CHAPTER 2

LITERATURE REVIEW

2.1 Hypertension

2.1.1 Classification of blood pressure

Basically, blood pressure (BP) is classified into four stages as shown in Table 2.1. Pre-hypertension is not considered as a disease but the category reflects the high risk of a person in developing hypertension. At this particular level, the person mostly would not be prescribed with antihypertensive drug, in fact the person would be recommended to engage in a healthier lifestyle in order to reduce the risk of hypertension.

2.1.2 Prevalence of hypertension

Hypertension is recognised as a grievous global health problem due to the tremendous increase in the frequency of hypertensive patients as well as the catastrophic sequela caused by its occurrence (Ray *et al.*, 2004; Kearney *et al.*, 2005). Current report from World Health Organization (WHO) revealed the predominance of hypertension in 2008 which accounted approximately 40 % of adults aged 25 and above around the globe.

Across the WHO regions, the highest incidence of hypertension was reported in African region. With respect to gender, the prevalence of raised blood pressure in men was found to be slightly higher than women. Moreover, socioeconomic status of a country was also suggested to be related to the prevalence of hypertension. High-income countries showed low occurrence of hypertension. Studies revealed that factors including under-nutrition, infectious disease and lack of treatment contributed to the rise of hypertension in low- and middle-income countries (Mendez *et al.*, 2003; Damasceno *et al.*, 2009).

Table 2.1 : Blood pressure classification

BP Classification	SBP (mmHg)	DBP (mmHg)
Normal	< 120	and < 80
Pre-hypertension	120-139	or 80-89
Stage 1 Hypertension	140-159	or 90-99
Stage 2 Hypertension	≥ 160	or ≥ 100

Adapted from the 7th Report of Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure (Chobanian *et al.*, 2003).

2.2 Renin angiotensin aldosterone system (RAAS)

The renin angiotensin aldosterone system (RAAS) is responsible in regulating blood pressure in human body. RAAS involves a series of enzymatic reaction by the involvement of kidney, liver and lung (Figure 2.1). A drop in blood pressure will be detected by the kidney and followed by the release of renin into the blood stream. Renin, a circulating enzyme, does not affect the blood pressure by itself. Instead, it plays role in catalysing the conversion of angiotensinogen to angiotensin I (Haber, 1976). Angiotensinogen is an inactive peptide produced by the liver.

Angiotensin converting enzyme (ACE) (EC 3.4.15.1) is widely distributed in the body and can be found in the blood (Skeggs *et al.*, 1956), kidney (Erdös and Yang, 1967), lung (Ng and Vane, 1967), brain (Ganten *et al.*, 1971), intestine (Ward *et al.*, 1980) and prostate (Erdös and Skidgel, 1986). The wide distribution throughout the body indicates that ACE plays an important role in RAAS (Igić and Behnia, 2003). ACE is responsible in converting angiotensin I to angiotensin II through a dipeptide cleavage and simultaneously involves in the inactivation of bradykinin. Table 2.2 describes the distribution of ACE in the human body.

Angiotensin II is a potent vasoconstrictor, acting directly on vascular smooth muscle cells (Folkow *et al.*, 1961). Upon formation, angiotensin II interacts with the sympathetic nervous system both peripherally and centrally to increase vascular tones (Zimmerman *et al.*, 1984). Besides, angiotensin II stimulates adrenal cortex to secrete aldosterone and induced posterior pituitary to secrete antidiuretic hormone (ADH). This leads to volume expansion through sodium retention via aldosterone (Biron *et al.*, 1961) and fluid retention via ADH (Padfield and Morton, 1977). Hence, inhibition on ACE has been viewed as a therapeutic target for treating hypertension.

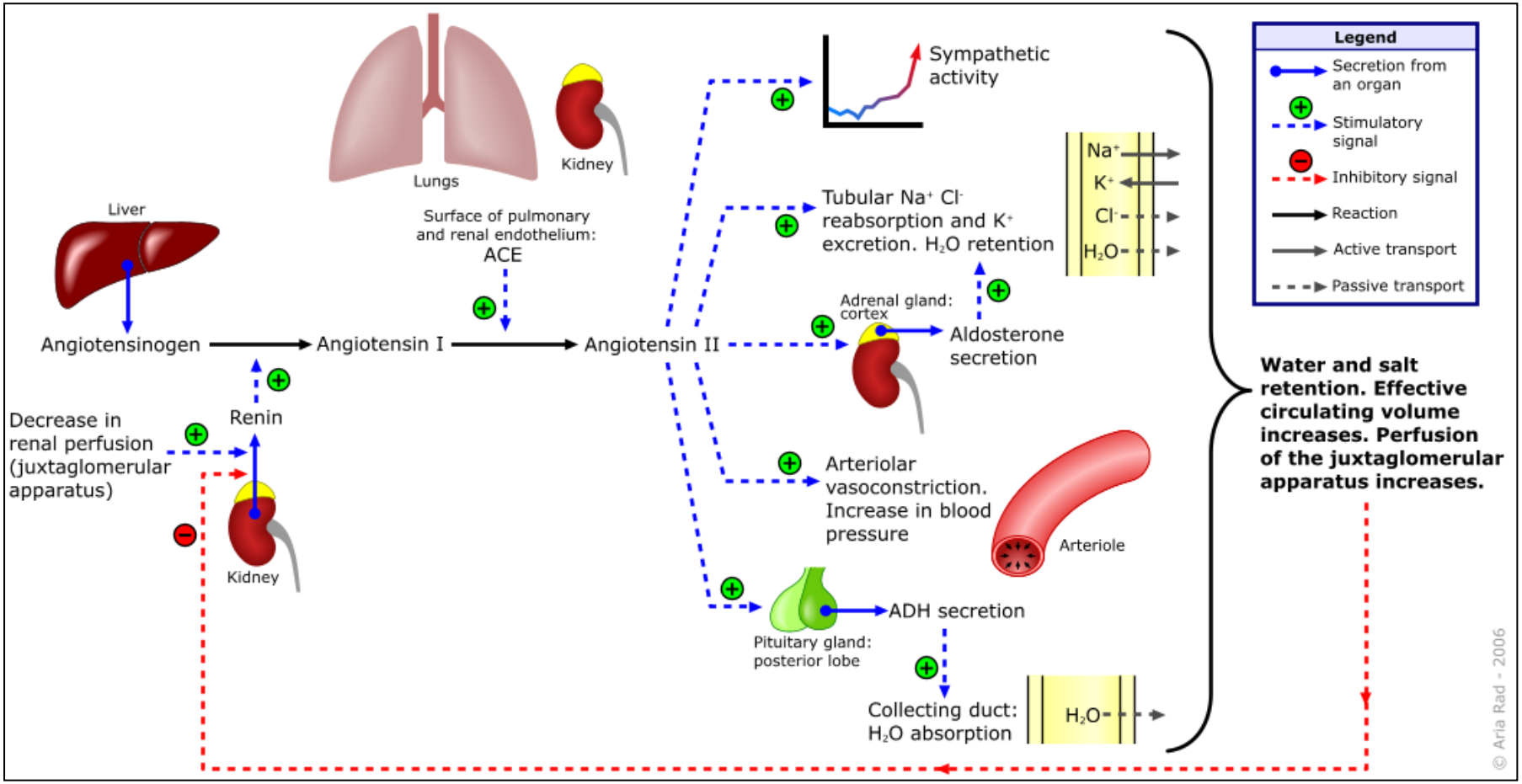

Figure 2.1 : Enzymatic and hormonal pathways in RAAS

Retrieved from http://en.wikipedia.org/wiki/Renin-angiotensin system on 30th July 2010.

Table 2.2 : Distribution of angiotensin I-converting enzyme (ACE) in human body

Source of ACE	Membrane-bound	Soluble
Epithelial cells		
Microvilli; brush border of placenta, kidney, intestine, choroid plexus	+	-
Neuroepithelial cells		
Subfornical organ, pallidonigral dendrites, median eminence, etc.	+	-
Male genital tract		
Testes	+	-
Prostate, epididymis	+	+
Seminal plasma	-	+
Body fluids		
Blood, urine, lung, amniotic fluid, cerebrospinal fluid, lymph	-	+

Adapted from Erdös (1990).

2.3 ACE inhibitory assay

Discovery of ACE by Skeggs and his associates (1956) led to the earliest knowledge that inhibition of ACE by ethylenediaminetetraacetic acid (EDTA) decreased the formation of angiotensin II. Inactivation of ACE alternatively results in the production of bradykinin, a potent vasodilator peptide. The vasodilatory effect is exerted via stimulation of specific endothelial B2 receptors which causes the release of nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor (Hornig *et al.*, 1997). Thereby, these findings brought researchers to basic concept of lowering blood pressure through ACE inhibition.

Most of the methods for *in vitro* screening of ACE inhibitory activity used Hippuryl-Histidyl-Leucine (HHL) as the synthetic substrate. The ACE inhibitory assay using the tripeptide was originally described by Cushman and Cheung (1971) and had undergone several modifications by later researchers (Quirós *et al.*, 2005; Ma *et al.*, 2006). Among a few synthetic ACE substrates, HHL structure represents the closest resemblance to angiotensin I (van Platerink *et al.*, 2007).

Another renowned assay was suggested by Holmquist *et al.* (1979) which utilized N-[3-(2-furyl)acryloyl]-l-phenylalanylglycylglycine (FAPGG) as the synthetic substrate. FAPGG was found to possess high solubility, high stability and the value of K_m was eight times less than HHL. Upon hydrolysis of the substrate by ACE, furylacryloylphenylalanine and glycylglycine are produced. The release of the peptides is observed at a wavelength of 340 nm over time. The absorbance versus time curve obtained is direct reflecting the ACE activity. Assay performed using FAPGG is more rapid than HHL due to less step required (Shalaby *et al.*, 2006).

The latest synthetic substrate was proposed by Lam *et al.* (2007), in which they used 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG) to screen the ACE inhibitory

activity. The presence of ACE and aminoacylase hydrolyzes 3HB-GGG, thereby releases 3-hydroxybutyric acid (3HB) as the end product. The amount of 3HB produced is observed using F-kit which contains iodonitrotetrazolium chloride (INT) and measured at 492 nm. Modification of the assay has been done where F-kit was replaced by water soluble tetrazolium salt, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (WST-1) for the detection of 3HB (Lam *et al.*, 2008).

The wide range of ACE inhibitory assays involved various analytical methods including fluorometry (Oliviera *et al.*, 2000), radiochemistry (Ryan *et al.*, 1980; Meng and Oparil, 1996), spectrophotometry (Erickson *et al.*, 2003; Serra *et al.*, 2005), high-performance liquid chromatography (Umeki *et al.*, 2008) and capillary electrophoresis (Chang *et al.*, 2001). Basically, those methods were used based on availability of the instruments, easy manipulation and sensitivity.

2.4 ACE inhibitors

In recent clinical practise, hypertensive patients are treated by giving appropriate dose of anti-hypertensive agents such as ACE inhibitors, diuretics, beta blockers, angiotensin receptor blockers and calcium channel blockers. The paramount aim in treating hypertension is to bring down the blood pressure to its normal level. Most of the physicians prescribe thiazide-type diuretics as the initial therapy for hypertension. Nevertheless, the drugs are not compatible with individual having hypertension with other coexisting health problems.

Alternatively, ACE inhibitors are recommended as the first line drug for treating hypertensive patient associated with chronic congestive heart failure (Brunner-La Rocca *et al.*, 1999), myocardial infarction (Vaughan *et al.*, 1997), diabetes

(Pahor *et al.*, 2000; Ramos-Nino and Blumen, 2009) and chronic kidney disease (Maschio *et al.*, 1996). The additional health promoting benefit of ACE inhibitor was reported by Charrier and co-workers (2004). They discovered that inhibition of ACE might contribute in the treatment of hematopoietic toxicity caused by irradiation.

Earlier on, ACE inhibitors were discovered as bradykinin-potentiating peptides from the venom of the South American snake *Bothrops jararaca,* as well as other venomous snakes. Based on the previous findings, Cushman and co-workers developed Captopril, which became the first clinically-approved synthetic ACE inhibitor (Cushman *et al.*, 1977; Ondetti *et al.*, 1977; Cushman & Ondetti, 1999).

There is a variety of ACE inhibitor drugs available on the market bench and are used universally in treating hypertension (Table 2.3). The drugs include Captopril, Enalapril, Fosinopril, Lisinopril, Perindopril, Ramipril, Trandolapril and Zofenopril. There are several differences among ACE inhibitors with respect to their molecular structure, potency, bioavailability, plasma half-life and tissue affinity (Hernandez and Harrington, 2008).

Variation in molecular structure lies on the group which binds to the ACE inhibitors. Captopril and Zofenopril possess sulphydryl group in their molecular structure while Fosinopril has a binding of phosphinyl group. Other ACE inhibitors are known to adapt carboxyl as the binding group. Apart from that, some of the ACE inhibitors are categorized as prodrug in which they require biotransformation through body metabolism before being active. The consumption of synthetic ACE inhibitors was reported to bring side effects to human including cough, taste disturbance, angioedema and rashes of the skin (Atkinson and Robertson, 1979; Wilkin *et al.*, 1980; Gianos *et al.*, 1990).

Table 2.3 : List of synthetic ACE inhibitors available in the market

Drug	Binding group	Prodrug
Captopril	Sulphydryl group	No
Enalapril	Carboxyl group	Yes
Fosinopril	Phosphinyl group	Yes
Lisinopril	Carboxyl group	No
Perindopril	Carboxyl group	Yes
Ramipril	Carboxyl group	Yes
Trandolapril	Carboxyl group	Yes
Zofenopril	Sulphydryl group	Yes

The presence of sulphydryl binding group was suggested to attribute to skin rashes, taste disturbance and proteinuria (Reid, 1997). Earlier study showed the direct effect of ACE inhibitors towards activation of bradykinin receptor (Ignjatovic *et al.*, 2002) and that the receptor gene polymorphism was suggested to colligate with cough induced by ACE inhibitors (Mukae *et al.*, 2000).

2.5 The search of naturally occurring ACE inhibitory peptides

Divulgence of synthetic ACE inhibitors' side effects by clinical trials has inspired scientists to investigate a safer alternative. Numerous attempts have been made to explore natural products as hypertensive cure including ACE inhibitory peptides. ACE inhibitory peptides have been increasingly acknowledged since the bioactive peptides are less expensive and serve safer blood pressure lowering effect compared to conventional ACE inhibitor drug.

2.5.1 ACE inhibitory peptides from dairy products

Dairy products are considered as an excellent source of protein, minerals and vitamins. Accordingly, a research conducted by He *et al.* (2011) reported a significant relationship of dietary protein intake towards improving blood pressure. ACE inhibitory peptides have been discovered in cheese (Abubakar *et al.*, 1996), bovine milk (Pihlanto-Leppälä *et al.*, 1998), goats milk (Geerlings *et al.*, 2006), fermented milk (Nakamura *et al.*, 1995; Seppo *et al.*, 2003; Miguel *et al.* 2006 and Pihlanto *et al.*, 2010) and yoghurt (Chobert *et al.*, 2005).

Jäkälä and Vapaatalo (2010) suggested that the ACE inhibitory peptides derived from milk products possessed low molecular weight and short chain structure. At present, some of the peptide inhibitors of ACE have been successfully commercialized

such as Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP), which are marketed with the trademark of Calpis® and Evolus® respectively (Korhonen, 2009).

2.5.2 Plant-derived ACE inhibitory peptides

A report has suggested that increase dietary intake of plant protein found to exert a more beneficial effect on blood pressure compared to protein from animal (Elliott *et al.*, 2006). Erstwhile studies succeeded to discover anti-ACE peptides in several selected vegetables such as garlic (Suetsuna, 1998), spinach (Yang *et al.*, 2003), broccoli (Lee *et al.*, 2006), potato (Pihlanto *et al.*, 2008) and peas (Barbana and Boye, 2010).

Furthermore, rapeseed (Pedroche *et al.*, 2004), buckwheat (Ma *et al.*, 2006) and amaranth (Vecchi and Añón, 2009) also showed promising effect in improving blood pressure level. Interestingly, a study has suggested flower to be a potential hypertensive cure due to the presence of its ACE inhibitory peptides (Megías *et al.*, 2009).

2.5.3 ACE inhibitory peptides from marine products

Nowadays, scientists are highly interested to study marine products since they are not only considered as food sources but also crucial as the source for bioactive peptides. ACE inhibitory peptides have been derived from tuna (Kohama *et al.*, 1991), algae (Okino *et al.*, 1993), fish (Byun and Kim, 2001; Jung *et al.*, 2006), oyster (Je *et al.* 2005), hard clam (Tsai *et al.*, 2008) and sea cucumber (Zhao *et al.*, 2009). The ACE inhibitory nonapeptide discovered by Wang *et al.* (2008) was found to be resistance towards heat and pH. In fact, the peptide was found to possess good stability when subjected to gastrointestinal enzymes.

2.5.4 ACE inhibitory peptides extracted from mushrooms

The broad medicinal values of mushrooms have prompted the search for ACE inhibitory peptides. Ohtsuru *et al.* (2000) have proposed three peptides from digested *Grifola frondosa* with inhibitory activity against ACE. Accordingly, Choi *et al.* (2001) reported a novel peptide from the same mushroom with the IC_{50} value and amino acids sequence of 0.097 mg and Val-Ile-Glu-Lys-Tyr-Pro, respectively.

Later, Lee *et al.* (2004a) announced the discovery of novel tripeptide ACE inhibitor from the fruit bodies of *Tricholoma giganteum*. Due to low molecular weight properties, it gave credit to the tripeptide to hinder degradation by stomach's enzymes as well as easily absorbed in the intestine. *Pholiota adiposa* was claimed to be a good source for alternative antihypertensive agents by the reason of its anti-ACE properties (Koo *et al.*, 2006).

2.6 Introduction to mushroom

2.6.1 Definition of a mushroom

Mushrooms belong to the kingdom of Fungi, one of the five major kingdoms of living organisms on earth. The term 'mushroom' is used according to the definition of Chang and Miles (1992) as a macrofungus with a distinctive fruiting body which can be either epigeous or hypogeous, and are large enough to be seen with naked eyes as well as to be picked up by hand.

2.6.2 Nutritional and medicinal value of mushrooms

Anthropologists claimed that the humanity's use of mushrooms has been initiated in Palaeolithic era, where Old Stone Age people only ate wild plant foods since cultivation of crops had not yet developed during that time. The discovery of

archaeological evidence has vindicated mushrooms utilisation during primitive human life (Samorini, 1992; Akers *et al.*, 2011).

Humankind has gained benefits from mushrooms for millennia. It was learnt that people from earlier time consumed mushrooms for nutritional value in order to complete their diet intake. Mushrooms constitute variety of nutritive compounds such as carbohydrates, proteins, fats, minerals, vitamins and fibre (Kanwar *et al.*, 1990; Chang and Buswell, 1996; Singh and Singh, 2002).

Mushrooms have been used extensively in food preparation as flavour enhancers especially in Japanese and Chinese cooking. Mushrooms such as *Lentinula edodes* and *Cordyceps militaris* were incorporated in cooking to boost the appetite without using artificial flavouring agents. The occurrence of natural pleasant taste was caused by the synergistic effect of flavour 5'-nucleotides with monosodium glutamate (MSG)-like components (Yamaguchi and Ninomiya, 2002).

Instead of consumption as a source of food, medicinal mushrooms have been exalted for their therapeutic potential since the early days of civilization. Ancient Egyptians believed mushrooms were gifts from the god Osiris and could bring immortality by eating them. In the Asian region, *Ganoderma lucidum*, *Trametes versicolor*, *Hericium erinaceus* and *Grifola frondosa* were among the famous medicinal mushrooms which have been valued by Japanese as well as Chinese folks for long time ago (Halpern, 2007).

2.7 *Ganoderma lucidum*

2.7.1 Taxonomy and characteristics

Ganoderma lucidum (Curtis) P. Karst is a basidiomycete mushroom, which has significant interactions with human beings for centuries in terms of medicinal and

nutritional purposes. *Ganoderma* is the largest genus in Aphyllophorales order and more than 300 species have been discovered (Bhosle *et al.*, 2010). The taxonomy of *G. lucidum* is shown in Table 2.4.

According to Wasser (2005), *G. lucidum* is called by different names in different regions. In China and Korea, people called the mushroom as Lingzhi which means 'herb of spiritual potency' while the Japanese named it as Reishi. The word *Ganoderma* is originally derived from Greek where 'ganos' means brightness while 'derma' means skin. The word *lucidum* originated from Latin word which means shiny. As the name suggests, it reflects to the varnished appearance of the mushroom's fruiting bodies.

Worldwide, *G. lucidum* can be found in Asia, Europe, Africa, Americas as well as in Australia region. The major distribution of the mushroom is reported to be in China, Japan and Korea. Mostly found on decaying woods, the fruiting bodies are made of hard woody dark reddish kidney-shaped basidiocarp (Figure 2.2). Due to the tough properties of the fruiting bodies, *G. lucidum* is categorized as non-edible mushroom.

During young stage of growth, the varnished surface appears to be red, bright yellow and white. When the mushroom matures, the yellow and white shades disappear and result in a dark reddish brown surface. The underside of the basidiocarp forms pores that are made of opened tubes. Normally, the surface of the pores is white in colour during active growth. A structure called basidium is borne in these tubes and it consists of two haploid nuclei. Both nuclei will fuse and undergo meiosis resulting in the formation of four haploid nuclei, referred as basidiospores. The basidiospores of Ganodermataceae have a unique characteristic among other polypores as it exhibited the presence of distinct double wall (Donk, 1964).

Table 2.4 : Taxonomy of *G. lucidum*

Kingdom	Fungi
Phylum	Basidiomycota
Class	Basidiomycetes
Order	Polyphorales
Family	Ganodermataceae
Genus	*Ganoderma*
Species	*lucidum*

Figure 2.2 : *Ganoderma lucidum*

2.7.2 Medicinal properties and bioactive compounds

According to Chinese folklore, *G. lucidum* has been regarded as the panacea to treat a broad range of diseases. Numerous modern scientific studies have been performed to support the claim. Bioactive compounds were identified as the constituents that demonstrated favourable effects in improving human health. Discovery of the valuable compounds gave impetus to researchers to carry out a more comprehensive investigation. Table 2.5 describes some of the *G. lucidum* therapeutic effects and bioactive compounds which have been discovered.

2.7.3 Growth stages

Ganoderma lucidum growth generally can be described in three phases which are the stages of spore, mycelia and fruiting bodies. The growth in these different stages is described briefly in Figure 2.3.

The life cycle of *G. lucidum* starts with the release of a million spores by mature fruiting bodies. Under optimal condition, the air-borne spores will germinate into fine filaments known as hyphae. Later on, the mass of hyphae will fuse and intertwine to each other to form mycelium. Mycelium is the essential part of the mushroom where it plays vital role in nutrients intake during the organism's growth. Enzymes will be excreted by the mycelium into the environment in order to break down the complex organic compounds of the substrate. The products of the catalysis are in the form of simpler compounds, hence will be easily absorbed through the hyphae cell wall. The mycelium will continuously grow until an ideal environment triggers the sprouting of fruit body.

Table 2.5 : Therapeutic effects and bioactive compounds of *G. lucidum*

Therapeutic effects	Bioactive compounds
Anti-oxidant activity	Polysaccharides (Jia *et al.*, 2009; Yang *et al.*, 2010) Phenolic compounds (Heleno *et al.*, 2012)
Anti-herpetic activity	Acidic protein bound polysaccharide (Eo *et al.*, 1999) Proteoglycan (Liu *et al.*, 2004)
Anti-cancer and anti-tumour activity	Ganodermanondiol (Gao *et al.*, 2002) Ganoderiol F (Gao *et al.*, 2006; Zhang *et al.*, 2009) Lucialdehydes B and C and Ganodermnonol (Gao *et al.*, 2006) Ganoderic acid T (Tang *et al.*, 2006) Ganoderic acid Me (Wang *et al.*, 2007) Ganoderic acids F, K, B, D and AM1 (Yue *et al.*, 2010) Polysaccharides (Zhao *et al.*, 2010)
Immunomodulation	Protein LZ-8 (Kino *et al.*, 1989) Polysaccharides (Chen *et al.*, 2004; Ji *et al.*, 2007; Guo *et al.*, 2009; Huang and Ning, 2010) Membrane-associated glycoprotein (Lim *et al.*, 2004)
Hepatoprotective	Polysaccharide (Zhang *et al.*, 2002) Proteoglycan (Yang *et al.*, 2006) Peptides (Shi *et al.*, 2008)
Anti-HIV activity	Ganoderic acid α, B, C1 and H, ganoderiol A, B and F and ganodermanontriol (El-Mekkawy *et al.*, 1998)
Anti-inflammatory activity	Ganoderic acids A, F, DM and TQ (Akihisa *et al.*, 2007)
Anti-androgenic activity	Ganoderol B (Liu *et al.*, 2007)
Cholesterol lowering effect	Ganoderol A and B, ganoderal A and ganoderic acid Y (Hajjaj *et al.*, 2005)
Anti-diabetic activity	Polysaccharides (Zhang and Lin, 2004; Jia *et al.*, 2009)
Anti-hypertensive activity	Ganoderic acids Y, F, H, B, K, S and D (Morigiwa *et al.*, 1986) Mycelium extracts (Compounds were not reported) (Kabir *et al.*, 1988; Lee and Rhee, 1990)

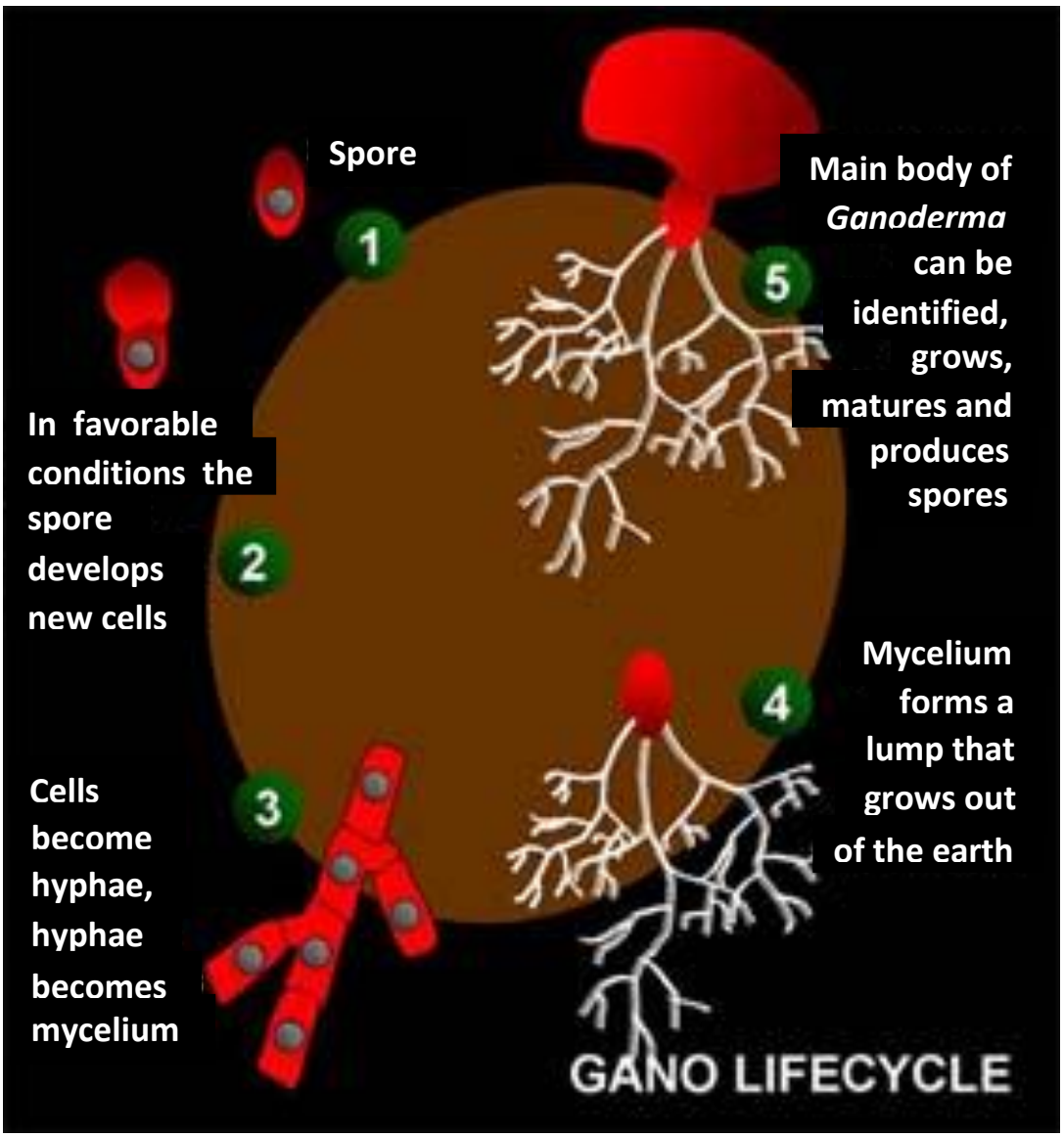

Figure 2.3 : Life cycle of *G. lucidum*

Retrieved from http://www.hbp.usm.my/1b/GT/ganoderma.htm on 9th June 2011.

2.7.4 Cultivation of mycelia

Ganoderma lucidum fruiting bodies showed a good anti-hypertensive potential (Abdullah *et al.*, 2011) and could be developed as medicinal products. However, the production of the fruiting bodies is time consuming and might not be tolerated with the market demands. Traditional basidiocarp cultivation acquired at least 3 to 5 months for the fruit bodies to form. Besides, it was difficult to control the quality of the fruiting bodies produced due to inconsistency of weather, substrates used as well as pests threat (Tang *et al.*, 2007).

Recent study has suggested the anti-hypertensive properties exhibited by cultured mycelia of *G. lucidum* (Lee and Rhee, 1990). With respect to rapid production which approximately 6 times faster than the fruiting bodies, cultivation of mycelia appeared to be an alternative approach to produce therapeutic compounds of *G. lucidum*. At present, high attention has been given to cultivate mycelia by submerged cultivation since such technique allowed mycelia to disperse more uniformly in the substrate media. Besides, formation of sporophores was found to hasten in submerged cultivation than in solid spawn (Song and Cho, 1987; Yang and Liau, 1998).

Furthermore, submerged cultivation is highly advantageous since it is able to enhance the production of mycelia biomass as well as bioactive compounds (Fang and Zhong, 2002; Berovič *et al.*, 2003). Mycelia biomass seems to be the prolific resource for various therapeutic proteins including proteoglycan (Sarangi *et al.*, 2006) and polysaccharide peptides (Wang *et al.*, 1995; Chan and Yeung, 2006.