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ANTI-TUMOUR ACTIVITY OF EXO-POLYSACCHARIDES PRODUCED BY AN ISOLATE OF *GANODERMA LUCIDUM* OCCURRING IN SOUTH INDIA

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ABSTRACT

Ganoderma lucidum is a popular medicinal mushroom worldwide because of its fascinating therapeutic properties. Investigations carried out in our laboratory have demonstrated the antitumor activity of the extracts, polysaccharides and the terpenoids isolated from fruiting bodies of G. lucidum occurring in south india. In this study, we examined the antitumor activity of the exopolysaccharides produced by a G. lucidum isolate in submerged culture. The exopolysaccharides were isolated from the culture broth by repeated ethanol precipitation, treatment with Sevag's reagent for the removal of protein, dialysis and lyophilisation. The antitumour activity was tested on Swiss albino mice implanted with Dalton's lymphoma ascites (DLA) cell line. The exopolysaccharides at doses of 5, 10 and 25 mg/kg body weight achieved 80, 84 and 86% tumor growth inhibition respectively. The standard reference drug, cyclophosphamide at a dose 25mg/kg body weight showed 94% tumor inhibition. The results thus showed that exopolysaccharides produced by the G. lucidum isolate possessed excellent antitumor activity. The finding suggests the potential therapeutic use of exopolysaccharides produced by G. lucidum.

KEY WORDS

Antitumor activity, Ganoderma lucidum, Exopolysaccharides, Submerged culture

INTRODUCTION

Among the treatment options for cancer, chemotherapy is considered as the most effective treatment option. However, drug resistance and dose limiting toxicities are some of the limitations of chemotherapy. A large number of chemotherapeutic drugs derived from synthetic as well as natural sources have been employed in the treatment of cancer for a long time. Most of the chemotherapy drugs currently in use have severe side effects in patients, hence search for chemotherapy drugs with least toxicity to the host cells, is continued. Discovery of safe and nontoxic Chemotherapeutic agents would benefit millions suffering from cancer.

Mushrooms have attracted great deal of interest as a source of biopharmaceuticals in recent years. They have been demonstrated to possess great potential in developing useful anticancer bioactives [1,2]. *Ganoderma lucidum* is popularly known as Linghzi or Reishi, is considered as the most important medicinal mushroom that has contributed greatly for the treatment of ancient populations in South East Asia (3). In traditional Chinese medicine Linghzi was considered as the most effective herbal drug for the treatment of a large number of diseases. The extracts of the fruiting bodies and mycelia of this mushroom have been reported to possess profound antitumor property.

Ganoderma lucidum has been reported to contain nearly 400 chemical constituents (4). The major components are polysaccharides and triterpenes. *Ganoderma* polysaccharides has been identified as a major bioactive component in a large number of medicinal preparations approved by Chinese FDA and Its

clinical efficacy, safety and long-term tolerability have been demonstrated (5). Polysaccharides isolated from the fruiting bodies of *G lucidum,* have been reported to possess significant antitumor activity (6). In this study, we examined the antitumor activity of exopolysaccharides produced by *G lucidum* in submerged culture.

MATERIALS AND METHODS

Animals

Male Swiss albino mice weighing 25±3g were purchased from Small Animal Breeding Center, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, India. They were kept under controlled environmental conditions with free access to standard food and water. The animal experiments were carried out following the guidelines of CPCEA (Government of India) and with the approval of Institutional Animal Ethics Committee (IAEC).

Cell line

Dalton's lymphoma ascites (DLA) cell lines were obtained from Cancer Institute, Adayar, Chennai. The cells were maintained in mice by intraperitoneal inoculation of viable cells.

Isolation and maintenance of *G. lucidum culture*

Pure culture of ***G. lucidum*** was isolated from the fruiting bodies growing on *Caesalpinia coriaria* Wild trees by standard microbiological methods. The culture of the isolate was maintained on potato- dextrose- agar medium.

Culturing the isolate for exopolysaccharides production

Mycelia of G. *lucidum* were grown on glucose peptone nutrient medium (Glucose 20g, Peptone 2g, KH_2PO_4, 5g, $MgSO_4$ 2.5g, Yeast extract 2g Maltose 10g, Distilled water- 1000ml). 100 ml medium was poured into 500 ml Erlenmeyer flasks and sterilized at 121^{0}C for 30 minutes. The medium was inoculated with 10-day old culture of *G. lucidum* seed culture. The inoculated flasks were kept on a shaker (150rpm) and grown at 25-27^{0}C for 15 days. After 15 days submerged growth, the culture was filtered and the mycelium separated. The culture broth was passed through several layers of muslin cloth and then Whatman No 1. filter paper. The volume of the culture filtrate was reduced to one tenth of its original volume at low temperature using a rotary vaccum evaporator and used for the isolation of exopolysaccharides.

Isolation of polysaccharides

Isolation of total exopolysaccharides from *G. lucidum* culture broth was carried out by the method of Mizuno (7) with some modifications. Culture broth was precipitated by the addition of 4x volume of chilled ethanol, and kept at 4^{0}C for 48 hours. It was then centrifuged at 10,000 rpm for 20 minutes. The supernatant was discarded, and pellet was dissolved in distilled water and again treated with ethanol. This process was repeated thrice. Finally, the pellet was dissolved in distilled water and treated with Sevag's reagent (8) (chloroforms: n-butanol; 4:1) several times to remove the protein and then dialyzed against deionised water for 48 hours. The polysaccharide fraction was finally lyophilized. The yield of polysaccharides obtained from culture broth was 245mg/L. To determine polysaccharide nature of the fraction, it was tested for Anthrone reaction (9) using glucose as standard. The presence of protein was confirmed by Bradford reagent (10). For determining the monosaccharides composition, 5mg of the total exopolysaccharides was hydrolysed by 2M Trifluroacetic acid (TFA) at 100 ^{0}C overnight followed by evaporation to dryness. Residual TFA removed by two evaporation cycles with 0.5ml methanol and finally residue was dissolved in 0.5ml 20% isopropanol. The residue was analysed by paper and thin layer chromatography using n-butanol: acetic acid: water (2:1:1) solvent system. The chromatograms were sprayed with aniline diphenylamine phosphoric acid reagent (2ml aniline, 2g diphenylamine and 10ml of 85% phosphoric acid in 100ml of acetone) and heated at 100^{0}C for 10 minutes.

Determination of antitumor activity

Swiss albino mice 25 ± 3 g were divided into five groups of six animals in each group. Viable DLA Cells (1×10^6) in 0.1ml PBS were transplanted subcutaneously onto the hind limb of mice. Group I injected with DLA cells alone and group II treated with cyclophosphamide 40mg /kg orally were maintained as control and standard drug treatment respectively. Group III, IV, V were administered with exopolysaccharides at concentrations of 5, 10, 25 mg/kg body weight orally 24 hours after tumor implantation and continued for once daily 10 consecutive days. The tumor development in animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier callipers twice a week for 5weeks. The tumor volume was calculated using the formula; V=4/3

$\pi r_1^2 r_2$. Where, r_1 is the minor radius and r_2 is the major radius. At the end of the fifth week, animals were sacrificed under anaesthesia, tumor extirpated and weighed. The percent inhibition was calculated using the formula (1-T/C) ×100; where C is the average tumour weight of the control group and T that of the treated groups.

Statistical analysis

All the values are expressed as mean ± SD. Experimental data were analysed for statistical significance using one-way ANNOVA.

RESULTS AND DISCUSSION

Isolation of polysaccharides.

The isolation of polysaccharide from the culture broth of *G.lucidum* was accomplished by ethanol precipitation. Polysaccharides nature was confirmed by Anthrone reaction and protein content was confirmed by Bradford's reagent. The results are presented in Table-1.

Table 1: Polysaccharide and protein content of the Exo polysaccharides isolated from *G .lucidum* in submerged culture

Content in Percentage	
Polysaccharides	66.6
Protein	27.6

Table 2: Antitumor activity of Exopolysaccharides of *G.lucidum*

Group	Volume on 5th week (cm^3)	% inhibition
Control	1.985 ± 0.765	-
Cyclophosphamide(25mg/kg)	0.164± 0.0566	94
Exopolysaccharides (5mg/kg)	0.395 ± 0.0350***	80
Exopolysaccharides(10mg/kg)	0.341 ± 0.0195***	84
Exopolysaccharides(25mg/kg)	0.285 ± 0.0250***	86

All values are expressed as mean ± SD (n = 6, ***P < 0.01 and *P < 0.05 compared to control were considered significant).

Paper chromatography analysis showed the Rf values of standards monosaccharide were 0.657 (D-galactose) 0.668 (D-mannose) 0.651 (D-glucose) and 0.661 (D-fructose). The TLC analysis showed the R_f values of standards were D-galactose (0.571), D-mannose (0.642), D-glucose (0.635), D-fructose (0.642).

The paper chromatography analysis showed that the hydrolysis product of exo polysaccharides produced a spot with the Rf value of 0.651 which coincided with the R_f value of glucose standard. This observation was further confirmed by TLC analysis. In TLC analysis exo-polysaccharides showed identical spots with R_f value of 0.635 which coincided with the R_f value of glucose. Thus, glucose was identified as the monosaccharide component of exo polysaccharides produced by G. *lucidum.*

Antitumor activity

The exopolysaccharides isolated from *G.lucidum* showed significant antitumor activity against solid tumor induced by DLA cell lines. The exopolysaccharide was observed to inhibit 86% tumor growth at a dose of 25 mg/kg body weight, after five weeks (Table 2). Even at a low dose of 5mg/Kg body weight the exopolysaccharides showed 80% inhibition of tumor growth. The standard reference drug cyclophosphamide at a dose of 25mg/kg body weight inhibited 94% tumor growth. However, cyclophosphamide caused drastic reduction in body weight of treated animals compared to exopolysaccharides treatment (Fig 1). Mortality rate in cyclophosphamide administered group was very high (Fig 2).

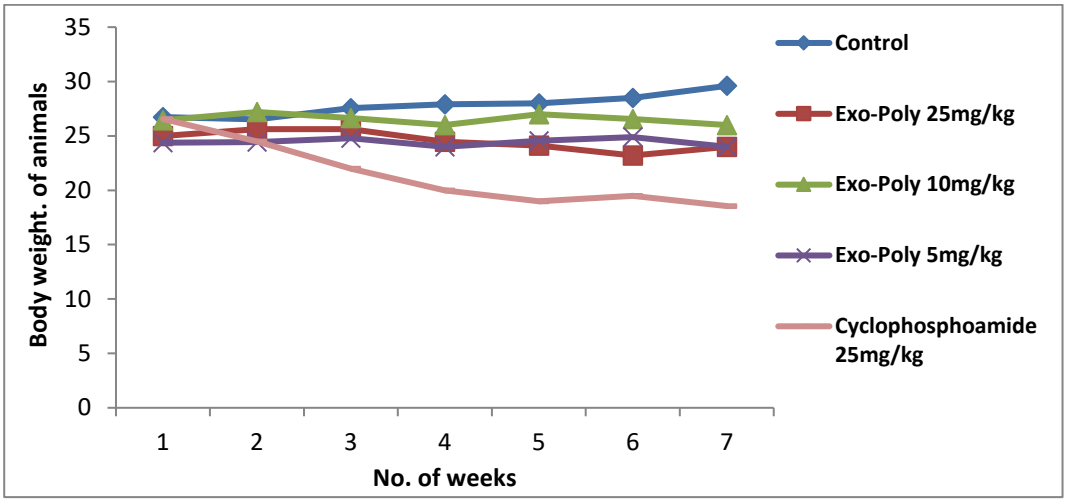

Figure 1: Body weight of animals under treatment

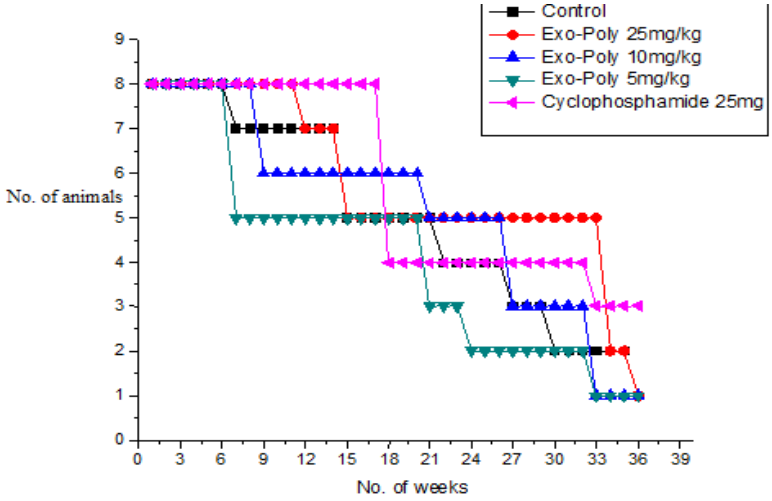

Figure 2: Mortality of animals during treatment

A number of edible and medicinal mushrooms derived products are used in traditional medicines for the treatment of diseases such as hypertension, arthritis, diabities, hyperchlosterimia, hepatitis and cancer. Among the medicinal mushrooms used in traditional therapy, G *lucidum* is the most interesting because of its fascinating therapeutic effects (11). *Ganoderma lucidum* popularly known as Reishi or Linghzi is known as mushroom of immortality in China and has been used for several medicinal purposes in Traditional Chinese Medicine for more than 4000years. This mushroom contains a wide variety of bioactive constituents such as terpenoids, steroids, phenols, glycoproteins, and polysaccharides etc (12). Polysaccharides and triterpenes are the major biologically active chemical constituents of *G.lucidum.* Polysaccharide protein complexes are well known for their immunomodulatory and anticancer properties (13). Exopolysaccharides isolated from *G applanatum* when tested for its bioactivies exhibited selective activity against tumor cells and immunostimulatory effect. Cultivation of *G.lucidum* is a long time taking method, hence submerged fermentation of the mycelia is the alternative process for the production of bioactives from this mushroom (14).

The results of the current investigations reveal that exo-polysaccharides produced by *G.lucidum* in Submerged culture show significantly high antitumor activity. Earlier studies indicate that the amount of protein present in carbohydrate is directly proportional to the antitumor activity of mushrooms. The present study support this observation as evident from the significant high protein content of *G.lucidum* exopolysaccharides. The antitumor effect might be mediated through immunomodulating effect.

Exopolysaccharides at a concentration of 10mg/kg and 25mg/Kg almost completely inhibited the tumor growth in the animals. At both these treatments no, visible tumor growth could be found, at the end of the experiment. The standard drug, cyclophosphamide at a concentration of 25mg/Kg also inhibited the tumor growth as effectively as exopolysaccharide at its lowest concentration (5mg/Kg). Cyclophosphamide is an extensively used chemotherapeutic drug for treating cancer patients. However, the exopolysaccharides even at its lowest concentration is found to have equivalent activity as cyclophosphamide. This suggests the possibility to develop *G.lucidum* exopolysaccharide as a drug for cancer treatments or as an adjuvents in cancer therapy. Cyclophosphamide has been found to cause severe toxic effects during the course of treatments. An added advantage of exopolysaccharide as a drug is that it doesn't cause any detectable toxicity in treated animals.

Majority of *Ganoderma* products available in the world today were developed from the fruiting bodies of the mushroom. The current investigation shows that exopolysaccharides produced by the *G lucidum* isolates would be an alternative source of bioactive antitumor polysaccharides.

CONCLUSION

Current investigations reveal that exopolysaccharides produced by *G lucidum* possessed excellent antitumor activity. The finding suggests the potential therapeutic use of *G lucidum* exopolysaccharides in cancer treatment.

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Conflict of interest

The authors declare that there is no conflict of interest in this study.

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