



Original Research Article

***In vivo* Antioxidant and Enzymatic Activity of *Ganoderma lucidum* (Curt.: fr.) P. Karst. on Mammary Cells of DMBA Induced *Sprague dawley* Rats**

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The popular mushroom *Ganoderma lucidum* (Reishi) is a bracket fungus and has been widely used for the promotion of health and longevity in Asian countries. The dried powder of *G. lucidum* was popular as a cancer chemotherapy agent in ancient China. *G. lucidum* clearly demonstrates anticancer activity in experiments with cancer cells and has possible therapeutic potential as a dietary supplement or alternative therapy for breast and prostate cancer. In our investigation, thirty six female Sprague dawley rats were evenly divided into six groups. From group I to VI. And tamoxifen treated as positive control. And on 120th day the animals were sacrificed, then blood, liver and kidney samples were isolated. The extracts produced significant effects on mammary carcinoma as evidenced by decreased ($p < 0.01$) enzymatic and non-enzymatic reaction such as superoxide dismutase, catalase, Glutathione peroxidase, reduced glutathione, lipid peroxidation (T Bars method), Vitamin C and E. Also decreased ($p < 0.01$) mitochondrial and glycolytic enzymes was significantly observed. *G. lucidum* has the potential activity against mammary carcinoma probably by its antioxidant and enzymatic activity.

Introduction

Oxidative stress is known to be a major contributor to increased cancer risk. Free radicals and reactive oxygen species (ROS) are generated as by-products of metabolic processes involving redox enzymes and bioenergetics electron transfer, and exposure to some exogenous chemicals. Cancer cells generate increased levels of free radicals relative to normal cells, further contributing to cancer progression (Dreher and Junod, 1996). More and more evidence suggests that this cancer-inducing oxidative damage might be prevented or limited by using anti-oxidants.

Anti-oxidants may mediate their effect by directly reacting with ROS, quenching them or chelating the catalytic metal ions (Sun *et al.*, 2002). *Ganoderma lucidum* a basidiomycete white rot fungus belonging to the Ganodermataceae family has been widely used for medicinal purposes to promote health and longevity in China, Japan and Korea for thousands of years (Noguchi *et al.*, 2005). The mushroom is considered to be a popular folk medicine for prevention or treatment of various diseases including hepatitis, hypertension, hypercholesterolemia, gastric cancer,

arthritis and bronchitis. Recent studies on this fungus have also revealed its various positive biological activities, including antitumour and hypoglycemic activity or anti-inflammatory effects and cytotoxicity towards hepatoma cells. The dried powder of *G. lucidum* is currently used worldwide as dietary supplement (Russell and Paterson, 2006). This research was focused on the potential anti oxidant effect of *G. lucidum* on mammary cells of SD rats.

Materials and Methods

Fungal material

The fruit body of *G. lucidum* was collected from the substrate of *Cocos nucifera* of Aadudhurai, Mayiladudhurai district. The fungal organism was identified and confirmed by standard manuals (Arora, 1979; Dickinson and John Lucas, 1979; Gilbertson and Ryvardeen, 1986; Gilbertson and Ryvardeen, 1987; Jia *et al.*, 2009; Parker, 1996).

Preparation of CMC *G. lucidum* extract

The fresh fruit body was air-dried, chopped and ground into coarse powder. Dried 200g of powder was dissolved in 750ml of 1% Carboxy methyl cellulose solvent for 72 hrs. Then centrifuged at 3000 rpm for 10 min. The centrifuged extracts (Supernatant) were again re-centrifuged and filtered with Millipore filter (Dandan Liua *et al.*, 2009). The extract was concentrated under reduced pressure below 50°C through rotary evaporator (RE 200, Bibby Sterling, UK). The concentrated extract was collected and air-dried for complete evaporation of CMC.

Chemicals

The DMBA was purchased from Sigma chemicals, Mumbai, India. And it was prepared by the company when the order is

placed and is shipped on dry ice. Upon arrival, DMBA was stored at -20°C to prevent its decomposition. 25mg/kg of DMBA was dissolved in 1ml of olive oil. The solution was used within 20 min after its preparation. Standard drug Tamoxifen citrate 10mg/kg was dissolved in 0.2 ml peanut oil vehicle. Estimation of enzymes by using semi auto analyzer Photometer 5010 v₅₊ using standard enzymatic kits procured from Piramal Healthcare limited, Lab Diagnostic Division, Mumbai, India.

Experimental animal

Female Sprague-dawley rats were purchased from Shri Venkatesh Animal House, Bangalore, India having age of 21 days and 70 gm body weight was used throughout the study. All rats were kept at room temperature of 22°C under 12 hr light/12 hr dark cycles in the animal house. Rats were fed with standard food. Animals were fed with commercial pellet diet and water *ad libitum* freely throughout the study. All animal procedures were performed after approval from the IAEC (institution of animal ethical committee) and in accordance with the recommendations for the proper care and use of laboratory animals. (CPCSEA/685)

Drug administration

Before the induction of DMBA, the Estrous cycle was monitored daily by vaginal cytology assay performed at 0800-0900h. A glass rod was inserted into the Vagina and gently touched against the vaginal wall. The Vaginal cells were then smeared onto the drop of 0.9% normal saline placed on the glass slide. The smear was observed under light microscope.

Thirty days before the induction of tumour, CMCGL extract was administered by single gastric intubation at the following doses of

250, 500mg/kg. At the end of the 30th day Tumour was induced by administrating DMBA (25 mg/ kg) was induced by single gastric intubation in 1ml olive oil and then the treatment was continued up to a period of 120 days.

Animal allotment

In each model of mammary cancer, thirty six female Sprague dawley rats were evenly divided into six groups.

- Group I : Normal saline
- Group II : Inducer (DMBA (25 mg/ kg)
- Group III : Inducer (DMBA (25 mg/ kg) + Cancer control (Tamoxifen Citrate)
- Group IV : Inducer (DMBA (25 mg/ kg) + CMCGL Extract 250 mg/kg
- Group V : Inducer (DMBA (25 mg/ kg) + CMCGL Extract 500 mg/kg
- Group VI : Extract alone 500 mg/kg

Experimental procedure

After treatment the time of latency period (the number of days between the NMU injection and the appearance of the first Tumour in each rat) was noted. At the end of the 120th day the animals were anaesthetized and blood was collected by Retro orbital bleeding then centrifuged and the serum was collected then the animals were sacrificed, Kidney and liver samples were isolated and washed with normal saline and stored for 12 h for in vivo antioxidant studies.

The separated liver and kidneys were homogenized with motor driven Teflon coated homogenizer in ice-cold (10% w/v) 0.1 M Tris-HCl buffer pH 7.4 to get 10% homogenate. The homogenate was centrifuged at 10000 rpm for 10 min at 5°C. The supernatant was collected and used for following in vivo studies.

Enzymatic anti-oxidant activity

Estimation of Superoxide dismutase (SOD) Activity (Jim Kimbrough and Tim Momol, 2000):

In a test tube, 0.5ml of supernatant tissue homogenate was taken. To this 1.5ml of carbonate buffer (pH 10.2), 0.5ml of 0.1Mm EDTA and 0.4ml of epinephrine was added and the OD was taken at 480nm. Epinephrine was added just before taking the OD. The activity of SOD was expressed as units/min/mg protein. One unit of the enzyme is defined as the amount of enzyme, which inhibits the rate of adrenaline auto oxidation by 50%.

Estimation of Catalase activity (Sinha, 1972):

The assay mixture contained 4 ml of hydrogen peroxide, 5 ml of phosphate buffer and 1 ml of homogenate. One ml portion of the reaction mixture was withdrawn and blown into 2 ml of dichromate/acetic acid reagent at 1 min intervals. Then the mixture was incubated for 30 min later the OD was measured at 570 nm. The activity of catalase was expressed as μ mole of H₂O₂ consumed/min/mg protein.

Estimation of glutathione peroxidase (GPx) activity (Rotruck *et al.*, 1973):

The reaction mixture consist of 0.2 ml each of EDTA, sodium azide, H₂O₂, 0.4 ml of phosphate buffer, 0.1 ml homogenate/mitochondria and was incubated at 37°C at different time intervals. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged at 2000 rpm. To this 0.5 ml of supernatant, 4 ml of disodium hydrogen phosphate and 0.5 ml DTNB were added and the colour developed was read at 420 nm immediately. The activity

of GPx was expressed as μ moles of glutathione oxidized/min/mg protein.

Estimation of reduced glutathione (GSH) activity (Ellman George, 1959; Moron *et al.*, 1979):

One ml of homogenate was precipitated with 1 ml of TCA and the precipitate was removed by centrifugation. To 0.5 ml of supernatant, 2 ml of DTNB was added and the total volume was made up to 3 ml with phosphate buffer. The absorbance was read at 412 nm. The level of glutathione was expressed as μ g/mg protein.

Non-Enzymatic Anti-Oxidant Activity

Assay of lipid peroxidation (LPX) (Desai, 1984):

Lipid peroxidation in brain was estimated colorimetrically by thiobarbituric acid reactive substances TBARS and hydroperoxides by the method of Niehaus and Samuelsson (1968) and Jiang *et al.* (1992) respectively. In brief, 0.1 ml of tissue homogenate Tris-HCl buffer, (pH 7.5) was treated with 2 ml of (1:1:1 ratio) TBA-TCA-HCl reagent thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA) and placed in water bath for 15 min, cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. The level of lipid peroxides was expressed as nmoles of MDA formed/mg protein.

Estimation of ascorbic acid (vitamin C)

To 0.5 ml of homogenate, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To this one ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37°C for 3 hrs. Then 1.5 ml of sulphuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 min. The

colour developed was read at 520 nm. The level of ascorbic acid was expressed as μ g/mg protein.

Estimation of vitamin E (King, 1965)

To 1 ml of homogenate, 1 ml of ethanol was added and thoroughly mixed. Then 3 ml of petroleum ether was added, shaken rapidly and centrifuged. 2 ml of supernatant was taken and evaporated to dryness. To this 0.2 ml of bathophenanthroline was added. The assay mixture was protected from light and 0.2 ml of ferric chloride was added followed by 0.2 ml of o-phosphoric acid. The total volume was made up to 3 ml with ethanol. The colour developed was read at 530 nm. The level of vitamin E was expressed as μ g/mg protein.

Preparation of liver and kidney homogenate:

The separated liver and kidneys were homogenized with motor driven Teflon coated homogenizer in ice-cold 0.1M Tris-HCl buffer pH 7.4 to get 10% homogenate. This homogenate is used for the estimation of in vivo antioxidant levels and lipid peroxidation estimation.

Estimation of Mitochondrial TCA cycle enzymes

Isolation of mitochondria and microsomes

A 10% w/v homogenate was prepared in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and centrifuged at 600 x g for 10 min. The supernatant fraction was decanted and centrifuged at 15,000rpm for 5 min. The resultant mitochondrial pellet was then washed and resuspended in the same buffer. The post mitochondrial fraction was further centrifuged at 105,000 x g for 60 min. The microsomal pellet was suspended in 0.05 M Tris-HCl buffer, pH 7.5 containing 0.15 M KCl.

The purity of mitochondrial and microsomal fractions was assessed by measuring the activities of succinate dehydrogenase and glucose-6-phosphate dehydrogenase respectively.

Assay of Isocitrate dehydrogenase (Mehler *et al.*, 1951)

0.1ml of tris-HCL, 0.2ml of Trisodium Isocitrate, 0.3ml of manganese chloride and 0.2ml of mitochondrial suspension and 0.2ml of NADP⁺ 0.2ml of water were added. After 60 min of incubation, 1ml of DNPH was added followed by 0.5 ml of EDTA and kept at room temperature for 20 min. then 10ml of NaOH was added and the colour developed was read at 390 nm. A standard containing α -ketoglutarate was run simultaneously. The Isocitrate dehydrogenase activity was expressed as nmoles of α -ketoglutarate liberated/min/mg protein.

Malate Dehydrogenase L-malate : (NAD oxidoreductase) (King, 1965)

To 0.3ml of buffer, 0.1ml of NADH and 0.1ml of oxaloacetate were added and the total volume was made up to 2.9ml with water. The reaction was started by adding 0.1ml of mitochondrial suspension. The change in optical density was measured at 350 nm in an interval of 15 seconds for 5 minutes in a spectrophotometer. The enzyme activity was expressed as nmoles of NADH oxidized/min/mg of total protein under incubation condition.

Glycolytic enzymes

Aldolase D-Fructose-1-6-Bis Phosphate D-Glyceraldehyde-3-Phosphate Lyase (Okawa *et al.*, 1979)

The incubation sample contained 0.25 ml of substrate, 0.25 ml of hydrazine sulphate,

1ml of Tris-HCL buffer and requisite amount of preparation to make it up to 2ml. it was incubated at 37°C for 15min. the reaction was terminated by the addition of 10% TCA and the tubes were centrifuged. An aliquot of the supernatant was transferred to the tubes containing 1ml of 0.75N NaOH. The tubes were left at room temperature for 10min. 1 ml of dinitrophenyl hydrazine reagent was added, incubated at 37°C for 60min. the colour developed after the addition of 7ml of 0.75N NaOH solution was read using a green filter (540 nm). Colour was developed with aliquots of standard DL-glyceraldehyde solution by treating in a similar manner. The enzyme activity is expressed as nmoles of glyceraldehyde formed/min/mg protein.

Gluconeogenic enzymes

Glucose-6-phosphate phosphohydrolase (King, 1965)

The incubation mixture in a total volume of 0.1 ml contained 0.3 ml of buffer, 0.5 ml of substrate, and 0.2ml of enzyme solution. Incubation was carried out at 37°C for 60min. the reaction was terminated by the addition of 1ml of 10% TCA solution. The suspension was centrifuged and the phosphorus content in the supernatant was estimated by the method (Subbarow, 1925). The enzyme activity is expressed as nmoles of Pi liberated/min/mg protein.

Fructose-1, 6-Diphosphatase Fructose-1,6-Diphosphate Phosphohydrolase (Gancedo and Gancedo, 1971)

The assay medium in a final volume of 2ml contained 1.2ml of buffer, 0.1ml of substrate solution, 0.25ml of MgCl₂, 0.1 ml of KCl solution, 0.25ml of EDTA solution and 0.1ml of enzyme. The incubation was carried out at 35°C for 15min. the reaction was terminated by the addition of 1ml of

TCA. Then estimation was carried out.

Statistical analysis

All the data were statistically evaluated and the significance of various treatments was calculated. All the results were expressed as mean standard error of mean (S.E.M) and comparison between the groups were made by Analysis of variance (ANOVA), followed by student t test. A value of $P < 0.01$ was considered significant.

Result and Discussion

Antioxidants donate an electron to stabilize a free radical. When a free radical is neutralized it is not capable of damaging our cells. There are two sources of antioxidants; our body makes some of them, including superoxide dismutase and several glutathione compounds. Mushrooms contain several unique polysaccharides that possess antitumorogenic effects (Ghoneum, 1999).

Enzymatic activity

Dietetic treatment using a *Ganoderma* mycelium-derived polysaccharide extract can be used to suppress the formation of colonic aberrant crypt foci in rats, possibly via reducing the oxidative damage induced by ROS (Lu *et al.*, 2003; Lu *et al.*, 2001). In addition, an amino-polysaccharide fraction of *G. lucidum* called 'G009' inactivates hydroxyl radicals and superoxide anions and reduces DNA strand breaks in a dose-dependent manner (Lee *et al.*, 2001). In this study polysaccharides have been shown to enhance the host's immune response by stimulating the production of macrophages, NK cells, and T-lymphocytes. Like triterpenes, polysaccharides can act as an anti-oxidant by reducing oxidative damage induced by ROS and preventing DNA strand breaks. Protein bound polysaccharides from *G. lucidum* (You and Lin, 2002), methanolic extracts from *G. tsugae* (Yen and Wu, 1999)

and ethanolic extracts from *G. lucidum* (Lakshmi *et al.*, 2003) have exhibited superoxide and hydroxyl radical scavenging activity. In our presentation, super oxide dismutase (SOD) and catalase (CAT) activity and levels of reduced glutathione (GSH) and lipid peroxidation as equivalents of malondialdehyde (MDA) formed were determined.

Non-Enzymatic activity

A hot water extract of *G. lucidum* has exhibited antioxidative effect on mouse liver and kidney lipid peroxidation (Shieh *et al.*, 2001) and extracts have also been found to reduce strand breakage in DNA caused by UV induced photolysis of hydrogen peroxide (Kim and Kim, 1999). Phenols have also been identified as a major constituent in methanolic extracts of mushrooms as naturally occurring antioxidants (Mau *et al.*, 2002). *Ganoderma* species were also found to possess higher antioxidant activity, reducing power, and scavenging and chelating abilities than the other mushrooms studied. There have been few investigations of the antioxidant activities of triterpenes from *Ganoderma* species (Zhu *et al.*, 1999). *Ganoderma lucidum* medicinal mushrooms that produce polysaccharides respond to environmental factors directly and for some, the nutritional conditions determine the degree of exopolysaccharide formation.

In an animal study (diabetic rats), nonenzymic and enzymic antioxidants levels increased and lipid peroxidation levels decreased with *G. lucidum* treatment (Jia *et al.*, 2009). However, a direct link has not been established between the antioxidant properties of *G. lucidum* and its immunomodulatory and anticancer effects, and whether lingzhi acts as an antioxidant or pro-oxidant may depend on concentration and environment.

Antioxidant activity

Table.1 In vivo antioxidant activity of CMCGL extract on mammary carcinoma of SD rats
Enzymatic Reaction

S.No.	Parameters	Control	Only DMBA (25 mg/ kg bw)	DMBA Tamoxifen Citrate (10mg/kg bw) ⁺	DMBA CMCGL (250mg/kg bw) ⁺	DMBA CMCGL (500mg/kg bw) ⁺	ONLY CMCGL (500mg/kg bw)
1.	Superoxide dismutase (units/mg of tissue)	26.71±4.664	36.96±5.885	43.65±2.650	34.78±4.121	39.92±4.212	39.68±8.593
2.	Catalase	57.97±12.114 ^c	274.29±47.968	286.98±14.434	144.49±20.581 ^c	158.40±16.356 ^c	357.60±30.607 ^a
3.	Glutathione peroxidase (moles of glutathione)	2.73±3.144	1.93±0.308	1.51±0.536	1.15±0.157	1.53±0.124	2.35±0.135
4.	Reduced Glutathione (mg of GSH/ mg of tissue)	14.56±1.073	18.10±1.827	22.42±1.356	21.60±5.124	20.08±1.291	28.00±2.462 ^b

Values are expressed as the mean ± S.D; Statistical significance (p)calculated by one way ANOVA followed by dunnett's ^cP< 0.001, ^bP < 0.01, ^aP < 0.05 calculated by comparing treated group with Induced group

Non-Enzymatic Reaction

Values are expressed as the mean ± S.D; Statistical significance (p)calculated by one way ANOVA followed by dunnett's ^cP< 0.001, ^bP < 0.01, ^aP < 0.05 calculated by comparing treated group with Induced group

Determination of mitochondrial and glycolytic enzymes estimation

Table.2 Effect of CMCGL extract on the mitochondrial and glycolytic enzyme activation of mammary carcinoma

S.No.	Parameters	Control	Only DMBA (25 mg/ kg bw)	DMBA Tamoxifen Citrate (10mg/kg bw) ⁺	DMBA CMCGL (250mg/kg bw) ⁺	DMBA CMCGL (500mg/kg bw) ⁺	ONLY CMCGL (500mg/kg bw)
1.	Isocitrate dehydrogenase_ KIDNEY	0.25±0.401	0.15±0.054	1.29±1.430	0.88±0.245	1.07±0.922	1.43±0.874 ^a
2.	Isocitrate dehydrogenase_LIVE R	0.29±0.227	0.08±0.037	1.22±1.389	0.75±0.264	1.01±0.845	1.31±0.889 ^a
3.	Malate dehydrogenase_ KIDNEY	0.91±0.476	0.93±0.088	0.88±0.698	0.91±0.261	0.72±0.163	1.10±0.298
4.	Malate dehydrogenase_LIVE R	0.08±0.048	0.48±0.405	0.49±0.717	0.08±0.043	0.39±0.447	0.07±0.027
5.	Aldolase	0.01±0.011	0.02±0.008	0.03±0.038	0.03±0.029	0.01±0.004	0.04±0.032
6.	Glucose-6- phosphatase	0.03±0.006	0.05±0.004	0.03±0.006	0.04±0.006	-0.02±0.048^c	0.03±0.023
7.	Fructose-1,6-di phosphatase phosphorous	0.04±0.011	0.03±0.002	0.04±0.000	0.04±0.010^a	0.03±0.009	0.04±0.005 ^a

Values are expressed as the mean ± S.D; Statistical significance (p)calculated by one way ANOVA followed by dunnett's ^cP< 0.001, ^bP < 0.01, ^aP < 0.05 calculated by comparing treated group with Induced group

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