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IMMUNOMODULATORY AND THERAPEUTIC EFFECT OF SEPARATED GALLIC ACID FROM *CALVATIA CRANIIFORMIS* MUSHROOM IN MAMMARY GLAND TUMOR BEARING MICE

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ABSTRACT : To identify the anticancer effect of gallic acid fraction from alcoholic extract of *Calvatia craniiformis* mushroom in white mice with mammary gland tumor (M-22) as a model for treatment of mammary gland tumor in human. Sixty albino mice were injectedwith M22 cells. Tumor growth was appear after 7-10 days in 50 mice. Tumor size was measured twice a week to after administration of gallic acid of *C. craniiformis* in 0.2mg/kg, 0.4mg/kg, 0.8 mg/kg to M_{22} tumor-bearing mice to determine relative tumor volume and growth inhibition (GI%). Administration of gallic acid of *C. craniiformis* in 0.2mg/kg, 0.4mg/kg, 0.8 mg/kg to M_{22} tumor-bearing mice show significant differences ($P \leq 0.05$) in tumor size compared with control group. Tumor size development was significantly slower in all treated group compared with control ($P<0.05$). The most effective dose for last days was 0.8mg/kg treated group compared with control, the tumor size was 35.12mm^3 compared to 240.15 mm^3 and 91.24 mm^3 for second and third dose and 3855.80 mm^3 for control group. Gallic acid (0.2 mg/kg, 0.4 mg/kg) leads to gradual increase in tumor size until 27 days after inoculation, when the size was reduced significantly after 10 doses, in the last day of experiment the size was 109.00 mm^3; 41.34 mm^3 compared with 1734.23 mm^3 in control group.

Gallic acid (0.8 mg/kg) leads toslow growth in tumor until 27 days after inoculation, when the size was reduced significantly after 10 doses, in the last day of experiment the size was 15.88 mm^3 compared with control group. Significant difference between gallic acid treated group and control group ($P>0.05$). Tumor inhibition (GI%) increase significantly for all doses with time ($P>0.05$). The most effective doses was (0.4mg/kg, 0.8 mg/kg) in which GI% was 87.06 and 83.91%, respectively, while in 0.2mg/kg, GI% was 71.91%. There was significant difference in body weight (BW) among treated groups with 0.2 mg/kg; 0.4 mg/kg and 0.8 mg/kg ($P < 0.05$) compared with control. Gallic acid fraction of *C. craniiformis* was highly efficient in tumor growth inhibition, causing reduction in the tumor size clinically. Antitumor activity of *C. craniiformis* alcoholic extract was dose and duration dependent.

Key words : *C. craniiformis*, gallic acid, M22, immunomodulation, Iraq.

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 (Lee *et al*, 2014; Maji *et al*, 2017). 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) (Shavit *et al*, 2009; Suganya *et al*, 2014). 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 â-glucans called Grifolan, a branched â-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 (Mao *et al*, 2007). Hot water extracts from seven edible mushrooms, including Shiitake and Maitake, showed marked host-mediated antitumor activity against Sarcoma 180 cancer. Lentinan, a protein-free polysaccharide (â-1,3-d-glucans and â-1,6-d-glucans) derived from the fruit body of Shiitake was approved for the treatment ofgastric 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 (Shavit *et al*, 2009).

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 hasanti-inflammation and a definite anti-tumor effect. *C. craniiformis* significantly inhibits the growth of Yoshida sarcoma in cell culture and increase the survival time of mice with Leukaemia 1210 (Umezawa *et al*, 1975). Subsequent investigations have focused on the anti-tumor properties of calvatic acid, which may represent a model for the synthesis of more specific glutathione transferase-P1-1 inhibitors with possible therapeutic relevance (Coetze and Wyk, 2009).

Aims of the study : To identify the anticancer effect of separated gallic acid from alcoholic extract of *C. craniiformis* mushroom in white mice with mammary gland tumor (m-22).

MATERIALS AND METHODS

Collection and identification of mushroom

Calvatia 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 and was authenticated by professor Salman Kamel Jabor. *C. craniiformis* belongs to the fungal kingdom Mycota, Class Agaricomycetes, family Lycoperdaceae. Fig. 1 represents the form of fungus discovered in Iraq by our team, which is part of the active ingredients used in the treatment.

Preparation alcohol extract of *C. craniiformis*

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°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 wastreated 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

Fig. 1 : The form of *C. craniiformis* mushroom discovered in Iraq.

appeared, the top layer is Diethyl etherlayer which had been neglected and the bottom layer is aaqueous 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 (Bellini *et al*, 2006). 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. Gallic acid was determine by High Performance Liquid Chromatography (HPLC).

Determination of acute toxicity effect for Gallic acid extracted from *C. craniiformis*

To determine any possible toxic effects for Gallic acid extracted from *C. craniiformis*, Up-and down method was followed for determination LD_{50} according to the following equation (Dixon, 1980).

$LD_{50} = Xf + Kd$

Xf : last dose administered

d : difference between dose levels

k : tabular value

According to acute toxicity study, alcoholic extract was administered in the following doses : 0.2 mg/kg, 0.4mg/kg, 8mg/kg.

Experimental animals

Sixty 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, (15) mice for each one. Three groups received extract and one

group for control receiving Dimethyl sulphoxide (DMSO).

Inoculation of M22 in mice

After complete growth, M22 cells were harvested from RPMI 1640 Medium and 0.1 ml of cells was inoculated intraperitoneally to 60 albino mice to establish a solid tumor model (King *et al*, 1982). Tumor growth was developed well in 50 mice after 7-10 days. All 50 mice was treated with gallic acid for 30 days with 3 doses in the form of single dose each 48h. The experiment ending with the death of last mouse from the control group given doses of gallic acid. Tumor size was measured twice a week during the duration of the experiment usingspecial caliber and take the measurement analogy (latitude and longitude) and extracted tumor size (Wolff and Rodin, 1978; Liu *et al*, 2007).

RESULTS

As shown in Fig. 2, administration of gallic acid extract of *C. craniiformis* in 0.2mg/kg, 0.4mg/kg, 0.8 mg/kg to M_{22} tumor-bearing mice show significant differences (P≤0.05) in tumor size compared with control group. Tumor size development was slower among treated group compared with control. Tumor size in 24 day (250.25mm), 27day (243.21mm) and 30 day (240.15mm) compared with control 1250.34 mm; 2550.70 mm; 3855.80mm) respectively during the same period of time as shown in Table 1.

Tumor size development was significantly slower among 0.4mg/kg treated group compared with control (P<0.05). Tumor size in 24 day (156.23mm), 27 day (125.27 mm) and 30 day (91.24mm) compared with control. Tumor size development was significantly slower among 0.8mg/kg treated group compared with control (P<0.05). Tumor size in 24 day (92.89mm), 27 day (51.87mm) and 30 day (35.12mm) compared with control.

Tumor size development was significantly slower in all treated group compared with control (P<0.05). The most effective dose for last days was 0.8mg/kg treated group compared with control, the tumor size was

Table 1 : Effect of different doses of gallic acid on M22 tumor size of mice.

Day of administration	Doses of Gallic acid (mg/Kg)			
	Control	0.2	0.4	0.8
	SE ± mean	SE ± mean	SE ± mean	SE ± mean
0	^{gA} 220.88±1.44	^{bA}220.06±2.28	^{aA}220.38±5.81	^{aA} 221.12±2.37
9	^{gA}237.65±4.33	^{bB}220.12±5.79	^{aA}220.16±5.81	^{aB}222.33±1.03
12	^{fA}477.86±31.87	^{bA}210.11±22.82	^{aB}210.02±5.81	^{bB}211.34±0.06
15	^{eA}682.15±1.00	^{aB}295.23±2.54	^{abB}191.13±5.84	^{cC}184.33±0.01
18	^{dA}963.81±0.65	^{aB}291.93±5.95	^{bC}186.22±8.52	^{dD}144.15±2.30
21	^{dA}981.53± 11.41	^{bB} 250.96±5.75	^{cC}174.82±2.32	^{eD} 114.45± 3.18
24	^{cA}1250.34±28.86	^{Bb} 250.25±28.81	^{dcC}156.23±2.78	^{fC}92.89± 1.86
27	^{bA}2550.70±28.92	^{Bb} 243.21±0.05	^{dC} 125.27±0.03	$^{f D}$ 51.87± 0.92
30	^{bA}3855.80±28.92	^{Bb} 240.15± 0.05	^{eC} 91.24±17.35	$^{f D}$ 35.12±0.92

- small letters in column indicate significant difference (P<0.05)
- small letters in rows indicate significant difference (P<0.05)

Table 2 : Effect of different doses of gallic acid on M22 relative tumorsize of mice compared with control group.

Day of administration	Doses of Gallic acid (mg/Kg)			
	Control	0.2	0.4	0.8
	SE ± mean	SE ± mean	SE ± mean	SE ± mean
0	^{gA} 100±1.15	^{cA}100±0.00	^{aA}100±0.00	^{aA}100±0.00
9	^{gA} 107.59±4.04	^{cAB}99.96 ±0.00	^{aB}99.96±2.30	^{aB}100.00±1.45
12	^{fA} 222.40±5.77	^{cB}95.44 ±7.44	^{aB}95.16±1.15	^{aB}97.31±1.15
15	^{eA} 280.00 ± 4.61	^{aC}134.09±5.00	^{bC}86.60±2.88	^{bC}83.38±1.73
18	^{dA} 436.00±5.77	^{aB} 129.85±2.30	^{bC}84.38±1.15	^{cD}65.29±2.88
21	^{dA} 444.39 ±2.30	^{bC}113.47±1.73	^{cC}79.21±2.88	^{dD}51.77± 0.75
24	^{cA} 566.09 ±2.30	^{bC}113.65±1.73	^{dC}70.79±2.30	^{eD}42.47±1.15
27	^{bA} 1155.34 ±2.88	^{bC}110.45±1.15	^{eC}56.76± 0.5	^{fD} 23.45±1.73
30	^{bA} 1734.23± 2.7 8	^{bC}109.00±1.16	^{eC}0.4±41.34±	^{fD}15.88± 1.53

- small letters in column indicate significant difference (P<0.05)
- small letters in rows indicate significant difference (P<0.05)

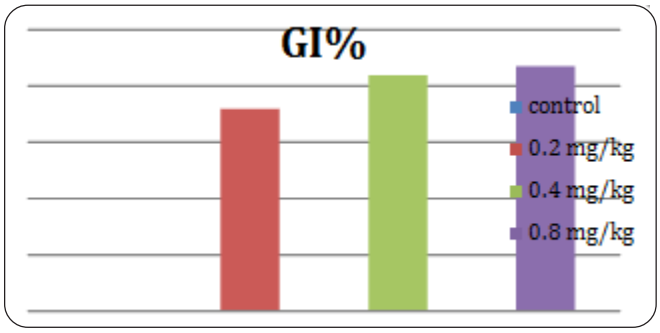

Fig. 2 : Effect of Gallic acid on GI% of mice bearing M22 in last day of experiment.

among treated group at the first day of experiment was 27.21gm, 27.22gm compared with 29.80gm for control. Significant difference in BW among 0.4 mg/kg treated. Significant increase in BW in day 24 was 29.91gm and in day 28 was 30.69gm compare with 27.21 gm at day 1. Significant difference in BW among 0.8 mg/kg treated group. Significant increase in BW in day 24 was 27.16 gm and in day 28 was 28.38gm compare with day 1.

DISCUSSION

Relative tumor volume (RTV) and growth inhibition (GI%) were used for evaluation of tumor size changes.

Table 3 : Effect of different doses of gallic acid on body weight of M22 tumor bearing mice.

Dose / Day	Control	0.2mg/Kg	0.4 mg/Kg	0.8mg/Kg
	SE ± mean	SE ± mean	SE ± mean	SE ± mean
0	^{eA}29.80 ± 0.02	^{cB}27.21± 0.00	^{dB}27.21± 0.01	^{eC}26.24±0.17
4	^{dA}31.22 ± 0.06	^{cB}27.22 ± 0.01	^{dB}27.21±0.00	^{deC}25.93 ±0.005
8	^{cA}32.58± 0.24	^{cB}27.33 ±0.01	^{bB}27.28± 0.02	^{deC} 26.42± $^{0.30}$
12	^{cA}32.34 ± 0.09	^{cB}27.31 ±0.01	^{bB}27.32± 0.02	^{cdeC}26.57± 0.28
16	^{cA}32.35 ±0.09	^{cB}27.32 ± 0.00	^{cC}28.33± 0.02	^{cdD} 26.72±0.21
20	^{cA}32.31 ± 0.04	^{bB}28.35 ± 0.05	^{cB} 28.36± 0.03	^{bC} 27.37± 0.03
24	^{bA}32.26 ± 0.02	^{bB}28.36 ± 0.11	^{bC}29.91± 0.03	^{bD}27.16 ± 0.005
28	^{aA}34.34 ± 0.05	^{aB}29.19 ± 0.06	^{aC}30.69± 0.17	^{aD}28.38±0.14

- small letters in column indicate significant difference (P<0.05)
- small letters in rows indicate significant difference (P<0.05)

35.12mm^3 compared to 240.15 mm^3 and 91.24 mm^3 for second and third dose and 3855.80mm^3 for control group. Tumor size in tumor bearing mice treated with gallic acid show gradual increase with time, but it is smaller than control group, as shown in Table 2. Gallic acid (0.25mg/kg, 0.4 mg/kg) leads to gradual increase in tumor size until 27 days after inoculation, when the size was reduced significantly after 10 doses, in the last day of experiment the size was 109.00 mm^3, 41.34 mm^3 compared with 1734.23 mm^3 in control group.

Gallic acid (0.8 mg/kg) leads to slow growth in tumor until 27 days after inoculation, when the size was reduced significantly after 10 doses, in the last day of experiment the size was 15.88 mm^3 compared with control group. Significant difference between gallic acid treated group and control group (P>0.05). Tumor inhibition (GI%) increase significantly for all doses with time (P>0.05) as shown in Fig. 1. The most effective doses was (0.4mg/kg, 0.8 mg/kg) in which GI% was 87.06 and 83.91% respectively, while in 0.2mg/kg, GI% was 71.91%.

As shown in Table 3, there was significant difference in body weight (BW) among treated group (P < 0.05) compared with control. Body weight decrease at the 24 day and 28 day; in 0.2 mg/kg treated group (28.38 gm; 29.19 gm) compared with control (34.34 mg). Body weight

Current results indicate that tumor size depends on the dose of gallic acid. The best dose was 0.8 mg/kg in which GI% was 87.06%. Indirect effect of *C. craniiformis* was probably due to immunostimulatory substances enhance the activation of T cells and secretion of INFã and IL2, which facilitate the activation of cytotoxic T-cells and natural killer cells leads to destruction of tumor and reduction of its size (Rowan *et al,* 2003). Current study come in line with other studies suggest that mushrooms contain anti tumor substances and enhance the activity of immune systemmainly NK cells (Rowan *et al*, 2003; Jameel *et al*, 2018).

The NK cells are activated by different stimuli such as direct involvement of NKR by stress induced tumor molecules, various cytokines such as IL-1, IL-2, IL-12, IL-15, IL-18, IL-21 and type I IFNs (Zamai *et al*, 2007). On the stimulation of cytokine, the NK cells are converted into lymphokine-activated killer (LAK) cells. These LAK cells propagate, produce cytokines and up-regulate the effectors or adhesion molecules like perforin, NKp44, granzymes, Fas ligand (FasL) and TRAIL. These LAK cells adhere to the target cells and recognize them and increase tumor lysis activity (Nouroz *et al*, 2016). Tumor necrosis factor (TNF) family ligands express themselves on the surface of NK cells. NK cells control the tumor

by continued T cell antitumor response through cross talk with dendritic cells (Thiery *et al*, 2011). Evidence showed that cytokines such as IL-2, IL-15, IL-12, IL-18 and CD40 enhance NK cell cytotoxicity against tumor target cells and the production of IFN-y by NK cells (Lauwerys *et al*, 2000; Lusty *et al*, 2017). Current study come in line with other study done by X. AL-Mosawy *et al* (2011) reported increase in the number of intra tumor infiltration of$CD16^+$ NK cell and antibody specific for cell cytotoxicity after treatment with mushroom extract. On the other hand, Huang *et al* (2011) reported that Human hepatoma (Hep3B) cell-transplanted mice when administered the mushroom extract daily for 8 weeks, a significant reduction in tumor size and increase in T cell numbers; IL-12, IFNã and TNF-α secretion; NK cell activity and phagocytic ability were observed. The present explanation about the activity of extract to inhibit tumor growth through apoptosis and induction of DNA fragmentation of tumor cells come in line with Huang *et al* (2011). On the other hand, AL-Mosawy *et al* (2011) reported excessive tumor infiltrated monocytes and macrophages and neutrophils and presence of intra tumor necrotic aggregations surrounded by fibrous tissue as indication of tumor size reduction.

Antiangiogenic effect was another possible effect of extract, which leads to hypoxia and apoptosis of tumor cells, which come in accordance with Kunz and Ibrahim (2003) and Liu *et al* (2006) mentioned that gallic acid has antiangiogenic effect that prevent angiogenesis and thus prevent development of new blood vessels for tumor nutrition, which in turn leads to reduction of blood supply and starting of cell death due to ischemia. Similar finding was reported by Patel and Goyal (2012). Current results come in agreement with Liu *et al* (2006) reported that gallic acid have antiangiogenic on human Glioma cells.

CONCLUSION

Calvatia craniiformis gallic acid is one of the biological remides available and non-toxic and have antiangiogenic effect and immunomodulatory effects, causing tumor growth inhibition and this effect was dose dependent.

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