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RESEARCH PAPER

Evaluation of the anti-oxidant and antiangiogenic activities of nanoemulsion prepared from *Boswellia carteri* essential oil

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ABSTRACT

Objective(s): Boswellia essential oil possesses bioactive compounds with therapeutic properties. The present study was conducted to evaluate the anti-oxidant and antiangiogenic activities of nanoemulsion prepared from *Boswellia* essential oil.

Materials and Methods: In this study, an oil-in-water nanoemulsion was prepared using the ultrasonic method and Boswellia essential oil (the oil phase) and Tween 80 surfactant, and water (the aqueous phase). Droplet size, dispersion index, and zeta potential of the prepared nanoemulsion were evaluated, and the ability of the nanoemulsion to inhibit DPPH free radicals was measured. Also, the angiogenic activity of the nanoemulsion was investigated using the chicken chorioallantoic membrane (CAM) model.

Results: The formulated nanoemulsion revealed particles with a spherical shape, average size of 58.29 nm, a dispersion index of 0.29, and a zeta potential of -28.87. Transmission electron microscopy (TEM) image of the nanoemulsion shown that the particles were almost uniformly spherical. The anti-oxidant activity of Boswellia essential oil, enclosed in O/W emulsion, was confirmed via the DPPH free radical inhibition assay with an IC_{50} of 61.92 µg/mL. In addition, the nanoemulsion was shown to inhibit the growth of new vessels in the CAM model, indicating anti-angiogenic effects.

Conclusion: Our findings suggest that due to anti-oxidant and anti-angiogenic effects, nanoemulsion loaded with Boswellia essential oil can be used as a therapeutic agent.

Keywords: *Antiangiogenic, Anti-oxidant, Boswellia, Nanoemulsion*

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INTRODUCTION

Plants contain secondary metabolites with widespread biological properties of clinical and pharmaceutical importance, including essential oils, which have long been used as perfumes, flavorings, and therapeutic compounds [1-5]. Essential oils can enter the human body via different ways such as breathing, eating, and absorption through the skin. Since essential oils are fat-soluble compounds, they can enter the body via the plasma membrane of skin cells and then travel all over of the body through small capillaries [6-8]. *Boswellia carteri* is a plant attracting the interest of doctors and nutritionists because of its diverse biological properties. Traditionally, the gum of some species of this plant is used in different countries to treat rheumatism and other inflammatory diseases, including Crohn's disease and ulcerative colitis [9, 10]. Many studies have verified the anti-cancer [11], anti-inflammatory [12], immunomodulatory [13], antimicrobial [14], antiviral [15], and anti-diabetic effects of several *B. carteri* species [16]. *Boswellia carteri* is a medicinal plant that is used in different forms, most commonly as essential oils [17]. Since essential oils are highly lipophilic compounds, they have low solubility, reducing their absorption and therapeutic effectiveness. Hence, encapsulating these compounds as nanoemulsion formulations can improve their therapeutic effectiveness, solubility, and pharmacokinetic [18, 19] . Using these formulations improves the physical and chemical properties, as well as water solubility and stability of payloads [20, 21]. Designing effective nanoemulsion formulations for drugs has always

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been a major challenge because of limitations such as drugs' instability or hydrophobicity [22]. The essential oils and extract of medicinal plants have successfully been used to synthesize nanoparticles [23-25]. The use of nanoemulsions may help obviate these challenges as they not only solve water solubility problems but also offer specific targeting of cancer cells and overcoming multidrug resistance. Nanoemulsions can be modified using different natural ligands to target specific molecules on the surface of tumor cells or escape from multidrug resistance mechanisms [26-28]. Multifunctional nanoemulsions have been shown to effectively reduce tumor growth and metastasis while causing minimal toxicity against healthy cells. Since cancer cells are surrounded by vascular tissues, nanoemulsions can easily pass through these barriers due to their small size and release drugs into tissues [29]. Extracellular matrix (ECM) is essential for tumor growth, cancer cell migration, tumor invasion, and metastasis [30]. Delivery of oxygen and nutrients to the tumor is generally achieved by simple diffusion, but when the tumor grows larger than 2.0 mm^3, oxygen level decreases, leading to hypoxia and the formation of new blood vessels [31]. Therefore, by inhibiting angiogenesis, the growth and proliferation of cells can be reduced. So far, various inhibitors of angiogenesis have been identified; however, the use of these inhibitors faces obstacles such as toxicity, drug resistance, and drug delivery problems [32].

Studies show that the use of *B. carteri* essential oil is effective in preventing and/or treating a wide range of cancers [33]. The presence of triterpene boswellic acids seems to be responsible for the anticancer activities of *B. carteri* essential oil [34]. The aim of this study was to prepare nanoemulsions of *B. carteri* essential oil in order to improve its solubility and bioavailability. We also investigated the anti-oxidant and angiogenic activities of these nanoemulsions.

MATERIALS AND METHODS

Synthesis of B. carteri essential oil nanoemulsion

In order to synthesize *B. carteri* oil nanoemulsion, as an oil-in-water nanoemulsion, Tween 20 and Tween 80 were used as surfactants and ethylene glycol as an auxiliary solvent. The nanoemulsion was synthesized using the ultrasonic method with a power of 150 watts for three minutes. For this, equal volumes (100 µl) of Tween 80 and 20 were first added into a 50 mL Falcon tube. One milliliter of *B. carteri* essential oil was added to the surfactant mixture, and then 0.5% (v/v) ethylene glycol was added to the tube along with distilled water. The total volume of the nanoemulsion solution was 50 mL. The resulting solution was then transferred to an ultrasonic device with a power of 150 watts for three minutes to prepare the nanoemulsion. Finally, the size and morphology of particles in the solution were analyzed using dynamic light scattering (Nano-ZS, Malvern, UK) and electron microscopy (JEOL, Japan) to confirm the formation of the nanoemulsion.

Dynamic light scattering

This technique determines the distribution of particles in solutions and suspensions based on interactions between light and particles. In other words, time-dependent changes in the light scattering properties of nanoparticles in the suspension are proportional to particles' diameter. This method offers a fast and non-destructive method to determine the size of particles in the range of several nanometers to microns.

Nanoemulsion structure under transmission electron microscopy

B. carteri essential oil nanoemulsion was initially diluted in distilled water, and then the samples were absorbed on the copper grids covered with carbon for one minute and stained with phosphotungstic acid for 10 minutes at room temperature. Finally, the grids were mounted on an electron microscope (JEOL, Japan) for imaging.

DPPH free radical inhibition assay

The free radical scavenging activity of the nanoemulsion prepared from *B. carteri* essential oil was measured using the DPPH radical inhibition method. For this purpose, DPPH free radicals were first produced in the laboratory by preparing a 0.1 mM DPPH solution in ethanol. Next, different concentrations of the nanoemulsion were prepared by the serial dilution method in a volume of 500 µl. To measure the DPPH free radical inhibition activity of the nanoemulsion, an equal volume of free radicals was added to different concentrations of the nanoemulsion. The absorbance of the resulting solution was measured at a wavelength of 517 nm. Distilled water and BHA were used as negative and positive controls, respectively [35]. The procedure was carried out in triplicate.

Procedure of angiogenic activity using the CAM method

For this purpose, 50 fertilized eggs of the Ross breed were purchased from Toos Poultry Company and randomly divided into five experimental groups, including two control groups and three experimental groups treated with different concentrations of the nanoemulsion. Fertilized eggs were first disinfected with 70% ethanol and placed in a research incubator at a temperature of 38 degrees Celsius and a relative humidity of 65%. Two days later, a small window was opened on each of the eggs by removing a part of the egg shell under a completely sterile condition. The window was then blocked by glue and sterile paraffin, and the eggs were transferred to the incubator [36]. On the eighth day, the windows were opened again and a sterile sponge was placed on the allantoic membrane. The sponges were treated with different concentrations of nanoemulsion including, 0, 25, 50 and 100 µg/ml of prepared from *B. Carteri* oil. Three replicates were made for each concentration. The windows were closed again and the eggs were returned to the incubator. On the twelfth day, the samples were photographed using a stereo microscope and the number and length of blood vessels were analyzed with the help of Image J software.

Statistical analysis

The anti-oxidant and angiogenic activities of the nanoemulsion were compared with that of the standard group using appropriate statistical tests. Initially, all ODs (absorbance) obtained from the samples were transferred to specific formulas, and the resulting values were entered into SPSS software. The one-way ANOVA test was used to compare means with the least significant differences (LSD) method. Error bar values on graphs, average standard deviations, and 95% confidence levels were considered for calculations.

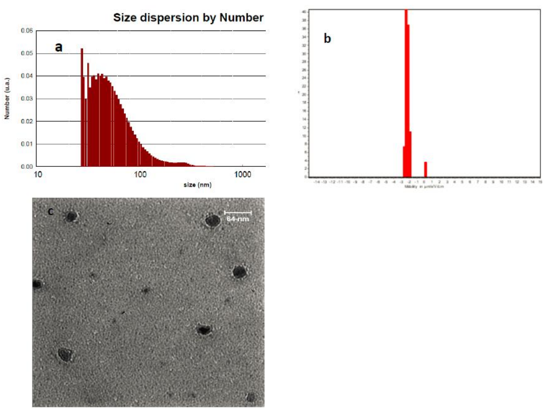

Fig. 1. (a) Average particle size according to the number of nanodroplets. (b) Zeta potential of the nanoemulsion prepared by *B. carteri* essential oil. (c)TEM image of the nanoemulsion synthesized by *B. carteri* essential oil

RESULTS

Nanoemulsion characterization

The results showed that the nanoemulsion had an average particle size of 58.2 nm with a PDI of about 0.29, indicating the uniform dispersion of the nanodroplets (Fig. 1a).

The value of zeta potential was obtained as -29 mV (Fig. 1b), which was very close to the stability range, confirming the good sustainability of the nanoemulsion.

The observation of size under an electron microscope revealed consistency with the data obtained from the experimental measurement of particles' characteristics. As it can be seen, the particles were almost uniformly spherical and had diameters smaller than 60 nm (Fig. 1c).

DPPH free radical inhibition activity

As shown in Fig. 2, the synthesized nanoemulsion was able to inhibit DPPH free radicals in a concentration-dependent manner. The rate of free radical inhibition was 7% at a concentration of 7.8 µg/ml, which increased to 50% at the concentration of 62 µg/ml, indicating the high anti-oxidant capacity of the nanoemulsion with a median concentration (IC50) of 62 µg/ml.

CAM test findings

Investigating the morphology of blood vessels after treatment with *B. carteri* essential oil nanoemulsion (Fig. 3a) showed that the number

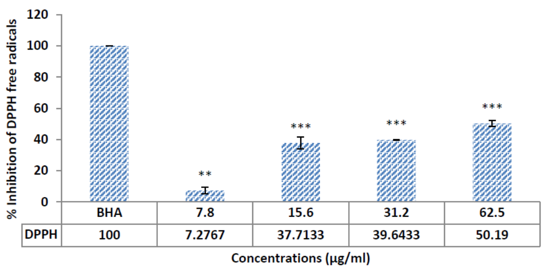

Fig. 2. DPPH free radical inhibition activity of the nanoemulsion at different concentrations

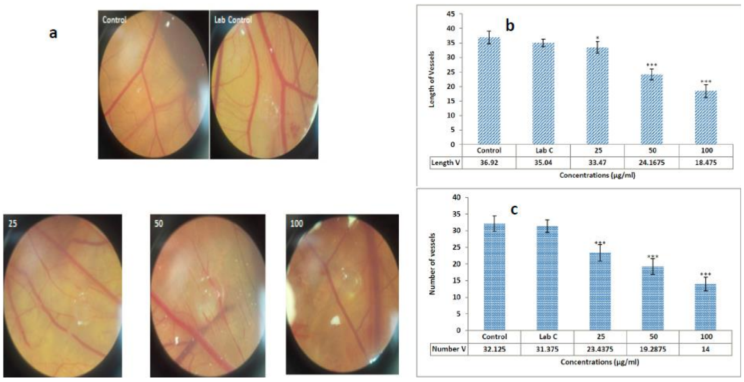

Fig. 3. (a) Changes in the rate of angiogenesis in chicken chorioallantoic membrane following treatment with *B. carteri* essential oil nanoemulsion. (b) Decreased average length of blood vessels in the groups treated with different concentrations of the nanoemulsion compared to the control group. (c)The average number of blood vessels significantly decreased in the groups treated with different concentrations of the nanoemulsion compared to the control group (***; P<0.001)

of blood vessels decreased significantly with increasing the concentration of the nanoemulsion. As shown in the figure, a decrease in the density of primary and secondary vascular branches is evident. As shown in Fig. 3b, the average length of vessels was not significantly different between the two control groups. In the experimental groups and with an increase in the concentration of the nanoemulsion from 25 to 100 µg/ml, the length of vessels significantly decreased. The average length of vessels in the samples treated with the 25 µg/ml concentration of the nanoemulsion was significantly different compared to that of in the control sample (P<0.05), and this difference became more significant at the highest concentration (100 µg/ml) of the nanoemulsion (P<0.001). Fig. 3c shows that the number of blood vessels depended on the concentration of the nanoemulsion. There was no significant difference in the number of blood vessels between laboratory control samples and the control samples. There was also a significant difference in the number of blood vessels between the experimental groups treated with different concentrations of the nanoemulsion (P<0.001), showing a remarkable dose-dependent reduction in all groups. The number of vessels was 23 at the concentration of 25 µg/ml and 10 at the concentration of 100 µg/ml.

Fetal growth parameters in nanoemulsion-treated samples

In this study, we assessed the effects of the nanoemulsion on fetal growth parameters (height and weight). The height of the embryos treated with different concentrations of the nanoemulsion was measured and compared with that of control samples. As shown in Fig. 4a, the average distance from the head to the seat in the control samples was 37.8 mm, showing no significant difference compared with the average height (37.66) of the fetuses in laboratory control samples. In the samples treated with 25 µg/ml of the nanoemulsion, the average height decreased to 34.4 mm, indicating a significant difference compared to the average height in control samples (P<0.05). An increase in the concentration of

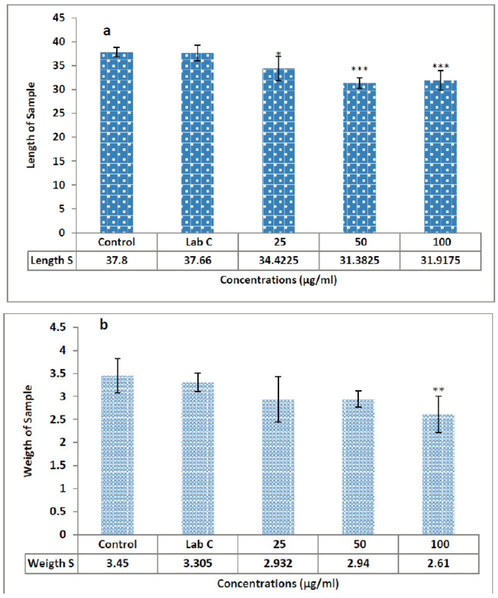

Fig. 4. (a) Reduction of the average distance from the head to the seat in the embryos treated with different concentrations of the nanoemulsion compared to the control group. (b) The average weight of embryos in the groups treated with different concentrations of nanoemulsion significantly decreased compared to the control group (*; P<0.05 and **; P<0.01***; P<0.001)

the nanoemulsion to 50 and 100 µg/ml further decreased the average height to 31 mm, which was significantly different compared to the control ($P<0.001$). So, it can be said that the nanoemulsion significantly decreased the height of the embryos at a concentration-dependent manner.

In this study, the weight of the embryos treated with nanoemulsion was evaluated and compared to the control. The average weight of embryos was 3.45 g in the control sample and 3.3 g in laboratory control samples, showing no significant difference (Fig. 4b). The average weights of the embryos treated with 25 and 50 µg/mL concentrations of nanoemulsion did not show a significant difference compared to the control, but increasing the concentration to 100 µg/mL led to a significant reduction in the average weight of the embryos compared to the control group ($P< 0.001$).

DISCUSSION

Essential oils and herbal extract have been used for many years due to their therapeutic properties [37-39]. Today, different encapsulation methods have been developed to overcome these drawbacks and increase the solubility and stability of these compounds, enabling them to be used in different medicinal and pharmaceutical fields [40-42]. Nanoscience can be used as carriers of plant-derived active compounds, including essential oils [19, 43-45]. The advantages of nanoemulsions include the ease of synthesis, high stability, high solubility, and cost-effectiveness [46]. There are different methods for the synthesis of nanoemulsions. In addition, different surfactants and cosurfactants can be used to synthesize nanoemulsions, and these differences can affect the biological properties of these nanomaterials [47]. Among the synthetic methods developed, ultrasound-based techniques, which are considered high-energy methods, are among the most commonly used methods as they are fast, simple, and cheap [48, 49]. In the present study, *B. carteri* essential oil was used to synthesize an oil-in-water nanoemulsion using the ultrasonic method, where Tween 20 and Tween 80 were utilized as surfactants and ethylene glycol as the cosurfactant.

Characterization of the nanoemulsion showed that the droplets had a diameter of about 50 nm. The zeta potential of the prepared nanoemulsion was obtained as -29 mV, which is very close to the stability range, indicating the stability of the nanoemulsion. In terms of morphology, the nano-droplets were spherical. So far, nanoemulsions have been synthesized using different essential oils employing various methods, and the synthesis method and the composition of essential oils are assumed to affect the biological properties of nanoemulsions, including their anti-oxidant activity, which was assessed in this study using the DPPH free radical scavenging assay. However, the *in vivo* anti-oxidant capacity of these compounds cannot be accurately predicted based on their *in vitro* free radical scavenging activity. The DPPH free radical inhibition assay is among the methods commonly used to measure the anti-oxidant capacity of different compounds *in vitro*. In this study, the level of DPPH free radical inhibition was investigated for different concentrations of the nanoemulsion produced, and the results showed that the nanoemulsion was able to inhibit DPPH free radicals with an IC_{50} of about 60 µg/mL, reflecting high anti-oxidant power. In comparison, in a study conducted in 2011 investigating the essential oils of different *B. carteri* species, the highest capacity (28%) for neutralizing DPPH free radicals was achieved at the 1000 µg/ml concentration [50]. In this study, the comparison of the free radical inhibition power of *B. carteri* essential oil with the nanoemulsion of *B. carteri* essential oil showed that the transformation of the essential oils into nanodroplets (i.e., nanoemulsion) increased the anti-oxidant activity of the compound. This study shows a change in the biological characteristics of the encapsulated essential oil. Therefore, it can be said that encapsulating essential oils in nanodroplets can improve some of their biological properties. In a study, DPPH free radical scavenging activity increased when nanoemulsion was synthesized from the ethanolic extract of *Phyllanthus urinaria* encapsulated in palm kernel oil [51]. In another study, the DPPH free radical scavenging ability of the nanoemulsion synthesized from Chinese five finger plant (*Vitex negundo* L.) was evaluated, reporting an IC_{50} of 23 µg/mL indicating a higher free radical inhibitory power compared to the nanoemulsion produced in the present study [52].

Uncontrolled cellular proliferation due to the genetic mutations activating oncogenes or inactivating tumor suppressor genes can lead to cancer [53]. The growth of tumor tissues requires active angiogenesis and production of new vascular beds to supply enough blood to the tumor [54]. Dramatic physiological, structural, and functional changes in the microenvironmental components of tumors can alter angiogenesis, oxygenation, pH, perfusion, and metabolic status, contributing to tumor progression [55]. Physiological barriers, such as hepatic and renal endothelium, and premature destruction due to enzymolysis or hydrolysis prevent therapeutic agents from reaching target cancerous cells[56]. In addition, multi-drug resistant mechanisms, such as the high expression of P-glycoprotein (Pgp), as well as the complexity of the tumor microenvironment are among the major causes of the failure of conventional chemotherapy [57]. In addition, anticancer drugs have poor aqueous solubility and high hydrophobicity, compromising their ability to target cancerous tissues [58]. Therefore, drug delivery systems, such as nanoemulsions, which have a high capacity for being loaded with hydrophobic drugs, are easily produced, have long-term stability, and are modifiable with imaging ligands, seem to be promising drug carrier platforms. Such technologies help selectively target cancer cells and co-deliver therapeutic and diagnostic materials to the tumor site, increasing the success of treatment in early stages [59]. In various studies, the anti-angiogenic effects of nanoemulsions synthesized from different compounds have been shown; however, these studies are infrequent. In the present study, the anti-angiogenic activity of the nanoemulsion synthesized by *B. carteri* essential oil was confirmed in the CAM assay. As mentioned, the decrease in the amount of vessels and as a result the decrease in blood supply in the process of embryogenesis can cause a decrease in the growth of the fetus, including a decrease in the weight of the fetus. Similarly, in a study in 2013, the CAM test was used to evaluate the anti-angiogenic effects of the nanoemulsion synthesized by betulin, and the results showed a decrease in angiogenesis in the presence of the nanoemulsion , which was consistent with our findings [60]. In a 2018 review, the anti-angiogenic effects of memecylaene oil-in-water nanoemulsion (size: 59 nm) synthesized by the ultrasonic method were evaluated, and the results showed that this nanoemulsion in the therapeutic range could significantly suppress angiogenesis, supporting our observation regarding the suppressive effects of *B. carteri* nanoemulsion on angiogenesis [61]. In another study in 2020 and consistent with our finding, the angiogenesis activity of lemon essential oil nanoemulsion was investigated using the CAM assay, and the results showed that the nanoemulsion inhibited angiogenesis as evidenced by a reduction in the length and number of blood vessels [62].

CONCLUSION

Today, encapsulating volatile and less soluble compounds, such as essential oils, and turning them into nanodroplets can overcome the hurdles limiting their clinical use. In this study, *B. carteri* essential oil nanoemulsion was synthesized in order to improve the stability and solubility, as well as the biological properties of *B. carteri* essential oil. Tween 20 and Tween 80 surfactants and ethylene glycol, as a cosurfactant, were used to produce *B. carteri* essential oil nanoemulsion by the ultrasonic method. The resultant was nanodroplets with a size of 58.29 nm, a dispersion index of 0.29, and a zeta potential of -29. The anti-oxidant activity of the nanoemulsion was confirmed using the DPPH free radical scavenging assay with an average concentration of 61.92 µg/ml. In addition, the nanoemulsion reduced the length and number of blood vessels, as well as the height and weight of fetuses as shown in the CAM assay. The results of this study highlight the applicability of the nanoemulsion prepared from *B. carteri* essential oil as a potential therapeutic agent.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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