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Comparative Antitumor Activity of Different Solvent Fractions from an *Auricularia auricula-judae* Ethanol Extract in P388D1 and Sarcoma 180 Cells

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The objective of this study was to evaluate and compare the antitumor activity of different solvent fractions (ethanol, dichloromethane, ethyl acetate, butanol and water) of the *Auricularia auricula-judae* 70% ethanol extract on the P388D1 macrophage and sarcoma 180 cells. A dose-dependent antitumor activity of each solvent fraction (from 0.01 mg/m*l* to 0.3 mg/m*l*) was shown against both cell types. These cytotoxic effects of all the tested fractions were confirmed on the MTT and SRB assays, without statistical differences each other. IC_{50} value of dichloromethane fraction was 94.2 μg/m*l* against sarcoma 180 cells lower than any other solvent fractions. The potent antitumor effect of the dichloromethane (DCM) fraction was also found against solid tumor in BALB/c mice. The splenomegaly and higher splenic index were found in tumor-bearing mice, with the DCM fraction returning to the negative control values. Thus, the results indicated the dichloromethane fraction may have potential ingredients as antitumor candidates.

Key words: *Auricularia auricula-judae*, Antitumor, Solvent fractions, P388D1 macrophage cell, Sarcoma 180 cell

INTRODUCTION

Medicinal mushrooms have been in the interest of many researchers for their pharmacological effects as well as for their nutritional value. Among medicinal mushrooms, basidiomycetes contain highly potent polysaccharides and protein complexes that have as antitumor, immunomodulation, anticardiovascular diseases and hypocholesterolemia, antiviral, antibacterial and antiparasitic activities. Antitumor activities of these mushrooms have been extensively investigated due to recent chemotherapeutic application of some antitumor drugs derived from natural sources and will continue to occupy an important role in modern cancer therapy (Wasser and Weis, 1999). The species of agaricus, pleurotus, lentinus, ganoderma, grifola, volvariella, auricularia and tremella genera are the most popular in mushroom industry for their medicinal and nutritional values.

Auricularia auricula-judae is well known as wood ear or tree ear or black fungus. Fruit bodies of *Auricularia auricula-judae* are rich in carbohydrates, protein and minerals (Ca, P and Fe). Polysaccharides from *Auricularia auricula-judae* extract include carbohydrate mainly composed of rhamnose, xylose and glucose and smaller amount of mannose, galactose and arabinose, uronic acids, sulfate groups, N and ash (Chen *et al*., 2008) and its active constituents are beta (1-3) and (1-6) D glucans. Polysaccharide and methanol extracts from *Auricularia auricula-judae* have been reported to have antitumor activity, anticoagulant, anti-lipidemic and anti-cholesterol, and antiplatelet aggregation (Chen *et al*., 2008; Misaki *et al*., 1981; Yoon *et al*., 2003). However, the reports on antitumor activities of different solvent fractions of *Auricularia auricula-judae* extracts are scarce. Therefore, this study aimed to determine the antitumor activity of the solvent fractions of the 70% ethanol extract and compared the effect among the different fractions of *Auricularia auricula-judae* mushroom and also examined the efficacy of *in vitro* stronger antitumor active solvent fraction against solid tumor sarcoma 180 in mice.

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MATERIALS AND METHODS

Chemicals/reagents. RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 i.u/m*l*)

and streptomycin (10 mg/m*l*), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue (TB), sulphorhodamine B (SRB), 50% trichloroacetic acid (TCA), 1% acetic acid, 10 mM unbuffered tris Base (PH 10.5) and doxorubicin were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, U.S.A.). Analytical grade chemical were all used in this experiment.

Preparation of Auricularia auricula-judae extract. The mesh of *Auricularia auricula-judae* was extracted with 70% ethanol (EtOH) at 100°C for 6 h. The supernatant was collected by filtration (70 mm, Advantec, Toyo Roshi Kaisha Ltd., Japan). The filtrate was taken in a boiling bottle and placed in water bath (Buchi Water Bath B-480, Tokyo Rikakikai Co. Ltd., made in China) at 70°C and EtOH was collected by using Buchi Rotavapor R-114 at 10 rpm and Eyela CCA-1111 (Tokyo Rikakikai Co. Ltd., made in China). Thereafter, EtOH extract was re-suspended in water using a Soxhlet extractor and then successively fractionated (Fig. 1) with the same volume of dichloromethane (DCM), ethyl acetate (EtOAc), butanol (BuOH), and water fractions from 70% EtOH extract at room temperature. Various fractions were concentrated in a vacuum concentrator (BioTron, BioTron Inc., Korea) at a controlled temperature (< 50°C). The various fractionated extracts were dissolved in sterile PBS and sonificated as well as final sample solutions were made for test.

Cell culture. The P388D1 macrophage cells and sarcoma 180 cells were obtained from the Korean Cell Line Bank (Seoul, Korea). All these cells were cultured in RPMI-1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 i.u./m*l*) and streptomycin (10 mg/m*l*). All cell cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2.

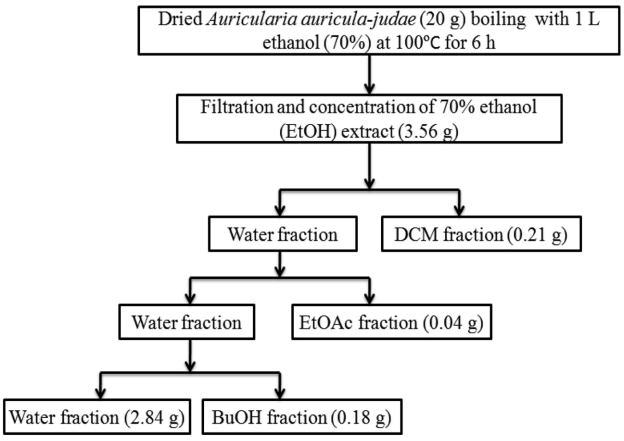

Fig. 1. Schematic diagram of fractionations of *Auricularia auricula-judae* ethanol extracts. EtOAc, ethyl acetate; BuOH, butanol; DCM, dichloromethane; EtOH, ethanol; and water fractions.

Determination of cell viability by MTT and SRB assay. The mitochondrial dependent reduction of MTT and SRB assay was determined by rapid colorimetric assay that measured the cell respiration, as an indicator of cell viability as previously described by Mosmann (1983) and SRB assay by Skehan *et al*. (1990). Briefly, for MTT assay, cells (1×10^5 cells/well) were cultured in 96-well plates and treated at various concentrations (1, 0.3, 0.1, 0.03 and 0.01 mg/m*l*) of different solvent fractions of *Auricularia auricula-judae* for 24 h at 37°C with 5% CO_2. Thereafter, 50 μ*l* of MTT solution (2 mg/m*l*) was added and incubated at 37°C with 5% CO_2 for 4 h. Then supernatant was aspirated and the insoluble formozan product dissolved in 200 μ*l* DMSO (Sigma-Aldrich Chemical, USA). For SRB assay, cells (1×10^5 cells/well) were cultured in 96-well plates for 24 h at 37°C with 5% CO_2. The cultured cells were treated with solvent fractions of *Auricularia auricula-judae* extracts and incubated further for 24 h. The supernatant was discarded after 1 h of 50% TCA addition and washed 5 times with distilled water, and then added 100 μ*l* of SRB solution. Afterwards, SRB solution was discarded and washed 5 times with 1% acetic acid, and then added 100 μ*l* of Tris base. The OD was measured using micro plate reader (Associates of Cape Cod, Inc., East Falmouth MA, USA) at 540 nm, yielding absorbance of binding SRB dye to basic amino acids of cellular proteins, which directly correlates to cell number. The inhibition of tumor cell proliferation was calculated using the following Formula:

$$\text{Inhibition rate (\%)} = \{1 - (\text{OD value of sample} / \text{OD value of control})\} \times 100$$

Doxorubicin was used as a positive control. IC_{50} concentrations required to inhibit growth by 50% were calculated from survival curves using the Bliss method.

In vivo tumor model and treatment. Seven week old male BALB/c mice were purchased from Orient Co. Seuol, South Korea (Charles River Technology) and weights were 22~24 g. The animals were maintained at 20~25°C with the relative humidity of 55 ± 10% and 12 h light/dark cycle under specific pathogen free condition. The feed and water were given ad libitum. The experimental protocols of animal were approved by the Institutional Animal Care and Use Committee of Kyungpook National University. After 1 week acclimatization of mice, the sarcoma 180 cells (2×10^6 cells/0.2 m*l*/mouse) were suspended in normal saline (0.85% NaCl) and inoculated subcutaneously into the mice. The development of visible tumor at 6th day of inoculation, the animals were divided into three groups (6 animals in each group) and another one normal control group (6 animals) was not inoculated tumor cells and administered with 0.85% saline p.o. to the negative control and normal control,

dichloromethane fraction (DCM) (100 mg/kg b.w., p.o.) to the treated group for 10 days and intra-peritoneal administration of doxorubicin (3 mg/kg b.w., i.p.) to the positive control group for once. After 7 days of last treatment, all mice were sacrificed and their tumor masses were measured and calculated for the per cent (%) of growth inhibition and tumor volume (cm^3) as the following formulae (Lee *et al*., 2003).

Inhibition of tumor growth (%) = (1 − T/C) × 100%,

where T is the tumor growth of treated groups and C is the control mice (1).

Tumor Volume (cm^3) = $4/3\pi(a^2b)/2$,

where a is the short diameter (mm^2) and b is the long diameter (mm^2) (2)

Calculation of splenic index. The spleens were collected gently from normal and tumor-bearing sacrificed mice and weighed immediately. The results were indicated as spleen

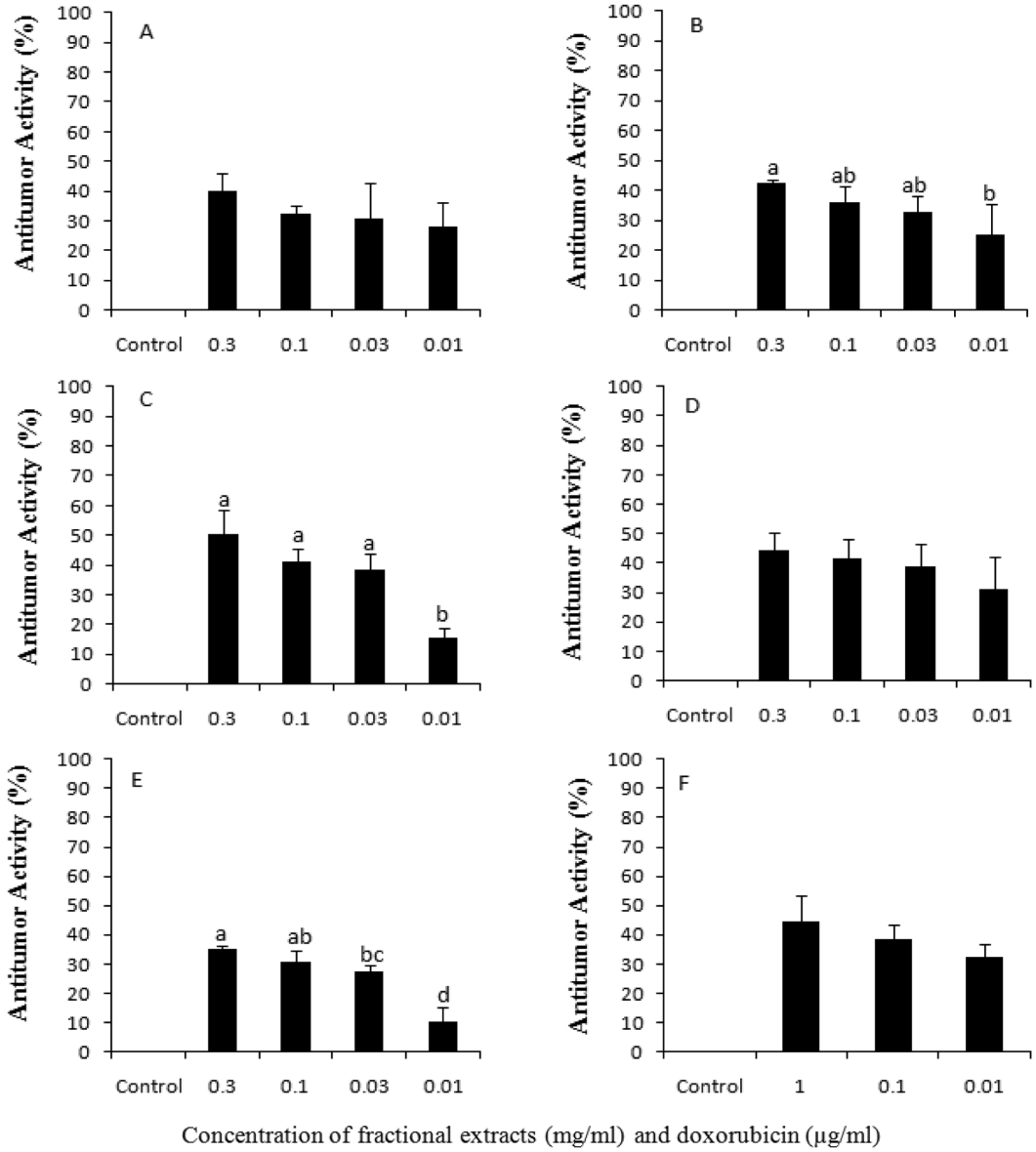

Fig. 2. Anti-tumor activity of solvent fractions on P388D1 cells; A. Ethyl acetate (EtOAc), B. Butanol (BuOH), C. Dichloromethane (DCM), D. Ethanol (EtOH) and E. Water fractions of *Auricularia auricula-judae* ethanol extract at the various concentrations (0.3 mg/ml, 0.1 mg/ml, 0.03 mg/ml and 0.01 mg/ml) and F. Doxorubicin as a positive control at different concentrations (1 μg/ml, 0.1 μg/ml and 0.01 μg/ml) on P388D1 cell by MTT assay. All values are presented as percentages of the results from control, and are expressed as mean ± SD of three independent (triplicate wells) experiments and the different alphabet superscripts differ significantly at $P < 0.05$.

weights per unit body weight, referred to as the spleen index (mg/g body weight) (Lee *et al*., 2003).

Statistical analysis. All values were expressed as the mean ± S.D. and statistical analysis was done by the one way analysis of variance (ANOVA) using SAS program. GraphPad Prism program was used to obtain for IC_{50} values. P-values less than 0.05 were considered to be significant.

RESULTS

In vitro antitumor effects by the MTT and SRB assay. The cytotoxic activities of the solvent fractions (EtOAc, BuOH, DCM, EtOH and water fractions) of the *Auricularia auricula-judae* 70% ethanol extract were revealed in P388D1 macrophage and sarcoma 180 cells using MTT and SRB tests. A significant dose-dependent inhibition ($p < 0.05$) of proliferative tumor cells was observed in all tested

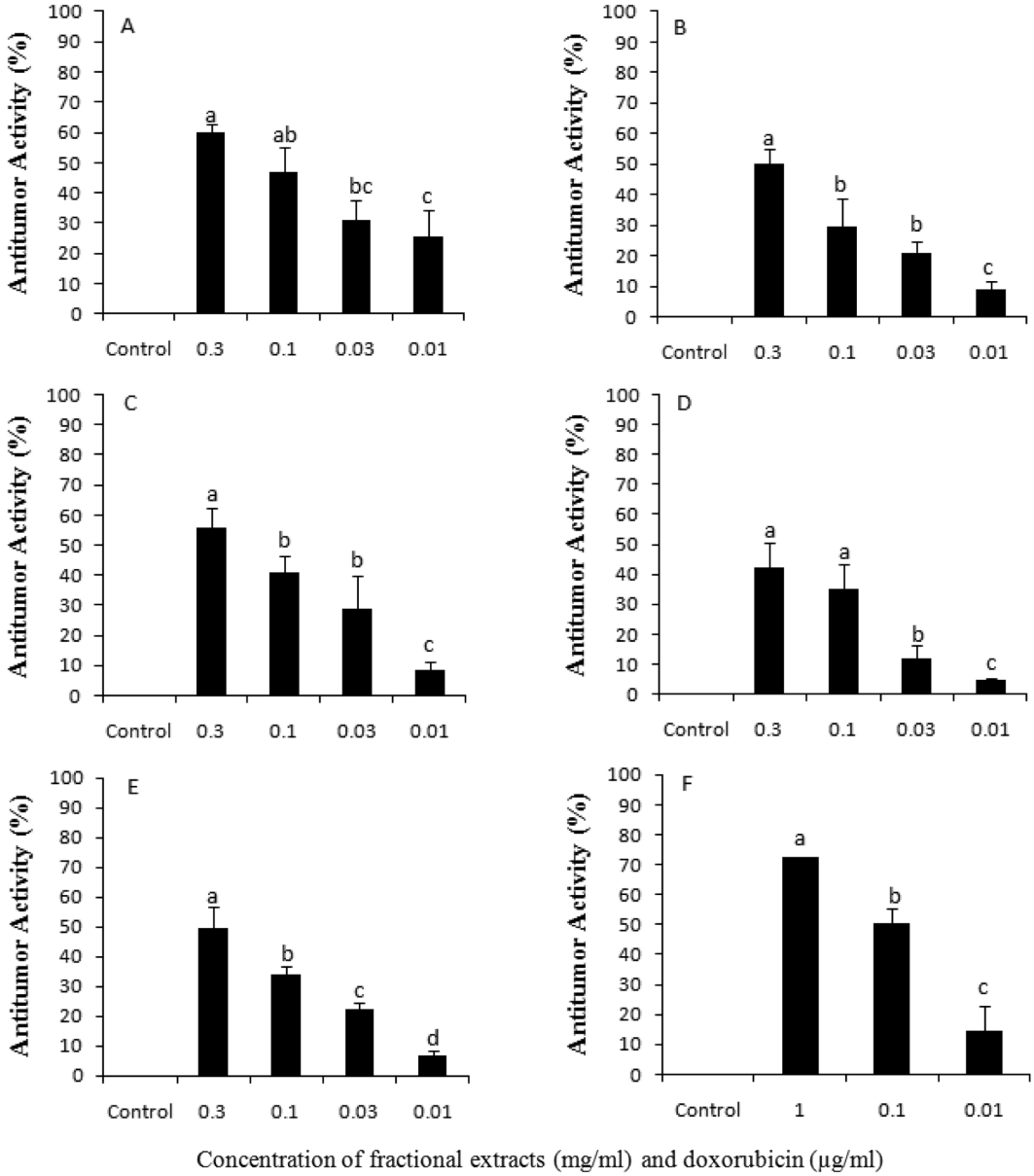

Fig. 3. Anti-tumor activity of solvent fractions on sarcoma 180 cells; A. ethyl acetate (EtOAc), B. butanol (BuOH), C. dichloromethane (DCM), D. ethanol (EtOH) and E. Water of ethanol extract from *Auricularia auricula-judae* at the various concentrations (0.3 mg/m*l*, 0.1 mg/m*l*, 0.03 mg/m*l* and 0.01 mg/m*l*) and F. Doxorubicin as a positive control at different concentrations (1 μg/m*l*, 0.1 μg/m*l* and 0.01 μg/m*l*) on Sarcoma 180 cell line by MTT assay. All values are presented as percentages of the results from control, and are expressed as mean ± SD of three independent (triplicate wells) experiments and the different alphabet superscripts differ significantly at $P < 0.05$.

Table 1. Comparative estimation of inhibitory activities solvent fractions of *A. auricula-judae* extract between MTT and SRB assays

Solvent fractions (1 mg/m*l*)	P388D1 cells		Sarcoma 180 cells	
	MTT assay	SRB assay	MTT assay	SRB assay
EtOAc	42.78 ± 0.78$^{\#}$	46.01 ± 2.12$^{*\#}$	69.61 ± 2.19	69.08 ± 4.03$^{\#}$
BuOH	48.13 ± 3.16$^{\#}$	51.17 ± 2.07$^{\#}$	65.74 ± 2.44	67.53 ± 1.30$^{\#}$
DCM	53.95 ± 7.67$^{\#}$	51.71 ± 3.71$^{\#}$	73.97 ± 1.11	72.67 ± 2.47$^{\#}$
EtOH	46.57 ± 6.32	47.42 ± 6.33	65.71 ± 9.14	59.67 ± 1.97
Water	37.23 ± 0.87	37.93 ± 1.70	64.48 ± 3.51	58.39 ± 4.59
Dox (1 μg/m*l*)	44.33 ± 9.11	46.04 ± 2.76	72.54 ± 0.26	72.81 ± 2.09

All values are presented as percentages of the results from control, and are expressed as mean ± SD of three independent (triplicate wells) experiments, * is expressed as significant different that compared to MTT values with SRB values of the respective fraction in same cell line (1 mg/m*l*) at $P < 0.05$ and $^{\#}$ is expressed as significant differences of solvent fractions than water fraction in same cell line.
EtOAc, ethyl acetate; BuOH, butanol; DCM, dichloromethane; EtOH, ethanol; Dox, doxorubicin (positive control).

solvent fractions and doxorubicin in both P388D1 macrophages (Fig. 2) and Sarcoma 180 cells (Fig. 3).

The highest cytotoxic effect was observed by the DCM solvent fraction followed by the BuOH, EtOH, EtOAc, and water fraction respectively at doses of 1 mg/m*l* in P388D1 cells (Table 1) by both MTT and SRB tests. While the antitumor responses were differed in sarcoma 180 cells from P388D1 cells, the DCM fraction was followed by the EtOAc, BuOH, EtOH and water fraction in terms of their antitumor activities (Table 1) by both tests. The comparative cytotoxic measurements between the mitochondrial reductase enzymes activity and cellular protein mass (MTT and SRB) revealed no significant differences for all solvent fractions assayed in both cell lines except ethyl acetate fraction. Ethyl acetate solvent fraction showed significantly higher inhibition in SRB assay than MTT in P388D1 cell. The result of cytotoxic measures of estimation in tumor cells between MTT and SRB assays are presented in Table 1.

Furthermore, the IC_{50} values were determined among various solvent fractions of *Auricularia auricula-judae* ethanol extract and the obtained IC_{50} values are presented in Table 2. Lower concentration of IC_{50} values were found by 28.2 μg/m*l* of water fraction in P388D1 cells and 94.2 μg/m*l* of dichloromethane fraction in sarcoma 180 cells, while

Table 2. IC_{50} values of different solvent fractions of *Auricularia auricula-judae* extract on tumor cell lines

Cell lines	Fractions of *Auricularia auricula-judae* (μg/m*l*)					Doxorubicin (ng/m*l*)
	EtOAc	BuOH	DCM	EtOH	Water	
P388D1	143.80	94.62	38.32	44.03	28.19	100.00
Sarcoma 180	108.90	134.10	94.20	133.00	102.30	95.00

Data are presented as IC_{50} values by MTT assay from three independent experiments, performed in triplicate on tumor cell lines, obtained by nonlinear regression using the GRAPHPAD Prism program.
EtOAc, ethyl acetate; BuOH, butanol; DCM, dichloromethane; EtOH, ethanol; Dox, doxorubicin (positive control).

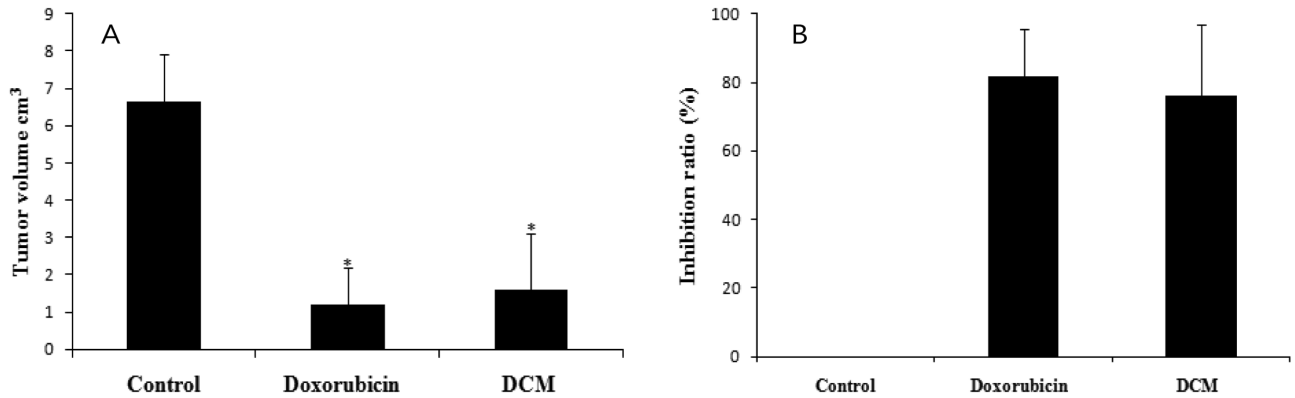

Fig. 4. *In vivo* antitumor effects of DCM (dichloromethane) fraction from 70% ethanolic *A. auricula-judae* extract on tumor volume (A) and inhibition of tumor volume (B) of sarcoma 180 solid tumor-bearing BALB/c mice. All values are presented as cm^3 (A) and % (B) and are expressed as mean ± SD. * superscripts differ significantly at $P < 0.05$ compared to control.

Table 3. Effect of dichloromethane (DCM) fraction from 70% ethanolic *A. auricula-judae* extract on sarcoma 180 solid tumor in BALB/c mice

Groups	Body weight (g)	Tumor weight (g)	Inhibition rate (%)	Complete regression
Control	26.00 ± 1.27	1.89 ± 0.15	00.00	0/6
Doxorubicin	26.67 ± 1.63	0.96 ± 0.76	49.23	2/6
DCM	27.5 ± 1.38	1.08 ± 0.84	42.62	2/6

Each value is presented as mean ± S.D. (n = 6/group). Control (0.85% saline), doxorubicin (Positive control, 3 mg/kg body weight), DCM (dichloromethane fraction, 100 mg/kg body weight).

100 ng/m*l* and 95 ng/m*l* of doxorubicin were observed for P388D1 and sarcoma 180 cells respectively.

***In vivo* antitumor effect of dichloromethane (DCM) fraction.** *In vivo* antitumor effect was determined in BALB/c mice by dichloromethane (DCM) fraction from 70% ethanolic *A. auricula-judae* extract which exhibited the strongest cytotoxic activity in cell culture than other fractions. The DCM fraction significantly ($P < 0.05$) reduced the tumor size in comparison to control group (Fig. 4A) and inhibition was found by 76.13% (Fig. 4B), where there were no significant differences between the positive control (doxorubicin) and DCM fraction. On the other hand, the remarkable inhibition of tumor weight and growth were observed in both the positive control and DCM fraction groups than the negative control group, while complete regression of tumors was found by 33.33% in the positive control and DCM groups (Table 3). There were no significant variations of body weight between the tested groups.

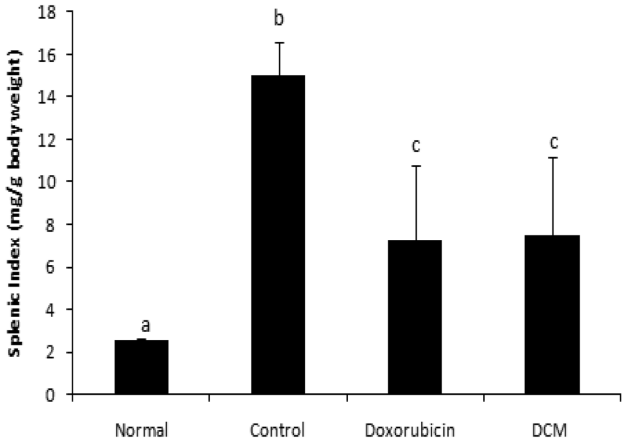

Fig. 5. Effect of dichloromethane (DCM) fraction from 70% ethanolic *A. auricula-judae* extract on spleen on sarcoma 180 solid tumor-bearing mice and non-tumor-bearing mice without DCM. All values are presented as mg/g body weight of mice and are expressed as mean ± SD. The different alphabet superscripts differ significantly at $P < 0.05$.

Spleen index. Splenomegaly was found in sarcoma 180 solid tumor-bearing mice (Figure not shown). The spleen index was significantly greater than non-tumor-bearing and treatment groups (Fig. 5). The sizes of spleen were increased approximately by six folds, three folds and three folds in control, positive control and DCM groups of tumor-bearing mice respectively than normal (non-tumor-bearing) mice.

DISCUSSION

Different antitumor activity might arise from different mechanisms of action by different chemical components of the solvent fractions from the ethanol extract, hence chemical modifications of polysaccharides and other components. Chemically modified polysaccharides of mushrooms exhibited potent antitumor activity, while water insoluble and alkali soluble polysaccharides had little or no antitumor activity (Wasser, 2002). The modified alkali insoluble β-glucan of *Auricularia auricula-judae* showed potent antitumor activity, while original alkali insoluble β-glucan had no inhibitory effect (Misaki *et al*., 1981). The results of these solvent fractions were comparable to previous reports observed by other mushrooms extracts (Jagetia and Rao, 2006; Song *et al*., 2008; Wang *et al*., 2008).

The difference cytotoxic effect observed by the solvent fractions might be due the difference in the ability of the solvents in concentrating the active ingredient of the cytotoxic compound of the plant extract. However, the cytotoxic effects observed in the two cell types differ for the same solvent fraction, which suggests also the possibility of different mechanisms of inhibition by these cells. This result indicates the cytotoxic activities of extract depend not only on the nature of extract component and its solvent but also on the type of tumor cell line (Ait Mbarek *et al*., 2007). The most potent cytotoxic effect observed by dichloromethane solvent fraction of *Auricularia auricula-judae* ethanolic extract was comparable to dichloromethane extract of guduchi (Jagetia and Rao, 2006) which showed higher cytotoxic effect compared to the other solvent fractions. The antitumor activity of ethyl acetate part of alcohol extract from seeds of *Livistona chinensis R.Br*, has shown similar effects with DCM solvent fraction and its mechanism has been associated with reducing VEGF protein secretion and inhibited the expression of Flk-1 mRNA and protein (Wang *et al*., 2008). Besides the difference in the dose response of antitumor activity of the solvent fractions in the two cells, and lower anti-tumor activity of the various solvent fractions were also observed in P388D1 cell.

The results of MTT and SRB assays agree with previous report of no significant differences between both colorimetric measurements (Henriksson *et al*., 2006). However, the difference observed for ethyl acetate solvent fraction for the two assays is not known. Spleen is an important hematopoietic organ in mice that contains both types of granular and

agranular leukocytes i.e. granulocytes, monocytes and lymphocytes. It plays a vital role in humoral immunity of the mice body. Splenomegaly and higher spleen index of tumor-bearing mice were reported that cause the paraneoplastic syndromes due to influence of splenic cytokines or humoral factors by the presence of tumor (Yoneda *et al*., 1991).

In conclusion, in the present study we have demonstrated the cytotoxic effects of the solvent fractions of *A. auricula-judae* ethanol extract inhibiting the growth proliferation of P388D1 and sarcoma 180 tumor cells. A dose-dependent response of anti-tumor activity was found for all solvent fractions tested. There is not observed difference between MTT and SRB assays on cytotoxic estimation. On the basis of IC_{50} values, the strong anti-tumor activity was observed by DCM solvent fraction against sarcoma 180 cells *in vitro* as well as the potent activity was also found against solid tumor in BALB/c mice. The splenomegaly and higher splenic index were observed in tumor-bearing mice.

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