



Protective effect of methanolic extract of *Ganoderma lucidum* P. Karst. Reishi from South India against doxorubicin-induced cardiotoxicity in rats

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SUMMARY

Doxorubicin is a powerful anticancer antibiotic extensively used in the treatment of several types of cancers. Long-term administration of this drug results in cumulative dose related cardiotoxicity due to enhanced production of free radicals leading to oxidative stress. Our earlier investigations have demonstrated significant antioxidant, anti-inflammatory and antitumour properties of *Ganoderma lucidum* extracts. We extended our investigations to evaluate the protective effect of *Ganoderma lucidum* extract against doxorubicin-induced cardiotoxicity. Administration of 3 doses of doxorubicin, 6 mg/kg body weights, i.p. per each dose, alternative days, showed clear signs of cardiotoxicity in rats. The drug enhanced serum creatine kinase (CK) activity and lipid peroxidation in tissue drastically. The drug also induced significant decrease in GSH level and activities of CAT, SOD and GPx. Administration of methanolic extract of *G.lucidum* (500 and 1,000 mg/kg body weight) significantly increased the level of GSH and activities of CAT, SOD and GPx. Activity of CK was significantly lowered in a dose dependent manner. The treatment also caused significant decrease in lipid peroxidation (MDA). The results thus indicated that methanolic extract of *G.lucidum* prevented oxidative stress caused by doxorubicin administration and the increase in serum CK activity and lipid peroxidation in the tissue. The experimental findings suggest the therapeutic potential of *G.lucidum* as adjuvant in cancer chemotherapy.

Key words: Medicinal mushrooms; *Ganoderma lucidum*; Doxorubicin; Cardiotoxicity

INTRODUCTION

Cancer is one of the leading causes of death in the world despite newly developed tools for treatment and diagnosis. Several methods exist for the treatment in modern medicine, which include chemotherapy, radiotherapy, and surgery. Chemotherapy is the sheet anchor of therapy in leukemia's, advanced lymphomas, choriocarcinoma and other widely disseminated malignancies. However most of the chemotherapeutants are not only cytotoxic to cancer

cells, but also to healthy cells. This results in a narrow therapeutic index, with adverse events frequently limiting optimal anticancer therapy. The anthracycline antibiotics are among the most active anticancer agents used against a wide range of solid and haemopoietic malignancies (Naidu *et al.*, 2002). Doxorubicin is a powerful anthracycline antibiotic originally isolated from *Streptomyces peucetius* var *caesius* and used for the treatment of many human neoplasm, including acute leukemia, lymphomas, stomach, breast and ovarian cancers, Kaposi's sarcoma, and bone tumors (Van Acker *et al.*, 1993; Shan, 1996). The major toxicity of doxorubicin is acute bone marrow suppression. However, the long-term clinical usefulness is limited by a cumulative

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dose-related cardiotoxicity, that manifests itself as congestive heart failure (Mott, 1997).

Therefore it is essential to identify the agents that can protect doxorubicin induced cardiotoxicity without interfering its ability to kill cancer cells, in order to sustain the use of this drug in clinical practice. It is believed that doxorubicin induced cardiomyopathy, at least partially, is caused by increased oxidant production in the heart and a great deal of evidence supports this hypothesis (Doroshov *et al.*, 1983; Kang *et al.*, 1996; Yen *et al.*, 1996; Kang *et al.*, 1997; Kottamraju *et al.*, 2000).

The practice of using macrofungi especially mushrooms to treat a variety of diseases is prevalent in many countries. They are traditionally used in China and Japan for medicinal purposes (Jong and Birmingham, 1992). *Ganoderma lucidum* (W.Curt.: Fr.) Karst. (Ganodermataceae), commonly known Reishi, is a wood rotting fungus generally found growing on trees and stumps. Reishi occurs in most part of the world (Hobbs, 1995). *Ganoderma lucidum* occurring in South India has been reported to have significant antitumor, anti-inflammatory, antinociceptive and nephroprotective properties (Jones and Janardhanan, 2000; Sheena *et al.*, 2003a; 2003b). Intraperitoneal injection of the tincture of *G. lucidum* or the alcohol extract of its mycelium increased contraction amplitude of the *in situ* rabbit heart and produced a cardiostimulant effect and bradycardia in anesthetized rats (Jong and Birmingham, 1992). Our recent investigations have found that extracts of *G.lucidum* occurring in South India possessed significant antioxidant properties (Jones and Janardhanan, 2000). The present study is addressed to evaluate the protective effect of the methanolic extract of *G.lucidum* against doxorubicin induced cardiotoxicity in rats.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats were purchased from the Small Animal Breeding Center, Kerala Agricultural

University, Mannuthy, Kerala and were kept for a week under environmentally controlled conditions with free access to standard food (Lipton, India) and water. Rats weighing 280 - 300 g were used for the experiments.

Chemicals

Doxorubicin hydrochloride (Adrim) was purchased from Dabur Research Lab Pvt Ltd. Mumbai. The other chemicals and reagents used were of analytical grade.

Preparation of the extract

Fruiting bodies of the *G. lucidum* were collected from the outskirts of Thrissur District, Kerala, South India. The type specimen was deposited in the herbarium of Centre for Advanced Studies in Botany, University of Madras, Chennai, India (HERB.MUBL.3175). The fruiting bodies were cut into small pieces, dried at 45-50°C for 48 h, and powdered. Two hundred gram of powdered material was extracted with petroleum ether using a Soxhlet apparatus. The defatted material was air dried, then extracted with boiling in 70% methanol for 8 h. The solvent was removed and the extraction repeated once again. The extracts were combined, filtered through Whatman No.1 filter paper. The solvent evaporated at low temperature under vacuum and the concentrated extract was finally lyophilized. The methanolic extract thus obtained (8 g) was employed in the experiments. The extract was solubilised in distilled water.

Determination of doxorubicin induced cardiotoxicity

Animals were divided into 4 groups of 6 animals each. Group 1 treated with distilled water was kept as normal. Group 2 treated with doxorubicin (6 mg/kg body weight, i.p) was kept as control. Group 3 and 4 were treated with *G. lucidum* extract 500 and 1,000 mg/kg orally 1 h before doxorubicin injection. The doxorubicin injection and the administration of the extract were repeated on alternative days (total 3 doses). The animals were

sacrificed after the third dose of doxorubicin, blood was collected directly from the heart. Serum was used for the determination of creatine kinase activity (CK) by the method of Moss and Henderson (1999). Heart was removed and stored at -70°C until analyses could be completed. The heart was homogenized in 50 mM phosphate buffer (pH 7) to give a 10% homogenate (W/V). Supernatant was used for the determinations of superoxide dismutase (SOD) by the method of Mc Cord and Fridovich (1969), glutathione peroxidase (GPx) by the method of Hafemann *et al.* (1974) levels of reduced glutathione (GSH) by the method of Moron *et al.* (1979) and malondialdehyde (MDA) by the method of Ohkawa *et al.* (1979) using 1,1,3,3-tetramethoxypropane as the standard. The protein content was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Histopathological examination

A portion of the heart was fixed in 10% formalin and then embedded in paraffin. Microtome sections 6 μm thickness was prepared from each heart tissue and stained with hematoxylin-eosin. The sections were evaluated for the pathological parameters of cardiotoxicity such as elongation of cardiomyocytes, increase intercellular spaces, myocyte nuclear swellings etc.

Statistical analysis

Experimental data were expressed as mean \pm SD. The significant difference among the four groups was assessed using one-way ANOVA. If found significant the extract treated group was compared

with that of the control group by Dunnett's-*t* test. Significance was acceptable to a level of $P < 0.05$.

RESULTS

The results of the present study indicated that intraperitoneal administration of doxorubicin (6 mg/kg body weight, i.p) 3 doses on alternate days induced clear signs of cardiac toxicity in rats. The animals in the control group are more sick, weaker and suffered from diarrhea as compared to the normal and extract pretreated group of animals. Administration of *G. lucidum* extract (500 and 1,000 mg/kg) to animals prior to doxorubicin challenge increased the heart GSH levels compared to animals that were injected with doxorubicin alone. Doxorubicin treatment produced significant decrease in SOD (4.35 ± 0.36 U/mg protein), GPx (25.78 ± 3.66 U/mg protein) in the control animals, compared to the activities of SOD (6.28 ± 1.47 U/mg protein) and GPx (48.53 ± 7.29 U/mg protein) in the normal group (Table 1). The activity of SOD and GPx in the *G. lucidum* extract treated group was comparable to normal. The product of lipid peroxidation measured as MDA in heart tissue was higher in doxorubicin treated group (1.00 ± 0.12 nmol/mg protein) compared to normal (0.34 ± 0.02 nmol/mg protein) (Table 2).

The MDA level was found to be near normal in extract treated group of animals. Administration of methanolic extract of *G. lucidum* (500 and 1,000 mg/kg) significantly lowered CK activity in a dose dependent manner (Table 3). The activities of serum CK in the treated groups (500 and 1,000

Table 1. Levels of GPx and SOD in the heart of rats treated with doxorubicin (6 mg/kg body wt., i.p), doxorubicin + methanolic extract of *G. lucidum* (GLME) (500 mg/kg and 1,000 mg/kg body wt., p.o) and normal rats

Groups	Treatments (mg/kg)	SOD (U/mg protein)	GPx (U/mg protein)
Normal	-	6.28 ± 1.47	48.53 ± 7.29
Doxorubicin	6	$4.35 \pm 0.36^*$	$25.78 \pm 3.66^*$
Doxorubicin + GLME	500	4.84 ± 0.17^b	41.18 ± 1.71^a
Doxorubicin + GLME	1,000	7.83 ± 0.35^a	50.24 ± 0.62^a

Values are mean \pm S.D, n=6 animals. * $P < 0.05$ (Isd) significantly different from normal. ^a $P < 0.01$ (Dunnett's *t* test) significantly and ^bnon-significantly different from control group.

Table 2. Levels of GSH and lipid peroxidation (MDA) in the heart of rats treated with doxorubicin (6 mg/kg body wt., i.p), doxorubicin+methanolic extract of *G. lucidum* (GLME) (500 mg/kg and 1,000 mg/kg body wt., p.o) and normal rats

Groups	Treatments (mg/kg)	GSH (nmol/mg protein)	MDA (nmol/mg protein)
Normal	-	4.18 ± 0.37	0.34 ± 0.02
Doxorubicin	6	2.37 ± 0.14*	1.00 ± 0.12*
Doxorubicin + GLME	500	3.48 ± 0.27 ^a	0.43 ± 0.06 ^a
Doxorubicin + GLME	1,000	4.43 ± 0.25 ^b	0.29 ± 0.03 ^a

Values are mean ± S.D, n=6 animals. * $P < 0.05$ (Isd) significantly different from normal. ^a $P < 0.01$ significantly and b non-significantly (Dunnett's-*t* test) different from control group.

Table 3. Serum CK in the heart of rats treated with doxorubicin (6 mg/kg body wt.,i.p), doxorubicin + methanolic extract of *G. lucidum* (GLME) (500 mg/kg and 1,000 mg/kg body wt., p.o) and normal rats

Groups	Treatments (mg/kg)	CK (IU/l)
Normal	-	131.70 ± 39.46
Doxorubicin	6	588.33 ± 194.08*
Doxorubicin + GLME	500	423.30 ± 79.60 ^b
Doxorubicin + GLME	1,000	186.50 ± 36.50 ^a

Values are mean ± S.D, n=6 animals. * $P < 0.05$ (Isd) significantly different from normal. ^a $P < 0.01$ (Dunnett's-*t* test) significantly and b non-significantly different from control group.

mg/kg) were 423.30±79.60 and 186.5±36.50 IU/l respectively. The activity was reduced by 68.3% in the 1,000 mg/kg extract treated group of animals.

Histopathological examination

Histopathological examination of heart tissue sections of doxorubicin alone treated animals showed elongation of cardiomyocytes, increased intercellular spaces and myocyte nuclear swellings. However, sections of heart tissue of animals pretreated with *G. lucidum* extract was found to retain almost normal cardiac texture (Fig. 1).

Preliminary phytochemical analysis of the extract indicated that polysaccharides and terpenes were the major components of the extract. This conclusion was based on the positive reaction of the extract to anthrone test (Yemm and Wills, 1954) and to phenol-sulphuric acid reagent (Dubois *et al.*, 1956) and thin layer chromatography analysis (TLC) of the extract (Harbone, 1973).

DISCUSSION

The results of the study reveal that the methanolic

extract of *G. lucidum* possess significant cardioprotective activity against doxorubicin induced cardiotoxicity in rats. Doxorubicin is an anticancer drug with a wide range of clinical uses (Childs *et al.*, 2002). The drug is used alone and in combination with other drugs for the treatment of tumors including malignant lymphomas and leukemia. However, its use continues to be limited by a potentially fatal dose related cardiotoxicity caused partially by its ability to generate free radicals and the increase production of free radicals results in oxidative stress. Doxorubicin induced generation of oxygen radicals may occur in several ways. During redox cycling of doxorubicin, superoxide anion radical (O_2^-) is formed which is then dismutated into H_2O_2 and O_2 by superoxide dismutase. Further more, doxorubicin is a very strong chelator of transition metal ions. The doxorubicin-iron complex is an even better radical generator than doxorubicin itself. Doxorubicin may even release bound non-heme iron, OH radicals and other reactive oxygen species that are able to initiate lipid peroxidation (Minotti, 1989). In mice and rats, Doxorubicin significantly increased MDA levels in myocardial

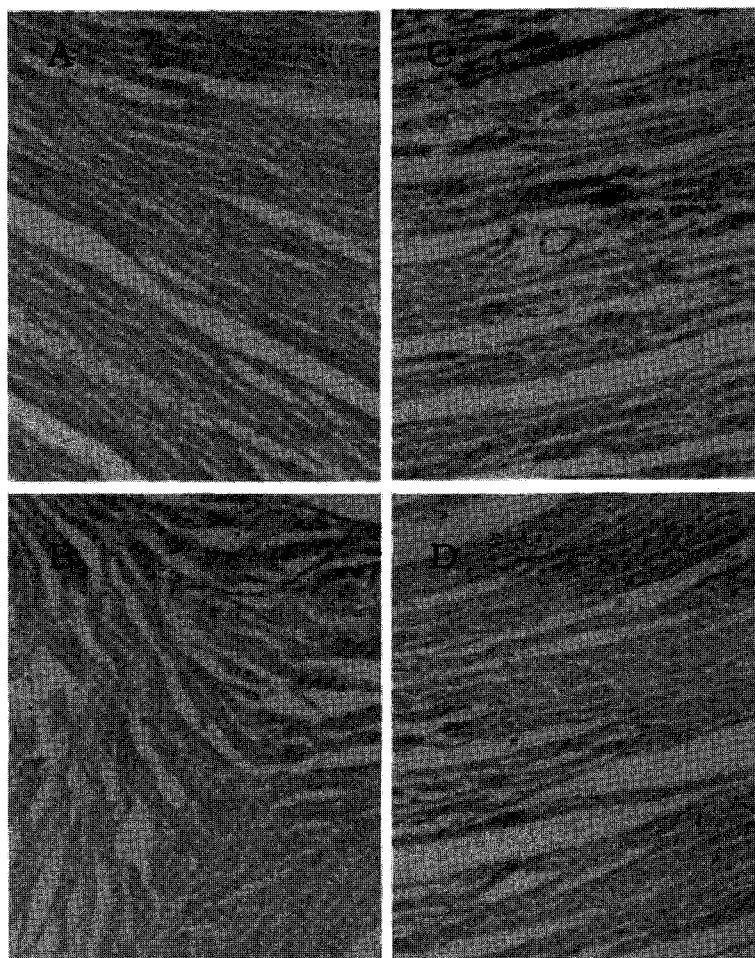


Fig. 1. Effect of methanolic extract *G. lucidum* (GLME) against doxorubicin induced cardiotoxicity in rats. Heart sections were stained with H&E. a) Normal; b) Doxorubicin (6 mg/kg); c) Doxorubicin + GLME (500 mg/kg) and d) Doxorubicin + GLME (1,000 mg/kg). Magnification $\times 20$.

tissues (Naidu *et al.*, 2002).

The antioxidant system consists of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSH), and glucose-6 phosphate dehydrogenase. These enzymes render protection against toxic oxygen radicals produced during normal metabolism and after oxidative insult. In our study, doxorubicin treated rats showed increase in heart tissue MDA levels with decrease in levels of SOD and GPx. Methanolic extract of *G. lucidum* prevented the doxorubicin-induced changes in MDA level and decline of antioxidant enzyme

activities. There is a direct correlation between GSH depletion and enhanced lipid peroxidation.

The experimental results reveal that methanolic extract of *G. lucidum* is capable to prevent cardiotoxicity caused by doxorubicin administration. The protective effect is mainly due to the capacity of the extract to restore cardiac antioxidant defense system. Our earlier investigations have shown that methanolic extract of *G. lucidum* occurring in South India possessed significant antioxidant and antitumor activities (Jones and Janardhanan, 2000). The experimental findings thus suggest the potentials

of South Indian *G. lucidum* mushroom for its therapeutic use as adjuvant with cancer chemotherapeutic agent. It is well established that GSH exhibits a broad spectrum of biological activities and plays a crucial role in cellular antioxidant defenses. The GSH antioxidant system consists of an array of non-enzymatic and enzymatic reaction pathways involved in the neutralization of reactive free radicals. GSH level was decreased after the administration of doxorubicin as compared with the normal values. Serum CK activity, which is a marker of cardiotoxicity was higher in control animals and the activity was significantly reduced by the treatment with the extract especially at a dose of 1,000 mg/kg body wt. The results thus suggest the cardioprotective effect of methanolic extract of *G. lucidum*. The histopathological observations of the heart tissue of animals treated with the extract also support this conclusion.

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