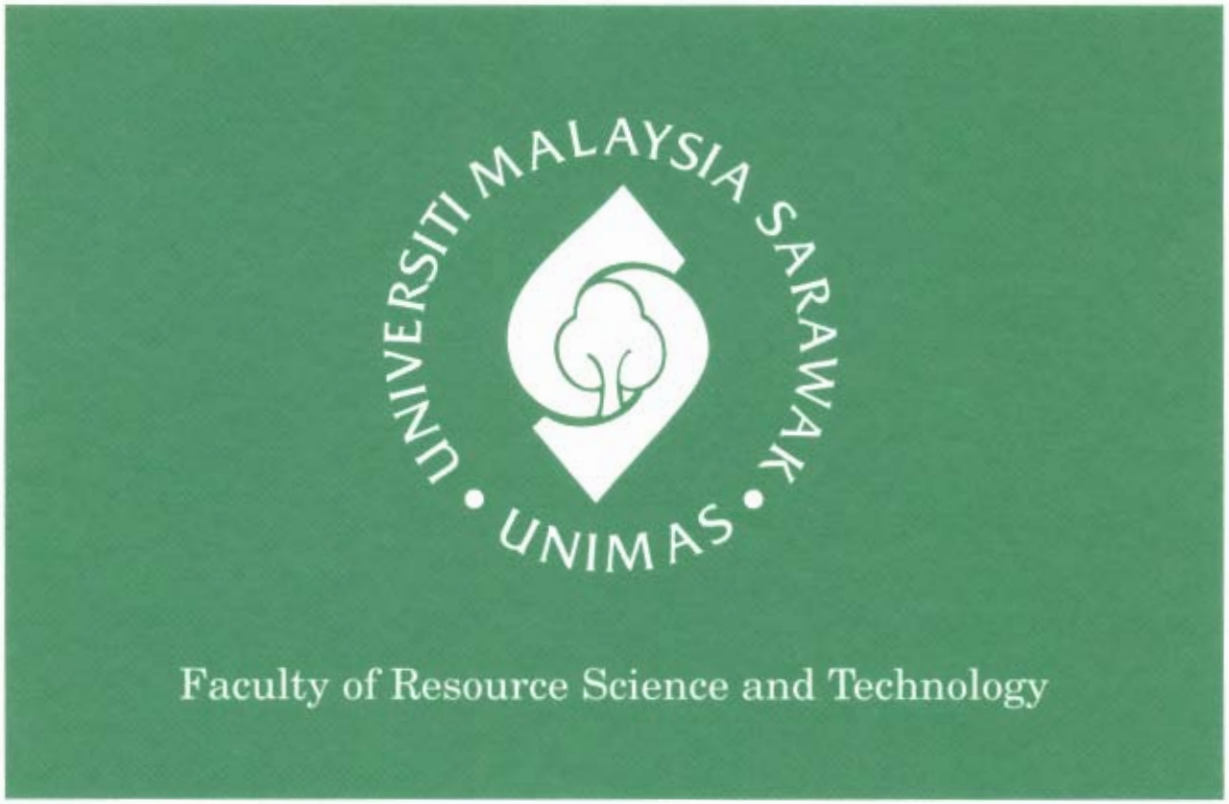

THE ISOLATION AND CHARACTERIZATION OF TRITERPENOID ACIDS FROM *Ganoderma spp.*

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Bachelor of Science with Honours
(Resource Chemistry)
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This project is submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning

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The Isolation and Characterization of Triterpenoids Acids from *Ganoderma* spp. (KOSA1K5).

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Abstract - *Ganoderma* spp. (KOSA1K5) were isolated and cultivated in 1 L malt extract broth (MEB). 17.6 mg extracellular triterpenoids acids and about 20.53 mg intracellular triterpenoids acids were obtained from *Ganoderma* spp. (KOSA1K5). The triterpenoids acids were then subjected to Thin Layer Chromatography (TLC). The triterpenoid acids crude extract from extracellular yielded 3 spots with R_f values 0.56, 0.75 and 0.86 in methanol: hexane (1: 0.5) solvent system. For intracellular tritepernoid acids crude extract, there were also 3 spots present with R_f values 0.41, 0.62 and 0.74 in methanol: hexane (3: 2) solvent system. Identification of isolated triterpenoid acids was carried out using UV-Vis and FT-IR. Toxicity studies of the isolated triterpenoid acids were conducted against *Artemia salina* indicates that both triterpenoid acids extracts have low toxicity against *Artemia salina*.

Keywords: *Ganoderma* spp., Triterpenoids acids

Abstrak - Ganoderma spp. (KOSA1K5) telah dikultivasikan di dalam 1L MEB. Pengekstrakan asid triterpenoid telah di jalankan di mana sebanyak 17.6 mg ekstrak kasar telah diperolehi daripada bahagian luaran Ganoderma (KOSA1K5) iaitu media pengkulturan yang digunakan dan sebanyak 20.53 mg ekstrak kasar diperolehi daripada mycelia Ganoderma (KOSA1K5). Analisis Kromatografi Lapis Nipis (KLN) telah dijalankan ke atas kedua-dua ekstrak kasar di mana ekstrak asid tritepenoid daripada bahagian luaran Ganoderma (KOSA1K5) memberikan 3 titik dengan nilai R_f 0.56, 0.75 and 0.86 dengan sistem perlart metanol: heksana (1:0.5). Bagi ekstrak asid tritepenoid daripada mycelium Ganoderma (KOSA1K5), 3 titik diperolehi dengan nilai R_f 0.41, 0.62 and 0.74 *dalam sistem pelarut metanol : heksana (3 : 2). Asid triterpenoid dikenal pasti dengan lebih lanjut dengan menggunakan FT-IR dan UV-Vis. Ujian ketoksikan ekstrak asid triterpenoid di jalankan di mana kedua-dua ekstrak menunjukkan kesan ketoksikan yang rendah terhadap Artemia salina.*

Kata kunci : Ganoderma spp., asid triterpenoid

CHAPTER 1

INTRODUCTION

Ganoderma spp., a fungus is an edible Chinese mushroom which is known as wood degrader fungus that usually grows on log or tree strumps. It is a medicinal fungus that has a long history of use in folk medicine. The fungus *Ganoderma lucidum* also known as "*Lingzhi*" in Chinese, "*Reishi*" in Japanese, and "*Youngzhi*" in Korean is a member of the genus *Ganoderma* and has been traditionally used as a popular folk medicine for the promotion of health in the Orient. For example, as early as in 100 before century, Lingzhi was cited in the Shen Nong's Herbal Classic (widely considered as the oldest book on oriental herbal medicine and the foundation of traditional Chinese medicine) for enhancing "vital energy" and promoting "longevity". This "mushroom of longevity" has been deemed as the most exalted medicine in ancient China (Shiao et al., 1994). The genus *Ganoderma*, however, was established in the West by a Finnish botanist, P. Karsten, in 1881 (Ryvarden, 1991).

During the past two decades, modern research has revealed that many bioactive components have been identified from its fruit bodies, mycelia, spores, and culture media. Pharmaceutically active compounds from *Ganoderma* spp. include triterpenoids, proteins, steroids, alkaloids, nucleotides, lactones, and fatty acids (Wasser et al., 1999, Kim et al., 1999, Mizushina et al., 1999). Among these ingredients, triterpenes and polysaccharides have attracted considerable attention as they have been shown to possess diverse and potentially significant pharmacological activities.

Although the fruit body of *Ganoderma* spp. had been utilized as medicine for several years in China, the spores of *Ganoderma* spp. were realized and utilized only in the 20th century. The spores of *Ganoderma* spp. also contain a large amount of bioactive substances like the fruit body of *Ganoderma* spp. (Min et al., 1998). Recent studies on this fungus have demonstrated that the spores of *Ganoderma* spp. show significant antitumor activity (Zhu et al., 2000) and anti-human immunodefiency virus-1 protease activity (Min et al., 1998). However, these effects are closely related to the status of the sporoderm. The breaking of the spores of *Ganoderma* spp. can improve the release of activity, and no effect is observed is not broken (Zhu et al., 2000).

Ganoderma spp. has been used for the treatment of a wide range of ailments and chronic diseases, such as migraine, hypertension, arthritis, bronchitis, asthma, anorexia, gastritis, hemorrhoids, diabetes, hypercholesterolemia, nephritis, dysmenorrheal, constipation, lupus erythematosis, hepatitis, and cardiovascular problems (Shiao et al., 1994, Jong and Birmingham, 1992). Significantly, *Lingzhi* has been demonstrated by recent scientific studies to possess anti-cancer (Wang et al., 1997) including leukemia (Lieu et al., 1992, Jarvis, 1994), anti-ageing (Gan et al., 1998) and antimicrobial activities (Eo et al., 2000, Yoon et al., 1994), including anti-human immunodeficiency virus (HIV) activity (El-Mekkawy et al., 1998, Kim et al., 1997).

Several products of *Ganoderma* spp have recently undergone clinical trials and became available as syrup, injection, tablets, tincture, bolus of powdered medicine and honey, both in solution and a mixture (Wasser and Weiss, 1997).

CHAPTER 2

LITERATURE REVIEW

Polypores are a large group of terrestrial fungi of the phylum Basdiomycota (basidiomycetes), and they along with certain Ascomycota are a major source of pharmacologically active substances. There are about 25 000 species of basidiomycetes, of which about 500 are members of the Aphyllophorales, a polyphyletic group that contains the polypores which is easily found in Southeast Asia countries and tropical region (Sudirman and Mujiyati, 2001). Many of these fungi have circumboreal distributions in North America, Europe, and Asia and broad distributions on all inhabited continents and Africa; only a small number of the most common species with the most obvious fruiting bodies (basidiocarps) have been evaluated for biological activity. An estimated 75% of polypore fungi that have been tested show strong antimicrobial activity, and these may constitute a good source for developing new antibiotics. Numerous compounds from these fungi also display antiviral, cytotoxic, and antineoplastic activities.

Ganoderma spp. is a basidiomycete, lamella less fungus belonging to the family of polyporaceae. There are about 214 species of *Ganoderma* have been recorded from over than 2,500 species of *Ganoderma* exist in the world (Walting et al., 1999). Generally, this saprophytic fungus has an inner brown layer which covered with spines that pierce on outer layer. The fruit bodies of *Ganoderma* is basically thick bracket, soft cork and solitary or in small groups. When it is getting old, it is usually

becoming woody and hardening. The cap colour ranges from yellow to black (Hamuro et al., 1974). Basically, the mycelium, spores as well as the fruit bodies contribute in medicinal usage (Teow, 1986).

In nature, it grows in densely wooded mountains of high humidity and dim lighting. The tropical climate in Malaysia results to the conducive growth of *Ganoderma* especially in oil palm area. This fungus has caused basal stem rot which is considered as serious disease of oil palm tress in Southeast Asia countries (Elliot and Uchida, 2003).

Mushrooms are the fruiting body and reproductive structure of a higher order fungus organism. The actual mushroom "tree" is a fine thread-like network called mycelium. This mycelium is for the most part subterranean, living in soil, logs and other organic litter. *Ganoderma* spp. rise out of the mycelium when the right nutrients are amassed and the right environmental conditions are present. They release spores at maturity. The wind spreads them and when they land on the right spot, the cycle starts over again (Mizuno, 1985).

Unlike green plants, which produce many of their own nutrients by photosynthesis, *Ganoderma* spp. capable to get their nutrients from dead organic matter or soil. It takes the nutrients from the dried trunk or trees that they grow on their survival. *Ganoderma* spp. and their mycelium are nature's original recyclers (Liu et al., 1979).

Triterpenoids are biologically active compound presence in ganoderma. Triterpenes or triterpenoids are bitter components of *Ganoderma* spp. that have received considerable attention owing to their well-known pharmacological activities (Kim and Kim, 1999). Since the first isolation of two new bitter triterpenes, ganoderic acids A and B, from the dried epidermis of *G. lucidum* in 1982 by Kubota et al. (Kubota et al., 1982), more than 130 oxygenated triterpenes (mostly lanostane-type triterpenes) have subsequently been isolated from the fruiting bodies, spores, mycelia and culture media of Lingzhi (Kim and Kim, 1999, Chairul and Hayashi, 1994). It should be noted that *Ganoderma* spp. is the only known source of these bioactive ganoderic acids.

In general, triterpenes have been reported to possess significant bioactivities, such as anti-oxidation (Ahmad, 1995), hepatoprotection (Kim et al., 1999), anti-allergy (Tasaka et al., 1988), antihypertension (Kimura and Tamura, 1988), cholesterol reduction (Komodo et al., 1990), as well as inhibiting platelet aggregation (Su et al., 1999), due to the inhibition of enzymes such as galactosidase, angiotension coverting enzyme, cholesterol synthase, and many more.

Recent research work that demonstrated the anticancer, anti-oxidative and anti-viral activities of triterpenes or triterpenoids included, a triterpene fraction from the mycelia of *G. lucidum* was reported by Liu et al. to inhibit the growth of human hepatoma cells via suppressing protein kinase C and activating mitogen-activated protein kinases and C2-phase cell cycle arrest.

Zhu and co-workers (Zhu et al., 1999) recently studied the anti-oxidative activities of *G. lucidum* extracts and found that the triterpene fraction exhibited the highest effect by testing the ingredients against pyrogallol induced oxidation on erythrocyte membrane and Fe (II)-ascorbic acid induced lipid peroxidation in liver mitochondria. The major ingredients of the triterpene fraction consisted of ganoderic acids, lucidenic acid B and ganodermanotriol.

Min et al. reported that a number of triterpenes isolated from the spores of *G. lucidum*, such as ganoderic acid A (**Figure 1**), luciumol B, ganodermanodiol, ganodermanontriol and ganolucidic acid, showed significant anti-HIV-1 protease.

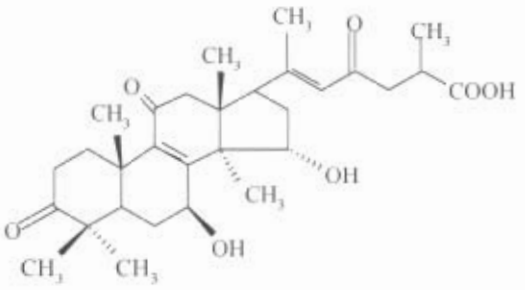

Figure 1. Molecular structure of Ganoderic acid A.

Recent investigation by Niu and the co- workers on the bioactive compounds of triterpenoids of *Ganoderma fornicatum* have led to the isolation of two novel triterpenoids, forcinatins A (**Figure 2**) and forcinatins B (**Figure 3**), which represents a novel carbon of 3, 4-seco-25, 26, 27-trinorlanostane (2004). Furthermore, these

compounds have been evaluated for their in vitro inhibitory activity against platelet aggregation (Niu et al., 2004).

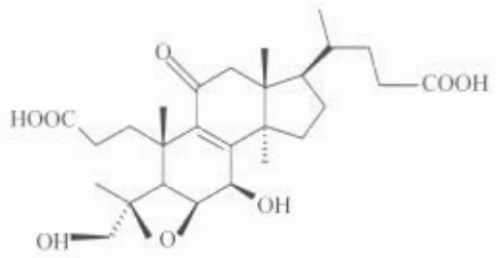

Figure 2. Molecular structure of forcinatins A (tritepenoid acid).

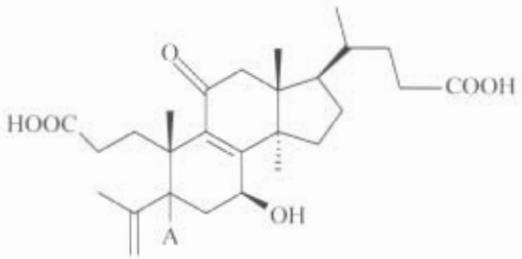

Figure 3. Molecular structure of forcinatins B (triterpenoid acid)

The bioactive triterpenes or triterpenoids isolated from *Ganoderma* spp. are shown in **Table 1**.

Table 1: Bioactive triterpenes and triterpenoids isolated from *Ganoderma* spp.

Sources	Triterpenes / triterpenoids	Usages	References
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acids A, B, H, C1 • Ganodermanondiol • Luciomol B • Ganolucidic acids	Anti HIV	Wang et al., 2006 Liu et al., 2002 Hiue and Di, 2004
Fruiting body of *G. lucidum*	• Ganoderan B	Anti diabetic	Teow, 1997.
Fruiting body of *G. lucidum*	• Ganoderic acids	Inhibitory effects on platelet	Liu et al., 2002
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acids C2, D	Anti histamine	Wang et al., 2006
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acid A, B, C, D • Lucidenic acid B • Ganodermanontriol	Anti oxidant	Hiue and Di, 2004
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acids A, B, G, H	Antinociceptive	Wang et al., 2006
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acids B, D, F, H, K, S, Y	Hypotensive activities	Teow, 1997
Fruiting body of *G. lucidum* and *G. tsugae*	• Ganodestrone • Ganoderic acids R, S, T, • Ganoderenic acid A	Liver function stimulant	Wang et al., 2006
Fruiting body of *G. lucidum* and spores of *G. lucidum*	• Ganoderic acids K, F, S	Inhibitory activity of angiotensin converting enzyme	Wang et al., 2006

OBJECTIVE

As *Ganoderma* spp. is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. The main goals of this research work were to use submerged cultivation for the production of *Ganoderma* spp. biomass, and to evaluate the potential of antimicrobial effects of triterpenoids produced by this method.

CHAPTER 3

MATERIAL AND METHODS

3.1 Preparation of *Ganoderma* spp. culture.

Ganoderma spp. was obtained from the Plant Pathology Laboratory Culture Collection at University Malaysia Sarawak. The culture then had been inoculated in plate containing malt extract broth and incubated at 25° C for seven days. The experimental media was prepared by dissolving 17 gram Malt Extract Broth (MEB) powder with 1 L of distilled water in a culture bottle and autoclaved. The mycelia agar was taken from the Malt Extract Agar (MEA) plate by cutting it in square shape (0.5 cm x 0.5 cm) using surgical blade and was inoculated in MEB media at constant temperature 25 ° C for 30 days. All the prepared cultures will be used for subsequent further research studies.

3.2 Extraction of extra cellular triterpenoids

250 mL of broth was extracted with 250 mL of methanol. The mixture solution was extracted with 100 mL of chloroform. The combined chloroform was concentrated under reduced pressure to a volume of 50 mL. The solution was then extracted with 70 mL saturated aqueous sodium hydrogen carbonate, $NaHCO_3$ 5%. The combined aqueous layer was acidified with hydrochloric acid, HCl 6.0 M to pH 2 – 3 under ice cooling. The solution was extracted with chloroform (150 mL x 3). The combined chloroform was concentrated under reduced pressure to 100 mL. Then, it is evaporated until dryness to yield a yellowish to white powder.

3.3 Extraction of intracellular triterpenoids

The intracellular or mycelium was filled with deionised water for 24 hour. After that, mycelium was mashed to release the extract by using stomacher. Then, the mashed mycelium was filtered using vacuum filter. The solution was used to proceed for the extraction process. 250 mL of broth was extracted with 250 mL of methanol. Then, the mixture solution was extracted with 100 mL of chloroform. The combined chloroform was concentrated under reduced pressure to a volume of 50 mL. After that, the solution was extracted with 70 mL saturated aqueous sodium hydrogen carbonate, $NaHCO_3$ 5%. The combined aqueous layer was acidified with hydrochloric acid, HCl 6.0 M to pH 2 – 3 under ice cooling. The solution was extracted with chloroform (150 mL x 3). The combined chloroform was concentrated under reduced pressure to 100 mL. Then, it evaporated until dryness to yield a yellowish to white powder.

3.4 Component separation

Quantitative separation of triterpenoid acids was carried out by using Thin Layer Chromatography (TLC). This method was used to determine the number of components that consist in a compound. 0.25 mm silica gel plate was used for TLC. Capillary tube was used to spot the samples onto TLC plate. The plates were developed using suitable solvent with different polarity in order to find solvent system that gave the best separation of components on TLC plate. The plate was visualized under UV light and the retention factor was determined.

3.5 Identification

Triterpenoid acids were identified using UV-vis and FT – IR.

3.6 Bioassay

3.6.1 Brine shrimp cytotoxicity test

Brine shrimp toxicity test established by McLaughin (1991). 2 mg of sample was dissolved in 2 mL of methanol. From this solution, 500, 50, 5 µL was pipetted using micropipette into test tubes in triplicates. The solvent was removed using rotovapor. This was followed by addition of 5 mL of seawater, resulting the final concentration of 100µg/L, 10µg/L, 1µg/L. 2 ml of sample was transferred into each NUNC multidish. 10 *Artemia salina* were added into each NUNC multidish to perform cytotoxicity test. The observation and the reading were carried out for 24 hours. After 24 hours contact, amount of survivors were counted. Control sample was performed similar way.

CHAPTER 4

RESULT AND DISCUSSIONS

4.1 Extraction and isolation of *Ganoderma* spp. (KOSA1K5) sample

The extraction of *Ganoderma* spp. using chloroform ($CHCl_3$) was obtained and produced the triterpenoid acids crude with the weight of yield shown in **Table 2.**

Table 2: The weight of the triterpenoids acids crude extracts from *Ganoderma* spp. (KOSA1K5).

Extracts	Weight (mg)
Extracellular layer	17.6
Intracellular layer	20.53

After solvent extraction, the isolated triterpenoids acids were subjected to Thin Layer Chromatography (TLC). The best separation of the isolated triterpenoids acid components from both intracellular and extracellular sample was obtained using methanol: hexane solvent system in ratios of 3: 2 and 1: 0.5 respectively. Separation of components for intracellular and extracellular samples show presence of three spots on each TLC plate. The retention factor (R_f) values of each spot are given in **Table 3** and **4**. **Figure 4** and **5** show the visualization of spots in TLC plates.

Figure 4: The spots of triterpenoid acids from extracellular in solvent system methanol: hexane (1: 0.5)

Spot	Retention factor, R_f
1	0.50
2	0.75
3	0.86

Table 3: R_f values for compounds observed in KOSA1K5 extracellular crude extract in the solvent system of methanol: hexane (1:0.5).

Figure 5: Extract intracellular sample in methanol: hexane (3:2)

Spot	Retention factor, R_f
1	0.41
2	0.62
3	0.74

Table 4: R_f values for compounds observed in KOSA1K5 intracellular crude extract in the solvent system of methanol: hexane (3: 2).

4.2 FT-IR spectroscopic characterization

In the spectrum obtained from extracellular triterpenoid acids, there is a band of hydroxyl group at 3323 cm^{-1}. The FT-IR measurement revealed the presence of alkanes with anti-symmetric and symmetric C-H absorption bands between 2852 cm^{-1} - 2924 cm^{-1}. The spectrum also supports the presence of carbonyl group as C=O band appears at 1712 cm^{-1}. The band of alkenes group was observed at 1452 cm^{-1}. Apart from these identical functional groups, saturated C-C band show absorption at 1258 cm^{-1} (**Figure 6**).

The intracellular of Ganoderma (KOSA1K5), indicates the presence of hydroxyl group at 3437 cm^{-1}. Strong absorption at 2927 cm^{-1} and 2855 cm^{-1} corresponds to the absorption by C-H single bond stretching motion that indicates the presence of alkanes group. The carbonyl group band was also identified at 1712 cm^{-1}. The band of alkenes group was observed at 1457 cm^{-1} Saturated C-C bond show several absorption in the 1000 cm^{-1} -1300 cm^{-1} range (**Figure 7**).

Further identification step should be carried out in order to elucidate the structure of the compound of interest.