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Research Article

Evaluation the Potential of Novel Antitumor Candidates on Ehrlich Ascites Tumor-Bearing Mice

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ABSTRACT

In our previous work fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) as well as sulfated oligosaccharides (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) were prepared. The objective of this study was to elucidate the antitumor activity of prepared compounds against Erlich ascites carcinoma cells (EAC) bearing mice through monitoring the tumor volume and life span of the mice using ascites tumor and solid tumor models. The results revealed that all the tested compounds when supplemented at 1/10 of their LD_{50} (median lethal dose), showing antitumor potential and caused increased in the life span of mice. Otherwise, the antitumor potential in simultaneous treatment groups was higher than the groups in which treatment was started 10 day post tumor inoculation. Compound 3C and Maltose SO_4 showed highest activity on reduction tumor volume from 5.30±0.60 CC in control mice to 2.20±0.19 and 2.00±0.18 CC respectively, while the positive drug, doxorubicin treated group revealed reduction of tumor volume from 5.30±0.60 to 1.10±0.14 CC. Otherwise 3C and Maltose SO_4 also showed the most highest survival rate (39.00±2.70 and 40.00±3.40 days, respectively) with the increase of life span 77% and 82% respectively compared to control, while doxorubicin showed increased in survival rate by 86% (41.00±4.30 days) as compared to control. It is obviously from the present study that the tested compounds especially 3c and Maltose SO_4 possessed antitumor activity and prolong the life span of mice bearing tumor.

Keywords: Antitumor, thiopyrmidines, sulfated oligosaccharides, Erich ascites carcinoma.

INTRODUCTION

There is a need for chemotherapeutic agents for treatment of neoplastic diseases that are safe for therapeutic use and that exhibit selective toxicity with respect to the pathological condition. Furthermore, there is a need for chemotherapeutic agents with modified or improved profiles of activity. The search therefore continues to develop new antitumor agents has been extensively studied[1].

It is well known that, pyrimidine and fused heterocyclic pyrimidine derivatives have attracted a great deal of interest in particular 4-hydrazinopyrimidine derivatives, which were tested for their medicinal, bactericidal and fungicidal activity[2-5]. Pyrimidine and heterocyclic annulated pyrimidine derivatives attracted great interest due to the wide variety of interesting biological activities observed for these compounds, such as anticancer[2], antiviral[6], anti-HIV-1 activity [7], anti-inflammatory[8] and antimicrobial activity[9].

Sulfated oligosaccharides, such as heparan, heparan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate, are important ingredients of extracellular matrix. Recently, many sulfated oligosaccharides have been extracted from bacteria, plants and animals for studying their effects as anti-tumor agents[10]. Furthermore, some experimental studies suggested that the anti-thrombotic activity play an important role in the antitumor effects of sulfated polysaccharides. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumor cells by the inhibition of tissue factor, thrombin, thrombus formation and platelet aggregation[11]. Sulfated oligosaccharides could suppress the proliferation and metastasis of tumor cells by inhibiting heparanase and directly bind to growth factors to inhibit the growth of tumors[12].

Owing to the above facts, the aim of the present work is to study the antitumor effect of previously synthesized thiopyrimidine acyclic nucleosides and thioglycoside analogs as well as sulfated oligosaccharides (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) against Erlich ascites carcinoma cells (EAC) bearing mice through monitoring the tumor volume and life span of the mice.

MATERIALS AND METHODS

Previously prepared fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c)[13] as well as

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Table 1: In *vivo* acute toxicity ((LD_{50}) of fused pyrimidine and thiopyrimidine nucleoside analogs as well as sulfated oligosaccharides.

Compounds	LD_{50} µg/Kg b.w.
2	132.00±15.50
3b	108.00±12.75
3c	80.00±10.00
4a	120.00±13.70
4c	160.00±17.50
Maltose SO_4	600.00±65.75
Raffinose SO_4	1006.00±115.00
Stachyose SO_4	800.00±85.00
Chondroitin-6-sulfate	1210.00±125.00
Maltohexaose SO_4	680.00±70.00

Values are mean ± SE

sulfated oligosaccharides (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) were used[14]. All chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Animals

Male Swiss albino mice (body weight 20±2 g) were purchased from animal breeding center of National Research Centre, Egypt. They were kept for a week under environmentally controlled conditions (constant temperature 25-27 °C, with 12 h light/dark cycle) for one week prior to starting the experiments, and they were provided with water and commercial diets. All experimental procedures were carried out in accordance with the protocol was approved by the institutional animal care and ethics guidelines of National Research Centre, Egypt.

Cell line

Ehrlich's ascites carcinoma (EAC) cell line was obtained from National Cancer Institute, Cairo University, Egypt. The cells were maintained by intraperitoneal inoculation of $1x10^6$ viable cells in mice.

Assay of acute toxicity

The acute toxicity of the tested compounds was determined *in vivo* according to Prieur *et al.* [15]; Ghosh [16]. Briefly, adult Swiss albino mice fasted for 12 h were randomly divided into groups of 10 per group. Each group was separately administrated once intraperitoneal (i.p.) in a value of 1 ml/kg b.w. gradually increasing doses (0-500 µg/kg b.w.) for the fused pyrimidine and thiopyrimidine nucleoside analogs and (0-1500 µg/kg b.w.) for sulfated oligosaccharides. Control animals received the vehicle alone. The fasted mice in both test and control groups were then provided with food and water immediately after the administration. Mortality of the animals was observed up to one month post-treatment. LD_{50} (the median lethal doses) of each compound was determined (the dose resulted in 50% mortality of the animals).

Antitumor activity

The antitumor activity of the tested compounds was determined using ascites tumor and solid tumor models.

Ascites tumor model

Animals were divided into groups of 10 animals in each group. All the animals were injected i.p. with 1×10^6 viable EAC cells in PBS (aspirated from 15 day old EAC ascites tumor in mice). After 24 h of tumor inoculation the tested compounds were administered i.p. at dose of 1/10 of their LD_{50} values and continued for 10 consecutive days. The group administered with vehicle alone (DMSO) was maintained as control. Doxorubicin (2 mg/kg body weight, i.p., for 10 days) was used as the standard reference drug. The mortality rate was noted in each group and the percent increase in life span (ILS) was calculated as described by Ahluwalia *et al.* [17]; Joy *et al.* [18].

Effect of tested compounds when administered simultaneous with tumor inoculation.

Viable EAC cells (1×10^6) in 0.1 ml PBS were transplanted subcutaneous into the right groin of mice. Tested compounds were administered i.p., at 1/10 of their LD_{50} values 24 h post tumor implantation and extended for 10 consecutive days. The control group was treated with vehicle (DMSO) and the standard reference group was treated with doxorubicin (2 mg/kg body weight, i.p, 10 days). The tumor development on animals in each group was determined by measuring the diameter of tumor growth in two perpendicular planes using vernier calipers on every fifth day. The tumor volume was calculated by measuring the radii of the tumor at two different planes as mentioned by Ma *et al.* [19]; Mary *et al.* [20].

Effect of tested compounds when administered after tumor development.

Antitumor activity of the compounds was tested after tumor development in mice. Solid tumor development in mice was induced as described earlier. After 13 days of tumor transplantation animals were divided into groups of 10 animals each and were administered i.p. at 1/10 of their LD_{50} values for 10 consecutive days. The group treated with vehicle was maintained as control and the standard reference group was treated with doxorubicin (2 mg/kg body weight, i.p., 10 days). Tumor diameter was measured using a vernier caliper on every fifth day and volume was calculated.

Statistical analysis

Values are recorded as mean ± S.E. The data were analyzed by Student's *t*-test; differences below the 0.5 level ($P<0.05$) was considered as statistically significant.

RESULTS

Acute toxicity of compounds

As shown in table 1, the median lethal doses (LD_{50}, the dose resulted in 50% mortality of the animals) of each compound was determined. Fused pyrimidine and thiopyrimidine nucleoside analogs compounds 2, 3b, 3c, 4a and 4c showed marked acute activity. The concentrations required by compounds 2, 3b, 3c, 4a and 4c for 50% mortality of the animals were found to be 132, 108, 80, 120 and 160 µg/kg body weight, respectively.

Sulfated oligosaccharides (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose

SO_4) showed marked acute activity. 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 600, 1006, 800, 1210 and 680 µg/kg body weight, respectively.

Antitumor activity

Antitumor activity of the tested compounds was determined using ascites tumor and solid tumor models.

Ascites tumor model

As shown in Table 2. In the ascites tumor model the administration of prepared compounds (2, 3b, 3c, 4a and 4c) significantly increase (P<0.05) the life span of the animals as compared with control group (animals in the EAC injected alone) except compound **4c**, which revealed insignificant change. There is a gradual increase in life span using the tested compounds from compound 4c, 2, 4a, 3b to compound 3c. The increases in life span were 9, 32, 45, 64, and 77% respectively. The standard reference drug (doxorubicin 2 mg/kg body weight) exhibited 86% increase life span of the animals. The animals in the EAC control group were dead after 22 days. It is clear from the data that compound **3c** was the best compound exerting a significant anticancer activity compared with doxorubicin.

On the other hand, the administration of sulfated oligosaccharide compounds (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) significantly increase (P<0.05) the life span of the animals as compared with control group (animals in the EAC injected alone). There is a gradual increase in life span using the prepared compounds from compound Chondroitin-6-sulfate, Raffinose SO_4, Stachyose SO_4, Maltohexaose SO_4 to compound Maltose SO_4 follows the order Maltose SO_4 > Maltohexaose SO_4 > Stachyose SO_4 > Raffinose SO_4 > Chondroitin-6-sulfate. Maltose SO_4 was the best compound exerting a significant anticancer activity. The increases in life span were 82, 41, 54, 27, and 73% respectively in Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4. Doxorubicin exhibited 86% increase life span of the animals. All the animals in the EAC injected alone group were dead after 22 days (table 2).

Effect of the tested compounds when administered simultaneous with tumor inoculation

All the tested compounds of fused pyrimidine and thiopyrimidine nucleoside analogs or sulfated oligosaccharide possessed significant antitumor activity against solid tumor models. i.p. administration of the of fused pyrimidine and thiopyrimidine nucleoside analogs in simultaneously treated groups showed significant antitumor activities against solid tumor and reduce the tumor volume (Figure 1). Among the tested compounds, 3c show the highest antitumor activity. The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 5.30 CC. The tumor volume reduced to 2.20 CC when treated with compound 3c, and to 2.60 CC when treated with compound 3b. While reduced to 4.70, 3.60 and 3.20 CC when treated separately with compound 4c, 2 and 4a respectively.

Table 2: Effect of treatment with fused pyrimidine and thiopyrimidine nucleoside analogs compounds as well as sulfated oligosaccharides on the survival of ascites tumor harboring mice inoculated with EAC cell line.

Compounds	Survival time (days)	% increase in life span of animals
Control	22.00±1.80	0
Doxorubicin	41.00±4.30[a]	86
2	29.00±2.50[a]	32
3b	36.00±2.70[a]	64
3c	39.00±2.70[a]	77
4a	32.00±1.90[a]	45
4c	24.00±2.30	9
Maltose so$_4$	40.00±3.40 [a]	82
Raffinose so$_4$	31.00±2.50 [a]	41
Stachyose so$_4$	34.00±3.00 [a]	54
Chondroitin-6-sulfate	28.00±3.20 [a]	27
Maltohexaose so$_4$	38.00±3.70 [a]	72

Values are mean ± SE.; n =10 mice; [a]P<0.05 significant with respect to control.

On the other hand, i.p. administration of the sulfated oligosaccharide in simultaneously treated groups showed significant antitumor activities against solid tumor and reduce the tumor volume (Figure 2). Among the compounds, Maltose SO_4 show the highest antitumor. The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 5.30 CC. The tumor volume reduced to 2.00 CC when treated with Maltose SO_4, and to 2.50 CC when treated with Maltohexaose SO_4. While reduced to 3.30, 2.90 and 4.00 CC when treated separately with Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate respectively.

Effect of tested compounds when administered after tumor development

The fused pyrimidine and thiopyrimidine nucleoside analogs or sulfated oligosaccharides were also highly effective against development solid tumor. The treatment with the fused pyrimidine and thiopyrimidine nucleoside analogs for 10 consecutive days after tumor development showed reduction in the volume tumor (Figure 3). The tumor volume of animals without treatment on the 30th day after tumor inoculation was found to be 6.00 CC. The tumor volume reduced to 2.90 CC when treated with compound 3c, to 3.25 CC when treated with compound 3b, and to 3.50 CC when treated with compound 4a. While reduced to 4.00 and 5.27CC when treated separately with compounds 2 and 4c respectively. From the forgoing results we noticed that administration of fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) shows more antitumor activity in simultaneous treated groups than the groups in which treatment was started 10 days after tumor inoculation.

The sulfated oligosaccharide compounds were also highly effective against development solid tumor. The treatment with the compounds for 10 consecutive days after tumor development showed reduction in the volume tumor (Figure 4). The tumor volume of animals without

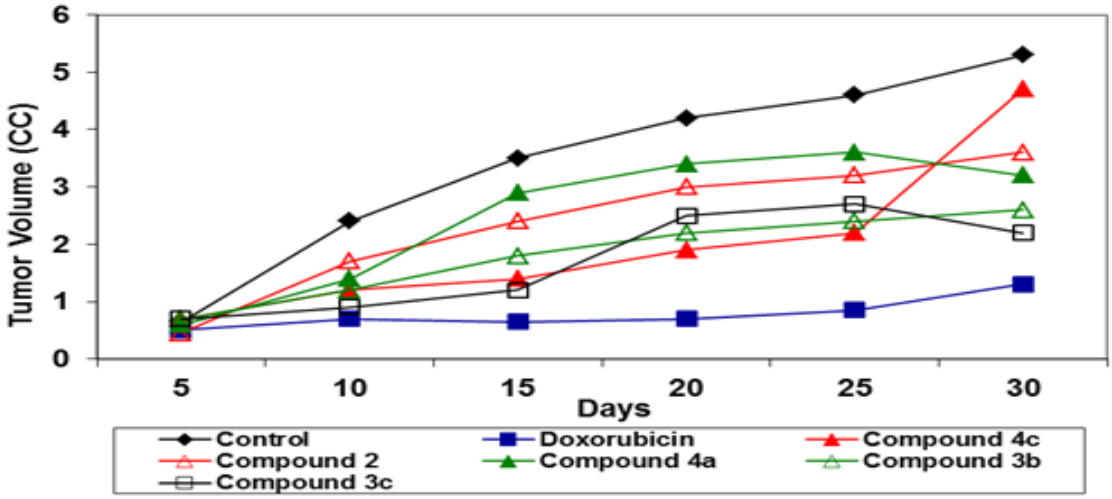

Figure **1**: Effect of the fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) administration on solid tumor development (simultaneous treatment).

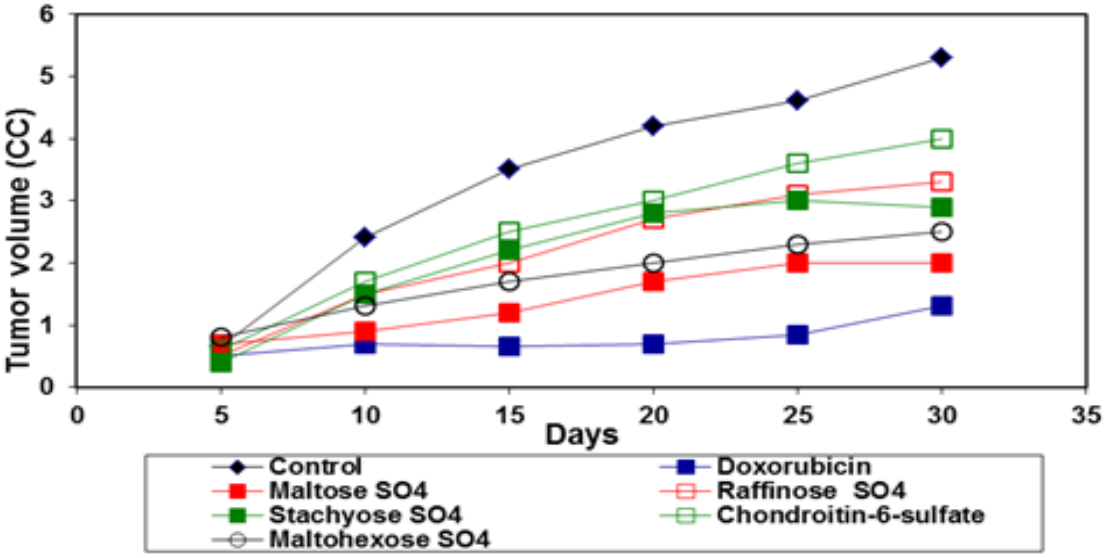

Figure 2: Effect of the tested sulfated oligosaccharides (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) administration on solid tumor development (simultaneous treatment).

treatment on the 30th day after tumor inoculation was found to be 6.00 CC. The tumor volume reduced to 2.70 CC when treated with Maltose SO_4, to 3.00 CC when treated with Maltohexaose SO_4, and to 3.50 CC when treated with Stachyose SO_4. While reduced to 3.80 and 4.80 CC when treated separately with Raffinose SO_4 and Chondroitin-6-sulfate respectively. From the forgoing results we noticed that administration of sulfated oligosaccharide compounds (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) shows more antitumor activity in simultaneous treated groups than the groups in which treatment was started 10 days after tumor inoculation.

DISCUSSION

Cytotoxicity is one of the chemotherapeutic targets of antitumor activity [21]. Most of the clinically used antitumor agents possess significant cytotoxic activity in cell culture systems. In our previous work, the *in vitro* cytotoxic activity study against cancer cell lines revealed that the fused pyrimidine and thiopyrimidine nucleoside analogs were potent cytotoxic agents in the order 3c > 3b > 4a > 2> 4c [13]. Otherwise, sulfated oligosaccharides has shown that the growth inhibitory potency against cell lines follows the order Maltose SO_4 > Maltohexaose SO_4 > Stachyose SO_4 > Raffinose SO_4 > Chondroitin-6-sulfate [14]. The higher cytotoxic activity of both tested groups against cell lines partially explains its significant antitumor activity against ascites and solid tumors bearing mice.

In the present study the fused pyrimidine and thiopyrimidine nucleoside analogs as well as sulfated oligosaccharide were examined for their *in vivo* antitumor action against Erlich ascites carcinoma bearing mice

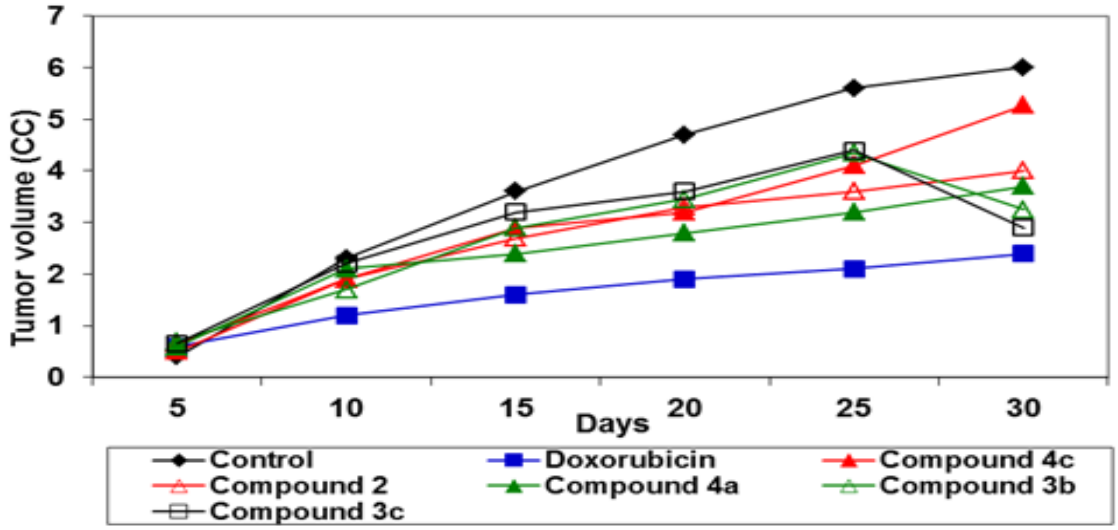

Figure 3: Effect of fused pyrimidine and thiopyrimidine nucleoside analogs (2, 3b, 3c, 4a and 4c) administration on solid tumor development (after 10 days treatment).

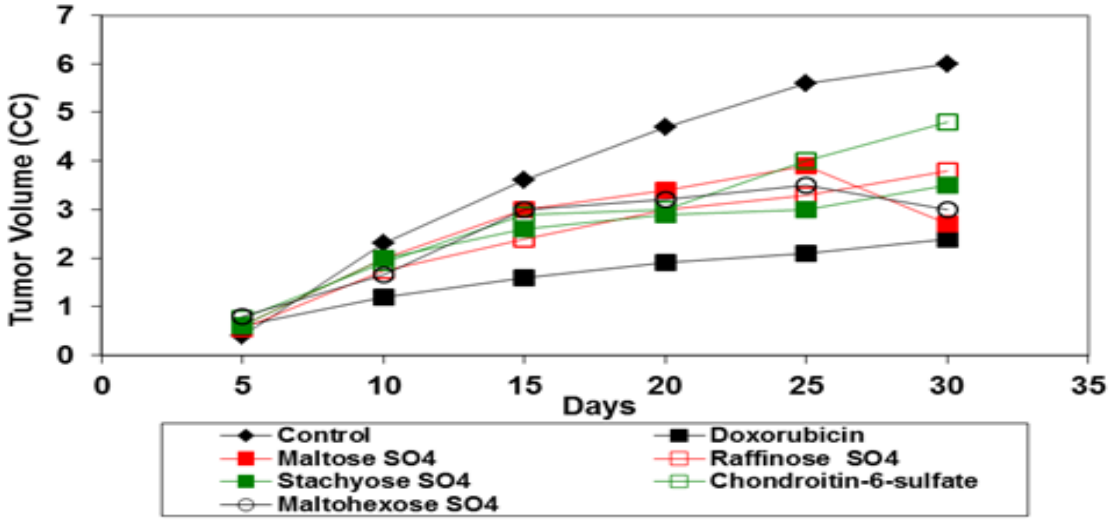

Figure 4: Effect of the different compounds of sulfated oligosaccharide compounds (Maltose SO_4, Raffinose SO_4, Stachyose SO_4, Chondroitin-6-sulfate, Maltohexaose SO_4) administration on solid tumor development (after 10 days treatment).

through monitoring the tumor volume and life span of the mice. All the investigated compounds exhibit a relevant antitumor activity and showing potency near doxorubicin cytotoxic activity specially compound 3c and Maltose SO_4. The preliminary evaluation of *in vivo* antitumor activity of tested compounds, in ascetic and solid Ehrlich tumor bearing mice showed noticeable activity near that of doxorubicin in increasing the life span of treated animals; furthermore, in case of solid tumor-bearing mice, the data suggest that the treatment with these compounds resulted in a significant tumor mass reduction in comparison with control animals.

In EAC ascites tumor bearing mice, a regular rapid increase in ascetic tumor volume was observed. Ascetic fluid is the direct nutritional source for tumor cells and increase in ascetic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells [22], this was completely noticed in the control groups in comparison to the treated groups. So it may be concluded that these synthesized compounds by decreasing the nutritional fluid volume and arresting the tumor growth increases the life span of EAC-bearing mice. Otherwise, the tested compounds significantly reduced the solid tumour development in mice. The tumour reduction was higher in animals treated simultaneously than after tumor development.

Antitumour activity of these compounds is through regulation of free radicals generation with affecting on the proliferation, differentiation and apoptosis of cancer cells [14]. Interestingly the carcinogenesis involves mainly

three steps namely initiation, promotion and progression. The implication of free radicals in different steps of carcinogenesis is well documented [23,24]. In our earlier studies we found that fused pyrimidine and thiopyrimidine nucleoside analogs as well as sulfated oligosaccharide were possess hepatoprotective effect [13,25]. The free radical hypothesis supported the fact that the regulation of free radicals production can effectively inhibit carcinogenesis.

In conclusion, the present study reveals that the investigated compounds exhibit a relevant antitumor activity comparable to the activity of commonly used anticancer drug, doxorubicin especially compound 3c and Maltose SO_4. They may be considered future drug candidates for cancer therapy.

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