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Research Article

Anticarcinogenic activity of rosemary loaded solid lipid nanoparticle against solid tumor in mice

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ABSTRACT

The present research conducted to evaluate the in vivo anticancer activity of rosemary extract (ROS) loaded solid lipid nanoparticles (SLNs) in a mice model bearing Ehrlich solid carcinoma (EAC). Tumor-bearing mice were haphazardly distributed to four distinct groups (6 animals / group) plus normal control group (5 total groups). Mice inoculated with EAC cells in the second group received SLNs only. In the third and fourth groups, two different doses (200 and 400 mg/kg) of the formulated ROS loaded SLNs were given to the animals. Hematological and biochemical alterations were evaluated in all animal groups after four weeks of treatment. Mortality rate, Tumor size, Tumor Necrosis Factor-alpha (TNF-α) were estimated in addition to flow cytometery analysis. Our results indicated that animals treated with ROS loaded SLN showed a marked reduction in tumor size, mortality rate and TNF- α level as compared to EAC group. In conclusion, Rosemary loaded SLN shows promising anticancer efficacy and displayed great potential as a therapeutic agent in cancer treatment, this could serve as an initiative for developing new cancer therapeutics.

1. Introduction

Rosmarinus officinalis (Rosemary) is a well-known spices herb, belongs to the *Lamiaceae* family widely distributed in the Mediterranean area. The pharmacological potential of ROS has confirmed in other studies [1–3]. ROS extract was found to be antimutagenic in the Ames tester strain TA102 [4]. Moreover, many previous studies reported the antiproliferative effects of ROS extracts on various human cancer cell lines, namely leukemia, liver, breast, prostate, and lung cancer [5]. The activities of the ROS leaves extract are due to its components that have high antioxidant activity such as butylated hydroxytoluene, butylated hydroxyanisole, carnosol, carnosic acid, ursolic acid, and rosemarinic acid [6,7].

One major factor influencing a drug's in vivo efficacy is how it is delivered. Recently, researchers have extensively used naturally synthesized nanoparticles as delivery systems in cancer therapy for the inhibition and treatment of tumor [6]. Solid lipid nanoparticles (SLNs) are one type of nanocarriers that are a desirable choice due to their ease of preparation, their ability to regulate release kinetics and efficiently load drugs. Moreover, it increases the bioavailability of the drugs, leads to enhancement of blood circulation duration, and maintains specific delivering to tumor site leading to an enhancement in drug efficacy and lowered toxicity [8,9]. Additionally, SLNs are made up of a solid lipid core that allows ROS leave extract to be effectively captured and released in a regulated manner. No previous studies to date on the anticancer effects of ROS loaded SLNs in vivo. So, in the present study, formulated ROS leave extract loaded on SLNs are prepared to evaluate its anticancer affect in vivo using mice bearing Ehrlich ascites carcinoma as a tumor model.

2. Materials and Methods

This research was carried out in agreement with Animals Ethics Committee of Fayoum University, Egypt.

2.1. Chemicals

All of the chemicals were acquired from Sigma in St. Louis, Missouri.

2.2. Preparation of rosemary Plant Extract:

The leaves of *Rosmarinus officinalis* were obtained from a local plant farm at Giza, Egypt. A taxonomist from the Botany Department of the Faculty of Science at Fayoum University, Fayoum identified and authenticated the plant components. Leaves were carefully washed, air dried at room temperature, and grinded to powder. Solvent extraction by alcohol was used to create an extract from rosemary leaves. In brief, the rosemary leaves were dried for four days in the shade before being ground up in a blender. 500 mL of 70% ethanol was used to extract each 100 g of plant material at room temperature. After 48 hours, a rotary evaporator was used to filter, evaporate, and dry the extract at 40°C while under vacuum. The organic solvents were subsequently evaporated. The dried extracts were stored at 4°C until used [10].

2.3. Preparations of solid lipid nanoparticles (SLNs) loaded with rosemary leaves extract (ROS-SLNs)

Hot homogenization method was used to prepare the SLNs according to Abd-Elrazek & Elnawawy [11]. Melted Glycerol Mono Stearate (GMS) (5%) with polysorbate 80 (1.5 % w/v) with lipoid 0.5% (hydrophilic surfactant) solution as the aqueous phase, and rosemary extract as the lipophilic phase. At 70°C, GMS was melted, and the medication was added while being stirred by hand. The lipid phase was mixed using a high-shear mixing apparatus for 15 minutes while 10 ml of a hot aqueous surfactant solution comprising polysorbate 80 and lipoid was added. Under constant swirling, solid lipid nanoparticles were generated by dispersing the heated o/w microemulsion drop by drop into ice-cold water (2-3 °C) in a beaker. After the microemulsion had formed, it was still being stirred. The SLN dispersion was stirred for 15 minutes, then high-speed homogenised for 5 minutes at 15000 RPM, and finally ultra-sonified for 15 minutes. Nanoparticle characteristics varied with the quantity of lipid used, the duration of mixing, the speed of homogenization, and the amount of surfactant used.

2.4. Particle Size and Zeta Potential

Nawah scientific company in Egypt used a Malvern Zetasizer Nano ZS90 photon correlation spectrometer to measure the particle size and zeta potential of the ROS-SLNs (Malvern Instruments, Worcestershire, UK). Disposable polystyrene cells and disposable plain folded capillary zeta cells were used to evaluate size and zeta potential, respectively, at 25 °C, following proper dilution with original dispersion preparation liquid.

2.5. Experimental animals

A total of healthy thirty adult male Swiss Webster mice weighting (18 -20 gram) were obtained from Cairo University's National Cancer Institute's animal housing. They were housed in clean sterile cages at controlled room temperature and under 12-hr light/dark cycle with free access to food and tap water.

2.6. Preparations of tumor-bearing mice and experimental design

The National Cancer Institute at Cairo University provided the Ehrlich Ascites Carcinoma (EAC)-bearing mouse (Giza, Egypt). Mice harboring tumors were generated by intramuscular injection of 0.2 mL of Ehrlich tumor cell solution (containing approximately 2×10^6 viable cells) into the left flank. The tumor was allowed to grow for approximately 15 days, then mice were haphazardly distributed to 5 different groups (6 animals / group) as follows: Group I: negative control group that was not induced with tumor and received 0.9% saline solution orally (1ml/kg). Group II: EAC group received 0.9% saline solution orally (1ml/kg). Group III: Solid lipid nanoparticles (SLNs), EAC bearing mice received 1 ml of SLN solution orally. Group IV: rosemary loaded SLN (ROS-SLNs) (200mg /kg), EAC bearing mice received 1ml of rosemary loaded SLNs orally once daily [12]. Group V: rosemary loaded SLN (ROS-SLNs) (400mg /kg), EAC bearing animals received 1ml of rosemary loaded SLNs orally once daily [12]. All the treatments were continued once daily for 4 weeks consecutively. Tumor size was measured every four days using a digital caliper and a formula of (A x B2 x 0.5), where A is the diameter that is largest and B is the diameter that is perpendicular to it [8]. The survival rate and tumor volume were also recorded for each tumor-bearing animal.

2.7. Sample collection

All of the animals were given sodium thiopental (0.5%) to induce anaesthesia before being slaughtered through cervical dislocation at the experiment end. Blood samples were collected from all groups and used for serum separation.

2.8. Hematological analysis

Using an automated Sysmex Kx-21N (CHU Habib Bourguiba Sfax), blood platelet count, hemoglobin, white blood cell count, and red blood cell count were recorded.

2.9. Tumor Necrosis Factor Alpha Level Determination (TNF-α).

Following the manufacturer's instructions, TNF-α-specific monoclonal antibodies were used in a sandwich enzyme immunoassay with quantitative analysis (R&D systems, Minneapolis, USA).

2.10. Flow cytometry analysis

Flow cytometry analysis was carried out as described by Pozarowski and Darzynkiewicz [13]. PBS (phosphate-buffered saline) in 0.5 mL was used to suspend about 10^6 cells. After that, the cells were preserved by putting this solution into centrifuge tubes that were chilled and held 4.5 mL of 70% ethanol. The cells suspended in ethanol were centrifuged at 300g for five minutes. After resuspending the cell pellet in 5 mL of PBS, centrifuging the mixture at 300 g for 5 minutes. The cell pellet was incubated for 10 minutes at 37°C in the dark after being suspended in 1 mL of propidium iodide (PI) staining solution. A flow cytometer was used to quantify cell fluorescence.

2.11. Statistical analysis

Version 25 of the Statistical Package Software System was used to do statistical analysis on all data, which were reported as mean + S.E.M. One way ANOVA was used to assess significant differences in means, and the Duncan posthoc test was then used to determine the significant differences between the treated groups. A significance level of less than 0.05 was applied.

3. Results

3.1. ROS-SLNs characterization

Images taken with a transmission electron microscope (TEM) showed that most nanoparticles are almost round (Figure 1A). The size of the nanoparticles seen by TEM matched well with the size of the particles measured by a particle size analyzer. ROS-SLNs values for the polydispersity index (PDI) showed that the particle size distribution was unimodal. The smallest size of a particle was 488.9 nm, and its PDI was 0.579. (Figure 1B). The ROS-SLNs that were made had a zeta potential of -33.3 mV, which shows that they are very stable (Figure 1B).
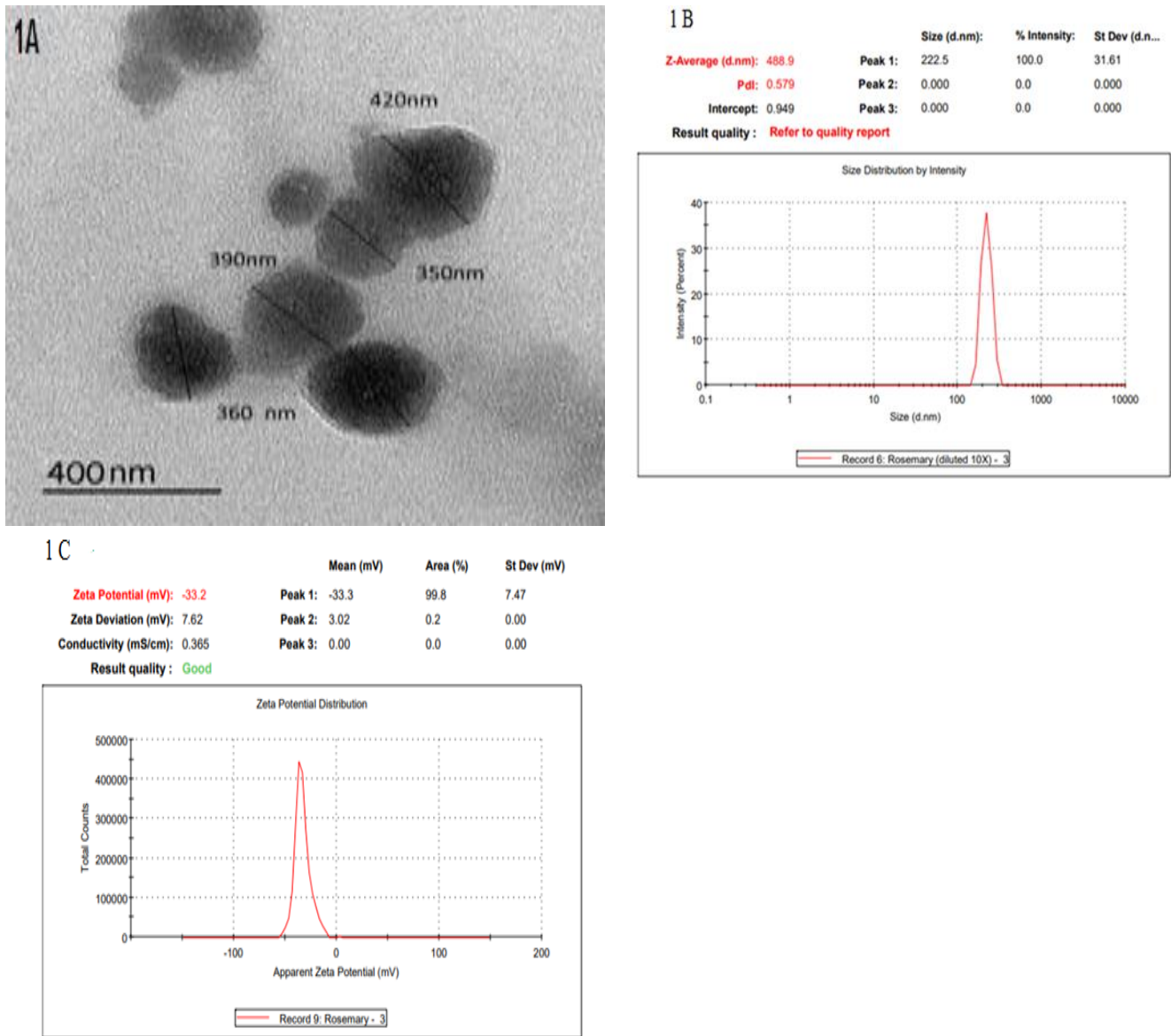

Fig. 1 Characterization of ROS-SLNs (A) A representative micrograph of ROS-SLNs using TEM showing The least particle size was 488.9 nm, (B) zeta potential of ROS-SLNs, (C) the hydrodynamic diameter of 100% ROS-SLNs using DLS. TEM, transmission electron microscope; XRD, DLS, dynamic light scattering; ROS-SLNs, rosemary loaded solid lipid nanoparticles.

3.2. Result of the vivo study

In all tested parameters, no significant difference was recorded between Positive control group (group II), and the group of animals bearing EAC

that received SLN only (group III).

3.2.1. Mortality rate

Results of the present study showed that the mortality percent among different groups of treated mice were recorded in the following order, positive control & SLN (60%) > R200 (40%)> R400 (35%).

3.2.2. Changes in tumor size.

After two weeks from tumor injection, the size of tumor was approximately the same in all injected groups. Tumor size in positive control (untreated) & SLN mice showed noticeable increase as the period extended (Table 1). Tumor size showed noticeable increase after only one week from treatment with the descending order, positive control as well as SLN, then R200, R400, while through the second, third and fourth week of treatment tumor size showed decrease among treated groups. At the end of fourth week animals treated with R200 and R400 showed a significant (P < 0.05) decrease in tumor size compared with positive control.

Table (1): Showing tumor size changes during the period of experiment in different experimental animal groups.

	+ve control		SLN		R200		R400	
0 time	44.57	3.99	48.07	2.12 [a]	43.43	4.09	38.64	3.92
1st week	149.21	7.67 [a]	150.86	5.16 [a]	83.57	2.19*[a]	60.00	6.08*[a]
2nd week	211.50	14.69 [b]	209.71	5.72 [b]	47.43	4.77*[b]	34.71	1.84*[b]
3rd week	224.50	13.37	218.71	9.52	39.43	2.19*	27.43	1.80*[c]
4th week	311.50	10.44*[d]	317.71	8.95*[d]	28.36	2.01*[d]	19.00	1.69*[d]

*p: significant difference from control group within the same raw (P < 0.05). [a]p: signficaant difference between 0 time and the 1st week (P < 0.05). [b]p: signficaant difference between the 1st week and the 2nd week (P < 0.05)within the same column. [c]p: signficaant difference between the 2nd week and the 3rd week (P < 0.05) within the same column. [d]p: signficaant difference between the 3rd week and the 4th week (P < 0.05) within the same column.

3.3. Hematological analysis

3.3.1. Hemoglobin content, RBCs, WBCs, and platelets count

The data in table (2) reported that positive control showed significance (p<0.05) drop in both hemoglobin content and RBCs count as compared to negative control group, while treated groups with rosemary alone (R200 & R400 groups) showed a significant increase (p<0.05) in both hemoglobin content and RBCs count in comparison to positive control group. It was observed that treatment with the low dose (R200 group) is more pronounced with insignificant (p> 0.05) difference in-between.

Animals in the positive control group showed a significance (p<0.05) decrease in platelets count comparing with the negative control group. A marked improvement in platelets count was also observed in rosemary alone treated groups (low and high doses) as compared with all treated groups, moreover, treatment with high dose of rosemary (R400) showed the most pronounced effect as shown in table (2) On the other hand, the data reported in table (2), WBCs count showed a significance (p<0.05) increase in positive control group in comparison to negative control group. While, animals received treatment with rosemary low and high doses showed a marked improvement in WBCs count comparing with negative control group. No significant difference was recorded between the group of animals treated with rosemary at the high dose (R400) and the negative control group.

Table (2): The effect of high and low doses of rosemary extract loaded SLNs on TNF-α, Mortality rate, hematological parameters (HB%, RBCs, WBCs, and platelets' counts) in mice blood bearing the Ehrlich Solid Tumor (EST).

	Control	+ve control	SLN	R200	R400
Mortality rate (%)	0.00 [a]	62.00 [d]	61.00 [d]	40.00 [c]	35.00 [b]
HB%	11.05±0.01[d]	10.18±0.03[bcd]	9.6±0.02[bc]	10.45±0.02[cd]	10.2±0.03 [bcd]
RBCS (10^6/µl)	5.80±0.02[b]	5.40±0.02[ab]	5.40±0.02[ab]	5.78±0.02[b]	5.43±0.02 [ab]
WBCS (cells/µl)	5175±21[ab]	9275±23[c]	8225±16[d]	8425±20[dc]	5050±11[ab]
PLts (10^3/µl)	531±0.7 [d]	406±1.9 [a]	461±1.2 [acd]	423±1.1 [ac]	499±2.2 [cd]
TNF (pg\ml)	15.00±0.02[a]	78.00±0.04[b]	76.20±0.02[b]	55.20±0.02[c]	53.00±0.03[d]

The groups that share the same letter are non-significant in-between within the same raw (P < 0.05).

3.4. Serum level of Tumor Necrosis Factor Alpha (TNF-α).

The data reported in table 2 showed that a significant (P<0.05) rise in TNF-α levels in the positive control group relative to the negative control group. In contrast to the positive control group, treatment with rosemary alone at the two tested doses resulted in a significant (P<0.05) decrease in TNF-α levels.

3.5. Cell cycle phase's percentage by Flowcytometery analysis:

There are various phases in the traditional cell cycle: G0, G1, S, G2, and M. In the G0 phase, cells are dormant. Because they are going through changes associated with cell division, cells in other phases are referred to be cycling. As shown in Fig (2), when compared to the control, the positive control group demonstrated a significant (P < 0.05) rise in the percentage value of the G2/M-cells and a significant (P < 0.05) drop in the percentage value of the G0/G1-cells. However, both the low and high doses of rosemary treatment demonstrated a significant (P < 0.05) increase in percentage value of the G0/G1-cells (cell cycle arrest at G0/G1 phase) and showed a significant (P < 0.05) decrease in percentage value of the G2/M-cells (stops cell cycling during G2/M).
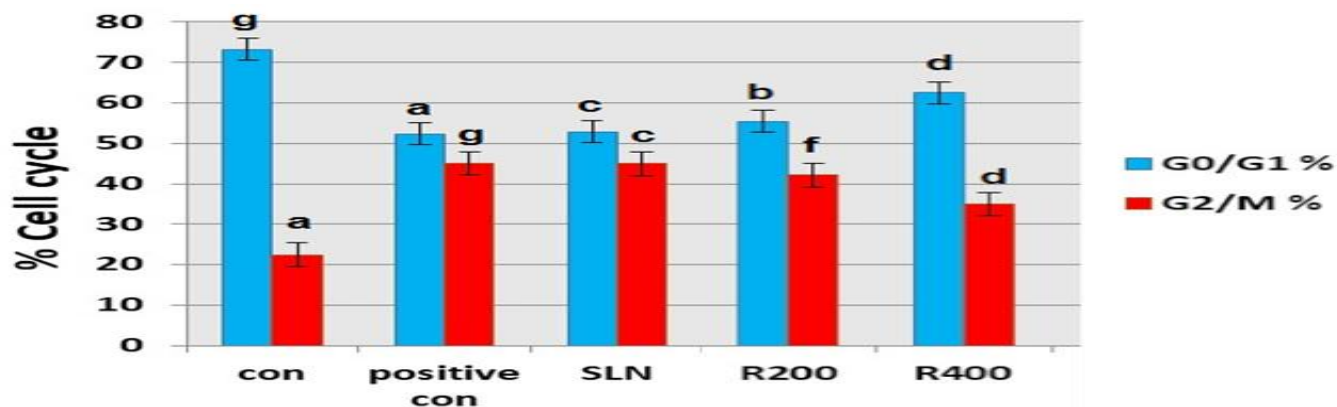
Fig. (2): The effect of rosemary loaded SLNs high and low doses on cell cycle phase's percentage in mice bearing the Ehrlich Solid Tumor (EST). The groups that share the same letter are non-significant in-between.

4. Discussion

Cancer is the main cause of mortality worldwide, especially in developing countries. It is an involved disease with numerous risk factors, including poor nutrition, oxidative stress, and genetic mutation. The anticancer effects of Rosemary (*Rosmarinus officinalis*) polyphenols have been investigated by several research groups [14,15]. Recent studies have used nanomaterial's delivery systems that are synthesized naturally for the inhibition and treatment of several solid tumors [16]. Solid lipid nanoparticles (SLNs) attracted more attention among other polymeric nanoparticles. It has many advantages such as its ability to control drug release, drug targeting and to increase physical stability as well as the ease of large-scale production. In addition, it exhibits high drug loading capacity and low toxicity [17]. Therefore, the application of ROS-loaded SLNs should be a worthwhile and promising strategy in cancer treatment.

Hence, the current study focused on study the anticarcinogenic potency of ROS- SLNs in vivo using Ehrlich solid tumor (EAC). The result of the present study indicated a significant dose-dependent reduction in the tumor size and mortality rate in animals received ROS-SLNs compared to untreated EAC groups. These data were consistent with the results of other recent studies that proved the anticancer activity of ROS leave extract. Singletary & Rokusek [18] reported that ROS extract possess chemo-preventive activities in 7,12dimethylbenz (a) anthracene-induced mammary tumors in rats. When ROS was given orally in water for 15 weeks in a nude mouse model of skin cancer produced by 7,12-dimethylbenz(a) anthracene (DMBA), the tumor weight and width significantly decreased as compared to the mice treated with DMBA alone [19].

Tumor growth in nude mice xenografted with SW620 colon cancer cells significantly decreased after receiving 1 mg/mL of ROS (in the drinking water) for 32–35 days [20]. Using athymic nude mice xenografted with HCT116 colon cancer and given ROS (100 mg/kg/day) dissolved in olive oil for four weeks, a similar reduction in tumor size was seen [21]. Treatment of mice xenografted with 22RV1 prostate cancer cells with ROS (100 mg/kg/day) for 22 days also resulted in a significant reduction in tumor volume [14]. The most important polyphenols in the plant extract, carnosol, carnosic acid (CA), and rosmarinic acid (RA), are responsible for the potent anticancer effects of ROS extract [14,20]. These compounds have been shown to induce apoptosis within cancer cells [22].

Regarding the combination with anticancer drugs, ROS extract was previously reported to exhibit a strong cooperative antitumor effect in combination with cisplatin on ovarian cancer cells in vitro [23,24] and increased significantly its antitumor efficacy in n tumor-bearing mice [25]. Moreover, rosemary extract displays a synergistic antitumor effect in combination with the anticancer drug 5-Flurouracil, in colon cancer therapy [26]. Regarding results of hematological analysis, treatment with ROS -SLNs at the two tested doses showed a marked elevation in hemoglobin content, platelets count, white blood cells, and red blood cells, respectively when compared with untreated EAC group. This finding is in line with Habtemariam [27] who observed the potency of rosemary *Diterpenes*, *Carnosic* acid and Carnosol to regulate inflammation and establish a suitable environment for erythropoiesis. Administration of ROS-SLNs at the two tested doses resulted in a significant reduction in TNF-α as compared with untreated EAC group. This effect might be attributed to the presence of Carnosic acid (found in rosemary) that has been reported to have antioxidant and antiinflammation properties [28,29].

Conclusion

In conclusion, ROS exerted anticancer effects, furthermore, their formulations in SLN nanoparticles enhance its therapeutic effects and ameliorate its efficacy because it allows ROS leave extract to be effectively captured and released in a regulated manner.

Conflict of interests
No conflict of interests to be declared.

Acknowledgements

Author Contributions

All authors contributed to this work. N.I. Said and D.H. Elsayed prepared the samples and completed the experimental measurements. Both D.H. Elsayed, A. A. Said, H. R. Hamad, and A. M. Abd-Elrazek shared writing and followed the performance of the experiments. A. M. Abd-Elrazek helped the first

author complete the sample preparation. A. M. Abd-Elrazek with N.I. Said completed the paper writing, analyzing the data, and validation. A. M. Abd-Elrazek followed the revision and submission of the manuscript for publication.

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