Immunohistochemical Detection of Caspase 8 Expression and Apoptotic index Activities of *Calvatia Craniiformis* Crude Extract in Balb/C Mice Inoculated with H22 Cells

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Summary:

Objectives:

The aim was to identify Immunomodulatory, apoptosis inducing and anticancer activities of aqueous and alcoholic extracts of *Calvatia craniiformis* regarding the size of tumor mass and immunohistochemical detection of caspase 8 expressions and apoptotic index in mice inoculated with H22 mouse hepatocellular carcinoma cells as therapeutic experimental system for human hepatocellular carcinoma.

Materials and methods:

Forty-eight Balb/C albino mice were injected in legs with H22 cells. Tumor size was measured twice a week. Immunohistochemistry used for evaluation of Caspase-8 protein expression and apoptotic index was determined.

Results:

Administration of aqueous extract of *C craniiformis* in (0.3,0.6,1.2)mg/kg and alcoholic extract in (0.25,0.5,1)mg/kg to H22 tumor-bearing mice show significant difference (P≤0.01) in tumor size compared with control group . Inhibitory activity of aqueous and alcoholic extracts was dose and duration dependent. Effective doses in reduction of tumor size were 1.2mg/kg for aqueous and 1mg/kg for alcoholic extract in which H22 tumor mass reduced in size for up to 87.9 % .

H22 bearing mice treated with *C craniiformis* aqueous and alcoholic extracts shows significant increase (P ≤ 0.05) in apoptotic index % compared with untreated control group.

Conclusions:

Extracts of *C craniiformis* were highly efficient in tumor growth inhibition, causing reduction in the tumor size clinically and increase the expression of caspase 8 gene product in tumor tissue, causing increase apoptotic index of H22 tumor cells taken from legs of transfected animals causing the loss of legs due to necrosis in the bones. Antitumor activity of C *craniiformis* aqueous and alcoholic extract was dose and duration dependent

Keywords: *C craniiformis*,H22 cells, Caspase 8,Apoptosis Index

Introduction:

Turned the attention of researchers in recent years to the importance of the use of certain medicinal herbs and part of Soil fungi in an attempt to treat cancer [1]. In the fight against cancer; hospitals in Japan use compounds derived from mushrooms that have been approved. These cellular compounds and the secondary metabolites derived from edible mushrooms have a significant advantage because they are Biological Response Modifiers (BRM) [2]. BRM are compounds that stimulate the body's own response systems and mechanisms to fight disease, yet they do not harm the body or place additional stress on it. BRM are immunostimulants, they stimulate the body's response to fight all kinds of pathogens, infections, cancer, and other diseases) and adaptogens (they increase the body's own resistance to stress and trauma . In Japan, an immunomodulator compound derived from Maitake β-glucans called Grifolan, a branched β-1,3-d-glucan extracted from *Grifola frondosa* was found to promote tumor regression and necrosis, and was approved to be used in the treatment of cancer[3]. Hot water extracts from seven edible mushrooms, including Shiitake and Maitake, showed marked host-mediated antitumor activity against Sarcoma 180 cancer[2] . 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 defenses like increasing natural Killer cells [2].

The biological characteristics of *Calvatia craniiformis* extracts was 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 antitumour effect. *C craniiformis* significantly inhibits the growth of Yoshida sarcoma in cell culture and increase the survival time of mice with Leukaemia 1210[4] . Subsequent investigations have focused on the antitumour properties of calvatic acid, which may represent a model for the synthesis of more specific glutathione transferase-P1-1 inhibitors with possible therapeutic relevance.[5]

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[6]. Activation of caspases requires proteolytic processing at conserved internal aspartic residues to generate a heterodimeric enzyme consisting of the large and small subunits[7]. This protein is involved in the programmed cell death induced by Fas and various apoptotic stimuli. The N-terminal FADD-like death effector domain of this protein suggests that it may interact with Fas-interacting protein FADD[7, 8].

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 were crucial for the induction of nuclear translocation of NF-κB[9]. 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[10].

The aim of the present study was to identify Immunomodulatory, apoptosis inducing and anticancer activities of aqueous and alcoholic extracts of *Calvatia craniiformis* regarding the size of tumor mass and immunohistochemical detection of caspase 8 expression and apoptotic in-

dex in mice inoculated with H22 mouse hepatocellular carcinoma cells as therapeutic experimental system for human hepatocellular carcinoma.

Materials and methods:

Collection and Identification of mushroom:

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) represents the form of fungus discovered in Iraq by our team and figure (2) represents the cross-section with clearly appeared brown color 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 grove***
B) Cross-section of *C craniiformis in laboratory*
C) Sagittal section of *C craniiformis in laboratory*

Preparation of aqueous and alcohol extract of *C craniiformis*:

For the preparation of the aqueous extract, 50 gram of soft plant was weight and added in to 500 ml of distilled water, and then put 5-10 minutes in a blender for mixing until homogeneity. Extract was put in to a clean sterile bottles and transferred to the Shaker for an hour, then centrifuged for 10 minutes (2000 rpm / min), get rid of the sediment and then taking supernatant which then distributed in the clean dry dishes, and left in an incubator to get the dry extract to prepare concentrations used in the study [11]. *C craniiformis* crude aqueous extract gave 5 gm out of 75 grams dry *C craniiformis*, i.e. the extraction ratio was 6.66 % of crude *C craniiformis*. The extract was dark brown to black color, thick and little viscous.

For preparation of alcoholic extract from raw mushroom, Soxhlet is used. Twenty 20 g of dry powder was took and placed in Thimble, then put Thimble in the space provided in the Soxhlet device and hexane was added to remove fat and chlorophyll and conducted extraction for 12 hours at a temperature (40-60 ^{o}C) which is the temperature of the evaporation of solvent used. Then after that, the powder was transferred to Reflex device with 70% alcohol methanol for three hours, and then the extract was filtered by piece of gauze and filter paper then incubated for 24 hours for evaporation of alcohol.

Alcoholic extract was treated by HCl 1% in a Reflex for a period of half an hour, and then was filtrated by Whattman 1.Diethyl ether was added to the filtrate in separating funnel and left for 24 hours. two layers were appeared , the top layer is Diethyl ether layer which had been neglected, and the bottom layer is a aqueous layer that picked . PH of aqueous layer was raise for PH 8 by adding ammonia, which is weak base. Then after the aqueous extract was incubated to get rid of chloroform ,the final form of extract was obtained [12]. Crude alcoholic extract gave 5 gm of 50 g ,i.e. extraction ratio was 10% of raw material , the resulting extract have yellowish-brown color, thick and little viscous .

Determination of Acute Toxicity Effect for *C craniiformis* :

To determine any possible toxic effects for aqueous and alcoholic extract of Calvatia, Up-and down method was followed for determination LD50 according to the following equation[13]:

$LD50 = Xf + Kd$

Xf: 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	last dose administered (Xf)	midterm lethal dose (LD50)
Aqueous	25	ooxo	-439	100	85 mg\kg
Alcoholic	50	oxxx	1.5	200	177 mg\kg

O: the survival animal within 24 hours of injection

X: the death of animal within 24 hours of injection.

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

Experimental animals:

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 in accordance with the recommendation for the proper use and care of laboratory animals. Mice were divided in to four groups, (6) mice for each one. Three groups received extract and one group for control receiving Dimethyl sulphoxide (DMSO).

Cell culture:

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 [14].

Tumor-bearing laboratory animals:

After complete growth, H22 cells were harvested from RPMI 1640 Medium and 0.1 ml of cells was transplanted in leg of (48) Balb/C albino mice to establish a solid tumor model. [15]. The experiment ending with the death of last mouse from the control group given doses of aqueous and alcoholic extract. tumor size was measured twice a week during the duration of the experiment using special caliber and take the measurement analogy (latitude and longitude), and extracted tumor size [16, 17].

Immunohistochemistry for Detection of Caspase 8:

Immunohistochemistry (IHC) used for evaluation of apoptosis. The procedure of IHC was preformed according to manufacturer's instruction, using polyclonal rabbit anti-mouse caspase 8 IgG, ab25901 recognises the p18 form of Caspase-8[18]. Secondary antibodies ,Biotinylated goat anti-rabbit IgG . final results visualized by using Immunohistochemistry detection kit , Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit ab80436[19]

The primary antibody diluted by the common antibody diluent 1:50.Optimal antibodies concentration may vary depending on specimen and preparation method, thus optimization 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. Positive control was obtained by using tonsil tissue [20].

The expression of 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 semi quantitatively using a scoring system for both intensity and extent of staining in 10 microscopic fields which was 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) [21].

final results for apoptotic index were expressed as Mean± SE [22].Apoptotic index % was determined according to the following equation[23]:

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

Statistical analysis: 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 8expression (AI %) between groups that composed from continuous variables. 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 0.3mg/kg,0.6mg/kg,1.2mg/kg to H_{22} tumor-bearing mice show significant differences (P≤0.01) in tumor size compared with control group. Among given doses , 1.2mg/kg was effective dose causing reduction in tumor size in last day of experiment(36th),in which tumor size was 960mm^3 compared with 1564.57 mm^3 for 0.6mg/kg and 3559.20 mm^3 for 0.3mg/kg while in control group the tumor size was 5747.05mm^3.

Administration of methanolic extract of *C craniiformis* in 0.25mg/kg,0.5mg/kg,1mg /kg to H_{22} tumor-bearing mice show significant difference (P≤0.01) in tumor size compared with control group . A significant difference in tumor size throughout the period of experiment was reported. Tumor size was minor increased in treated group compared with control. In last day of experiment(36^{th}), tumor size was 1167.20 mm3 in group treated with 1 mg/kg compared with 1332.64mm3 for 0.5mg/kg and 2076.33mm3 for 0.25mg/kg while in control group the tumor size was (7747.04) mm^3 as shown figure (3).

Among given doses , 1 mg/kg was effective dose causing reduction in tumor size in last day (36^{th}),in which tumor size was 1167.28 mm3 compared with 1332.64mm3 for 0.5mg/kg and 2076.33mm3 for 0.25mg/kg while in control group the tumor size was (7747.05) mm^3 as shown figure (3). Inhibitory activity of aqueous and alcoholic extract was dose and duration dependent. The extract of *C craniiformis* was effective in reduction of H22 tumor size at dose 1.2mg/kg for aqueous extract and 1mg/kg for alcoholic in which H22 tumor mass was reduced in size for up to 87.9%mg/kg as shown in figure(2&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 tumor in the leg of mouse compared with H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis in the bones .

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 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-c) shown that H22 bearing mice treated with *C craniiformis* alcoholic extract using three consecutive doses (0.25, 0.5 ,1) mg/kg shows significant inhibition of tumor in the leg of mouse compared with H22 bearing mouse in control group that loss the leg inoculated with H22 Cells due to necrosis in the bones. As shown in table(3) and figure (5-B) H22 bearing mice treated with *C craniiformis* alcoholic extract using three consecutive doses (0.25, 0.5,1) mg/kg shows significant increase (P ≤ 0.05) in the caspase 8 expression and apoptotic index %(28.16%, 25.06, 24.53%) compared with control group (18.30 %).

As shown in table (4), 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 0.3mg, the intensity of expression was strong in (33.33%) and very strong in (66.67%).while at 0.6mg and 1.2mg the intensity of expression was strong in (33.33%) and very strong in (100%) .significant difference (P ≤ 0.001) in caspase 8 expression was reported. In H22 bearing mice treated with C craniiformis alcoholic extract using three consecutive doses (0.25, 0.5 ,1) mg/kg show increase in caspase 8 expression in a dose dependent manner . At 0.25mg , the intensity of expression was strong in (33.33%) and very strong in (66.67%).While at 0.5mg , the intensity of expression was strong in (33.33%) and very strong in (100%) . At 1mg , the intensity of expression was strong in ((16.67%) and very strong in (83.33%) . Significant difference (P ≤ 0.001) in caspase 8 expression was reported.

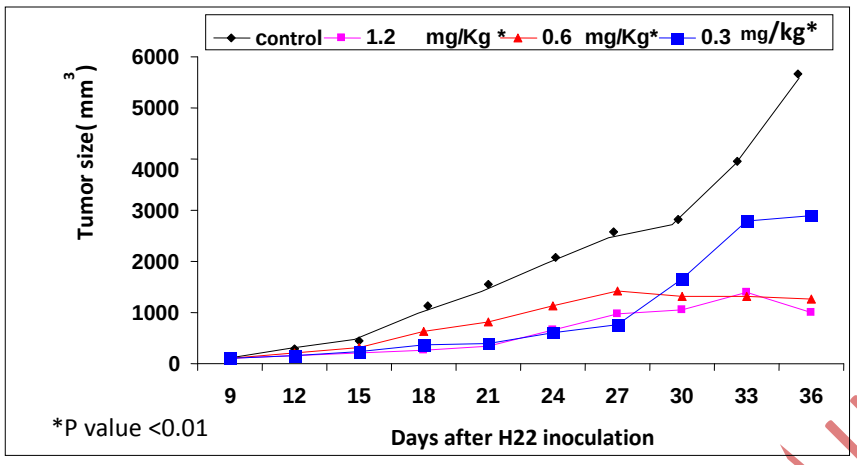

Figure (2): Tumor size follow up in mice inoculated with H22 murine tumor cells treated with aqueous extract of *C craniiformis.* significant difference in tumor size (p value<0.01) in the leg of inoculated mouse compared with H22 bearing mouse in control group that loss the legs inoculated with H22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

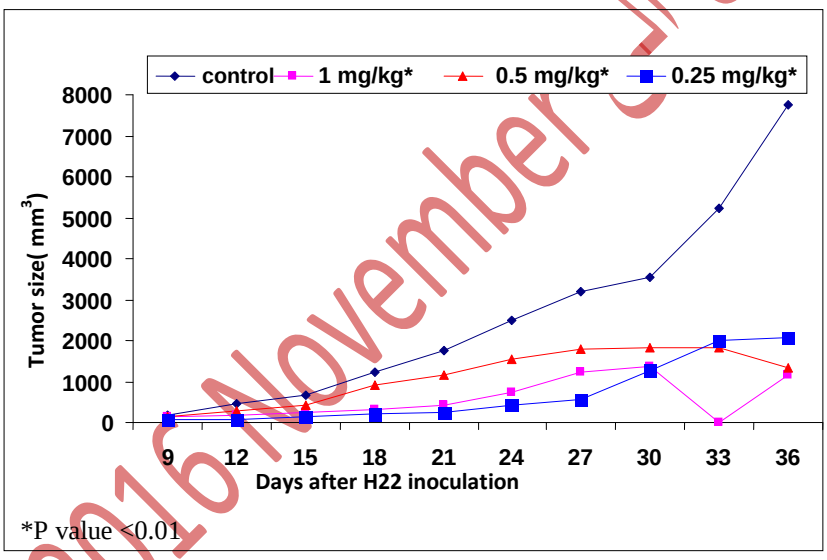

Figure (3): Tumor size follow up in mice inoculated with H22 cells treated with alcoholic extract of *C craniiformis.* Significant difference in tumor size (p value<0.01) of mouse inoculated legs compared with H22 bearing mouse in control group that loss the legs inoculated with H22 Cells due to necrosis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

Table (2): The effect of different doses of *C craniiformis* aqueous extract in AI (%) of H22 bearing mice.

Dose(mg/kg)	Apoptosis index %	ANOVA (P value)
Untreated Control group	19.32	P ≤ 0.05
0.3	25.34	
0.6	26.70	
1.2	27.21	

Table (3): The effect of different doses of *C craniiformis* alcohol extract in AI % of H22 bearing mice.

Dose(mg/kg)	Apoptosis index %	ANOVA (P value)
Untreated Control group	18.30	P ≤ 0.05
0.25	24.53	
0.5	25.06	
1	28.16	

Table (4): Effect of different doses of C craniiformis extracts in Caspase 8 expression of H22 bearing mice.

Score	Control group	H22 Bearing mice treated with aqueous extracts			H22 Bearing mice treated with alcoholic extracts		
		Caspase 8 expression according to the dose			Caspase 8 expression according to the dose		
		0.3mg	0.6mg	1.2mg	0.25 mg	0.5 mg	1 mg
O	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 animals	6(100%)	6(100%)	6(100%)	6(100%)	6(100%)	6(100%)	6(100%)
P value		<0.001			<0.001		

Figure (4): Effect of aqueous and alcoholic extracts of *C carniiformis on* H22 tumor development in legs of inoculated mice.

Figure 4-A: control group inoculated with H22 cancer cells (Left) ,lossing of leg due to bone necrosis and increased tumor size. compared with normal uninoculated mouse (right). increased tumor size,leading to lossing of leg due to bone necrosis and destruction

Figure 4-B: H22 cells bearing mouse treated with aqueous extract of C carniiformis, shows inhibition of the tumor growth (right arrow) compared with untrated control group inoculated with H22. increased tumor size. (left) leading to lossing of leg due to bone necrosis and destruction

Figure 4-C: H22 cells bearing mouse treated with alcoholic extract of C carniiformis ,mouse shows inhibition of tumor size (left arrow) compared with untreated H22 inoculated mouse(right)

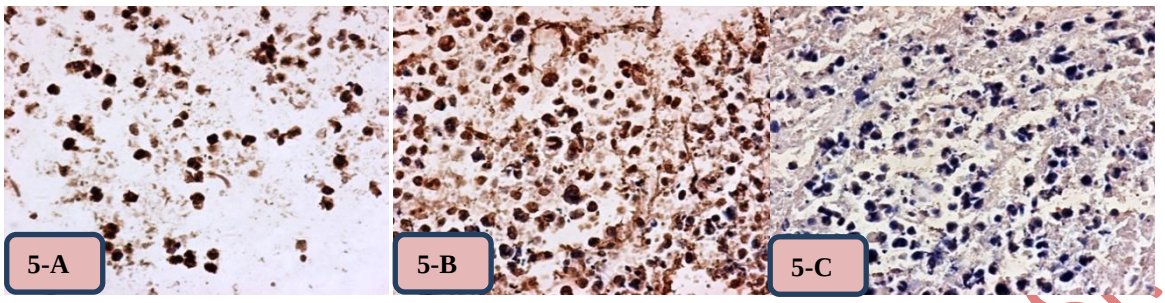

Figure (5): Immunohistochemical staining tissue sections from leg of H22 murine hepatocellular carcinoma bearing mouse show cellular expression of caspase 8:
(5-A) Tissue sections from leg of H22 bearing mouse treated with crude aqueous extract of *C craniiformis*. Up to 40% Cells with positive staining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

Discussion:

The intake of mushrooms proved to be effective in cancer prevention, growth inhibition and also has high anti-tumor activity and a preventive effect in tumor metastasis[24], anticancer activities due to Lentinan[25] .National Cancer Institute [26] reported that a number of medicinal mushrooms possessed promising antioxidant and anticancer properties. Several mushroom derived compounds are now increasingly used as adjuvant to standard radio and chemotherapy.

In current study, administration of aqueous and alcoholic extracts of *C craniiformis* in 0.3mg/kg,0.6mg/kg,1.2mg/kg and in 0.25mg/kg,0.5mg/kg,1mg /kg respectively to H22 tumor-bearing mice show significant differences ($P \leq 0.01$) in tumor size compared with control group in a dose and duration dependent manner. Among given doses , 1 mg/kg of alcoholic extract was effective dose causing reduction in tumor size in last day (36th),in which tumor size was 1167.28 mm3 compared with (7747.05) mm3 among control group. This come in line with others stated that the intake of mushrooms proved to be effective in cancer prevention, growth inhibition and also has high anti-tumor activity and a preventive effect in tumor metastasis[24]. Its contain different bioactive polyphenolic compounds. These compounds act as effective antioxidants based on their excellent ability to scavenge free radicals and act as reducing agents. Different groups of desert truffles and mushrooms showed strong antioxidant activities based on their high polyphenolic and ergosterol contents [27] . It was claimed that the puffball antioxidant capacity is attributed to the presence of various chemicals such as ascorbic acid, carotenoids, esterified phenolics, and free- and nonflavonoid phenolics and flavonoids[27] as well as ergosterol such as ergosteryl 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 [28] and selenium which protect cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well [29] [30].

The aqueous extract of *C craniiformis* was effective in reduction of H22 tumor size at dose 1.2mg/kg and 1mg/kg for alcoholic in which H22 tumor mass was reduced in size for up to 87.9%mg/kg . The reduction in tumor size prove the presence of restriction in the tumor growth ,angiogenesis inhibi-

tion and apoptosis induction as well as increase in activity of Immune system for fighting against cancerous cells. This come in line with the fact that β-D-glucans which is one of important constituent of *C craniiformis* have the ability to inhibit tumor growth through inhibition of DNA polymerase and have the ability to modify oncoprotein gene expression [31]. 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 tumor mass and stating destructive effects[32].β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors. Mushrooms 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 tumor inhibition than could be induced by a single polysaccharide[33] . A protein bound polysaccharide stimulates the functional maturation of macrophages and have an ability to scavenge active oxygen species which is widely prescribed for cancers of digestive organs like stomach, esophagus colon etc. [25].

H22 bearing mice treated with *C craniiformis* aqueous extract using three consecutive doses (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* alcoholic extract using three consecutive doses (0.25, 0.5,1) 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 %) .

Increase of AI% in H22 bearing mice indicate cytotoxic effects of *C craniiformis* on tumor cells . This cytotoxic effects started by induction of apoptotic process. Apoptosis can be induced *via* 2 different pathways. The extrinsic pathway is triggered by ligation of death receptors such as CD95 and recruition of caspase-8 to the death-inducing signaling complex [21, 34]. 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[35].

One of possible pathways in H22 tumor inhibition is the activation of tumor 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 tumors[36]. TRAIL selectively induces apoptosis in cancer cells whilst normal cells are refractory [37]. 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[38] forming Death Inducing Signal Complex (DISC).This recruitment and clustering results in procaspase-8 dimerization, 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 [39, 40] . DR4 and DR5 expression on cancer cells has been suggested be one reason for TRAIL's selective anti-tumor properties [41].

C craniiformis Lectins play a vital role in inhibition of tumor growth. β-D-glucans which is one of important constituent of *C craniiformis* have the ability to inhibit tumor growth through inhibition of DNA polymerase and have the ability to modify Oncoprotein gene expression [31]. 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 tumor mass and stating destructive effects[32]. β-glucan caused direct enhancement of the colony-forming units granulocytes/macrophages (CFU-GM) response of bone marrow cells progenitors and activates

alternative complement pathway [42]. antitumor β-glucan induced the release of IL-1, IL-6 and TNF-α from macrophages[43].

There are several reports of mushrooms 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 tumor inhibition than could be induced by a single polysaccharide[33] . 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, esophagus colon and others [25]. The puffball Calvatia candida contain alkaloids, steroids and terpenoids, and have potent antioxidant activities[28]. *C craniiformis* contain different bioactive polyphenolic contents and ergosterol compounds, ascorbic acid, carotenoids, esterified phenolics, and nonflavonoid phenolics and flavonoids [27]. 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 sp. These include, ergosterol such as ergosteryl ester, 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 protect cells from damage that might lead to chronic diseases and help to strengthen the immune system, as well[29]. Ergothioneine which is one of important constituent of *C craniiformis* have the ability to protect cells from distraction via antioxidant activity[44], in contrast to Lectins which have inhibitory effects on mitotic activity of tumor cells [11, 31]. Gallic acid, containing C craniiformis which is one of the types of phenols, as well as a longer parts tannin appears to works as antioxidant that helps in protecting human cells from damage caused by oxidative stress processes [45]. Glucooligosacharide present in *C craniiformis* have anticancer therapeutic effects due to its activation for T lymphocytes and NK cells as well as phagocytic cells and increase in production of Tumor necrosis factor (TNF-α) as well as increase macrophages cytotoxicity against tumor cells via perforin – granzyme system [12, 31, 46, 47].

Increase in expression of caspase 8 leads to increase AI% and reduction in tumor 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 tumor cells after binding with tumor 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 components of *C craniiformis* have cytoprotective action and have the ability to maintain the cells from damage[45]. 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 characterized 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[48, 49] as well as activation of alternative complement pathway[42] .

As in another studies on *C. versicolor* mushroom , the possible anti-tumor activity of *C craniiformis* may achieved due to various mechanisms mainly by Inhibition of DNA of tumour cells , Enhancement of cytokine production , Antitumour activity in wide range of animal systems , Tumour 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 ; antimetastic activity ; Immunoprotective effects during radiation and chemotherapy[48].

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 increase production of cytokines, including interleukins (ILs) IL12 , tumor necrosis factor alpha (TNF)-α ; interferon gamma (INF)-γ and the ability to modulate the differentiation capacity of CD4+ T cells to mature into TH1 and/or TH2 subsets. Evidences indicates that mushrooms active substances induce TH differentiation toward TH1 more than TH2subset and induced most TH1-specific cytokines (IL-2, IFN-γ, and LT) and TH2-specific cytokine (IL-4) in tumor bearing animals [42]. Thus regulated cytokine production and possessed both anti-tumor and immunopotentiating activities. 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.

Various experimental evidences demonstrated that the anti-tumor action of mushroom polysaccharides is due to the enhancement and potentiation of cell-mediated immune system through the regulation of immunomodulatory cytokines and activation of the complement system and Natural Killer Cells (NK cells)[50] . However, the mechanism of anti-tumor actions of PSP from most fungi is still not clear. It is accepted that anti-tumor polysaccharides enhance various immune responses, and act as biological response modifiers [50]. PSP are nonspecific immunopotentiators and exert 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 [51] also has favorable effect on the activation of leucocyte chemotactic locomotion and phagocytic activity [48].

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 can 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 apoptosis of activated T cells. Unlike normal liver, which appears to selectively remove only activated CD8+ T cells, enhanced apoptosis determine for both CD4+ and CD8+ T cells via Fas–FasL dependent pathway. [52, 53].

Conclusions:

Extracts of *C craniiformis* were highly efficient in H22 tumor growth inhibition, causing reduction in the tumor size clinically and increase the expression of caspase 8 gene product in tumor tissue. This effect causing increase in apoptotic index of H22 tumor cells taken from legs of inoculated animals , causing protection of H22 inoculated legs from losing compared with untreated control group which loss their legs due to necrosis and destruction in the bones . Antitumor activity of *C craniiformis* aqueous and alcoholic extract was dose and duration dependent. These finding 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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