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Research Article

ANTI-CANCER EFFECT OF SOME PREPARED SULFATED OLIGOSACCHARIDES ON THREE DIFFERENT HUMAN CANCER CELL LINES

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ABSTRACT

Inhibitors of tumor angiogenesis and metastasis are rapdly emerging as important new drug candidates for cancer therapy. In our pursuit to develop new potential anticancer leads sulfated oligosaccharide we synthesized and characterized maltose SO_4, raffinose SO_4, stachyose SO_4 and maltohexaose SO_4. Cytotoxic evaluations of these compounds over a panel of three human cancer cell lines including liver cancer HepG2; breast cancer MCF-7 and lung cancer A549 were carried out with investigation the ability of these compounds in inhibiting tumor growth, metastasis, and angiogenesis. The sulfated compounds exploited potent to moderate growth inhibitory activity in the three cell lines, in particular maltohexaose SO_4 exhibited superior potency to doxorubicin (IC_{50} = 4.20, 4.70 and 6.80 µg/ml, respectively in HepG2, MCF-7 and A549 against 4.50, 4.80 and 7.00 for doxorubicin respectively). On the other hand, the unsulfated oligosaccharides have no cytotoxicity activity. The anticancer activity of these compounds was accompanied by over production of free radicals allowed tumor cells death as monotoring by significantly increased in the activity of superoxide dismutase (SOD) and the levels of hydrogen peroxide (H_2O_2), and nitric oxide (NO), while the activities of catalase (CAT), glutathione peroxidase (GSH-Px) and the level of reduced glutathione (GSH) were significantly lowered with decline the total protein, DNA and RNA content. Furthermore, the prepeared compounds resulted in reduction in the activity of tyrosine kinase (TRK) and cytochrome P450 2E1 (CYP 2E1) which implicated in the development of cancers. Also, the compounds decreasing the level of vascular endothelial growth factor (VEGF) as a marker of angiogenesis and inhibition in a metastatic as measured by reduction in the activity of heparanase and elastase enzymes. In conclusion, the results suggested that the synthesized compounds can be used as good candidate for novel therapeutic strategies for cancer especially maltohexaose SO_4 which possessed significant anticancer activity through regulation angiogenesis and metastasis of cancer.

Keywords: Sulfated oligosaccharide – anticancer – angiogenesis - metastasis -antioxidants

INTRODUCTION

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges, with the incidence of some malignancies continuing to increase [1]. For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects [2]. Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

When patients with cancer are treated with a cytotoxic agent, the pharmacological goal is to deliver as much active drug as possible to the molecular target in the cancer cells; causing sufficient molecular damage lead to cell death [3]. Numerous complexes with biological activity act as anticancer agents have been investigated, however many of them are not suitable for therapeutic use due to their toxic, carcinogenic and mutagenic properties. The use of chemotherapeutic drugs in cancer therapy involves the risk of life threatening host toxicity. The search therefore continues to develop the drugs which selectively act on tumor cells [4].

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 [5]. On the basis of this finding, the development of drugs that inhibit angiogenesis has become an attractive approach to cancer therapy [6]. 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 tumours 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 [7].

Any compound which can inhibit one of the steps in the cascade will be useful in the inhibition of tumor metastasis and tumor growth.

Sulfated oligosaccharides, such as heparan, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, are important ingredients of extracellular matrix (ECM). Recently, many sulfated oligosaccharides have been extracted from bacteria, plants and animals [8] for studying their effects as anti-tumour agents. Furthermore, some experimental studies suggested that the anti-thrombotic activity play an important role in the antitumour effects of sulfated polysaccharides. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumour cells by the inhibition of tissue factor, thrombin, thrombus formation and platelet aggregation [9]. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumour cells by inhibiting heparanase and directly bind to growth factors to inhibit the growth of tumours [10, 11].

However, other studies suggested that the anti-metastatic and anti-thrombotic activities of sulfated polysaccharides were unrelated [12, 13]. Clinical trials have indicated that sulfated polysaccharides influenced the survival in animals and patients with advanced malignancy favourably but without venous thromboembolism [14]. Besides thrombin, sulfated polysaccharides bind to a wide range of proteins, such as growth factors and cell adhesion molecules. As a consequence, it is more likely that the anti-cancer mechanisms of sulfated polysaccharides are not purely an anti-thrombotic effect. Owing to the above facts, the aim of the present work is to synthesize sulfated oligosaccharide such as maltose SO_4, raffinose SO4, stachyose SO_4, and maltohexaose SO_4 that can be inhibiting tumor growth, metastasis, and angiogenesis on different cell lines including liver cancer HepG2; breast cancer MCF-7 and lung cancer A549.

MATERIALS AND METHODS

Chemicals

Dimethylsulphoxide (DMSO), doxorubicin (Doxo) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were purchased from Merck (Darmstadt, Germany). Maltose, raffinose, stachyose and maltohexaose were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Preparation of sulfated oligosaccharides

Sulfation of different oligosaccharides (maltose, raffinose, stachyose, and maltohexaose to produce maltose SO_4, raffinose SO_4, stachyose SO_4 and maltohexaose SO_4) was done as described by Parish [15]. Briefly, one volume of a solution of sulfur trioxide-pyridine complex in dimethyl formamide was added to a suspension of oligosaccharide in dimethyl formamide and pyridine (2 vol : 3 vol). The mixture was heated at 80°C for 2 h. The supernatant was decanted while still warm, and the sticky residue was washed thoroughly with methanol three times. After decanting the residual methanol, the product was dissolved in water and neutralized (to pH 6) with barium acetate (~ 0.7 g in 5 ml of water) with vigorous stirring. After centrifugation (3000 x *g*), the overlying liquid was decanted and the precipitated barium sulfate pellet was washed thoroughly with water. The overlying liquid and washings were combined and applied to a column (2.5 X 14 cm) of DOWEX 50W-X8-400 cation exchange resin. The column was eluted with water until the eluate was neutral. The eluate was stirred and neutralized (to pH 7) with sodium acetate. The solution was diluted with acetone and centrifuged (1750 x *g*) to separate the product. The pellet was finely pulverized by crushing under methanol, stirred while still under methanol, and then the solid was filtered and washed several times with methanol to give the sulfated oligosaccharide. The resultant sulfated oligosaccharides were not contaminated with barium ion (determined by microanalysis and flame ionization) or nitrogen (microanalysis). The homogeneity of sulfated oligosaccharide preparations was also assessed by electrophoresis of samples in 30% polyacrylamide gels using the discontinuous buffer system of Laemmli in the absence of SDS. Sulfated oligosaccharides were visualized in the polyacrylamide gels by toluidine blue staining.

Nuclear magnetic resonance (NMR)

NMR spectra were recorded as ^{1}H-NMR spectra at 27°C with a Jeol EX 300 MHz NMR spectrometer employing standard Bruker NMR software. ^{1}H spectra were referenced to DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) in D_2O as external standard. Coupling constants are reported in Hz and chemical shifts (δ) in ppm.

Cell lines and culturing

Anticancer activity screening for the synthesized compounds utilizing 3 different human tumor cell lines including human liver cancer cell line HepG2, breast cancer cell line MCF-7 and human lung cancer cell line A549 were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/ml) and streptomycin (100 µg/ml) at 37 °C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50 x 10^6 were grown in a 25 cm² flask in 5 ml of complete culture medium.

In Vitro antitumor activity of *unsulfated and sulfated oligosaccharide*s on different cell lines

For *in vitro* cytotoxicity evaluation of unsulfated and the prepared sulfated oligosaccharides, against human HepG2 hepatocellular carcinoma, MCF-7 adenocarcinoma breast cancer and A549 lung adenocarcinoma, cells were plated at a concentration of 0.65 x 10^5 cells per well, in complete culture medium in 96 – well flat – bottomed culture plates (Falcon) for 24 h to assure total attachment. Then 20 µl of various concentration of test compounds (0, 10, 20, 40, 80 or 160 µg/ml) and the standard reference drug (Doxorubicin) were added to the cells suspended in 0.10 ml of DMEM medium after washing the cells several times with phosphate buffered saline (PBS) (0.20 M, pH 7.4), the control cells without the test compounds were also cultured, then the plate was incubated for 24 h at 37 °C, in a humidified 5% CO_2 atmosphere. Cells survival was evaluated at the end of the incubation period with MTT colorimetric assay according to Mosmann [16]. This test is based on the selective ability of living cells not dead cells to reduce the yellow soluble salt of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple-blue insoluble formazan precipitate. The viable cell number is proportional to the production of formazan salts. The crystals of formazan were dissolved in 10 % DMSO and the optical density was measured spectrophotometrically. After incubation, media were removed and 40 µl MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 200 µl of 10 % DMSO/well and plate was shacked gently for 10 min at room temperature. The absorbance was determined photometricaly at 570 nm using microplate ELISA reader (Microplates reader, Asys Hitech, Austria), where the optical density is directly proportion to the number of living cells in the culture. The experiments were performed in six replicates for each compound and the results were normalized to the control value and expressed as percentage of control. The compound concentrations which give 50% growth inhibition are referred to as the IC_{50} and values were obtained mathematically from the concentration response curve using a computer program for probit analysis. IC_{50} calculations were performed using Microsoft Excel and Microcal Origin software for PC.

Biochemical assays

The cells in culture medium were treated with 20 µl of 1/10 of IC_{50} values of the compounds or the standard reference drug, Doxorubicin [17, 18] then incubated for 24 h at 37 ºC, in a humidified 5% CO_2 atmosphere. The cells were harvested and homogenates were prepared in saline using a tight pestle homogenizer until complete cell disruption. The supernatants obtained after centrifugation of cell homogenates was used for biochemical analysis including.

Antioxidant enzyme assays

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) were determined as described by Paglia and Valentine [19]; Aebi [20]; Marklund and Marklund [21], respectively.

Oxidative stress assays

The levels of hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) were determined by methods of Wolf [22]; Granger et al. [23]; Ellman [24], respectively.

Estimation of nucleic acids and protein

Nucleic acids (DNA and RNA) and total protein were precipitated and measured in cell homogenates. Total DNA was extracted and assayed according to the method described by Zhou et al. [25], total RNA was extracted and assayed according to the method adopted from the method provided by Hybaid/AGS (Germany), and total cellular protein was assayed by the method of Lowry et al. [26].

Tyrosine kinase assay

The effect of synthesized compounds on the level of tyrosine kinase (TRK) was determined in cell 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.

Cytochrome P450 2E1 (CYP 2E1) assay

The effect of synthesized compounds on the level of Cytochrome P450 2E1 (CYP 2E1) was determined in cell 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.

Determination of heparanase (HPSE) activity

Determination of heparanase activity in cell 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 cell 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 [27]. The elastase activity was determined as U/mg protein.

Estimation of VEGF concentration

VEGF concentration 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.

Statistical analysis

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

RESULTS

Preparation of sulfated oligosaccharides

To produce a range of sulfated oligosaccharides for *in vitro* testing, all oligosaccharides were sulfated under conditions that resulted in maximum sulfation to produce maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4 by substitutionof all free hydroxyl groups of an oligosaccharide (Figure. 1).

Maltose SO_4, 4-*O*-α-D-Glucopyranosyl-D-glucose sulfate

^{1}H-NMR (300 MHz, DMSO-d_6): δ 13.70 (1H, s, 5-OH), 7.97 (2H, d, J = 8 Hz, H-2′,6′), 6.88 (2H, d, J = 8 Hz, H-3′,5′), 6.78 (1H, s, H-3), 4.83 (1H, d, J = 7.5 Hz, H-1′′), 4.66 (1H, d, J = 7.5 Hz, H-1′′′), 4.01 (1H, t, J = 9 Hz, H-2′′) and 3.85–3.20 (11H, m).

Raffinose SO_4, α-D-Galactosylsucrose sulfate

^{1}H-NMR (300 MHz, DMSO-d_6): δ 13.10 (1H, s, 5-OH), 7.95 (2H, d, J = 8 Hz, H-2,6′), 7.16 (2H, d, J = 8 Hz, H-3′,5′), 6.95 (1H, s, H-3), 6.79 (1H, d, J = 1.8 Hz, H-8), 6.45 (1H, d, J = 1.8 Hz, H-6), 5.04 (1H, d, J = 7.6 Hz, H-1′′), 4.63(1H, d, J = 1.6, H-1′′′), 3.85 (3H, s, 4′ –OCH3), 3.88–3.21 (11H, m) and 0.98 (3H, d, J = 5.9 Hz, CH3-6′′′).

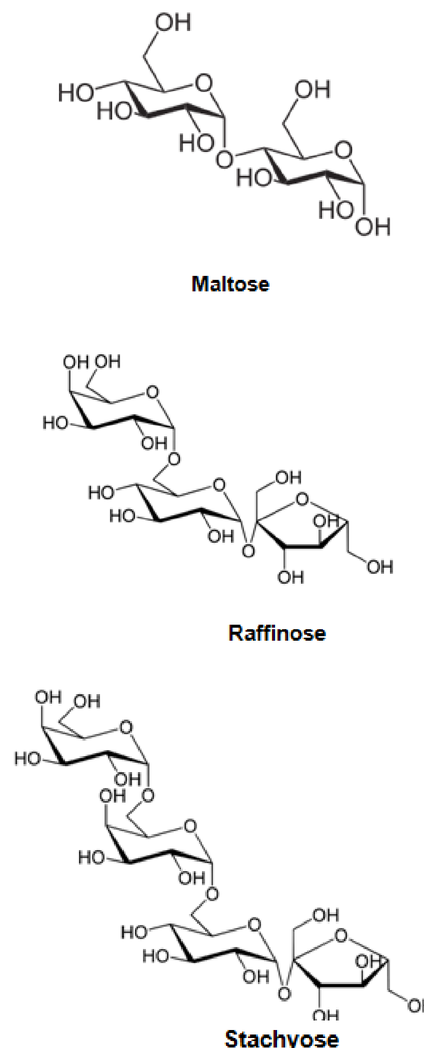
Stachyose SO_4, β-D-Fructofuranosyl-*O*-α-D-galactopyranosyl-(1→6)-*O*-α-D -galactopyranosyl-(1→6)-α- D glucopyranoside sulfate

^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.13 (1H, s, 5-OH), 7.95 (2H, d, J = 8.4 Hz, H-2′,6′), 6.95 (2H, d, J = 8.4 Hz, H-3′,5′), 6.87 (1H, s, H-8), 6.78 (1H, s, H-3), 5.13 (1H, d, J = 7.30 Hz, H-1′′), 4.66 (1H, d, J = 8 Hz, H-1′′′), 4.02 (1H, t, J = 9 Hz, H-2′′) and 3.70–3.22 (11H, m).

Maltohexaose SO_4, α-D-Glucopyranosyl-(1->4)-α-D-glucopyranosyl-(1->4)-α-D-glucopyranosyl-(1->4)-α-D-glucopyranosyl-(1->4)-α-D-glucopyranosyl-(1->4)-D-glucopyranose sulfate

^{1}H-NMR (300 MHz, DMSO-d_6): δ 13.22 (1H, s, 5-OH), 7.95 (2H, d, J = 8.8 Hz, H-2′,6′), 6.96 (2H, d, J = 8.8 Hz, H-3′,5′), 6.86 (1H, s, H-8), 6.85 (1H, s, H-3), 6.48 (1H, d, J = 2.1 Hz, H-6), 5.08 (1H, d, J = 6.9 Hz, H-1′′) and 3.77–3.15 (6H, m).

Once a range of sulfated oligosaccharides had been synthesized they were examined in a range of biological assays.



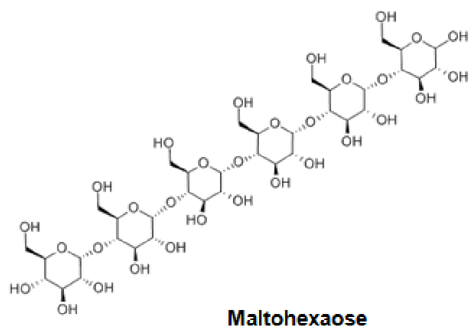


Fig. 1: **Structure of different sulfated oligosaccharides. H = SO_3**

In Vitro antitumor activity of the sulfated oligosaccharides on different human cell lines

Chemotherapy is a major approach for both localized and metastasized cancer. Therefore, the unsulfated and the synthesized sulfated oligosaccharides (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) were screened for their *in vitro* cytotoxicity and growth inhibitory activities against human HepG2 hepatocellular carcinoma, MCF-7 adenocarcinoma breast cancer and A549 lung adenocarcinoma, in comparison with the activity of the known anticancer Doxorubicin as a reference drug. The cytotoxicity activities of the tested compounds were expressed as the median growth inhibitory concentration (IC_{50}) which is the dose that reduces the cell survival to 50%.

It is evident that the HepG2, MCF-7and A549 cells showed normal growth in our culture system and DMSO did not seem to have any noticeable effect on cellular growth. Although all the unsulfated oligosaccharides have no cytotoxicity activity, all of the sulfated compounds show antitumor activities in the three cell lines. A gradual decrease in viability of cancer cells was observed with increasing concentration of the tested compounds, in a dose-dependent inhibitory effect. In case of HepG2 the median growth inhibitory concentrations (IC_{50}) after 24 h for maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4 were 22.00, 15.00, 8.00, 4.20 µg/ml, respectively with IC_{50} values ranging from 5.20 to 22.00 µg/ml. The IC_{50} values for MCF-7 cells after 24 h from treatment with the tested compounds was 26.00 µg/ml for maltose SO_4, 22.00 µg/ml for raffinose SO_4, 14.00 µg/ml for stachyose SO_4 and 4.70 µg/ml for maltohexaose SO_4. The IC_{50} values for A549 after 24 h for maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4 were 38.00, 33.00, 19.00, 6.80, µg/ml, respectively.

It is clear from the data that, the comparison of the cytotoxicity against HepG2, MCF-7 and A549 cell lines (Table 1) of the tested compounds has shown that the growth inhibitory potency follows the order maltohexaose SO_4 > stachyose SO_4 > raffinose SO_4 > maltose SO_4. Maltohexaose SO_4 was the best compound exerting a significant cytotoxic effect in the three cell lines compared with doxorubicin (the commonly used anticancer drug).

Table 1: Effect of sulfated oligosaccharides on the growth of HepG2, MCF-7 and A549 cancer cells

Compounds	IC_{50} (µg / ml)		
	HepG2	MCF-7	A549
DOX	4.50±0.33	4.80±0.42	7.00±0.65
Maltose SO_4	22.00±2.32	26.00±3.00	33.00±3.40
Raffinose SO_4	15.00±1.61	22.00±2.30	38.00±4.00
Stachyose SO_4	8.00±0.76	14.00±1.50	19.00±1.88
Maltohexaose SO_4	4.20±0.50	4.70±0.55	6.80±0.80

IC_{50} concentration (µg/ml) providing 50% cell killing effect. Values are mean ± S.E (n = 4).

Biochemical assays

As shown in Tables 2, 3 and 4 in general treatment of the three cell lines with different compounds (at the 1/10 of IC_{50} values) or doxorubicin resulted in a significant increase in the activity of SOD and level of H_2O_2 higher than those of control cancer untreated cells, accompanied with a significant depletion in the activity of CAT, and GSH-Px, as well as the level of GSH. These changes were in the order of maltohexaose SO_4 > stachyose SO_4 > raffinose SO4 > maltose SO_4 which is in the accordance with the order of cytotoxicity activity of the tested compounds, indicating an increase in the cellular levels of reactive oxygen species.

These results indicate that the antitumor effect of the present compounds may be exerted at least partly by production of reactive oxygen species. Furthermore, the level of total protein and nucleic acids were significantly lower than of control cancer untreated cells, while the level of NO was significantly higher in the cancer cells treated with most compounds as compared to control cells in the order of maltohexaose SO_4 > stachyose SO_4 > raffinose SO_4 > maltose SO_4. The highest activity was found for maltohexaose SO_4, which resulted in the highest SOD activity and H_2O_2 and low activities of CAT and GSH-Px as well as GSH level than the other tested compounds which showed the highest antitumor activity.

Table 2: Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH), hydrogen peroxide (H_2O_2) and on the level of total protein, nucleic acids (RNA and DNA) and nitric oxide (NO) in HepG2 treated cells

Compounds	SOD U/mg Protein	CAT U/mg protein	GSH-Px U/mg Protein	GSH nmol/mg protein	H_2O_2 nmol/mg protein	Protein (µg/10^6 cells)	RNA (µg/10^6 cells)	DNA (µg/10^6 cells)	NO (µmol/mg protein)
DMSO	40.30±4.75	7.60±0.70	9.30±1.00	40.00±5.00	15.70±1.60	110.50 ± 12.30	15.30 ± 1.60	8.50 ± 0.80	1.90 ± 0.16
Dox	130.80±15.65 ^{a}	2.96±0.22 ^{a}	4.40±0.40 ^{a}	21.60±2.40 ^{a}	47.50±5.70 ^{a}	33.60 ± 3.70^{a}	3.40 ± 0.40^{a}	2.50 ± 0.30^{a}	4.20 ± 0.37^{a}
Maltose SO_4	80.00±7.20 ^{b}	5.50±0.60^{b}	6.36±0.65 ^{b}	30. 20±2.80^{a}	30.70±3.20^{a,b}	55.00 ± 5.20^{a,b}	5.00 ± 0.48^{a,b}	5.00 ± 0.45^{a,b}	3.00 ± 0.29^{a,b}
Raffinose SO_4	88.60±9.40^{a,b}	4.00±0.42^{a,b}	6.00±0.66^{a}	28. 20±3.00^{b}	33.60±4.00 ^{b}	45.20 ±4.40^{a,b}	4.60 ± 0.48^{a,b}	4.80 ± 0.50^{a}	3.70 ± 0.35^{a}
Stachyose	100.00±11.50^{a}	3.22±33^{a}	5.50±0.60^{a}	26.18±2.22^{a}	44.00±4.80^{a}	40.70 ±	4.20±	4.20 ±	4.00 ±

SO$_4$						5.40^{a,b}	0.33^{b}	0.33^{a,b}	0.36^{a,b}
Maltohexaose SO$_4$	120.20±11.30^{a}	2.60±0.25^{a}	4.90±0.50a	23.20±1.60^{a,b}	46.20±3.80^{a}	36.50 ± 3.60a	3.00 ± 0.25^{a,b}	3.80 ± 0.30^{a,b}	4.20 ± 0.40^{a,b}

Data are expressed as means ± S.E. of four separate experiments. a and b is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

Table 3: Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH), hydrogen peroxide (H2O2) and on the level of total protein, nucleic acids (RNA and DNA) and nitric oxide (NO) in MCF-7 treated cells

Compounds	**SOD U/mg Protein**	**CAT U/mg protein**	**GSH-Px U/mg Protein**	**GSH nmol/mg protein**	**H_2O_2 nmol/mg protein**	**Protein (µg/10^6 cells)**	**RNA (µg/10^6 cells)**	**DNA (µg/10^6 cells)**	**NO (µmol/mg protein)**
DMSO	42.60±4.35	8.10±0.77	8.90±0.90	42.00±4.70	16.30±1.60	90.50 ± 10.00	17.00 ± 1.50	9.00 ± 0.83	1.94 ± 0.18
Dox	140.80±14.75 ^{a}	3.00±0.24 ^{a}	4.20±0.40 ^{a}	19.80±1.90 ^{a}	50.65±6.00 ^{a}	30.50 ± 3.40^{a}	3.20 ± 0.29^{a}	2.80 ± 0.30^{a}	4.40 ± 0.40^{a}
Maltose SO$_4$	95.15±9.30^{a,b}	5.62±0.52^{a,b}	7.40±0.72^{a}	38. 60±4.20^{a,b}	33.00±2.80^{a,b}	66.40 ± 6.30^{a,b}	6.60 ± 0.70^{a,b}	5.80 ± 0.65^{a,b}	3.50 ± 0.30^{a}
Raffinose SO$_4$	105.00±12.00^{a}	5.00±0.47^{a}	7.25±0.70^{a}	36.20±3.70^{a,b}	37.30±4.20^{a,,b}	57.50 ± 6.20^{a,b}	4.80 ± 0.52^{a,b}	5.00 ± 0.46^{a}	3.70 ± 0.38^{a}
Stachyose SO$_4$	120.00±12.30^{a,b}	4.80±0.42 ^{b}	6.90±0.75 ^{b}	30. 60±3.25^{b}	40.00±4.40^{a,b}	44.20 ± 4.80^{a,b}	4.30± 0.30^{a,b}	4.40 ± 0.50^{b}	3.80 ± (
Maltohexaose SO$_4$	130.70±12.00 ^{a}	4.25±0.30^{b}	6.60±0.70 ^{b}	20. 30±2.20^{a}	46.60±4.60^{a}	30.60 ± 3.30^{a}	3.80 ± 0.35^{a}	3.00 ± 0.35^{a}	4.00 ± 0.36^{a}

Data are expressed as means ± S.E. of four separate experiments. a and b is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

Table 4: Effect of treatment with the prepared compounds on the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), as well as the levels of reduced glutathione (GSH), hydrogen peroxide (H2O2) and on the level of total protein, nucleic acids (RNA and DNA) and nitric oxide (NO) in A549 treated cells

Compounds	SOD U/mg Protein	CAT U/mg protein	GSH-Px U/mg protein	GSH nmol/mg protein	H_2O_2 nmol/mg protein	Protein (µg/10^6 cells)	RNA (µg/10^6 cells)	DNA (µg/10^6 cells)	NO (µmol/mg protein)
DMSO	45.00±4.25	6.30±0.66	9.60±1.10	43.00±4.60	16.80±1.70	120.30 ± 13.00	17.00 ± 1.80	9.20 ± 0.86	2.00 ± 0.18
Dox	147.60±15.00 ^{a}	2.56±0.24 ^{a}	5.00±0.46 a	23.50±2.60 a	48.50±5.30 a	36.60 ± 3.70a	3.00 ± 0.37^{a}	3.30 ± 0.36a	4.70 ± 0.77a
Maltose SO$_4$	80.30±7.20^{a}	4.20±0.40^{a}	7.20±0.68^{a}	40.00±4.40^{a}	27.60±3.25^{a}	65.20 ±6.60^{a,b}	7.80 ± 0.80^{a}	6.60 ± 0.70^{a,b}	2.80 ± 0.25^{a}
Raffinose SO$_4$	90.60±9.00^{a,b}	4.30±0.35	6.90±0.70^{a}	35.40±3.60^{a}	30.00±2.80 ^{a}	57.50 ± 6.00^{a}	5.30 ± 0.60^{a}	6.00 ± 0.60^{a}	3.00 ± 0.26^{a}
Stachyose SO$_4$	92.00±8.20 ^{b}	3.90±0.40^{b}	6.20±0.70 ^{b}	33. 00±2.80^{b}	38.00±3.60^{a,b}	44.20 ± 5.80^{b}	4.20± 0.30^{b}	5.20 ± 0.55^{b}	3.20 ± 0.30^{a,b}
Maltohexaose SO$_4$	110.00±11.20^{a}	3.30±0.25^{b}	6.00±0.50 ^{b}	30.00±3.00^{b}	47.80±5.20^{a,b}	35.00 ± 4.00^{a,b}	4.20 ± 0.44^{a,b}	4.00 ± 0.47^{a,b}	4.00 ± 0.44^{a,b}

Data are expressed as means ± S.E. of four separate experiments. a and b is significant difference from control and doxorubicin groups respectively at ($p < 0.05$).

The treatment of cancer cells (HepG2, MCF-7 and A549) with doxorubicin or sulfated oligosaccharides (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) at a dose of 1/10 of the IC_{50} values resulted in significantly inhibitory potential against TRK (Figure 2) and cytochrome P450 (CYP) 2E1 (Figure 3) in the three cell lines as compared with control (DMSO treated).

In case of TRK, the treatment of HepG2 cells with maltose SO_4, raffinose SO_4, stachyose SO_4 and maltohexaose SO_4 resulted in 27, 43, 56 and 72% respectively inhibitory potential comparing with the control (DMSO treated) cells. In case of MCF-7 cells treatment the inhibitory potential was 13, 32, 40.50 and 71% respectively. While In case of A549 cells the inhibitory potential was 33, 37, 56 and 64% respectively. On the other hand, treatment of HepG2, MCF-7 and A549 cells with doxorubicin resulted in 83, 82 and 82 % inhibitory potential against TRK respectively as compared with DMSO treated cells.

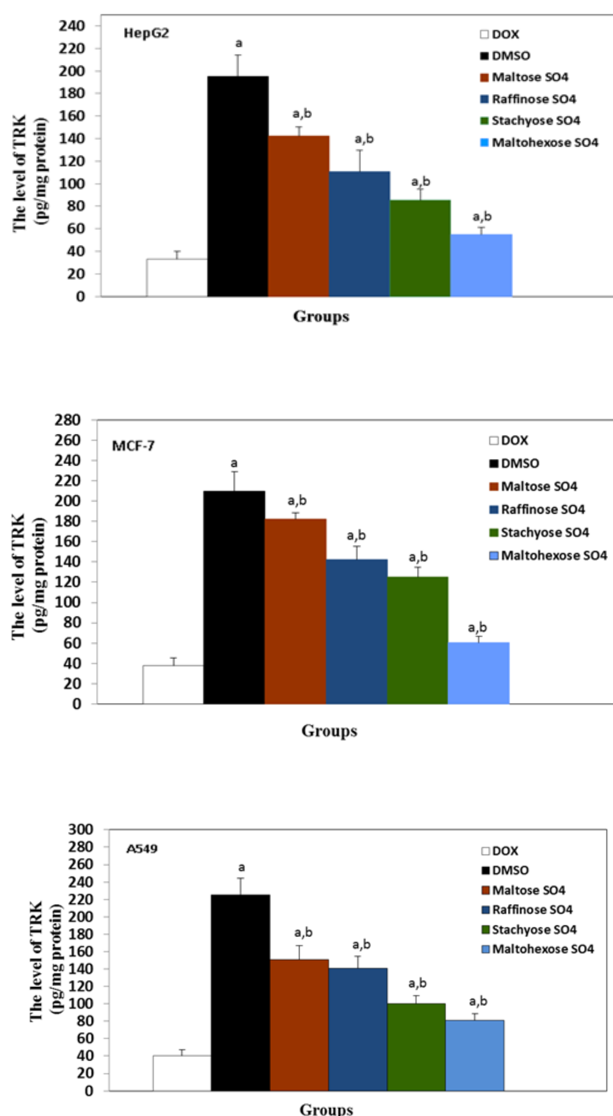


Fig. 2: Effect of treatment with the prepared sulfated oligosaccharides (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) on the level of protein kinase (TRK) in HepG2, MCF-7 and A549. Data were expressed as mean ± S.E. (n=4), [a] and [b] is significant difference from doxorobucin and DMSO – cancer treated cells respectively at ($p < 0.05$)

In case of cytochrome P450 (CYP) 2E1, the treatment of HepG2 cells with maltose SO_4, raffinose SO_4, stachyose SO_4 and maltohexaose SO_4 resulted in 49, 61, 75 and 80% respectively inhibitory potential comparing with the control (DMSO treated) cells. In case of MCF-7 cells treatment the inhibitory potential was 54, 58, 71 and 76% respectively. While In case of A549 cells the inhibitory potential was 38, 45, 52 and 70% respectively. On the other hand, treatment of HepG2, MCF-7 and A549 cells with doxorubicin resulted in 88, 85 and 84 % inhibitory potential against CYP 2E1 respectively as compared with DMSO treated cells.

It is clear from the forgoing results that, maltohexaose SO_4 was the most potent inhibitors against both TRK and CYP 2E1 as compared to cancer cells reach near the effect of doxorubicin and this can explain their anticancer effects which in consistent with their cytotoxicity results.

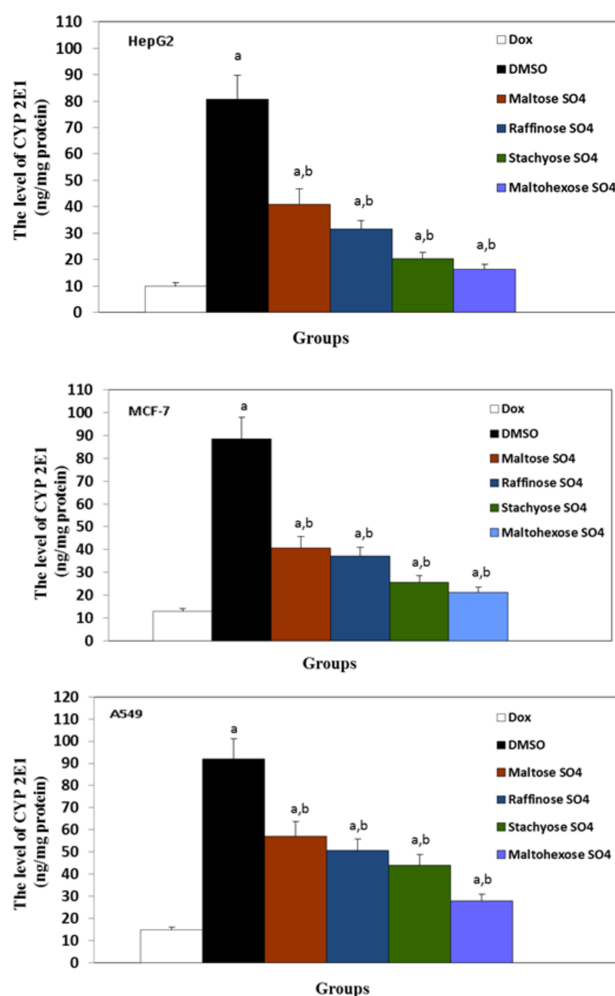


Fig. 3: Effect of treatment with the prepared sulfated oligosaccharides (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) on the level of (CYP) 2E1 in HepG2, MCF-7 and A549. Data were expressed as mean ± S.E. (n=4), [a] and [b] is significant difference from doxorobucin and DMSO - treated cancer cells respectively at ($p < 0.05$)

Several studies suggested that targeting the activity of heparanase and elastase might be a beneficial antitumor therapy for liver cancer. In the present study, results showed that the activities of heparanase and elastase enzymes were very highly significantly increased in cancer cells (HepG2, MCF-7 and A549). The treatment of the cancer cells with sulfated oligosaccharide compounds (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) at the safe doses, resulted in decrease in the activity of heparanase activity of the both enzymes especially in maltohexaose SO_4 treated cells (Table 5).

VEGF has become one of the most common targets in delaying angiogenesis. In this study, it was found that the level of VEGF was very highly significantly increased in cancer cells (HepG2, MCF-7 and A549). The treatment of the cancer cells with sulfated oligosaccharide compounds (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) at the safe doses, resulted in decrease in VEGF levels in all the compounds especially maltohexaose SO_4 (Table 5). This reduction in VEGF level after protector administration could be related to the anti-angiogenic actions.

Table5: Effect of treatment with the prepared sulfated oligosaccharides (Mmaltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) on the activity of heparanase and elastase as markers for metastasis and on the level of VEGF as a marker for angiogenesis in HepG2, MCF-7 and A549 cancer cell lines

Compounds	Heparanase (U/mg protein)			Elastase (U/mg protein)			VEGF (Pg/mg protein)		
	HepG2	MCF-7	A549	HepG2	MCF-7	A549	HepG2	MCF-7	A549
DOX	2.0±0.25	3.5±0.3	4.6±0.4	0.21±0.03	0.31±0.04	0.5±0.06	22.0±2.6	30.0±3.3	33.0±3.5
DMSO	6.0±7.0[a]	7.7±0.7[a]	9.2±0.9[a]	1.08±0.08[a]	1.09±0.09[a]	1.3±0.1[a]	55.8±6.0[a]	80.2±8.0[a]	85.0±9.0[a]
Maltose SO_4	4.7±0.6[a,b]	6.2±0.6[a,b]	7.7±0.8[a,b]	0.95±0.09[a]	0.96±0.08[a]	1.2±0.12[a]	50.0±5.0[a,b]	58.0±6.0[a,b]	60.0±6.5[b]
Raffinose SO_4	4.2±0.42[a,b]	5.3±0.55[a,b]	6.8±0.77[a,b]	0.89±0.09[a]	0.9±0.09[a]	1.1±0.1[a]	38.0±4.0[a,b]	45.0±5.0[a,b]	50.0±5.7[a,b]
Stachyose SO_4	3.3±0.37[a,b]	4.8±0.5[a,b]	6.0±0.7 2[a,b]	0.75±0.07[a,b]	0.76±0.07[a]	0.96±0.09[a]	32.0±3.5[a,b]	40.0±4.4[a,b]	44.0±4.7[a,b]
Maltohexaose SO_4	2.2±0.3[b]	3.6±0.33[b]	5.4±0.6[a,b]	0.37±0.04[b]	0.38±0.04[b]	0.6±0.06[b]	23.0±2.7[b]	33.9±3.5[b]	36.0±4.0[a,b]

Data were expressed as mean ± S.E. (n=4), [a] and [b] is significant difference from doxorobucin and DMSO - treated cancer cells respectively at ($p < 0.05$).

DISCUSSION

The treatment of tumour therapy remains an important and challenging therapeutic problem; the majority of the solid tumours are currently not curable by chemotherapy. Half of all cancer patients fail to respond to chemotherapy or relapse from the initial response and ultimately die from their metastatic disease [28]. The aim of most cancer chemotherapeutic drugs currently in clinical use is to kill malignant tumour cells by inhibiting some of the mechanisms implied in cellular division. Early approaches of selectively inhibit tumour growth were generally disappointing in clinical studies. The investigation of tumour growth inhibitors is a major obstacle in the medical field [29]. For these reasons, the development of novel antitumour drugs is still necessary and has very much demand. In the present study, we evaluated the anti-cancer effect of sulfated oligosaccharides on tumour biology.

After synthesis of a range of sulfated oligosaccharides maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4, they were examined for their cytotoxicity activity. The results of cytotoxicity tests revealed that, although all the unsulfated oligosaccharides have no cytotoxicity activity, most of the tested sulfated compounds exploited potent to moderate growth inhibitory activity in the three cell lines (HepG2, MCF-7 and A549), especially maltohexaose SO_4 which showed suprior effect comparing to doxorubicin. To elucidate the mechanism by which the prepared sulfated oligosaccharides exert their antitumor activities, we estimated the activities of the free-radical-metabolizing enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) as well as the levels of the oxidative stress parameters including hydrogen peroxide (H_2O_2), nitric oxide (NO) and reduced glutathione (GSH) in the three cell lines HepG2, MCF-7 and A549 treated with the prepared compounds comparing to the reference standard doxorubicin. Additionally, the effect of these compounds on the levels of total protein and nucleic acids was determined.

The antitumor activities of these compounds were accompanied by increases in SOD activities of tumor-treated cells compared to control cells. This means that these compounds can cause H_2O_2 production. The H_2O_2 produced should be rapidly removed through the activation of CAT and GSH-Px. The present results show that activities of CAT and GSH-Px and the level of reduced GSH are lowered in groups treated with compounds compared to control cells. Consequently, the excess H_2O_2 produced in tumor cells with the compounds can not be removed. In other words, the accumulation of H_2O_2 and other free radicals in tumor cells should be partly the cause of tumor cell killing. Thus, the results of the present study are consistent with the hypothesis that the prepared compounds exert their antitumor effects because they produce reactive oxygen species (ROS). Moreover, the results showed that treatment with these compounds lead to an increased in the level of NO and according to Blanco et al. [30] an increase in NO level leads to apoptosis (programmed cell death), whereas an increase in ROS leads to necrosis (cell death), so, the way a tumor cell dies reflects the radical balance in the system. From our data it is observed that the compounds having higher SOD activity with lowering CAT, and GSH-Px activities are those having high antitumor activity.

In addition our results showed that the increase in the NO level was accompanied with depletion the levels of total protein and nucleic acids compared to control and this can be explained by the fact that several cytotoxic effects, including reactions with proteins and nucleic acids. The main targets of NO in proteins are the SH group [31] and Fe of active sites [32]. In the nucleus, NO has been shown to cause mutations of genes [33], and inhibition of DNA repair enzymes [34], and to mediate DNA strand breaks [35]. NO has been shown to mediate apoptosis [36].

Our results are inconsistent with Bienvenu et al. [37] who reported that most chemotherapeutic agents cause cells to over generate ROS and thus, are capable of inducing apoptosis, and causing oxidative damage to DNA, proteins and lipids. The cascade of signals mediating apoptosis often involves a ROS intermediate messenger, and ROS can short circuit the pathway, bypassing the need for upstream signals for cell suicide. Huang et al. [38] reported that regulation of free radical-producing agents may also have important clinical applications.

Protein tyrosine kinases are enzymes involved in many cellular processes such as cell proliferation, metabolism, survival and apoptosis. Tyrosine kinase activation has long been known to be an important mechanism underlying tumor development, proliferation and spread ([39, 40]. Blocking tyrosine kinase activity have, therefore, been attractive targets of anti-cancer therapies [41]. Our result demonstrated that, the administration of sulfated oligosaccharide compounds (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) in the three cell lines resulted in significantly inhibitory potential against TRK. Maltohexaose SO_4 was the most potent inhibitors against TRK as compared to doxorubicin treated cells in he three cell lines especially in HepG2 cell line which reach to its level in doxorubicin treated cells, and this can explain their anticancer effects. On the other hand, human CYP 2E1 is responsible for activation of carcinogenic nitrosamines such as dimethyl and diethylnitrosamine [42]. CYP 2E1 mRNA and protein are expressed in peripheral human lung and bronchial mucosa, human A549 lung cells, and in human liver. There is precedence for CYP 2E1 inhibition by naturally occurring compounds [43]. The administration of sulfated oligosaccharide compounds (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) in the three cell lines, resulted in significantly decrease in the level of CYP 2E1 in the three cell lines as compared with control untreated cancer cells in descending order of HepG2, MCF-7, A549. The data revealed that there were consistent between the inhibition of TRK and CYP 2E1 expression and cytotoxicity for the tested compounds.

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 tumours 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 [44]. Heparanase is a heparan sulfate (HS) degrading endoglycosidase participating in extracellular matrix degradation and remodeling [45]. Heparanase seems to modulate two critical systems involved in tumor progression,

namely vascular epidermal growth factor (VEGF) expression and epidermal growth factor receptor (EGFR) activation. Neutralizing heparanase enzymatic and non-enzymatic functions is therefore expected to profoundly affect tumor growth, angiogenesis, and metastasis [46]. 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 [47]. In addition, elastase is another broad-range proteolytic enzyme thought to be a tumor promoter involved in increasing tumor cell invasiveness by facilitating cell motility and transendothelial migration as it has the ability to degrade basement membrane and ECM glycoproteins such as elastin, fibronectin, as well as adhesive molecules and junctional cadherins [48]. Moreover, elastase considered to be protease that is able to degrade insoluble elastin, a structural component of elastic tissues such as blood vessel, skin, lung, liver and breast tissues [49]. Furthermore, Taniguchi et al. [50] 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 the present study, results showed that the activities of heparanase and elastase enzymes were very highly significantly increased in cancer cells (HepG2, MCF-7 and A549). The treatment of the cancer cells with sulfated oligosaccharide compounds (maltose SO_4, raffinose SO_4, stachyose SO_4, maltohexaose SO_4) at the safe doses, resulted in decrease in the activity of heparanase activity of the both enzymes especially in maltohexaose SO4 treated cells. This finding confirmed that sulfated oligosaccharides are effective antimetastatic compounds and there being a reasonably good correlation between the antimetastatic activity of these compounds and their heparanase and elastase inhibitory activity.

Tumor angiogenesis performs a critical role in tumor progression through which the tumor establishes an independent blood supply, consequently facilitating tumor growth and favoring the transition from hyperplasia to neoplasia [51]. This process is regulated through a balance of pro- and anti-angiogenic factors and researchers have found out that VEGF seems to be the most potent and predominant angiogenic cellular factor sustaining tumor growth [52]. The demonstration that prepared oligosaccharides can reduce tumor vascularity was a critical finding because it supports the *in vitro* angiogenesis inhibition. In this study, it was found that the treatment of the three cancer cell lines with sulfated oligosaccharide compounds (maltose SO_4, raffinose SO4, stachyose SO_4, maltohexaose SO_4), causes significant decrease in VEGF levels especially maltohexaose SO_4 in the three cell lines. This reduction in VEGF value after the administration of oligosaccharides could be related to the anti-angiogenic actions. According to [51] inhibiting tumor angiogenesis, can block one of the fundamental requirements for tumor growth. As the most potent angiogenic factor, VEGF has become one of the most common targets in delaying angiogenesis [53]. Of course there is the additional possibility that sulfated oligosaccharides are inhibiting *in vivo* angiogenesis not only by blocking angiogenic growth factor action, but also via heparanase inhibition. Heparanase activity has been implicated in several aspects of neovascularization, such as degradation of the endothelial ECM during endothelial cell migration and the release of heparan sulfate bound angiogenic factors associated with the ECM. In addition, sulfation was found to be essential for biological activity because unsulfated compounds were inactive in all assays. With increasing sulfation as in maltohexaose SO_4, there was a steady increase in the ability to inhibit angiogenesis and anti-metastasis activities.

CONCLUSIONS

In conclusion, sulfated oligosaccharide such as maltose SO_4, raffinose SO4, stachyose SO_4, maltohexaose SO_4, especially maltohexaose SO_4 may be potent anticancer agents for inclusion in modern clinical trials acting by binding to a wide range of proteins, such as growth factors and cell adhesion molecules. As a consequence, sulfated oligosaccharides could affect the proliferation, differentiation, apoptosis and metastasis of cancer cells. In future, further study will be carried out on the effect of the prepared oligosaccharides for inhibiting of metastasis and angiogenesis of tumor in the experimental animals carrying liver cancer induced chemically.

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