

**ANALYSIS OF BIOLOGICALLY
ACTIVE CONSTITUENTS FROM WATER AND
METHANOL SOLUBLE EXTRACTS
OF REISHI MUSHROOM
(*Ganoderma lucidum*)**

by

GOH YUNG SHIN

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of the requirements for the degree
of Bachelor of Health Sciences (Biomedicine)**

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CERTIFICATE

This is to certify that the dissertation entitled
**“Analysis of biologically active constituents from
water and methanol soluble extracts of reishi mushroom
(*Ganoderma lucidum*)”**

is a bonafide record of research work done by

Miss Goh Yung Shin

Signature of supervisor:

..........

Name and address of supervisor:

Assoc. Prof. Syed Waliullah Shah
Lecturer
School of Health Sciences
Universiti Sains Malaysia
Health Campus
16150 Kubang Kerian, Kelantan

Date:

...07/05/06.....

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LIST OF ABBREVIATIONS

CIK	Cytokine induced killer
DNA	Deoxyribonucleic acid
FT-IR	Fourier transform infrared spectrometer (spectroscopy)
GC	Gas chromatography
GC/MS	Gas chromatography-Mass spectrometry
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
HSV	Herpes simplex virus
INF-γ	Interferon-gamma
IL	Interleukin
KBr	Potassium bromide
LC-MS	Liquid chromatography-Mass spectrometry
M-CSF	Macrophage colony-stimulating factor
MS	Mass spectrometry
NIR	Near infrared
NMR	Near magnetic resonance
ppm	Parts per million
RP-HPLC	Reverse phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-alpha

ABSTRACT

Ganoderma lucidum is one of the treasures of Chinese medicine. It has attained an unparalleled reputation in the Orient as the ultimate herbal substance. In this study, a rapid and efficient method for the extraction of bioactive molecules in *Ganoderma lucidum* is described. This method is based on the extraction of *Ganoderma lucidum* with hot water, methanol and a 1:1 v/v mixture of hot water-methanol. The FT-IR spectra of *Ganoderma lucidum* and its extracts were recorded in potassium bromide (KBr) over the range of 4,000-400 cm^{-1} . The UV-Visible spectrophotometric measurements were performed in water and methanol over the range of 200-800 nm. Bioactive elements in the acid-digested *Ganoderma lucidum* samples were determined by flame atomic absorption spectroscopy (AAS). The FT-IR spectra of *Ganoderma lucidum* and its extracts exhibited characteristic absorption bands with significant difference in their number and position. The infrared spectral bands indicated presence of functional groups attributed to polysaccharides, amino acids, vitamins, terpenes and enzymes moieties. The UV-Visible spectra, however, could not give much information on the structure of the compounds extracted, due to very complex nature of biological molecules. The concentration of elements determined in *Ganoderma lucidum* by flame AAS was found for Na 12.5 ± 0.001 mg/L, K 210.4 ± 0.003 mg/L, Ca 23.9 ± 0.001 mg/L, Mg 7.1 ± 0.005 mg/L, Zn 1.9 ± 0.001 mg/L and Fe 1.2 ± 0.004 mg/L.

ABSTRAK

Ganoderma lucidum adalah sejenis herba yang merupakan harta tak ternilai dalam perubatan Cina. Kajian tersebut berkisarkan tentang pengekstrakan molekul bio-aktif daripada *Ganoderma lucidum* dengan menggunakan suatu kaedah yang cepat dan berkesan. Kaedah tersebut adalah berdasarkan kepada pengekstrakan *Ganoderma lucidum* dengan menggunakan tiga jenis pelarut, iaitu air suling panas, metanol dan juga campuran air suling panas dengan metanol pada nisbah 1:1 v/v. Spektra FT-IR bagi *Ganoderma lucidum* dan ekstraknya telah direkodkan dalam kalium bromida (KBr) pada julat 4,000-400 cm^{-1} . Pengukuran oleh spektrofotometer UV-Vis telah dilakukan dengan menggunakan air suling dan metanol pada julat 200-800 nm. Elemen bio-aktif di dalam sampel *Ganoderma lucidum* yang telah dicernakan dengan asid pekat telah dikenalpasti dengan menggunakan spektroskop penyerapan atom (AAS). Spektra FT-IR bagi *Ganoderma lucidum* dan ekstraknya memiliki julat penyerapan yang unik dengan perbezaan yang ketara dari segi bilangan and posisi mereka. Spektra FT-IR tersebut melambangkan kehadiran kumpulan berfungsi bagi polisakarida, amino asid, vitamin, terpenes dan juga enzim. Namun begitu, spektra yang diperolehi daripada spektrofotometer UV-Vis tidak membekalkan banyak maklumat tentang struktur sebatian yang telah diekstrakkan. Ini mungkin adalah disebabkan oleh sifat semula jadi molekul-molekul biologi tersebut yang kompleks. Kepekatan bagi elemen-elemen daripada *Ganoderma lucidum* yang telah dianalisis dengan menggunakan AAS adalah seperti berikut: Na 12.5 ± 0.001 mg/L, K 210.4 ± 0.003 mg/L, Ca 23.9 ± 0.001 mg/L, Mg 7.1 ± 0.005 mg/L, Zn 1.9 ± 0.001 mg/L dan Fe 1.2 ± 0.004 mg/L.

CHAPTER 1

INTRODUCTION

1.1 Overview of Reishi Mushroom (*Ganoderma lucidum*)

Reishi is the Japanese name for the *Ganoderma* family of mushrooms. In Chinese, they are called “ling-zhi”. Reishi mushroom comes from the family Basidiomycetes and the genus and species are *Ganoderma lucidum*. The Latin word *lucidum* means “shiny” or “brilliant” and refers to the varnished surface of reishi mushroom’s reddish colored cap. This wood-decomposing fungus has a circular kidney-shaped cap divided by concentric growth rings, resembling a ram’s horn. The color is shiny, lacquered red to reddish-brown. The fruiting body is very woody, as is the stem. The spores are white to brown which may differ according to age, but the spore print is brown.

Ganoderma lucidum is a mushroom, a higher order organism of the world of fungus. It belongs to the Polypore group and the primary nutrients are from dead organic matter or soil. Polypores, commonly known as bracket or shelf fungi, are conspicuous mushrooms that grow off the sides of trees. As long as nutrients are available, the mycelium of *Ganoderma lucidum* can be considered perennial and will live for many years. At least once a year, mushrooms emerge from the mycelial network. Mushrooms, which act as the reproductive organ of the fungus is the means by which spores are created. The wind spreads the spores and when they land in suitable locations, the cycle recommences (Willard, 1991).

1.2 Historical Perspectives of Reishi Mushroom

Reishi mushroom is one of the treasures of Chinese medicine. Over the centuries it has gone by many names including “Lucky fungus”, “Happy herb”, “Celestial herb”, “Herb of spiritual potency”, “Holy mushroom”, “Ten-thousand-year mushroom”, “Miraculous chi”, “Auspicious herb” and “Good omen plant”.

Reishi mushroom has attained an unparalleled reputation in the Orient as the ultimate herbal substance. For over three thousand years it has been the most sought-after natural product by mountain sages as well as by the emperors and empresses of all Eastern nations (Zangari, 1998). The virtues of Reishi mushroom are believed to have been known since Chinese great antiquity. Seng Nong, the legendary herbalist, who was the emperor of the Han Dynasty, is said to have discovered the curative virtues of plants by tasting hundreds of species. “Seng Nong’s Herbal Classic” is considered to be the earliest manuscript on “Chinese Materia Medica” and is still today the foundation of “Traditional Chinese Medicine” and in general all Oriental Medicine.

The 2000 years old medicinal book “Seng Nong’s Herbal Classic” describes Reishi mushroom in details. According to the Seng Nong’s Pharmacopeias, 365 species of roots of grass, woods, furs, animals and stones are classified into 3 categories. The first category, called superior, includes herbs effective for multiple diseases and mostly responsible for maintaining and restoring the body balance. They have no unfavorable side effects. The second category, middle, comprises tonics and boosters and their consumption must not be prolonged. The third category, low, must be taken usually in small dosages and for specific

ailments. This category includes some poisonous herbs. Reishi, ranked number one of the superior medicines, was the most exalted medicine in ancient times. A “superior herb” was defined as a substance that serves to maintain life, causes no side effects by continuous usage, and promotes radiant health and long life by helping to harmonize various functions of the body, mind and spirit. Seng Nong said about Reishi that “if eaten customarily, it makes your body light and young, lengthens your life and turns you into one like the immortal who never dies.” Thus Reishi was traditionally called “The mushroom of immortality” (Zangari, 1998).

Reishi has been used in Traditional Chinese Medicine for more than 4,000 years to treat liver disorders, hypertension, arthritis, and other ailments. The Chinese have always regarded the mushroom as having special properties. Reishi mushrooms are regarded as “spirit medicine” because they are believed to nourish the “shen” or spirit. Therefore, they are considered particularly important in vegetarian diets and regarded as a medicinal food that promotes longevity. Reishi also has immune potentiating properties.

According to Asian tradition, Reishi is a nourishing tonic to the three treasures (*Jing, Qi and Shen*), which helps to build body resistance, and is detoxifying, aphrodisiac, and relaxing (Zangari, 1998). In the Taoist tradition, Reishi is said to enhance spiritual receptivity. It was used by monks to calm the spirit and mind. It is also considered as a symbol of feminine sexuality. Himalayan guides sometimes employ the Reishi to combat high altitude sickness. Mayan Indians have used Reishi to fight and prevent communicable diseases (Kosma, 2001).

1.3 Classification and Cultivation of Reishi Mushroom

There are many varieties of Reishi mushroom and they are classified by color into six types namely the Red Reishi (*Akashiba*), the Black Reishi (*Kuroshiba*), the Blue Reishi (*Aoshiba*), the White Reishi (*Shiroshiba*), the Yellow Reishi (*Kishiba*) and the Purple Reishi (*Murasakishiba*). Among these six types of Reishi mushroom, the red variety is the most potent and most commonly used (Kosma, 2001).

In 1972, researchers at Kyoto University in Japan, successfully cultivated Reishi mushroom in the laboratory. From a single species, *Ganoderma lucidum*, all six colors of Reishi mushroom could be grown by varying the temperature, humidity, carbon dioxide content and the available nutrients. Thus, it had been shown that the six types of Reishi mushroom are to be one species (Willard, 1991).

Wild Reishi is extremely rare and suffers from adverse environmental conditions, such as, insect infestation, lack of proper nutrients, non-optimum temperatures, and lack of humidity. Only one or two mushrooms can be found on a hill. The timing of the harvest cannot be controlled, and the mature Reishi has already lost most of its digestible active ingredients due to damage by insects and weather. The dried mushrooms may not have the potency of the fresh mushrooms (Willard, 1991).

Artificial culture and cultivation of *Ganoderma lucidum* were attempted initially by Henmi et al., in 1937. However, the cultivation attempts did not give satisfactory results both quality-wise and quantity-wise. Yukio Naoi from the

Kyoto University found ways to cultivate Reishi mushroom in quantity by using pots containing sawdust (Mizuno, 1997). Since the discovery of this method and with further amelioration, the production of Reishi raised more than ten folds in the last ten years. Modern bioengineering technology has made Reishi available to the general public in large quantities. The quality can be carefully controlled by providing the best conditions and sufficient nutrients (Willard, 1991). Today, an estimated 4,300 tons of Reishi is produced yearly in the world, with China producing 3,000 tons followed by Korea, Taiwan, Japan, Thailand, United States of America, Malaysia, Vietnam, Indonesia and Sri Lanka.

1.4 Cultivation and Utilization of *Ganoderma lucidum* in Malaysia

Cultivation of *Ganoderma lucidum* in Malaysia was initially attempted by Teow in 1984 by using sawdust in polypropylene or polyethylene bags in sheds under palm oil trees. Subsequently, better hybrids were obtained through cross-breed-lug which only required 40 to 45 days of incubation for its cultivation. The annual production of *Ganoderma lucidum* in Malaysia is estimated to be around 300 tons (Teow, 1997).

The fruiting bodies of *Ganoderma lucidum* (basidiocarps) are sliced and brewed and are taken as a tonic or tea. It may also be powdered or extracted with chemical solvents and the finished product is then processed into capsules (Teow, 1997).

1.5 Objectives of Present Study

Previous studies of *Ganoderma lucidum* have mainly concentrated on the medicinal properties of the mushroom. A limited information is available on the biologically active constituents of *Ganoderma lucidum*. Therefore, the objectives of this study are:

- 1) To extract biologically active constituents from *Ganoderma lucidum* by using various solvents
- 2) To conduct preliminary studies on the structure elucidation of these biologically active constituents by applying spectroscopic techniques
- 3) To determine the content of bioactive metals in *Ganoderma lucidum*

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal Effects of *Ganoderma lucidum*

Ganoderma lucidum is the most revered herbal substance in Asia, certainly ranking as the elite substance for the attainment of radiant health, longevity and spiritual attainment (Zangari, 1998). Since *Ganoderma lucidum* has been known to have many functions, it has been the subject of a great deal of research in recent years.

Ganoderma lucidum is used very widely. According to the concept of Chinese medicine, *Ganoderma lucidum* can penetrate into and work on the five key human organs, so it can be served for impairment of the heart, lung, liver, pancreas, and kidney. *Ganoderma luicudm* can cure the sickness of various human systems including respiratory, circulatory, digestive, neuronal, endocrine and locomotory. It differs from any other medicines that are used to cure specific disease, or supplementary nutrients that only address particular body requirements. *Ganoderma lucidum* helps regulate entire body mechanism and metabolism to ensure that all organs are functioning properly (Concord, 2003).

Sissi *et al.* (2004) investigated the effects of LingZhi supplementation on a range of biomarkers for antioxidant status, coronary heart disease risk, DNA damage, immune status and inflammation as well as markers of liver and renal toxicity. The results showed no evidence of liver, renal or DNA toxicity with LingZhi intake.

2.1.1 Anti-androgenic Activity

The ethanol extract of *Ganoderma lucidum* showed inhibitory activity on both isoenzymes (Type 1 and Type 2) of 5 α -reductase and suppression effects of ventral prostate growth induced by testosterone in castrated rat (Liu *et al.*, 2006). The activity-guided fractionation and TLC analysis suggested that the active principles in vivo were triterpenoids which might be a useful ingredient in the treatment of benign prostatic hyperplasia (Liu *et al.*, 2006). Fujita *et al.* (2005) reported that the extract of *Ganoderma lucidum* possess the strongest 5 α -reductase inhibitory activity.

2.1.2 Immuno-modulating and Anti-tumor Activity

Ganoderma lucidum is one of the most commonly used herbs in Asia and preclinical studies have established that the polysaccharide fractions of *Ganoderma lucidum* may have potential immuno-modulating effect in patients with advanced colorectal cancer (Chen *et al.*, 2005). Zhu *et al.* (2005) demonstrated that *Ganoderma lucidum* polysaccharides can modulate cytokines production, granzyme B and perforin in cytokine induced killer (CIK) cells thus confirmed that *Ganoderma lucidum* polysaccharides was a promising biological response modifier and immune potentiator.

According to Chen *et al.* (2004), a fraction of Reishi polysaccharide has been shown to activate the expression of IL-1, IL-6, IL-12, IFN- γ , TNF- α , GM-CSF, G-CSF and M-CSF. The expression of IL-1, IL-6 and TNF- α was able to stimulate the inflammatory response. The expression of IFN- γ and TNF- α was relevant to the anti-tumor activity, and that of GM-CSF, G-CSF and M-CSF was possibly

associated with hematopoiesis. Wang *et al.* (2001) extracted *Ganoderma lucidum* with water and identified a fucose-containing glycoprotein fraction that stimulates spleen cell proliferation and cytokine expression.

Muller *et al.* (2006) reported that *Ganoderma lucidum* extract has a profound activity against leukemia, lymphoma and multiple myeloma cells and may be a novel adjunctive therapy for the treatment of hematological malignancies.

Hsiao *et al.* (2004) reported that both the fruiting bodies and mycelium filtrate of *Ganoderma lucidum* can yield strong inhibitory effects on *ras*-induced cell transformation, with the latter seemed to be more effective.

The hot water extract of the spores of *Ganoderma lucidum* was shown to have a stimulating effect on concanavalin A-induced mitogenic activity of T lymphocytes. The polysaccharide fraction has been isolated by ethanol fractionation, anion-exchange, and size exclusion chromatography and has been identified as a branched β -D-(1 \rightarrow 3)-glucans (Bao *et al.*, 2002).

Kuo *et al.* (2005) found that *Ganoderma lucidum* mycelia can stimulate moderate levels of TNF- α , IL-6 and IFN- γ release in human whole blood suggesting the possible beneficial effect of cytokine induction by *Ganoderma lucidum* mycelia on innate immunity.

Cheung *et al.* (2000) demonstrated the presence of neuro-active compounds in *Ganoderma lucidum*. The *Ganoderma lucidum* extract can induce neuronal differentiation and prevent nerve growth factor-dependent neurons from apoptosis suggesting the possible neuro-protective effect.

2.1.3 Cancer Management

Ganoderma lucidum is used as a supplementation in cancer patients to reduce side effects during chemotherapy or radiotherapy, to prolong survival and minimize metastasis, to improve quality of life as well as to prevent occurrence or recurrence (Chang, 1997).

Sheena *et al.* (2003) had revealed that the methanolic extract of *Ganoderma lucidum* could help prevent nephrotoxicity manifested consequent to cisplatin (an anti-cancer drug) chemotherapy. The effect is mainly due to the capacity of the extract to restore renal antioxidant defense system.

2.1.4 Anti-microbial Activity

The methanolic extracts of all fruiting bodies of *Ganoderma spp.* inhibited the growth of *Bacillus subtilis* and also gave trace inhibition to *Rhodotorula mucilaginosa* suggesting that *Ganoderma* is a useful source for exploiting the anti-microbial compounds (Sudirman, 1997). Another report reveals that *Ganoderma lucidum* has the anti-bacterial activity, against *Staphylococci*, *Streptococci*, and *Bacillus pneumoniae* (Hsu *et al.* 1986).