Cellular and Molecular Biology

Smina *et al. Cell. Mol. Biol.*2016, 62 (5): 55-59
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.5.9

Original Research

Ganoderma lucidum total triterpenes attenuate DLA induced ascites and EAC induced solid tumours in Swiss albino mice

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Abstract: *G. lucidum* total triterpenes were assessed for its apoptosis-inducing and anti-tumour activities. The ability of the total triterpenes to induce apoptosis was evaluated in Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC) cell lines. Total triterpenes were found to be highly cytotoxic to DLA and EAC cell lines with IC_{50} values 5 ± 0.32 and 7.9 ± 0.2 µg/ml respectively. Total triterpenes induced apoptosis in both cell lines which is evident from the DNA fragmentation assay. Anti-tumour activity was accessed using DLA induced solid and EAC induced ascites tumour models in Swiss albino mice. Administration of 10, 50 and 100 mg/kg b. wt. total triterpenes showed 11.86, 27.27 and 40.57% increase in life span of animals in ascites tumour model. Treatment with 10, 50 and 100 mg/kg b. wt. total triterpenes exhibited 76.86, 85.01 and 91.03% inhibition in tumour volume and 67.96, 72.38 and 77.90% inhibition in tumour weight respectively in the solid tumour model. The study reveals the significant dose-dependent anti-tumour activity of total triterpenes in both models. Total triterpenes were more active against the solid tumour than the ascites tumour. The anti-oxidant potential and ability to induce cell-specific apoptosis could be contributing to its anti-tumour activities.

Key words: Anti-tumour, apoptosis, cytotoxicity, anti-proliferation, mushrooms.

Introduction

Cancer is a complex disease that is triggered by various factors including genetic changes, diet, environmental and lifestyle modifications. Even though there are hundreds of drug available in the market against this deadly disease, various side effects especially their dose limiting toxicity towards the normal cells restrict their extensive clinical usage. Search is continuing for novel cell-specific and completely safe chemo therapeutic compounds. Many pharmaceutical substances with potent and unique health-enhancing properties have been isolated from natural products. Mushrooms contain a variety of physiologically functional compounds that are capable of developing into potential medicines or dietary supplements. Experimental evidence also supports that regular incorporation of certain powdered medicinal mushrooms or its extracts in the diets of animals can have a cancer prevention effect and restriction of tumour metastasis (1,2).

Ganoderma lucidum is a highly important medicinal mushroom that has been used extensively in Chinese traditional medicine. Crude extracts of *Ganoderma* species are used as remedies for the treatment of a number of ailments including cancer. A variety of *Ganoderma* extracts were found to arrest the cell cycle and prevent the growth of different cancer cell lines (3-5). Earlier studies on polysaccharides isolated from mycleia of *G. lucidum* revealed its growth inhibiting activity in Sarcoma-180 in Balb/c mice (6). It is also reported that co-administration of *G. lucidum* polysaccharides with cyclophosphamide in mice enhanced its anti-tumour activity (6). In this study, we have evaluated the apoptosis-inducing and anti-tumour activities of total triterpenes isolated from *G. lucidum*. Cytotoxicity and the ability of the total triterpenes to induce apoptosis were evaluated in various mouse cancer cell lines like Dalton's lymphoma ascites (DLA) and Ehrlich's ascites carcinoma (EAC). Anti-tumour activity was accessed using DLA induced solid and EAC induced ascites tumour models in Swiss albino mice.

Materials and Methods

Isolation of total triterpenes

Total triterpenes were isolated from the fruiting bodies of *G. lucidum* as previously described (7). Briefly, dried and powdered fruiting bodies of *G. lucidum* (100 g) were extracted with ethanol. The extract was concentrated (9 g) and dissolved in chloroform. The chloroform soluble fraction was separated and the solvent completely recovered, the residue (3 g) was loaded on silica gel column (3 cm×60 cm) and eluted with petroleum ether, chloroform, methanol and various combinations of these solvents [petroleum ether (Fr. 1–26), chloroform:petroleum ether 1:1 (Fr. 27–50), chloroform (Fr. 51–150), chloroform:methanol 9:1 (Fr. 151–165), methanol (Fr. 165–180)]. Each fraction was analysed for the presence of triterpenes using Liebermann–Burchard reagent (acetic anhydride–con. H2SO4). The fractions were also spotted on TLC plate and analysed for the presence of triterpenes using the spray reagent anisaldehyde–H2SO4. The fractions that answered the tests for triterpenes (8) were combined together and

Received, April 22 2016; **Accepted**, April 28 2016; **Published** April 30 2016

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concentrated to get the total triterpene fraction (TT) (1.5 g). The total triterpenes (TT) thus obtained were used for further studies.

Animals

Swiss albino mice (25±2g) used for the studies were purchased from Small Animal Breeding Station, Mannuthy, Kerala, India and were housed in well-ventilated cages under controlled conditions of light and humidity and provided with normal mouse chow (Sai Durga Food and Feeds, Bangalore, India) and water *ad libitum*. All the animal experiments were carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India, and by the approval of Institutional Animal Ethical Committee (149/99/CPCSEA dated 23-10-2009).

Cell lines

Ehrlich's ascites carcinoma (EAC) and Dalton's lymphoma ascites (DLA) were obtained from Cancer Institute, Adayar, Chennai and were maintained *in vivo* in Swiss albino mice by intra-peritoneal inoculation of 1×10^6 viable cells.

Cytotoxicity analysis in DLA and EAC cells

The *in vitro* cytotoxicity of total triterpenes in DLA and EAC cell lines was determined by trypan blue exclusion method (9). Briefly, 1×10^6 viable cells suspended in phosphate buffered saline (PBS) (0.2 M, pH 7.4) and various concentrations of total triterpenes (1-12 µg/ml) in a final volume of 1 ml were incubated at 37^0 C for 3 h. After the incubation, 10 µl of cell suspension was mixed with 90 µl of trypan blue solution (0.16%). The total number of cells present in 10^4 cc was counted with simultaneous determination of viability using a differential cell counter or haemocytometer. Percentage cytotoxicity was calculated after comparing with the untreated control using the formula; Cytotoxicity (%) = $(T_D - C_D)/ T_T \times 100$; where T_D - number of dead cells in the treated group, C_D - number of dead cells in the control group, T_T - total number of cells in the treated group.

Determination of apoptotic activity using DNA ladder assay

The ability of total triterpenes to induce apoptosis in DLA and EAC cell lines was determined using DNA ladder assay. DLA or EAC cell lines (2×10^6 cells) suspended in 1 ml PBS (0.2 M, pH 7.4) were incubated with various concentrations of the total triterpenes (5 and 10 µg/ml) for 4 h in a 5% CO_2 incubator at 37^0 C. The cells were washed with PBS, and the pellet was re-suspended in 100 µl lysis buffer (50 mM Tris–HCl, pH 8, 10 mM EDTA, 0.5% N-lauryl sarcosine, 0.5 mg/ml proteinase K) and incubated for 1 hour at 50° C. Ten microliter of RNase (1 mg/ml in Tris-NaCl buffer) was added to the lysate and further incubated for 1 h at 50° C. The DNA samples were resolved on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in Tris–Borate–EDTA buffer. The bands were visualized and photographed using a Gene Genius Bioimaging System.

Determination of anti-tumour activity

Ascites tumour model

Male Swiss albino mice (25±2 g) were divided into six groups of six animals each. Group 1 comprises of control animals that receive only intra-peritoneal injection of EAC cells and no other drug treatment. Group 2 animals received 25 mg/kg b. wt. cyclophosphamide, a standard reference drug, after tumour induction. Group 3, 4 and 5 animals received 10, 50 and 100 mg/kg b. wt. total triterpenes respectively after tumour induction. Group 6 animals received 250 µl of sunflower oil after tumour induction, which was used as the vehicle to administer total triterpenes. To induce ascites tumour, all the animals were injected with 1×10^6 viable EAC cells in PBS (aspirated from 15days old EAC ascites tumour in mice) to the peritoneal cavity. After 24 h of tumour cell inoculation, cyclophosphamide (25 mg/kg b. wt.) and total triterpenes (10, 50 or 100 mg/kg b. wt.) were administered orally, once daily, for 10 consecutive days. The mortality rate was noted in each group and the percentage increase in life span (ILS) was calculated using formula % ILS = (1-C/T)×100; where 'T' is mean survival time of treated group and 'C' that of the control group (10).

Solid tumour model

Male Swiss albino mice (25 ± 2 g) were divided into six groups of six animals each. Group 1 comprises of control animals that received only subcutaneous injection of DLA cells and no other drug treatment. Group 2 animals received 25 mg/kg b. wt. cyclophosphamide after tumour induction. Group 3, 4 and 5 animals received 10, 50 and 100 mg/kg b. wt. total triterpenes respectively after tumour induction. Group 6 animals received 250 µl of sunflower oil after tumour induction, which was used as the vehicle to administer total triterpenes. To induce solid tumour, $1x10^6$ viable DLA cells in 0.1 ml PBS were transplanted subcutaneously into the right groin of mice. Cyclophosphamide (25 mg/kg b. wt.) and total triterpenes (10, 50 or 100 mg/kg b. wt.) and were administered orally, 24h after tumour implantation, and continued for 10 consecutive days. The tumour development on animals in each group was determined by measuring the diameter of tumour growth in two perpendicular planes using vernier callipers twice a week for 5 weeks. The tumour volume was calculated using the formula $4/3\ \pi r_1^2 r_2$ 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 using diethyl ether, tumour extirpated and weighed. Percentage 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 group (11).

Statistical analysis

All values are expressed as mean ± standard deviation (S.D.). Statistical evaluation of the data was done by one way analysis of variance (ANOVA) followed by Bonferroni's test using InStat Graph pad software. $p<0.05$ was considered as significant with respect to control group.

Results and Discussion

Screening of compounds for their cytotoxicity in cancer cell lines is the preliminary step in evaluating its efficacy as chemo therapeutic drug. The cytotoxicity of total triterpenes was evaluated using trypan blue method. The total triterpenes showed remarkable cytotoxic activity against DLA (Fig. 1A) and EAC (Fig. 1B) cell lines. The concentration of total triterpenes required for 50% death of the DLA and EAC cell lines (IC_{50}) was found to be 5 ± 0.3 µg/ml and 7.9 ± 0.2 µg/ml respectively.

A variety of external agents including cytotoxic drugs can induce apoptosis in living cells. The apoptosis-inducing ability of total triterpenes was assessed by DNA ladder assay. Total triterpenes were highly effective in inducing apoptosis in DLA and EAC cells as evident from the fragmentation of DNA occurred in the treated cells. Fig. 2 indicates the laddered electrophoretic pattern of fragmented DNA in treated cells. In the control group, there was no fragmentation indicating the absence of apoptosis. Treatment with total triterpenes induced apoptosis leading to the laddering of DNA. These results confirmed the significant cytotoxicity of total triterpenes against DLA and EAC cell lines.

The total triterpenes possessed significant anti-tumour activity against EAC induced ascites and DLA induced solid tumour models. In ascites tumour model, the percentage increase in life span of standard and total triterpenes treated groups (10, 50 and 100 mg/kg b. wt.) is given in table 1. Total triterpenes, when administrated at a dose of 100 mg/kg body weight, increased the average life span of tumour bearing animals to 40.57% ($P < 0.001$), compared to the control group. Administration of 10 and 50 mg/kg body weight triterpenes also showed 11.86% and 27.27% increase in life span of animals. The standard reference drug cyclophosphamide (25 mg/kg b. wt.) exhibited 42.86% ($P < 0.001$) increase in life span compared to the control group. On 21st day of cell line administration, all the animals in the control group were dead. However, the mortality rate was decreased significantly in total triterpenes and standard drug-treated groups (Table 1). All the animals in the standard and 100 mg/kg. b. wt. total triterpenes treated groups were alive until 25th day after tumour inoculation.

Total triterpenes also possessed significant anti-tumour activity in solid tumour model (Fig. 3). Tumour volumes of the control, vehicle and treated groups at different days after tumour induction are represented in fig. 4. A significant reduction ($P < 0.001$) in the tumour volume and weight was observed in the treated groups at the end of the 5th week compared to the control group

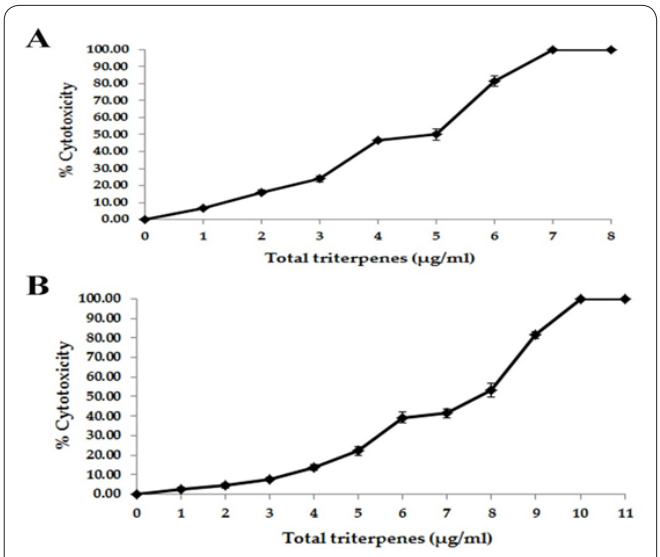

Figure 1. Cytotoxicity of total triterpenes A) Effect on DLA cell line; B) Effect on EAC cell line; Values are mean ± S.D., (n=3).

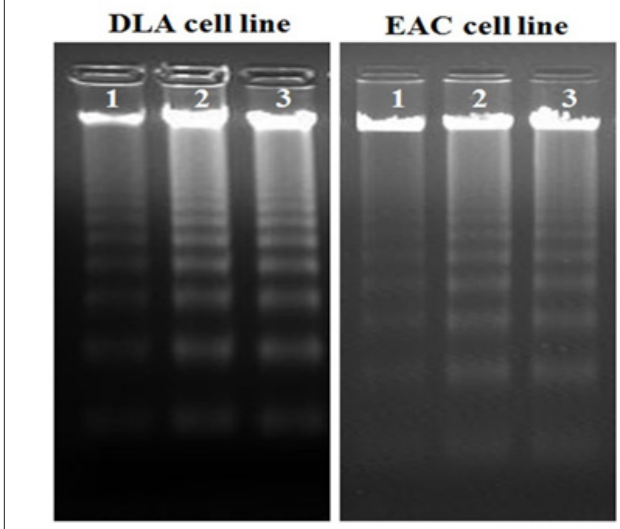

Figure 2. Induction of apoptosis by total triterpenes. DNA fragmentation observed in DLA and EAC cell lines Lane 1: Control, Lane 2: cells treated with 5 µg/ml total triterpenes, Lanes 3: cells treated with 10 µg/ml total triterpenes.

Table 1. Effect of total triterpenes on EAC and DLA induced ascites and solid tumours in mice.

Treatments	EAC induced ascites tumour		DLA induced solid tumour	
	Survival time (days)	Mortality (on 21st day)	Tumour volume (cm^3)	Tumour weight (g)
Control	17.33 ± 1.75	6/6	0.256 ± 0.07	3.02 ± 0.21
Cyclophosphamide (25 mg/kg b.wt)	30.33 ± 3.14***	0/6	0.016 ± 0.003***	0.70 ± 0.08***
TT (10 mg/kg b.wt)	19.67 ± 3.56 ns	3/6	0.059 ± 0.002***	0.97 ± 0.18***
TT (50 mg/kg b.wt)	23.83 ± 2.14**	1/6	0.038 ± 0.004***	0.83 ± 0.16***
TT (100 mg/kg b.wt)	29.17 ± 2.48***	0/6	0.023 ± 0.008***	0.67 ± 0.12***
Vehicle (250 µl)	17.50 ± 1.52 ns	6/6	0.238 ± 0.05ns	2.55 ± 0.60 ns

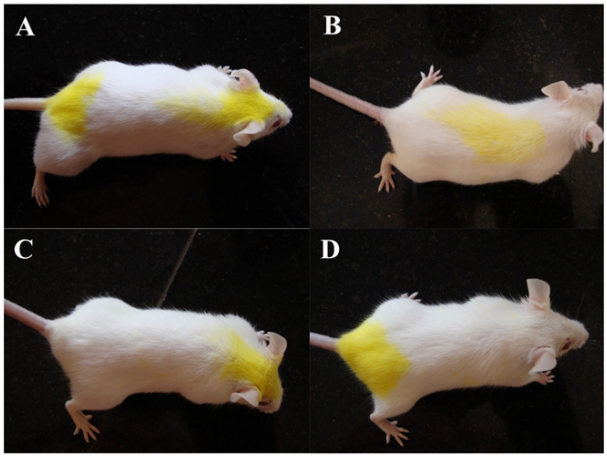

Figure 3. Effect of total triterpenes on DLA induced solid tumour. Representative images of animals from each group A) Control mouse; B) Mouse treated with 25mg/kg b. wt. cyclophosphamide; C) Mouse treated with 50mg/kg b. wt. total triterpenes; D) Mouse treated with 100mg/kg b. wt. total triterpenes.

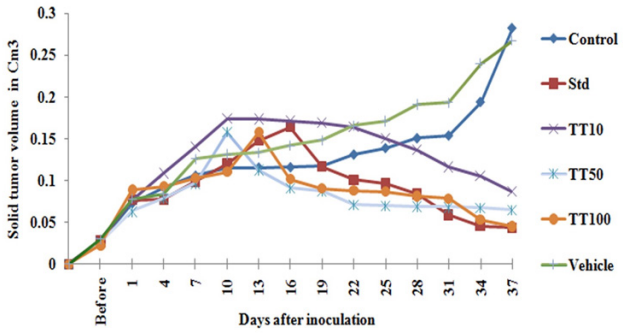

Figure 4. Effect of total triterpenes on solid tumor volume. Values are mean ± S.D., n=6., TT- total triterpenes (mg/kg b.wt.).

(Table 1). The administration of total triterpenes (10, 50 and 100 mg/kg b. wt.) showed 76.86, 85.01 and 91.03% inhibition in tumour volume and 67.96, 72.38 and 77.90% reduction in tumour weight respectively. The standard reference drug cyclophosphamide (25 mg/kg b. wt.) showed 93.82% inhibition of tumour volume and 76.80% reduction of tumour weight.

Thus, the present study reveals the significant dose-dependent anti-tumour activity of total triterpenes against EAC cell line induced ascites and DLA cell line induced the solid tumour. Total triterpenes were more active against the solid tumour than the ascites tumour. Higher concentration of total triterpenes (100 mg/kg b. wt.) showed almost the same activity as standard reference drug cyclophosphamide against both solid and ascites tumour. The survival rate and reduction of tumour volumes in treated animals highly recommend its potential as an anti-tumour agent. Earlier investigations on the anticancer or antitumor activity of *Ganoderma* extracts and triterpenes using *in vitro* and *in vivo* models also support the outcomes of the present study. Su et al. (3) reported the cytotoxic activity of lanostanoids from *G. tsugae* and found activity against three cancer cell lines. Gonzalez et al. (5) observed apoptosis in human promyelocytic leukaemia HL-60 cells exposed to three lanostanoids isolated from *G. concinna*. Studies by Kimura et al. (12) described that triterpenoid fraction from the fruiting bodies of *G. lucidum* inhibited the primary solid tumour growth as well as secondary metastatic tumour growth in the liver of Lewis lung carcinoma (LLC) – implanted mice.

There is increasing evidence that antioxidants can inhibit proliferation of certain types of cancer. Earlier studies conducted on total triterpenes revealed its potent *in vitro* and *in vivo* antioxidant activity (7). There are also many reports that phytochemicals present in medicinal plants express anti-tumorigenic activity by inducing apoptosis (13). It is clear from the results of the present study that total triterpenes induced apoptosis in both DLA and EAC cells. Moreover, total triterpenes were also found to protect normal cells from radiation-induced oxidative stress and apoptosis (14,15). The anti-oxidant potential of triterpenes and its ability to induce cell-specific apoptosis could be the reason behind its anti-tumour activities. Earlier studies also reported that triterpene-enriched mycelial extract from *Ganoderma lucidum* induced G2 phase arrest in Huh-7 hepatoma due to the deficiency in M-phase promoting factors, resulting from the down-regulation of PKC activity and activation of the c-Jun N-terminal kinase (JNK) and p38 MAPKs (16). More studies have to be performed to find the exact mechanism by which total triterpenes from the fruiting bodies prevent tumour development.

From the current study, it is clear that total triterpenes effectively prevented the formation of solid and ascites tumour induced by DLA and EAC cell line in Swiss albino mice. It was highly cytotoxic towards various cell lines assessed. The total triterpenes were also found to be highly capable of inducing apoptosis in tumour cells, leaving a path to develop more chemotherapeutic drugs from the total triterpenes.

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