INTRODUCTION

Mitochondria play a central role in energy-generating processes within the cell. Apart from this important function, they house the multiple metabolic pathways, such as β-oxidation of fatty acids and the TCA and urea cycles, control intracellular Ca^{2+} metabolism and signaling, regulation of thermogenesis and serve as the gatekeeper of the cell for programmed cell death (apoptosis) (Scheffler, 1999). Mitochondria are the main source of the superoxide radical and other reactive oxygen species (ROS) generation (Chance et al., 1979). Normally, ROS are decomposed or their peroxidation products are neutralized by natural defense. However, under conditions of increased ROS generation they may accumulate exerting a potent damaging effect on the cell and the whole organism (Raha and Robinson, 2000). ROS can damage mitochondrial macromolecules either at or near the site of their formation. Therefore, apart from as a source of ROS, the mitochondria themselves can be damaged by ROS leading to partial or full damage or dysfunction. A rapidly expanding body of literature recently suggests that mitochondrial dysfunctions play pivotal roles in various diseases including neurodegenerative disorders, cardiac damage, liver damage, diabetes and aging (Scatena et al., 2007).

A pathogenetic role for mitochondrial dysfunction has been invoked in a vast number of illnesses without sufficient experimental support to precisely establish the molecular pathophysiological mechanisms. The complexity and heterogeneity associated with this organelles making difficulty in getting evidence to elucidate the exact mitochondrial pathophysiology. Mitochondrial damages are associated with mutations to its DNA, decreased production of ATP, formation of free radicals, and alterations in cellular calcium handling. These events may eventually lead to the opening of the mitochondrial permeability transition pore, an event linked to apoptotic cell death (Scatena et al., 2007). The typical structural and functional characteristics may make the mitochondria a valuable target for many drugs (Szewczyk and Wojtczak, 2002). These unique aspects of mitochondria stimulated us to study the interrelationships between drugs and mitochondria.

The preservation of mitochondrial function is important for maintaining overall health of the cell and also the organism. Mitochondrial targeting of antioxidants to preserve the genomic and structural integrity of mitochondria and to increase the functional life span is a promising new area that may overcome problems in bioavailability and tissue distribution of antioxidants

(Sheu et al., 2006). The small molecule antioxidants such as L-carnitine, ascorbate, α-tocopherol, reduced coenzyme Q_{10}, urate and glutathione and N-acetyl cysteine are chain-breaking antioxidants with a capacity to repair oxidizing radicals directly (Sheu et al., 2006). DL-α-Lipoic acid is well known as a powerful biological antioxidant and its therapeutic potential has been explored extensively in the protection of mitochondria against aging and chemical induced oxidative stress (Shay et al., 2009). Recently drugs such as the mitochondria targeted versions of CoQ10 and vitamin E, 4-hydroxy-2,2,6,6-tetramethylpiperidin-N-oxide (TEMPOL) and Salen-Mn (III) complex of o-vanillin (EUK-134) have been successfully synthesized and partially tested in term of their antioxidant and mitochondrial protective properties (Smith et al., 2003).

However, data from well-designed randomized trials to issue the general recommendation for people to take antioxidant supplements in order to prevent the oxidative stress induced disease is still insufficient and the area of sub-cellular, i.e. mitochondria-specific delivery of drugs is still in its infancy. Hence, search for the development of new safe and effective mitochondrial protective antioxidants is of major importance. Medicinal mushrooms are well known as promising remedy against various life threatening diseases including cancer and AIDS (Wasser, 2011). These important properties of mushrooms are derived from the various pharmacologically important chemical constituents present in them. Triterpenes, proteins and polysaccharides are some of the potent bioactive molecules and they represent the most diverse and yet important group of compounds found in medicinal mushrooms (Cheung, 2010).

Although medicinal mushrooms have been found useful against a large number of diseases, investigations into mushrooms as a source of natural antioxidants have been made only recently (Ferreira et al., 2009). *Ganoderma lucidum* (Fr.) P. Karst is one of the most important medicinal mushrooms exploited for its scientific value. Many bioactive compounds found in *G. lucidum* have properties conducive for normalizing and balancing the body, and as a result, they can enhance health and aid in relief of a multitude of diseases. The majority of the studies on the *G. lucidum* are related to its antitumour and antiviral effects, while the antioxidant properties associated with this fungus have only recently become apparent (Mau et al., 2002). Polysaccharides, triterpenes, sterols, lectins and proteins are some of the major active constituents that were isolated from *G. lucidum*. *G. lucidum* has been shown to be safe and prevents many chronic diseases in clinical practice (Zhou et al., 2005).

G. lucidum was considered as the "elixir of life" by emperors and sages during most of China's long history, and has been glorified in Chinese literary classics, with a reputation as a tonic to prolong life matching that of ginseng. However, adequate experimental evidences are lacking. Wang et al. (2004) showed that *G. lucidum* can prevent age-related deteriorations in learning and memory and decreased amyloid β -peptide accumulation of brain in aged senescence-accelerated prone mouse (SAMP8). *G. lucidum* has also been reported to attenuate Aβ-induced synaptotoxicity, the main reason behind Alzheimer's disease (Lai et al., 2008). Recently, some novel ergosterol derivatives such as ganodermasides A, B, C and D were isolated from *G. lucidum*, showed to extend the replicative life span of yeast (Weng et al., 2010, 2011). Such observations imply that the *G. lucidum* could play an important role in the anti-aging. However, detailed studies are necessary to evaluate the exact anti aging mechanism of *G. lucidum.*

Since the mitochondria play the major role in aging (Raha and Robinson, 2000), the protection of mitochondria by the *G. lucidum* received major attention. Previous investigations have demonstrated that *G. lucidum* occurring in South India possessed significant antioxidant, antitumor, anti-inflammatory and antimutagenic properties (Jones and Janardhanan, 2000; Lakshmi et al., 2003). But there are no studies related to the protective effect of this mushroom against mitochondrial oxidative stress and dysfunction. This prompted us to evaluate the effect of *G. lucidum* on the oxidative stress induced mitochondrial dysfunction and declined cellular energy status in various models. In the current study, the 70% ethanol extract, protein bound polysaccharides and total triterpenes isolated from the *G. lucidum* were evaluated for their effects on the mitochondrial enzymes, mitochondrial antioxidant status, ROS levels, and mitochondrial membrane potential ($\Delta\psi mt$) of various organs of rats during aging as well as chemical induced cardiac and hepatic damage. The *in vitro* antioxidant activities, toxicity studies, and phytochemical analysis of the extract, protein bound polysaccharides and total triterpenes were also undertaken.

OBJECTIVES OF THE PRESENT STUDY

1. Evaluation of antioxidant activity of aqueous-ethanolic (70% ethanol) extract and protein bound polysaccharides and total triterpenes isolated from the *Ganoderma lucidum*.

2. Evaluation of the effect of *Ganoderma lucidum* extract on mitochondrial oxidative stress and dysfunction during aging.
3. Evaluation of the effect of protein bound polysaccharides isolated from the *Ganoderma lucidum* on mitochondrial oxidative stress and dysfunction in old aged rats.
4. Evaluation of the effect of total triterpenes isolated from the *Ganoderma lucidum* on mitochondrial oxidative stress and dysfunction in old aged rats.
5. Evaluation of the effect of *Ganoderma lucidum* extract on chemical-induced mitochondrial oxidative stress and dysfunction

   5.a. Evaluation of the effect of *Ganoderma lucidum* extract on isoproterenol-induced cardiac mitochondrial oxidative stress and dysfunction

   5.b. Evaluation of the effect of *Ganoderma lucidum* extract on acetaminophen-induced hepatic mitochondrial oxidative stress and dysfunction.

   5.c. Evaluation of the effect of *Ganoderma lucidum* extract on carbon tetrachloride-induced hepatic mitochondrial oxidative stress and dysfunction
6. Toxicity studies of ethanol extract, protein bound polysaccharides and total triterpenes isolated from the *Ganoderma lucidum.*
7. Preliminary phytochemical analysis of ethanol extract, protein bound polysaccharides and total triterpenes isolated from the *Ganoderma lucidum*.

METHODS

Fruiting bodies of *G. lucidum* growing on the *Caesalpinia coriaria* trees were collected. The fruiting bodies were cut into small pieces, dried at 40-50°C for 48 h and powdered and extracted with 70% ethanol on a boiling water bath for 48 h for getting aqueous ethanolic extract. Protein bound polysaccharide fraction was isolated from aqueous extract of *G. lucidum* by ethanol precipitation method. Assay for the polysaccharides was done by anthrone and phenol sulphuric acid tests and protein by Lowry's method. The total triterpene fraction was isolated and purified from the ethanol extract of *G. lucidum* by silica gel column chromatography and the confirmation was done by thin layer chromatography and chemical test of triterpenes.

The *in vitro* free radical scavenging activities of the extract, protein bound polysaccharides and total triterpenes were determined by different *in vitro* antioxidant assays. The superoxide, hydroxyl, DPPH and ABTS radical scavenging activities of the drugs were evaluated. The reducing abilities of the drugs were determined using ferric reducing antioxidant power (FRAP) assay and the ability to inhibit lipid peroxidation was estimated by the Fe^{2+}-EDTA- ascorbate system.

The effect of *G. lucidum* against aging induced mitochondrial damage was evaluated by using male albino rats of Wistar strain of different ages such as young aged (3-4 months), middle aged (13-15 months) and old aged (above 24 months). The aqueous ethanol extract of *G. lucidum* (50 and 250 mg/kg) was given orally to rats of different ages once daily for 15 days. The effect was compared with that of DL-α-lipoic acid (100 mg/kg) which was taken as the positive control. The effect was evaluated using mitochondria isolated from the heart, brain, skeletal muscle, liver and kidney through differential centrifugation in sucrose medium. The mitochondrial innate antioxidants such as manganese-superoxide dismutase (Mn SOD) and glutathione peroxidase (GPx) as well as levels of reduced glutathione (GSH), lipid peroxidation (as levels of MDA) were assayed by standard methods and the ROS level by the fluorescence method using dichlorodihydrofluorescein diacetate (H_2DCFDA) and mitochondrial membrane potential ($\Delta\Psi$mt) by the fluorometric analysis using Rhodamine123 (Rh123). To assess the extent of mitochondrial damage, the activities of Kreb's cycle enzymes such as isocitrate dehydrogenase (ICDH), α- ketoglutarate dehydrogenase (α-KGDH), succinate dehydrogenase (SDH), and malate dehydrogenase (MDH) as well as mitochondrial complex I (NADH: ubiquinone oxidoreductase), complex II (Succinate ubiquionone oxidoreductase), complex III (Ubiquinol-cytochrome c oxidoreductase), and complex IV (Cytochrome c oxidase) were determined.

The protein bound polysaccharide isolated from *G. lucidum* was evaluated for its effect against mitochondrial damage during aging. The protein bound polysaccharide (25 and 50 mg/kg) was administered orally once daily for 15 days to old aged male Wistar rats keeping DL-α-lipoic acid (100 mg/kg) as the positive control. The methods used are the same as used in the case of the extract and the activities of Mn SOD, GPx, ICDH, α-KGDH, SDH, MDH, complex I, II, III and IV. The levels of GSH, MDA, ROS and $\Delta\Psi$mt were determined in all the groups of animals.

The effect of total triterpens isolated from *G. lucidum* was also evaluated for its effects against age related oxidative stress and mitochondrial damage. Old aged Wistar rats were administrated total triterpens (2.5 and 5 mg/kg) suspended in olive oil once daily for 15 days keeping DL-α-lipoic acid (100 mg/kg) as the positive control and the extend of protection was estimated by determining the activities of Mn SOD, GPx, ICDH, α-KGDH, SDH, MDH, complex I, II, III and IV and the levels of GSH, MDA, ROS and ΔΨmt in all the groups of animals.

Evaluation of the effect of *G. lucidum* on the chemical induced oxidative stress and mitochondrial dysfunction was determined by using well known mitochondrial toxins such as isoproterenol (ISO), acetaminophen (APAP) and carbon tetrachloride (CCl_4) in male Wistar rats of middle age (13-15 months). Subcutaneous injection of ISO was shown to cause myocardial necrosis in experimental animals which resembles myocardial infarction (MI) in human being (Rona et al., 1959) and it also induced mitochondrial dysfunction (Punithavathi and Prince, 2010). Similarly, studies have shown that liver of animals challenged with APAP and CCl_4 leads to mitochondrial dysfunction (Donnelly et al., 1994; Tang et al., 2006).

In the ISO induced cardiac damage model, the mitochondrial dysfunction was induced by the subcutaneous injection of ISO (85 mg/kg) at an interval of 24 h for 2 days to different groups of animals which were pretreated with aqueous-ethanol extract of *G. lucidum* **(**100 and 250 mg/kg body weight) orally for 15 days keeping α-tocopherol (100 mg/kg body weight) as the positive standard. The extend of cardiac damage was assessed by determining the activities of cardiac markers such as serum creatine kinase (CK) and lactate dehydrogenase (LDH) 24 hrs after the last ISO administration. The mitochondrial damage was determined by estimating the activities of Mn SOD, GPx, ICDH, α-KGDH, SDH, MDH, complex I, II, III and IV and the levels of GSH, MDA, ROS and ΔΨmt in the heart of all the animals.

In the APAP induced hepatic damage model, the mitochondrial dysfunction was induced by a single dose of APAP (3g/kg body weight). The aqueous-ethanol extract of *G. lucidum* **(**100 and 250 mg/kg body weight) was given orally for 15 days before the APAP challenge. α-tocopherol (100 mg/kg body weight) was employed as the positive standard. Extend of hepatic damage was assessed by determining the activities of hepatic markers such as serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and alkaline

phosphatase (ALP) after 3 h of APAP administration. The extend hepatic mitochondrial damage was determined by estimating the activities of Mn SOD, GPx, ICDH, α-KGDH, SDH, MDH, complex I, II, III and IV and the levels of GSH, MDA, ROS and ΔΨmt.

Similarly in the CCl_4 model, the mitochondrial dysfunction associated with hepatic damage was induced by a single dose of CCl_4 in paraffin oil (1:5 v/v, 1.5 ml/kg, i.p). Before the administration of CCl_4, animals were orally administrated with *G. lucidum* (100 and 250 mg/kg body weight) for 15 days and α-tocopherol (100 mg/kg body weight) was used as standard. Extend of hepatic damage and mitochondrial dysfunctions were estimated after 24 h of CCl_4 administration by similar methods as employed in APAP model.

Acute and subacute toxicity studies of the aqueous ethanol extract, protein bound polysaccharides and total triterpenes isolated from the *G. lucidum* were carried out using female Swiss albino mice. In acute study, mortality after a single dose of the different drugs were noted and in subacute toxicity study, changes in body weight, haematological parameters and markers of liver and kidney functions were evaluated after 30 days of oral administration of the drugs.

Preliminary phytochemical analysis was carried out to determine the chemical constituents in the extract, protein bound polysaccharides and total triterpenes using standard methods. The HPTLC analysis of the extract and the triterpenes and the HPLC analysis of the protein bound polysaccharides were also performed.

RESULTS

The *in vitro* antioxidant assays showed that the extract, protein bound polysaccharides and total triterpenes from *G. lucidum* possessed significant antioxidant activity and free radical scavenging ability. They showed significant ferric reducing power, inhibition of lipid peroxidation induced by Fe^{2+}-ascorbate system, scavenging the DPPH radicals, hydroxyl radicals generated from Fe^{3+}-asccorbate-EDTA-H_2O_2 system and ABTS radicals generated by the reaction between 2,2'- azinobis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS). However, the aqueous ethanol extract *G. lucidum* and the protein bound polysaccharides possessed higher antioxidant activities than that of the total triterpenes, except in the case of lipid peroxidation. The water insoluble nature of total triterpenes made them unable to perform the hydroxyl radical and superoxide radical scavenging assays. However, all the tested samples showed a dose

dependant activity in all these assays. Butylated hydroxyanisole (BHA), the synthetic antioxidant used as the positive standard in the experiments showed potent antioxidant activities.

The effects of *G. lucidum* extract against age induced mitochondrial damage showed that the antioxidant status and activities of mitochondrial dehydrogenases and respiratory chain complexes were declined significantly ($p < 0.01$) with advancing age. There was also significant decrease in the ΔΨmt and increase in the ROS level in the middle and old aged rats compared to that of young rats. Treatment with *G. lucidum* extract effectively ameliorated the oxidative stress and cellular energy status by enhancing the innate antioxidant enzymes, mitochondrial dehydrogenases and respiratory chain complexes and the levels of GSH and ΔΨmt and by reducing the levels of lipid peroxidation and ROS in the mitochondria of heart, brain, skeletal muscle, liver and kidney of middle aged and old aged rats than that of young aged rats. The effect of *G. lucidum* was non-significant in the young aged group where as it was significant in the middle and old aged groups. A similar effect was also observed in the case of DL-α-lipoic acid treatment.

The effects of the major components from *G. lucidum* extract such as the protein bound polysaccharides and total triterpenes were evaluated for their roles against age associated mitochondrial dysfunction and oxidative stress. The protein bound polysaccharides showed a significant ($p < 0.05$) increase in the activities of innate antioxidants, Kreb's cycle dehydrogenases, respiratory chain complexes and increased the levels of GSH and ΔΨmt and could reduce the levels of lipid peroxidation and ROS in the mitochondria of old aged rats. Similarly, the total triterpenes from *G. lucidum* extract could also protect the mitochondria from age induced oxidative stress by enhancing the antioxidant status, mitochondrial enzymes, respiratory chain complexes and ΔΨmt and by reducing the ROS level and lipid peroxidation. Where as, the treatment with olive oil alone for 15 days could not produce any effect against aging. However, the extract possessed higher effect than the protein bound polysaccharides and total triterpenes. No dose dependency was observed among the doses of extract (50 and 250 mg/kg), protein bound polysaccharides (25 and 50 mg/kg) and total triterpenes (2.50 and 5.0 mg/kg). The enhanced effect observed in the case of extract might be due to the synergetic effect of several components in it.

In the ISO induced cardiac mitochondrial damage model, the results clearly showed that ISO induced cardiac damage as evident by the significantly ($p < 0.01$) increased activities of serum CK and LDH. There was also significant ($p < 0.01$) decline in the antioxidant status, mitochondrial dehydrogenases, respiratory chain complexes and ΔΨmt and increase in the ROS levels in the cardiac mitochondria by ISO challenge, but the administration of the aqueous ethanol extract of *G. lucidum* can significantly ($p < 0.05$) ameliorated the oxidative stress associated with the cardiac damage by reducing the activities of CK and LDH and by enhancing the antioxidant status, activities of mitochondrial enzymes, ΔΨmt and by directly reducing the mitochondrial ROS levels. The histopathological analysis also supported the results obtained.

APAP and CCl_4 treatments significantly ($p < 0.01$) enhanced the activities of SGPT, SGOT and ALP compared to normal group which clearly suggested the hepatic damage. There was also significant decline in the antioxidant status, mitochondrial enzymes and levels of ΔΨmt in the control group of animals. The aqueous ethanol extract of *G. lucidum* significantly ($p < 0.05$) protected the liver mitochondria by reducing the SGPT, SGOT, ALP and ROS levels and by enhancing the antioxidant levels and mitochondrial enzymes. In the ISO, APAP and CCl_4 models, no dose dependency was observed between the doses of *G. lucidum* extract. The positive standard (α-tocopherol) used also showed significant ($p < 0.05$) protection against mitochondrial damage induced by these chemicals. The histopathological analysis also supported the results obtained.

The acute toxicity study showed that the aqueous-ethanol extract of *G. lucidum* up to the dose of 2500 mg/kg body weight, protein bound polysaccharide up to the dose of 500 mg/kg body weight and the total triterpene fraction up to the dose of 50.0 mg/kg body weight orally did not produce any symptoms of toxicity such as changes in skin fur, eyes, diarrhea or vomiting tendency and behavior changes with in 30 min after the administration or mortality for 14 days. In subacute toxicity studies, treatment with the different concentrations of the extract (50 and 250 mg/kg), protein bound polysaccharides (25 and 50 mg/kg) and total triterpene fraction (2.5 and 5.0 mg/kg) did not produce any statistically significant change in the hematological or biochemical parameters compared to the normal group of animals. The histopathological examination of the liver and kidney tissues of the treated animals also supported the non toxic nature of these components.

Phytochemical screening of the aqueous ethanol extract of *G. lucidum* showed the presence of polysaccharides, triterpenes and proteins as components in the extract. The extract was found to have 9% total carbohydrate content and 40% protein in it. HPTLC analysis showed the presence of 19 peaks in the extract. The protein bound polysaccharide isolated was a mixture of polysaccharide and protein in the ratio 60:18. Thus, it can be called as protein-bound polysaccharide or polysaccharide-protein complex. Further, the monosaccharide present in the polysaccharide-protein complex was found to be glucose and the amino acids in the polysaccharide-protein complex were found to be aspartic acid, glutamic acid, alanine and threonine. The triterpenes fraction isolated was observed to contain a number of triterpenes and the HPTLC analysis showed a total of 14 peaks corresponding to 14 different constituents.

In summary, the present study demonstrates that *G. lucidum* and its major chemical components such as polysaccharides and triterpenes significantly ameliorated the oxidative stress and protected the mitochondrial damage associated with aging. *G. lucidum* could also significantly prevent the cardiac and hepatic damage by protecting the mitochondria and by enhancing the cellular energy status. However, further studies are needed to elucidate the exact mechanism. The experimental finding indicated that the aqueous-ethanol extract of *G. lucidum* and the polysaccharides and triterpenes isolated from the extract are nontoxic and effective to protect the mitochondrial damage due to oxidative stress and hence would be effective against various mitochondria mediated diseases.

The thesis has been divided into the following 9 chapters.

Chapter 1: Review of literature

Chapter 2: Materials and method

Chapter 3: Antioxidant activity of aqueous-ethanol (70% ethanol) extract of *G. lucidum*, protein bound polysaccharides and total triterpenes isolated from the extract.

Chapter 4: Evaluation of the effect of *Ganoderma lucidum* extract on mitochondrial oxidative stress and dysfunction during aging

Chapter 5: Effect of protein bound polysaccharides isolated from the *Ganoderma lucidum* on the mitochondrial oxidative stress and dysfunction in old aged rats.

Chapter 6: Effect of total triterpenes isolated from the *Ganoderma lucidum* on the mitochondrial oxidative stress and dysfunction in old aged rats.

Chapter 7: Effect of *Ganoderma lucidum* on the chemical induced mitochondrial oxidative stress and dysfunction

Chapter 7a: Effect of *Ganoderma lucidum* on the isoproterenol induced cardiac mitochondrial oxidative stress and dysfunction

Chapter 7b: Effect of *Ganoderma lucidum* on the acetaminophen induced hepatic mitochondrial oxidative stress and dysfunction.

Chapter 7c: Effect of *Ganoderma lucidum* on the carbon tetrachloride induced hepatic mitochondrial oxidative stress and dysfunction

Chapter 8: Toxicity studies of aqueous-ethanol extract of *G. lucidum*, protein bound polysaccharides and total triterpenes isolated from the extract.

Chapter 9: Preliminary phytochemical analysis of aqueous-ethanol extract of *G. lucidum*, protein bound polysaccharides and total triterpenes isolated from the extract.

Bibliography

List of publications

REFERENCES

Chance, B., Sies, H., and Boveris, A. (1979) Hydroperoxide metabolism in mammalian organs, Physiological reviews 59, 527.

Cheung, P. (2010) The nutritional and health benefits of mushrooms, Nutrition Bulletin 35, 292-299.

Donnelly, P. J., Walker, R. M., and Racz, W. J. (1994) Inhibition of mitochondrial respiration in vivo is an early event in acetaminophen-induced hepatotoxicity, Archives of toxicology 68, 110-118.

Ferreira, I. C. F. R., Barros, L., and Abreu, R. (2009) Antioxidants in wild mushrooms, Current Medicinal Chemistry 16, 1543-1560.