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# Anticancer, antiangiogenesis and antimetastasis properties of prepared sulfated oligosaccharides on chemically induced hepatocellular carcinoma in rats

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**ABSTRACT**

*Hepatocellular carcinoma (HCC) is a serious healthcare problem worldwide because of its increasing morbidity and high mortality rates. The present study aimed to investigate the chemopreventive effects of prepared sulfated oligosaccharide compounds(Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate and Maltohexaose $SO_4$)by studying their ability to inhibit, reverse or restrict the development of cancer through inhibiting the metastasis and angiogenesis of tumor in diethylnitrosamine (DENA) induced hepatocarcinogenesis in rats. To elucidate the mechanism by which the prepared sulfated oligosaccharidesexert their antitumor activities in the animals-bearing tumor the following parameters were determined including:aspartate and alanine aminotransferases (AST & ALT), alkaline phosphatase (ALP), total bilirubinas liver function test;hepatic tyrosine kinase (TRK) and cytochrome P450 2E1 (CYP 2E1) as marker for tumor progression;vascular endothelial growth factor (VEGF) and sialic acid (TSA) as markers of angiogenesis;heparanase and elastase as marker of metastasis. Liver histopathological analysis was also evaluated. Carcinogenic rats recorded drastic elevation in all parameters under investigation whichconfirmed by histopathological distortion in the tissue organization with hyperchromatism, hyperplasia, proliferating hepatocytes.Prepared sulfated oligosaccharides supplementation at 1/10 of theirmedian lethal dose, $LD_{50}$, significantly improved the biochemical and histopathological changes induced by DENA in the order of Maltose $SO_4$>Maltohexaose $SO_4$>Stachyose $SO_4$>Raffinose $SO_4$> Chondroitin-6-sulfate. Taken together, our results demonstrate that the tested compounds especially Maltose $SO_4$ and Maltohexaose $SO_4$ may be potent anticancer agents for inclusion in modern clinical trials after more investigations on higher animals.*

**Keywords:** Hepatocellular carcinoma, Chemoprevention, Sulfated oligosaccharides, Angiogenesis, Metastasis.

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## INTRODUCTION

Hepatocellular carcinoma (HCC)or liver cancer is the sixth most common cancer and the third leading cause of cancer mortality worldwide [1,2]. The majority of HCC cases are attributable to underlying infections caused by hepatitis B and C viruses [3]. However, several other risk factors, including alcohol consumption, obesity, iron overload, environmental pollutants, as well as several dietary carcinogens, such as aflatoxins and nitrosamines, have

been shown to be involved in its etiology [4]. Currently, there is no proven effective systemic chemotherapy for HCC. Considering the limited treatment and grave prognosis of liver cancer, chemoprevention has been considered to be the best strategy in lowering the current morbidity and mortality associated with this disease [5]. Egypt has the highest prevalence of HCV in the world with 20% of the population infected and seven million with chronic HCV liver disease [6]. Up to 90% of HCC cases in the Egyptian population were attributed to HCV. Once cirrhosis has developed, retrospective studies have suggested that patients will develop either hepatic decompensation or HCC at a rate of 2% to 7% per year [7].

It is now well established that solid tumor growth is critically dependent on the growth of new vessels from preexisting blood vessels surrounding the tumor, a process called angiogenesis[8]. On the basis of this finding, the development of drugs that inhibit angiogenesis has become an attractive approach to cancer therapy [9]. In addition, metastasis of cancer cells to distant sites is one of the major deciding factors in cancer outcome. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumors and its ability to metastasize. There is a cascade of events leading to the metastasis of tumors. These include separation from the primary site, circulation through blood or lymph, adhesive to the basement membrane (composed mainly of heparan sulfate, elastin, and collagen), invasion and proliferation at distant sites [10]. Any compound which can inhibit one of the steps in the cascade will be useful in the inhibition of tumor metastasis and tumor growth.

Clinically important antitumor substances such as vincristine, camptothecin analogues, doxorubicin, etoposide, taxol, bleomycin, and folic acid analogues (methotrexate), are all of natural origin, and considerable efforts have been made to improve their biological activities through structural modification. However, such efforts have been little rewarded from a viewpoint of dramatic alteration of the key biological characters, such as antitumor spectrum, type of action, and drug resistance except for antitumor potency and toxicity. This means that the fundamental biological characters originally present in the parent complexes are difficult to alter by simple chemical modification [11]. We therefore aimed to produce new kinds of compounds by creating new structures artificially that may have anticancer effects.

Our ongoing research program is to discover and develop tumor growthinhibitors and apoptosis inducers as potential new anticancer agents. In our previous research [12] wesynthesized and characterized sulfated oligosaccharides including Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate and Maltohexaose $SO_4$whichhadantitumor activity in liver HepG2; breast MCF-7 and lung A549 cancer cell lines and our results revealed that the synthesized compounds can be used as good candidate for novel therapeutic strategies for cancer through regulation angiogenesis and metastasis of cancer. The present study dealt with evaluation the effect of the previously prepared sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$)for inhibiting, reversing or restricting the development of cancer and inhibitionits metastasis and angiogenesis in the experimental animal carrying liver cancer induced by diethylnitrosamine.

## MATERIALS AND METHODS

### *Animals*

The animal care and handling was done according to the guidelines set by the World Health Organization, Geneva, Switzerland, and according to approval from the ethics committee for animals care at the National Research Centre, Egypt (ethic No. 10-230). Adult male Sprague-Dawley rats (180±20 g, body weight), were purchased from the animal house of National Research Centre, Egypt. The animals were housed under standard laboratory conditions (constant temperature 25-27 °C, with 12 h light/dark cycle) during the experimental period. The rats were provided with tap water and commercial diets. The rats were acclimatized to laboratory condition for 10 days before commencement of the experiment.

### Assessment of *in vivo* cytotoxicity of prepared sulfated oligosaccharides

The median lethal doses ($LD_{50}$) of the prepared compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) was determined *in vivo* according to Ghosh [13]. Briefly, adult male Sprague-Dawley rats fasted for 12 h were randomly divided into groups of 10 per group. Each group was separately administrated once daily for a period of 4 weeks with doses ranging from 0-2000 µg/kg b.w. of the compounds by oral gavagingin a value of 1 ml/kg body weight. Control animals received the vehicle alone. The fasted animals were then provided with food and water immediately after the administration. The mortality of the animals was

observed up to one month post-treatment. The $LD_{50}$ of the prepared compounds was calculated by using a computer program of probit analysis.

**Experimental design**

Diethylnitrosamine (DENA) and carbon tetrachloride ($CCl_4$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). DENA was dissolved in saline and injected in a single dose (200 mg/kg, i.p.) to initiate hepatic carcinogenesis, while $CCl_4$ was used in a single dose (2 ml/kg) by gavage as 1:1 dilution in corn oil to stimulate liver cell proliferation and regeneration [14]. The experiment continued for 32 weeks.

Adult male Sprague-Dawley rats were divided into groups with 10 animals in each group. Group 1 (untreated control group): animals were fed on a standard diet and given water throughout the course of the experiment. Group 2 (DENA treated group): Rats were injected with a single dose of DENA (200 mg/kg, i.p.) and 2 week later received a single dose of $CCl_4$ (2 ml/kg) by gavage as 1:1 dilution in corn oil for 32 weeks. Group 3 (DENA and sulfated oligosaccharides group): Rats from Group 2 were treated daily with sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate or Maltohexaose $SO_4$)by oral gavaging at dose of 1/10 of their $LD_{50}$ values and continued for 32 weeks.

At the end of the treatment protocol (32 weeks), animals were anesthetized with ether and blood samples were drawn from the orbital venous plexus.Serum was separated by centrifugation for 5 min at 1500 g and stored at -20**°C** until analysis.

All animals were sacrificed by decapitation and their livers were rapidly excised, weighed, washed with salineand blotted with a piece of filter paper.Portion of the liver was immediately fixed in 10% formalin for histological analysis according to Conn et al.[15] using a standard method of hematoxylin and eosin. Another portion of liver was homogenized using a Branson Sonifier (250 VWR Scientific, Danbury, Conn., USA) in cold sucrose buffer (0.25 M). All the investigation will carry out in fresh 10% homogenate. The freshly prepared homogenates were then centrifuged at 30,000 xg for 30 min at 4°C to obtain the supernatant, which used for biochemical assays and the protein level was determined as described by Lowry et al.[16]. Aspartate and alanin-aminotransferase (AST and ALT), alkaline phosphatase (ALP) activities as well as the level of total bilirubin (Bili) were determined spectrophotometrically according to the manufacturer's instructions, using reagent kits obtained from Biomerieux (France).

**Cytochrome P450 2E1 assay**

The effect of synthesized compounds on the level of cytochrome P450 2E1 (CYP 2E1)was determined in tissue homogenates based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of cytochrome P450 2E1 kit purchase from Cloud-Clone Crop. (Houston, TX 77082, USA). The microtiter plate provided in this kit has been pre-coated with an antibody specific to CYP 2E1. Standards or samples are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody specific to CYP 2E1. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain CYP 2E1, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of CYP 2E1 in the samples is then determined by comparing the O.D. of the samples to the standard curve.

**Tyrosine kinase assay**

The effect of synthesized compounds on the level of tyrosine kinase (TRK) was determined in the tissue homogenates based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of tyrosine kinase kit purchase from Glory Science Co., Ltd (Del Rio, TX 78840, USA) according to the manufacturer's instructions. In brief, add TRK to monoclonal antibody enzyme well which is pre-coated with human TRK monoclonal antibody, incubate; then, add TRK antibodies labeled with biotin, and combined with Streptavidin-HRP to form immune complex; then carry out incubation and washing again to remove the uncombined enzyme. Then add Chromogen solution A, B, the color of the liquid changes into the blue, and at the effect of acid, the color finally becomes yellow. The chroma of color and the concentration of the human TRK of sample were positively correlated and the optical density was determined at 450 nm. The level of TRK in samples was calculated as triplicate determinations from the standard curve and the percentage of TRK inhibition for each compound was calculated.

**Estimation of vascular endothelial growth factor concentration**

Vascular endothelial growth factor(VEGF) concentration in the serum was determined using ELISA kit obtained from Koma Biotech Inc., Korea. This assay depends on binding VEGF antigen to a specific immobilized antibody. The formed immune complex binds to avidin-peroxidase conjugate, and a color developed in proportion to the amount of VEGF bound which was measured at 450 nm.

**Total sialic acid (TSA)**

Total sialic acid (TSA) was estimated in the serum by periodate-resorcinol microassay as described by Surangkul et al.[17]. Briefly, 40 µl of standard NANA (2-10 µg/well) or serum samples were added to the wells of a 96-well microtiter plate. Then, 50 µl of 1.3 mM periodic acid was added to each well and mixed by shaking. The plate was placed on an ice box for 60 min, then 100 µl of 0.6 g/dl of resorcinol reagent was added and mixed again. The plate was covered with a glass plate and heated 80 $^{o}$C for 60 min in a water bath then removed until the contents cooled down to room temperature. Then, 100 µl of 95% tert-butanol was added and the contents were mixed once again. The absorbance at 620 nm was measured immediately by a microtiter plate reader.

**Determination of heparanase (HPSE) activity**

Determination of heparanase activity in tissue homogenates was determined in cell homogenates based on a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) of heparanase kit purchase from Glory Science Co., Ltd (Del Rio, TX 78840, USA) according to the manufacturer's instructions. The kit assay HPSE activity in the sample, use purified HPSE to coat mictotiter plat wells, make solid-phase antibody, then add HPSE to wells, combined HPSE which with enzyme labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add substrate solution, substrate becomes blue color and the reaction is terminated by the addition of a sulphuric acid and the color change is measured spectrophotometrically at a wavelength of 450 nm. The activity of HPSE in the samples is then determined by comparing the absorbance of the samples to the standard curve. The activity was determined as U/mg protein.

**Estimation of elastinolytic activity**

The elastase activity is determined in the tissue homogenates by its catalytic effect on the *N*-succinyl-trialanyl-*p*-nitroanilide substrate releasing *p*-nitroaniline (*p*NA) which is measured photometrically at 405 nm [18]. The elastase activity was determined as U/mg protein.

***Statistical analysis***

The results are reported as Mean ± Standard error (S.E.). Statistical differences were analyzed by one way ANOVA test followed by student's *t* test wherein the differences were considered to be significant at $p < 0.05$.

## RESULTS

The $LD_{50}$ (the median lethal dose resulted in 50% mortality of the animals) of each compound was determined. Compounds Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate andMaltohexaose $SO_4$ showed marked acute toxicity (Table 1). The concentrations required by Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$ for 50% mortality of the animals were found to be 600, 1006, 800, 1210 and 680 µg/kg body weight, respectively.

**Table 1: In *vivo* the median lethal dose ($LD_{50}$) of sulfated oligosaccharides**

| Compounds | $LD_{50}$ (µg/kg b.w.) |
|---|---|
| **Maltose $SO_4$** | 600 |
| **Raffinose $SO_4$** | 1006 |
| **Stachyose $SO_4$** | 800 |
| **Chondroitin-6-sulfate** | 1210 |
| **Maltohexaose $SO_4$** | 680 |

**The effect of prepared sulfated oligosaccharides on liver tissues**

DENA-treated rats showed significant ($p<0.05$) increase in serum AST, ALT and ALP activities, along with significant ($p<0.05$) increase in total bilirubin level compared to control. The administration of sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$)at a dose of 1/10 of the $LD_{50}$ values in the DENA-treated rats resulted in normalization in AST, ALT and ALP

activities as well as the total bilirubin level compared to DENA-treated group (Table 2). The treatment with the tested compounds has resulted in decreasing in the level of liver function test follows the order Maltose $SO_4$>Maltohexaose $SO_4$>Stachyose $SO_4$>Raffinose $SO_4$> Chondroitin-6-sulfate. It is clear that, Maltose $SO_4$ was the best compound in this series.

**Table 2: Effect of prepared sulfated oligosaccharides on serum AST, ALT, ALP activities and bilirubin level in different studied groups**

| Groups | AST (U/L) | ALT (U/L) | ALP (U/L) | Bili (mg/dl) |
|---|---|---|---|---|
| **Control** | 56.00±5.20 | 38.00±3.70 | 110.00±9.30 | 1.40±0.11 |
| **DENA** | 110.00±7.80[a] | 95.00±7.00[a] | 215.00±15.70[a] | 2.90±0.20[a] |
| **Maltose $SO_4$** | 62.00±6.30[b] | 41.00±4.00[b] | 120.00±11.00[b] | 1.60±0.15[b] |
| **Raffinose $SO_4$** | 92.00±8.00[a,b] | 72.00±5.20[a,b] | 180.00±16.00[a,b] | 2.60±0.19[a,b] |
| **Stachyose $SO_4$** | 85.00±7.50[a,b] | 66.00±5.40[a,b] | 166.00±12.60[a,b] | 2.50±0.20[a,b] |
| **Chondroitin-6-sulfate** | 100.00±8.66[a] | 82.00±6.50[a] | 195.00±16.00[a] | 2.80±0.23[a] |
| **Maltohexaose $SO_4$** | 70.00±6.90[a,b] | 50.00±4.70[a,b] | 125.00±13.20[a,b] | 2.00±0.20[a,b] |

*Results expressed as Mean ±S.E.; [a] Significantly different from normal control at $p < 0.05$.; [b] Significantly different from DENA - treated rats at $p < 0.05$.*

In this study histological examination of rat liver sections was consistent with the results obtained from biochemical studies. Liver of control animals as presented in (Figure 1a) revealed normal architecture of hepatic strands around the central veins. The liver showed intact hepatocytes with normal sinusoids in between. The hepatic cells are polygonal in shape with one or two rounded nuclei. Liver of rats treated with DENA alone showed distortion in the tissue organization with hyperchromatism, hyperplasia, proliferating hepatocytes (Figure 1b), both hepatic and portal with significant tumor thrombi within portal vessels, tumor cells are slightly larger have more irregular nuclei and numerous mitotic figures with malignant nuclei (Figure 1c). Some section showed megalocytosis, hyperchromatic nuclei as well as nuclear vacuolationand nuclear prominence, dissolution of hepatic cords which appeared as empty vacuoles aligned by strands of necrotic hepatocytes (Figure 1d).

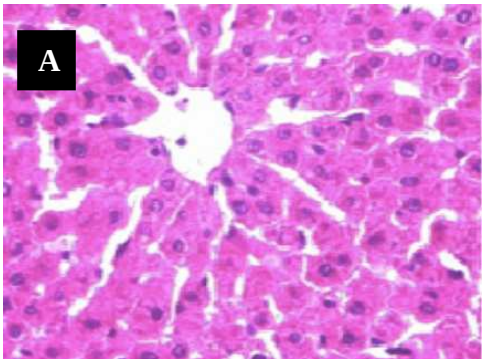


**Figure 1a: Liver section of normal control rats showed normal appearance with normal appearing hepatocytes cords, unremarkable pathological changes (H&E, 400x)**

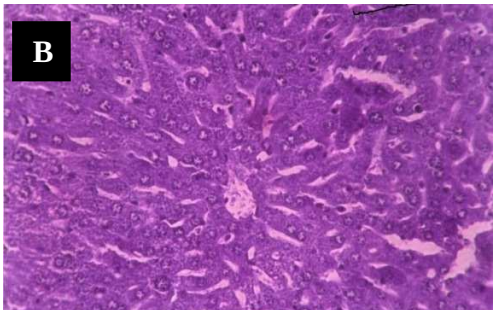


**Figure 1b: Photomicrograph of liver section from rats treated with DENA showing hyperchromatism, hyperplasia, proliferating hepatocytes (H&E, 200x)**

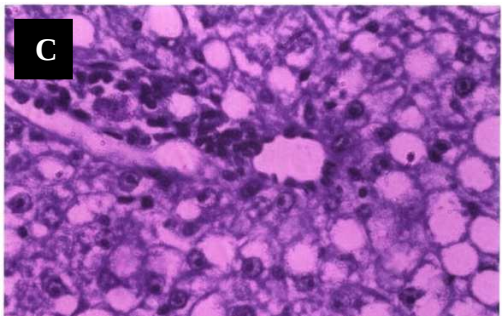


**Figure 1c:Section from liver section treated with DENA revealed loss architecture, both hepatic and portal with significant tumor thrombi within portal vessels, tumor cells are slightly larger have more irregular nuclei and numerous mitotic figures (H&E, 400x)**

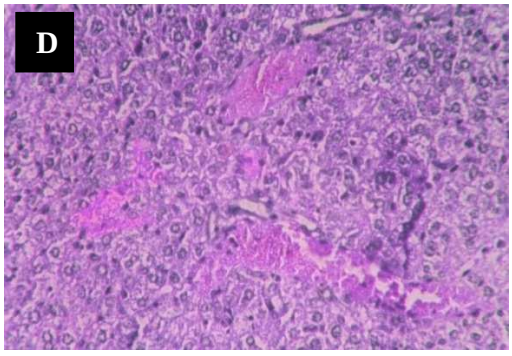


**Figure 1d: Liver section from animal treated with DENA showed megalocytosis, hyperchromatic nuclei as well as nuclear vesiculation and nuclear prominence (H&E,200x)**

Liver of the DENA-rats treated with Maltose $SO_4$ showed improvement in the hepatic pattern associated with normal hepatic architecture (Figure 1e). Liver sections from rats treated with Stachyose $SO_4$exhibited areas of aberrant hepatocellular phenotype with variation in nuclear size, hyperchromatism, and irregular sinusoids (Figure 1f).Liver sections from rats treated with Raffinose $SO_4$ showedmild ballooning degeneration of hepatic cells; mild infiltration of portal tract by inflammatory cells; mild parenchymal cells congestion; mild bile duct injury with proliferation (Figure 1g). Liver sections from rats treated with Chondroitin-6-sulfateexhibited severe ballooning degeneration of hepatic cells; severe infiltration of portal tract by inflammatory cells; moderate parenchymal cells congestion; moderate bile duct injury with proliferation; severe necrosis of hepatic cells; enlarged nuclei with increase number and prominence of nucleoli; fibrosis was observed in surrounding central veins, fibrous septa formation and cirrhotic changes; fatty changes (Figure 1h).

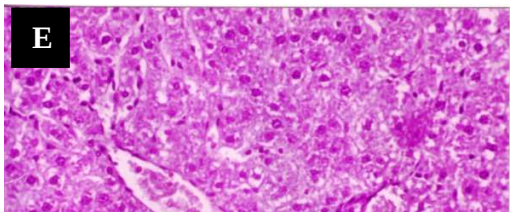


**Figure 1e:Liver of the DENA-rats treated with compound Maltose $So_4$ showed improvement in the hepatic pattern as previously seen in normal liver of control rats (H&E, 200X)**

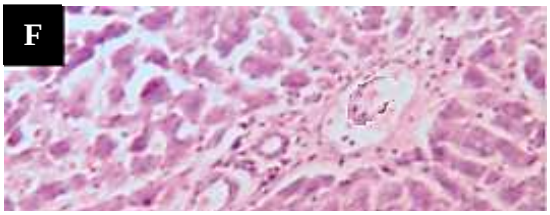


**Figure 1f:Liver of the DENA-rats treated with Stachyose $SO_4$,showing areas of aberrant hepatocellular phenotype with variation in nuclear size, hyperchromatism, andirregular sinusoids (H&E,250x)**

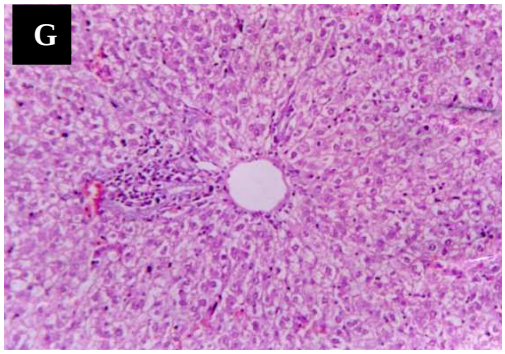


**Figure 1g:Liver of the DENA-rats treated with Raffinose $SO_4$, showed liver ballooning degeneration, mild infiltration of portal tract by inflammatory cells (H&E,250x)**

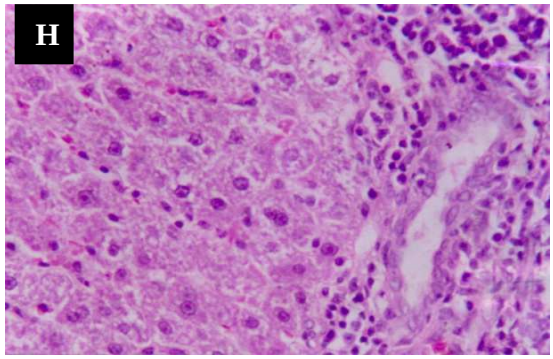


**Figure 1h:Liver of the DENA-rats treated with Chondroitin-6-sulfate,showed dense lymphocytic infiltration of portal tract by inflammatory cells, moderate necrosis, moderate ballooning degeneration of hepatic cells, enlarged nuclei with increase number and prominence of nucleoli (H&E, 250x)**

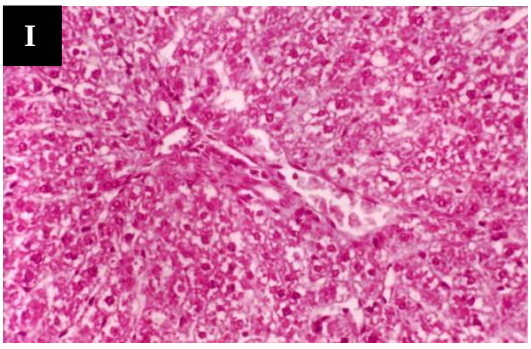


**Figure 1i:Liver of the DENA-rats treated with Maltohexaose $So_4$,showing hepatocytes maintaining near-normalarchitecture (H&E, ×250)**

Liver sections from rats treated with Maltohexaose $SO_4$improved the hepatocellular architecture with more regular and less altered hepatocytes when compared to group treated with DENA alone (Figure 1i). From the foregoing results it is clear that, there are improvement in the histological change in the order of Maltose $SO_4$>Maltohexaose $SO_4$>Stachyose $SO_4$>Raffinose $SO_4$> Chondroitin-6-sulfate.

**Effect of sulfated oligosaccharides on (CYP) 2E1 and TRK expression**

The effect of the prepared compounds on both CYP 2E1 and TRK which implicated in cancer growth was illustrated in Figure 2 and 3.The expression of CYP 2E1 and TRK were significantly increased in DENA-treated as compared to normal control. While the administration of sulfated oligosaccharides (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) at a dose of 1/10 of the $LD_{50}$ values in the DENA -treated rats resulted in significantly inhibitory potential against both CYP 2E1 and TRK for all the compounds comparing with the DENA group. Maltose SO4 was the most potent inhibitor against CYP 2E1 and TRK expression.

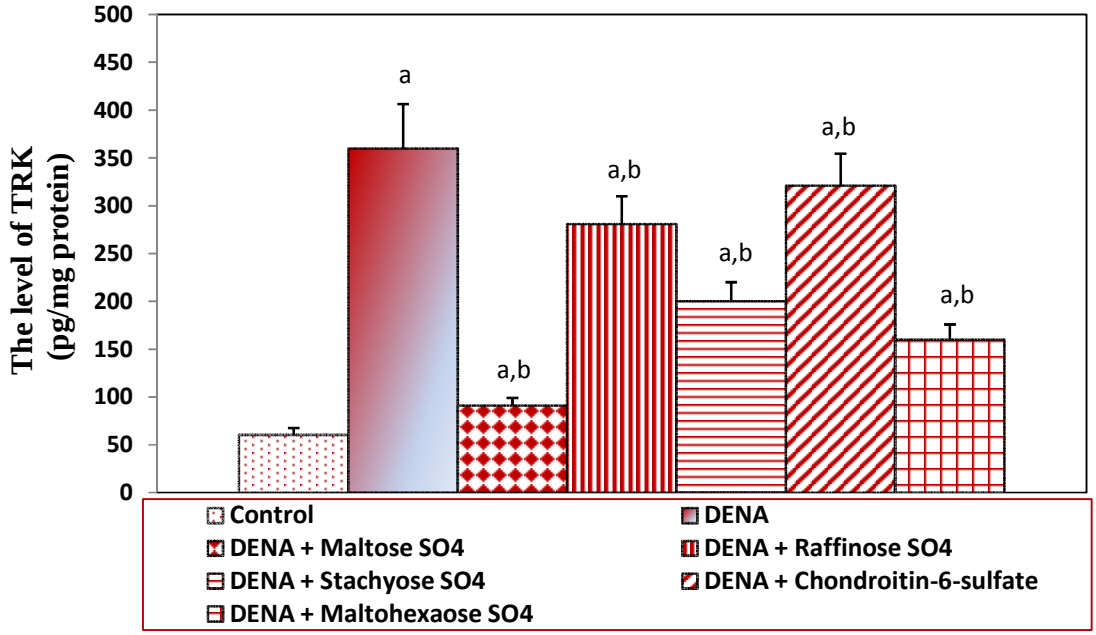


**Figure 2: Effect of treatment with the sulfated oligosaccharides on the level of hepatic protein kinase(TRK)**

*Data were expressed as mean ±S.E., [a] and [b] is significant difference from control and DENA - treated rats respectively at ($p < 0.05$)*

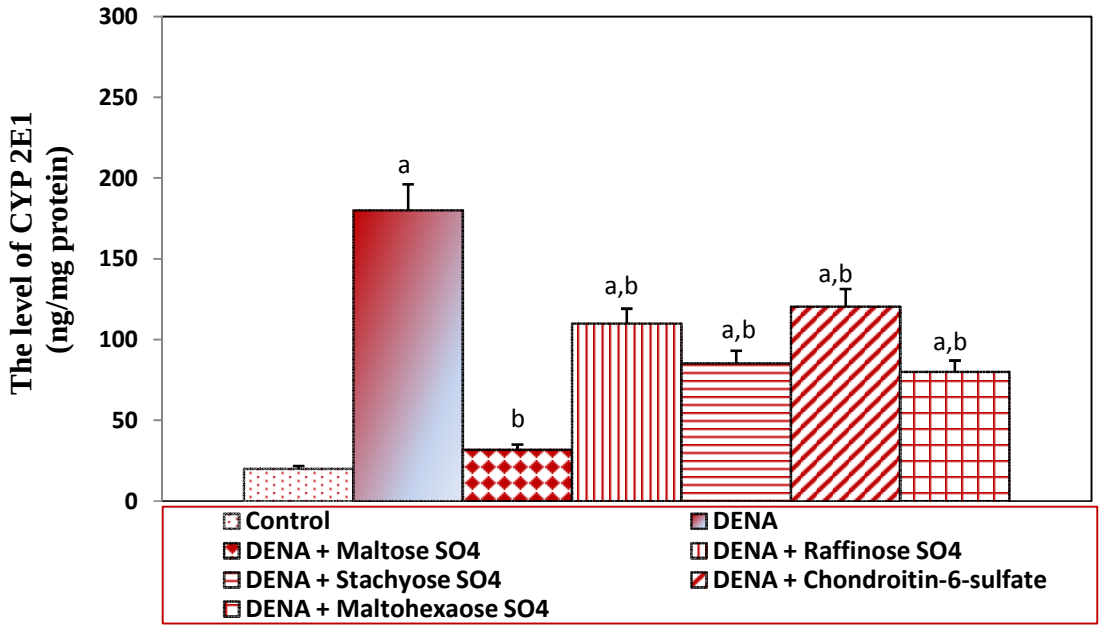


**Figure 3: Effect of treatment with the prepared sulfated oligosaccharides on the level of hepatic cytochrome P450 2E1 (CYP 2E1)**
*Data were expressed as mean ±S.E., [a] and [b] is significant difference from control and DENA - treated rats respectively at ($p < 0.05$)*

**Effect of sulfated oligosaccharides on VEGF and TSA**

The effect of the prepared compounds on VEGF and TSA as markers of angiogenesis was illustrated in Figure 4 and 5. In this study, it was found that the levels of both VEGF and TSA in the DENA-treated group was very highly significant increase as compared to control group while the treatment with sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) in the DENA-treated rats, causes decrease in the level of VEGF and TSA as compared with DENA-treated rats,while the VEGF and TSA level showed significant decrease in Maltose $SO_4$and Maltohexaose $SO_4$-treated group reaching to its control level.

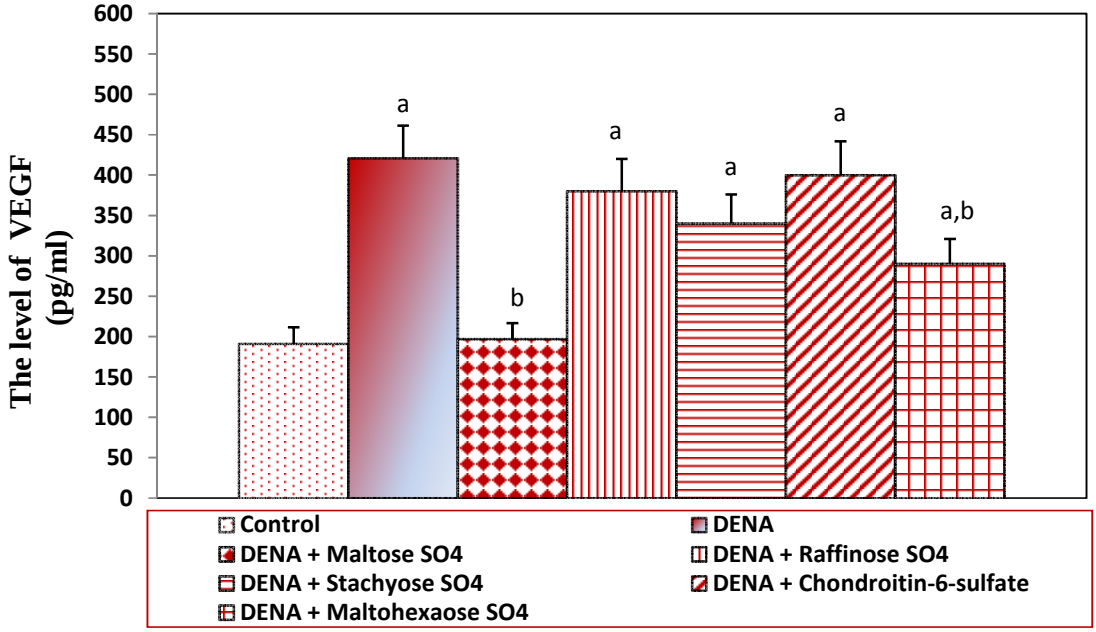


**Figure 4: Effect of treatment with the prepared sulfated oligosaccharides on the level of VEGF**
*Data were expressed as mean ±S.E., [a] and [b] is significant difference from control and DENA - treated rats respectively at ($p < 0.05$)*

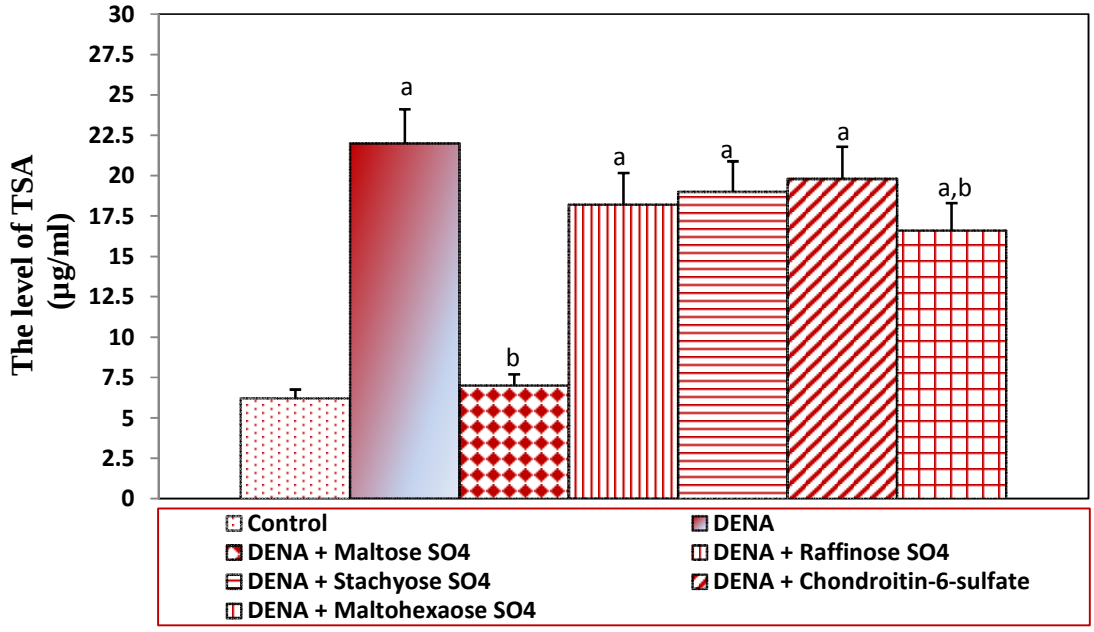


**Figure 5:Effect of treatment with the prepared sulfated oligosaccharides on the level of sialic acid (TSA)**
*Data were expressed as mean ±S.E., [a] and [b] is significant difference from control and DENA - treated rats respectively at ($p < 0.05$)*

**Effect of sulfated oligosaccharides on heparanase and elastase activity**

In the present study the activity of heparanase and elastase as marker for metastasis of tumor was investigated, the results showed that the activity of heparanase and elastase enzymes was very highly significantly increased in DENA-treated group. The treatment with sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) in the DENA -treated rats, resulted in decrease in the

activity of heparanase and elastase enzymes as compared with DENA-treated group especially Maltose $SO_4$ and **Maltohexaose $SO_4$** (Figure 6).

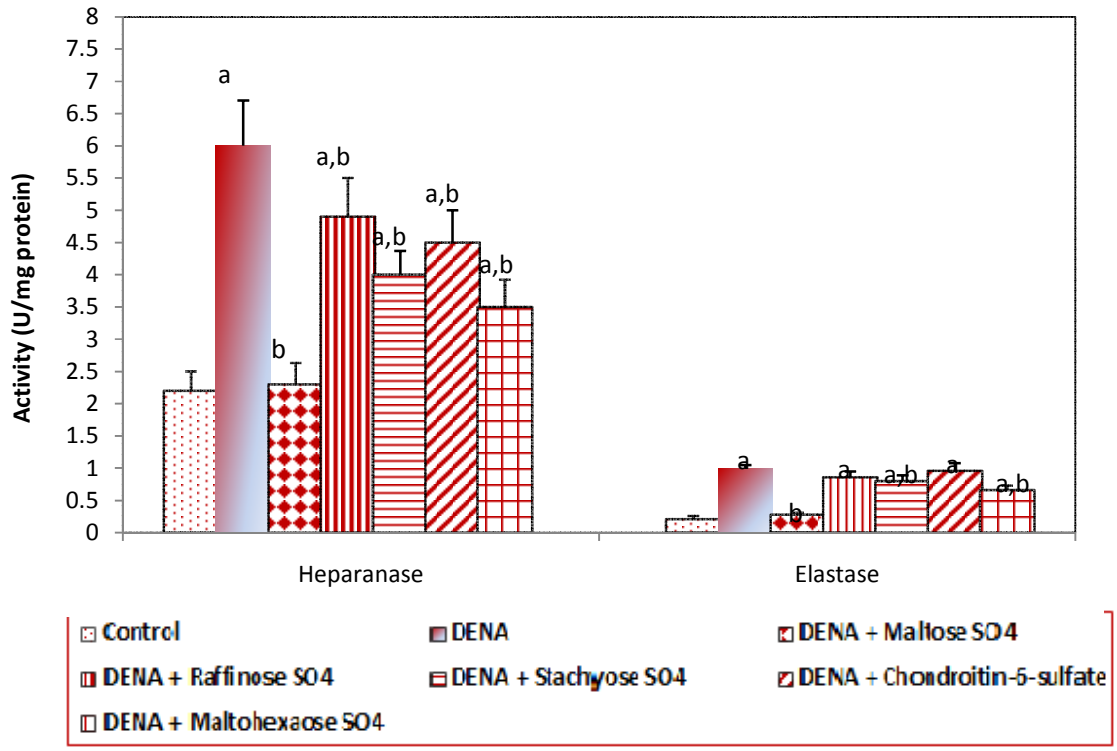


**Figure 6: Effect of treatment with the prepared sulfated oligosaccharides on the activity of heparanase and elastase**
*Data were expressed as mean ±S.E., [a] and [b] is significant difference from control and DENA - treated rats respectively at ($p < 0.05$)*

## DISCUSSION

Sulfated oligosaccharides, such as heparan, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, are important ingredients of extracellular matrix(ECM). Many sulfated polysaccharides have been extracted from bacteria, plants and animals[19]. Our group has recentlyreported that preparedsulfated polysaccharides (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$)exhibit significant anticancer against HepG2 liver cancer cells [12].

Experimental liver cancer in rodents induced by DENA, anenvironmental and dietary hepatocarcinogen [20], has been considered as one of the best characterized experimental models of HCC, allowing the screening of potential anticancer compounds on various phases of neoplastic transformation and development [21]. DENA-induced preneoplastic foci and preneoplastic and neoplastic nodule formation in rodents closely mimics HCC development in humans. Moreover, a cross-species comparison of gene expression patterns has established that DENA-induced liver tumors in rodents closely resemble a subclass of human HCC [22], which allows to extrapolate potential chemopreventive effects of a candidate agent in clinical setting.

In the present study, we have investigated the preventive effect of prepared sulfated polysaccharides including Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$ on the appearance of early hepatic preneoplastic events, utilizing a two-stage model ofhepatocarcinogenesisinitiated with DENA and promoted by carbon tetrachloride. An understanding of how cancer may be prevented is one of the key objectives of the recent researches. This can be achieved to some extent by using chemopreventive agents, naturally occurring or synthetic, that can suppress or prevent the processes of tumor development. Therefore, it is essential to identify agents as well as to evaluate their efficacy and to elucidate their mechanisms of action. In the present study, serum

obtained from tumor bearing rats showed significant increase in AST, ALT and ALP activities along with significant increase in total bilirubin compared to control animals.The elevation of these enzyme activities was indicative of the toxic effect of DENA on the liver tissue associated with sever histological distortions (Figure1b-d). It is known that N-nitroso compounds act as strong carcinogens in various mammals including primates [23]. DENA has been shown to be metabolized by cytochrome P-450 IIE1 (CYP 2E1) to its active ethyl radical metabolite, which could interact with DNA causing mutation and carcinogenesis [24]. Administration of sulfated oligosaccharides (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate orMaltohexaose $SO_4$) to DENAtreated rats showed restoration of AST, ALT and ALP activities and total bilirubin level towards normal especially in group treated with Maltose $SO_4$ and Maltohexaose $SO_4$. Such reverse in serumenzyme activities could be attributed to the ability of these compounds to inhibit CYP 2E1 activity (as shown in Figure 3), presumably by serving as a competitive inhibitor, leading to a decrease in the formation and/or bioactivation of these nitrosamines.This improvement in the biochemical parameters were accompanied withimprovement in the histopathological abnormalities especially in Maltose $SO_4$ and Maltohexaose $SO_4$(Figure 1e and i) which showing hepatocytes maintaining near-normal architecture while the other compounds revealed moderate improvement of hepatic histopathology over DENA group.

Tyrosine kinases play a critical role in the modulation of growth factor signaling. Activated forms of these enzymes can cause increases in tumor cell proliferation and growth, induce antiapoptotic effects, and promote angiogenesis and metastasis. In addition to activation by growth factors, protein kinase activation by somatic mutation is a common mechanism of tumor genesis [25]. In consistent with the above fact,our results showed that highly significant increase in the expression of TRK in DENA-treated group, while the administration of sulfated oligosaccharides (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) in the DENA-treated rats resulted in significantly inhibitory potential against TRK for all the tested compounds comparing with the DENA group. Maltose $SO_4$ and Maltohexaose $SO_4$ were the most potent inhibitor against TRK expression.

Tumor cell transformation is a multistage process. An *In Situ* tumor after a period of time abruptly sparks the formation of new blood vessels from the preexisting vasculature a process termed as angiogenesis or neovascularization. Tyrosine kinase plays an important role in this process [26]. This process though occurs normally during embryonic development, female reproductive cycle or wound healing is found as a crucial step in tumor transition from benign to malignant form, capable of spreading throughout the body [27].Antiangiogenic drugs stops new vessels from forming around a tumor and break up the existing network of abnormal capillaries that feeds the cancerous mass, thus shrinks the tumor by limiting blood supply[28]. To study the antiangiogenesis effect of the prepared compounds we measured the level of VEGF and ourresults showed that there was over production of VEGF after administration DENA. This is in concurrently with Torimura et al. [29] who stated that VEGF was over expressed intoxicated with DENA. Inhibition of tumor growth by neutralization of VEGF has been verified by treatment with the prepared sulfated compounds which showed decreased amount of VEGF in the cancer-bearing animals, thereby inhibiting the formation of new blood vessel and tumor growth in the order of Maltose $SO_4$>Maltohexaose $SO_4$>Stachyose $SO_4$>Raffinose $SO_4$> Chondroitin-6-sulfate.

It has been proposed that sialic acid appears to be highly sensitive marker for the progression of tumor growth and its angiogenesis [30]. Previously, Rachesky et al. [31] have been reported an increased level of glycoproteins in animals exposed to carcinogen diethylnitrosamine. In the present study, serum total sialic acid level was estimated and found to be significantly elevated ($p<0.001$) in DENA-treated rats (Figure5). The exact cause in rising of sialic acid levels in tumoregenesis is not known, however, various theories are attributed to such increment as: alterations in the cell surface during cell transformation, stimulation of the liver by tumor growth to synthesize glycoproteins or increased glycosylation [32]. Also, neoplastic transformation could lead to sialic acid elevation through the shedding of sialic acid from the tumor cell surface or possibly as a product of the tumor itself [33]. Administration of sulfated compounds leads to significant decrease in TSA level especially in Maltose $SO_4$ and Maltohexaose $SO_4$groups. Consequently, this suggests that these compounds played an important role against DENA-induced hepatocarcinogenesis by maintaining TSA status.

In the present study, results showed that the activities of heparanase and elastase enzymes were very highly significantly increased in DENA-treated group as compared with control. The treatment with sulfated oligosaccharide compounds (Maltose $SO_4$, Raffinose $SO_4$, Stachyose $SO_4$, Chondroitin-6-sulfate, Maltohexaose $SO_4$) resulted in decrease in the activity of both enzymes especially Maltose $SO_4$ and Maltohexaose $SO_4$ (Figure 6). In the meantime, several studies suggested that targeting the activity of heparanase and elastase might be a

beneficial antitumor therapy for liver cancer [34]. Few studies on the potency of heparanase as a marker for HCC were found in the literature [35]. Heparanase is a heparan sulfate (HS) degrading endoglycosidase participating in extracellular matrix degradation and remodeling. Apart of its well-characterized enzymatic activity, heparanase was noted to exert also enzymatic-independent functions, which include enhanced adhesion of tumor-derived cells and primary T-cells [36]. Heparanase seems to modulate two criticalsystems involved in tumor progression, namely vascular epidermal growth factor (VEGF) expressionand epidermal growth factor receptor (EGFR) activation. Neutralizing heparanase enzymatic and non-enzymaticfunctions is therefore expected to profoundly affect tumor growth,angiogenesis and metastasis [37]. A large number of publications clearly link heparanase expression to the process of tumorigenesis in a wide number of cancers, which were reviewed by Zhang et al. [38]. Collectively, they suggest that heparanase plays a fundamental role in sustaining the pathology of malignant diseases and therefore it may provide a potential target for anti-cancer therapy [39].

Elastase is another broad-range proteolytic enzyme thought to be a tumor promoter involved in increasing tumor cell invasiveness by facilitating cell motilityand transendothelial migration as it has the ability to degrade basement membrane and ECM glycoproteins such as elastin, fibronectin, aswell as adhesive molecules and junctional cadherins[40]. Moreover, elastase considered to be the only protease that is able to degrade insoluble elastin, a structural component of elastic tissues such as blood vessel, skin, lung, liver and breast tissues [41]. Furthermore, Taniguchi et al. [42] postulated that increased elastase destroy the barrier between tumor and the local circulatory system, either lymphatic or hematogenous, and result in at least loco-regional metastases.

In conclusion, the results of our study clearly indicate a beneficial effect of sulfated oligosaccharide compounds onchemically-induced rat liver tumorigenesis. To our knowledge, this is the first experimental evidence of the chemopreventive activity of sulfated oligosaccharide compounds. Under our experimental conditions, the tested compounds especially Maltose $SO_4$ and Maltohexaose $SO_4$ exert their antitumor activity through affecting the process of angiogenesis and metastasis and may be potent anticancer agents for inclusion in modern clinical trials after more investigations on higher animals.

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