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Processed Cheeses Fortified by *Laurus nobilis* L. Extract Nanoemulsion Ameliorate Hyperhomocysteinemia in Ehrlich Ascites Carcinoma Model

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Abstract

We aimed to evaluate the protective effect of processed cheeses fortified by *Laurus nobilis* L. extract nanoemulsion against hyperhomocysteinemia in Ehrlich ascites carcinoma (EAC) model. *Laurus nobilis* L. extract nanoemulsion was prepared via spontaneous emulsification procedure. Then, nanoemulsion was added by different loadings to supplement processed cheeses. The morphology of *Laurus nobilis* L. extract nanoemulsion was detected using transmission electron microscopy (TEM). Female Swiss albino mice received processed cheese for two weeks then inoculated once with EAC cells. After the end of the experiment, blood samples were collected for determination of serum superoxide dismutase (SOD) , thiobarbituric acid reactive substances (TBARS), B-cell lymphoma 2 (Bcl-2), matrix metallopeptidase-9 (MMP-9), Tumor necrosis factor-α (TNF-α), and homocysteine (Hcy). Results revealed that the formation of *Laurus nobilis* L. extract nanoemulsion in spherical shape in size around 50 -120 nm. Mice inoculated with EAC cells showed significant increase in serum Hcy, TNF-α, TBARS, and MMP-9 levels while there was a significant decrease in SOD activity and Bcl-2 level. Pretreatment with different concentrations of *Laurus nobilis* L. extract significantly attenuated oxidative stress, inflammation, and induced apoptosis compared to EAC group.

Keywords: *Laurus nobilis* L. extract nanoemulsion; Homocysteine; B-cell lymphoma 2; Ehrlich ascites carcinoma; HPLC

1. Introduction

Initiation of cancer causes uncontrolled alteration in cell growth and division in addition to impairment in apoptotic signals [1]. Previous studies reported the key role of oxidative stress in cancer initiation and progression. Oxidants enhance the alterations in DNA, cell proliferation, survival and invasiveness [2] On the other hand, cytokines such as TNF-α act as a major mediator of inflammation in the pathogenesis of many diseases, recently it is suggested to act as an endogenous tumor promoter [3–5]. Plasma homocysteine (Hcy) considered as a risk factor for different diseases [6] and cancer development [7]. Although chemotherapy showed effectiveness in treatment of advanced or metastatic stages of cancer, it may induce damage to normal cells and tissues [8]. Moreover under certain molecular mechanisms such as activation of enzymes responsible for drug detoxification and modification in the molecular targets of drugs; cancerous cells become resistant to chemotherapy [9].

Natural products are used in the treatment of different diseases including cancer and bacterial infection in addition to immunological disorders [10–12]. *Laurus nobilis* L., a part of lauraceae family, is widely cultivated in Europe and the Mediterranean, it has been used as a traditional medicine [13]. *Laurus nobilis* L. has antimicrobial properties as well as antioxidant , and anti-inflammatory [14–16]. The leaves of this plant have been used as a treatment of different diseases like arthritis, skin inflammation, rheumatic pains, and asthma [17].

Nanoemulsion extracts from natural products offer promising approaches for the protection of several life-threading diseases including cardiotoxicity [18], lung cancer [19], diabetes [20], Alzheimer's [21], nephrotoxic [22], and neurotoxicity [23]. Processed cheeses are a very popular and important dairy product, which is prepared by blending one or more varieties of natural cheese, plus emulsifiers, and sometimes other ingredients added

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such as herbs, vegetables, and spices. Recently, there was more attention in the progress of processed cheese manufacture due to its high nutritional value and its benefit for the human body to deliver specific nutrients or for people who suffer from some diseases.

The current study aimed to evaluate the role of processed cheese fortified with *Laurus nobilis* L. extract nanoemulsion in protecting against hyperhomocysteinemia in EAC model.

2.Materials and Methods

2.1. Materials

Dried leaves of *Laurus nobilis* L. were obtained from the herbal market of Giza, Egypt. Cheddar cheese, Ras cheese, Skim milk powder, and skimmed milk acid coagulated cheese (Karish cheese) were bought from a super market, Giza, Egypt. Emulsifying salts (Joha S9) were gotten from, Chemie GmbH, Ladenburg, Germany. 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) Folin- Ciocalteaureagent, and Gallic acid were gotten from Sigma Aldrich, Germany. Homocysteine (HPLC standard) and all other chemicals used in Hcy estimation were of HPLC grade and purchased from Sigma-Aldrich Company, St. Louis, MO, USA.

Sixty female Swiss albino mice (20-25 g) were achieved from the animal house of the National Research Centre (NRC), Giza, Egypt. Mice were let for one weak as an acclimatization period before beginning the experiment; water was available *ad libitum* and standard diet, for; mice were kept under constant environmental conditions at temperature 25±2º c. The guidelines of the ethical care and animal' treatment were followed the ethical committee' regulations of the National Research Centre (NRC).

2.2. Methods

2.2.1. Preparation of *Laurus nobilis. L* extract

The plant leaves were identified by horticulture professors at NRC, Giza, Egypt. Dried leaves were grounded, and 200 g of dried powder soaked and shacked in 2000 ml ethanol for 48 hours. The macerates were filtered by using Whatman no. 1 filter paper, and then the plant extract was concentrated at 40°C under vacuum by using a rotatory evaporator. The extract was stored at −20°C in dark glass bottles until further analyses and study application.

2.2.2.Characterization of *Laurus nobilis* extract nanoemulsion

The morphology of *Laurus nobilis L.* extract nanoemulsion was detected with transmission electron microscopy (TEM) (JEM-2010; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 200 kV. The TEM specimens were fabricated using insertion a drop of *Laurus nobilis* L. extract nanoemulsion on the grid of carbon-coated copper.

The droplet size of the prepared nanoemulsion of *Laurus nobilis* L. extract was examined using dynamic light scattering (DLS) with a ZetasizerNanoZS laser diffractometer (Malvern Instruments Ltd, Worcestershire, UK) working at 633 nm at 25°C and equipped with a backscatter detector (173°), which is appropriate to measure submicron particles. Zeta potential value was also determined via a ZetasizerNanoZS laser diffractometer. It is well known the value above +30 or below -30 is an indication for the good stability of the prepared nanoparticles. Thus, zeta potential value provides us a predication about the stability of the formed compounds.

2.2.3. Manufacture of processed cheese fortified with *Laurus nobilis. L* extracts nanoemulsion

Processed cheese was manufactured by using young Ras cheese (250 g), Karish cheese (500 g), matured cheddar cheese (250 g), skim milk powder (100 g), emulsifying salt (33 g), and water (400 g) as a base formula according to Youssef et al., [24]. Cheeses were minced and placed into processing pot of 2.5 Kg capacity, cooked with controlled agitation for 5 min at 90°C using direct injection steam. The hot melted cheese was filled into glass containers, allowed to cool to 45°C under controlled conditions before adding *Laurus nobilis* L. extract nanoemulsion. The cheese containers were divided into three groups, the 1st was served as control without adding *Laurus nobilis* L. leaves extract nanoemulsion, while the 2nd and 3rd groups were supplemented with 0.25 % and 0.50 %*of Laurus nobilis* L. extract nanoemulsion, respectively. Cheese containers were kept in the refrigerator until analysis study.

2.2.4.Estimation of total phenolic content of processed cheese

Total phenolic content of the processed cheese supplemented with *Laurus nobilis* L. extract nanoemulsion was determined by Folin–Ciocalteu method [25] and Gallic acid as standard. Cheese extract solution (0.5 ml) , de-ionized water (20 ml), and of the Folin–Ciocalteau reagent (625 µl) were added in a volumetric flask (25 ml) . After three min, a saturated solution of Na_2CO_3 (35%) (2.5 ml) was added. The absorbance was read at 760 nm (spectrophotometer Hitachi U-3210) after remaining one hour in the dark with intermittent shaking. Calibration curve was prepared using Gallic acid. Total phenol contents were expressed as mg Gallic acid /g dry weight.

2.2.5. Determination of antioxidant activity of processed cheese

2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging activity of the processed cheese content of *L. nobilis* nanoemulsion extract was assessed by method according to Bandoniene et al. [26] with some modifications. 100 µl methanol phenol extracts and 3.9 ml methanol DPPH were added in a cuvette; the absorbance was measured at 515 nm using the spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The percentage of DPPH radical scavenging of the samples, expressed as percentage inhibition of DPPH, were calculated as the following formula, where A_0 is the absorbance at 515 nm of the blank sample and A is the final absorbance of the test sample at 515 nm.

Inhibition (%) = 100 X (A-$A_\circ$) / $A_\circ$

2.2.6. Physiochemical analysis of processed cheese

Fresh processed cheese was analyzed for moisture, total Solids, total protein, fat, and ash content according to AOAC (2007). The carbohydrate values were measured by calculation. The pH values were examined by using a digital laboratory (Hanna Co., Italy). All of the analyses were run in triplicate.

2.2.7. Antioxidant activity of processed cheese fortified with Laurus nobilis L.extract nanoemulsion

Firstly**,** 10 g of cheese was placed into the 100 ml conical flask then 20 ml of methanol/water mixture (80:20) were added and shaking for 30 min in the ultrasonic water bath. The remaining solution was filtered and completed to 25 ml with the extraction solvent. Then the extracts were individually collected in glass closed containers for analysis.

2.2.8.Tumor transplantation

Ehrlich cell line was from Medical Biochemistry Unit, National Research Centre, Egypt. Mice were inoculated with 2.5×10^6 cells/ mouse intraperitoneally using a fine needle. Tumor was observed within two weeks from the inoculation of Ehrlich cells.

2.2.9.Experimental design

Sixty female Swiss albino mice were divided into six groups (10 mice each). Control group: healthy mice (received 250 µL vehicle / mice/ day orally for 14 days) , EAC group: healthy mice inoculated once with 2.5×10^6 cells/mouse intraperitoneally, prophylactic group I: healthy mice received processed cheese (3 mg/g body weight / day orally) according to Malti and Amarouch [27] for 14 days then inoculated once with 2.5×10^6 cells/mouse intraperitoneally , prophylactic group II: healthy mice received *Laurus nobilis* L. extract nanoemulsion only(3 mg/g body weight / day orally) according to [27] by oral administration for two weeks then inoculated once with 2.5×10^6 cells/mouse intraperitoneally, prophylactic group III: healthy mice received processed cheese fortified with *Laurus nobilis* L. extract nanoemulsion(3 mg/g body weight / day orally) [27] in a concentration of 0.25 (v/v) / daily for 14 days by oral administration then inoculated once with 2.5×10^6 cells/mouse intraperitoneally, prophylactic group IV: healthy mice received processed cheese fortified with *Laurus nobilis* L. extract nanoemulsion (3 mg/g body weight / day orally) [27] in a concentration of 0.5 (v/v) / daily by oral administration for 14 days then inoculated once with 2.5×10^6 cells/mouse intraperitoneally

After the experimental period ,fasting blood samples were withdrawn from the orbital vein and collected in clean tubes for the biochemical analysis.

2.2.10. Biochemical analysis

Serum antioxidant parameters: Serum TBARS levels , SOD activity and GSH were estimated as described previously [28–30] respectively using spectrophotometer.

Anti-apoptotic and inflammatory markers: Serum B-cell lymphoma 2 (Bcl-2), Tumor necrosis factor- α (TNF-α), and matrix metallopeptidase-9 (MMP-9) were determined using ELISA according to the manufacturer's instructions (Glory Science Co, Ltd, Del Rio, TX, USA).

2.2.10.1.Homocysteine (Hcy)

Serum Hcy was determined by high performance liquid chromatography (HPLC) system, (Agilent technologies 1100 series) as described previously [31].

Sample extraction: serum sample (400 µL) was mixed with trichloro acetic acid (32 µL) incubated for 30 min. in ice for protein precipitation, then centrifuged at 4000 rpm at 4°C for 20 min. The produced supernatant was taken and filtered through PVDF syringe filter with pore size 0.45µm. Twenty µL from the filtered solution were injected onto HPLC [31,32].

HPLC condition: The separation was completed through a reversed phase (RP) column (Agillent 5HC-C18(2) 250x0.46 mm) at 40°C; the mobile phase (MP) consists of sodium phosphate monobasic monohydrate 40 mmol/L, heptanesulfonic acid (8 mmol/L) , and methanol 18% (v/v) . The pH was adjusted to 3.1 using phosphoric acid; the mobile phase was filtered twice through Whatman membrane filter (Cellulose Nitrate , 0.45µm pore size) and then eluted at a flow rate 1 ml/min ; UV detection was set at 260 nm.

Serial dilutions of Hcy standard were injected onto HPLC and their peak areas were determined. The

concentration of each sample was calculated using Agilent Chem Station software for LC/MC and HPLC.

2.2.11. Statistical analysis

SPSS software version 12 was used and all data were expressed as mean ± standard error (SE) .data was analyzed by one-way analysis of variance (ANOVA) . The probability was considered significant when it less than or equal 0.05.

3. Results and Discussion

Nanoemulsions are establishing growing utilizing in the food and beverages industries for definite applications owing to their exclusive physicochemical as well as novel functional properties. A variability of fabrication techniques have been established to synthesis nanoemulsions, in addition these methods may appropriately be categorized for example low energy or high energy techniques [33]. Low energy methods reliance on the spontaneous creation of small oil drops inside oil water-emulsifier structures once the environmental conditions or solution are changed [34]. In contrast, High-energy techniques use mechanical devices able to producing strong disruptive forces which destruction the phases of oil and water as well as make possible the construction of small oil drops, as microfluidizers ,sonication and high-pressure valve homogenizers methods [35]. The smallest particle size was attainable via the high energy technique count on homogenizer kind, homogenizer working conditions such as (time, energy intensity, and temperature), sample preparation for example (emulsifier nature, oil kind, and comparative concentrations).

Laurus nobilis L. extract was prepared in the nanoemulsion form using good stabilizing agent; Tween 20 to act as a cover for the formed particles. The prepared solution was continuously stirred for 10 h with the aid of magnetic stirrer at 80 ^{o}C. Firstly, the extract was formed with yellow color. This yellow color was changed to milky solution when prepared in the small size (nanoemulsion state). The prepared extract nanoemulsion was characterized by means of TEM, DLS and zeta potential.

After *Laurus nobilis* L. extract nanoemulsion preparation and characterization, different concentrations were blended with cheese. These blended admixtures were injected to experimental mice as displayed in scheme 1. The Levels TBARS, GSH, SOD activity, α (TNF-α), 9 (MMP-9), Bcl-2 and Hcy were assessed.

3.1. The morphology and droplet size of the prepared Laurus nobilis L. extract nanoemulsion

Transmission electron microscopy was used to examine the structure morphology of the prepared extract nanoemulsion using low energy technique as revealed in (Fig. 1). The TEM images are taken at different magnification to illustrate the particle shape of *Laurus nobilis* L. extract nanoemulsion. The Fig. 1 (A, B) demonstrated that the formation of *Laurus nobilis* L. extract nanoemulsion in spherical shape in size around 50 -120 nm.

For further confirmation, particle size analyzer was carried out using dynamic light scattering (DLS) for determination of hydrodynamic particle sizes. As mentioned, the nanoemulsion of *Laurus nobilis* L. extract nanoemulsion was achieved after was stirring for 10 h *via* magnetic stirrer (700 rpm) at 80 ^{o}C using Tween 20 as surfactant for its high HLB value that favors preparation of oil-in-water nanoemulsion.

The overall particle size distribution of *Laurus nobilis* L. extract nanoemulsion, rather than just the particle diameter, is essential for numerous applications of nanoemulsion. Also, small molecule surfactant like Tween 20 acquires fast adsorbed onto emulsion droplet surface and therefore they are more active in decreasing droplet length than polymeric surfactants. Fig. 1C display the average size of the prepared *Laurus nobilis* L. extract nanoemulsion and it is depicted that the size is around 113 nm. On the other hand, zeta potential value of the synthesized nanoemulsion is -43 nm as shown in Fig. 1D.

The data obtained from DLS and zeta potential proved that the nanoemulsion is prepared with small size and in excellent stable form. The data is in accordance with eye observation for the formed nanoemulsion. It is observed that the originated nanoemulsion is stable with a long time with no noticeable for any precipitation.

3.2. Chemical composition of processed cheese with different ratios of L. nobilis leaves extract nanoemulsion

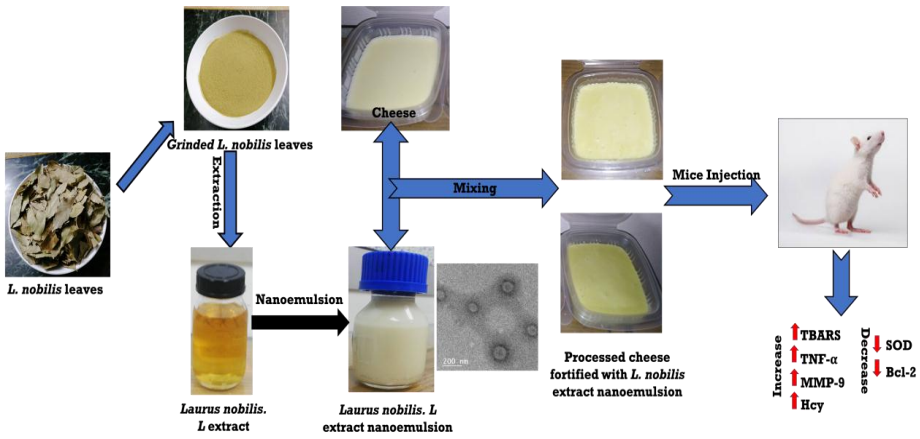
The chemical composition of various blends used in the manufacture of processed cheese by adding different ratios (0%, 0.25% and 0.50%) of *Laurus nobilis* L. extract nanoemulsion to the base formula is shown in Table (1) the total solids content of the processed cheese ranged from 39.44 to 39.63% in all treatments, including the control. The total solids content increased significantly ($p \leq 0.05$) with an increase in *Laurus nobilis* L. extract nanoemulsion in the base formula. This increase in total solids content was reflected in a decreased in the moisture. The highest total solids were observed with treatment supplemented with 0.50 %nanoemulsion extract compared with control treatment. In contrast, the fat percentage was not affected by the added of *Laurus*

nobilis L. extract nanoemulsion and the pH values decreased with increasing of leaves extract nanoemulsion in cheese treatments. These results are in harmony with. The date reported by El-Naggar et al., [6].The protein, ash, total phenol content, and antioxidant activity in the processed cheese also demonstrated in Table (1) increased to record the highest values of 19.47 %, 4.89 %, 125.56 mg/Kg cheese, and 45.08 %, respectively in treatment supplemented with 0.50 %nanoemulsion extract. This may be due to the high content of *Laurus nobilis* L. extract nanoemulsion during substitution with water in cheese base formula at different ratios, 0.25%, and 0.50%. The *Laurus nobilis* L. extract was high in protein and ash content and, these data in the agreement with Guenane et al., [36] and AL-Hashimi and Mahmood [37], who noted that the *Laurus nobilis* L. extracts may be a good source of minerals and crude protein to treat number of diseases and can be used as medicinal food. Previous studies confirmed that *Laurus nobilis* L. extract has an excellent total phenol content and high DPPH scavenging activity for that, it could be considered as a potential source for pharmaceutical and food industry [38,39].

Table 1: Physiochemical Analysis, Total Phenol Content Mg/Kg Sample and Antioxidant Activity by DPPH of Processed Cheese Control and Fortified with *Laurus Nobilis* L. Extract Nanoemulsion

Parameters	Control withoutnanoemulsion extract	Treatment with 0.25 %nanoemulsion extract	Treatment with 0.5 %nanoemulsion extract
Total solids%	$39.44^{c} \pm 0.038$	$39.58^{b} \pm 0.095$	$39.63^{a} \pm 0.577$
Moisture%	$60.56^{a} \pm 0.046$	$60.42^{b} \pm 0.020$	$60.37^{c} \pm 0.090$
Protein%	$19.07^{c} \pm 0.016$	$19.24^{b} \pm 0.043$	$19.47^{a} \pm 0.211$
Fat%	$10.50^{a} \pm 0.577$	$10.50^{a} \pm 0.517$	$10.50^{a} \pm 0.525$
Ash%	$4.83^{c} \pm 0.266$	$4..85^{b} \pm 0.242$	$4.89^{a} \pm 0.211$
Carbohydrate%	$5.04^{a} \pm 0.467$	$4.99^{b} \pm 0.441$	$4.77^{c} \pm 0.508$
pH	$5.87^{a} \pm 0.220$	$5.85^{b} \pm 0.280$	$5.83^{c} \pm 0.320$
Total phenol content (TPC)	$45.32^{c} \pm 3.17$	$110.98^{b} \pm 3.22$	$125.56^{a} \pm 3.05$
Antioxidant activity %	$8.50^{c} \pm 2.19$	$25.85^{b} \pm 2.27$	$45.08^{a} \pm 3.14$

Data expressed as mean of 3 replicates ± standard error. Means in the same row showing the same capital letters are not significantly different ($P \leq 0.05$).



Scheme 1: Steps for the Preparation of *Laurus Nobilis* L. *Extract* Nanoemulsion, Blending with Cheeseto Be Used as Mice Injection.

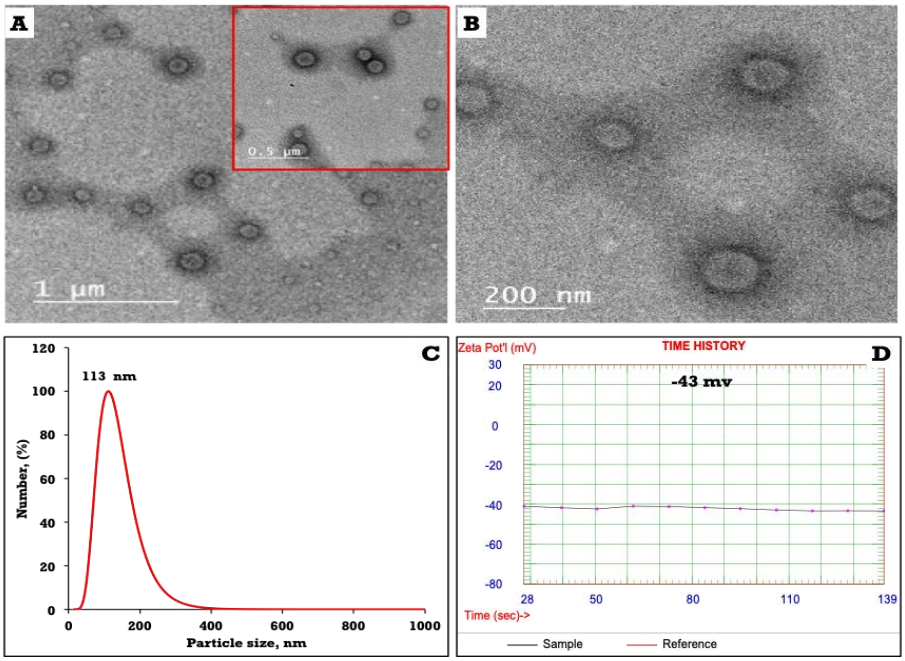


Figure 1: (A, B) TEM Images, (C) Average Particle Size and (D) Average Zeta Potential of the Prepared *Laurus Nobilis* L. Extract Nanoemulsion

EAC is a well-established spontaneous murine paradigm for studying biological aspects of tumor, tumor pathogenesis and evaluation of anti-tumorigenic agents [40].

Accumulation of genetic alteration such as tumor suppressors, oncogenes, and senescence genes resulting in neoplasia, in addition oxidant such as toxins release reactive oxygen species (ROS) which disturb the imbalance in the status of cellular redox thus forces normal cells to a more oxidized state. Taking together; these factors are involved in the development of many diseases including cancer [41].

In this study, intraperitoneal inoculation of EAC showed a significant increase in the levels of serum TBARS in EAC group compared to the control group and a significant decrease in serum GSH and SOD in EAC group compared to control (Fig. 2a, b, c).

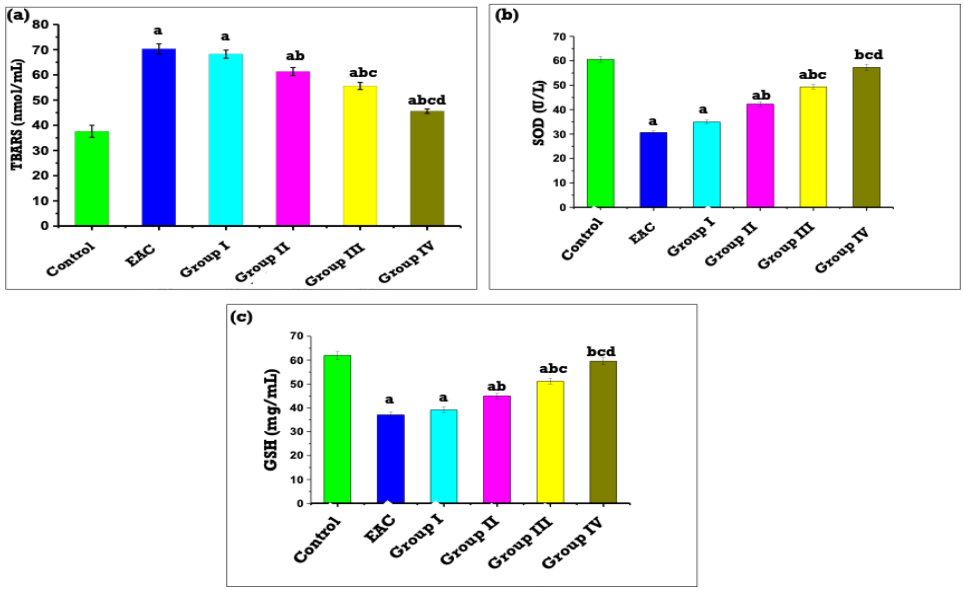


Figure 2: Oxidant and Antioxidant Parameters in Different Studied Groups , P: Significant Difference (<0.05), P^a Compared to the Control Group, P^b Compared to EAC Group, P^c Compared to Prophylactic Group II, P^d Compared to Prophylactic Group III.

Our results may be explained by the fact that under normal conditions, cells were able to protect cellular components from damage by reducing ROS via enzymatic and non-enzymatic antioxidants. However, under the stress induced by EAC cells and the massive release of ROS resulting in obstruction in cellular mechanisms, impairment in the scavenging of ROS [42], and destroying cellular macromolecules like proteins, lipids and nucleic acids resulting in cell death [43].

Medicinal plants represent a major attention for drug advancement and manufacturing ; it holds an important portion in drug-market all over the world [44–46].

In this work, we used *Laurus nobilis* L. *extract* alone or nanoemulsion in two different concentrations to find out the best formula protects against experimental EAC.

A significant decrease in the levels of cellular TBARS and a remarkable increase in SOD activity and GSH level in EAC groups pre-treated with different concentrations of *Laurus nobilis* L. extract nanoemulsion were observed compared to the EAC group (Fig. 2). It is worth to mention that the higher concentration (0.5 v/v) of *Laurus nobilis* L. extract nanoemulsion showed the best result compared to *Laurus nobilis* L. extract alone or in the lower concentration (0.25 v/v). These results suggested that *Laurus nobilis* L. extract significantly reduced the level of TBARS induced by EAC; and the nanoemulsion effectively increased its potential which is considered a useful step in cancer therapy [47]. Additionally, the observed increase in the level of antioxidants in EAC groups pre-treated with different concentrations of *Laurus nobilis* L. extract nanoemulsion may explain the role of antioxidant defense mechanisms that play a critical role in detoxifying superoxide radicals and H_2O_2. Our results were in agreement with [48] who reported that anti-oxidants prohibit release of ROS damage and might prevent progression of cancer.

Another important marker in this study was matrix metalloproteinase-9 (MMP-9) that has been involved in diverse roles in cancer growth and expansion [49]. MMP-9 is known to be a monitor for the tumor microenvironment, involved in the initiation, development, invasion, and metastasis of cancer through degradation of basement membranes and presenting cryptic peptide epitopes into the extracellular matrix, enhancing cellular invasion [50]. In this study, inoculation of EAC cells appeared a significant increase in MMP-9 levels compared to control (Fig. 3a). These results were in agreement with an *in vitro* study of [49] who showed that human breast cancer cells release MMP-9 showed high rate of development and invasion for pulmonary metastasis in a mouse orthotopic model of basal-like breast cancer which confirm the role of MMP-9 in tumor vascularization.

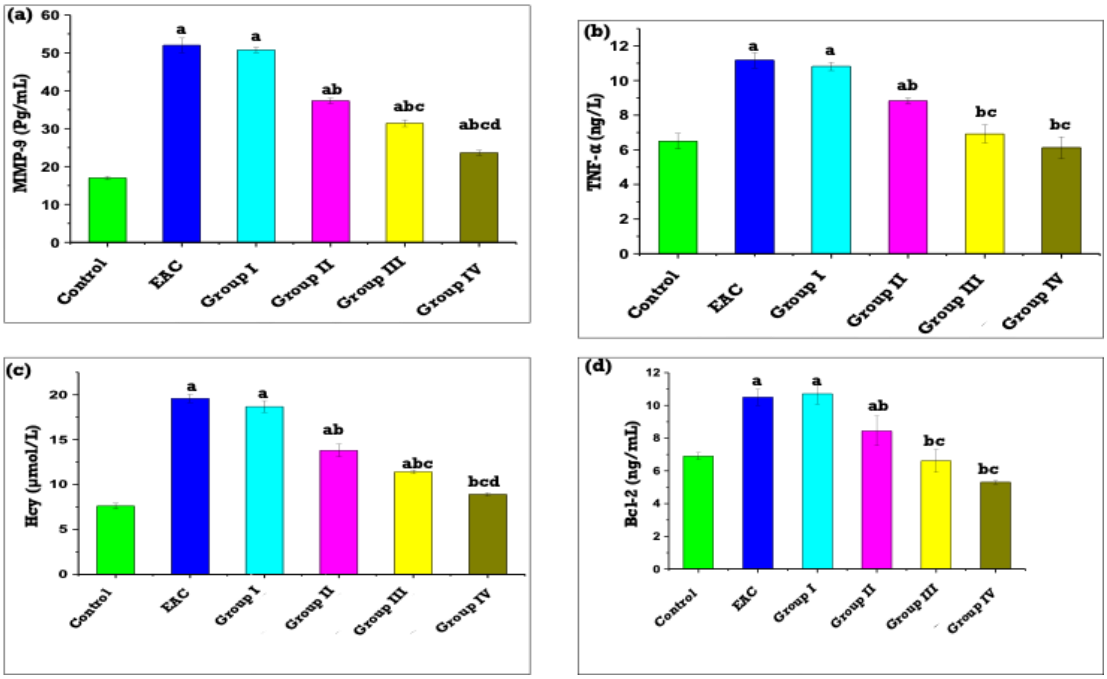


Figure 3: Serum Inflammatory and Anti- Apoptotic Markersin Different Studied Groups, P: Significant Difference (<0.05), P^a Compared to The Control Group, P^b Compared to EAC Group, P^c Compared to Prophylactic Group II, P^d Compared to Prophylactic Group III.

TNF-α suggested to play exceptional role in the host's immune defense, inflammation and homeostasis [51]. Cancer causes excessive releasing of cytokines such as TNF-α and interferon gamma (IFN-γ) which is produced by macrophages [52].

In the current study, a significant increase in serum level of TNF-α was observed in EAC group when compared to the control group (Fig. 3b);this result was in agreement with [53]. In several diseases that depend on oxidative stress ,excessive production of ROS is increased leading to stimulation of lipid peroxidation and hence elevation of inflammatory mediators such as TNF-α [54]. During cancer development; impairment in apoptosis is a crucial step in efficient therapeutic strategies; Bcl-2 protein family plays a major role in regulating apoptotic mechanisms [55]. In addition to the elevation of oxidative and inflammatory markers a significant increase in serum Hcy (Fig. 3c) was observed in EAC group compared to the control group.

Results obtained from this study showed that Bcl-2 levels were significantly increased in EAC group compared to control (Fig. 3d). Thus, the up-regulation of Bcl- 2 has been associated with various types of cancers, as breast cancer, prostate cancer and colon cancer [56]. Pro-apoptotic family members Bax and Bak are important for inducing permeabilization of the outer mitochondrial membrane (OMM) and the consequent release of apoptogenic molecules (such as Smac/Diablo and cytochrome c), which leads to caspase activation [55]. The anti- apoptotic family, including Bcl- 2 or Bcl- xL, inhibit Bax and Bak OMM permeabilization triggered by BH3- only proteins [57].

According to our results, another possible mechanism that may be involved in the progression of EAC is the initiation of extrinsic apoptotic pathway by TNF-α, this pathway is associated with the activation of the caspase-8 cascade which also transmits the apoptotic signal in mitochondria [58]. Also, the generation of reactive oxygen species (ROS) and Bcl-2 protein-regulated compartmentalization of apoptosis-inducing agents in the inter-membrane of the mitochondrial space [59].

In addition to the elevation of oxidative and inflammatory markers a significant increase in serum Hcy (Fig. 3c) was observed in EAC group compared to the control group. It was found that cancer patients are frequently associated with elevated circulating total homocysteine (tHcy) even though they are not treated with anti-folate drugs suggesting that the rapid proliferation rate of tumor cells would cause an elevation of circulating tHcy or an increase in the concentrations of Hcy in the cell medium. It has been assumed that rapid proliferation of tumor cells would deplete folate and inactivate the methionine synthase catalyzed re-methylation reaction. As a result, homocysteine would not be converted to methionine and become increased in a grade known as hyperhomocysteinemia [60].

Deficiencies in apoptosis are no more important than in cancer. Lack of apoptosis impinges on both the effectiveness of treatment and the survival of the patient [57]. Bcl- 2 is an anti- apoptotic protein expressed on the outer membrane of the mitochondria. Its overall role is to inhibit multidomain and BH- 3 only proteins, which prevent cytochrome c and Smac/Diablo release from the mitochondria, thus preventing apoptosis [57].Thus, our results revealed that pre-treatment with *Laurus nobilis* L. extract nanoemulsion showed a significant decrease in anti-apoptotic protein (Bcl-2) compare to the EAC group.

tHcy may be used as an accurate tumor marker for monitoring cancer patients during treatment, and hyperhomocysteinemia as a risk factor for carcinogenesis [7]. Pre-treatment with *Laurus nobilis* L. extract nanoemulsion with different concentration showed a significant decrease in serum Hcy level in comparison with the EAC group; *Laurus nobilis.* L. extract nanoemulsion in a concentration of 0.5 (v/v) showed the best result compared to other prophylactic groups. Our results were in agreement with [17] who reported that *Laurus nobilis* L. extract modulates inflammatory signaling by suppressing inflammation via reducing pro-inflammatory cytokine expression *in vitro* and *in vivo*.

1,8- cineole (51.73–68.48%) is the major component of *Laurus nobilis* L. extract nanoemulsion, it exert its anti- inflammatory and antioxidant effects [61]. This compound also has lipid-lowering properties in hypercholesterolemic zebrafish [62]. It was previously indicated that exposing neck squamous cell carcinoma to 1,8- cineol resulted in dose dependent proliferation inhibition and decreased Wnt/β catenin pathway activity [63].

However, the role of nanoemulsion forms in this study is appeared in all using biochemical parameters, thus when using the pure extract of *Laurus nobilis* L. (prophylactic group II); all parameters were significantly changed compared to control group as well as compared to EAC. While using *Laurus nobilis* L. extract nanoemulsion in two different concentrations appeared more potent than the extract alone (significant changes were observed in the results). Additionally, the high concentration (0.5 v/v) gave the best results to become more or less near the control group. These results elucidated the role of *Laurus nobilis* L. extract nanoemulsion as a promising agent in protecting against experimental EAC.

4.Conclusion

In the current study, *Laurus nobilis* L. extract nanoemulsion was successfully prepared *via* spontaneous emulsification procedure. The fashioned *Laurus nobilis* L. extract nanoemulsion was tested using TEM, DLS and zeta potential. The processes cheese was fortified by different concentrations of *Laurus nobilis* L. extract nanoemulsion. Furthermore, the protein, ash, total phenol content, and antioxidant activity of the prepared processed cheese enrich with *Laurus nobilis* L. extract nanoemulsion increased to the highest values of 19.47 %, 4.89 %, 125.56 mg/Kg cheese, and 45.08 %, respectively in treatment supplemented with 0.50 %nanoemulsion extract. Then, the prepared *Laurus nobilis* L. extract nanoemulsion was used as a novel protective strategy against inflammatory cascades that increase the progression of cancer which will help the researchers to explore critical mechanisms in cancer therapy.

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Declaration of Competing Interest

Authors declare no conflict of interest.

Ethical approval

Mice used in this study were kept under constant environmental conditions at room temperature. The guidelines of the ethical care and treatment of the animals followed the regulations of the ethical committee of the National Research Centre (NRC).

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الملخص العربى

الهدف من البحث هو تقييم التأثير الوقائي للأجبان المعالجة المدعمة بمستخلص مستحلب النانو من نبات اللورى ضد فرط الهوموسيستين في الدم في نموذج سرطان استسقاء إيرليش .(EAC) تم تحضير مستحلب النانو من نبات اللورى عن طريق عملية الاستحلاب التلقائي. بعد ذلك ، تمت إضافة مستحلب النانو بواسطة حمولات مختلفة لتكملة الأجبان المصنعة. تم الكشف عن الشكل الظاهرى لمستخلص مستحلب اللورى باستخدام المجهر الإلكتروني النافذ (TEM) .

تلقت إناث الفئران البيضاء الجبن المطبوخ لمدة أسبوعين ثم تم تلقيحها مرة واحدة بخلاياEAC المسببة لمرض السرطان الاستسقائى ، و بعد انتهاء التجربة ، تم تجميع عينات الدم لتحديد نشاط انزيم السوبر أوكسيد ديسميوتيز (SOD) ، المواد التفاعلية لحمض الثيوباربيتوريك (TBARS) ، وورم الغدد الليمفاوية B-cell 2 (Bcl-2) ، و الميتالوبرزتينيز (MMP-9) ، عامل نخر الورمى ألفا (TNF-α ، والهوموسيستين(Hcy) .

أوضحت النتائج أنه تم تكوين مستحلب نبات اللورى النانوى في شكل كروي بحجم حوالي 50-120 نانومتر، و أظهرت الفئران الملقحة بخلايا EACزيادة معنوية في مستويات Hcy و TNF-α و TBARS و MMP-9 في المصل بينما كان هناك انخفاض معنوي في نشاط SOD ومستوى Bcl-2 .

و قد أدت المعالجة المسبقة بتركيزات مختلفة من مستخلص اللورى إلى تخفيف الإجهاد التأكسدي والالتهاب والاستماتة مقارنة بالمجموعة المصابة بمرض السرطان .