

Cytotoxic Effect of a Mycelial extracts on Different Cell lines and Experimental animals

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Abstract—Mushroom polysaccharides play an important role as functional foods because they exhibit biological modulator properties such as antitumor, antiviral and antibacterial activities. Cytotoxic and antitumor activities of hot aqueous extracts of five macrofungi were studied. Hot water extracts showed *in vitro* cytotoxic activity against Breast carcinoma cell line (MCF7), Liver carcinoma cell line (Hep-G2) and Colon carcinoma cell line (HCT116). *Flammulina velutipes 6* polysaccharide extract had potential for tumor therapy. So, it was selected for *in vivo* studies. The antitumor activity of *F. velutipes 6* polysaccharide extract against Ehrlich carcinoma tumor in the mice model was significant. Significant regression of tumor growth was observed in the mice upon oral administration of 200 mg/kg *F. velutipes 6* polysaccharide extract for 10 days before tumor implantation with inhibition ratio of 99.34% (mean tumor volume was 0.006mm³), while 4 out of 15 mice showed complete tumor regression. While, group with oral injection after tumor implantation of *F. velutipes 6* polysaccharide extract inhibited tumor growth was even more effective than group injected with extract before tumor implantation. The inhibition ratio of 99.79 % (mean tumor volume was 0.002mm³), while 5 out of 15 mice showed complete tumor regression in this group.

Keywords—Cytotoxicity, Experimental animals, Mushroom, Polysaccharide, Tumor induction.

I. INTRODUCTION

Mushrooms have been consumed globally as tasty food and nutritional supplements. The medicinal power and nutritional value of several mushrooms are widely known. However, only since the last decade of the 20th century, it has been possible to isolate and partially characterize some biologically active antitumor substances [1].

The therapeutic properties of mushrooms are attributed mainly to their content of polysaccharides, which exhibit biological modulator properties such as antitumor, antiviral and antibacterial activities [2]–[6].

From a structural view point, polysaccharides are a diverse group of biological macromolecules that are found to occur in several organisms. They contain repetitive structural features and are polymers of monosaccharide residues joined to each other by glycosidic bonds. Therefore they differ structurally from proteins and nucleic acids and have the highest capacity

for carrying biological information since they have the greatest potential for structural variability [1],[2].

The effects of mushroom nutrition on clinical conditions have attracted great interest in the scientific community in the last decade in order to understand the molecular mechanism responsible for their action [7].

Protein-bound polysaccharides are macromolecules that consist of a central core protein to which are attached a number of polysaccharide chains. Polysaccharide peptide (PSP) and polysaccharide-K (PSK) from *Coriolus versicolor* are among the most widely studied protein-bound polysaccharides. However, such basidiomycetes also synthesize free polysaccharides that exhibit powerful biological activity [8, 9].

There are some reports on the production of free and protein-bound polysaccharides from basidiomycete strains such as *C. versicolor*, *Pleurotus ostreatus*, *Lentinula edodes* and *Ganoderma lucidum* [2], [10], [11].

Flammulina velutipes (*F. velutipes*) is a cultivated mushroom. Few studies, however, have been conducted on this species. An alkaline protease and the antitumor activities have been reported from this mushroom [12], [13]. Both methanolic and ethyl acetate extracts of this mushroom exhibited anti-hyperlipidemic and antioxidant activities [14]. As result of its perceived health benefits, *F. velutipes* has become one of the valuable mushrooms in China.

Therefore the aim of this study was a trial to produce polysaccharides from different mushroom species grown by submerged culture and studying their cytotoxic effect on human cancer cell lines” in vitro study”. Then the most potent extract was studied on animal model "in vivo"

II. MATERIALS AND METHODS

Fungal Strains:

Five mushroom strains *Lentinus edodes* LC2141, *Lentinus edodes* LC202, *Flammulina velutipes 5*, *Flammulina velutipes 6* and *Flammulina velutipes 13* were kindly obtained from Fujian Agriculture Univ., China. All strains were maintained on Potato Dextrose Agar (PDA) slants and incubated at 25°C for 7 days then stored in refrigerator at 4°C after growth for routine culture and storage purposes.

Culture Media and culture conditions:

All fungal strains were initially grown on PDA medium in Petri dishes for 7–8 days. The flask culture experiments were performed in a 250 ml flask containing 50 ml of mushroom fermentation medium (MFM). Each flask was inoculated with

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10 agar plugs 5 mm² of the agar plate culture with sterilized cork porer. MFM medium consisted of the following components (g/liter): glucose (35), peptone (5), yeast extract (5), KH₂PO₄·H₂O (1), MgSO₄·7H₂O (0.5) and vitamin B1 (0.05) [15].

Determination of dry weight:

The fungal mats were separated by filtration and washed several times with distilled water then dried at 70°C to a constant weight.

Preparation of crude hot water extract:

The mycelia of the five tested strains were dried at 60°C before analysis. The dried mycelia were pulverized and 5.0g of the powdered sample were extracted with 80% (v/v) ethanol for 24 h, and then filtered. The residues were dried and extracted with distilled water at 100 °C three times. The whole extract was filtered and centrifuged. After centrifugation (6000 rpm for 10min), The supernatant was concentrated by evaporation under reduced pressure and treated with three volumes of ethanol for precipitation at 4 °C overnight. The precipitate was obtained by centrifugation, and dried to give a crude extract [16].

Human tumor cell lines and in vitro cytotoxicity assay:

Human cancer cell lines which used in this study, were MCF7 (Breast carcinoma cell line), Hep-G2 (Liver carcinoma cell line) and HCT116 (Colon carcinoma cell line). They were obtained frozen in liquid nitrogen (-180oC) from the American Type Culture Collection. The tumor cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Cytotoxicity was determined using the MTT assay. For the assessment of the cytotoxic and cytostatic activities of all mushroom extracts, cells were seeded in 96-well flat-bottomed microtiter plates at a density of approximately (0.5X10⁵ cells/well), in complete RPMI-1640 Medium. After 24 h to ensure cell attachment, serial dilutions of the extracts in physiological saline were prepared. 100 µl of different concentrations of each tested extracts were added for 24 h at 37°C, in a humidified 5 % CO₂ atmosphere. After incubation, medium was removed, wells washed with 100ul PBS then 30 µl MTT solution/well was added and incubated for an additional 4 h. MTT crystals were solubilized by added 50µl of DMSO/well then the plate was shacked at room temperature. It was followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader after development of violet color. Control cells were treated with vehicle alone. For each compound concentration, 3 wells were used (triplicate wells were prepared for each individual dose). The average was calculated. The half maximal inhibitory concentration (IC₅₀) was determined after plotting the dose response curve for each conc. using Graph pad Prism software. The inhibition rate was calculated according to the formula below:

$$\text{Inhibition rate (\%)} = 1 - (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Antitumor activity "In vivo" experiments:

• Experimental Animals

The experimental animals used in this study were adult male albino mice weighing from 19-20 g purchased from the animal house colony of the National Cancer Institute (NCI), Cairo University, Egypt. Animals were housed under normal environmental conditions of standard temperature, humidity and diurnal environment of light and dark in suitable cages and kept on standard diet.

• Experiment design:

Animals were divided into five groups:

Group (1): Ten mice were considered as a negative control group which was not inoculated with Ehrlich tumor cells. Control mice were orally administered with physiological saline only.

Group (2): 15 mice were injected with Ehrlich carcinoma cells only (positive control).

Group (3): 15 mice-bearing Ehrlich carcinoma cells were orally administered with 0.2 ml of the hot water extract from mycelia of strain *Flammulina velutipes* 6 each for 10 days at a daily dose 200 mg/kg started after 24h of tumor implantation. "Treatment"

Group (4): 15 mice were orally administered with 0.2 ml of the hot water extract from mycelia of strain *Flammulina velutipes* 6 each for 10 days at a daily dose 200 mg/kg and then implanted with Ehrlich carcinoma cells. "Protective"

Group (5): 10 mice were orally administered with 0.2 ml of the hot water extract from mycelia of strain *Flammulina velutipes* 6 each for 10 days at a daily dose 200 mg/kg only.

• Solid tumor production:

Solid tumors were produced by intramuscular inoculation in the right thigh of the lower limb of each mouse with 0.1 ml of Ehrlich carcinoma cells, which contained 1 x 10⁶ viable Ehrlich carcinoma cells. Viability, assessed by the Trypan blue dye exclusion method, was found to be 95% or more.

• Evaluation of the antitumor activity:

Antitumor activity was assessed by measuring the change in tumor volume (TV/mm³), body weight (BW/g) and survival rate.

Tumor volume (Size) (TV) measurements:

The change in tumor volume (TV/mm³) was measured at different time intervals during the experiment time course using a Vernier caliper and calculated by the following equation: . The tumor volume was calculated using the formula:

$$\text{Tumor volume (mm}^3\text{)} = 4/3\pi a^2b/2,$$

Where

a is the smallest and b is the largest diameter (mm).

$$\pi = 22/7$$

Body weight changes:

Body weight (BW/g) and survival were monitored throughout the experimental time course.

Antitumor inhibition ratio (%) [17]:

$$=100 (1-T/C)$$

Where

C is the average tumor size (mm³) in control group.

T is the average tumor size (mm³) in Treatment group.

Statistical Analysis:

Statistical analysis of data was carried out by using one way analysis of variance (ANOVA) followed by homogenous subsets (Duncan) at confidence level of 5% (0.05) using the Statistical Package for the Social Science (SPSS) version 17. Duncan's multiple range tests were used to compare between means of treatments.

III. RESULTS AND DISCUSSION***In vitro* cytotoxicity assay:**

The treatment options of cancer including surgery, chemotherapy, radiation therapy, and palliative care often are expensive and have side effects. For example, most chemotherapeutic agents for the treatment of cancer destroy tumors and stop cancer progress but also damage healthy cells and tissues. Due to these side effects, researchers search for new antitumor substances from various natural sources, especially fungi [18]-[20]. More than 140 fungal metabolites have shown confirmed activity in tumor cell line bioassays [21].

Many kinds of polysaccharides have shown significant antitumor activities and low side-effects *in vivo*. Most reports conformed that mushroom polysaccharides exerted their antitumor action via activation of the immune response of the host organism, and mushroom polysaccharides were regarded as biological response modifiers (BRMs). However, in the past few years, studies related with the effects of polysaccharides on the tumor cells have increased. It has also been reported that polysaccharides have different antitumor activities *in vitro*, depending on their monosaccharide composition, protein contents, molecular mass, and chain conformation [22].

The antitumor activity of the tested polysaccharide extracts against the three carcinoma cell lines is shown in (table 1A-E). Cytotoxicity was measured and expressed as the survival fraction compared with untreated control cells, where the cell viability was determined, and IC₅₀ was determined from a range of concentrations shown in Figures 1. According to the *in vitro* inhibition ratio of MCF7 breast cell line by the five polysaccharide extract of mushrooms mycelia (*Lentinus edodes* LC2141, *Lentinus edodes* LC202, *Flammulina velutipes* 5, *Flammulina velutipes* 6 and *Flammulina velutipes* 13) at different concentrations (2.5, 5, 10 and 20 µg/ml) (fig.1), all extracts exhibited strong inhibition against cell growth at all concentrations, and there was also a dose-response relationship between concentration of the mycelial polysaccharides and suppression of MCF7 cell proliferation. The polysaccharide extract of *Lentinus edodes* LC2141 mycelia showed the highest significant antitumor effect as compared with other polysaccharide extracts from the tested mushroom

strains. It was noted that extract of *Lentinus edodes* LC2141 showed high inhibition ratio of 72.3 % at the concentration of 10µg/ml. While the extracts of *Lentinus edodes* LC202 and *Flammulina velutipes* 6 showed inhibition ratio of 60.77 % and 53.3% at the concentration of 20µg/ml. The extracts of both *Flammulina velutipes* 5 and *Flammulina velutipes* 13 exhibited no cytotoxic effect with all tested concentrations. The treatment of MCF7 cells with different concentrations of hot water extract of *L. edodes* LC2141, *L. edodes* LC202 and *F. velutipes* 6 dramatically inhibited the cell growth with IC₅₀ values of 7.5µg/ml, 4.5µg/ml and 5µg/ml respectively.

Also, the treatment of Hep-G2 liver cell line with the mushroom polysaccharide extracts at different concentrations revealed that all extracts exhibited strong inhibitory effect against cell growth at all concentrations (fig.2). The extracts of *L. edodes* LC202, *F. velutipes* 6, *F. velutipes* 13, *F. velutipes* 5 and *L. edodes* LC2141 exhibited high inhibition ratio of 67.88%, 67.60 %, 66.20%, 55.03% and 53.35% respectively at the concentration of 2.5µg/ml for *L. edodes* LC202 extract and 20µg/ml for the other strains. The polysaccharide extracts of *L. edodes* LC2141 and *L. edodes* LC202 inhibited the cell growth with IC₅₀ values of 5µg/ml. *F. velutipes* 6 and *F. velutipes* 5 showed cell inhibition with IC₅₀ values of 4µg/ml and 8µg/ml respectively.

Table 1: Inhibition ratios of different cancer cell lines with IPS extract of the five studied Mushroom strain using concentration range from 2.5 to 20µg/ml(A-E)

A

CONC. µg/ml	Inhibition ratio % with IPS extract of <i>Lentinula edodes</i> LC2141		
	MCF7	Hep-G2	HCT116
2.5	11.54	26.82	14.01
5	23.08	45.81	-1.91
10	72.31	48.04	4.46
20	66.92	53.35	59.24
IC ₅₀ (µg/ml)	7.5	5	18

B

CONC. µg/ml	Inhibition ratio % extract of <i>Lentinula edodes</i> LC202		
	MCF7	Hep-G2	HCT116
2.5	32.05	67.88	33.76
5	55.90	46.93	30.89
10	59.49	2.51	32.48
20	60.77	66.48	6.05
IC ₅₀ (µg/ml)	4.5	5	No cytotoxic effect

C

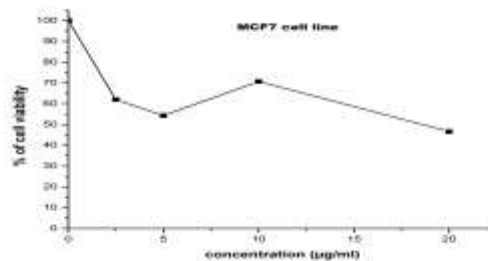
CONC. µg/ml	Inhibition ratio % extract of <i>F. velutipes</i> Fv5		
	MCF7	Hep-G2	HCT116
2.5	31.28	22.07	24.52
5	20.51	41.06	17.20
10	26.41	48.88	17.20
20	29.49	55.03	52.23
IC ₅₀ (µg/ml)	No cytotoxic effect	8	10

D

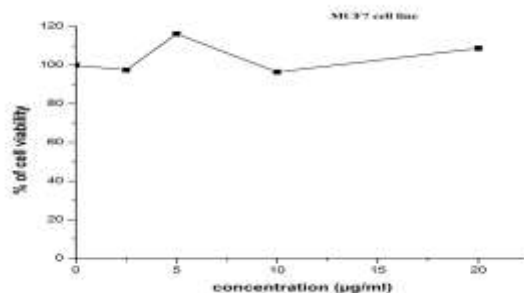
CONC. µg/ml	Inhibition ratio % extract of <i>F. velutipes</i> Fv6		
	MCF7	Hep-G2	HCT116
2.5	37.95	33.80	16.24
5	45.64	55.03	4.46
10	29.23	9.78	6.05
20	53.33	67.60	29.62
IC ₅₀ (µg/ml)	5	4	No cytotoxic effect

E

CONC. µg/ml	Inhibition ratio % extract of <i>F. velutipes</i> I3s		
	MCF7	Hep-G2	HCT116
2.5	2.56	51.40	-33.76
5	-16.15	46.09	-62.42
10	3.59	17.88	-81.53
20	-8.72	66.20	-81.53
IC ₅₀ (µg/ml)	No cytotoxic activity	7	No cytotoxic activity

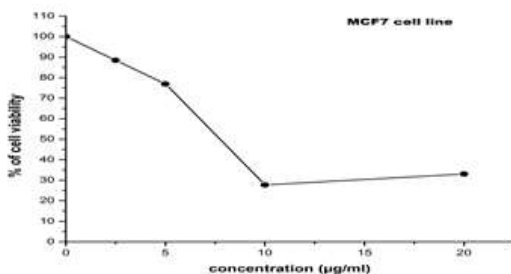


(D) MCF7 cell treated with Fv6

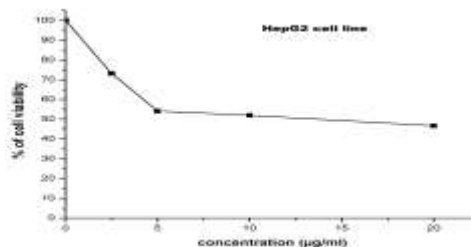


(E) MCF7 cell treated with Fv13

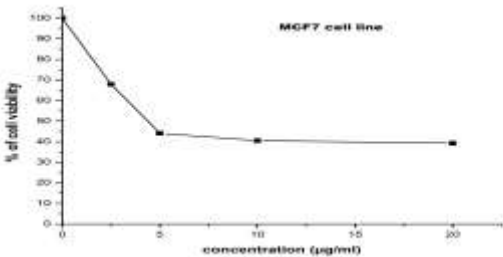
Fig. 1. (A-E) Inhibition ratios of MCF7 cell lines with IPS extract of the five studied Mashrom strain using concentration range from 2.5 - 20µg/ml



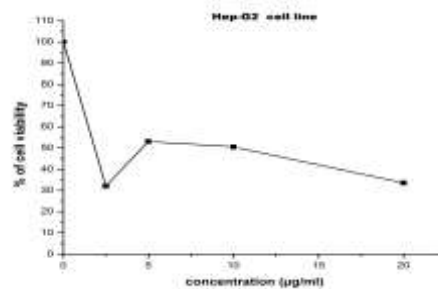
(A) MCF7 cell treated with LC2141



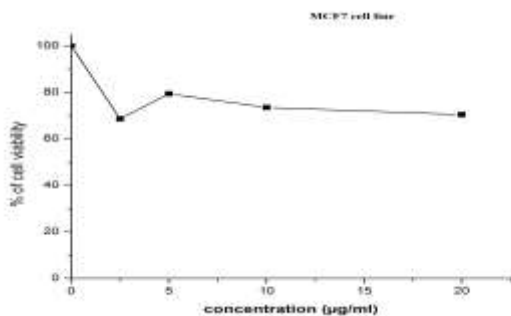
(A) Hep-G2 cells treated with LC2141



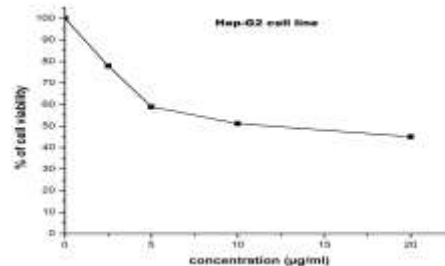
(B) MCF7 cell treated with LC202h



(B) HepG2 cells treated with LC202h



(C) MCF7 cell treated with Fv5



(C) HepG2 cells treated with Fv5

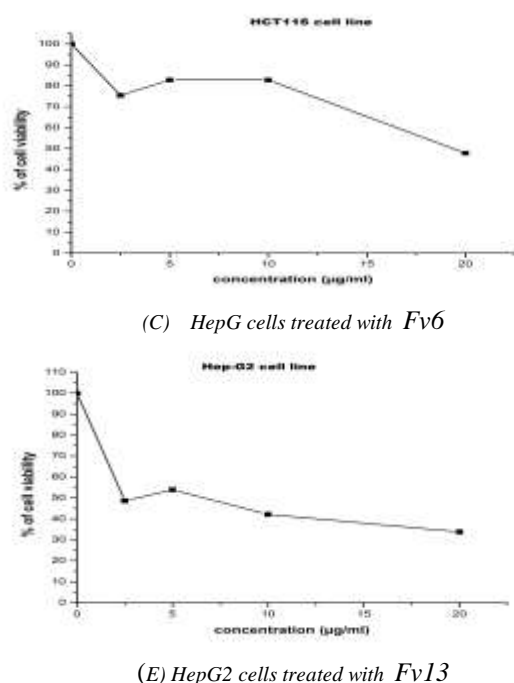


Fig. 2. (A-E) Inhibition ratios of HepG2 cell lines with IPS extract of the five studied Mushroom strain using concentration range from 2.5 - 20µg/ml

Only hot water extract of *L. edodes* LC2141 and *F. velutipes* 5 revealed strong inhibitions against HCT116 colon cell line with inhibition ratios of 59.24 % and 52.23% respectively at concentration of 20µg/ml (table 1). This result was consistent with previous investigations which confirmed that the polysaccharides or their complexes could have cytotoxicity or cytostatic effects on various tumor cell lines in vitro whereas they were less toxic on normal cells [23]-[25]. Also, our results was similar to reports observed by Ahmed et al., [26] that water extracts of *F. velutipes* fruiting bodies showed highest inhibition effect against three tumor cells (HepG-2, HCT 116 and HeLa) with IC50 ranging between 5-16 µg/ml

Our results demonstrated that mushroom extracts can be a good source for antitumor substances, and these results are consistent with reports indicating that mushrooms extracts had antitumor effects. For examples, low-molecular weight mushroom substances have been studied, and reported to interact with particular intracellular signaling pathways related to processes such as inflammation, cell differentiation and survival, apoptosis, angiogenesis, tumor progression and metastasis [27]. Many polysaccharide or polysaccharide protein complexes isolated from mushrooms exhibit antitumor activity [28]. Lentinan, a polysaccharide isolated from water extract of *L. edodes* has strong antitumor activities against stomach cancer (29, 30). Also, Finimundy et al. [31] reported high inhibitory activities against both HeLa and Hep G2 cell lines by water extracts of *L. edodes*

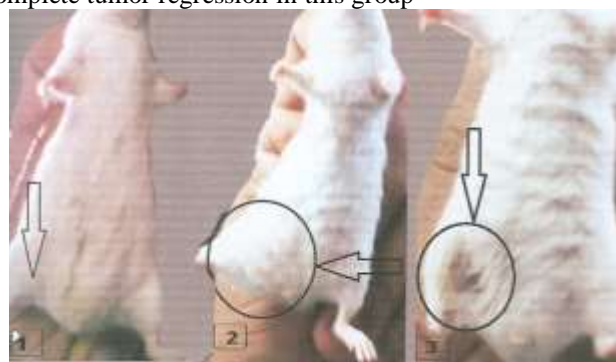
The difference in the antitumor activity may be attributed to their different molecular weights, monosaccharide composition, and charge characteristics. The results suggested that *F. velutipes* 6 polysaccharide extract had potential for investigation for antitumor activities. So, it was selected for in vivo studies

Tumor Inhibition Effects

The anti-tumor activity of a polysaccharide is usually believed to be a consequence of the stimulation of the cell-mediated immune response [32]. Mice transplanted Ehrlich carcinoma cells was used to evaluate the effects of *F. velutipes* 6 polysaccharide extract in vivo

The antitumor activity of *F. velutipes* 6 polysaccharide extract against Ehrlich carcinoma tumor in the mice model was significant. As shown in fig. 3, the mean tumor volume reached 0.942 mm³ in the saline-treated control 6 weeks after tumor implantation. In contrast, a significant inhibition of tumor growth was observed in tested mice upon oral administration of 200 mg/kg *F. velutipes* 6 polysaccharide extract for 10 days before tumor implantation with the inhibition ratio of 99.34% (mean tumor volume was 0.006mm³), while 4 out of 15 mice showed complete tumor regression

Group with oral injection after tumor implantation of *F. velutipes* 6 polysaccharide extract inhibited tumor growth was even more effective than group injected with extract before tumor implantation. The inhibition ratio of 99.79 % (mean tumor volume was 0.002mm³), while 5 out of 15 mice showed complete tumor regression in this group



Effects of polysaccharide extract from *F. velutipes* Fv19 on 1- Negative control group, 2- Positive control group, & 3- Mice administrated polysaccharide extract after tumor induction

Effect of the *F. velutipes* 6 polysaccharide on Body Weight in Mice

Before embarking on the experiments, the body weights of all the groups were 20.0 ± 2.0 g. There was no significant difference in body weight of each group ($p > 0.05$). During the experiments, the appetite and activity of each animal in these groups were better than the mice with solid tumor only. The results showed little change in final body weight in group injected with extract before tumor implantation while significant increase ($p > 0.05$) in final weight in group injected with extract after tumor implantation. Especially in the solid tumor group, many mice exhibited ruffled fur, lethargy and loss of appetite, which suggested some toxic effect of on mice. On the other hand, the body weights in the group injected with extract only showed no significant change ($p < 0.05$) in body weight as compared to those of the normal control group with increase in activity and survival of mice. The results showed that there was no significant toxic effect of the *F. velutipes* 6 polysaccharide to Ehrlich implanted mice. Also, water extract of *F. velutipes* 6 increased the survival rate of the mice- bearing

tumor and their activity in comparison with positive control

IV. CONCLUSION

This study demonstrated that hot water extract of the mycelia from five different strains of mushroom, revealed cytotoxic activity against different cancer cell lines. Cytotoxicity assay suggest the polysaccharide of *F. velutipes 6* exhibited strong antitumor activities so, it was chosen for *in vivo* experiment. Characterization of most active hot water extract and the immunomodulating activity of *F. velutipes 6* polysaccharide will be further investigated in future work.

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