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Anticancer activity of an ultrasonic nanoemulsion formulation of *Nigella sativa* L. essential oil on human breast cancer cells

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ABSTRACT

Nigella sativa L. (NS) is a plant renowned in traditional holistic medicine systems for almost 1400 years because of its remarkable antioxidant, antimicrobial, anti-inflammatory and anti-cancer properties. The essential oil of *N. sativa*, in particular, possesses these significant biological properties. However, *N. sativa* essential oil has many insoluble constituents with properties that have not been fully explored. Nanoemulsion-based insoluble formulations are a widely used carrier system for lipophilic materials. In the present study, we used ultrasonic emulsification, polysorbate 80 and water to formulate a highly stable *N. sativa* essential oil nanoemulsion (NSEO-NE). To optimize the NSEO-NE preparation, we changed the surfactant concentration, the oil–surfactant mixing ratio and the emulsification time. The droplet size distribution and morphology of the prepared NE was analyzed using dynamic light scattering and scanning electron microscopy, respectively. The droplet size of the NSEO-NE was approximately 20–50 nm in diameter. The anticancer properties of the NE preparation were studied using a modified methylthiazolyl-diphenyl tetrazolium bromide (MTT) assay as well as cellular uptake and nuclear morphological analyses. The NSEO-NE significantly reduced the viability of Michigan Cancer Foundation-7 (MCF-7) breast cancer cells. The nucleo-cytoplasmic morphological features of NSEO-NE-treated cells included cell membrane blebbing, cytoplasmic vacuolation, marginalization of chromatin, and fragmentation of the nucleus. The results clearly indicate that NSEO-NE induced apoptosis in MCF-7 cells. These findings support the potential application of NSEO-NE in breast cancer therapy, and also merit future translational research.

1. Introduction

Nigella sativa L. (of the Ranunculaceae family) is a well-known medicinal plant, widely used in various traditional medicine systems, including Ayurveda, Siddha and Unani. *N. sativa* is used to treat various chronic diseases, such as diabetes, hypertension, asthma, cancer and cardiovascular disease [1–3]. Previous studies of *N. sativa* (NS) have isolated the plants phytochemical constituents, and investigated the constituents *in vitro* and *in vivo* pharmacological effects. The NS seed contains various active phytochemical constituents, including thymoquinone, thymohydroquinone, ρ-cymene, carvacrol, t-anethole, 4-terpineol, longifoline, nigellicine, nigellidine, nigellimine and isoquinolines [4]. The NS seed constituents exhibited remarkable therapeutic properties, including antioxidant, antiparasitic, anticancer, antimicrobial, anti-inflammatory, analgesic and antipyretic properties [5–9]. Moreover, several studies suggested that NS seed extract can be used to suppress cough, retard carcinogenesis, disintegrate renal calculi, and treat polio, diarrhea, abdominal pain and flatulence [10–13]. Notably, the active ingredients in NS seeds play a major role in inhibiting carcinogenesis and induces cell death in various cancer cells, including cervical cancer, hepatic cancer, colon cancer, blood cancer, pancreatic cancer, skin cancer, renal cancer, fibrosarcoma, lung cancer, prostate cancer and breast cancer [14–26]. For example, dimethylbenz[a]anthracene, one of the active components of NS extract, induced skin carcinogenesis in mice; NS extract treatment delayed the onset of papilloma formation [16]. Salomi et al. reported that NS seeds contain certain fatty acids that exhibited 50% cytotoxicity in sarcoma-180 cells, Dalton's lymphoma ascites and Ehrlich ascites carcinoma at a concentration of 1.5, 3, and 1.5 μg, respectively [27]. *N. sativa* extract in combination with an oxidative stress agent exhibited significant anticancer activity in MCF-7 breast cancer cells [26]. Salim and Fukushima

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suggested that oral administration of NS volatile oil inhibits colon carcinogenesis and suppresses colon cancer cell proliferation in postinitiation stage rats [21]. Khan et al. found that NS extract suppressed $KBrO_3$-induced renal oxidative stress, toxicity and tumorigenesis [14]. The NS active ingredient thymoquinone induced p53-independent apoptosis through caspase-8 activation in myeloblastic leukemia HL-60 cells [18].

NS seeds have a bitter taste and are used as food additives in confectionery and other food products. Moreover, NS seeds are consumed with honey, and in baking products or pastries. NS seeds contain essential fatty acids, antioxidants, vitamins (Retinol, Thiamine, Riboflavin, Ascorbic acid and niacin) and minerals such as calcium, potassium, iron, magnesium, selenium and zinc [28,29]. The NS seeds contain unsaturated fatty acids, such as linoleic and oleic acid, and saturated fatty acids, including palmitic acid myristic, myristoleic, palmitoleic, margaric, margaroleic, stearic, linolenic, arachidic, eicosenoic, behenic and lignoceric acids [30]. Moreover, NS seeds contain sterols, including β-sitosterol, stigmasterol campesterol, lanosterol and Δ7-avenasterol [31]. The chemical composition of NS seed is suitable for the effective treatment of various diseases. However, NS seeds also contain essential oils and highly lipophilic compounds, and these insoluble compounds have not been studied well, either *in vitro* or *in vivo*.

Recently, nanoemulsion or mini-emulsion formulations have received a lot of attention because of their potential in wide variety of applications, including pharmaceutical, cosmetic and food industry applications. Nanoemulsions are ultrafine oil-in-water dispersions and have a droplet size range of 10–600 nm [37]. Nanoemulsions are produced by the mixing of two immiscible liquids with or without an emulsifier that contains ultrafine droplets. Nanoemulsions are transparent or opaque, usually very fluid and extremely fragile system by nature [32–35]. Previous studies suggested that nanoemulsions can be used as drug carriers, and can impart a long shelf-life to carried drugs for mosquito repellent, antimicrobial, anticancer, larvicidal and insecticidal activities [32–36]. A variety of plant-derived essential oils have been used to prepare nanoemulsions, including neem oil, eucalyptus oil, thyme oil, cashew nut shell liquid, citronella oil, basil oil, lemongrass oil, clove oil and *Stenachaenium megapotamicum* oil [32–36,38–41].

Numerous methods were used for formulation of nanoemulsion including high-pressure homogenization, microfluidization, phase inversion, spontaneous emulsification, solvent evaporation and ultrasonication [42]. Among these methods, ultrasonic emulsification is an easy, cost-effective, high energy, clean, fast and aseptic technique for nanoemulsion formulation [43]. Earlier studies demonstrated that ultrasonic emulsification method for nanoemulsion formulation [35,38,43,44]. Ultrasonic emulsification utilizes a probe that generates ultrasonic waves to disintegrate the macroemulsion by cavitation forces [43]. Two kinds of mechanisms are involved to nanoemulsion formulation in ultrasonic emulsification. First, an acoustic field generates combination of interfacial waves and instability leads to the eruption of oil phase into water medium in the form of droplets. Second, low frequency ultrasound waves disintegrate the droplets by cavitation near the interface. The extreme instability of primary droplets produces nanoemulsion with very small droplet size [44].

Breast cancer is one of the most common cancers in women worldwide; an estimated 1.7 million new cases were diagnosed in 2012. Breast cancer begins in the lining of the milk ducts and is the second most common cancer overall [45]. Breast cancer is treated using various methods, including surgery, chemotherapy and radiotherapy, but these methods are costly, mostly painful and can cause several side effects [46]. Despite advanced techniques in early diagnosis and systemic therapeutic agents, most breast cancers are resistant to drugs [47]. Hence, there is a need to develop novel cost-effective treatment methods or drugs with minimal side effects. In recent years, nanoscience and nanotechnology have caused a revolution in the diagnosis and therapy of diseases and regenerative medicine. The diagnosis and therapy of cancer at the cellular level using nanoparticles have resulted in the gradual development of innovative treatment modalities [48]. However, a nanoemulsion of NS seed essential oil has not been produced nor used for studies of cancer, *in vitro* or *in vivo*. In the present study, we formulated an NSEO nanoemulsion and assessed its anticancer properties in breast cancer cells. Moreover, NE can be used to improve delivery of hydrophobic compounds into cells. In this present study, we have prepared NSEO-NE using polysorbate 80 as an emulsifier by ultrasonication. Additionally, we used an *in vitro* system to evaluate the anticancer activity of NSEO-NE on MCF-7 human breast cancer cells.

2. Materials and methods

2.1. Oil extraction from N. sativa seeds

N. sativa seeds were purchased at a local market (Riyadh, KSA), and dried at room temperature in the absence of sunlight. The collected plant material was pulverized using a milling machine, and extracted with methanol using a Soxhlet apparatus as previously described [49]. The organic phase was evaporated under reduced pressure to obtain a residue. The residue was dried using a rotary evaporator to produce a oily substance/paste. The oil portion was separated for further nanoemulsion preparation.

2.2. Preparation of nanoemulsion

NS seed oil nanoemulsion was formulated using NS essential oil (3%), the non-ionic surfactant and emulsifier polysorbate 80 and water. The nanoemulsion was formulated by adding water to NS essential oil and surfactant in different proportions, 1:1, 1:2 and 1:3 (v/v). The mix was sonicated using a 20 kHz sonicator (Ultrasonic Processor, GEX 750, USA) with maximum power output of 750 W for 1 h at room temperature. The ultrasound probe with tip of 13 mm diameter was used for the generation of ultrasonic waves. During ultrasonication process heat energy generated was neutralized by keeping the sample container in an ice-bath. The formulated nanoemulsion morphology, droplet size and stability were analyzed, and the nanoemulsion was used further for *in vitro* studies.

2.3. Stability of nanoemulsion

The prepared NSEO-NE storage stability was studied by dynamic light scattering analysis (DLS). The prepared NSEO-NE by different ratios (1:1, 1:2 and 1:3 (v/v)) of oil and surfactant was storing at room temperature. The droplet size change was observed at different time intervals.

2.4. Characterization of nanoemulsion

The droplet size and polydispersity index (PDI) were analyzed using a Malvern Zetasizer Nano ZS-90 instrument. The nanoemulsion droplet size was characterized using a dynamic light scattering analysis (DLS). The average droplet size was calculated using software-generated measurements of intensity, volume, and number distributions. The turbidity of the prepared nanoemulsions was analyzed using a UV–Visible spectrophotometer at 600 nm absorbance (Model 2201, Systronics, India). The morphology and structure of NS essential oil nanoemulsion was carried out using scanning electron microscopy (SEM) and brightfield microscopy.