

Immunomodulatory and Chemo preventive activity of *Bacillus subtilis* sulphated Levan

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ABSTRACT: **Aim:** To investigate the possible immunomodulatory and chemopreventive effects of a bacterial polysaccharide drug (*Bacillus subtilis* sulphated Levan; BSL) for prevention of tumor development through antipromotion and antiprogession effects in vivo studies. The antipromoting effect of BSL, was assessed by estimating different aspects of such activities ;tumor necrosis factor- α (TNF- α) level, apoptotic and necrotic cell damage, DNA fragmentation, nitrous oxide (NO) and COX-2 (Cyclooxygenase-2) levels. Antiprogession mechanism was evaluated through recording of vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and histopathological examination. In addition, BSL was also compared with garlic which is known as a natural compound with chemopreventive action. **Results** No significantly elevation in the NO or TNF- α levels were recorded when compared with control on receiving BSL, while, garlic possessed lower significant inhibitory effect. Apoptosis and necrosis states were induced as a mode of cell death. It was concluded that BSL was a potent anti-inflammatory and anti-apoptotic agent. Furthermore, a significant DNA fragmentation inhibition (insignificant lesser extent and decrease in VEGF level was determined by receiving BSL and garlic. Also, histopathological examinations showed that, BSL maintained nearly normal liver architecture and inhibited hepatocellular preneoplas induced by the cancer promoting material used in the experiment (diethylnitrosoamine) (DEN). **Conclusion**, BSL activity as anti-tumor agent due to its antipromotion and antipropagation actions was proved.

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Key words: BSL, histopathological examination, antipromotion, antiprogession

Introduction

Cancer is a serious problem in Egypt. In Egypt, HCC was reported to account for about 4.7% of chronic liver disease (CLD) patients (Rahman *et al.*, 2001). The epidemiology of HCC (the most common type of liver cancer) is characterized by marked demographic and geographic variations. A number of different studies suggested that immunotherapeutical approaches will be successful for the treatment of this disease (Greten *et al.*, 1999; Butterfield, 2004). Carcinogenesis can be viewed as a process that involved accelerated, and abnormal, cellular changes in which the genes controlling proliferation, differentiation, and apoptosis are transformed under selective environmental pressures (Bertram, 2000). Genetic damage, from accumulated carcinogenic exposure, becomes evident during neoplastic transformation. Specific genes have been discovered that, when altered, may play a role in epithelial carcinogenesis. These include both tumor suppressor genes and proto-oncogenes, which encode proteins that are involved in cell cycle control, signal transduction, and transcriptional regulation. These affect different stages of carcinogenesis including initiation, promotion, and progression (Berenblum and Armuth, 1981; Heidelberger *et al.*, 1983 and

Soria *et al.*, 2003). The initiation phase is a rapid (within hours or days), irreversible event that occurs. Promotion phase is a protracted process that may require several years or decades to be established, this consists of the expansion of mutated cells to form an actively proliferating, multicellular premalignant lesion. During the progression phase, another irreversible event occurs over a relatively short period, perhaps less than 1 year, in which new clones with increased proliferative capacity, invasiveness, and metastatic potential are produced (Surh, 1999). Due to the fact that, the initiation and progression phases are irreversible and relatively transient events, the promotion phase of carcinogenesis may provide the best target for cancer prevention (Umar *et al.*, 2001).

Chemoprevention, by definition, is the use of agents to slow the progression of irreversible events or inhibit carcinogenesis, thereby lowering the risk of developing invasive or clinically significant disease (Hong and Sporn, 1997; Kelloff *et al.*, 2001). Consequently, an effective chemopreventive agent should intervene early in the process of carcinogenesis to eliminate premalignant cells before they become malignant (Smith *et al.*, 1995;

Wattenberg, 1995; Hong and Sporn, 1997; Kelloff *et al.*, 1999a).

B. subtilis produces and secretes large amounts of various proteins into the culture medium (Priest 1977). For the last two decades numerous attempts have been made to use this bacterium as an efficient host for the expression of proteins (Simonen and Palva, 1993). Levans are natural polymers of the sugar fructose found in many plants and microbial products. Levan is also produced by *B. subtilis* (natto), which is used to make fermented soybeans (Meng *et al.*, 2003). Commercial interest in the production of levan has intensified in recent years (Vina *et al.*, 1998). Levan has some potential pharmaceutical applications owing to its anticarcinogenic and hypocholesterolemic properties (Kim *et al.*, 1998). Levan has a number of effects on the immunologic system, including tumor suppression and enhancement of leukocyte antitumor activity (Pileggi and Khin, 1962).

In this study, we aimed to evaluate the potential of in vivo anti-cancer and immunomodulatory properties of purified bacterial polysaccharides (*Bacillus subtilis* sulphated levan).

2. MATERIALS AND METHODS:

2.1. Materials

2.1.1. Levan Sedimentation from Culture Liquid

Levan was isolated from culture filtrate of *Bacillus subtilis* after the stage of fermentation using sedimentation by ethanol. Ethanol (96%) was added to the culture filtrate in a ratio of 2 :1 and mixed for 24 hr at room temperature. Sediment was then separated from the culture filtrate by decantation and used for investigations as levan.

The production of levan was indicated after acid hydrolysis by chromatography (Tanaka *et al.*, 1978). The chromatography was sprayed with aniline phthalate (Block *et al.*, 1955). Sulphation of levan was carried out with chlorosulfonic acid (Hussein, 1994).

2.1.2. Garlic

Garlic was purchased from (Tomex, ATOS pharma, Cairo, Egypt) as tablets (200 mg) and administered, as a suspension in physiological saline, orally to the mice in a dose of 250 mg/kg.

2.1.3. Diethylnitrosoamine (DEN)

Carcinogenicity in the mice was induced by a single dose of intraperitoneal (i.p.) injection of DEN (200 mg/kg) and promoted by adding 0.05 % sodium phenobarbital to drinking water.

2.2. Methods

2.2.1. Experimental Design

This study was carried out on a total number of 200 adult male Swiss albino mice with average weight of 25-30 g obtained from the Laboratory Animal House

of NRC, Dokki, Egypt. The animals were acclimatized to the laboratory conditions with a lighting schedule of 12 hr light, temperature 24±1°C, and relative humidity 55±5% and were housed in Tarson Cages (8-10 mice per cage) for 1 week before the commencement of the experiment.

Mice were divided into seven groups (16 animals in each):

***Control gp.:** Mice that are normal healthy and untreated gp.

***Saline gp.:** Mice that orally received saline twice a week for 6 wk.

***BSL gp.:** Mice that received 80mg/kg body wt. BSL i.p. twice a week for 6 wk.

***Garlic gp.:** Mice that received 250mg/kg body wt. orally twice a week for 6 wk.

***DEN gp.:** Mice that received 200mg/kg body wt. i.p. once, in addition, 0.05% sodium Phenobarbital was added to the drinking water after 2 wk of DEN injection.

***BSL+DEN gp.:** Mice that received 80mg/kg body wt. BSL i.p. 24 hr before and after DEN injection followed by 80mg/kg body wt. BSL i.p. twice a week for six weeks.

***Garlic+DEN gp.:** Mice that received 250mg/kg body wt. garlic 24 hr before and after DEN injection followed by 250mg/kg body wt. garlic orally twice a week for six weeks.

On the 6th week, blood samples were collected. The animals were sacrificed; liver tissues were excised and rinsed in cold PBS to remove excess blood. One portion was preserved in 10% buffered formalin then embedded in paraffin for immunohistochemistry and pathological investigation. The other portion was used for the preparation of 20% (w/v) liver homogenate by homogenization in ice Ripa buffer, centrifuged using cooling centrifuge at 1000 xg for 10 min at 4°C. The supernatant was stored at -80°C for later assessment of MDA (Malondialdehyde), HDAC (Histon Deacetylase) and GST (glutathione-S-transferase) activity as well as GSH level.

2.2.2. Measurement of LD₅₀

The LD₅₀ of BSL was determined following Behrens and Karber (1935). Two Groups (8 in each) were used, the first group (control) received sterile saline i.p. The other group received BSL in a dose of 1g/kg body weight. Mortalities were recorded within the first 24 hr following the administration of BSL.

The LD₅₀ was determined according to the following formula:

$$LD_{50} = D_m - \sum (z \times d)/n$$

Where:

D_m = highest dose which kill all animals in the group

Z = the mean number of dead animals in two successive groups

D = the constant factor between two successive groups

n = the number of animals in each of the dose levels

2.2.3. Assays for estimation of BSL anti-promoting mechanisms and anti-inflammatory activities

a.. Estimation of nitric oxide level

Nitrite accumulation was used as an indicator of NO production in blood using an assay based on the Griess reaction (Gerhäuser *et al.*, 2003).

b. COX-2 immunohistochemistry

COX-2 was detected immunohistochemically by using polyclonal rabbit anti-COX antibody and polyclonal rabbit anti goat Ig/HRP. Fluorescent images were visualized using a fluorescent microscope (AxioStar plus, Zeiss, Goettingen, Germany) equipped with digital camera (PowerShot A20, Canon, USA).

c. Determination of TNF- α

The level of TNF- α was determined by using an ELISA using a monoclonal antibody to mice TNF- α . The TNF- α level in the animal's serum was determined using the standard curve equation

d. Apoptosis /necrosis stain

The type of the cell death was investigated using acridine orange/ethidium bromide staining (Kinner and Ma (2002)

2.2.4. Assays for estimation of BSL anti-progression Mechanisms

a. Determination of serum total protein

The determination of serum total protein was performed according to Dumas (1975), using kits of Bio-Analytix.

b. Quantitative DNA fragmentation analysis

This method is based on the notion that extensively fragmented double-stranded DNA. The protocol includes the lysis of cells and the release of nuclear DNA, a centrifugation step with the generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA, hydrolysis and colorimetric quantitation upon staining with DPA, which binds to deoxyribose (McConkey *et al.*, 1989).

Calculations

The percentage of fragmented DNA calculated using the formula:

$$\% \text{ Fragmented DNA} = (S+T)/(S+T+B) \times 100$$

Where,

S , T and B are the OD at 600nm of fragmented DNA in the S , T (fragmented) and B fractions, respectively.

The fragmented DNA released by cells undergoing apoptosis and lysis during the experiment was

recovered in the fraction S should therefore be taken in consideration in particular circumstances.

c. Determination of VEGF

The level of VEGF was determined by using ELISA technique as previously described in TNF- α experiment using monoclonal antibody to mice VEGF.

d. Determination of PDGF

Estimation of PDGF was performed using ELISA technique as explained in experiment of TNF- α level determination by using monoclonal antibody to mice PDGF, (Banchroft *et al.*, 1996).

2.2.4. Statistics Analysis

Results were expressed as mean \pm S.E. from $n = 6-8$. Statistical analysis was performed by Student's t-test using instate soft ware (Version 3.05). Statistical significance was accepted at the level of $P < 0.05$.

3. RESULTS

3.1. Statistical results of assays for estimation of BSL Anti-promoting mechanisms and anti-inflammatory Activities:

In the evaluation of NO level in mice serum the results were normalized to the total protein content in the serum using total protein assay as indicated in methods. For the determination of the total protein, a standard curve was plotted (Fig. 1) to be used in the calculation of the serum total protein.

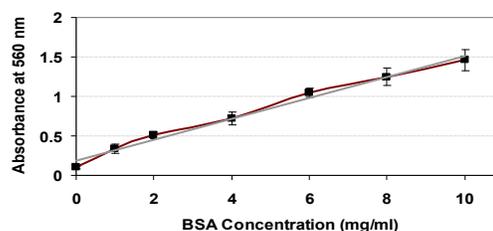


Figure 1: Standard curve for total protein (mg/ml) using BSA.

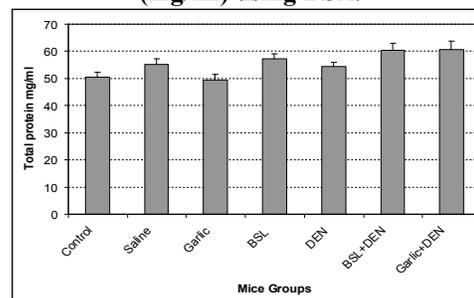


Figure 2: The determination of total protein concentration (mg/ml) in all animal groups. Results were expressed as Mean \pm S.E.

3.1.1. Estimation of nitric oxide level

NO is a highly diffusive hydrophobic molecule and is therefore a key signaling molecule in inflammation-driven diseases, including cancer. A standard curve of sodium nitrite was plotted in each experiment to calculate the nitrite content in the mice serum (Fig. 3).

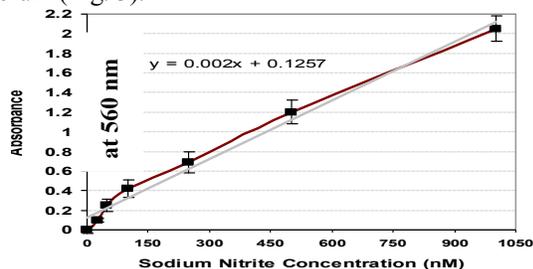


Figure 3: Standard curve for sodium nitrite using Griess assay.

The effect of BSL on NO level was performed through determination of the nitrite level in mice serum.

The results showed that NO level in control group and mice received saline, garlic, BSL, DEN, BSL+DEN and garlic+DEN were 21.5, 24.3, 23.1, 24.25, 28.8, 25.9 and 26.5 nmol nitrite/mg protein, respectively. These results demonstrated NO level in control and saline groups were not significantly altered by administration of garlic or BSL (Fig. 4).

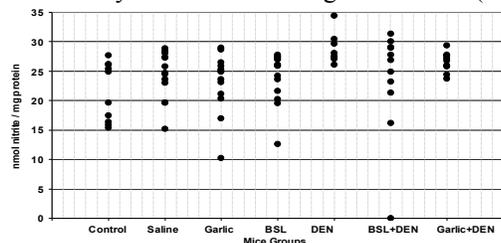


Figure 4: Determination of the nitrite level as an index of NO in mice serum (nmol nitrite/mg protein).

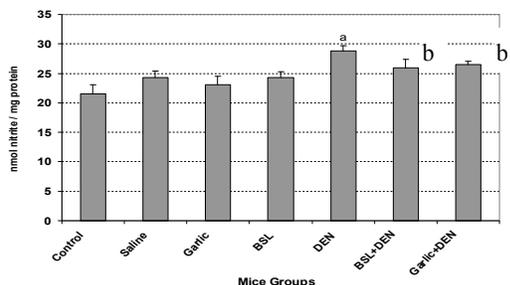


Figure 5: Determination of the nitrite level as an index of NO in mice serum. Results were expressed as Mean ± S.E.

a: Significantly different from control gp at ($P < 0.05$).

b: Significantly different from DEN gp at ($P < 0.05$).

Administration of DEN led to significant ($P < 0.05$) 1.3 times elevation in the NO when compared with control. Such elevation was not significantly affected by receiving BSL or garlic (Fig. 5).

3.1.2. Immunochemical evaluation of the promotion marker COX-2

Inhibition of COX-2 is recognized as one of the most feasible strategies for cancer chemoprevention and treatment, so evaluation of COX-2 expression in the animal liver immunohistochemically was performed. Immunohistochemical staining for COX-2 expression in the mice livers showed that, there was no COX-2 detection in control, saline, BSL and garlic treated groups where, aggressive COX-2 detection observed in DEN group. This very high COX-2 expression was not affected by BSL or garlic treatments in the mice that received DEN then treated with BSL or garlic (Fig. 6).

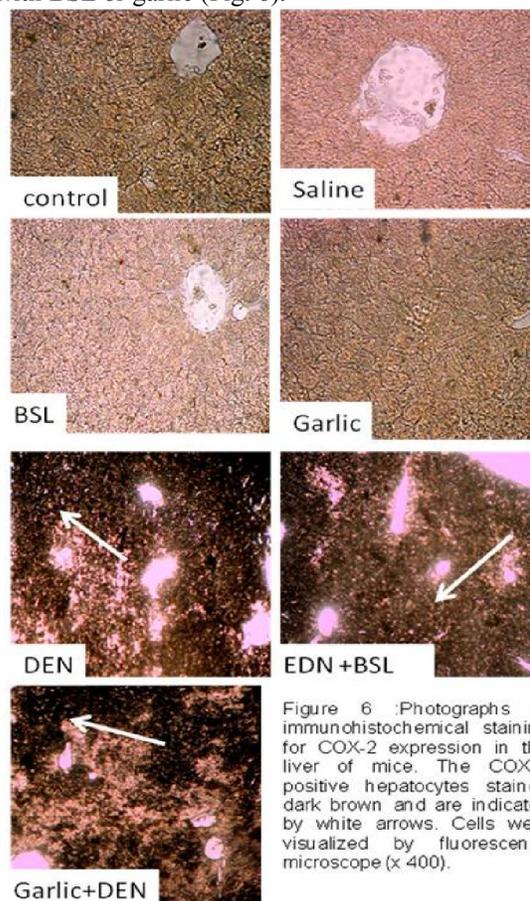


Figure 6: Photographs of immunohistochemical staining for COX-2 expression in the liver of mice. The COX-2 positive hepatocytes stained dark brown and are indicated by white arrows. Cells were visualized by fluorescence microscope (x 400).

1.3. Effect of BSL on TNF- α

The effect of BSL on TNF- α reflect its pro-inflammatory activity which play a dangerous role in cancer promotion. ELISA reader measured the level of TNF- α in all groups' sera as 580.5, 611, 1031.5,

698.8, 1611.3, 430.7 and 1002.1ng/ml in control, saline, garlic, BSL, DEN, BSL+DEN and garlic+DEN groups, respectively.

TNF- α level was significantly ($P<0.05$) elevated in garlic group to 1.7 times, whereas treatment with BSL did not significantly alter this value (Fig. 7). Administration of DEN significantly elevated TNF- α level to 2.8 times compared with control. This elevation was significantly ($P<0.05$) decreased by administration of BSL. Meanwhile, garlic induced a lower significant inhibitory effect on TNF- α level (Fig. 7).

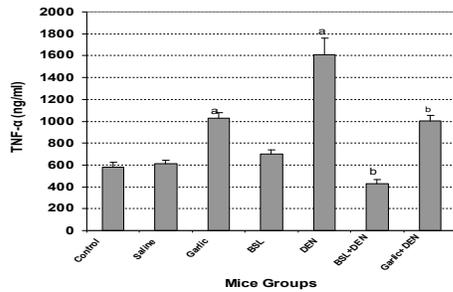


Figure 7 Effect of BSL on mice serum TNF- α level (ng/ml) Results were expressed as Mean \pm S.E.

a: Significantly different from control gp at ($P<0.05$).
b: Significantly different from DEN gp at ($P<0.05$).

1. 4. Apoptosis and necrosis staining

To investigate whether the liver cells of BSL treated mice underwent apoptosis or not, we studied the type of cell death using acridine orange/ethidium bromide staining to distinguish between apoptotic, necrotic, and viable cells. This experiment resulted in absence of apoptotic and necrotic cells in control, saline and garlic groups where, apoptotic cells appeared in slightly small number in BSL and DEN groups. The highly aggressive appearance of the apoptotic cells occurred in BSL+DEN group which showed complete absence of the necrotic cells. In the opposite side garlic+DEN treated group showed high presence of necrotic cells and no apoptotic cells appeared. The results indicate that hepatocytes of BSL treated mice had mainly undergone apoptosis with condensed chromatin and not necrosis and this demonstrates the apoptotic property of BSL and that garlic increases necrosis rather than apoptosis (Fig. 8).

2.2. Assays for estimation of BSL anti-progression mechanisms

Investigation of the possible anti-progression activity of the BSL was performed using a series of tests to estimate DNA fragmentation, VEGF, PDGF and histopathological examinations.

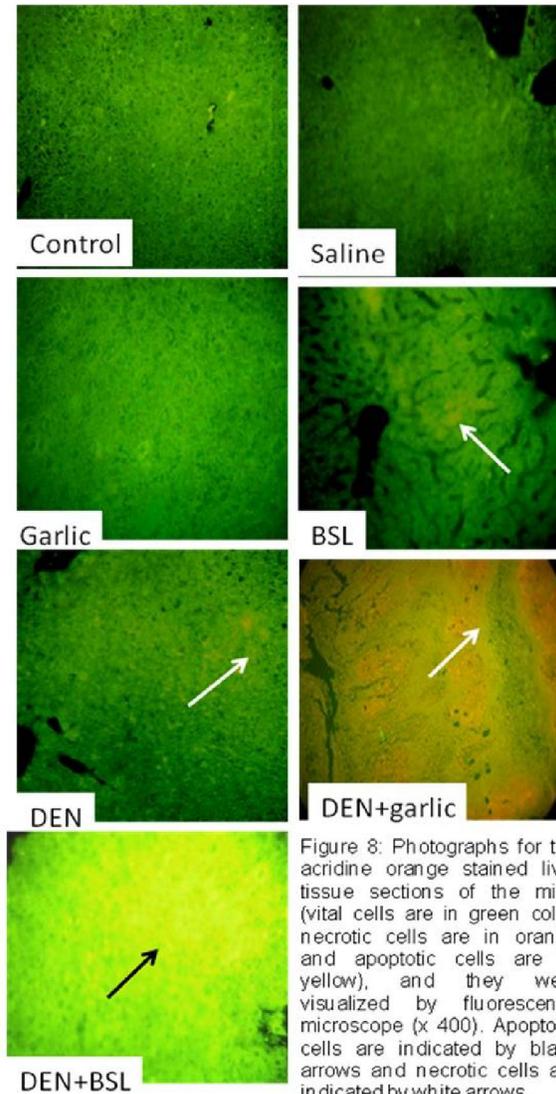


Figure 8: Photographs for the acridine orange stained liver tissue sections of the mice (vital cells are in green color, necrotic cells are in orange and apoptotic cells are in yellow), and they were visualized by fluorescence microscope (x 400). Apoptotic cells are indicated by black arrows and necrotic cells are indicated by white arrows.

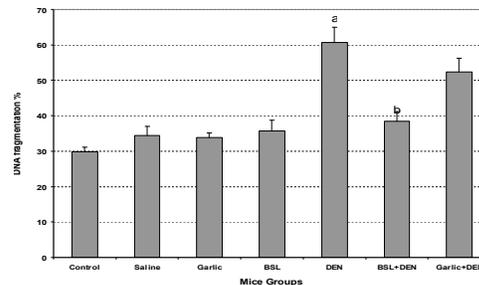


Figure 9 Effect of BSL in DNA fragmentation percentage in mice liver tissue homogenate. Results were expressed as Mean \pm S.E.

A: Significantly different from control gp at ($P<0.05$).
b: Significantly different from DEN gp at ($P<0.05$).

2.2.1. Effect of BSL on DNA fragmentation

Reactive oxygen species (ROS) generated in inflamed tissues can cause injury to cells and damage to DNA, which could also contribute to tumor development. DNA fragmentation is an index of DNA damage. DNA fragmentation experiment resulted in 29.78, 34.47, 33.9, 35.8, 60.7, 38.4 and 52.3 DNA fragmentation percentage in control, saline, garlic, BSL, DEN, BSL+DEN and garlic+DEN groups, respectively. All control, saline, garlic and BSL treated groups showed DNA fragmentation percentage of (29-35 %). While, administration of DEN leads to a 2 times elevation in the DNA fragmentation percentage compared with control group. Such elevation was significantly ($P<0.05$) decreased by receiving BSL and to lesser extent insignificantly by receiving garlic (Fig. 9).

2.2. 2. Estimation of VEGF level

VEGF is a growth factor essential to angiogenesis initiation and regulation. Upregulation of VEGF expression has been demonstrated to be strongly associated with tumor growth, angiogenesis, and increased resistance of liver cancer. VEGF level was estimated in different groups of mice (control, saline, garlic, BSL, DEN, BSL+DEN and garlic+DEN groups) recording 689.8, 805.1, 640.8, 798.5, 1271.6, 694.1 and 616.1 ng/ml, respectively. Accordingly, it was obvious that control and saline groups VEGF level was not significantly altered by administration of garlic or BSL (Fig. 10).

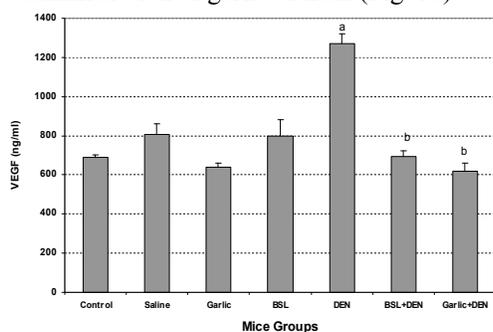


Figure 10 Effect of BSL on mice serum VEGF level (ng/ml) as measured by ELISA kit.

Results were expressed as Mean±S.E.

a Significantly different from control gp at ($P<0.05$).

b Significantly different from DEN gp at ($P<0.05$)

Administration of DEN significantly ($P<0.05$) increased VEGF level to 1.8 fold of the control. Such increasing was significantly normalized by receiving BSL and garlic as shown in figure (10).

2.2.3. Estimation of PDGF level

PDGF is an angiogenic growth factor that increased during neoangiogenesis observed in tumor. Control, saline, garlic, BSL, DEN, BSL+DEN and garlic+DEN groups showed 2887.7, 3208.7, 2510.8,

2987.5, 2257.9, 2848.3 and 2190.1ng/ml PDGF level, respectively. This demonstrated that PDGF level were not significantly altered by administration of BSL or garlic. DEN administration did not significantly affecting PDGF level, also treatment with garlic or BSL before and after DEN did not significantly altered PDGF level (Fig. 11).

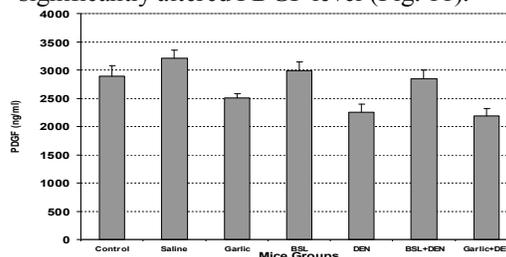


Figure 11 Effect of BSL on mice serum PDGF level (ng/ml) as measured by ELISA kit.

Results were expressed as Mean±S.E.

a Significantly different from control gp at ($P<0.05$).

b Significantly different from DEN gp at ($P<0.05$).

2.2.4. Histopathological Examination

There was no histopathological alteration observed in control and saline groups and the normal histological structure of the central vein and surrounding hepatocytes in the hepatic cords recorded in Table (1) and (Fig. 12). BSL group showed sever dilatation in the central vein as recorded in (Fig. 12) where, administration of garlic causes dilatation and congestion in the central veins. The portal areas showed sever dilatation of the portal vein associated with inflammatory cells infiltration surrounding the bile duct in the DEN group (Fig. 12). In BSL+DEN group there was mild dilatation in the central veins with diffuse Kupffer cells proliferation in between the hepatocytes (Fig. 12). Kupffer cells were proliferated in diffuse manner between the hepatocytes associated with dilation and congestion in the portal vein in garlic+DEN group (Fig. 12).

In BSL group portal veins showed sever dilatation. Where, in DEN group double nuclei were observed in multiple numbers of hepatocytes associated with karyomegaly degeneration in other hepatocytes also, there was constriction in the central zone of some nuclei in the hepatocytes as a stage for division and diffuse proliferation and hyperplasia were observed in Kupffer cells in between the degenerated and cytomegalic hepatocytes. The portal area showed dilatation in the portal vein with inflammatory cells infiltration surrounding the bile duct in BSL+DEN group. The hepatocytes showed double nuclei in the most of them with cytomegaly and karyomegaly, as well as, degeneration in garlic+DEN group (Fig. 13).

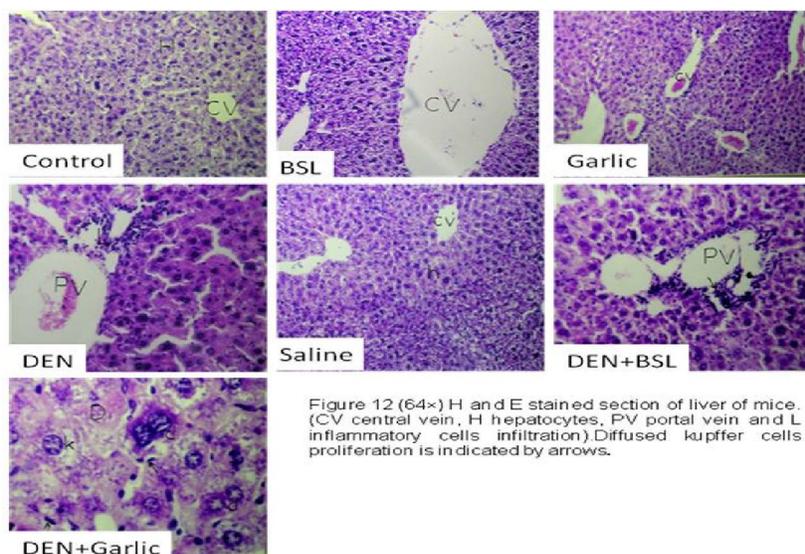


Figure 12 (64x) H and E stained section of liver of mice. (CV central vein, H hepatocytes, PV portal vein and L inflammatory cells infiltration). Diffused kupffer cells proliferation is indicated by arrows.

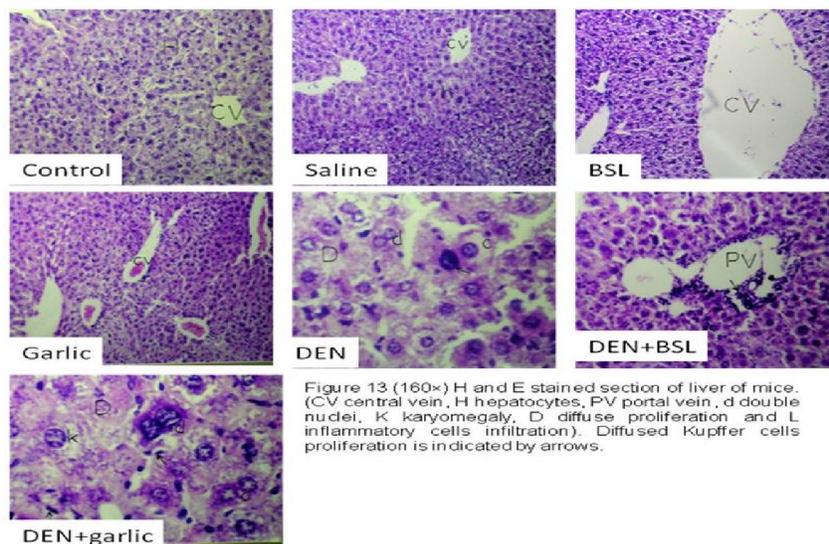


Figure 13 (160x) H and E stained section of liver of mice. (CV central vein, H hepatocytes, PV portal vein, d double nuclei, K karyomegaly, D diffuse proliferation and L inflammatory cells infiltration). Diffused Kupffer cells proliferation is indicated by arrows.

Table 1 Histopathological Alterations in the Different Groups of Mice's Liver sections.

Histopathological Alterations	Mice	Control group	Saline group	BSL group	Garlic group	DEN group	BSL + DEN group	Garlic +DEN group
Degenerative change (fatty degeneration)		-	-	-	-	+	-	+
Inflammatory cells infiltration in the portal area (portal infiltration)		-	-	-	-	++	+	-
Kupffer cells proliferation (kupffer cell hyperplasia)		-	-	-	-	+++	+	+++
Karyomegaly (nuclear enlargement)		-	-	-	-	+++	-	++
Cytomegaly		-	-	-	-	+++	-	++
Double nuclei (bi or multinucleation)		-	-	-	-	+++	-	++
Pyknosis Nuclear Chromatin & Hyperchromachia (hyperchromosia)		-	-	-	-	+++	-	-
Nuclear constriction for mitosis (mitosis)		-	-	-	-	+++	-	-
Dilated portal vein		-	-	++	-	++	+	+
Dilated central vein		-	-	++	+	++	+	-

+++ = Severe reaction, ++ = moderate reaction, + = weak, - = absent of the reaction

4.DISCUSSION

In recent years, the morbidity and mortality of cancer still reaches a high plateau and is a major public health problem worldwide. Chemoprevention, i.e. the use of either synthetic or naturally occurring agents to inhibit pre-cancerous events, has become recognized as a plausible, cost-effective and necessary approach to reduce cancer morbidity and mortality (Hong and Sporn, 1997; Wattenberg, 1997; Sporn and Suh, 2002). So, searching for new compounds for the treatment and prevention of cancer was the aim of numerous studies and the aim of our study.

Proliferation plays an important role in several steps of the carcinogenic process (Barret, 1993). It is involved in the fixation of a miscoding lesion in the newly made DNA (Ames and Gold, 1990). To investigate BSL *in vivo* activity as an antiproliferator and antiprogesser, DEN has been used as an effective experimental model in the field of carcinogenesis (Laughton *et al.*, 1989).

The potency of COX-2 inhibitors *in vivo* could be attributed to the inhibition of the enzyme in the tumor, as well as, in stromal cells, resulting in antiproliferative, pro-apoptotic actions within the tumor, and anti-angiogenic, pro-immune surveillance activities in endothelial and myeloid cells. The combination of COX-2 inhibitor with standard cancer chemotherapeutic and/or radiation may provide additional therapeutic paradigms in the treatment of various human cancers (Mazhar *et al.*, 2005).

The present results indicated that BSL was able to induce apoptosis rather than necrosis as mode of cell death in BSL treated mice when compared with untreated groups when liver tissue of mice stained with acridine orange. Modulation of the oxidative stress, inhibition of HDAC activity, NO inhibition and prevention of DNA damage which are the properties of BSL may lead to p53 regulation and/or caspases induction and this cause apoptosis induction in mice liver tissue since HDACs play an essential role in the regulation of apoptosis (Sambucetti *et al.*, 1999). Also, NO plays a critical role in apoptosis via nitrosylation of caspase-9 (Torok *et al.*, 2002). Mutations in genes that regulate apoptosis pathways are common in most cancers (Sun *et al.*, 2004). Animal studies have also demonstrated that, certain chemopreventive agents could induce apoptosis in tumor cells *in vivo* without affecting normal cells (Sun *et al.*, 2004). In accordance with our results, Gamal-Eldeen *et al.* (2009) reported that, different fractions of water-soluble polysaccharide extract derived from *S. latifolium* showed a disturbance in cell cycle including arrest in both S-phases in lymphoblastic leukemia (1301 cells). This

disturbance was associated with an induced-cell death due to apoptosis, but not necrosis. On the other hand, our results revealed that, garlic increases necrosis rather than apoptosis in mice liver and this may be due to increased TNF- α and NO. Since allicin the major component of garlic, induced tumoricidal activity and increased the production of TNF- α and NO in murine peritoneal macrophages in a dose-dependent manner (Kang, *et al.*, 2001). Allicin inhibits the apoptosis of macrophages in a depleted nutritional state through the MEK/extracellular signal-regulated kinase pathway (Cho *et al.*, 2006).

Liver infiltration by phagocytes, during liver injury, provided a source of ROS which cause damage to DNA, proteins and lipids when their generation exceeds the ability of the antioxidant systems to remove them (Simile *et al.*, 2005; Calvisi *et al.*, 2008). In the current study, BSL showed down regulation of DNA fragmentation in BSL treated mice before and after DEN injection when compared with DEN-induced mice, suggesting that, BSL prevents DNA damage. The decrement in the rate of oxidative DNA damage by BSL which could involve induction of the DNA repair system serves as a basis for chemopreventive mechanisms.

Angiogenesis, the development of new blood vessels from endothelial cells, is a crucial process in tumor pathogenesis as it sustains malignant cells with nutrients and oxygen (Fayette *et al.*, 2005). During angiogenesis, endothelial cells are stimulated by various growth factors, such as VEGF and FGF, and are attracted to the site where the new blood supply is needed by inflammatory cytokines and chemoattractants (Albini *et al.*, 2005; Presta *et al.*, 2005).

There is a tight interplay between innate immune and endothelial cells, inflammatory leukocytes (neutrophils, macrophages, and others) release a number of factors that influence endothelial cell behavior [VEGF, hepatocyte growth factor (HGF), MMP2, and IL-8]. Inflammatory leukocytes might provide the angiogenic stimulus in the initial phases of tumorigenesis, as well as growth stimulus permitting accumulation of further mutations that eventually render the tumor inflammation-independent and malignant (Heryanto *et al.*, 2004). Therefore targeting tumor angiogenesis is an attractive strategy to treat cancer. The production of VEGF is considered essential for angiogenesis and the migration of cancer cells and high VEGF expression level is associated with a wide array of malignancies.

VEGF mRNA expression is upregulated by a wide array of oncogenes (including H-ras and K-ras, src, p53, and C-jun) and growth factors (including epidermal growth factor [EGF], transforming growth

factor [TGF α , TGF β], insulin-like growth factor-1, and PDGF) In the current study, administration of BSL before and after DEN resulted in a significant decrease in VEGF level in mice serum compared with DEN-induced mice. This decreasing effect might be due to BSL inhibitory effect on NO and TNF- α since Shin *et al.* (2000) and Josko and Mazurek (2004) reported that, TNF- α was exerted its regulatory effect on both iNOs and VEGF and as line of evidence suggests NO activates the transcription factor hypoxia-inducible factor-1 alpha (HIF-1 α) (Sandau *et al.*, 2001a; Sandau *et al.*, 2001b; Sharp, 2001) which in turn targets VEGF and can promote angiogenesis (Ravi *et al.*, 2000). In the same line with our findings Bae *et al.* (2005) found that, polysaccharides isolated from *Phellinus gilvus* inhibited VEGF gene expression in the B16F10 melanoma cell line that constitutively express VEGF.

In the current study we found that, administration of garlic before and after DEN resulted in significant decrease in VEGF level compared with DEN-induced mice. The antioxidant activity of garlic might be responsible for this effect. Mousa *et al.* (2005), demonstrated inhibition of fibroblast growth factor-2 and VEGF-induced tube formation in human endothelial cells and inhibition of *ex vivo* neovascularization in chick chorioallantoic membrane assay by alliin. The anti-angiogenic effects of alliin were mediated, at least in part, by increase in cellular NO and p53 protein expression.

Over expression of growth factor and growth factor receptors such as EGF, PDGF, and others can result in enhanced proliferation by cancer cells (Masuda *et al.*, 2001). Many growth factors (i.e., EGF and PDGF) bind their receptors and generate large increases in ROS (Martin, 2006). So, we estimated PDGF level in mice serum and found that BSL and garlic showed no effect on PDGF level in the serum of treated mice.

Our findings were further supported by the histopathological examination of liver sections, which illustrated that liver tissue of DEN-treated mice showed damage, manifested as degenerative change, inflammatory cells infiltration in the portal area, Kupffer cells proliferation (Kupffer cell hyperplasia), karyomegaly (nuclear enlargement), cytomegaly, double nuclei, pyknosis nuclear chromatin and hyperchromachia, nuclear constriction for mitosis, dilated portal vein, dilated central vein. On the contrary, liver tissue of mice treated with BSL before and after DEN injection showed more or less normal hepatic lobular architecture and this effect was seen to a lesser extent in the animals treated with garlic before and after DEN injection compared to DEN induced mice.

Accordingly, these histopathological findings as an end-point biomarker primarily confirm the chemopreventive potential of BSL in inhibiting hepatocellular preneoplas induced by DEN.

Altogether, the results of the present study indicates that BSL could be represented as promising cancer chemopreventive agent against hepatocarcinogenesis, since it has tumor anti-promoting activity via its immunomodulatory activity as, it has anti-inflammatory and proliferative effect on the macrophage cells and apoptosis induction. Also, BSL had anti-progression properties through inhibition of proliferation of hepatocarcinoma cells and inhibition of angiogenesis.

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