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Immunomodulatory, Apoptosis Induction and Antitumor Activities of Aqueous and Methanolic Extract of *Calvatia Craniiformis* in Mice Transfected with Murine Hepatocellular Carcinoma Cells

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Keywords: *C. craniiformis*; H22 cells; Caspase-8; Apoptosis Index

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Abstract

OBJECTIVES: To evaluate the Immunomodulatory, apoptosis induction and antitumor effects of aqueous and methanolic extracts of *Calvatia craniiformis* regarding the size of tumour mass, caspase-8 expression and apoptotic index (AI%) in mice transfected with murine hepatocellular carcinoma cell line (H22) as an experimental therapeutic system for human hepatocellular carcinoma.

MATERIAL AND METHODS: Forty-eight Balb/C albino mice were transfected in legs with H22 cells. Tumour size was measured twice a week. Caspase-8 protein expression and apoptotic index determination evaluated by Immunohistochemistry.

RESULTS: Tumor size significantly differed between the two groups of mice transfected with H22 cells; the first was treated with *C. craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg and the second group was treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg compared with control group. The inhibitory activity of aqueous and methanolic extracts was dose and duration dependent. The size of the tumour mass was reduced up to 87.9% when treated with 1.2 mg/kg aqueous extract and 1 mg/kg for methanolic extract. Caspase-8 expression was increased in a dose-dependent manner among H22 bearing mice treated with *C. craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg. At 0.3 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.6 mg/kg and 1.2 mg/kg the intensity of expression was strong in (33.33%) and very strong in (100%) with a significant difference ($P \leq 0.001$). H22 bearing mice treated with (0.25, 0.5, 1.0) mg/kg *C. craniiformis* methanolic extract shows increased caspase-8 expression in a dose-dependent manner. At 0.25 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.5 mg/kg, the intensity of expression was strong in (33.33%) and very strong in (100%). At 1.0 mg/kg, the intensity of expression was strong in (16.67%) and very strong in (83.33%) with significant difference ($P \leq 0.001$). AI% of H22 bearing mice treated with *C. craniiformis* aqueous and methanolic extracts were significantly increased ($P \leq 0.05$) compared with the untreated control group. No significant difference was reported in AI% between aqueous and methanolic extracts treated groups.

CONCLUSIONS: Extracts of *C. craniiformis* were highly efficient in tumour growth inhibition, causing a reduction in the tumour size clinically and increase the expression of caspase-8 gene product in tumour tissue, causing increase apoptotic index of H22 cells taken from the legs of inoculated mice leading to loss of legs due to bone necrosis. Antitumor activity of *C. craniiformis* aqueous, and the methanolic extract was dose and duration dependent.

Introduction

The first written records on medicinal applications of plants date back to 2600 BC and report the existence of a sophisticated medicinal system in Mesopotamia, comprising about 1000 plant-derived medicines [1]. The Arabs preserved a large amount of the Greco-Roman knowledge during the dark and middle ages (i.e., 5th to 12th centuries), and complemented it with their medicinal expertise, and with herbs from Chinese and Indian traditional medicines [2].

Cellular compounds and the secondary metabolites extracted from edible mushrooms can be used for the treatment of cancer by acting as a

biological response modifier (BRM) [3]. BRM are immunostimulants which can be helpful in treating cancer (where targeted therapy often relies on the immune system being used to attack tumour cells) [4]. One of BRM, Maitake β-glucans called Grifolan, a branched β-1,3-d-glucan extracted from *Grifola frondosa* was found to promote tumour regression and necrosis and was approved to be used as anticancer therapy [5]. Aqueous extracts from Shiitake and Maitake, edible mushrooms showed increased host-mediated antitumor activity against Sarcoma 180 cancer [3]. Lentinan, a protein-free polysaccharide (β-1,3-d-glucans and β-1,6-d-glucans) derived from the fruit body of Shiitake was approved for the treatment of gastric cancer in Japan. Lentinan was found to be instrumental in activating macrophages to stimulate lymphocytes and other immune cell defences like increasing natural Killer cells [3].

The biological characteristics of *Calvatia craniiformis* extracts were studied extensively, as some of their compounds showed medical benefits because they contain active ingredients such as Calvatic acid, which has anti-inflammation and a definite antitumor effect. *C. craniiformis* significantly inhibits the growth of Yoshida sarcoma in cell culture and increase the survival time of mice with leukaemia 1210 [6]. Subsequent investigations have focused on the antitumor properties of calvatic acid, which may represent a model for the synthesis of more specific glutathione transferase-P1-1 inhibitors with possible therapeutic relevance [7].

The CASP8 gene encodes a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive proenzymes composed of a prodomain, a large protease subunit and a small protease subunit [8]. Activation of caspases requires proteolytic processing at conserved internal aspartic residues to generate a heterodimeric enzyme consisting of the large and small subunits [9]. This protein is involved in the programmed cell death induced by Fas and various apoptotic stimuli [10]. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with Fas-interacting protein FADD [9] [11].

Biochemically, caspase-8 was found to enter the complex of the inhibitor of NF-KB kinase (IKK) with the upstream Bcl10-MALT1 (mucosa-associated lymphatic tissue) adapter complex which was crucial for the induction of nuclear translocation of NF-κB [12]. Moreover, the biochemical form of caspase-8 differed in the two pathways. For the death pathway, the caspase-8 zymogen is cleaved into subunits that assemble to form the mature, highly active caspase heterotetramer whereas, for the activation pathway, the zymogen appears to remain intact perhaps to limit its proteolytic function but enhance its capability as an adapter protein [13].

The aim of the present study was to evaluate the Immunomodulatory, apoptosis induction and antitumor effects of aqueous and methanolic extracts of Calvatia craniiformis regarding the size of tumor mass, caspase-8 expression and apoptotic index (AI%) in mice transfected with murine hepatocellular carcinoma cell line (H22) as therapeutic experimental system for human hepatocellular carcinoma.

Materials and Methods

C. craniiformis obtained from groves of Al-Khalis region - Diyala province, Iraq. The classification of mushroom achieved in fungi research laboratory, Faculty of Agriculture, University of Baghdad, Iraq by professor Salman Kamel Jabr. *C. craniiformis* belongs to the fungal kingdom Mycota, Class Agaricomycetes, family Lycoperdaceae. Figure 1-A represents the form of fungus discovered in Iraq by our team, and Figure 1-B represents the cross-section with clearly appeared brown colour region filled with spores, which is part of the active ingredients used in the treatment.

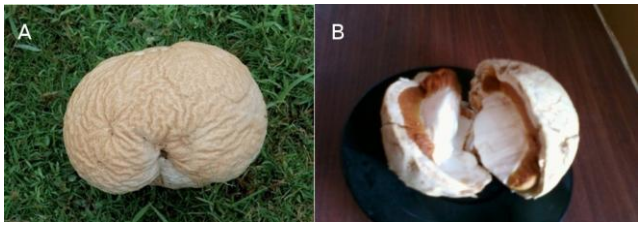

Figure 1: The discovered Calvatia craniiformis forms; A) C. craniiformis in the grove; B) Cross-section of C. craniiformis in laboratory

Fifty gram of soft plant was added to 500 ml of distilled water and then blended for 5-10 minutes until homogeneity. The extract was vibrated for an hour by shaker and then centrifuged for 10 minutes (2000 rpm/min). The sediment was discarded, and the supernatant was distributed in the clean, dry dishes and placed in an incubator for obtaining of the dry extract [14]. *C. craniiformis* crude aqueous extract gave 5 gm out of 75 grams of dry *material*, i.e. the extraction ratio was 6.66 % of crude *C. craniiformis*. The extract was dark brown to black colour, thick and little viscous.

Twenty gram of dry powder was taken and placed in a thimble. The thimble was placed in the Soxhlet device where the solvent, hexane was added to remove fat and chlorophyll. The extraction was conducted for 12 hours at a temperature (40-60^{o}C) for evaporation of solvent used. The obtained powder was transferred to the Reflex device with 70% methanol for three hours. The extract was filtered by a piece of gauze and filter paper then incubated for 24 hours for evaporation of methanol.

Methanolic extract was treated with HCl 1% in a Reflex for 30 minutes and filtrated by Whatman 1 filter paper. Diethyl ether was added to the filtrate in separating funnel and left for 24 hours. Two layers appeared, the top layer is the diethyl ether layer which had been neglected, and the bottom layer is an aqueous layer which picked. PH of the aqueous layer was raise for PH 8 by adding ammonia. Then after the aqueous extract was incubated to remove chloroform, the final form of the extract was obtained [15]. Methanolic extract gave 5 gm of 50 g, i.e. extraction ratio was 10% of the raw material. The resulting extract have yellowish-brown colour, thick and little viscous

To determine any possible toxic effects for *C. craniiformis*, Up-and down method was followed for determination LD50 according to the following equation [16]:

$$LD50 = Xf + Kd$$

Xf: the last dose administered

d: difference between dose levels

k: tabular value calculated from Table (1).

Table 1: the median lethal dose of alcohol and aqueous extract

Type of Extract	Difference between dose levels (d)	Death of the animal or to stay alive after 24 hours	The value of K tabular	The last dose administered (Xf)	Midterm lethal dose (LD50)
Aqueous	25	Ooxo	-439	100	85 mg\kg
Methanolic	50	Oxxx	1.5	200	177 mg\kg

O: the survival animal within 24 hours of injection; X: the death of the animal within 24 hours of injection.

According to acute toxicity study, aqueous extract was administered in the following doses: 0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg. Methanolic extract was administered in the following doses: 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg.

Forty-eight albino Bclb/C mice (weight 18-20 g) were purchased from Drug investigation department-ministry of health (Baghdad, Iraq). The mice were housed under normal condition and with free access to food and water. Animal experiments and animal care carried out according to protocols approved by the institutional committee for animal care and by the recommendation for the proper use and care of laboratory animals. Mice were divided into four groups, (6) mice for each one. Three groups received extract and one group for control receiving Dimethyl sulphoxide (DMSO).

Murine Hepatocellular carcinoma cells (H22) was received from Tongji Hospital in Tonji University, Joaqon Hughoin (China). The steps for implant tissue carried out under sterile conditions. RPMI (1640) medium used for cultivation of H22 hepatocellular carcinoma cell line and development of full growth [17].

After complete growth, H22 cells were harvested from RPMI 1640 Medium, and 0.1 ml of cells were transfected in the leg of (48) Balb/C albino mice to establish a solid tumour model [18]. The experiment ending with the death of the last mouse from the control group given doses of aqueous and methanolic extract. Tumour size was measured twice a week during the duration of the experiment using special calibre and take the measurement analogy (latitude and longitude), and extracted tumour size [19] [20].

Immunohistochemistry (IHC) used for evaluation of apoptosis. The procedure of IHC was performed according to the manufacturer's instruction, using polyclonal rabbit anti-mouse caspase-8 IgG, ab25901 recognises the p18 form of Caspase-8 [21]. Secondary antibodies, Biotinylated goat anti-rabbit IgG. Final results visualised by using Immunohistochemistry detection kit, Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit ab80436 [22]

The primary antibody diluted by the common antibody diluent 1:50. Optimal antibodies concentration may vary depending on specimen and preparation method. This optimisation has been done. Both positive and negative controls were included for each run of caspase-8 detection by IHC. The negative control was obtained by replacing the primary antibody with PBS buffer. The positive control was obtained by using tonsil tissue [23].

The expression of the caspase-8 protein was measured by counting the number of positive cells with brown (DAB) nuclear staining under light microscopy X40. For the evaluation of caspase-8 expression, immunostaining was assessed semiquantitatively using a scoring system for both intensity and extent of staining in 10 microscopic fields which were randomly selected and based on the estimated percentage of caspase-8 positive cells, staining results were divided into 5 scores , (0 = no expression, no positive cells; 1 = weak expression, less than 40% positive; 2 = moderate expression, 40-60% positive cells; 3 = strong expression , more than 60% but less than 100% positive cells; 4 = strong ^{high}, 100% positive cells) [24]. Final results for the apoptotic index were expressed as Mean ± SE [25] [26]. Apoptotic index % was determined according to the following equation [27]:

$$Apoptotic\ Index\ \% = \frac{Number\ of\ Apoptotic\ cells}{Total\ number\ of\ cells\ per\ field} \times 100$$

Statistical analysis was performed using SPSS version 16 software. One-way Analysis of Variance (ANOVA), used to find out the significance of differences in caspase-8 expression; AI % between groups that composed of continuous variables. Mann-Whitney test used to find out the difference between aqueous and methanolic extract AI% activity. The level of Significance at ($P < 0.05$) and ($P < 0.01$).

Results

As shown in Figure 2, Administration of aqueous extract of *C. craniiformis* in 0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg to H_{22} tumour-bearing mice shows significant differences (P ≤ 0.01) in tumour size compared with control group. Among given doses, 1.2 mg/kg was effective dose causing a reduction in tumour size in last day of the experiment (36th), in which tumour size was 960 mm^3 compared with 1564.57 mm^3 for 0.6 mg/kg and 3559.20 mm^3 for 0.3 mg/kg while in the control group the tumour size was 5747.05 mm^3.

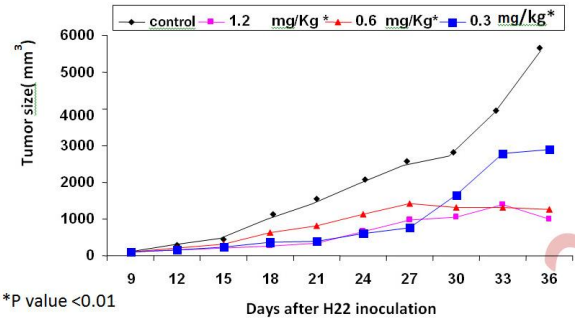

Figure 2: Tumor size follow up in mice transfected with H22 murine tumor cells treated with aqueous extract of C craniiformis; Significant difference in tumor size (p-value < 0.01) in the leg of inoculated mouse compared with H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones

Administration of methanolic extract of *C. craniiformis* in 0.25 mg/kg, 0.5 mg/kg, 1 mg/kg to H_{22} tumour-bearing mice shows a significant difference (P ≤ 0.01) in tumour size compared with control group. A significant difference in tumour size throughout experiment was reported. Tumour size was increased slightly in the treated group compared with control. In last day of the experiment (36th), tumour size was 1167.20 mm^3 in the group treated with 1 mg/kg compared with 1332.64 mm^3 for 0.5 mg/kg and 2076.33 mm^3 for 0.25 mg/kg while in control group the tumour size was 7747.04 mm^3 as shown Figure 3.

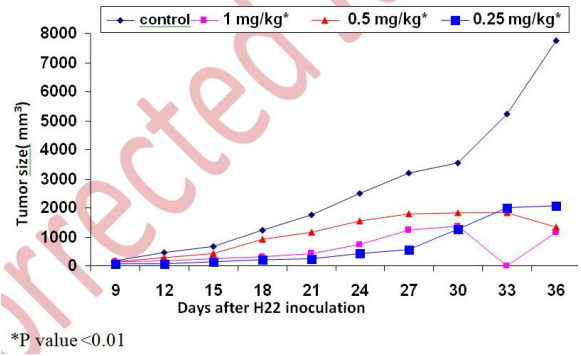

Figure 3: Tumor size follow up in mice transfected with H22 cells treated with a methanolic extract of C. craniiformis; the Significant difference in tumour size (p-value < 0.01) of mouse inoculated legs compared with the H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones

Among given doses, 1 mg/kg was effective dose causing reduction in tumor size in last day (36th), in which tumor size was 1167.28 mm^3 compared with 1332.64 mm^3 for 0.5 mg/kg and 2076.33 mm^3 for 0.25 mg/kg while in the control group the tumor size was 7747.05 mm^3 as shown in Figure 3. Inhibitory activity of the aqueous and methanolic extract was dose and duration dependent. The extract of *C. craniiformis* was effective in reduction of H22 tumour size at dose 1.2 mg/kg for aqueous extract and 1 mg/kg for methanolic in which H22 tumour mass was reduced in size for up to 87.9% mg/kg as shown in Figure 2 and 3.

Figure 4-B shown that H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6, 1.2) mg/kg shows significant inhibition of a tumour in the leg of the mouse compared with the H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones.

Table 2: The effect of *C. craniiformis* extracts on the apoptotic index of H22 bearing mice

Type of Extract	Dose (mg/kg)	Apoptosis index %	ANOVA (P-value)
Aqueous Extract	Untreated Control group	19.32	P ≤ 0.05
	0.3	25.34	
	0.6	26.70	
	1.2	27.21	
Methanolic Extract	Untreated Control group	18.30	P ≤ 0.05
	0.25	24.53	
	0.5	25.06	
	1	28.16	
Mann-Whitney test (p-value)	P > 0.05		

As shown in Table 2 and Figure 5-A, H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6 ,1.2) mg/kg shows a significant increase (P ≤ 0.05) in caspase-8 expression and hence in apoptotic index % (27.21%, 26.70%, 25.34%) compared with untreated control group (19.32%).

Figure 4: Effect of aqueous and Methanolic extracts of C carniiformis on H22 tumor development in the legs of inoculated mice; A) control group inoculated with H22 cells (Left), losing of leg due to bone necrosis and increased tumor size, compared with normal uninoculated mouse (right), increased tumor size, leading to loss of leg due to bone necrosis and destruction; B) H22 cells bearing mouse treated with aqueous extract of C carniiformis, shows inhibition of the tumor growth (right arrow) compared with untreated control group inoculated with H22, increased tumor size, (left) leading to loss of leg due to bone necrosis and destruction; C) H22 cells bearing mouse treated with methanolic extract of C carniiformis, mouse shows inhibition of tumor size (left arrow) compared with untreated H22 inoculated mouse (right)

Figure 4-C shown that H22 bearing mice treated with *C. craniiformis* methanolic extract using

three consecutive doses (0.25, 0.5, 1.0) mg/kg shows significant inhibition of a tumour in the leg of the mouse compared with the H22 bearing mouse in control group that loss the leg inoculated with H22 Cells due to necrosis in the bones. As shown in Table 2 and Figure 5-B H22 bearing mice treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg shows significant increase (P ≤ 0.05) in the caspase-8 expression and AI% (28.16%, 25.06%, 24.53%) compared with control group (18.30 %). No significant difference was reported in AI% between aqueous and methanolic extracts treated groups.

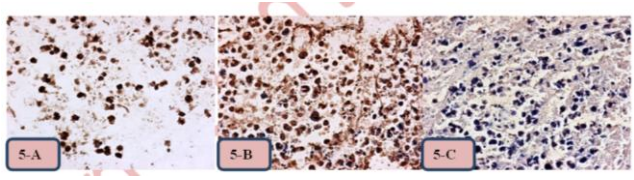

Figure 5: Immunohistochemical staining tissue sections from the leg of H22 murine hepatocellular carcinoma bearing mouse show cellular expression of caspase-8. 5-A: Tissue sections from the leg of an H22 bearing mouse treated with a crude aqueous extract of C. craniiformis. Up to 40% Cells with positive staining of caspase-8 expression stained with DAB chromogen (dark brown) counterstained with Mayer's hematoxylin (400X). 5-B: Tissue sections from the leg of an H22 bearing mouse treated with a crude methanolic extract of C craniiformis. Up to 100% of Cells with positive caspase-8 expression stained with DAB chromogen (dark brown) counterstained with Mayer's hematoxylin (400X). 5-C: untreated H22 hepatocellular carcinoma (control group).

As shown in Table 3, H22 bearing mice treated with *C. craniiformis* aqueous extract using three consecutive doses (0.3, 0.6, 1.2) mg/kg show increase in caspase-8 expression in a dose-dependent manner. At 0.3 mg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.6 mg and 1.2 mg the intensity of expression was strong in (33.33%) and very strong in (100%).

Table 3: Effect of *C. craniiformis* extracts on the caspase-8 expression of H22 bearing mice

Caspase-8 mRNA Expression Score	Control group	H22 Bearing mice treated with *C. Craniiformis* aqueous extract			H22 Bearing mice treated with *C. Craniiformis* methanolic extract		
		0.3mg	0.6mg	1.2mg	0.25 mg	0.5 mg	1 mg
0	1 (16.67%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
1	3 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
2	2 (33.33%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
3	0 (0%)	2 (33.33%)	0 (0%)	0 (0%)	2 (33.33%)	4 (66.67%)	1 (16.67%)
4	0 (0%)	4 (66.67%)	6 (100%)	6 (100%)	4 (66.67%)	2 (33.33%)	5 (83.33%)
Total No.(%) of Mice	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)	6 (100%)
P value			< 0.001			< 0.001	

A significant difference (P ≤ 0.001) in caspase-8 expression was reported. In H22 bearing mice treated with *C. craniiformis* methanolic extract using three consecutive doses (0.25, 0.5, 1.0) mg/kg show increase in caspase-8 expression in a dose-dependent manner. At 0.25 mg, the intensity of expression was strong in (33.33%) and very strong in (66.67%). While at 0.5 mg, the intensity of expression was strong in (33.33%) and very strong in (100%). At 1 mg, the intensity of expression was strong in (16.67%) and very strong in (83.33%). A significant difference (P ≤ 0.001) in caspase-8 expression was reported.

Discussion

The intake of mushrooms proved to be effective in cancer prevention, tumour growth inhibition and has high anti-tumour activity and a prevent tumour metastasis due to the high content of antioxidants thus, several mushrooms derived compounds are now increasingly used as an adjuvant to standard radio and chemotherapy [28] [29].

In current study, administration of aqueous (0.3 mg/kg, 0.6 mg/kg, 1.2 mg/kg) and methanolic extracts (0.25 mg/kg, 0.5 mg/kg, 1 mg/kg) of *C. craniiformis* to H22 tumor-bearing mice show significant differences (P ≤ 0.01) in tumor size compared with control group in a dose and duration-dependent manner. Among given doses, 1 mg/kg of methanolic extract was effective dose causing a reduction in tumour size in the last day (36^{th}), tumour size was 1167.28 mm^3 compared with 7747.05 mm^3 among the control group. This comes in line with [28], stated that as mushrooms contain different bioactive polyphenolic compounds, it effective in tumour growth inhibition and tumour metastasis. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents based on their high polyphenolic and ergosterol contents [30]. It was claimed that the puffball antioxidant capacity attributed to the presence of various chemicals such as ascorbic acid, carotenoids, esterified phenolics, and free and nonflavonoid phenolics and flavonoids [30] as well as ergosterol such as ergosterol ester, gallic, homogentisic, protocatechuic, p-hydroxybenzoic, and o- and p-coumaric acids, and other phenolic derivatives such as 3,4-dihydroxybenzaldehyde, ergothioneine, alkaloids, steroids, terpenoids [31] and selenium which protect cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well [32] [33].

The aqueous extract of *C. craniiformis* was effective in reduction of H22 tumour size at dose 1.2 mg/kg and 1 mg/kg for methanolic in which H22 tumour mass was reduced in size for up to 87.9% mg/kg. The reduction in tumour size proves the presence of a restriction in the tumour growth, angiogenesis inhibition and apoptosis induction as well as an increase in activity of the Immune system for fighting against cancerous cells. This comes in line with the fact that β-D-glucans which is one of

important constituent of *C. craniiformis* can inhibit tumour growth through inhibition of DNA polymerase and have the ability to modify Oncoprotein gene expression [34]. β-D-glucans, a protein-bound polysaccharide compound binds via specific receptors expressed as surface markers on phagocytic cells also play a vital role in stimulation and activation of phagocytic cells to invade tumour mass and stating destructive effects [35]. β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors. Mushrooms are containing more than one polysaccharide with antitumor activity. The responses to different polysaccharides are likely to be mediated by different cell surface receptors, which may be present only on specific subsets of cells and may trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumour inhibition that could be induced by a single polysaccharide [36]. A protein bound polysaccharide stimulates the functional maturation of macrophages and can scavenge active oxygen species which is widely prescribed for cancers of digestive organs like stomach, oesophagus colon etc. [37].

H22 bearing mice treated with *C craniiformis* aqueous extract (0.3, 0.6, 1.2) mg/kg shows significant increase in caspase-8 expression and hence in apoptotic index % (27.21%, 26.70%, 25.34%) compared with untreated control group (19.32%). H22 bearing mice treated with *C. craniiformis* methanolic extract (0.25, 0.5, 1.0) mg/kg shows significant increase in the caspase-8 expression and apoptotic index % (28.16%, 25.06, 24.53%) compared with control group (18.30%).

The increase of AI% in H22 bearing mice indicate cytotoxic effects of *C. craniiformis* on tumour cells. This cytotoxic effects started by induction of the apoptotic process. Apoptosis can be induced *via* two different pathways. The extrinsic pathway is triggered by the ligation of death receptors such as CD95 and recruiting of caspase-8 to the death-inducing signalling complex [24].

The intrinsic pathway is initiated by the release of cytochrome *c* from the mitochondria, which interacts with apoptosis protease activating factor-1 (APAF-1), caspase-9 and deoxyadenosine triphosphate to form the apoptosome complex. Links between the death receptor and the mitochondrial pathway exist at different levels [38].

One of the possible pathways in H22 tumour inhibition is the activation of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) which is a cytokine that is produced and secreted by most normal tissue cells. TRAIL plays a critical role in the NK cell-mediated and IFN-γ-dependent suppression of subcutaneous growth of TRAIL-sensitive tumours [10] [39]. TRAIL selectively induces apoptosis in cancer cells while normal cells are refractory [40]. TRAIL binds to Death Receptor (DR)-4 or -5 expressed on the plasma membrane of tumor cells ,resulting in recruitment of adapter molecules, Fas-Associated Death Domain (FADD), Procaspase-8 and 10 to the intracellular death domain (DD) of both receptors DR4 and DR5 [41] forming Death Inducing Signal Complex (DISC) [42]. This recruitment and clustering results in procaspase-8 dimerisation, activation, processing and release of active caspase-8 from the complex. Caspase-8, then, activates downstream the effector caspases including procaspase-3, -6, and -7, leading to activation of specific kinases resulting in classical apoptotic cell death [43]. DR4 and DR5 expression on cancer cells have been suggested be one reason for TRAIL's selective anti-tumour properties [44].

C. craniiformis lectins play a vital role in the inhibition of tumour growth. β-D-glucans which is one of the important constituents of *C. craniiformis* can inhibit tumour growth through inhibition of DNA polymerase and have the ability to modify Oncoprotein gene expression [34]. β-D-glucans, a protein-bound polysaccharide compound binds via specific receptors expressed as surface markers on phagocytic cells also play a vital role in stimulation and activation of phagocytic cells to invade tumour mass and stating destructive effects [35]. β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors and activated the alternative complement pathway [45]. Antitumor β-glucan induced the release of IL-1, IL-6 and TNF-α from macrophages [46].

Mushrooms are containing more than one polysaccharide with antitumor activity. The responses to different polysaccharides are mediated by different cell surface receptors, which may be present only on specific subsets of cells and may trigger distinct downstream responses. A combination of such responses involving different cell subsets could conceivably provide greater tumour inhibition that could be induced by a single polysaccharide [36]. A protein bound polysaccharide extracted from the mushroom displays various unique biological activities including the stimulation of functional maturation of macrophages and have an ability to scavenge active oxygen species which is widely prescribed for cancers of digestive organs like stomach, oesophagus colon and others [37]. The puffball *Calvatia candida* contains alkaloids, steroids and terpenoids, and have potent antioxidant activities [31]. *C. craniiformis* contain different bioactive polyphenolic contents and ergosterol compounds, ascorbic acid, carotenoids, esterified phenolics, and nonflavonoid phenolics and flavonoids [30]. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents. Different types of bioactive organic compounds showing antioxidant activities were isolated

from Tuber spp. These include ergosterol such as ergosterol ester, a wide range of phenolic acids such as gallic, homogentisic, protocatechuic, p-hydroxybenzoic, and o- and p-coumaric acids, and other phenolic derivatives such as 3,4-dihydroxybenzaldehyde. Mushrooms are the leading source of the essential antioxidant selenium, which protects cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well [32]. Ergothioneine which is one of important constituent of *C. craniiformis* can protect cells from distraction via antioxidant activity [47], in contrast to lectins which have inhibitory effects on the mitotic activity of tumour cells [14] [34]. Gallic acid, containing *C. craniiformis* which is one of the types of phenols, as well as a longer parts tannin, appears to works as an antioxidant that helps in protecting human cells from damage caused by oxidative stress processes [48]. Glucooligosacharide present in *C. craniiformis* have anticancer therapeutic effects due to its activation for T lymphocytes, NK cells and phagocytic cells leading to increase in production of TNF-α and increase macrophages cytotoxicity against tumour cells via perforin-granzyme system [15] [34] [49] [50].

Although, no significant difference was reported in AI% between aqueous and methanolic extracts treated groups, the increased expression of caspase-8 leads to increased AI% and reduction in tumour size in dose and duration-dependent manner. This primarily due to the effect of antioxidant and inhibitory compounds found in *C. craniiformis* which triggered the apoptotic signals in tumour cells after binding with tumour cells such as Lectins, Ergothioneine, β-glucan, Glucooligosacharide which are main chemicals appeared after conducting chemical analysis of the components of the head fruiting of *C. craniiformis* by our team. Some of these compounds have hematopoietic and immunomodulatory activities which bringing the importance of its use in vivo, particularly in experimental animals as the use of these component led to inhibition of cancerous cells. Gallic acid which is one of *C. craniiformis* components have cytoprotective action and can maintain the cells from damage [48]. Several major substances with immunomodulatory and/or antitumor activity have been isolated from *C. craniiformis*. These include mainly polysaccharides (in particular β-D-glucans), polysaccharopeptides (PSP), polysaccharide proteins, and proteins. Furthermore, other bioactive substances, including triterpenes, lipids, and phenols, have been identified and characterised in mushrooms with proven medicinal properties.

Polysaccharopeptides (PSP) present in *C. craniiformis* influence cancer metastasis in a number of ways: 1) by suppression of intravasation through the inhibition of tumor cells infiltration, 2) by suppression of tumor cell attachment to endothelial cells through the inhibition of tumor cell-induced platelet aggregation, 3) by suppression of tumor cell migration after extravasation through the inhibition of tumor cell mobility, and 4) by suppression of tumor growth after extravasation through the inhabitation of angiogenesis, the modulation of cytokine production and the augmentation of effector cell function [51] [52] and activation of alternative complement pathway [45].

As in another study on *C. versicolor* mushroom, the possible anti-tumor activity of *C craniiformis* may achieve due to various mechanisms mainly by Inhibition of DNA of tumor cells, Enhancement of cytokine production, antitumour activity in wide range of animal systems, Tumor cell killing effect, Inhibition of carcinogenesis, antioxidant effects, induction of apoptosis and antiproliferative effect; anti-invasion effects and anti-angiogenesis effects; tumouricidal and cytotoxicity effects; antimetastatic activity; Immunoprotective effects during radiation and chemotherapy [51].

The major immunomodulating effects of active substances derived from mushrooms include mitogenicity and activation of immune cells, such as hematopoietic stem cells, lymphocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, resulting in the increased production of cytokines, including interleukins (ILs) IL12, TNF-α; INF-γ and the ability to modulate the differentiation capacity of CD4+ T cells to mature into Th1 and/or Th2 subsets. Evidence indicates that mushrooms active substances induced TH differentiation toward Th1 more than Th2 subset and induced most Th1-specific cytokines (IL-2, IFN-γ, and LT) and Th2-specific cytokine (IL-4) in tumour-bearing animals [45]. Thus regulated cytokine production and possessed both anti-tumour and immunopotentiating activities. The main mechanism might be an anti-teratogenic effect attributed to free radical trapping and prevention of chromosome injury, coupled to an immunomodulating effect linked to the modulation of cytokines production and effect cell function.

The various experimental evidence demonstrated that the anti-tumour action of mushroom polysaccharides is due to the enhancement and potentiation of the cell-mediated immune system through the regulation of immunomodulatory cytokines and activation of the complement system and NK cells [53]. However, the mechanism of anti-tumour actions of PSP from most fungi is still not clear. It is accepted that anti-tumour polysaccharides enhance various immune responses, and act as biological response modifiers [53]. PSP is nonspecific immunopotentiators and exerts immunomodulatory actions by promoting the proliferation of T-lymphocytes, the activation of macrophages, NK cells, and Th cells, thereby inducing the production of antibody and interleukins [54] also has a favourable effect on the activation of leukocyte chemotactic locomotion and phagocytic activity [51].

The obvious effect of *C. craniiformis* is to stop the process of T lymphocytes apoptosis, a cells which responsible for fighting against viral infections who can dodge the immune system and also urged the liver cells to kill lymphocytes T effective, It was noted that the liver cells infected with HCV could urge or speed up the process of getting rid of activated T lymphocytes via apoptosis. Murine hepatocytes expressing a transgene encoding the HCV structural proteins core, envelope 1 (E1) and envelope 2 (E2) enhance the apoptosis of activated T cells. Unlike normal liver, which appears to remove only activated CD8+ T cells selectively, enhanced apoptosis determine for both $CD4^+$ and $CD8^+$ T cells via Fas–FasL-dependent pathway [55] [56].

In conclusion, extracts of *C. craniiformis* were efficiently inhibited H22 tumour growth, leading to a reduction in the tumour size clinically and increase the expression of caspase-8 gene product in tumour tissue. This effect is causing an increase in the apoptotic index of H22 tumour cells taken from legs of inoculated animals, causing protection of H22 inoculated legs from losing compared with an untreated control group which lost their legs due to necrosis and destruction in the bones. Antitumor activity of *C. craniiformis* aqueous and the methanolic extract was dose and duration dependent. These findings indicate the usefulness of *C. craniiformis* extracts as a novel antitumor agent for hepatocellular carcinoma, with its proved apoptosis induction through caspase-8 activation pathway.

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