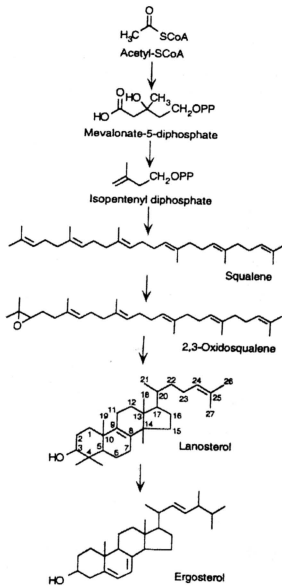


Figure 6.1: Synthesis of ergosterol in fungi (Griffin, 1994)

The fractions collected from the chromatography of both hexane and methanol crude extracts, together with the four isolated compounds were used to test for antibacterial and antifungal activity. The results showed that different combinations of compounds may give rise to different strengths of inhibition against the different types of test microorganisms. As a result, both synergistic and antagonistic effects were being observed in some of the fractions.

All fractions showed weak (7.0 to 8.9 mm) to moderate (9.0 to 10.9 mm) activity against one or more of the test microorganisms. *Candida albicans* was the most susceptible organism, with 35% of the fractions showing moderate activity against this opportunistic pathogen.

DEHP showed a moderate broad-spectrum inhibition against the test bacteria and fungi, while the other three ergosta-like sterols only exhibited mild activity against one or more of the test microorganism.

FUTURE WORK

The present study on the chemistry and biology of a locally grown *Ganoderma* species are concentrated in three important areas, namely:

- 1) Identification of two locally grown species based on their morphology of basidiospores and cultural characteristics. However, the identification of species can only be tentatively made. Host relationship and interfertility tests with homokaryons need to be carried out for complete identification of the species.
- 2) Extraction and isolation of terpenoids from *G. tsugae*. Both the hexane and methanol fractions that showed moderate activity should be further purified in order to identify the active compounds. Besides terpenoids, alkaloids were also found to be present in considerable amount. This group of compounds should be studied in future since many of the alkaloids have been known to possess interesting biological activities.
- 3) Screening for antibacterial and antifungal activities. Although most of the fractions only showed mild to moderate activity against one or more of the test microorganism, this result may indicate that this *in vitro* assay is not an effective biological assay system to screen for bioactive compounds from the *Ganoderma*

extract. Hence, an alternative *in vivo* biological assay system should be carried out in the future.

More importantly, from the findings of this study is the presence of DEHP. The source of contamination of DEHP should be investigated. This can be carried out by extracting the pasteurized substrate with hexane or ether and partially separate the extract before loading into GC-MS to detect the fragmentation peaks of DEHP. Alternatively, the experiment described in Chapter four need to be repeated for other batches from the same farm or other farm in order to look for DEHP.

The new brominated sterol is an interesting compound to look at. Further confirmation of the structure such as exact mass analysis need to be made. The biological aspect of this compound should also be further studied. The significant of the presence of bromine is not known. It will also be interesting to find the mechanisms of the bromine incorporated in the biosynthesis pathway of the sterol in producing **compound 1**.

CONCLUSION

This report intends to create an awareness of the possibility of contamination from the environment if care and considerations were not seriously taken into account. From the findings of this study, few possible suggestions can be made to overcome the presence of DEHP and bromine if this is truly a contamination:

1. time of pasteurization can be also shorten to prevent leaching of DEHP from the plastic onto the substrate.
2. use plastic bags which contain no or less concentration of DEHP.
3. change the method of cultivation by:
 - using logs rather than plastic bags.
 - cultivating the mycelium in submerged culture rather than solid substrate cultivation
4. fresh substrate should not be used immediately but need to be kept at least a month or compost before using.

The major terpenoids isolated from the fruit body of *G. tsugae* were identified as stellasterol (GM 3.6.5), ergosterol (GM 4.3), di-(2-ethylhexyl) phthalate (DEHP) and a new brominated ergosta-type sterol (GM 1.1). The brominated sterol has a novel structure, which may possess interesting activity in other bioassay system.