IN VITRO EFFECT OF GANODERMA LUCIDUM EXTRACTS ON HEMATOLOGICAL PARAMETER

By

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Dissertation submitted in partial fulfillment of the requirements for the degree of Bachelor of Health Sciences (Biomedicine)

March 2005

CERTIFICATE OF APPROVAL

This is to certify that the dissertation entitled,

"IN VITRO EFFECT OF GANODERMA LUCIDUM EXTRACTS ON HEMATOLOGICAL PARAMETER"

is the bonafide record of research work done by Che Shuraya Bt Che Ismail during the period from July 2004 to March 2005 under our supervision.

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ACKNOWLEDGEMENTS

(*In the name of Allah, the most Beneficent, the most Merciful*)

I would like to express my gratitude to the following individuals for their support, guidance and input in helping me to bring forth this project to reality:

- Prof. Madya Pim Chau Dam, my supervisor and chairperson of Biomedicine Program, School of Health Sciences, Universiti Sains Malaysia, for his patient, constant support and guidance throughout this project.
- Prof Syed Mohsin Syed Sahil Jamalullail, my co supervisor and Deputy Dean of Academic and Student Affairs, School of Health Sciences, Universiti Sains Malaysia, for his faithful guidance.
- Prof. Madya Dr. Syed Hatim Noor, Coordinator of Biostatistics and Research Methodology, PPSP, USM for his interest in my study and for helping to solve statistical analyzes of my project.
- En. Md. Lukmi Ismail, Senior Scientific Officer of School of Health Sciences, Universiti Sains Malaysia, for his invaluable advice and opinion.
- All staff of Unit Kemudahan Makmal (UKM) and scientific officers of School of Health Sciences, Universiti Sains Malaysia especially for Pn Herlina, Miss Juskasmini, En. Amri, En.Nik Fakurudin, En. Mohammad Azwan, Miss Jastina and others, who were involved in helping me with the technical aspects of this study.

- Miss Noraswati Bt Mohd Nor Rashid, research officer from Medicinal Plants Programme, Biotechnology Division, Forest Research Institute Malaysia (FRIM), for her a great help in providing an expert advice at the beginning of this research project on *Ganoderma* species identification.
- My grateful thanks to all the donors who were willingly participated in this study. Without them this study would not be possible.
- My Family, especially to my mother for her full support and encouragement throughout my study.
- My colleagues, for their great help and advice especially during the preparation of this dissertation.

Lastly, thanks to everybody who has helped me in carrying out this study either directly or indirectly.

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ABSTRACT (English Version)

Thrombosis is a pathologic condition that occurs when the body forms arterial or venous blood clots that are excessively large and obstruct blood flow. Heart attack, stroke or pulmonary embolism are examples of conditions when develop in blood vessels which may contribute to various symptoms and complications. Therefore the search for an effective agent that can secure full antithrombotic benefits while minimizing the antihaemostatic outcome should be pursued. In this study, in order to assess the effect of *Ganoderma lucidum* in inhibition of platelet aggregation, two types of extract were prepared from crude powder of commercially available products of *Ganoderma lucidum*. Both the two types of extracts were tested for their activities on blood cells *in vitro*. The effects of this mushroom were tested based on the changes in hematological parameters following the treatment with different doses of water and ethanol extract *Ganoderma lucidum* using hematology analyzer (Abbott Celldyn 4000). The test duration for the observation was set for 2 hours and the observation were made every 30 minutes intervals. From this study, the results showed that there were no significant difference of effect between different doses of water and ethanol extracts on MPV. However, there were significant differences of the effect of extracts of *Ganoderma lucidum* in different concentration on MPV at different time intervals. Antiplatelet aggregation activities by indirect evaluation of changes in blood cells parameters *in vitro* following the treatment with *Ganoderma lucidum* extract may not be fully explored or elucidated by the *in vitro* study. However, the

results from this preliminary study will assist in establishing the direction of an *in vivo* study, which will be followed. In order to look at the activities of platelet and the effects on other hematological parameters *in vivo*, a long-term prospective study should be explored to prove the potential use of *Ganoderma lucidum* mushroom as a prophylactic agent for prevents arterial thrombosis.

ABSTRAK (Malay Version)

Penyakit yang berhubung dengan trombosis seperti serangan jantung, renjatan, emboli pulmonari dan lain-lain merupakan penyebab maut yang paling kerap di negara maju. Trombosis adalah satu keadaan patologi yang berlaku akibat pembentukan bekuan darah pada salur darah arteri mahupun vena. Gangguan pada pengaliran darah ini membawa kepada meningkatnya aktiviti platelet iaitu pengaktifan, pembebasan granul, aggregasi dan aktiviti lain yang berlebihan. Oleh itu, pencarian satu agen yang efektif untuk mengatasi masalah ini adalah amat diperlukan. Untuk memenuhi keperluan ini, projek penyelidikan ini dijalankan untuk mengkaji kesan kulat merah atau nama saintifiknya *Ganoderma lucidum* ke atas parameter darah terutamanya mean platelet volume (MPV). Untuk tujuan ini, dua jenis ekstrak dihasilkan daripada serbuk komersial *Ganoderma lucidum* iaitu ekstrak air dan ekstrak etanol. Ekstrak- ekstrak ini telah dikaji ke atas sampel darah secara *in vitro* dengan menggunakan kepekatan yang berbeza. Setelah ekstrak tersebut dicampurkan ke dalam darah, parameter hematologi diukur dengan menggunakan penganalisa hematologi (Abbott Celldyn 4000) untuk selang masa yang tertentu iaitu parameter hematologi telah diukur setiap 30 minit selama 2 jam. Keputusan daripada kajian ini menunjukkan kesan yang nyata ke atas MPV untuk perbezaan di antara selang masa tertentu dan kesan yang terlalu kecil atau tiada kesan yang nyata untuk perbezaan antara kepekatan dan jenis ekstrak yang digunakan. Keputusan yang diperolehi daripada kajian ini mungkin dapat membantu kajian *in vivo* atau kajian lanjutan selepas ini. Kajian jangka

panjang dengan menggunakan kaedah yang lebih specifik juga perlu dilakukan untuk mengkaji kesan kulat ini ke atas sel darah terutamanya ke atas aggregasi platelet dan potensinya dalam mencegah trombosis.

1. INTRODUCTION

During last decades, several major advancements in medicine came from lower organisms such as fungi and yeasts. Antibiotics such as penicillin and tetracycline were derived from moulds are a major example of this advancement. In another development, cyclosporin derived from a species of yeast was used as a powerful immunosuppressive drug. Therefore it would be by no means unprecedented if *Ganoderma* mushroom, which has been claimed to have a lot of medicinal properties, will one day be used routinely in medical practice.

In Asia, mushrooms have been regarded as potent medicine for thousands of years. It also has a long history of its use in traditional Chinese medicine (Lu *et al*, 2004). There are various *Ganoderma* species like *Ganoderma lucidum*, *Ganoderma applanatum, Ganoderma tsugae, Ganoderma sinense, Ganoderma oregonens and others.* In this study, commercially available product of red *Ganoderma lucidum* was used to study its effect on the hematological parameters particularly on mean platelet volume (MPV).

Ganoderma lucidum is one of the treasures of Chinese medicine. For thousands of years, *Ganoderma lucidum*, a kind of medicinal fungi, has been highly regarded by the Chinese as the "Miraculous King of Herbs." (Mizuno, 1996). In the oldest chinese medical directory, "Shen Nong's Meteria medica" and "Pen-ts'ao Kanmu" (Compendium of Materia Medica) written respectively in the Han and Ming dynasties, both rated *Ganoderma lucidum* as a superior and the best among

all other Chinese herbs. This is because it has valuable properties of an adaptogen, which means that it is non-toxic, non-specific and has a normalizing effect on the body (Concord International Trading Pty Ltd).

Ganoderma lucidum is a rare mushroom and it is extremely hard to cultivate. In nature, it grows in densely wooded mountain of high humidity and dim lighting. The spores of *Ganoderma lucidum* have tough outer husks that germination is almost impossible and these accounts for its rarity (Matsumoto, 1979). In the past *Ganoderma lucidum* grow only in small quantities wildly and relatively rare, so that it was very expensive. However, in 1972, researchers at Kyoto University in Japan had successfully cultivated *Ganoderma lucidum* in the laboratory (Willard, 1991). Now, there are many companies actively cultivate this mushroom for commercial purposes and this make it available to almost everyone who wish to consume or use it.

Its effectiveness as a highly potent medicine have been demonstrated by over 30 years of modern scientific research in Japan, Taiwan, China, U.S.A., Canada, and Poland. Backed by 5,000 years of accumulated experience, *Ganoderma lucidum* can safely claim to be totally free from side effects (Kim *et al*, 1986). However, the wide range of potential health benefits that can be derived from *Ganoderma lucidum* is enormous and unmatched.

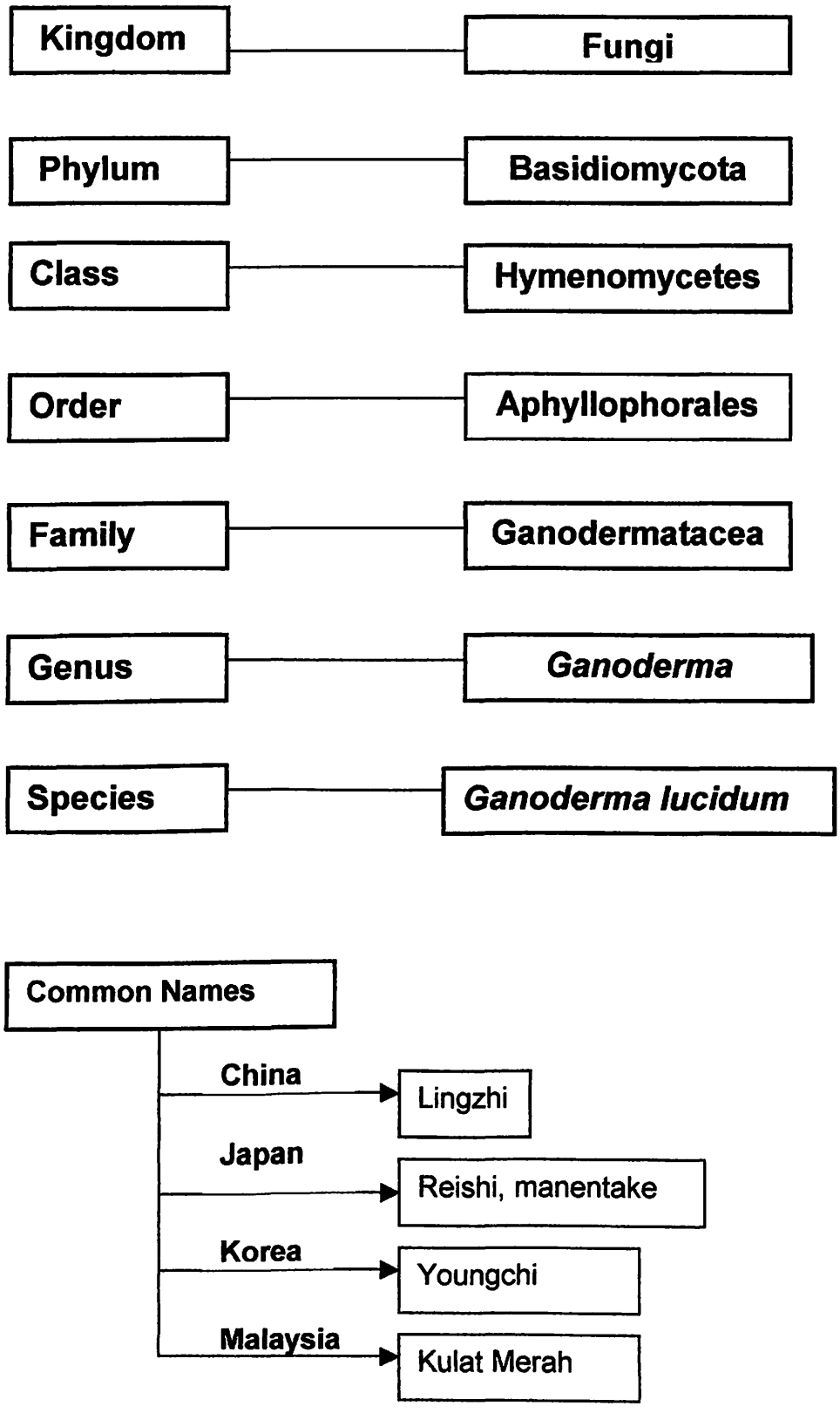

Figure 1: Taxonomy of *Ganoderma lucidum*

2. REVIEW OF LITERATURE

2.1 MORPHOLOGY

Ganoderma lucidum is the scientific name for this red mushroom. *Ganoderma lucidum* is also known as reishi or mannentake to the Japanese, lingzhi to the Chinese, youngchi in Korean and chaga to the Russian. It is a basidiomycete, lamellales fungus belonging to the family of polyporaceae. The Polyporales form a large group of diverse mushrooms. Most of them are wood decomposers whose spores are held in tubes. Many lack fully developed stems, and grow shelf-like or crust-like on wood, while some have more or less central stems and grow at the bases of trees, and a few appear to actually grow terrestrially (Kuo, 2004).

Figure 2: *Ganoderma lucidum*

The polypores fungi including *Ganoderma lucidum* can be easily distinguished from the other common poroid fungi because of their typically hard exterior, usual "non-mushroom" shape, and their usual growth on wood as wood decomposers. Some polyporologists place *Ganoderma* in a separate family, that is Ganodermataceae, because it has unusual spore ornamentation that is "double" wall properties and this characteristic is highly diagnostic (Kuo, 2004). *Ganoderma lucidum* mushrooms and their mycelium are nature's original recyclers. The fruiting bodies of this mushroom are very hard and woody, reddish-orange to black in color, have a shiny and varnished appearance on the surface of the cap. The mushroom caps are kidney shaped, 3-6 inches in width, with a slender stalk that attaches to the indentation in the side of the cap. The under side is creamy colored and porous (Maehr, 2004).

Figure 3: *Ganoderma lucidum* mushroom

Ganoderma lucidum actually possesses many different characteristics and it is dependent on the growth location (Jong and Birminghan, 1992). There are six different types of *Ganoderma lucidum* and it is differentiated by its color that is red, purple, blue, yellow, black and white. However, these mushrooms actually came from one species but grown under different condition (Zhao *et al*, 1979). *Ganoderma lucidum* is now available in capsule or tablet form, which makes it possible to avoid the bitter taste and standardize the dosage. The red variety of this mushroom is most commonly used and commercially cultivated. Beside that, the red type of *Ganoderma lucidum* is generally regarded as the work-strongest (Matsumoto, 1979).

The fruiting body of red *Ganoderma lucidum* is extremely bitter and this characteristic not found in any other mushroom. The bitter taste of this mushroom also was associated with many of its therapeutics characteristics. However, the relationship between bitterness and its pharmacological effects is not fully understood, the bitterness serves as a marker for pharmacological evaluation and classification of *Ganoderma* species. The bitterness of this mushroom varies in degree depending on the place of production, cultivation conditions and its strain (Jong and Birmingham, 1992).

2.2 MEDICINAL EFFECTS

Ganoderma lucidum is rich in mushroom nutriceutical components with potential therapeutic values. Chemical investigations on the fruiting bodies, spores and mycelia of *Ganoderma lucidum* reveal that they contain various bioactive substances. Active constituents of *Ganoderma lucidum* including polysaccharides that is a bioactive polygycans in *Ganoderma lucidum* and is found in all parts of the mushroom, organic germanium, triterpenoids, adenosine, LZ-8, and an array of amino acids besides numerous types of mineral. In addition to all of the ingredients in the fruit body, *Ganoderma lucidum* mycelium contains higher level of the ribonucleic acid (RNA), oleic acid, cyclooctasulfur and also LZ-8 (Mizuno, 1991; Yeung *et al*, 2004).

In the first Chinese herbal text, Shennong's Pharmacopeia written about 2400 years ago, *Ganoderma lucidum* was classified as a "superior herb" which is defined as one that serves to maintain life, promote radiant health and long life because of its normalizing action, and to cause no side effects, even when used continuously. Beside that, the ancient book said continuous consumption of *Ganoderma lucidum* makes your body light and young, lengthens your life and turns you into one like the immortal who never dies. Thus *Ganoderma lucidum* was traditionally called "the mushroom of immortality."

There were a lot of evidences to suggest that *Ganoderma lucidum* does have medicinal potential. The fruiting body of *Ganoderma lucidum* has been used for the prevention and treatment of various diseases in the Orient. It is a well-known traditional crude drug in the Orient to treat hepatopathy, chronic hepatitis, nephritis, gastric ulcer, hypertension, arthritis, neurasthenia, insomnia and bronchitis (Kim *et al*, 1980). In the Orient it was also used as talisman, in order to protect humans or homes against bad omen (Matsumoto, 1979).

As through the review, there were a lot of researches already done to demonstrate the medicinal effect of water and ethanol extracts of *Ganoderma lucidum*. However, most of those researches were centered on the ability of mushroom compound to enhance the immune system and exert anti-cancer actions. In modern clinical trials, the extract of *Ganoderma lucidum* show therapeutic efficacy in treating cancer and inhibits cell metastasis (Kim *et al*, 1980; Wang *et al*, 1993; Zhang *et al*, 2002).

Besides, the polysaccharide of *Ganoderma lucidum* seems to have promise as a new type of carcinostatic agent, which might be useful in immunotherapy; this is because this mushroom can give immunomodulatory effects (Morigiwa *et al.* 1986 and Mizuno, 1992). Zhang *et al,* 2002 have demonstrated, a bioactive fraction isolated from fruiting body of *Ganoderma lucidum* stimulated the activation and proliferation of mouse spleen B lymphocytes and may be effective in improving the immune response in tumor patients. Another medicinal property and potency of *Ganoderma lucidum* that has been proposed and studied was on its hypotensive

action. *Ganoderma lucidum* would limit blood pressure by blocking the renin-angiotensin system that is responsible for the hypertension and it does occur due to the presence ganoderic acid that exerts their hypotensive activities (Morigiwa *et al*, 1986).

Various studies were also done to see the effect of this mushroom on bronchitis (Dunham, 2000), anti-angiogenic (Cheng *et al,* 1986), hepatoprotective effect (Hirotani *et al*, 1986), anti-histamine release and anti allergies (Kohda *et al*, 1985), blood sugar level reducing properties (Hikino *et al*, 1985), lipolysis (Kubo *et al,* 1980), anti-HIV (Luu, 1992), anti-hyperlipidemic (Liu *et al,* 1988), analgesic (Kubota *et al,* 1982), radiation protective (Chu *et al,* 1988) and many others.

However, the primary objective of this study was to evaluate the effects of water and ethanol extracts of *Ganoderma lucidum in vitro* on blood parameters, particularly on platelet aggregation. As in the literature review, many articles have been reported that *Ganoderma lucidum* contains adenosine and this compound is responsible for the ability of this mushroom to inhibit platelet aggregation (Shimizu *et al*, 1985; Wang *et al*, 1989).

2.3 SIDE EFFECTS

Ganoderma lucidum is known generally to be safe for long term use. Kim *et al*, 1986 has reported that the extract of *Ganoderma lucidum* did not show any conceivable toxicity thus it was considered as a very safe mushroom to be consumed. However, this mushroom is a blood vessel dilator, which can accelerate blood circulation and also has the ability to inhibit platelet aggregation (Shimizu *et al*, 1985; Wang *et al*, 1991). As such, precautions should be taken to stop temporarily the usage of *Ganoderma lucidum* supplement during surgery or with severe cuts to avoid excessive bleeding. It is also not recommended for those taking anticoagulant medications. Pregnant or lactating women also should consult their physician before taking *Ganoderma lucidum* as a food supplement.

In general, *Ganoderma lucidum* is non-toxic, even when used at high therapeutic doses. Allergic reactions due to consumption of *Ganoderma lucidum* have not been reported. However, as with many herbal supplements, mild side effects may include dizziness, dryness of the mouth, throat and nasal areas, upset stomach or loose stools and abdominal upset might occur (Chang, 2004).

2.4 OVERVIEW OF HEMOSTASIS

The ability of the body to control the flow of blood following vascular injury is paramount to continued survival. The process of blood clotting and the subsequent dissolution of the clot, following repair of the injured tissue, is termed hemostasis. Hemostasis composed of four major events that occur in a set order following the loss of vascular integrity.

The initial phase of the process is vascular constriction. This limits the flow of blood to the area of injury. Next, platelets become activated by thrombin and aggregate at the site of injury, forming a temporary, loose platelet plug. The protein fibrinogen is primarily responsible for stimulating platelet clumping. Platelets clump by binding to collagen that becomes exposed following rupture of the endothelial lining of vessels. Upon activation, platelets release adenosine-5'-diphosphate, thromboxane A_2 (TXA_2), serotonin, phospholipids, lipoproteins, and other proteins important for the coagulation cascade. In addition to induced secretion, activated platelets change their shape to accommodate the formation of the plug. To ensure stability of the initially loose platelet plug, a fibrin clot forms and entraps the plug. Finally, the clot must be dissolved in order for normal blood flow to resume following tissue repair. The dissolution of the clot occurs through the action of plasmin (Guyton and Hall, 1996)

In order for hemostasis to occur, platelets must adhere to exposed collagen; release the contents of their granules and aggregate. The initial activation of platelets is induced by thrombin binding to specific receptors on the surface of platelets, thereby initiating a signal transduction cascade. The thrombin receptor is coupled to a G-protein that, in turn, activates phospholipase C-γ (PLC-γ). PLC-γ hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP_2) leading to the formation of inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of intracellular Ca^{2+} stores, and DAG activates protein kinase C (PKC). The collagen to which platelets adhere as well as the release of intracellular Ca^{2+} leads to the activation of phospholipase A_2 (PLA_2), which then hydrolyzes membrane phospholipids, leading to liberation of arachidonic acid. The arachidonic acid release leads to an increase in the production and subsequent release of thromboxane A_2 (TXA_2). This activated protein induces the release of platelet granule contents; one of which is adenosine diphosphate (ADP). ADP further stimulates platelets increasing the overall activation cascade; it also modifies the platelet membrane in such a way as to allow fibrinogen to adhere to two platelet surface glycoproteins, GPIIb and GPIIIa, resulting in fibrinogen-induced platelet aggregation (Pierce *et al*, 1999).

2.5 ANTITHROMBOTIC AGENT

Thrombotic events in high-risk patients for cardiovascular complications and stroke may be a consequence of increased platelet activation. Therefore, inhibition of platelet reactivity is important for prophylaxis of thromboembolic events. Aspirin

is the most commonly used antithrombotic agent in the prevention of vascular ischemic events (Halushka *et al*, 1995). This is because low-dose of aspirin as currently used blocks platelet production of prothrombotic thromboxane A_2 and allows endothelial synthesis of antithrombotic prostacyclin. By virtue of inhibiting the activity of cyclooxygenase, aspirin reduces the production of thromboxane A_2.

Aspirin also reduces endothelial cell production of prostacyclin (PGI_2), an inhibitor of platelet aggregation and a vasodilator. Localized to the site of coagulation is a balance between the levels of platelet-derived thromboxane A_2 (TXA_2) and endothelial cell derived PGI_2. This allows for platelet aggregation and clot formation but preventing excessive accumulation of the clot, thus maintaining blood flow around the site of the clot. Endothelial cells regenerate active cyclooxygenase faster than platelets because mature platelets cannot synthesize the enzyme, requiring new platelets to enter the circulation. Therefore, PGI_2 synthesis is greater than that of TXA_2. The net effect of aspirin is more in favor of endothelial cell-mediated inhibition of the coagulation cascade. This reflects the cardiovascular benefits to low dose administration of aspirin. However, there is a large group of patients with thrombotic disorders who do not respond to aspirin treatment (Altman *et al*, 2004).

Aspirin has reduced the incidence of vascular events by only 25% for a broad range of patients at risk for occlusive vascular diseases (Hirsh *et al*, 1995; Patrono, 1994). Prolonged usage of aspirin and ticlopidide as anti-thrombotic agent is also limited because of several adverse effects. Adverse reactions like gastric

bleeding, allergy like asthma, angioneurotic edema, urticaria, rashes, rhinorhoea and it also may aggravate chronic urticaria and other symptom may occur (Hillman and Ault, 1998)

2.6 PLATELET AGGREGATION INHIBITORY EFFECT

Ganoderma lucidum has been traditionally used for treatment of atherosclerosis, heart problems, high blood pressure, insomnia, blood clots and others. Some active compounds have been isolated from this mushroom as platelet aggregation inhibitor. It has recently been found that the water-soluble fraction of *Ganoderma lucidum* was able to suppress platelet aggregation with an inhibitory substance having been identified as adenosine. Its structure has been identified as both epimers of 5'-deoxy-5'methylsulphinyl adenosine (Shimizu *et al*, 1985). Adenosine seems to have a stronger effect on platelet aggregation induced by thrombin than on that induced by adenosine diphosphate (ADP) (Kasahara *et al*, 1987). These characteristics are in good agreement with what they have observed with the inhibitor obtained from *Ganoderma lucidum.* The content of adenosine in this fungus was found to be at least 40mg/l00 g of dried preparation. However, the adenosine content of different kinds of *Ganoderma lucidum* is different (Shiao *et al*, 1994). Experimental and clinical studies on inhibitory effect of *ganoderma lucidum* on platelet aggregation was also done previously by Toa and Feng (1990) from Tongji Medical University of Wuhan and the results suggested that this mushroom may have effective inhibitory agent of platelet aggregation.

In another study, Chen *et al* (2000) have found that Ganodermic acid S (GAS), isolated from *Ganoderma lucidum*, also was shown to have an inhibitory effect on platelet responses to various aggregating agonist such as collagen. Ganoderic acid is one of the most important components of this mushroom. These specific triterpenoids also helps to reduce blood platelets from sticking together and this effect is important in lowering the risk for coronary artery disease. GAS is an anionic amphiphile and it is highly soluble in platelet plasma membrane. Wang *et al* have found that GAS isolated from cultured mycelium of this mushroom could be inserted into the membrane of platelets. The morphologically altered membrane will occur and can contribute to inhibition of platelet aggregation (Wang *et al*, 1989).

This effect would be very significant since inhibition of platelet aggregation reduces the incidence of blood clots and strokes. However these findings are also significant in another ways. If *Ganoderma lucidum* does inhibit platelet aggregation patients with clotting disorders such as hemophilia or who are on anticoagulants therapy should be strongly advised against using *Ganoderma lucidum* as a complementary medicine. Long-term prospective studies are required to assess the effect of *Ganoderma lucidum* on inhibition of platelet aggregation. One of the possible uses of this mushroom is for prophylaxis of arterial thrombosis.

3. OBJECTIVES

The main objective of this study is to verify the effect of *Ganoderma lucidum* or red mushroom on blood parameters especially on inhibition of platelet aggregation.

-To investigate the effect and permeability of *Ganoderma lucidum* extracts on blood by focusing on hematological parameters especially on mean platelet volume (MPV).

-To compare the effect of water and ethanol extract of *Ganoderma lucidum* on mean platelet volume (MPV)

-To observe the effect of extracts of *Ganoderma lucidum* in different concentration on MPV at different time intervals.

4. MATERIALS AND METHODS

Below is a list of the equipments and materials that were used during the process of conducting my research. These equipments were obtained from the Laboratory Facility Unit (UKM- Unit Kemudahan Makmal) of Pusat Pengajian Sains Kesihatan (PPSK), Health Campus, Universiti Sains Malaysia (USM).

4.1 EQUIPMENTS

1. Electronic balance (Sartorius, BP221S)
2. Refrigerator (National, NR-B53FE)
3. Freeze dry (ilshin Lab Co. Ltd)
4. Hematology analyzer (Abbott CellDyn 4000)
5. Sonicator (Sonicor, SC-221)
6. Rotary evaporator (Heildoph Instrument, LABOROTA 4000)
7. Centrifuge (Hettich Zentrifugen, UNIVERSAL 32R)
8. Freezer (Flocchetti Frigoriferi Scientifici)
9. pH Meter (HANNA Instruments)
10. Hot air oven (BINDER, BD115)

4.2 MATERIALS

Distilled water, ethanol (98 %), beaker (250 ml, 500 ml), aluminium foil, glass rod, spatula, graduated cylinder (100 ml), filter paper, funnel, universal bottle and commercial product of *Ganoderma lucidum*.

4.2.1 *Ganoderma lucidum*

Dried powder in capsule from both stages of mushroom, the mushroom (fruiting stage) and the mycelium (vegetative stage) of *Ganoderma lucidum* were available commercially as food supplementary products. In this research project the mushroom crude (fruiting stage) of a particular brand of commercially available *Ganoderma lucidum* (Gano Excel Enterprise Sdn. Bhd) was selected for the study.

4.3 EXTRACTION PROCESS

Ganoderma lucidium comprises of over 200 active compounds (Bidleman, 2004). In order to isolate these compounds two types of extractions were done which were water extract and ethanol extract. In this study, sonicator was used to mix and to facilitate in dissolving the crude of *Ganoderma lucidum* in water and ethanol solvent. This step was critical as to ensure that those very small particles will be fully dissolved in the mixture. The break down and mixing process were done by means of ultrasonic at a very high frequency or intensity. High intensity ultrasonic generation is sufficiently powerful to achieve useful liquid processing in a wide variety of applications including to increase the solubility of the compounds.

4.3.1. Water Extraction

A water-soluble extract of *Ganoderma lucidum* was prepared as follows, about 10 gram of the fine particles of this mushroom from the capsule were accurately weighed out and placed in a beaker and then mixed with 100 ml of water. The beaker was covered with aluminium foil and the mixtures were placed in the sonicator for 10 minutes. After that, the mixtures were spun in centrifuge at 600 rpm for 10 minutes and the supernatant was obtained as water-based extraction solution. The undissolved particles were separated by filtration and these steps were repeated until 50 gram of *Ganoderma lucidum* powder was used. The separated filtrate and sediment were placed in a refrigerator to freeze them, before subjected to freeze drying in a vacuum concentrator.

4.3.2 Ethanol Extraction

For ethanol extraction, about 5 gram of the fine particles from the freeze dried sediment were accurately weighed out and placed in a beaker and then mixed with 100 ml of ethanol. The beaker was covered with aluminium foil and the mixtures were placed in the sonicator for 10 minutes. After that, the mixtures were spun in centrifuge at 600rpm for 10 minutes and the supernatant was obtained as ethanol-based extraction solution. The undissolved particles were removed by filtration and the procedures were repeated until 45 gram of *Ganoderma lucidum* powder was used. The sediment was then discarded. The filtrate was combined, and then rotaevaporated using rotary evaporator at 40°C until it concentrated to

1/10 of the original volume. Finally, concentrated ethanol extract was allowed to dry in hot air oven at 40°C until it was fully dried to obtain the ethanol extract of *Ganoderma lucidum*.

4.3.3 Principle of Separation Technique

Centrifugation was a process used to separate or concentrate materials suspended in a liquid medium. The theoretical basis of this technique was the effect of gravity on particles (including macromolecules) in suspension. Two particles of different masses settled in a tube at different rates in response to gravity. Particles experiencing a greater centrifugal force had faster sedimentation rates and thus were preferentially pulled toward the bottom of the centrifuge tube. The centrifugal force generated was proportional to the rotation rate (rpm) of the rotor and the distance between the rotor center and the centrifuge tube.

4.3.4 Freeze Drying

Freeze-drying was the process whereby water was removed from a material under freezing temperatures and a high vacuum. During freeze drying, water was transformed directly to gas phase from solid phase, which was ice, without going through liquid phase. This process of going directly from a solid to a gas is called sublimation. The process required a large amount of energy to obtain the very cold temperatures and high vacuum environment needed for this process.

Freeze drying was a very long process, often taking days or even weeks, depending upon the initial concentration of solids of the material to be dried. In this study, the samples were placed in an appropriate container for the freeze drying process. The operating temperature and pressure were set at 55°F and 200atm respectively. In this study the freeze-drying process took about 4 days to complete. Powdered extract was obtained from this process and stored in the refrigerator before being used in the dosage preparation.

4.4 PREPARATION OF PHOSPHATE BUFFERED SALINE (PBS)

SOLUTION A: 0.1M KH_2PO_4

13.6g of KH_2PO_4 was dissolved in approximately 600mL of distilled water and made up to 1 liter with distilled water

SOLUTION B: 0.1M Na_2PO_4

14.2g 0f Na_2PO_4 was dissolved in approximately 600mL of distilled water and made up to 1 liter with distilled water

Solution A and B were stored at 4°C

Preparation of pH 7.3 Buffered Saline

100 ml of a mixture of 23.6 ml of solution A and 76.4 ml of solution B were added to every 900 ml of unbuffered saline (0.90%w/v). Following that, the pH of the solution was checked using pH meter. Adjustments were made to obtain a correct pH using 0.1M HCl or 0.1M NaOH (Green *et al*, 1987).

4.5 DOSE PREPARATION

4.5.1 Materials

Ganoderma lucidum extract powders, phosphate buffer saline (PBS), dimethyl sulphoxime (DMSO), electronic balance (Sartorius BP221S), spatula, glass rod, EDTA tube (5 ml), plain tube, blood specimen, beaker (50 ml), syringe and needle (10 ml), gauze, pipette 1000μl, 20μl (Gilson Pipetman), blue and yellow tips.

4.5.2 Dosage Preparation

In this preliminary study, dosages were chosen based on recommendation by the manufacturer Gano Excel Enterprise Sdn. Bhd., the company that commercially *Ganoderma lucidum* crude for this study were obtained. Based on the instruction recommended by the manufacturer, the consumers have to take two capsules per day of this mushroom food supplement for general health maintenance. One capsule contains about 300 mg of dried powder of *Ganoderma lucidum*. Therefore, two capsules of *Ganoderma lucidum* contained about 600 mg of crude mushroom powder. It was assumed that normal adults have total blood volume of approximately 5 liters in their body.

Based on the above assumptions, the following calculations were performed:

5000 ml = 600 mg (daily)

So 1 ml = $\frac{600mg}{5000\ ml}$ = 0.12 mg/ml blood

Three different doses were used in this study; 0.12 mg/ml, 0.012 mg/ml and 0.0012 mg/ml. Two types of extracts were prepared and used in this study. They were water and ethanol extract of *Ganoderma lucidum*.

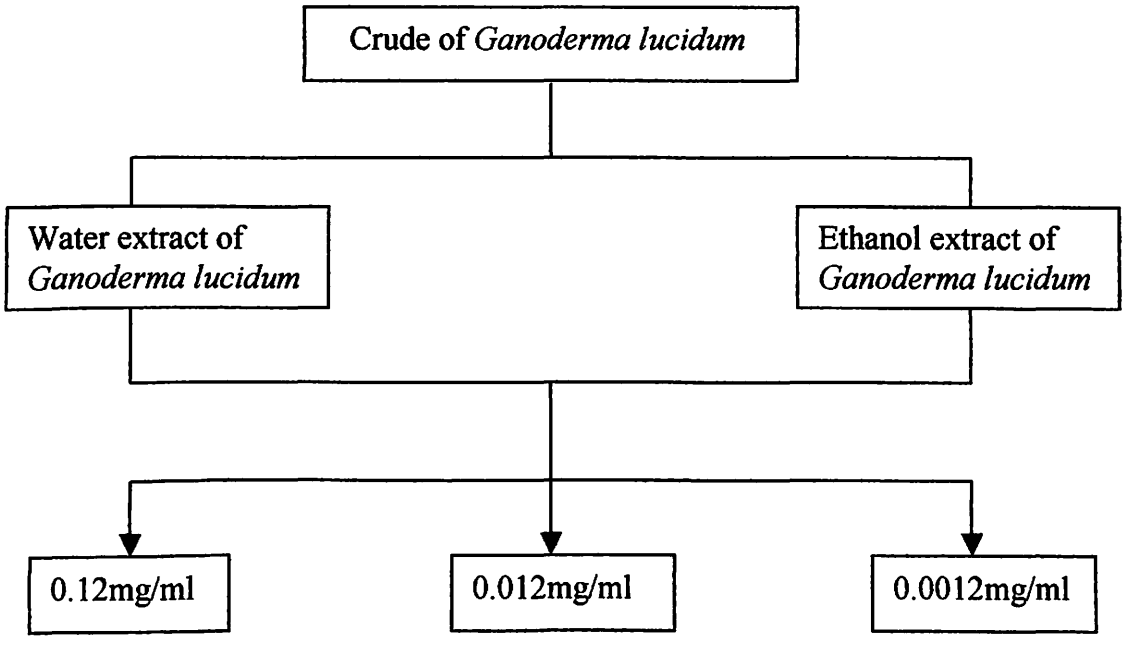

Figure 4: Dosage prepared from water and ethanol extract of *Ganoderma lucidum*

4.5.3 0.12mg/ml of water and ethanol extract

The dose 0.12 mg/ml is equivalent to the concentration of 12 mg per one milliliter of PBS. In the dosage preparation, about 0.12 gram of the freeze-dried powder of *Ganoderma lucidum* were accurately weighed out and placed into a 10ml volumetric flask and dissolved with 10 ml of PBS. The solution was mixed until all of the extract was completely dissolved in the PBS. Then, the mixture was transferred into universal bottle. These same steps were repeated for preparation of 0.12 mg/ml of ethanol extract.

4.5.4 0.012mg/ml of water and ethanol extract

The dose 0.012 mg/ml is equivalent to the concentration of 1.2 mg per one milliliter of PBS. In the dosage preparation, about 0.012 gram of the freeze-dried powder of *Ganoderma lucidum* was accurately weighed out and placed into a 10ml volumetric flask and dissolved with 10 ml of PBS. The solution was mixed until all of the extract was completely dissolved in the PBS. Then, the mixture was transferred into universal bottle. These same steps were repeated for preparation of 0.012 mg/ml of ethanol extract.

4.5.5 0.0012mg/ml of water and ethanol extract

The dose 0.0012 mg/ml is equivalent to the concentration of 0.12mg per one milliliter of PBS. In the dosage preparation, about 0.0012 gram of the freeze-dried