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The low molecular weight heparins (LMWHs) and the purified heparinase from *Paenibacillus polymyxa* acting as antitumour agents on RD tumor cell line

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Abstract

A novel *Paenibacillus polymyxa* strain isolated from agricultural soil showed an ability to produce of heparinase. The heparinasewas purified to homogeneity by sequential chromatographic steps involved ammonium sulfate precipitation,ion exchange on DEAE-cellulose column and gel-filtration on Sephadex G-150 with a final specific activity of 7.52U/mg, 37.5% a yield and 50.1fold of purification.The cytotoxic effect of the purified heparinase and low molecular weight heparins (LMWHs) produced in reaction mixture of heparinase with heparin was tested *invitro* against Rhabdomyosarcoma cell line (RD) and the normal cell Rat embryo fibroblast (REF) and found that the proliferation was significantly reduced with concentrations increasing of heparinase until 450µg/ml and in a time-dependent manner but in the higher concentrations there is no significant difference and for all exposure times on RD cell line, low molecular weight heparins (LMWHs) also showed cytotoxic effect for all exposure times with a significant effect.While the heparinase and low molecular weight heparins showed no significant effect during different periods time of exposure in comparison with the control against REF cells. Thus the heparinase and the generated low-molecular-weight heparins by hepatinasehave a promising applications as antitumour agents.

Keywords: Heparinase; Paenibacillus polymyxa; Antitumour; RD Tumor

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1. Introduction

Paenibacillus polymyxa (formerly *Bacillus polymyxa*) is a Gram-positivebacterium, facultative anaerobic, capable of fixing nitrogen, non-pathogenic and endospore-forming *Bacillus* (Lal and Tabacchioni, 2009; Eastman et al., 2014), originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993. It is widely distributed and have been isolated from various environmental samplessuch as soil, water, milk, feces, and decaying vegetables, forage and insect larvae, plant roots, and marine sediments[1] and clinical samples (Timmusk et al., 2005; Spadden and Gardener, 2004). Some strains produce the antibiotic polymyxin (Quinn et al., 2012). Biofilms of *P. polymyxa* growing on plant roots have been shown to produce exopolysaccharides which protect the plants from pathogens (Montes et al., 2004).

Heparin, also known as unfractionated heparin (UFHs), is an acidic polysaccharide, characterized by a disaccharide repeating unitof hexosamine and uronic acid (L-iduronic or D-glucuronic acid) connected through 1–4 linkages. Heparin is highly heterogeneous in its composition dueto the varying degree of modification of the functional groups inthe disaccharide unit (Sasisekharant et al., 1993). Heparin is widely used as blood anticoagulant, anti-inflammatory agent, normalizing prothrombin and thromboplastin times (Pulsawat and Khanitchaidecha, 2012; Christopherson et al*.*, 2002).

Heparinase is an inducible enzyme that created by many microorganisms for utilization the heparin as a sole source of carbon, nitrogen, and sulfur (Linhardt et al., 1981). Heparinase (EC4.2.2.7) is an enzyme that catalyzes the chemical reaction by cutting polysaccharides chains that contain 1,4-linked D-glucuronate or L-iduronate residues and 1,4-alpha-linked 2-sulfoamino-2-deoxy-6-sulfo-D-glucose residues to produce oligosaccharides chains that terminated at their non-reducing ends with 1,4-linked D-glucuronate or L-iduronate residues and 1,4-alpha-linked 2-sulfoamino-2-deoxy-6-sulfo-D-glucose residues (Sasisekharan et al., 1996; Kim et al., 2004). The heparinases have many applications such as quality control in heparin processing, formation of low molecular weight heparins, disaccharide or oligoaccharide from unfractionated heparin, diagnosis of plasma heparin (Sasisekharan et al., 1996). The heparinase also entered in pharmaceutical industry by formation of low-molecular-weight heparins that have an important role as antithrombotic and antitumour agents (Banga and Tripathi, 2009). The objective of this research was to obtain heparinase-producing bacteria isolated from agriculture soils besides to purification of heparinase and investigation the cytotoxic effects of heparinase and low molecular weight heparins (LMWHs) produced by heparinase on viability of cancer cell line (RD).

2. Materials and methods

2.1. Samples collection

Twenty-five agricultural soil samples were collected from different locations by using pre-sterilized sample bottles and sterile spatula from garden in Al-Mustansiriya university.

2.2. Isolation heparinase producers

0.1 g of soil sample was added to 1.5 ml of medium which consisted of (per liter): 14 g of tryptone, 1.0 g of yeast extract, 3.5 g of K_2 HPO_4, 2 g of $MgCl_2$, and 1.0 g of heparin,mannitol, 8·0; NH_4NO_3, 2·5; Na_2HPO_4, 0·5; chitin, 0·6; trace salt solution ($NaSO_4.2H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CaCl_2$) modification the medium that described by Banga and Tripathi (2009). The cultures were held in sterile 24-well microtiter trays, sealed with plastic wrap to minimize evaporation, at 45°C, without shaking for 48hour. From those samples which exhibited evidence of microbial growth, 0.1 was spread on the surface of blood agar and MacConkeys agar, then incubated at 45°C for three days.

2.3. Bacteriological analysis

The bacterial isolates were observed for morphological characters and identified by using the tests guided by Berge's Manual of Systemic Bacteriology. Suspicious isolates *Paenibacillus* were found to be Gram-positive, spore-forming, rod-shaped, facultativly anaerobic, motile, catalase-positive and had a growth temperature range of 20°C to 55°C (Holt, 1994; Forbes et al*.,* 2002). Further, *Paenibacillus polymexa* isolates were confirmed by using Vitek 2 system by using Vitek GNI card (bio Merieux, France) according to the manufacturer's instructions.

2.4. Heparinase production

The biologically pure isolates obtained were tested for production of heparinase activity by culturing the cells in 20 ml of liquid medium containing (per liter): 14 g of tryptone, 1.0 g of yeast extract, 3.5 g of K_2 HPO_4, 2 g of $MgCl_2$, and 1.0 g of heparin, Mannitol, 8·0; NH_4NO_3, 2·5; Na_2HPO_4, 0·5; Chitin, 0·6; trace salt solution ($NaSO_4.2H_2O$, $CoCl_2.6H_2O$, $CuSO_4.5H_2O$, $FeSO_4.7H_2O$, $CaCl_2$) (modification the medium that described by Banga and Tripathi (2009), adjusted to pH 8.0 with NaOH. The cultures were incubated at 45°C., with rotary shaking at 65 rpm. After 48 hours of cultivation, the cells were collected by centrifugation at 8000×g for 20 minutes and the supernatant was tested for residual heparin by the toluidene blue metachromatic assay and used as crude extract.

2.5. Heparinase assay

Reaction mixtures containing 20 µl of the present enzyme solution (suitably diluted), 80 µl of 50 mM citrate-phosphate buffer, pH, 7.4 and 20 µl of 100 ng per ml of heparin (sodium salt) are sealed to prevent evaporation and held at 45° C. for 30 minutes. Then 20 µl of 0.025 mg/ml toluidene blue solution is added and the absorbance of the solution is determined at 620 nm. The total amount of heparin remaining in the reaction mixtures is calculated from their absorbance by reference to a series of standard solutions of known heparin concentration. One Unit of enzyme activity is taken as the amount required to degrade 1.0 mg of heparin per hour at 45° C (Pulsawat and Khanitchaidecha, 2012).

2.6. Estimation of protein content

The protein content of the enzyme was determined by using Bradford dye method with BSA as a standard (Bradford, 1976).

2.7. Heparinase purification

The selected isolate was grown in the medium that described above at 45° C., with rotary shaking at 65 rpm. After 72 hours of cultivation, the cells were collected by centrifugation at 8000×g for 20 minutes and the resulting supernatant was subjected to fractionation with ammonium sulfate at different saturation ratio ranging from 30 to 80% saturation. The sample was left overnight at 4°C and the precipitate was collected by centrifugation at 8000 rpm for 15 min and dissolved in 50 mM citrate-phosphate buffer, pH, 7.4 then dialyzed overnight against the same buffer. The sample was loaded onto a column of DEAE-cellulose (2.5x25cm) equilibrated with 10 m Mcitrate-phosphate buffer, pH, 7.4 and eluted with gradient of 0.05-0.35 M NaCl prepared in the same buffer. The active fractions that showing the highest heparinase activity were pooled and applied to Sephadex G-150 column (2x80 cm) that was pre-equilibrated with 10 m M citrate-phosphate buffer, pH, 7.4 . The column was eluted with same buffer. Protein concentration at 280 nm and heparinase activity were estimated and the active fractions were pooled for further studies.

2.8. Cytotoxicity assay

Rhabdomyosarcoma (RD) and Rat embryo fibroblast (REF) were obtained fromthe center Biotechnology research center of Al-Nahraine University. The cells were grown in RPMI-1640 medium containing 10% fetal calf serum .When the cells are in the exponential phase ,the cells be in full activity so these cells were collected after dissociation the monolayer with 2-3 ml trypsin/versine for 5-15 min., then200µl of cell suspension reseeding the cells in 96 well microtiter plate to be incubated in CO_2 incubator at 37ºC for 24 hour and get adhering monolayer that treated later with 200µl of different concentration of purified heparinase (600,450, 300 and 150) µg/ml and low molecular weight heparins (LMWHs) produced in reaction mixture of heparinase with heparin were prepared in serum free media (SFM),separately, then re-incubated the plates for additional 48h and 72h. Five replicate wells were used for each concentration besides to LMWHs and negative control wells were treated with SFM. After the exposure time was finished, the medium was removed from the plate and washed with Phosphate buffer saline (PBS) and 100µl of crystal violet solution was added to each well, incubated for 4 hours . Discarded the excess of the dye and the optical density of each well was read by micro-ELISA reader at 490nm (Freshney, 2001).

2.9. Statistical analysis

The results were statistically analyzed to determine the significance effect among the concentrations of enzyme besides to LMWHs and their effect on tumor cell line and normal cells. The comparison between groups has based on analysis of variance test (ANOVA). Significance between control and samples was determined using students F- test. A Pvalue ≤0.05 was considered statistically significant.

3. Results

3.1. Isolation of *Paenibacillus polymexa* and heparinase production

Among 25 soil samples were cultured the liquid medium containing heparin and found that 4 samples gave growth after incubation period. The bacterial isolates subjected to macroscopic, microscopic, physiological and biochemical tests and demonstrated that belong to *Paenibacillus polymexa*. Thus *Paenibacillus polymexa* was isolated with isolation percentage of (16%). These 4 *Paenibacillus polymexa* isolates that revealed an ability to produce heparinase enzyme by growing in the liquid medium recultured in the same medium after changing the pH of the medium to 8 and found that *Paenibacillus polymexa* S_3 produced higher level (0.88U/ml) of heparinase in comparison with the other isolates, thus this isolate was chose as the efficient heparinase producer (Figure 1).

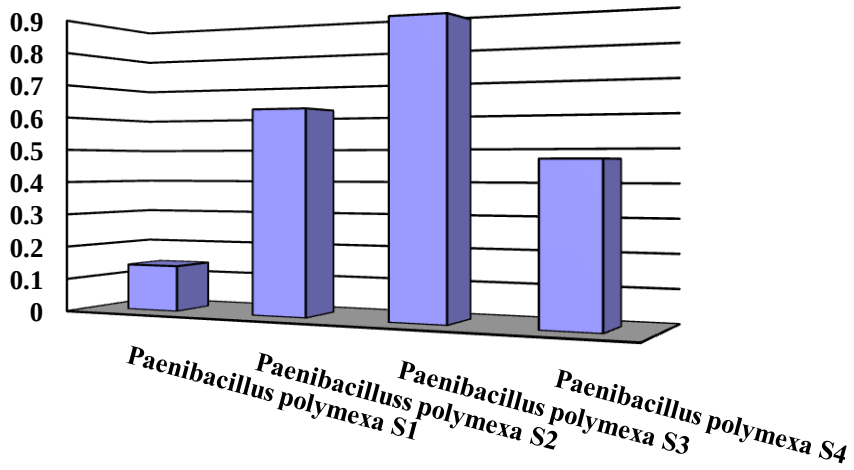

Figure1. Specific activities of *Paenibacillus polymexa* isolates

3.2. Purification of heparinase

Heparinase was purified to homogeneity by sequential chromatographic steps involved ammonium sulfate precipitation, ion exchange on DEAE-cellulose column and gel-filtration on SephadexG-150. An ammonium sulfate fractionation with 75% saturation increased the specific activity to 208.13 U/mg. After dialysis step, the sample was passed through DEAE-cellulose column and eluted with an increasing gradient concentrations of sodium chloride ranging from 0.05 to 0.35M. The elution process showed two protein peaks with heparinase activity in the second peak (Figure 2), in this stage heparinase was purified witha

yield of 43.9% and 17.3 fold of purification. The active fractions was applied to Sephadex G-150 and eluted with10 mM citrate-phosphate buffer, pH, 7.4. The elution led to appear two peaks of protein and the heparinase activity was located in the second protein peak (Figure 3) with a yield of 37.5% and 50.1 fold of purification. The key results from the purification process are summarized in Table 1.

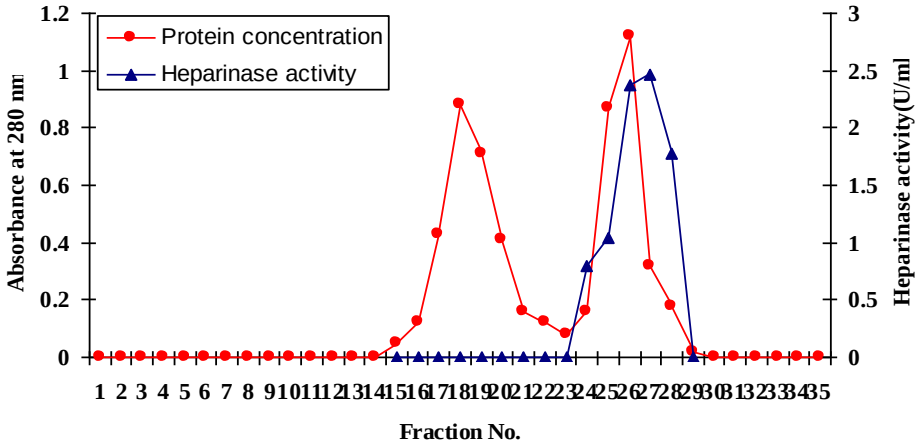

Figure 2. Purification of heparinase from that *Paenibacillus polymexa* S_3 by using ion exchange chromatography DEAE-cellulose column

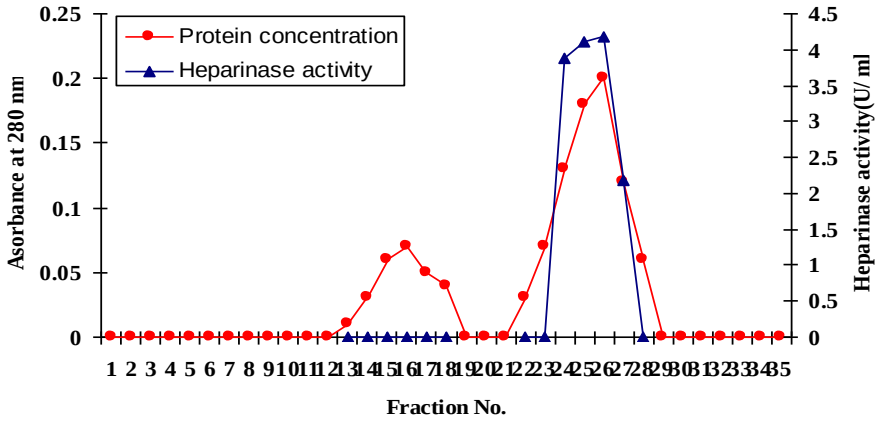

Figure 3. Purification of heparinase from that *Paenibacillus polymexa* S_3 by using gel filtration chromatography on Sephadex G-150 column

Table 1. Summary of heparinase purification from *Paenibacillus polymexa* S_3

Purification step	Size (ml)	Heparinase activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/ mg)	Total activity	Purification fold	Yield (%)
Crude extract	100	0.88	1.14	0.15	88	1	100
$(NH_4)_2SO_4$ precipitation	30	1.85	1.02	1.81	55.5	12	63
DEAE-cellulose	16	2.42	0.93	2.60	38.7	17.3	43.9
Sephadex G-150	8	4.13	0.68	7.52	33.04	50.1	37.5

3.3. Cytotoxicity assay

The results that revealed in Figure 4 show the effect of purified heparinase and low molecular weight heparins (LMWHs) producedin reaction mixture of heparinase with heparinon cancer Rhabdomyosarcoma (RD) and Rat embryo fibroblast (REF) with heparinase concentrations (600,450,300 and 150) µg/ml and exposure period (24,48,72) hrs. The cytotoxicity of the purified heparinase was increase with concentrations increasing until 450µg/ml and in a time-dependent effect on viability of RD cells with significant effect at level ($P<0.05$) in comparison with control but in the concentration that higher than 450µg/ml there is no significant difference at level ($P>0.05$) and for all exposure times. Also low molecular weight heparins (LMWHs) produced in reaction mixture of heparinase with heparin showed cytotoxic effect for all exposure timeswith significant effect at level ($P<0.05$) in comparison with control.

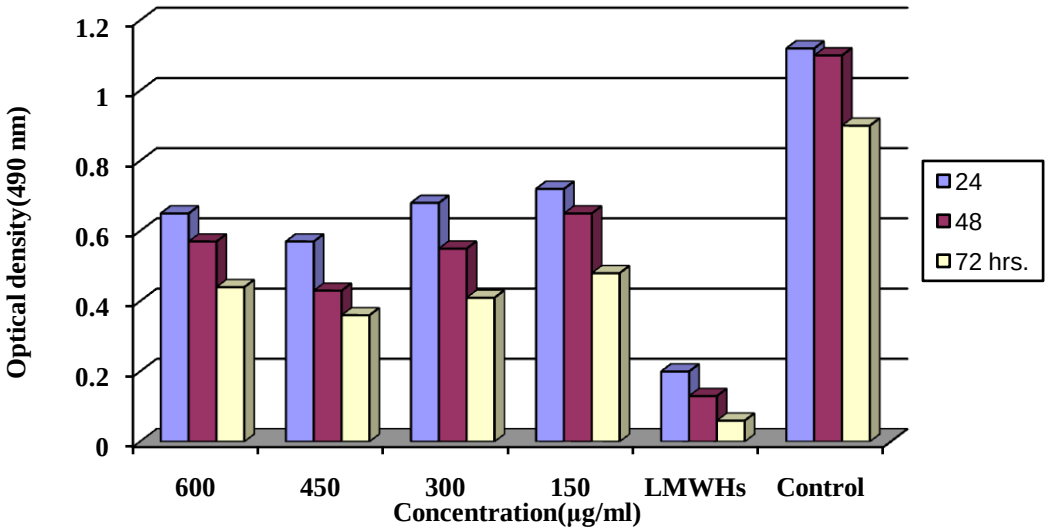

Figure 4. Effect of purified heparinase from *Paenibacillus polymyx* and LMWHs produced by heparinase on cancer cell line (RD) after (24, 48 and 72) hrs.

The effect of purified heparinase and low molecular weight heparins (LMWHs) produced by heparinaseon normal (REF) at 24, 48 and 72 hrs of exposure was illustrated in Figure 5. All heparinase concentrations (600,450,300 and 150) µg/ml and low molecular weight heparins (LMWHs) revealed no significant effect at level (P>0.05) during different time of exposure as compared with control.

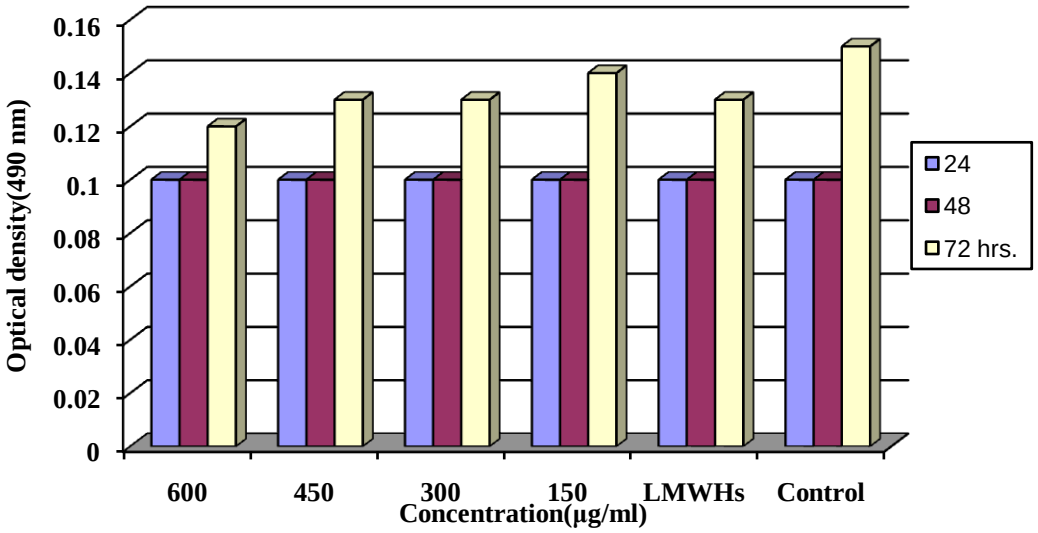

Figure 5. Effect of purified heparinase of *Paenibacillus polymyx* and LMWHs produced by heparinase on normal cell line (REF) after (24, 48 and 72) hrs

4. Discussion

The efficiency in heparin degradation (indirectly determination of heparinase producing) increased with a decrease of metachromasia formation which could be observed from conversion of blue color to purple or purple-red color. This assay was used to screen heparinase producing isolates also heparinase activities were depending on growth, heparinase production and enzyme secreted rates of isolates (Pulsawat and Khanitchaidecha, 2012). Heparinase production by *Flavobacterium heparinum* in complex protein digest medium, with heparin employed as the inducer and in defined medium containing glucose, ammonium sulfate, basal salts, L-methionine, and L-histidine was assayed and revealed that the growth rate in the defined medium was 0.21 h^{-1}, which is 40% higher than that in complex medium (Linhardt et al., 1981). *Aspergillus oryzae* isolated from soil sample had the ability to produce both extracellularly and intracellularly heparinases in its late log phase of growth and the heparin was used as the inducer and the enzyme was harvested at the beginning of exponential phase of fungal growth (Banga et al., 2008). *Flavobacterium*

heparinum, Eubacterium saburreum, E. alactolyticum and a *Peptostreptococcus* sp. isolated from cases of endometritis in cows, were capable of degrading heparin (Joubert, 1985). Heparinase production with a strain of *Aspergillus flavus* (MTCC-8654) was dependent upon heparin, the inducer; chitin, structurally similar to heparin and NH_4NO_3, the nitrogen source so that heparinase production was doubled by statistical optimization in a cost-effective manner (Banga and Tripathi, 2009). In a study reported by Banga (2010) found that heparinase production by *Aspergillus flavus* and *Acinetobacter calcoaceticus* in the structural medium contained heparin, mannitol, chtin and ammonium nitrate in the basal production medium enhanced heparinase production where heparin was used as a sole carbon and nitrogen source.

A novel type of heparinase was purified to apparent homogeneity from *Bacteroides stercoris* HJ-15, isolated from human intestine with a specific activity of 19.5 Unit/mg by using sequenced steps of QAE-cellulose, DEAE-cellulose, CM-Sephadex C-50, hydroxyapatite, and Hi Trap SP chromatographies (Kim et al., 2000). Heparinase I was purified from *Flavobacterium heparinum* by hydroxylapatite, gel filtration and chromato focusing chromatographies (Yang et al., 1985). The heparinases of *Aspergillus flavus* and *Acinetobacter calcoaceticus* were purified depending on their physical properties by using anion and cation exchange liquid chromatographies according to the type of the microbe (Banga, 2010).

The treatment with heparin (unfractionated heparin, UFH) and low molecular weight heparin (LMWH) significantly reduced metastasis of primary or solid tumors and they have high effect in inhibition stages of the metastatic cascade than in the influence on the primary tumors (Long et al., 2011). Liu et al. (2005) reported that the heparinase may be acting on tumor cells by two mechanisms, the first includes the treatment of cells with heparinase leads to lose the cells their surface HLGAG coat which may affect on their ability to grow or metastasize, the second includes the treatment of cells with heparinase leads to generate of distinct HLGAG fragments and these fragments may be affect on tumor cell function and suggested the heparinase was found to inhibit primary tumor growth and significantly reduce metastasis while the fragments generated from the heparinase treatment of a cancer cell can be used to prevent or treat the growth of a tumor as well as preventing metastasis. On the other hand Mousa and Petersen (2009) showed that the treatment with heparin and low-molecular-weight heparins (LMWH) led to prevent and treatment of thromboembolic events in cancer patients and this result increased the benefits of LMWH in hypercoagulation and thrombosis as well as in various processes involved in tumour growth and metastasis. Castelli et al. (2004) revealed that the heparin oligosaccharides that contain less than 10 saccharide led to inhibit the biological activity of basic fibroblast growth factor (bFGF), while heparin oligosaccharides that contain less than 18 saccharide residues led to inhibit the binding of vascular endothelial growth factor (VEGF) to its receptors on endothelial cells since the heparins may interfere with various aspects of cancer proliferation, angiogenesis, and metastasis formation.

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