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Biological Evaluation of Different Molecular Weight Dextran and Oligosaccharides Produced by Immobilized *Leuconostoc paramesenteroides* Dextransucrase.

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ABSTRACT

The optimal process for synthesis of different molecular weights (M.W.) of dextran (40,000-100,000) was examined by using different units of *Leuconostoc paramesenteroides* immobilized dextransucrase (69-103.5 U). Dextran with M.W. 100,000 (compound 1) obtained with 103 U of the immobilized enzyme and used for 6 cycle with 92% conversion of glucose to dextran. While 90 U and 69 U of the enzyme were enough to synthesis dextran with 70,000 and 40,000 M.W. (compounds 2 and 3) which can be used for 6 cycle with 88% and 80% conversion respectively. To establish the optimal process for synthesis of isomalto-oligosaccharide (IMO), we systematically examined the reaction condition of the immobilized enzyme (69 U) by changing the sucrose maltose ratios. The optimal condition for long- chain IMO (DP 10) was achieved with double concentration of sucrose to maltose with 63.9% conversion. Biological evaluation of these compounds (1-4) revealed that, compounds 3 and 4 have fibrinolytic activity while oligosaccharide with DP10 (compound 4) can be used as a prebiotic. Also, the antiproliferative effect of the four compounds has been assessed against four human cancer cell lines comparing with commonly used anticancer drug, doxorubicin. The results revealed that, while compounds (1-4) had no effect on lung A549 and colon HCT116 cell lines. Compounds 1 and 2 had anticancer effect against liver HepG2 and breast MCF-7 cell lines through down regulation of urokinase and histone deacetylase activities as markers for cancer growth and metastasis. So, compounds 1 and 2 acting as promising anticancer agents against liver and breast cancers.

Keywords: Oligosaccharides; Dextran; Dextransucrase; *Leuconostoc paramesenteroides*; Immobilization; Biological evaluation

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INTRODUCTION

Dextran is a long chain polymer of D-glucose mainly linked with α - (1 \rightarrow 6) linkages and side chains having α -(1 \rightarrow 2), α -(1 \rightarrow 3), α -(1 \rightarrow 4) linkages depending upon the producing strain [1-3]. Dextran applications depend on its molecular weight [4]. For clinical purposes, two dextran products are available in most countries, one with MW of ca 70,000 Daltons (Dextran 70) and the other with a M.W. of ca 40,000 Daltons (Dextran 40) [5]. Dextran plays a key role in different industries such as petroleum, mining, food and also in gel permeation chromatography [4,6]. It has several clinical applications such as blood volume expanders, heparin substitutes and also for treatment of anemia [7-9]. Dextran also used as thickener for jam and ice cream, improves moisture retention, crystallization of sugar and maintaining the flavor of various food items [10-13].

Oligosaccharides have been accorded prebiotic status and have evinced worldwide interest from the food and pharmaceutical sectors to match the over whelming consumer preference for healthier foods [14]. Isomalto-ligosaccharides (IMOs) are commercially important oligosaccharides and have many biological functions such as promotion of the growth of *bifidobacteria* in the large intestine of humans and animals and reduction of the carcinogenic effect of sucrose [15]. The access to pure oligosaccharides for research relies on the chemical and enzymatic synthesis of oligosaccharides [16]. The production efficiency by chemical synthesis is very low because of tedious and selective protecting and deprotecting group manipulations as compared with the enzymatic approach which is more selective [17].

Dextranase is an industrially important enzyme either for its synthesis of dextran (which has many important industrial and medical uses) or due to its theoretical and practical aspects in understanding the mechanism of glucan synthesis and its ability to synthesize a wide variety of oligosaccharides by glucosyl transfer reactions to acceptors [14,18]. Dextranase from *L. mesenteroides* is a difficult enzyme to immobilize [19]. Covalent immobilization of the enzyme either had low yields or low stability problem [19,20]. Alginate immobilization is the only method giving rise to high immobilization yield, easy recovery and operational and storage stabilities [21-24].

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 [25]. For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects [26]. Therefore, needing the better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer with some natural products. Urokinase plasminogen activator (uPA) is a serine protease that involved in many physiological functions and it has been implicated in cancer invasion and metastatization [27,28]. In addition, histone deacetylases (HDACs) are a class of enzymes playing an important role in gene expression [29]. Because it has been reported that its inhibition brought about cell-cycle arrest and induced differentiation [30], HDACs inhibition is considered a target for new type of treatment of cancers [31].

Considering above, this study was designed to make significant use of the previous prepared *L. paramesenteroides* immobilized dextran sucrose enzyme for the continuous production of different M.W. of dextran. Also we systematically examined the reaction condition of the immobilized enzyme by changing the ratio of sucrose to maltose. The resulting products of dextran with different M.W. and oligosaccharides were tested for their biological activities as prebiotic, fibrinolytic, anticoagulant, antimicrobial and antitumor agents.

MATERIALS AND METHODS

Chemicals

Fetal bovine serum (FBS) and L-glutamine, were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, streptomycin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Nutrient agar, nutrient broth, potato dextrose broth, potato dextrose agar, MRS broth and MRS agar media were purchased from Difco company (Cypress, TX 77410, USA). Penicillin (1,000,000 IU) and Fluconazole (150 µg) antibiotics were purchased from Pfizer company (France). All other chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

Microorganisms

The bacterial strain *Leuconostoc paramesenteroides* was isolated locally; *Lactobacillus helveticus*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* were attained from Chr. Hasen's Lab. Inc., Denmark, grown on MRS medium [32]. The pathogenic bacterial strains *Escherichia coli*, *Staphylococcus aureus* and the pathogenic yeast *Candida albicans* were obtained from the clinical laboratory of Nozha International Hospital. Cairo, Egypt. The cultures were maintained on nutrient agar for bacteria and potato dextrose agar for yeast then stored at 4°C and sub-cultured monthly.

Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing 4 different human cancer cell lines including breast MCF-7; liver HepG2, lung A549 and colon HCT116 cancer cell lines which obtained from the American Type Culture Collection (Rockville, MD, USA). The 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% CO₂. Cells at a concentration of 0.50 x 10⁶ were grown in a 25 cm² flask in 5 ml of complete culture medium.

Dextranucrase production

The culture medium for enzyme production was defined as follows (g/l): Sucrose, 100; yeast extract, 2.5; magnesium sulfate heptahydrate, 0.2; dipotassium hydrogen phosphate, 5.0. The initial pH of the medium was adjusted to pH 7.0 before sterilization at 121°C for 15 min. The culture was incubated at 25°C in static incubator for 24 h [33].

Immobilization of dextranucrase in alginate beads

Enzyme immobilization was performed with an enzyme preparation obtained by subjecting the culture filtrate to ultrafiltration after pretreatment with dextranase to remove the contaminated dextran. The ultrafiltrate was lyophilized and then entrapped with 2% calcium alginate gel beads [34] as previously described [23] which will be used in the present work.

Enzyme assay

Dextranucrase activity was determined by measuring the initial rate of fructose production using the dinitrosalicylic acid method [6].

Production of dextran

The dextran with different molecular weight were produced by incubating the immobilized enzyme (69, 90, 103 U) each in 5 ml CaCl₂ (0.05%) with 5 ml Na-acetate buffer (0.2 M) with 2.5 g sucrose and 0.1 g dextran (100,000) at 30°C for 24 h. Two volumes of absolute ethanol was added to each of the reaction mixtures and centrifuged at 7000 xg for 10 min to precipitate the polysaccharides, dried and weight [35]. The beads were washed with acetate buffer and repeated use. The conversion yield was based on the glucose equivalent.

Production of oligosaccharides

To confirm the synthesis of oligosaccharides by the acceptor reaction of immobilized dextranucrase, the immobilized enzyme (69 U/reaction) was mixed with different substrate ratio (sucrose : maltose 1:0.5; 1:2; 1:4 and 1:6), finally 7.5 g in 5 ml CaCl₂ (0.05%) and 5 ml Na-acetate buffer (0.2 M) with a total volume 25 ml at 30°C, pH 5.4 for 24 h. Two volumes of absolute ethanol were added to each reaction mixture, centrifuged at 7000 xg for 10 min to precipitate the oligosaccharides. Each one dried and weight. After each batch reaction lasting for 24 h, the beads were thoroughly washed with buffer prior to the successive batch.

Determination of molecular weight of dextran

This was performed viscometrically as described by Lazic et al. [36].

Determination of the degree of polymerization of oligosaccharides

The degree of polymerization (DP) was determined by examination of the reducing power of the oligosaccharides before and after hydrolysis leads to determination of the degree of polymerization [37].

Biological evaluation

Determination of prebiotic activities

The basis for the determination of the prebiotic activity depends upon comparison of the densities of growth of the probiotic beneficial bacteria *Lactobacillus helveticus*, *Lactobacillus reuteri* and *Lactobacillus acidophilus* with that of the pathogenic *E. coli* and *St. aureus* as they were grown on MRS-medium (contain the studying samples of the compounds 1-4 as a carbohydrate source). Experimentally, the three probiotics were grown on the MRS-medium, while *E. coli* and *St. aureus* were grown on nutrient broth medium, both at 37°C for 24 h. Aliquots of 0.1 ml of each of the resulted bacterial culture was used as an inoculum for 10 ml of the studied medium containing the studied samples as carbohydrate source. Such medium was prepared at a concentration of 150 mg carbohydrate source per 10 ml MRS-base medium. After incubation at 37°C for 24 h, the resulted bacterial growth was measured at 625 nm against a blank composed of un-inoculated medium [38]. The prebiotic activity was calculated as Prebiotic Index (I) which represents the ratio of bifidogenic bacteria growth to the pathogenic *E. coli* growth.

Fibrinolytic activity

This was performed by exposing a plasma clot to the effect of the investigated samples (at suitable concentration) [39]. The lysis percentage of the plasma clots at 37°C were recorded with each sample and compared with that of standard hemoclar.

Anticoagulant activity

The activities of the investigated samples were performed by using sodium heparin assay [39].

Antimicrobial activity

The four compounds 100,000(1); 70,000(2); 40,000(3) dextran and oligosaccharide with DP 10(4) were *in vitro* evaluated for their antimicrobial activity against two species of bacteria (*E. coli* and *St. aureus*) and one yeast species (*C. albicans*) by well diffusion method [40]. The experiment was performed using a culture at 37 °C for 24 h on 10 ml of nutrient broth for bacteria and 48 h on potato dextrose broth for yeast.

Antiproliferative activity

This was evaluated *in vitro* for the prepared compounds (1-4) using the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay according to the previous reported standard procedure [41].

Determination the uPA activity

This was done by using assay Max human uPA ELISA kit (Assay pro, USA), in the lysate of cancer cells treated with the four compounds.

Determination of HDAC activity

This was done by using a colorimetric assay kit (BioVision, Mountain View, kit no. K331-100) in the lysate of cancer cells treated with the compounds (1-4).

Statistical analysis

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

RESULTS AND DISCUSSION

One of the most applications of the immobilized dextransucrase is to produce different molecular weight of dextran and also oligosaccharides. Many trials were done (data not shown) to obtain different M. W. The production of high molecular weight of dextran with immobilized dextransucrase results in extremely viscous solutions, which makes recovery of the support difficult at the end of the synthesis, when all sucrose has been converted. However, in the presence of acceptors such as low M.W. dextran or maltose, the molecular weight of synthesized dextran is considerably reduced and the support is readily recovered [42].

In this work, different units of *L. paramesenteroides* immobilized dextransucrase (69-103.5 U) were used to synthesis different M.W. of dextran (40,000-100,000). Dextran with M.W. 40,000 was obtained by 69 U of the immobilized enzyme and can be used for 6 cycles with 80% conversion to dextran after that the recovered dextran decreased to 29% (7th cycle) (Figure 1). While to prepare dextran with M.W. 70,000 higher units of the immobilized dextransucrase (90 U) will be required (Figure 1). Also, the results indicated that the immobilized enzyme can be used for 6 cycles with 88% conversion of glucose to dextran then decline to 50.85% at the seventh cycle. One hundred and three U of the immobilized enzyme was used to get dextran with M.W. 100,000 and the results also indicated the durability of the enzyme in repeated use (Figure 1). The immobilized enzyme used for 6 cycles with 92% conversion of glucose to dextran. Martinez-Espindola and Lopez-Munguia [3]; Qader and Aman

[21] reported that maximum dextran production achieved with 10% sucrose concentration which agree with our results.

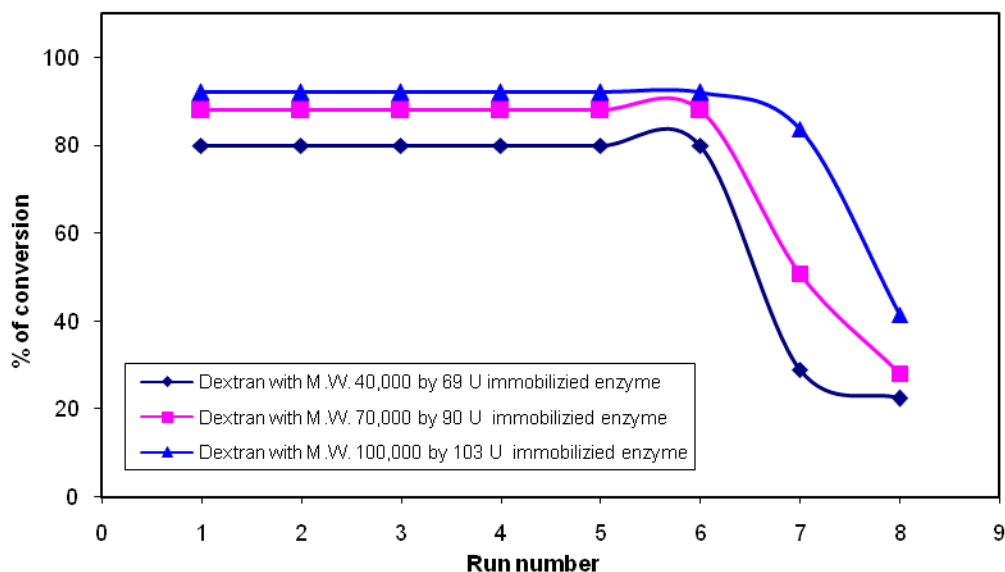


Figure 1: The preparation of dextran with different molecular weights (M.W.)

To confirm the synthesis of oligosaccharides by acceptor reaction of the immobilized dextransucrase, the amount of acceptor products were studied as the ratio of sucrose to maltose which varied as 1:0.5, 1:2, 1:4 and 1:6 with final concentration of 7.5 g substrate, the enzyme solution retaining 69 U immobilized on calcium alginate beads. This behavior of acceptor reaction products using maltose with different ratio revealed to produce oligosaccharides with different DP. The first product of this reaction with sucrose: maltose ratio (1:0.5) was oligosaccharide with DP 10. The efficiency of this synthesis using the immobilized enzyme is 63.9% and this immobilized enzyme can be used 3 times with 63.9 % then decreased to 40.9% in cycle 4 (Table 1). Our results agree with Lee et al. [43] who reported that when sucrose fraction was doubled against maltose, oligosaccharides with long chain IMO was obtained.

Generally, it was observed that with increasing maltose concentration (sucrose: maltose 1:2), higher conversion to oligosaccharide (96.7%) was attained and it could be used for 5 cycles and the conversion decreased to 73.7% and 56% at 6 and 7 cycle respectively with DP 4. This value was higher than obtained (about 90%) by Paul et al. [44], when maltose is used as acceptor. Also, it is considerably higher than the maximum yield (45%) obtained by using glucose as acceptor [4].

With sucrose maltose ratio (1:4), higher conversion (91.7%) was obtained till the 6 cycle with decrease the degree of polymerization (DP3). These results were similar to those reported by some authors [20,45,46]. Also, by increasing sucrose maltose ratio to 1:6 the conversion

decreased to 63.9% and can be used for 6 cycles with constant yield (DP3). Therefore, the best condition for long chain IMO was determined with the ratio of sucrose to maltose (1:0.50).

Table 1: Effect of different ratio of sucrose to maltose concentrations on the biosynthesis of low M.W. oligosaccharides

Ratio of Sucrose : maltose	Run No.	Weight (g)	% of conversion	DP
1:0.50	1	4.79	63.90	10
	2	4.79	63.90	
	3	4.79	63.90	
	4	3.07	40.90	
1:2	1	7.25	96.70	4
	2	7.25	96.70	
	3	7.25	96.70	
	4	7.10	94.70	
	5	7.10	94.70	
	6	5.53	73.70	
	7	4.20	56.00	
1:4	1	4.79	91.70	3
	2	4.79	91.70	
	3	4.79	91.70	
	4	4.70	91.70	
	5	4.70	91.70	
	6	4.70	91.70	
	7	3.00	45.70	
1:6	1	4.79	63.90	3
	2	4.79	63.90	
	3	4.79	63.90	
	4	4.70	62.70	
	5	4.70	62.70	
	6	4.70	62.70	
	7	3.00	40.00	

- DP: Degree of polymerization

- Sucrose to maltose ratio with a total 7.5 g were reacted with 69 U immobilizes dextranucrase 24 h at 30°C.

It is well known that some of poly and oligosaccharides have many applications in food and pharmaceutical fields. In this work, biological evaluation of the prepared compounds revealed that oligosaccharides with DP 3 and 4 are not prebiotics, while oligosaccharides with DP10 can be used as prebiotic. Our results agree with others previous studies [43,47], as they found that iso-maltose and iso-maltotriose not prebiotics because they are hydrolyzed in the small intestine and by increasing the amount of sucrose small oligosaccharides were converted by dextranucrase into oligosaccharides larger than iso-maltotriose which could be used as prebiotics. While the high molecular weight of dextran (compounds 1-3; 100,000; 70,000; 40,000 respectively) had no prebiotic activity (Table 2).

Table 2: Prebiotic activity of different molecular weight of dextran and oligosaccharides

Absorbance Sample no.	<i>L. acidophilus</i>	<i>L. reuteri</i>	<i>L. helveticus</i>
I ₁	0.79	0.65	0.37
I ₂	0.82	0.73	0.47
I ₃	0.68	0.48	0.22
I ₄	4.64	3.84	2.75

I = prebiotic index = ratio of bifidogenic bacteria growth / pathogenic *E. coli* growth.

- Negative effect when equal or less than 1.0.

- Positive effect when equal more than 1.0 significantly.

- Compounds: 1(dextran 100,000), 2(dextran 70,000), 3(dextran 40,000), 4(IMO DP 10).

Fibrinolytic activities of the various compounds (1-4) were investigated and compared with hemoclar (reference drug). The results indicated that compounds 3 and 4 showed fibrinolytic activities (75% lysis of plasma clot) which similar with the reference drug while compounds 1 and 2 had no effect. These results agree with that reported by Hashem et al. [23].

Unfortunately, the results showed that all the prepared compounds failed to have either antimicrobial or anticoagulants activities.

The antiproliferative activities were expressed by median growth inhibitory concentration (IC₅₀) and provided in Table 3. The activity of compounds (1-4) was evaluated against human breast MCF-7; liver HepG2, lung A549 and colon HCT116 cancer cell lines using MTT colorimetric assay, in comparison with doxorubicin as reference drug. The results revealed that although compounds (1-4) did not exert any activity against lung A549 and colon HCT116 cancer cell lines, they displayed potent growth inhibitory activity against liver HepG2 and breast MCF-7.

Table 3: Cytotoxicity (IC₅₀, µg/ml) of tested compounds 1-4 against human cancer cell lines as measured with MTT assay method

Compound	Cell line			
	HepG2	MCF-7	A549	HCT116
Doxorubicin	20.10 ± 2.00	24.00±2.50	25.50±2.70	19.25±2.00
1	60.50±5.80	88.80±9.00	NA	NA
2	28.60±4.00	30.60±4.60	NA	NA
3	75.70±7.76	NA	NA	NA
4	NA	NA	NA	NA

Data are expressed as means ± S.E. of four separate experiments.

NA : no activity.

For HepG2 cell line, compounds 1, 2 and 3 were exerted antiproliferative activity with IC₅₀ values of 60.50±5.80, 28.60±4.00 and 75.70±7.76 µg/ml respectively, it is clear that, compound 2 had IC₅₀ value near to the value of the doxorubicin (20.10±2.00 µg/ml). On the

other hand, in MCF-7 cell line only compounds 1 and 2 had antiproliferative activity with IC₅₀ values of 88.80±9.00 and 30.60±4.60 µg/ml respectively, it is clear that, compound 2 was more potent than compound 1. From the foregoing results, it is clear that compound 2 was the best compounds, exerting a significant antiproliferative effect on HepG2 and MCF-7 cell lines compared to doxorubicin (Table 3).

To identify the mechanism of action responsible for the antiproliferative activity of compounds, the activity of uPA expressed in hepatic HepG2 and breast MCF-7 cancer cell lines were estimated. In case of HepG2 cells, compound 4 had no effect on the expression of uPA, while compounds 1, 2 and 3 showed 30, 55 and 26% inhibition. In case of MCF-7 cells, compound 3 and 4 had no effect on the expression of uPA while compounds 1 and 2 showed 20 and 35% inhibition. From the results, compound 2 exhibited a good activity in HepG2 and MCF-7 near to the activity of doxorubicin (92% and 82%, respectively) (Figure 2). In both HepG2 and MCF-7 the inhibition of uPA activity of the tested compounds was in accordance with the antiproliferative activity. The previous results were in consistence with the report of Bickerstaff [34] who mentioned that urokinase inhibition considerably slowing the tumor growth and its metastasis.

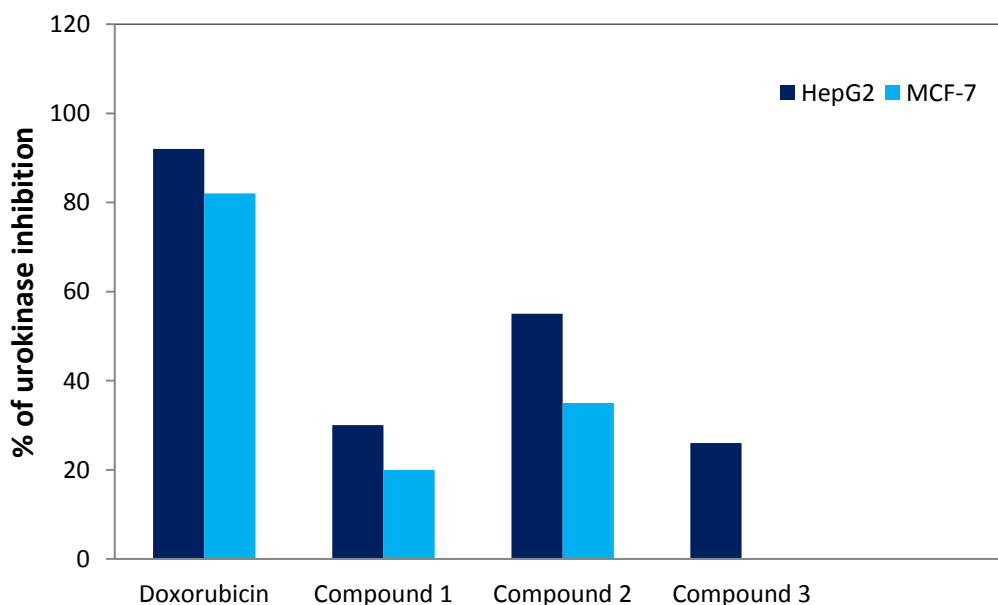


Figure 2: The percent of uPA inhibition of the compounds and doxorubicin in HepG2 and MCF-7 cell lines comparing to the control cancer cells

Beside the role urokinase system in extracellular matrix degradation allowing tumor progression and metastasis, extensive experimental evidence has been accumulated over the last years documenting the relevance of urokinase in multiple aspects of the neoplastic evolution, including tumor cell proliferation, adhesion and migration, intravasation and extravasation, growth at the metastatic sites and tumor neoangiogenesis [48]. From the foregoing results we can identify that the urokinase inhibition was a suitable target for anti-cancer therapies by our compounds.

Histone deacetylase (HDAC) inhibitors are a new class of targeted anticancer agents, which are potent inducers of growth arrest, differentiation, and/or apoptotic cell death of transformed cells *in vitro* and *in vivo* [49]. HDACs and histoneacetyl transferases can, by reversible acetylation, modify the structure and function of histones and proteins in transcription factor complexes, which are involved in the regulation of gene expression, as well as many non-histone proteins that are involved in regulating cell proliferation and cell death [49]. Thereby, we examined the effect of compounds (1-4) as histone deacetylase inhibitors in an attempt to use them in treatment of cancer.

In this work the activity of HDAC in the lysate of hepatic HepG2 and breast MCF-7 cancer cells treated with compounds (1-4) as well as Trichostatin, as a known inhibitor was measured and the data were calculated as percentage of inhibition as compared with control cancer cells. The results revealed that while treatment of liver HepG2 and breast MCF-7 cells with Trichostatin resulted in 60% and 45% inhibition respectively, the treatment with compounds 1-3 resulted in 28, 40 and 19% respectively in hepatic HepG2 cells with no effect of compound 4. Similarly, the treatment of breast MCF-7 cancer cells with the compounds show that only compounds 1-2 caused inhibition of the activity of HDAC by 22 and 29% respectively (Figure 3). These results agree with that obtained by Esawy et al. [50] who reported that Levan (HMW) have anticancer activity.

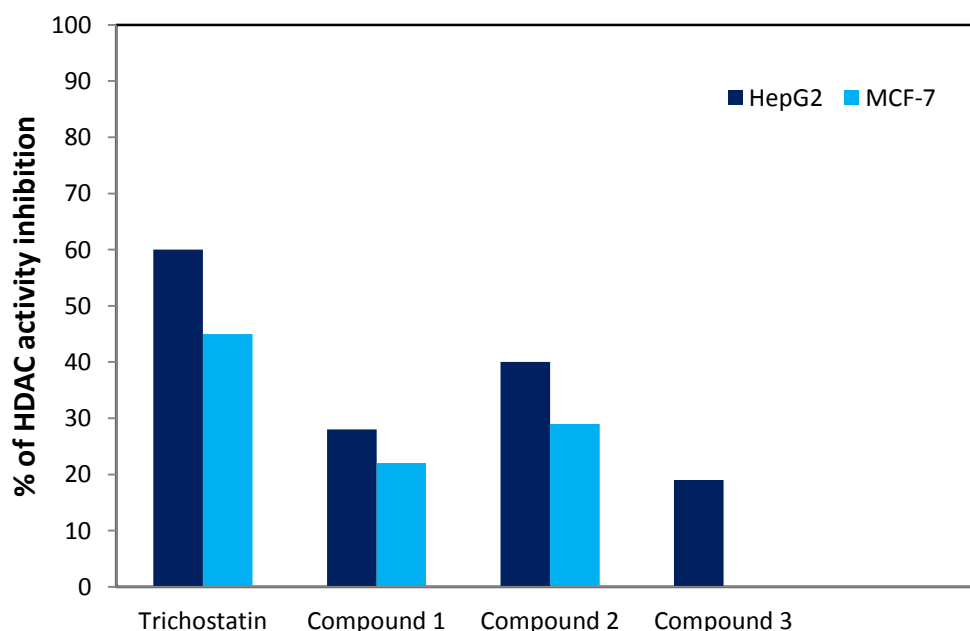


Figure 3: The HDAC activity in HepG2 and MCF-7 cells after treatment with the compounds and Trichostatin, as a known inhibitor. The data were compared with the HDAC activity of the control cancer cells

CONCLUSION

Taken together, these findings suggested that there are correlation between the antiproliferative activity of the compounds and inhibition of the urokinase and histone deacetylase activities. The tested compounds exert anti-carcinogenic activity in liver HepG2 and breast MCF-7 cancer cell lines through down regulation the activity of these enzymes which may reduce the cell proliferation and resulted in significant growth inhibition.

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