

INTRODUCTION

Natural products not only provide valuable bioactive components but also are valuable sources to produce chemical blue prints that provide lead information for developing useful synthetic compounds. Mushrooms are macrofungi with distinctive fruiting body, which can be either hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang and Miles, 1992). The number of mushroom species on the earth is estimated to be 140 000, suggesting that only 10% are known. Assuming that the proportion of useful mushrooms among the undiscovered and unexamined mushrooms will be only 5%, which implies 7000 yet undiscovered species will be of possible benefit to mankind (Hawksworth, 2001). The medicinal use of mushrooms has a very long tradition in the Asian countries, whereas their use in the Western hemisphere has been increasing only in the recent years. Mushrooms are increasingly being evaluated for their nutritional value and acceptability as well as their pharmacological properties. Mushrooms contain a large number of biologically active components that impart health benefits and protection against degenerative diseases. They have been traditionally used in China, Japan and South eastern countries for the treatment of a variety of diseases including cancer. Some of the most recently isolated and identified compounds originating from the medicinal mushrooms have been shown promising anti-fungal, antiviral, anti-bacterial, anti-oxidant, anti-inflammatory, immunomodulatory, antitumour, cardioprotective, antibacterial, antiparasitic, hypolipidemic, hepatoprotective, antidiabetic, anti-thrombotic properties and many other medicinal applications.

Ganoderma is a polypore (Aphyllophoromycetideae) white rot fungus, normally found growing on rotten wood and living trees. Species of *Ganoderma* is a highly ranked herbal medicine. Generally all types of *Ganoderma* species are described as beneficial to all viscera and are non-toxic (Liu, 1999). *Ganoderma lucidum* (Curt.:Fr) P. Karst, commonly known as Reishi or Ling Zhi, was recognized as a superb herb in Oriental medicine with most extensive and effective healing powers. *Ganoderma*

lucidum is known as 'mushroom of immortality' and has been regarded as a panacea for numerous types of diseases in Chinese folklore. This has been attributed to its demonstrated efficacy as a popular remedy to treat hepatopathy, chronic hepatitis, hepatic carcinoma, hypertension, arthritis, diabetics, bronchitis, asthma, anorexia, mushroom poisoning, gastric ulcer, neurasthenia, inflammation, hypertension, cardio vascular diseases and its action as anti-bacterial, anti-oxidant, anti-tumor, anti-viral, and anti-HIV agent (Hsu, 1986; Jong and Birmingham, 1992; Kim and Kim, 1990; Eo et al., 1999; Liu, 1999). In clinical studies, *G. lucidum* products have been widely used as a single agent or in combination with other herbal medicines or chemotherapeutic drugs for many years, mainly in Asian countries. Our previous investigations have demonstrated significant antioxidant and antitumor, antiinflammatory, antinociceptive, antimutagenic, anti-carcinogenic, cardio protective, and nephroprotective effects of extracts of *G. lucidum* (Jones and Janardhanan, 2000; Sheena et al., 2005).

Identifying the active fractions or ingredients responsible for the biological activities and their mechanism of action is of great importance for developing pharmaceutical products from the mushroom. The fruiting bodies of *Ganoderma lucidum* are rich in a variety of organic compounds such as polysaccharide, triterpenes, amino acids, sterols, lipids, peptidoglycans, proteins, adenosine, lectins, alkaloids and trace minerals (Ma et al., 2002, Koyama et al., 1997, Chyr and Shiao, 1991). Polysaccharides and triterpenes are the major pharmacologically active chemical components of *G. lucidum*. Triterpenes are the bitter components of *G. lucidum*. Over 150 triterpenoids are known from *Ganoderma* sp. till recently. They include the following classes: ganoderic (highly oxygenated C₃₀ lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic, ganolucidic acids, applanoxidic acids, lucidones, ganoderals and ganoderols (Boh et al., 2007; Nishitoba et al., 1986). It has been reported that some of the physiological effects and distinct properties of *G. lucidum* are depended upon the strains and the

cultivating conditions (Nishitoba et al., 1986). Preliminary studies also indicated that triterpene composition of *G. lucidum* fruiting bodies vary according to the area in which they are growing (Min et al., 2000). Investigations have revealed that triterpenes are responsible for some of the major medicinal properties of *G. lucidum* and its demonstrated therapeutic efficiency (Patterson, 2006; Wasser, 1997; Hattori, 2001; Huie and Di, 2004; Kim and Kim, 1999; Lin et al., 2003; Min et al., 2000).

Cancer is the second largest single cause of death in developed countries, claiming more than 6 million lives every year world wide. The human body is continuously and unavoidably exposed to a plethora of structurally diverse chemicals with established carcinogenic activity in animal models and/or mutagenic activity in short-term tests (Maron and Ames, 1983). As the great threat to human life by cancer continues to increase, the pursuit for discovering new anticancer drugs has been a compelling urgency. The discovery of new effective and safe anticancer drugs will benefit millions suffering from this disease. Almost every conceivable approach has been attempted to acquire clinically effective natural and synthetic anticancer agents. Eventhough *Ganoderma* triterpenes have received considerable attention for their pharmacological activities, including anti-hypertensive, hypocholesterolemic, anti-histamine, anti-complement, anti-HIV and hepatoprotective properties (Boh et al., 2007; Patterson, 2006; Wasser, 1997; Kim and Kim, 1999), the extensive range of medical use of triterpenes isolated from *G. lucidum* occurring in South India, especially their potential therapeutic use in the field of chemo and radiotherapy of cancers has not yet been fully understood. At present, *G. lucidum* is a health food supplement to support cancer patients, yet the evidence supporting the potential of direct *in vivo* anticancer effects should not be underestimated (Yuen and Gohel, 2005).

Damage to DNA is likely to be a major cause of cancer and other degenerative diseases. Unrepaired or misrepaired DNA damage leads to genetic instability, mutation, chromosomal aberration and may finally

leading to neoplasm. Thus, protecting living system from genotoxic agents is of significant importance. Environmental stresses, including ionizing radiation and the consequent generation of reactive species, cause damage to DNA. Ionising radiation is well known to be both mutagenic and carcinogenic. Ionising radiations induce damage to DNA by direct ionisation and also through generation of hydroxyl radicals that attack DNA resulting in single strand breaks, double strand breaks, base and sugar modifications and oxidative damage to sugar and base residues, which can be converted into DNA strand breaks later. Modification of base in DNA can lead to mutation, either directly or during attempts by the cell to replicate or repair damaged DNA. Although, radiotherapy is the most common and effective tool for cancer treatment; radio-sensitivity of normal tissues especially adjacent to the tumor which are unavoidably exposed to radiation, limit the therapeutic gain. Hence protection of normal cells from the deleterious effects of radiotherapy is of significant importance in cancer treatment. The identification of a novel, non-toxic, effective and convenient compounds to protect human against radiation induced normal tissue injuries, especially the damages to DNA, is of paramount importance. The current investigations are undertaken to evaluate the anti carcinogenic and geno protective effects of total triterpenes isolated from *G. lucidum* occurring in South India.

OBJECTIVES

1. Isolation of total triterpenes from the fruiting bodies of *Ganoderma lucidum*.
2. Evaluation of *in vitro* and *in vivo* antioxidant activity of total triterpenes.
3. Evaluation of anti-inflammatory and anti-arthritic activities of total triterpenes.
4. Evaluation of anti tumor and apoptotic effect of total triterpenes on various tumor and cancer cell lines .
5. Evaluation of anti carcinogenic activity of total triterpenes.

6. Evaluation of effect of total triterpenes on radiation induced oxidative damage

and genotoxicity.

7. Evaluation of effect of total triterpenes against radiation induced DNA damage

and apoptosis in splenic lymphocytes *in vitro*.

8. Evaluation of toxicity of total triterpenes.

METHODS

Isolation of total triterpenes:

Ninety days old fresh fruiting bodies of *G. lucidum*, growing on *Caesalpinia coriaria* Wild., were collected from Thrissur district, Kerala, South India. Samples of dried and powdered fruiting bodies of *G. lucidum* (100g) were extracted with ethanol. The extract was concentrated (9g) and suspended in chloroform. The chloroform soluble fraction was separated and the solvent completely recovered, the residue (3g) was loaded on silica gel column (3 × 60cm) and eluted with petroleum ether, chloroform, methanol and various combinations of these solvents. The fractions that answered the tests for triterpenes (Harborne, 1973) were combined together and concentrated to get the total triterpene fraction (1.5g). The total triterpenes thus obtained was used for further studies.

***In vitro* and *in vivo* antioxidant activity:**

The direct antioxidant activity of the total triterpenes isolated from *G. lucidum* was evaluated using various *in vitro* antioxidant assays, including the oxygen radical absorbance capacity (ORAC) assay, the DPPH radical scavenging assay, the ABTS⁺ radical scavenging assay, the ferric reducing antioxidant power (FRAP) assay, the superoxide radical scavenging activity assay and the inhibition of lipid peroxidation. Effect of total triterpenes administration in the activities of *in vivo* antioxidant enzymes in blood and tissue were analysed using Swiss albino mice.

Anti-inflammatory and anti-arthritic activity:

Inflammation is increasingly recognized as an important component of tumorigenesis. Rheumatoid arthritis (RA) may contribute to an increased risk for certain types of cancer and for a poor prognosis of malignancy. Anti-inflammatory activity of the total triterpenes was determined by both acute and chronic models using Swiss albino mice. In acute model, the inflammation was induced using carrageenan and in chronic model using formalin. For evaluating the antiarthritic activity, arthritis was induced in Wistar rats using Freund's complete adjuvant (FCA).

Anti tumor and apoptotic effect on various tumor and cancer cell lines:

The antitumor activity of the total triterpenes was determined using both ascites and solid tumor model in Swiss albino mice. In ascites tumor, Ehrlich's ascites carcinoma cells (1×10^6) were injected intra peritoneally and the increase in life span of the control and total triterpenes treated animals were compared. In solid tumor model, Dalton's lymphoma ascites cells used were injected into the thigh of animals and % decrease in tumor volume and weight after treatment was determined. Ability of total triterpenes to induce apoptosis in DLA, EAC cell lines were determined using DNA laddering assay. Cytotoxicity of total triterpenes in MCF-7 cells was determined using MTT assay. Induction of apoptosis in MCF-7 cell lines was assessed by flow cytometric assay. Effect of total triterpenes on intracellular ROS levels was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) assay and expression of genes involved in apoptotic pathway (Bcl-xl, Caspase 3) were determined using Western blot analysis.

Anti carcinogenic activity:

The anticarcinogenic activity of the total triterpenes was determined using dimethyl benz [a] anthracene (DMBA) induced mammary adenocarcinoma and skin tumour models. Two-stage skin carcinoma in Swiss albino mice was induced by topical application of DMBA and the promoter (croton oil).

After treatment average number of papilloma per mice, percent of animals with papilloma and tumour latency period were recorded and compared with the untreated control. Mammary tumours were induced by oral administration of 7,12-dimethyl benz[*a*]anthracene (DMBA) in female Wistar rats. Average number of tumours per tumour-bearing rat, percentage of animals with tumour and tumour latency period were recorded for a period of 17 weeks.

Protection against radiation induced oxidative damage and genotoxicity:

The effect of total triterpenes on γ -radiation-induced DNA strand breaks in pBR 322 plasmid DNA, human peripheral blood lymphocytes *ex vivo*. Radiation-induced damage to plasmid DNA was determined using electrophoresis. pBR 322 DNA was exposed to 25 Gy γ -radiations in the presence and absence of different concentrations of the total triterpenes. Radiation-induced damage to DNA in the human blood lymphocytes was measured as strand breaks using alkaline single cell gel electrophoresis or "comet assay". The total triterpenes was also examined for its potential to prevent γ -radiation induced membrane damage using rat liver mitochondria and microsomes. *In vivo* radioprotective effect of total triterpenes was determined using Swiss albino mice, with pre treatment of total triterpenes for 7 days, followed by a whole body exposure to γ -radiation. Protection of radiation induced DNA strand breaks in peripheral blood leukocytes and bone marrow cells were assessed using comet assay. Lipid and protein peroxidation levels in liver and brain homogenates after irradiation were estimated. The activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the level of reduced glutathione (GSH) in liver and brain homogenates were analysed. Formations of the micronuclei in mice bone marrow cells *in vivo* were also evaluated to estimate the indexes of both chromosome breakage and chromosome loss.

Protection against radiation induced DNA damage in splenic lymphocytes:

Protection of normal cells from the deleterious effects of radiation by total triterpenes isolated from *Ganoderma lucidum* (Fr.) P. Karst was evaluated with the help of mouse splenic lymphocytes *in vitro*. Effect of the total triterpenes on cell viability was analyzed using the MTT assay. The effect of the total triterpenes on DNA damage and apoptosis induced by radiation was analyzed using the comet assay, DNA ladder assay and flow cytometry. The effect of the total triterpenes on intracellular ROS level and endogenous antioxidant enzyme activity in splenic lymphocyte were determined to ascertain potential radioprotective mechanisms.

Toxicity studies:

Acute and subacute toxicity studies of the total triterpenes were carried out using Swiss albino mice. In acute study, mortality after the administration of a single high dose of triterpenes was noted. In chronic study, changes in body weight, haematological parameters, liver and renal function enzymes were evaluated after the administration of the triterpenes for a period of 30 days. Small portions of the selected tissues of liver and kidney of treated animals were also examined for histopathological changes.

RESULTS

The total triterpenes efficiently scavenged the free radicals generated in the *in vitro* systems. The total triterpenes successfully scavenged DPPH⁺ (IC₅₀ 41.45 ± 5.2 µg/ml), ABTS⁺ (IC₅₀ 30 ± 5.4 µg/ml) and superoxide radicals (IC₅₀ 25 ± 3µg/ml), showed significant ferric reducing activity (IC₅₀ 6 ± 0.5 µg/ml) and was highly effective in reducing the *in vitro* lipid peroxidation (IC₅₀ 84.62 ± 4.1 µg/ml). ORAC value for the total triterpenes from *G. lucidum* was found to be 3515 ± 23 micromoles of Trolox per gram which indicated its high radical scavenging ability. Activities of the antioxidant enzymes in blood and tissue were increased by the administration of total triterpenes to Swiss albino mice *in vivo*. The ability

of total triterpenes to scavenge the free radicals and to enhance body's antioxidant defence systems indicates its potential use as an antioxidant.

The total triterpenes showed significant protection from both acute and chronic inflammation and also helped to reduce the chronic symptoms of Freund's complete adjuvant induced arthritis in rats and restored the antioxidant enzymes in the blood almost to the normal level. The total terpenes, at concentrations 10, 50 and 100 mg/kg b. wt, administered orally showed 41.86%, 62.79% and 79.07% inhibition of chronic and 36.96%, 54.35%, 76.09% inhibition of acute inflammation. The anti-inflammatory activity of the triterpenes at 100mg/kg was higher than that of the standard drug (diclofenac) (71.74% and 76.74%) in both chronic and acute models. In Freund's complete adjuvant induced arthritis, the paw thickness of total triterpenes treated groups was found to be decreased without leading to a chronic inflammation phase, when compared to untreated control group. Free radicals have long been implicated in changes in connective tissues on inflammation and arthritis. The administration of triterpenes effectively modulated the antioxidant levels in blood and helped to counteract with the adverse effects of free radicals produced in arthritis rats.

The total triterpens possessed significant antitumor activity against DLA induced solid tumor and EAC induced ascites models. In solid tumor model, a significant reduction in the tumor volume and weight were observed in the treated groups at the end of the 5th week compared to the control group. The percentage inhibition of 10, 50 and 100 mg/kg b. wt total triterpenes administration was 76.86, 85.01 and 91.03% in tumour volume and was 67.96, 72.38 and 77.90% in tumour weight respectively. In ascites tumor model, total triterpene administrated at a dose of 100 mg/kg body weight increased the average life span of tumour bearing animals to 29.16 ± 2.4 days where as in control group average life span was 17.33 ± 1.8 . Total triterpenes was also found to induce apoptosis in EAC, DLA and MCF- 7 cell lines as evident from DNA laddering and Flow

cytometric assays. MCF-7 cells when treated with total triterpenes showed an increase ($p < 0.001$) in apoptotic cells as evident from Flow cytometric assay. MTT assay revealed its cytotoxicity towards MCF-7 cell lines. Treatment with total triterpenes enhanced the intracellular ROS levels which may be one of the reasons for its cytotoxicity towards MCF-7 cells. Total triterpenes was also found to up regulate the expression of Caspase 3 protein and down regulate the expression of Bcl-xl genes in MCF-7 cells.

Topical application of *G. lucidum* total triterpenes inhibited mouse skin tumour initiated by DMBA and promoted by croton oil. The tumour incidence in the control group of animals treated with DMBA and croton oil was 87.5%, 18 weeks after the application of DMBA. There was a significant reduction in the percentage of tumour incidence in total triterpenes treated animals. The application of total triterpenes (5mg, 10mg and 20mg) 1 hour before each croton oil application showed only 62.5%, 37.5% and 12.5% tumor incidence at 18th week. The average number of tumour (1mm diameter) per animal in the control group was 4.88 at 18th week after DMBA application, while in the 5 mg, 10 mg and 20 mg total triterpenes treated groups, the average number of tumor per animal was 1.87, 0.88 and 0.38 respectively. The tumor latency period in the control groups was 66.75 days. Total triterpenes (5 mg, 10 mg and 20 mg) applied 1 hour before each croton oil increased the tumor latency period to 94.75, 104.0 and 113.13 days respectively. In mammary tumor model, administration of the total triterpenes significantly reduced the incidence of mammary tumor. DMBA-alone treatment induced 100% tumour incidence whereas in animals treated with 100 mg/kg b.wt. total triterpenes, the percentage of tumour incidence decreased significantly to 16.67%. An average of 2.83 mammary tumours per animal was observed in the DMBA-alone treated group, after 17 weeks of DMBA administration. Treatment with triterpenes (100 mg/kg b.wt.) showed an average of 0.17 tumor. Tumour latency period in the control group of animals was 79.83 days and it was extended to 120 days in the case of animals treated with 100 mg/kg b.wt. total triterpenes. Treatment with the total triterpenes

significantly inhibited tumour growth. Animals treated with 100 mg/kg b.wt. total triterpenes showed 67.83% of inhibition in tumor weight compared to the control group.

Treatment with total triterpenes showed a concentration dependent reduction in the open circular form, thus retaining the intact supercoiled form in pBR 322 DNA. 50 µg total triterpenes could retain 98.87% of supercoiled form. Total triterpenes showed a significant protection against the DNA strand breaks, as revealed by comet assay, in human blood lymphocytes. This was evident from the reduction of parameters % tail DNA, tail length, tail moment and olive tail moment that were considered as the indices of DNA damage. Lipid peroxidation, being a chain reaction, is one of the most important organic expressions of oxidative stress. The total triterpenes (100 µg/ml) was able to reduce TBARS formation ($P < 0.001$) as well as lipid peroxidation ($P < 0.001$), which are considered as the indices of membrane damage, to normal levels in rat liver mitochondria and microsomes. Triterpenes when administered *in vivo*, was also found to be highly effective in restoring the antioxidant enzyme activities and GSH level in liver and brain of irradiated mice. Triterpenes were also effective in reducing the levels of lipid peroxidation and protein oxidation to near normal values in both liver and brain tissues of *in vivo* treated mice. There was significant increase in the number of micronucleated polychromatic ($2.65 \pm 1.02\%$) and normochromatic ($0.76 \pm 0.24\%$) erythrocytes in the bone marrow cells of animals after exposure to 2.5 Gy radiation. Treatment with 100 mg/kg b.wt of the total triterpene considerably decreased this radiation induced micronuclei formation to $0.87 \pm 0.61\%$ and $0.16 \pm 0.20\%$ respectively. The P/N ratio was significantly decreased in 2.5 Gy treated group of animals (0.70 ± 0.13). But it restored to normal level in the 100 mg/kg b.wt total triterpenes treated animals (1.44 ± 0.29). The results thus revealed the significant protection of *Ganoderma* triterpenes against radiation induced oxidative stress.

In the *in vitro* studies conducted in splenic lymphocytes, *Ganoderma* triterpenes were found to have no effect on cell viability, as revealed by MTT assay, indicating that they are non-toxic to splenic lymphocytes. Total triterpenes were found to be highly effective in preventing DNA laddering, even at low concentrations (25 µg/ml). The comet assay demonstrated that the *Ganoderma* triterpenes effectively prevented DNA damage as evident from the reduction of parameters % tail DNA, tail length, tail moment and olive tail moment. Flow cytometry assay revealed a reduction in apoptotic cells. In irradiated splenocytes, $50.84 \pm 3.4\%$ ($P < 0.001$) of cells were found to be in the apoptotic phase. Treatment with triterpenes (100 µg/ml) reduced the percentage of apoptotic cells to $25.80 \pm 5.1\%$ ($P < 0.001$). A significant decrease the formation of intracellular ROS was also observed in 100 and 200 µg/ml triterpenes ($P < 0.001$) treated splenocytes. The activities of endogenous antioxidant enzymes (SOD, GPx and GR) were found to be significantly increased ($P < 0.001$) following treatment of splenocytes with 100 µg/ml total triterpenes.

Acute toxicity studies indicated that total triterpenes isolated from *G. lucidum* did not produce any symptoms of toxicity, behavioural change and mortality of animals in all the tested doses. Even at a high dosage of 5000 mg/ kg b. wt, no toxic effect was observed. In sub acute model, three different concentrations (100, 250 and 500 mg/ kg b. wt) of total triterpenes were given orally to the animals and no significant change ($P > 0.05$) in the haematological and biochemical parameters were observed compared to the normal group of animals. Liver marker enzymes such as GOT, GPT, ALP and kidney function test such as serum urea and creatinine did not show any significant ($P > 0.05$) increase in the treated group. Histopathological examination of liver and kidney of treated animals did not show any pathological manifestations in treated animals. The animals were absolutely healthy and devoid of any adverse reactions, throughout the treatment period.

In summary, the findings of the present study indicate that total triterpenes from *G. lucidum* is an excellent antioxidant and is capable to defend well against inflammation and arthritis. The total triterpenes possessed good antitumor and apoptotic activity against various tumor and cancer cell lines and showed significant anticancer activity against DMBA induced mammary and skin tumor. *Ganoderma* terpenes also exhibited significant protection to normal cells against radiation induced oxidative stress as well as DNA damages indicating its genoprotective activity. The findings reveal the potential therapeutic use of this mushroom derived component as a genoprotective and anticancer agent.

The thesis has been divided into following 10 chapters:

Chapter 1: Review of literature

Chapter 2: Materials and methods

Chapter 3: Isolation of total triterpenes from *Ganoderma lucidum*

Chapter 4: Antioxidant activity of total triterpenes

Chapter 5: Anti-inflammatory and anti-arthritic activity of total triterpenes

Chapter 6: Anti tumor and apoptotic effect of total triterpenes on various tumor and cancer cell lines

Chapter 7: Anti carcinogenic activity of total triterpenes

Chapter 8: Effect of total triterpenes on radiation induced oxidative damage and genotoxicity

Chapter 9: Effect of total triterpenes against radiation induced DNA damage and apoptosis in splenic lymphocytes *in vitro*

Chapter 10: Toxicity studies of total triterpenes isolated from *Ganoderma lucidum*

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