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Inhibitory effect of the partially purified protein from *Raphnus sativus* roots and low-molecular-weight heparin on Ehrlich ascites carcinoma bearing mice

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ABSTRACT

A partially purified protein from the roots of *Raphnus sativus (R. sativus)* and low-molecular-weight heparin (LMWH) were tested for their abilities to inhibit the growth of Ehrlich ascites carcinoma cells (EAC) intraperitoneally implanted in albino mice. 90 mice were randomly divided into 6 groups. The antitumor effect of the partially purified protein and LMWH were assessed by estimating serum transforming growth factor (TGF-β1) and vascular endothelial growth factor (VEGF). Also, tumor volume, median survival time (MST), and total lipids, DNA and RNA in liver tissues, liver function tests and the redox status were estimated. Serum TGF-β1 & VEGF levels were highly significantly increased ($p < 0.005$) in the tumorized mice compared to control group and restored to their normal levels after treatment with LMWH and the purified protein ($p < 0.005$). Also, the erythrocytes content of glutathione (GSH) were showed to be highly significantly decreased ($p < 0.005$) in tumorized groups compared to control groups and significantly increased after treatment with LMWH and the purified protein ($p < 0.05$). In tumorized mice, the median survival time (MST) was 12.25 ± 2.5 days, while, MST of tumorized mice treated with the purified enzyme and LMWH were 19.00 ± 4.69 and 15.75 ± 4.11 days and the increase in life span percent were 155.1% and 128.6% respectively. It can be concluded that, the partial purified protein from the roots of *R. sativus* and the LMWH have anti-angiogenic activity against EAC cells in Swiss albino mice.

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1. Introduction

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Oxidative damage to DNA strands followed by mutation and alterations in gene expression are the principal mechanisms by which ROS contribute to carcinogenesis [1]. Therefore, protective and beneficial roles of superoxide dismutase (SOD) have been demonstrated both preclinically and clinically in combating a broad range of diseases, including ischemic-reperfusion injury, inflammation, and cancer [2]. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as SOD and catalases. The SODs are found in prokaryotes and eukaryotes, where they catalyze the disproportionation of the superoxide radical anion ($O.^{2-}$) in cellular processes detoxifying reactive oxygen species [3].

SOD, an enzyme that catalyzes the degradation of $O.^{2-}$ to oxygen and hydrogen peroxide, was discovered approximately 40 years ago. SOD was presumably the most potent anti-ROS and was initially thought to be potentially a dream drug. In humans three SOD enzymes exist, SOD1 is a cytoplasmic Cu/Zn-SOD, SOD2 is a mitochondrial Mn-SOD and SOD3 is an extracellular Cu/ZnSOD. Many studies report the purification of Mn-SOD from animal blood and plants like watermelon, pea, garlic and pearl millet [4]. The tumor cells were inhibited by the addition of these exogenous isolated Mn-SODs[4]. Manganese superoxide dismutase (Mn-SOD) is one of the major enzymes responsible for the defense against oxidative damage due to ROS in the mitochondria [5].

Raphnus sativus (*R. sativus*) is an annual herb, consumed as vegetable. It belongs to the family Brassicaceae. It has been used in folk medicine as a natural drug against many toxicants. It decreases blood glucose levels in diabetic rats [6] and improves the histopathology of colon mucosa in the rats fed high fat diet [7]. Recently, Habib and Othman reported that SOD from *Raphnus sativa* upregulate erythrocytes glucose uptake in diabetic patients [8]. Radish extract improves the antioxidant status of male mice and can overcome or, at least, significantly diminish mycotoxin (Zearalenone) effects [9].

Heparin and low molecular weight heparin (LMWH) are commonly used for the prevention or treatment of thromboembolic complications in cancer patients [10]. Based on earlier observations linking the anticoagulant therapy with heparin to an improved survival of cancer patients, several prospective clinical trials were launched with the aim to test heparin as a potential anticancer treatment [11]. Preclinical analysis of heparin provides evidence for its anti-metastatic activity in a variety of animal models [12]. Heparin is a member of a class of acidic polysaccharides called glycosaminoglycans and consists of alternating residues of uronic acid and hexosamine covalently bound to serine residues of the serglycin core protein [13].

In addition, tumor inhibition by different low molecular weight heparins (LMWH) has also been studied [14]. Experimental evidence from various animal models consistently supports the ability of heparin to attenuate metastasis. Moreover, this protective effect was not the result of an inhibition of the growth of primary tumors, but rather the prevention of the spreading of cancer through metastases.

Therefore, the goal of that research was to study the anticancer and anti-metastasis effect of the SOD-like activity enzyme (natural protein partially purified from *R. sativa*) compared with low molecular weight heparin (*Fraxiparine*, MW 4.5 KDa) on EAC-bearing mice.

2. Materials & methods

2.1. *Preparation of* R. sativus *extract*

About 300 g of root tissues of *R. sativus* were homogenized with 1 L ice-cold tris buffer pH 7. The resulted supernatant was precipitated with 80% ammonium sulfate and the precipitated protein was passed through calcium phosphate gel, which then eluted with serial concentrations of NaCl solutions. The protein after elution with 0.4 and 0.8 M saline solution was dialyzed against distilled water and then concentrated using polyethylene glycol. The concentrated partially purified enzyme was reconstituted with Tris buffer and applied onto sephadex G100 column. The purified protein concentration and its SOD activity were assayed according the methods of Lowry et al. [15] and DeChatelet et al. [16] respectively.

2.2. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) of the purified enzyme*

Protein sample was mixed with 20 μl of SDS–PAGE loading buffer (250 mM Tris–HCl, 10% SDS, 0.5% bromophenol blue, 50% glycerol pH 6.8), and incubated at 100 °C for 5 min. The samples were loaded on a 10% SDS–polyacrylamide gel. The gel was stained with Coomassie blue and inspected visually for protein bands [17].

2.3. *Animals and tumor cell line*

All experiments were performed on adult female Swiss albino mice purchased from Theodore Bilharz Research Institute, Giza, Egypt, with an average body weight of 25–30 g. Mice were housed in steel mesh cages (10mice/cage) and maintained for two weeks acclimatization period on commercial standard diet and tap water. The mice were randomly divided into 6 groups (10 animals each), according to the following scheme:

Group I: Normal mice saline-treated group (control): Each mouse was intraperitoneally injected with daily 200 μl of the physiological saline solution for 10 days.

Group II: Normal mice-treated with the purified SOD: Healthy mouse treated intraperitoneally for ten days with a daily dose of 200 μl purified protein (4 mg/kg BW/day) [18].

Group III: Normal mice-treated with low molecular weight heparin (LMWH): Healthy mouse treated intraperitoneally for ten days with a daily dose of LMWH (*fraxiparine*, average MW 4.5 KDa) was obtained from Glaxo Smith Kline (Brentford, Middlesex, UK) (1000 IU/Kg/BW/day [19]) in 200 μl.

Group IV: EAC-bearing mice saline treated: Each mouse was intraperitoneally injected once with 1x106 tumor cells. After

24 h of tumor inoculation, the mouse was intraperitoneally injected with daily 200 μl of the physiological saline solution for 10 days.

Group V: EAC-bearing mice treated with the purified SOD: Each mouse inoculated intraperitoneally with 1 x 106 tumors cells, after 24 h treated intraperitoneally for 10 days with a daily dose of (200 μl) purified protein (4 mg/kg BW/day).

Group VI: EAC-bearing mice treated with low molecular weight heparin: Each mouse inoculated intraperitoneally with 1 × 106 tumors cells, after 24 h treated intraperitoneally for 10 days with a daily dose of LMWH (*fraxiparine*) (1000 IU/Kg/BW/day).

2.4. Samples

One day after the last treatment, ten mice of each group were sacrificed & the other 5 mice of each group remain for determination of the median survival time. The ascetic fluids containing EAC cells were collected and their volumes were measured. Liver samples were quickly dissected, rinsed with isotonic saline and dried. 0.25 g of these tissues was homogenized in ice cooled saline and diluted to yield a 5% (w/v). The supernatants were used for biochemical analysis. Blood samples were also collected by cardiac puncture from deeply ether anaesthetized mice and their sera were used for subsequent analysis.

2.5. SOD-like activities of the purified enzyme

SOD-like activities of the different concentrations of the purified protein were assayed by the method of DeChatelet et al. [20].

2.6. EAC cells cytotoxicity in vitro

The cytotoxicity was determined using trypan blue exclusion by the method of Maclimans et al. [26]. After treatment with the purified protein and the LMWH, 0.2 ml of 0.32% trypan blue was mixed with 0.2 ml of EAC cells and incubated for 10 min at 37 °C. The number of viable tumor cells, unstained cells, was counted within 5 min after incubation using a homocytometer.

2.7. Determination of median survival time (MST) and increase in life span (ILS %)

The mortality was monitored by recording percentage increase in life span and median survival time by the method of Gupta et al. [21].

2.8. Biochemical tests

According to the method of Schneider [22], nucleic acids were extracted from liver homogenate. DNA content was determined colorimetrically in the extract using diphenylamine procedure described by the method of Dische and Schwatez [23] and RNA content was measured by the orcinol procedure described by the method of Mejbaum [24]. Total lipids in liver tissues were determined by the method of Knight et al. [25]. Serum Alanine transaminase (ALT) was colorimetrically determined by the method of Reitman and Frankel [26]. Serum albumin concentration was determined by the method of Doumas [27]. Erythrocyte reduced glutathione (GSH) content was determined by the method of Beutler et al. [28]. Malondialdehyde (MDA) level was determined in the blood by the method of Stocks and Donnandy [29]. Superoxide dismutase (SOD) activity in serum was assayed by the method of DeChatelet et al. [30]. Serum alkaline phosphatase (ALK-P) activity was determined by the method described by El-Aaser and EL-Merzabani [31]. Serum Gamma-glutamyl transferase (γ-GT) activity was determined by the method of Szasz et al. [32]. Nitric oxide (NO) activity was estimated in serum by the method of Berkels et al. [33] using a commercially available assay kit (Egyptian American Company for laboratory services, Egypt). TGF-β1 was assayed using a commercially available assay ELISA kit (Sigma Aldrich, USA). VEGF was assayed using a commercially available assay ELISA kit (Sigma Aldrich, USA) following manufacturer's guidelines.

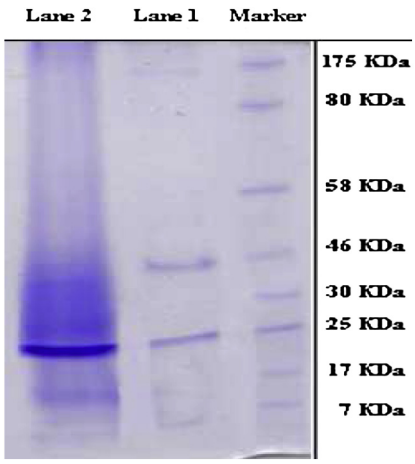

Fig. 1 – SDS-PAGE for the *Raphnus sativa* extract (lane 1) and prestained broad range protein marker (marker) and standard SOD-enzyme (lane 2).

2.9. Statistical analysis

The statistical analyses of the results were carried out using Instate software computer program, version 2.03 (Graph pad, USA). The one tailed *p*-values tables were used for statistical analysis. A difference was said to be significant, and highly significant, when the corresponding level of probability (*p*) was ≤ 0.05 and ≤ 0.005, respectively. Correlation coefficient (*r*) was used for measuring the relationship between two variables. The correlation is weak at $r = 0.50$, moderate at $r = 0.50–0.75$ and strong at $r = 0.80–1.00$.

3. Results

R. sativa extract shows a protein concentration of 52.5 mg/100 ml extract, several protein fractions are seen in the electropherogram of R. sativa extract and an expected Mn-sod enzyme with a molecular mass of 25 KDa was detected [5] as shown in Fig. 1.

The in vitro study reveals that the partial purified protein has SOD-like activity and its activity increased with increasing

Table 1 – In vitro SOD-like activities and cytotoxicity of the partial purified protein (52.2 mg protein/100 mL) and LMWH (IU) against EAC-cells.

Conc. (μl/mg)	SOD (% inhibition)	Cytotoxicity% of SOD	Cytotoxicity% of LMWH
50 μL/mg	69.4%	70.6%	51.5%
100 μL/mg	89.8%	85.0%	67.2%
150 μL/mg	96.2%	98.8%	79.0%
200 μL/mg	98.0%	99.1%	86.8%

the concentration and reached 98% inhibition at 200 μl/mg of the extract solution. Also, the cytotoxicity of that protein and LMWH on viable EAC cells is tested The percentages of dead cells increased with increasing the concentration of the enzyme and LMWH and reached 99% and 86% respectively (Table 1).

DNA and RNA contents in liver tissues of untreated EAC-bearing mice (group IV) are highly significantly elevated ($p < 0.005$) compared to the corresponding control (group I). However, the levels of total lipids are highly significantly decreased ($p < 0.005$) in liver tissues of the same group compared to those of the control. These parameters restored its normal levels ($p < 0.005$) after treatment with the partial purified SOD like activity protein and LMWH (groups V and VI) (Fig. 2). In Table 2, SOD activity in serum of the treated mice (groups V and VI) is highly significant ($p < 0.005$) increased than normal and tumorized untreated groups (I, II, III and IV). However, the levels of nitric oxide (NO) in sera and MDA in RBCs of untreated EAC-bearing (group IV) are highly significant increased ($p < 0.005$), however erythrocytes GSH content is highly significantly decreased ($p < 0.005$) than the corresponding controls. These parameters maintained its normal values when treated with the SOD extract and LMWH (groups V and VI).

In untreated EAC-bearing mice (group IV), the total ascetic volume and serum levels of (TGF-β1) and (VEGF) are highly significantly elevated ($p < 0.005$) compared to those of the controls. These values return to the normal levels ($p < 0.005$) after treatment with SOD extract and LMWH (groups V and VI) (Table 3).

In Fig. 3, the serum albumin level is highly significantly decreased ($p < 0.005$) in untreated EAC-bearing mice compared to that of the control. However, the activities of γ-GT, ALP and SGPT in serum of that group are much elevated than that of the controls. The activities of γ-GT, ALP and SGPT remain within normal value after treatment with SOD extract and LMWH (groups V and VI). In untreated EAC-bearing mice, the median survival time (MST) was 12.25 ± 2.5 days, while, the median survival time of EAC-bearing mice treated with the purified enzyme and LMWH were 19.00 ± 4.69 and 15.75 ± 4.11 days and the increase in life span percent were 155.1% and 128.6% respectively (Table 4). Table 5 shows the different correlations between the different parameters in all groups.

4. Discussion

The present work aimed to study the potent effects of a protein partially purified from *R. Sativus* roots and low molecular weight heparin (*fraxiparine*) on TGF-β1 and VEGF in EAC-bearing mice. The Ehrlich tumor was initially described as spontaneous murine mammary adenocarcinoma. It is a rapidly growing carcinoma with very aggressive behavior and is able to grow in most strains of mice. In ascetic form, it has been used as transplantable tumor model to investigate the antitumor effect of several substances [34].

In the present study, the partial purified protein showed high SOD-like activities (about 98% inhibition) as shown in (Table 1). Such results confirm the tendency of such protein to consume $O2\cdot$ radical produced during photosensitization and act as free radical scavengers. Ben Salah-Abbès reported that, the extract of radish contains many antioxidant compounds that protect against the toxicity resulting from mycotoxin (Zearalenone) [35]. In addition, the *in-vitro* cytotoxic activity of natural protein and heparin against EAC cell lines explains its significant antitumor activity against ascites as shown in (Table 1).

In contrast, the levels of total lipids in liver tissues of groups V and VI showed high elevation after treatment as shown in Fig. 2, which confirms that, these materials prevent tumor growth and metastases.

The present study showed higher levels of RNA and DNA and total volume of ascetic fluid in EAC-bearing mice compared to the corresponding controls. These elevations accompanied by a decrease in levels of total lipids compared to the control (Fig. 2), confirming the existence of a catabolic state accompanying the growth of the tumor cells [36]. This increase in DNA and RNA levels and high replication of the tumor cells needs high energy; so tumor cell growth is usually consuming the energy sources including lipids and proteins. These explanations are confirmed by the established negative correlation between DNA and both total lipids and albumin (Table 5). Habib et al. reported that, the tumor cell growth requires high energy, therefore the catabolic effect of the tumor cell negatively correlated with the total lipids in liver tissues of the tumorized mice [8]. After treatment with the partially purified protein, these parameters restored their normal levels (groups V and VI) by inhibiting the tumor DNA synthesis [37]. These results agree with the results of Esmat [38]. In EAC-bearing hosts, regular rapid increase in ascetic fluid volume was observed. The ascetic fluid is the direct nutritional source for tumor cells, and the faster increase in ascites fluid with tumor growth could possibly be a mean to meet the nutritional requirements of tumor cells [39]. The results of the present study confirm the previous finding in that the rate of the tumor growth was significantly inhibited in the tumorized mice after treatment with the natural extract and heparin as shown in Fig. 2.

Glutathione, a potent inhibitor of neoplastic process, plays an important role as an endogenous antioxidant system that is found particularly in high concentration in liver and is known to have key function in the protective process [40]. In the present study, the mean levels of GSH were significantly elevated in EAC-bearing mice treated with the natural SOD-like activity protein and heparin compared to those of EAC-bearing untreated mice ($p < 0.05$), indicating that the used material can be implicated in the redox cycle involved in GSH production [41]. Reduced glutathione is central to the cellular antioxidant defenses and acts as an important cofactor for antioxidant enzymes [42].

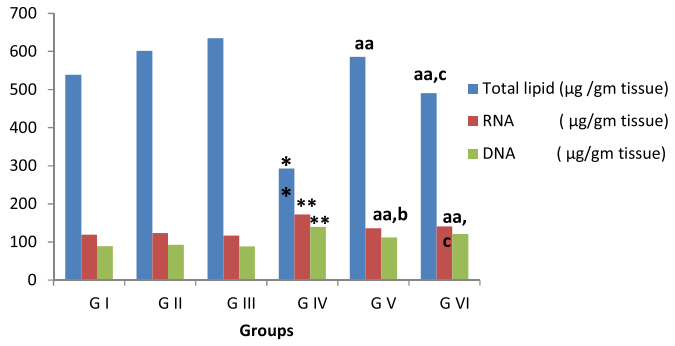

Fig. 2 – Total Lipids, DNA and RNA contents in liver tissues of mice of groups (I – VI). (*) Significant ($p < 0.05$) (**) highly significant ($p < 0.005$) compared to group GI. (^{a}) Significant ($p < 0.05$) (^{aa}) highly significant ($p < 0.005$) compared to group GIV. (^{b}) Significant ($p < 0.05$) (^{bb}) highly significant ($p < 0.005$) compared to group II. (^{c}) Significant ($p < 0.05$) (^{cc}) highly significant ($p < 0.005$) compared to group III. Group I: Normal mice saline-treated (control): Group II: Normal mice-treated with the partial purified SOD: Group III: Normal mice-treated with low molecular weight heparin (LMWH): Group IV: EAC-bearing mice saline treated: Group V: EAC-bearing mice treated with the purified SOD: Group VI: EAC-bearing mice treated with low molecular weight heparin.

MDA, the end product of lipid peroxidations was reported to be higher in carcinomatous tissue than that in the non diseased organs. The level of MDA reflects the extent of membrane lipid peroxidation and hence cell membrane damage and correlated with advanced clinical stages and the impairment is related to tumor progression. Moreover, it has been claimed that MDA acts as a tumor promoter and co-carcinogenic agent because of its high cytotoxicity and inhibitory action on protective enzymes. Low levels of MDA indicate inhibition of lipid peroxidation. The biochemical determination of MDA serves to indicate lipid peroxide formation. Zhang et al., reported that, low-molecular-weight heparin (LMWH) could significantly decrease MDA content and increase SOD activity in ischemic brain [43]. It is clear from this study that *R. Sativa* inhibits lipid peroxidation and provides protection by strengthening the antioxidants effect by glutathione [44].

One mechanism that macrophages use to exert their cytostatic and cytolytic effects on the target tumors is by the release of nitric oxide (NO) by the activation of the cytosolic, NADPH-dependent enzyme inducible NO synthase (iNOS) [50]. In the present study, EAC-bearing mice showed a highly significant elevation in nitric oxide (NO) level compared to normal mice. In contrast, EAC-bearing mice treated with the natural SOD-like activity protein and LMWH showed a highly significant reduction in nitric oxide (NO) level compared to EAC-bearing untreated mice. Such results confirm the tendency of the natural purified protein and LMWH to reduce the cytotoxicity effect of EAC cells. In the present study, EAC-bearing mice treated with natural SOD-like activity protein showed an increase of 155.1% in their life span and those treated with the low molecular weight heparin showed an increase of 128.5% compared to the tumorized untreated mice (Table 4).

Table 2 – SOD activity and blood levels of GSH, nitric oxide, and MDA in RBCs of mice of groups (I – VI).

Parameters group	MDA (Mole/1 ml RBCs)×1000	Nitric oxide (NO) µmol/L	GSH (ml Mole/liter RBCs) × 1000	SOD (inhibition %)
G I	4.38 ± 1.256	43.3 ± 5.2	2711.5 ± 605.4	39.2 ± 11.8
G II	4.73 ± 1.62	41.2 ± 6.1	2624.1 ± 765.3	56.0 ± 7.7*
G III	5.58 ± 0.71	44.3 ± 5.6	2765.9 ± 444.9	49.3 ± 5.3
G IV	7.99 ± 1.17**	91.3 ± 5.0**	1165.2 ± 580.9**	40.2 ± 8.4
G V	5.56 ± 1.24^{a}	65.0 ± 7.5^{aa,bb}	2009.2 ± 365.3^{a}	82.1 ± 3.8^{aa,bb}
G VI	5.40 ± 1.47^{a}	71.5 ± 6.9^{aa,cc}	2040.9 ± 236.1^{a,c}	74.3 ± 9.5^{aa,cc}

The results represented as M ± SD Number of mice = 9.
(*) Significant ($p < 0.05$) (**) highly significant ($p < 0.005$) compared to group GI.
(^{a}) Significant ($p < 0.05$) (^{aa}) highly significant ($p < 0.005$) compared to group GIV.
(^{b}) Significant ($p < 0.05$) (^{bb}) highly significant ($p < 0.005$) compared to group II.
(^{c}) Significant ($p < 0.05$) (^{cc}) highly significant ($p < 0.005$) compared to group III.
Group I: Normal mice saline-treated (control):
Group II: Normal mice-treated with the partial purified SOD:
Group III: Normal mice-treated with low molecular weight heparin (LMWH):
Group IV: EAC-bearing mice saline treated:
Group V: EAC-bearing mice treated with the purified SOD:
Group VI: EAC-bearing mice treated with low molecular weight heparin.

Table 3 – Total ascetic volume and serum levels of TGF-β1 and VEGF in mice of groups (I – VI).

Parameters group	Ascetic volume (ml)	VEGF (pg/ml)	TGF-β1 (pg/ml)
G I	–	122.2 ± 24.7	102.7 ± 12.4
G II	–	122.3 ± 24.0	115.6 ± 21.5
G III	–	127.5 ± 31.3	105.4 ± 15.5
G IV	8.1 ± 1.4	720.2 ± 26.4**	568.6 ± 28.8**
G V	0.8 ± 0.7aa	273.7 ± 65.6aa, bb	233.0 ± 44.9aa, bb
G VI	2.0 ± 2.1aa	337.6 ± 60.9aa, cc	296.4 ± 49.7aa, cc

The results represented as M ± SD Number of mice = 9.
(*) Significant ($p < 0.05$) (**) highly significant ($p < 0.005$) compared to group GI.
(a) Significant ($p < 0.05$) (aa) highly significant ($p < 0.005$) compared to group GIV.
(b) Significant ($p < 0.05$) (bb) highly significant ($p < 0.005$) compared to group II.
(c) Significant ($p < 0.05$) (cc) highly significant ($p < 0.005$) compared to group III.
Group I: Normal mice saline-treated (control):
Group II: Normal mice-treated with the partial purified SOD:
Group III: Normal mice-treated with low molecular weight heparin (LMWH):
Group IV: EAC-bearing mice saline treated:
Group V: EAC-bearing mice treated with the purified SOD:
Group VI: EAC-bearing mice treated with low molecular weight heparin.

Table 4 – Median survival time of treated and untreated EAC-bearing mice.

Group	Median survival time (Day)	Percentage increase of life span
Tumor	12.25 ± 2.5	.....
Treated (SOD)	19.00 ± 4.69	155.1%
Treated (H)	15.75 ± 4.11	128.5%

Values are expressed as mean ± SD ($n = 5$).

All of the interactions between the hemostatic system and tumor cells are involved in the promotion of angiogenesis [45]. Furthermore, several of the steps in the development of angiogenesis can be suppressed by UFH (UnFractionated Heparin), LMWH, and non-anticoagulant molecules [46]. The complex interactions of these various mechanisms were recently reviewed by Ruf [47]. Thus prominent roles in angiogenesis involve the release of growth factors such as VEGF (VascularEndothelial Growth Factor) [47].

The suppression of angiogenesis has been promoted as one of the mechanisms whereby anticoagulants such as UFH and LMWH may suppress tumor growth and metastasis. In experimental models assessing endothelial capillary tube formation and cell proliferation in cultures have shown that UFH and various LMWHs can suppress angiogenesis, although not all to the same extent [48]. Ratner reported an association of thrombosis with the anti-angiogenic therapy which further highlights the complex interaction of angiogenesis with the hemostatic system [49].

Ghosh et al. indicated that tumor volume and its growth rate increase with increasing angiogenesis and VEGF level of the host and the rate of VEGF secretion is positively correlated well with tumor volume [50]. The present results agree with these observations. After treatment, the tumorized mice which were treated with the partial purified protein with SOD like activity andLMWH (groups V and VI),VEGF levels showed a highly significant decrease compared to that of the tumorized untreated mice. The latter result confirms the anti-angiogenic properties of the LMWH in addition to their antithrombotic properties. This is because short heparin fragments have been shown to inhibit the binding of VEGF to its receptors on endothelial cells[51].

TGF-β1 (Transforming growth factor-β1), is a multifunctional cytokine that regulates cell proliferation, differentiation and extracellular matrix production [52]. Also, TGF-β1 may contribute to tumor pathogenesis by direct support of tumor growth and influence on local microenvironment, resulting in immunosuppression for all cells of the immune system, induction of angiogenesis, and modification of the extracellular matrix [53]. Vozenin-Brotons et al. reported that, SOD could significantly reduce TGF- β 1 expression, thus demonstrating that SOD might be proposed as a potent antagonist of this

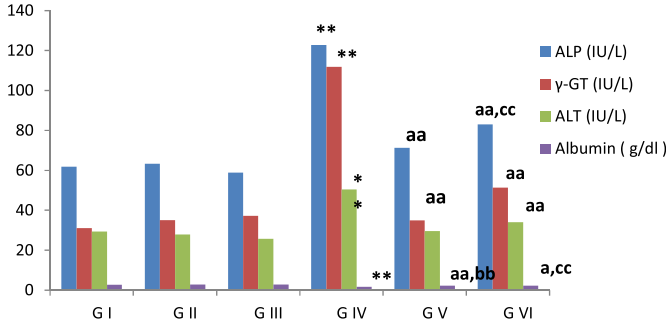

Fig. 3 – Serum levels of albumin and Serum activities of ALT, ALP and γ-GT in mice of groups (I – VI). (*) Significant ($p < 0.05$) (**) highly significant ($p < 0.005$) compared to group GI. (a) Significant ($p < 0.05$) (aa) highly significant ($p < 0.005$) compared to group GIV. (b) Significant ($p < 0.05$) (bb) highly significant ($p < 0.005$) compared to group II. (c) Significant ($p < 0.05$) (cc) highly significant ($p < 0.005$) compared to group III. Group I: Normal mice saline-treated (control): Group II: Normal mice-treated with the partial purified SOD: Group III: Normal mice-treated with low molecular weight heparin (LMWH): Group IV: EAC-bearing mice saline treated: Group V: EAC-bearing mice treated with the purified SOD: Group VI: EAC-bearing mice treated with low molecular weight heparin.

Table 5 – Correlations between the different parameters of mice of groups (I – VI).

Parameters	GSH	SGPT	γ-GT	Alb	Tumor volume	Total lipid	NO
DNA (μg/gm tissue)	$r = -0.63$	$r = 0.9$	$r = 0.72$	$r = -0.81$	$r = 0.77$	$r = -0.55$	$r = 0.88$
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
MDA (Mole/1 ml RBCs)	$r = -0.30$	$r = 0.58$	$r = 0.65$	$r = -0.51$	$r = 0.61$	Ns	$r = 0.54$
	$p < 0.05$	$p = 0.0001$	$p < 0.0001$	$p < 0.001$	$p < 0.05$		$p < 0.001$
VEGF (pg/ml)	$r = -0.69$	$r = 0.84$	$r = 0.88$	$r = -0.82$	$r = 0.93$	$r = -0.52$	$r = 0.91$
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$
Albumin (g/dl)	$r = 0.72$	$r = -0.65$	$r = -0.71$	.....	$r = -0.86$	$r = 0.41$	$r = -0.84$
	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	.....	$p < 0.0001$	$p < 0.05$	$p < 0.0001$

(p) Probability, (r) correlation coefficient.
Significant ($p \leq 0.05$), highly significant ($p \leq 0.001$).
Correlation is considered weak at $r = 0.50$, moderate at $r = 0.50–0.75$ and strong at $r = 0.80–1.00$.

major fibrogenic growth factor [54]. In the present study, TGF-β1 was highlysignificantly elevated in EAC-bearing mice compared to normal mice (Table 3). However, after treatment asin groups V and VI, TGF-β1 level was highly significantly decreased compared to that of the tumorized untreated mice. These results confirm the abilities of the LMWH and the natural SOD-like activity protein to downregulate the level of TGF-β1 and reduce the growth and metastasis of EAC cells.

In the present results, the activities of GPT, ALP and γ-GT were highly increased in the sera of the untreated EAC-bearing mice and the level of albumin was decrease (Fig. 3). The reduction in the activities of both γ-GT and SGPT after treatment with both natural SOD-like activity protein and LMWH compared with those of the tumorized untreated mice can confirm the anti-tumor effect of the two materials. Similar to that, reduction of the activities of the all elevated enzymes ($p < 0.005$) to near normal levels in animals treated with both Jasminum sambac and 5'fluorouracil were observe [55].

Most experimental models and cell culture studies have concluded that SOD has anti-proliferative and tumor suppressor effects [56]. This may be one of the mechanisms by which *R. sativus* extract kill the EAC cell line. Furthermore, El-Sayed reported that, SOD activity was enhanced after treatment of the tumor cells with metal complexes having SOD-like activity, such type of treatment expresses excessive amount of H2O2 which implicated in EAC cell killing but not the normal cell because the latter cell contains sufficient catalase and/or peroxidase activities to degrade H2O2 [57]. Also, the use of substances with SOD-like activities can cause a pronounced activation of blood phagocytes. Moreover, Habib et al. extracted a similar metalloprotein with MnSOD-like activity from the roots of *R. sativus* and showed that, such extract can activate the immune system to kill the EAC cell line [58].

Liu et al., showed the effects of heparin–superoxide dismutase conjugate (heparin–SOD) on carbon tetrachloride (CCl4)-induced acute liver failure and hepatic fibrosis. Biochemical indicators, such as glutamic pyruvic transaminase (SGPT), GSH (glutathione), and malondialdehyde (MDA) were determined 24 h after CCl4 treatment. The development of CCl4-induced acute liver failure altered the redox state with a decreased hepatic GSH and increased formation of lipid peroxidative products, which were partially normalized by treatment with heparin–SOD or heparin + SOD. Compared with non-treated groups, the acute liver injury of heparin–SOD group was significantly reduced activities of SGPT, MDA, and increased activities of GSH. Also, in the present study, level of MDA & SGPT were elevated and the level of erythrocyte GSH reduced in the tumorized mice, while after treated with the partially purified extract and low molecular weight heparin, these parameter were partially normalized [59].

Natural SOD-like activity protein is economical and of low toxicity and this is perhaps the advantage over synthetic agents which exhibit normal tissue toxicity.

5. Conclusion

From the above observations one can conclude that, the naturally and the partially purified protein from *R. sativus* roots and the LMWH (*fraxiparine*) can be used to inhibits EAC cell growth possibly by many mechanisms including their antioxidative and anti-angiogenic effects which was observed in serum of the tumor bearing mice. The mechanism may involve protection of the tumor suppressor genes from the toxic effects of free radicals. Moreover, the role of *R. sativus* extract in the enhancement of the immune system function cannot be neglect.

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