WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 5.990
Volume 4, Issue 7, 2122-2148. Research Article ISSN 2277– 7105

POTENTIAL ANTITUMOR AND ANTIOXIDANT EFFECT OF GARLIC (*ALLIUMSATIVUM*) OIL IN FEMALE MICE INJECTED WITH EHRLICH ASCITES CARCINOMA CELLS

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Article Received on
13 May 2015,

Revised on 08 June 2015,
Accepted on 01 July 2015

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ABSTRACT

Objectives: This study was undertaken to investigate the antitumor and antioxidant potential of garlic oil (GO)in vivo using Ehrlich Ascites Carcinoma (EAC) cells. **Methods:** Female Swiss albino mice were divided into 6 groups: G1: (Healthy control), G2:(Healthy GO) received oral dose of GO (100 mg/Kg/day), G3: (Tumor bearing mice TBM) mice were injected intramuscularly with (2.5 x 10^6 EAC/ml) to form solid tumors. G4:(GO Protection)mice were orally pretreated with GO(100 mg/Kg/day) for two weeks then injected with (2.5 x 10^6 EAC/ml), G5:(GO Treatment) mice were injected with (2.5 x 10^6 EAC/ml), then treated with GO(100 mg/Kg/day) for two weeks andG6: GO(Protection + Treatment)mice were pretreated with GO(100 mg/Kg/day) for two weeks, then injected with (2.5 x 10^6 EAC/ml) and continued to receive GO at the same dose. **Results:** The antitumor potential of GO was evident through the significant prolongation of lifespan of (TBM); up to (53.57%),regression in tumor growth rate(-36.38 %) and inhibition of histonedeacetylase enzymes (-12%). GO significantly elevated the depleted oxidative stress markers including (TAC), (GSH), (GST) and (CAT) and also significantly reduced the marked elevation of malondialdehyde level in TBM (-33.7%).Moreover, GO significantly improved liver function and hematological parameters in TBM. These results were confirmed by histopathological examination of the liver. **In conclusion**, GO administration significantly inhibited tumor growth and histonedeacetylation. Also, GO attenuated the severity of oxidative damage accompanying tumor development, all while exercising meyloprotectiveand hepatoprotectiveproperties.

KEYWORDS: Garlic oil, EAC, antioxidant, HDAC, epigenetic, tumor bearing mice.

INTRODUCTION

Cancer is a multifactorial heterogeneous disease. It is a major cause of morbidity and mortality worldwide.[1] Carcinogenesis is a multistep process involving three distinct stages namely: initiation, promotion, and progression. The dysregulated cellular evolution during carcinogenesis drives cells to acquire six phenotypic hallmarks of cancer; the ability to proliferate and replicate autonomously, resist cytostatic and apoptotic signals, induce tissue invasion, metastasis, and angiogenesis thereby initiating the transformation of a normal cell to a malignant phenotype.[2]

Alterations in tumor suppressor genes or oncogenes are not always due to mutations. They may also be due to transcriptional regulation by epigenetic mechanisms, including DNA methylation or demethylation and/or histone acetylation or deacetylation.The balance between histone acetylation and deacetylation, mediated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively, is usually well regulated, but the balance is often upset in diseases such as cancer.[3]

Targeting multiple molecular pathways that are prone to be deregulated during carcinogenesis is the major focus in cancer prevention and treatment.[2] Prevention is undeniably the sensible maneuver towards the ultimate goal of cancer control. Intervention with chemopreventive agents at the early stage in carcinogenesis is theoretically more rational than attempting to eradicate fully developed tumors with chemotherapeutic drugs. Most cancer chemotherapeutants severely affect the host's normal cells. Hence the use of natural products now has been contemplated as of exceptional value in the control of cancer and its eradication program.[4]

Garlic (*Allium sativum*) is a member of the Alliaceae family, which also includes onions, leeks, scallions or chives. Garlic is rich in sulfur-containing compounds, which contribute to its characteristic odor, taste and beneficial health effects. It has been used as a spice as well as a medicine since prehistoric times in various cultures.[5]

Garlic contains water-soluble and oil-soluble organosulfur compunds (OSCs). Oil-soluble OSCs such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide (DATS) and

ajoene are produced during the decomposition of allicin, which is released upon crushing garlic and other plants of the Alliaceae family.[6]

Human beings for several centuries are dependent on medicinal plants to cure various diseases. Developments in the area of nutrition during the last few decades have revealed their therapeutic benefits.[7] Garlic has proven beneficial health effects including antibacterial, anti-inflammatory, hypolipidemic, hypoglycemic, antifungal and anti-atherosclerotic properties. While these effects are well known, the exact mechanisms of action havenot yet been fully established.[8-9] This study was designed to investigate the antitumor and antioxidant potential of garlic oil in vivo using Ehrlich Ascites Carcinoma (EAC) bearing- mice.

MATERIAL AND METHODS

1. MATERIALS

1.1. Garlic Oil

Pure GO was purchased from the National Research Center Cairo, Egypt.

1.2. Tumor cell line

The murine Ehrlich Ascites Carcinoma (EAC) cells were kindly provided by the National Cancer Institute, Cairo University, Egypt.

1.3. Animals

Because Ehrlich Ascites Carcinoma cells were reported to show greater initial growth and total cell count in female mice than male mice,[10] the present study used female mice as experimental animals. One hundred and twenty healthy adult female Swiss albino mice weighing between 20-25g and were obtained from Oncology Unit, National Cancer Institute, Cairo University, Egypt. Mice were maintained on standard commercial pellets diet[11] and tap water *ad libitum*, and kept individually in stainless steel cages in constant environmental conditions.

1.4. Chemicals

- Trypan blue dye used to assess cell viability was purchased from El- Gomhouria Company, Cairo, Egypt.
- Kits used for HDAC assay were obtained from Biovision company, USA.
- Kits used for the determination of other biochemical measurements were obtained from Biodiagnostic company, Egypt.

2. METHODS

2.1.Determination of the bioactive components derived from garlic oil

GC/MS analysis was performed and the compounds were identified by comparing their peaks with the Wiley library.[12]

2.2. EAC cells preparation

EAC cells were maintained in vivo by weekly intra-peritoneal injection of($2.5x10^6$ cells/mouse)in a female Swiss albino mouse according to the method recommended by the Egyptian National Cancer Institute, Cairo University. This tumor is characterized by its moderate rapid growth which could not kill the animal due to the accumulation of ascites before about 14 days after transplantation. Cells were harvested, and their total number and viability were determined by direct counting using Trypan blue stain on a bright line hemocytometer. The desired concentration of tumor cells(2.5×10^6 cells per 0.2 mL) was obtained by dilution with physiological saline (0.9% NaCl).[13]Solid Ehrlich carcinoma was induced by inoculation of ($2.5x10^6$) cells in the right thigh of each animal.[14]

2.3.Animal trial

A total of 120 female Swiss albino mice were used in this study. Animals were randomly assigned to 6 groups as follows:

- **Group I:** (Healthy control):Mice in this group were orally administered with 0.2 ml vehicle oil (corn oil)daily for two weeks.
- **Group II:** (Healthy GO): Mice in this group were orally administered with GO (100 mg/kg body weight) dissolved in 0.2 ml corn oil for two weeks. [15]
- **Group III:** (TBM):Each mouse in this group was injected intramuscularly with (2.5×10^6 EAC/ml)in the right thigh to form a solid tumor.[16]
- **Group IV**: GO Protection (GO Pr) mice were orally pretreated with GO(100mg/Kg body weight/day)dissolved in 0.2 ml corn oil for two weeks, then were injected intramuscularly with ($2.5 x10^6$ EAC/ml) in the right thigh to form a solid tumor.
- **Group V:** GO Treatment (GO Tr): Mice were injected intramuscularly with($2.5 x10^6$ EAC/ml)then orally treated with GO(100 mg/Kg body weight /day)dissolved in 0.2 ml corn oil for two weeks.

- **Group VI**: GO (Pr+ Tr) Mice were pretreated with GO (100 mg/Kg body weight /day)dissolved in0.2 ml corn oil for two weeks then injected intramuscularly with(2.5 x10^6 EAC/ml)and continued to receive GO treatment at the same dose for two weeks.

2.4.Blood sample collection

At the end of experimental period10 mice from each group were scarified after 12 hours fasting with water *ad libitum*. Blood samples were collected in two tubes. The first one contained Ethylene Diamine Tetra Acetic acid (EDTA) for collecting blood immediately used for the determination of hematological measurements, the determination of reduced glutathione(GSH) concentration and Histone Deacetylases (HDACs) activity. In the second tube, blood was allowed to stand for 15 minutes at temperature of 37ºc, then was centrifuged at 4000 rpm for 20 min by EBA8 centrifuge (obtained from china) for the separation of serum. Serum was removed and kept in plastic vials at -20ºc until used for biochemical analyses.

2.5. Tissues Sampling

Liver and tumor were separated and cleaned, rinsed and washed by saline solution then blotted on filter paper to remove water residue. Solid tumors were weighed immediately. Part of the liver and tumor were stored frozen at -20ºc until used for tissue biochemical analyses. Another portion of the liver was kept in 10% formalin for histopathological examination. In non-tumor bearing mice (NTBM) groups, part of muscle tissue was homogenized and used for the different biochemical assays.

2.6.Tumor assessment

The effect of GO on tumor growth inhibition and host's survival time was examined by studying the following parameters:

2.6.1. Determination of Tumor volume

The size of solid tumor was measured using Vernier caliper to measure the two axes; the tumor volume was calculated using the following formula:[17]

Tumor volume(mm^3) = 0.52 (length × $width^2$)

Where length is the greatest longitudinal diameter and width is the greatest transverse diameter.

2.6.2. Measurement of Tumor growth response

Tumor growth inhibition ratio

(T/G %)was recorded using the following formula:[16] (T/G %)= (Mean tumor weight of TBM group - Mean tumor weight of treated group /Mean tumor weight of TBM group) x100.

2.6.3. Measurement of lifespan

Ten mice from each group were kept alive to measure the mean survival time. The percentage of increased life span (% ILS) was calculated using the following equation.[10]

ILS (%) = [(MST of treated group/MST of EAC group) -1]x 100

(MST) Mean Survival Time= $\frac{\text{(day of first death + day of last death)}}{2}$

2.7.Biochemical measurements

2.7.1. Assessment of Histone Deacetylases (HDACs) activity

The activity of HDACs was measured in blood and tissue samples, using a colorimetric assay kit (BioVision, kit number K331-100). [18] The procedure involves the use of the HDAC colorimetric substrate (Boc-Lys(Ac)-pNA), which comprises an acetylated lysine side chain and is incubated with a sample containing nuclear extract. Deactivation sensitizes the substrate, and treatment with the lysine developer produces a chromophore, which can be analyzed using a colorimetric plate reader.

2.7.2. Assessment of oxidative stress markers

Oxidative stress measurements included parameters in blood, serum, liver and in tumor tissue. Oxidative stress markers measured in blood included reduced glutathione (GSH) content.[19] While serum measurements included, total antioxidant capacity (TAC),[20] glutathione S-transferase (GST) activity[21] and catalase (CAT)enzyme activity.[22] Estimation of malondialdehyde (MDA) level as one of the main end products of lipid peroxidation by the thiobarbituric acid test.[23] However, Liver and tumor tissue homogenates' measurements included: (GSH) concentration,[19] (GST),[21] and (CAT),[22] enzyme activities and (MDA) level.[23]

2.7.3. Assessment of Liver function

Liver function tests included: aspartate aminotransferase (AST) and alanine aminotransferase (ALT)activities,[24] alkaline phosphatase (ALP) activity,[25] total protein(TP),[26] albumin, globulin and A/G ratio.[27]

2.7.4. Hematological Measurements

Complete blood count and measurements of blood indices were assessed immediately after mice were sacrificed.[28]

2.8.Histopathological Examination

Hepatic morphology was assessed by light microscopy. Part of the liver was sliced and tissue was fixed in 10% buffered-neutral formalin for 6 hours. Fixed liver tissue was processed and embedded in paraffin. Sections of 4 mm in thickness were subjected to Hematoxylin and Eosin (H&E) staining before examination.

2.9.Statistic alanalysis

Data were analyzed using one-way analysis of variance (ANOVA) using the Statistical Package for Social Science (SPSS) program, version 17.0 followed by Newman–Keuls post hoc test for multiple comparisons. Differences were considered significant at ($p < 0.05$) level.

3. RESULTS

3.1. Bioactive components derived from GO

On applying GC mass and recognizing the molecular ion peaks followed by comparing them with Wiley library we found that the final product contained major garlic oil essential components, including 35.1% DADS , 27.5% DATS, 17.5% DAS, and minor amounts of many other volatile compounds (fig. 1,2).

3.2.Effect of GO on tumor assessment

The incidence of solid tumors in G3, G4, G5 and G6 was 100%. The tumors were very prominent and fast growing in G3, while G4, G5 and G6 showed a relatively smaller and slower tumor growth compared to G3(fig.3).

As shown in table (1), regarding the tumor weight, GO pretreatment and/ or treatment caused a significant reduction, ***(P < 0.05)***.The reduction was (24.9 %), (37.5%) and (49.5%) for G4, G5 and G6respectively, when compared with G3. Pretreatment of mice with GO also resulted in a noticeable regression of tumor volume (– 31.2 %),***(P < 0.05)***. Moreover, marked and progressive tumor suppression in G5 and G6 was also recorded with a percentage of reduction in tumor volume, (– 40.4 %) and (– 46.3 %) respectively, when compared with the G3, ***(P < 0.05)***.

Similarly, results illustrated in table (2), show the significant inhibition of tumor growth (T/G %) in G4, G5 and G6. It was (12.52 %), (29.02 %) and (36.38 %) when compared to G3 in the same order. There was a significant increase in mean survival time (MST) and increased lifespan (% ILS) of GO treated TBM groups as compared with G3,***(P<0.05).***The administration of GO to mice in G4, G5 and G6caused a remarkable prolongation of MST; (31 days), (39 days) and (43 days) respectively, when compared with G3 (28 days).

3.3.Effect of GO administration on Histone Deacetylases (HDACs) activity

Table (3) show HDAC activity measured in the isolated peripheral blood mononuclear cell (PBMC) lysates as well as the nuclear extracts of the liver and tumor tissue homogenates. There was a significant increase in the activity of HDAC in G3 that reached (132.3±0.823µM/ µg protein) in PBMC, (158±1.155 µM/ µg protein) in liver and (175.3±1.059 µM/ µg protein) in tumor. Conversely, GO decreased the activity of HDAC and subsequently increased the acetylation of histone proteins. This was presented as a significant reduction in HDAC in G4 PBMC to become (130.7±1.251 µM/ µg protein), in liver (153±1.764 µM/ µg protein) and in tumor (171.2±1.476µM/ µg protein), ***P < 0.05***.G5 and G6exhibited a more pronounced significant reduction in the activity of HDAC in PBMC(121.1 ±1.449 µM/ µg protein), and (116.4±1.646 µM/ µg protein), in Liver (146±1.563 µM/ µg protein)and (143.2±1.476 µM/ µg protein)and in tumor (150.1±1.969µM/ µg protein) and (143±1.414 µM/ µg protein), ***P < 0.05.***

3.4.Effect of GO administration on oxidative markers

3.4.1. In blood

In this study, table (4) displays the oxidative stress markers. Regarding the TAC, oral administration of GO to healthy mice in G2 did not induce any significant change as compared to G1, ***P <0.05***. Nevertheless, there was a significant reduction (- 34.8 %) in serum TAC in TBM of G3 as compared to G1***p< 0.05***. On the other hand, when compared with G3, mice in G4, G5 and G6 had significantly elevated serum TAC (30.5%), (38.7%) and (46.7%), ***P<0.05*** respectively.G6 showed the most promising improvement as indicated by the rise of the level of TAC to near normal.

A significant elevation of free radicals in TBM groups was observed and evidenced by increased lipid peroxidation. The most remarkable result was the upsurge of MDA level in G3. The elevation in lipid peroxidation -exemplified by MDA content- as a result of tumor cell inoculation reached (5.8± 0.33 mmol/L). However, in groups 4, 5 and 6 the values were

significantly lowered by administration of GO and recorded (4.85 ± 0.5 mmol/L), (4.29 ± 0.5) and (3.84± 0.34 mmol/L) respectively, ***P < 0.05.***

The blood concentration of the non-enzymatic antioxidant GSH was also dramatically decreased in G3(- 53%) when compared with G1. But it increased in groups 4, 5, and 6 with levels rising by (16.6%), (46.9%), and (66.3%) respectively when compared with G3, ***P < 0.05.***

The serum activities of the enzymatic antioxidants CAT and GST were also distinctly decreased in G3 with levels falling by (- 47.8%) and (-56.9%) for CAT and GST respectively when compared with G1, ***P<0.05***. CAT activity in serum of groups 4, 5, and 6 was substantially elevated when compared withG3. The increments mounted up to (19.7%), (56%) and (68%) respectively,***P<0.05***. Compared to G3, GO considerably increased the activity of GST in serum of groups 4, 5, and 6 as well by (12.9 %), (42.5 %) and (63.8 %) respectively, ***P<0.05***.

3.4.2. In liver and tumor tissue homogenates

Table (5) and (6) illustrate oxidative stress markers measured in liver and tumor tissue homogenates, respectively. In liver tissue homogenate, GSH content was considerably decreased inG3 relative to G1 (-54.8%). GO administration resulted in a substantial elevation of GSH content in groups 4, 5 and 6 compared to G3by (46.6 %), (62.8%) and (73.5%), ***P<0.05***. likewise, in tumor tissue homogenate of G3, measured GSH content was significantly lowered by (-55.8%), ***p<0.05*** in comparison with G1. However, GO was able to promptly correct this demotion in G4 (17.1%), G5 (41.3%) and G6 (53.6%).

Regarding the MDA level in G3, we found that there was more than a double fold increment in its level in liver (5.234 ± 0.44 mmol/ g tissue) and more than a triple fold increase in its level in tumor tissue homogenates (6.044 ± 0.36). Predictably, hepatic MDA in groups receiving GO was significantly ***P<0.05***, lowered and recorded(4.335 ± 0.484 mmol/ g tissue),(3.658 ± 0.295 mmol/ g tissue) and (3.024 ± 0.148 mmol/ g tissue) for groups 4, 5 and 6 respectively, when compared with G3. Similarly, in tumor tissue homogenate, the MDA concentration has fallen to(4.75 ± 0.41 mmol/ g tissue), (4.73 ± 0.32 mmol/ g tissue) and (4.278 ± 0.28 mmol/ g tissue) in the same order, ***P < 0.05.***

As for CAT and GST activities measured in liver tissue homogenate, theywere found to be significantly lowered in G3 by (-54.3%) and (-36.6%) correspondingly, when compared with G1, ***p<0.05***.On the other hand, both enzymes' activities were improved in mice of groups 4, 5 and 6 when compared with G3. In G4, CAT but not GST was significantly elevated by (17.2%) and (2.4%),***p<0.05***. Whereas, in G5 and G6 CAT was expressively increased by (57.5%) and (77.9%) while GST by (12.2%) and (23.3%), when compared with G3***p<0.05***. In the same way, the activities of CAT and GST in tumor tissue homogenate mirrored that in liver tissue. The activities of both enzymes were elevated as a response to GO administration to TBM groups.

3.5.Effect of GO on Hematological Measurements

Table (7) demonstrates the effect of GO administration on hematological parameters in healthy and TBM groups. The mean values of all measured parameters in G2 did not significantly differ from G1, ***P > 0.05***. However, there were significant decreases in RBCs count (-17.5%) Hb concentration (-13.1%), HCT (-17.5%), MCV (-16.4%), MCH (-6.3%), MCHC (-14%)and lymphocytes % (-24.1%)in TBM ofG3 when compared with G1, ***P < 0.05.*** Conversely, mice in G3 exhibited a significant rise in total WBCs count (48.5%) when compared with G1, ***P < 0.05.***

Pretreatment of mice in G4 with GO resulted in a significant reduction in WBCs count (-12.1%) and elevation of Hb concentration (8.4%) when compared with G3,***P < 0.05.*** There was a noticeable improvement in other hematological parameters but it wasn't statistically significant at ***P < 0.05.*** On the other hand, GO administration to mice in G5 caused a significant improvement in RBCs count (10.9%), Hb concentration (12.5%), HCT (9.2%), MCV (8.4%), MCH (4.4%) and MCHC (12.5%). The decline in total WBCs count was also significant (-25.6%) while the increase in lymphocytes % did not reach a statistical significance at ***P < 0.05.*** In G6, GO was able to significantly raise RBCs count (14.6%), Hb concentration (16.3%), HCT (10.9 %), MCV (10.9 %), MCH (5.2%) and MCHC (15%), ***P<0.05***, in comparison to G3. Finally, GO administration restored the elevated total WBCs to near normal value.

3.6. Effect of GO administration on Liver function

Table (8) counts the effects of GO administration on the liver function of healthy and TBM groups. We found that GO administration in healthy GO group did not alter any values of the measured parameters significantly when compared to G1,***P < 0.05***. However, EAC injection

in G3induced a markedly significant elevation of the serum activities of the enzymes; AST (55.7%), ALT (118.4%) and ALP (38.2%) when compared with G1. A statistically significant reduction in the level of total protein (-14.9%) and albumin (-37.7%) were recorded as well, $P < 0.05$.

Garlic oil was able to alleviate some of these deleterious effects. When compared with G3, mice in G4 showed a significant reduction in the serum AST and ALT but not ALP activities by (-11.3%), (-13.1%) and (-2.5%), respectively, $P < 0.05$. In G5, GO led to a significant drop in AST (-14.4%), ALT(-20.5%) and ALP (-13.4%) activities. Also, G6 showed a considerable reduction in these enzymes' activities; (-18.2%), (-27.8%) and (-15.9%) for AST, ALT and ALP in that order.

Mice in groups 5 and 6 but not 4 exhibited a significant increase in total protein concentration by (9.9%), (14.1%) and (0.7%) respectively, when compared with G3, $p < 0.05$. The most favorable result followed the administration of GO in G6 as serum total protein level was brought back to near normal level. Albumin concentration was also improved due to GO administration to TBM. The elevation was significant in G5 (29.6 %) and G6 (41.2 %) but was not statistically significant in G4 (0.9 %) when compared with G3, $p < 0.05$.

Regarding the inflammatory protein globulin, there was a significant rise in its level in G3 (32.8 %) when compared with G1, thereby decreasing the A/G ratio between the two groups by (- 53.1%). A slight demotion of serum globulin and a promotion of the A/G ratio were observed in G4 in comparison with G3, however, neither was statistically significant, $p < 0.05$.

Alternatively, in G5 and G6 the decrement in the globulin concentration was (-9.34%) and (-12.46%) respectively, while the significant rise in the A/G ratio recorded (42.9 %) and (61.3%) when compared with G3, $p < 0.05$.

3.7.Histopathological Examination of liver

The effects of GO on liver of healthy and TBM was assessed by histopathological examination (fig. 4 A-F). Healthy control and healthy GO groups showed no detectable pathological changes. Yet, in liver of TBM group, high grade of metastasis was observed. Analysis confirmed that tumor inoculation induced hepatic necrosis and hepatocyte degeneration whereas GO pretreatment and/or treatment resulted in notable improvement.

Table (1): Effect of GO administration on tumor weight (g) and tumor volume (mm^3) in TBM groups

Parameters / Groups	Tumor weight (g)	Tumor Volume (mm^3)
Healthy Control	---	---
Healthy GO	---	---
TBM	$3.057 \pm^{a}$ 0.483	$4.065 \pm^{a}$ 0.686
GO (Pr)	$2.294 \pm^{b}$ 0.182	$2.795 \pm^{b}$ 0.686
GO (Tr)	$1.909 \pm^{c}$ 0.231	$2.423 \pm^{b,c}$ 0.588
GO (Pr) + (Tr)	$1.543 \pm^{d}$ 0.312	$2.183 \pm^{c}$ 0.548

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p < 0.05$) (Pr): Protection, (Tr): Treatment

Table (2): Effect of GO administration on Lifespan and Tumor growth inhibition in TBM groups

PTI (days)	Mortality			
	TBM	**GO (Pr)**	**GO (Tr)**	**GO (Pr) + (Tr)**
12	0/10	0/10	0/10	0/10
19	1/10	1/10	0/10	0/10
20	1/10	1/10	0/10	0/10
22	1/10	2/10	0/10	0/10
24	2/10	2/10	0/10	0/10
29	4/10	2/10	1/10	1/10
30	4/10	3/10	1/10	2/10
32	7/10	4/10	2/10	2/10
37	10/10	6/10	3/10	2/10
39		6/10	5/10	2/10
41		6/10	5/10	5/10
43		10/10	7/10	5/10
48			10/10	6/10
55				7/10
57				10/10
MTW	5.03	4.4	3.57	3.2
MST	28	31	39	43
ILS%		10.71 %	39.28 %	53.57%
T/G%		12.52 %	29.02 %	36.38 %

PTI: Post Tumor inoculation MTW: Mean Tumor Weight (g)
MST: Mean Survival Time (days) ILS%: Increase in life Span %
T/G %: Tumor growth inhibition

(Table 3): Effect of GO administration on histone deacetylase (HDAC) activity in peripheral mononuclear cells (PMNC), liver tissue and tumor tissue of healthy and TBM groups

Parameters / Groups	HDAC in PBMC µM/µg protein	HDAC in Liver tissue µM/ µg protein	HDAC in Tumor tissue µM /µg protein
Healthy control	111.5 [a]± 2.173	128.6[a]± 0.966	131.6 [a]± 1.35
Healthy GO	103.9 [b]± 1.286	129.2[a]± 1.549	128.5[b]± 1.509
TBM	132.3[c]± 0.823	158[b]± 1.155	175.3[c]± 1.059
GO (Pr)	130.7[d]± 1.251	153[c]± 1.764	171.2 [d]± 1.476
GO (Tr)	121.1[e]± 1.449	146[d]± 1.563	150.1 [e]± 1.969
GO (Pr) + (Tr)	116.4 [f]± 1.646	143.2 [e]± 1.476	143[f]± 1.414

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p < 0.05$)

(Table 4): The effect of GO administration on oxidative stress markers in blood of healthy and TBM groups

Parameters / Groups	Total antioxidant capacity in serum mM/L	Glutathione in plasma mg/dl	Catalase in Serum U/L	Malondialdehyde in Serum mmol/L	GST in serum U/L
Healthy control	1.26 [ae]± 0.021	154.97 [a]± 8.81	717.256 [a]± 11.29	2.81[a]± 0.403	75.6 [a]± 3.31
Healthy GO	1.278 [a]± 0.009	171.49 [b]± 2.89	792.677 [b]± 3.73	2.34 [b]± 0.188	89.84 [b]± 2.45
TBM	0.827 [b]± 0.077	72.79 [c]± 6.075	374.38 [c]± 9.29	5.8 [c]± 0.33	32.61 [c]± 6.24
GO (Pr)	1.079 [c]± 0.116302	84.84 [d]± 4.16	448.26 [d]± 15.63	4.85 [d]± 0.51	36.83 [d]± 4.67
GO (Tr)	1.14809 [d]± 0.060817	106.94 [e]± 8.023	584.103 [e]± 18.73	4.29 [e]± 0.5	46.47 [e]± 4.14
GO (Pr) + (Tr)	1.214 [e]± 0.018	121.02 [f]± 11.92	628.968 [f]± 17.74	3.84 [f]± 0.34	53.42 [f]± 6.07

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p < 0.05$)

(Table 5): The effect of GO administration on oxidative stress markers in liver tissue of healthy and TBM groups

Parameters / Groups	Glutathione in liver mg/g tissue	Catalase in liver U/g tissue	Malondialdehyde in liver mmol/ g tissue	GST in Liver U/g Tissue
Healthy control	178.46 ^{a}± 8.178	4.297 ^{a}± 0.224	1.639 ^{a}± 0.205	783.97 ^{a}± 17.46
Healthy GO	182.523 ^{a}± 2.48	4.541 ^{b}± 0.14	1.593 ^{a}± 0.261	796.39 ^{a}± 18.37
TBM	80.609 ^{b}± 2.99	1.963 ^{c}± 0.256	5.234 ^{b}± 0.44	496.882 ^{b}± 22.92
GO (Pr)	118.175 ^{c}± 13.43	2.301 ^{d}± 0.16	4.335 ^{c}± 0.484	508.972 ^{b}± 21.78
GO (Tr)	131.198 ^{d}± 12.68	3.093 ^{e}± 0.137	3.658 ^{d}± 0.295	557.416 ^{c}± 21.99
GO (Pr) + (Tr)	139.856 ^{d}± 12.7	3.493 ^{f}± 0.304	3.024 ^{e}± 0.148	612.828 ^{d}± 22.43

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p< 0.05$)

(Table 6): Effect of GO administration on oxidative stress markers in Tumor tissue of healthy and TBM groups

Parameters / Groups	Glutathione in Tumor mg/g tissue	Catalase in Tumor U/g tissue	Malondialdehyde in Tumor m mol/ g tissue	GST in Tumor U/g Tissue
*Healthy control	144.55 ^{a}± 6.09	4.398 ^{a}± 0.46	1.475 ^{a}± 0.18	258.02 ^{a}± 11.61
*Healthy GO	145.09 ^{a}± 6.92	4.373 ^{a}± 0.08	1.433 ^{a}± 0.23	247.34 ^{a}± 7.93
TBM	63.87 ^{b}± 5.96	1.329 ^{b}± 0.38	6.044 ^{b}± 0.36	153.25 ^{b}± 19.26
GO (Pr)	74.8 ^{c}± 2.55	1.904 ^{c}± 0.26	4.75 ^{c}± 0.41	163.87 ^{b,c}± 19.35
GO (Tr)	90.26 ^{d}± 9.48	2.424 ^{d}± 0.26	4.73 ^{c}± 0.32	167.63 ^{b,c}± 16.89
GO (Pr) + (Tr)	98.12 ^{d}± 18.3	2.928 ^{e}± 0.28	4.278 ^{d}± 0.28	170.12 ^{c}± 19.26

* in healthy mice groups, normal muscle tissue is used to be compared with tumor tissue in TBM groups
Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p< 0.05$)

(Table 7): Effect of GO administration on hematological parameters in healthy and TBM groups

Parameters / Groups	RBCs count ($x10^6$ /µL)	WBCs count ($x10^3$ /µL)	Hb concentration g/dl	HCT %	MCV (g/dl)	MCH (pg)	MCHC (g/dl)	Platelet count ($x10^3$ /µl)	lymphocytes %
Healthy control	4.626 ±[a] 0.114	8.93 ±[a] 0.567	11.81 ±[a,e] 0.409	45.10[a]± 1.105	51.66[a]± 1.563	14.88[a]± 0.373	26.37[a]± 0.679	709[a]± 19.465	32.75[a]± 4.611
Healthy GO	4.566 ±[a] 0.139	8.97 ±[a] 0.531	12.96 ±[b] 0.327	44.515[a]± 1.349	52.33[a]± 1.284	14.78[a]± 0.609	26.35[a]± 0.554	709.2[a]± 23.408	32.83[a]± 3.949
TBM	3.817 ±[b] 0.109	13.26 ±[b] 0.575	10.26 ±[c] 0.600	37.21[b]± 1.078	43.17[b]± 1.253	13.95[b]± 0.726101	22.68[b]± 1.323	708.3[a]± 28.990	24.87[b]± 2.845
GO (Pr)	3.861 ±[b] 0.236	11.65 ±[c] 0.494	11.12 ±[d] 0.439	37.65[b]± 2.299	43.67[b]± 2.682	14.46±[a,b,c] 0.476562	24.59[c]± 0.959	709.5[a]± 35.021	25.815[b,c]± 1.059
GO (Tr)	4.233 ±[c] 0.052	9.87 ±[d] 0.630	11.54 ±[a] 0.241	40.618[c]± 1.727	46.8[c]± 1.778	14.57[a,c]± 0.58319	25.51[d]± 0.504	709[a]± 19.759	26.705[b,c]± 4.951
GO (Pr) + (Tr)	4.373 ±[d] 0.084	9 ±[a] 0.469	11.93 ±[e] 0.305	41.265[c]± 0.516	47.88[c]± 0.592	14.67[a,c]± 0.66675	26.09[a,d]± 0.882	709.9[a]± 37.179	28.85[c]± 3.407

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p< 0.05$)

(Table 8): Effect of GO administration on liver function of healthy and TBM groups

Parameters / Groups	AST in serum U/L	ALT in serum U/L	ALP in serum U/L	Total protein in Serum (g/dL)	Albumin in Serum (g/dL)	Globulin (g/dL)	A/G ratio
Healthy control	16.20[a]± 0.53	31.71[a] ±1.38	94.35[a]± 4.49	6.98[a]± 0.42	4.72[a]± 0.29	2.255	2.09
Healthy GO	16.37[a]± 0.36	31.153[a]± 0.69	93.808[a]± 4.3	6.98[a]± 0.36	4.78[a]± 0.43	2.199	2.17
TBM	25.23[b]± 0.67	69.24[b]± 2.53	130.35[b]± 7.9	5.94[b]± 0.26	2.94[b]± 0.22	2.996	0.98
GO (Pr)	22.38[c]± 0.55	60.18[c]± 2.91	127.07[b]± 6.32	5.98[b]± 0.53	2.97[b]± 0.25	3.01	0.98
GO (Tr)	21.60[d]± 0.6	55.05[d]± 1.67	112.89[c]± 3.68	6.53[c]± 0.58	3.81[c]± 0.33	2.716	1.4
GO (Pr) + (Tr)	20.64[e]± 0.64	49.96[e]± 2.47	109.62[c]± 7.47	6.78[ac]± 0.27	4.15[d]± 0.35	2.622	1.58

Data is represented as mean ± SD.
There is no significant difference between means having the same letter in the same column ($p< 0.05$)

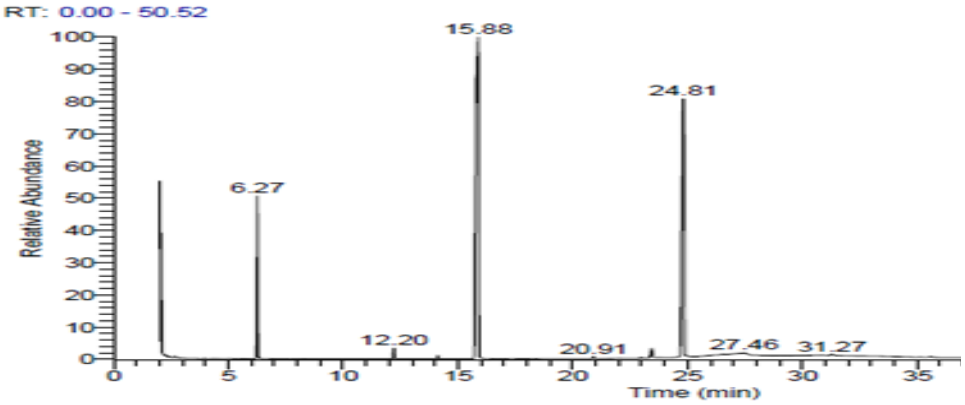

Fig. (1): GC –MS analysis of garlic oil showing retention time of main components.

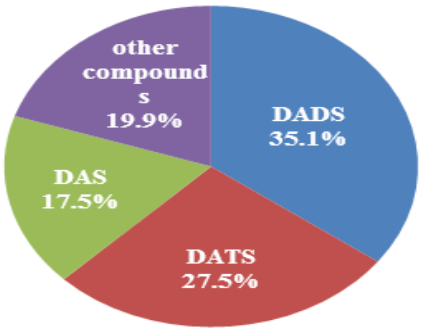

Fig. (2): Bioactive compounds derived from GO and given on GC-MS analysis

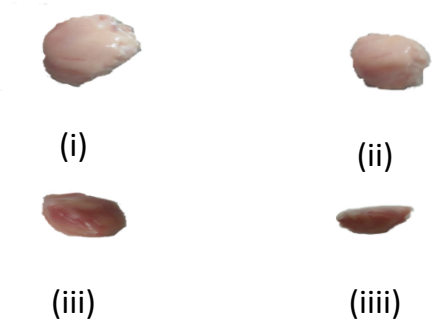

Fig. (3):
(i): Solid tumor excised from TBM.
(ii): Solid tumor excised from GO (Pr)
(iii): Solid tumor excised from GO (Tr)
(iiii): Solid tumor excised from GO (Pr) +(Tr)

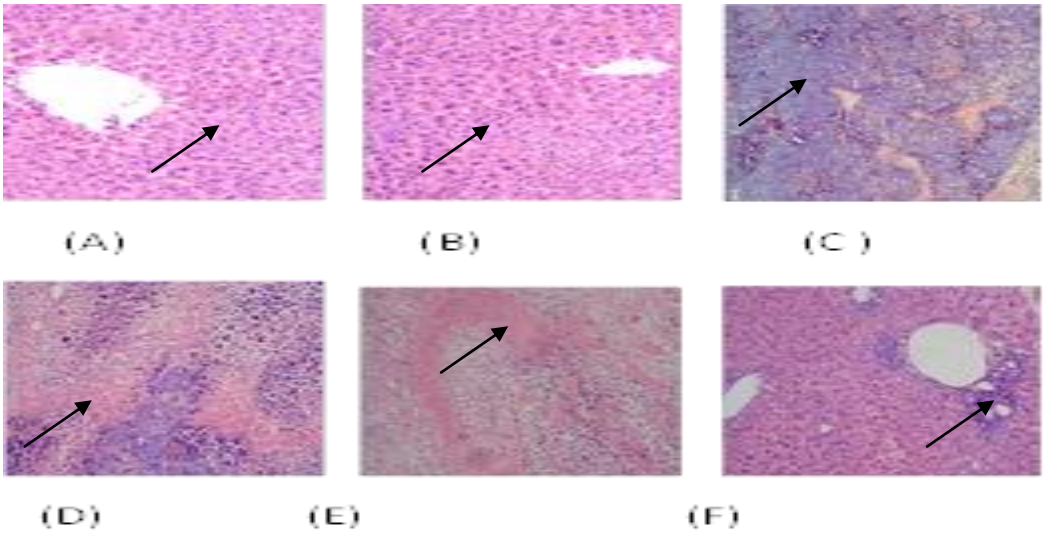

Fig. 4 (A-F): Liver sections of mice in experimental groups, stained with (H&E- x40)

(A): (Healthy control): normal liver tissue of healthy animals showing normal hepatocytes with single nuclei.

(B): (Healthy GO): normal liver tissue of healthy animals showing normal hepatocytes with single nuclei. (C): (TBM): high grade of metastasis: malignant darkly stained cells densely arranged in masses and invading hepatocytes.

(D): (GO Pr): relatively normal hepatocytes surrounded by scattered focal neoplastic cells.

(E): (GO Tr): No metastasis, with marked tissue repair and minimal area of necrosis. Apoptotic cells are observed.

(F): (GO Pr +Tr):No metastasis or apoptotic cells with only inflammation observed near portal vein surrounded by normal hepatocytes.

4. DISCUSSION

4.1. Bioactive components derived from GO

Garlic oil contains many bioactive components with varying amounts. The concentrations of each of them depend on the extraction and distillation techniques. DADS is essentially the main components found in most oil preparations followed by DATS and other allyl compounds. Our results of GC MS analysis are close to many studies found in literature.[29]

4.2.Antitumor effect of GO

GO's antitumor effect was possibly achieved through retarding the rate of tumor growth or via the induction of cancer cell apoptosis. The present findings are in line with the main concept of cancer research that evaluation of any tested substance as an antitumor agent, depends on extension of the survival time of cancer patients. The prolongation in the lifespan of TBM by GO can be considered indicative of its antitumor potential. These observations are consistent with the previous findings.[30]

It merits note that a more pronounced effect was observed in (G6) represented by an increased prolongation in the lifespan of mice and a higher tumor inhibition ratio compared to mice in (G4) and (G5).

4.3.Inhibitory effect of GO on Histone Deacetylases activity

Various studies in cancer cell lines and tumor tissue revealed changes in the acetylation levels and the expression of the HDAC enzymes. [31] HDACs regulate the expression and activity of numerous proteins involved in both cancer initiation and progression. Several diseases, especially cancer, are caused by aberrant epigenetic alterations in addition to genetic mutations. Chromatin remodeling by histone acetylation and/or deacetylation is an example of epigenetic regulation.[32]

HDACs bind to and deacetylate a variety of cellular proteins including transcription factors implicated in control of cell growth, differentiation and apoptosis. The acetylation of histones by histone acetyltransferases (HAT) changes their charge from positive to negative, which reduces their interaction with negatively-charged DNA. This increases accessibility for the transcriptional machinery, resulting in transcriptional activation. This series of the events can be reversed by deacetylation by HDACs. Epigenetic changes caused by imbalances between HATs and HDACs resulting in hyper acetylation of core histone.[32]

In recent years, a lot of effort has been put into the development of HDAC inhibitors and on the development of small molecule enzyme inhibitors like DNA-methyltransferase inhibitors or inhibitors of histone modifying enzymes. These may reverse misregulated epigenetic states and be implemented in the treatment of cancer or other diseases, e.g., neurological disorders. Today, several epigenetic drugs are already approved by the FDA and the EMEA for cancer treatment and around ten histone deacetylase (HDAC) inhibitors are in clinical development.[3]

It was suggested that GO inhibits the proliferation of malignant tumor cells by a mechanism tightly coupled with HDAC. Induction of histone acetylation was reported previously in cancer cells treated with the garlic compounds diallyl disulfide and S-allyl mercapto cysteine.[33,34]

The effects of DADS and one of its metabolites, allylmercaptan (AM) was explored on HDAC activity: using nuclear extracts of Caco-2 cells. 200 **µM** DADS decreased HDAC activity by 29% and AM at the same concentration was more efficient (92% inhibition).[35]

DADS induced differentiation and inhibited the growth of HL-60 cells through increasing the expression of acetylated histone H3 and H4in vitro and in vivo.[36]Several garlic-derived OSCs were screened for their ability to inhibit HDAC activity in vitro, it was found that AM was the most potent HDAC inhibitor in a dose-dependent response.[37]

4.4. Antioxidant effect of GO

Reactive oxygen species (ROS) play an important role, in both health and disease. They are extremely reactive and unstable molecules that can damage cell membrane lipids, proteins, and DNA and cause oxidative injury. Several studies had shown that free radicals could cause extensive chemical modifications and alterations in DNA and nucleoproteins, including modified bases and sugars and even DNA strand breaks and conformational changes in proteins. Nevertheless the reinforcement of endogenous antioxidant may be particularly important when free radical generation is enhanced.

The decrease in the TAC in TBM by the excessive production of free radicals which require detoxification by the endogenous antioxidants caused their cellular stores to be depleted. In this study, low levels of antioxidants in the liver and blood of TBM may be ascribed to increased use to scavenge lipid peroxides and to prevent the accumulation of superoxide anions that are capable of traversing membranes causing deleterious effects at sites beyond the tumor.

The present data revealed marked depletion in GSH content as well as the activity of the antioxidant scavenger enzymes, GST and CAT in the liver tissues of TBM. GSH, a potent inhibitor of the neoplastic process, plays an important role as an endogenous antioxidant system. It is found in particularly high concentration in the liver and is known to have a key function in the protective process against damage by free radicals.[38]

GSH is a cofactor for several antioxidant enzymes and thus can protect cells against oxidative stress by conjugation of toxic metabolic intermediate representing a detoxification reaction. Therefore, cells or animals become more sensitive to toxic intermediates after treatment with

a GSH depleting agent. The antioxidant effect of GO may be associated with possibly the increase in GSH content.[39]

Hepatic glutathione level was drastically lowered in EAC bearing mice vs. healthy animals. It significantly rose after DADS treatment. DADS showed a selective beneficial effect on normal liver cells, where it nullified the changes induced by carcinogenesis.[40]

It was reported that during cancer growth, glutathione redox (GSH/GSSG) decreases in the blood of Ehrlich ascites tumor–bearing mice, mainly due to an increase in blood GSSG levels as a result of oxidative stress. This increase may be caused by an increase in peroxide production by tumor cells that can lead to GSH oxidation within the red blood cells and increased GSSG release from different tissues into the blood. After GSH has been oxidized to GSSG, the recycling of GSSG to GSH is accomplished mainly by Glutathione reductase using NADPH as its source of electrons. GSH is present in high concentration in the cell to protect it from free radical attack. Early studies hypothesized that the enzyme inactivating action of ROS or lipid peroxides can overcome enzyme synthesis capacity. [41] Significant decreases in the levels of GSH and inhibition of antioxidant enzymes activities as a result of tumor growth in EAC bearing mice were also previously reported.[42-45]

It is clear that dietary intake of naturally occurring antioxidants may be the most sensible means to develop prevention strategies for biochemical alterations and diseases risk factors associated with free radicals formation. GO has different protective pathways explained in endogenous antioxidant system including scavenging ROS, protection of endothelial cell integrity by inhibition of lipid peroxidation induced injury, enhancement of GSH level, improving cellular scavenging enzymes such as CAT.[46]

The hepatoprotective effect of GO can also be explained partly by the inhibition of cytochrome P450 gene (CYP2E1) and the induction of GST by its content of OSCs. GSTs are detoxification enzymes, which have been recently considered as either phase I or phase II enzymes that catalyze the conjugation of a wide variety of electrophile agents and carcinogens with GSH. This reaction is the first step in the formation of mercapturic acids, a pathway resulting mostly in the elimination of potentially toxic compounds. GSTs are also involved in the metabolism of several types of anticancer drugs are overexpressed in many human persistent tumors. Chemopreventive effects of garlic constituents are associated with

increased levels of GSH and with both significant increase of GST activity in rats treated with DADS.[43]

DADS and DAS selectively inhibited CYP2E1-mediated metabolic activities. Both had suppressive activities of CYP2E1 and potent inhibitory effects on the induction of colon and liver cancer induced by chemical carcinogen.[39]

An in vivo study showed that GO, DADS, DATS but not DAS significantly increased the activities of hepatic GST and GSH reductase. Hepatic GSH levels in rats administered GO, DAS, DADS and DATS were not different from that in the control group ($p< 0.05$) however, there was an increase in their levels in blood in the order DATS (84%) > GO (50%) > DADS (47%) > DAS (2%).[47]

SOD, CAT, and GPx are involved in the clearance of superoxide and hydrogen peroxide and thus protecting the cells against the resulting lipid peroxidation. SOD catalyzes the diminution of superoxide into H_2O_2, which has to be eliminated by GPx and/or CAT which is widely distributed in all tissues. However, when the oxidative damage is extreme as a result of tumor growth, ROS scavenging enzymes such as SOD and CAT are degraded. The inhibition of CAT activity in different tissues of mice-bearing Ehrlich tumor as a result of tumor growth was also reported by others.[36]However, the results of this study demonstrate the ability of garlic to nullify all of these deleterious effects. This is confirmed by several studies.[46, 48-49]

4.5.Effect of GO on Hematological Measurements

The early effects of EAC on T lymphocytes of the host account for a dramatic decrease in the number of T helper (Th) cells in TBM. [50] A significant increase in lymphocytes was observed in garlic treated group which indicates its immune stimulating effect.[51]

Myelo suppression and anemia are the most common problems encountered in cancer and are augmented by most modes of therapy. In experimental animals, anemia occurs mainly due to the reduction in RBC or hemoglobin production, and this may occur either due to iron deficiency or due to hemolytic or other myelopathic conditions. Treatment with GO brought back the hemoglobin content, and RBC and WBC counts to near normal. These results clearly demonstrate the protective effect of GO on the hemopoietic system. These results are confirmed by several studies.[52-54]

4.6. Effect of GO administration on Liver function

The elevation in liver enzymes could potentially be attributed to the release of these enzymes from the cytoplasm into the blood circulation after rupture of the plasma membrane and cellular damage. Serum AST, ALT and ALP levels have been considered as biomarkers in the diagnosis of hepatic damage. Nevertheless, the reversal of these parameters -as seen in groups that received GO - alludes to the possibility of restoring the normal functional status of the liver. Garlic prevented liver damage by maintaining the integrity of the plasma membrane, thereby suppressing the leakage of enzymes.

The resulted increase in serum total protein following GO administration is possibly due to the prevention of protein oxidation. Its ability to reduce free radical-induced oxidative damage in the liver is suggested as the cause of its hepatoprotective properties. Serum protein is a fairly labile biochemical system, precisely reflecting the condition of the organism and the changes happening to it under the influence of internal and external factors. These results are in agreement with previous research.[55]

The amount of albumin and globulin and their ratio paints a picture of liver's activities. Since albumin synthesis decreases in cases of liver malfunctions with a usual decline in the A/G ratio, an increase in albumin level in mice receiving GO and the rise of the A/G ratio in these groups signify that it affects the induction of the liver activity.[56]

In a previous study, AST, ALT and ALP in the EAC control group were significantly increased as compared to the normal group ***P<0.01***. The total protein content was found to be significantly declined in the EAC control group when compared with the normal group ***P<0.05***. [44]

In another study, treatment of animals with (100 mg/Kg/day) GO prior to chemically inducing hepato toxicity resulted in a significant reduction in the serum AST, ALT and ALP activities by 33%, 62% and 49% respectively.[48]

4.7.Histopathological Examination of liver

Mice pretreated with GO exhibited marked improvements in liver histopathology and a reduced extent of liver injuries. The combination of pretreatment and treatment with GO indeed prevented hepato cellular injuries potentiated by tumor inoculation. It is noteworthy that tumor induced necrosis and hepatocyte degeneration were both almost completely blocked by GO administration. Histopathological observations were in correlation with

biochemical measurements carried out in our study that further support the antitumor effect of GO.

In a recent study, garlic minimized the histopathologicaland electron microscopic alterations in EAC bearing mice. They concluded that this may be due to an immune-stimulatory effect, reduced lipid peroxidation processes, and/or enhancement of anti-oxidant action by garlic.

CONCLUSION

GO has been found to contain a large number of potent bioactive components with antitumor properties, largely allyl sulfide derivatives. GO influenced a number of molecular mechanisms in carcinogenesis including: retardation of tumor growth rate through scavenging of free radicals and modification of histone acetylation. In addition to inhibiting the primary tumor, GO further inhibited the metastasis in liver. Besides, the antitumor effect of GO was more pronounced when used in a combination as a chemo preventive and a chemotherapeutic agent rather than a single approach. GO corrected the abnormalities in liver function and the hematological parameters.

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