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ORIGINAL RESEARCH PAPER

Melissa officinalis extraction with nanoencapsulation By chitosan as an ecofriendly compound

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ABSTRACT

The bioactive compounds in extracts are prone to degradation by oxidation, heat, or light. Nanoencapsulation is one of the best techniques to keep the properties of these chemical compounds. The aim of this study was the extraction of *Melissa officinalis (MO)* and nanoencapsulation of the extract via chitosan as a biodegradable polymer. In this research, extraction of MO was investigated using various extraction methods and nanoencapsulation with *MO* extract was carried out via ionic gelation technique. The effectiveness of the extracts was evaluated by measuring the total phenolic content (TPC), antioxidant activity, and extraction efficiency of the solid contents. The highest efficiency was achieved for microwave-assisted extraction with the utmost values in each parameter. (TSC) was 22.81% and amounts of the TPC and antioxidant activity were 311.94 mg Gallic acid and 36 mg diphenyl picryl hydrazyl (DPPH) per 1g of the plant, respectively. Morphology study by field emission scanning electron microscopy (FE-SEM) indicated spherical shape nanoparticles with a diameter of 25nm. The size of the nanoparticles was evaluated by the Dynamic Light Scattering (DLS) technique for various concentrations of the used extracts in the encapsulation process. For 1.0, 3.0, and 5.0 mg /mL concentration, mean diameters were 24, 118, and 145 nm, respectively. Results indicated that microwave-assisted extraction was the best extraction method for *MO* and the encapsulation of *MO* extract could be created successfully with different particle sizes for the protection of bioactive compounds. Since *MO* is a beneficial herbal plant, the development of this research is recommended.

Keywords: *Nanoparticles, Melissa officinalis, Extract, Ionic gelation*

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INTRODUCTION

Herbal medicine is defined as the usage of medicinal herbs to prevent and treat several diseases and its broad scope of application has ranged from traditional and popular drugs in most countries to standard herbal extracts [1]. Using herbs as medicine is the oldest recognized human health care which has been common in all cultures throughout history. Medicinal herbs are easy-to-consume and available to everyone. In comparison with chemical drugs, herbal medicine incurs lower costs and excludes the harms of chemical medications as being naturally produced [2]. Among such plants, *Melissa officinalis* (lemon balm) is highly appreciated due to its high therapeutic properties possessing multiple anti-oxidant compounds. This plant originates from the mint family and is a massively grown annual plant that grows vertically reaching a height of approximately one meter. Its soft and tufted heart-shaped leaves have a length of almost 2 to 8 centimeters [3]. *MO* has revealed promising characteristics such

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as anti-oxidant, soothing, stimulating, anti-parasitic, anti-contractile, anti-tumor, sedative, and hypnotic effects [4]. An anti-inflammatory drug normally contains proteins that can inhibit protein biosynthesis in cancer cells. The biological activity of such medicines is related to their essential oils. They also have demonstrated diverse attributes such as anti-herpes, antiviral, antiviral immunodeficiency virus (HIV), antimicrobial, anticancer, anti-stress, anti-anxiety, anti-depressant, anti-Alzheimer and anti-inflammatory properties in addition to the treatment of sleep disorders [5]. The main compounds in this plant are gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, ellagic acid, isoquercetin, quercetin, routine, and kaempferol which are introduced as bioactive compounds. Several new oral formulations have been identified naming Orsen and Orsen glycoside isolated from a polar extract of the plant's stem and leaves [6, 7]. In 2004, Sousa et al. examined the anti-tumor and anti-oxidant properties of *MO via* an in-vitro cytotoxic test using MTT which indicated that this plant is effective in the treatment of some human cancer cells to a great extent [8]. In 2003, Akhondzadeh et al. applied *MO* extract to cure patients with mild to moderate Alzheimer's[4]. Their results revealed that *MO* extract contains valuable bioactive compounds that could lead to a noticeable effect on the patient's cognitive function while attenuating their inconvenience and stress. Separation of the Antifungal bioactive compounds *via* nanotechnology was reported [9]. There are various techniques for the extraction of bioactive compounds from all medicinal herbs. These methods are divided into two categories: conventional and unconventional methods. Soxhlet, maceration, and extraction by hydro distillation are the main conventional methods. Most of these techniques are carried out based on the extracting power of different solvents during heating or mixing. In soxhlet extraction, a small amount of dry sample is placed in a thimble which was placed in a distillation flask containing the solvent of particular interest. During heating and distillation, some of the bioactive compounds will be damaged or rearranged. Maceration is a common method in which a suitable solvent is used for extraction during shaking for a long time. Hydrodistillation is a traditional method for the extraction of bioactive compounds and essential oils from plants. During hydrodistillation at a high extraction temperature, some volatile components may be lost [10]. Long-time extraction, inquiry of high-purity solvent, low extraction selectivity, and thermal decomposition of the bioactive compounds are the major challenges of conventional extraction. To overcome these complications, some new extraction techniques are introduced [11]. Microwave-assisted, ultrasound-assisted, enzyme-assisted, pulsed electric field-assisted, pressurized liquid, as well as supercritical fluid extraction methods, are some new extraction methods that are defined as unconventional extraction procedures [12]. Since, these methods include less hazardous chemical material, use of renewable feedstock, design to prevent degradation and time analysis for pollution prevention, and inherently safer chemistry for the prevention of accidents, some of these methods are known as "green techniques" [11]. The microwave-assisted extraction is considered a novel method for extracting soluble products into a fluid for a wide range of materials. Frequency range from 300 MHz to 300 GHz is used to create electromagnetic fields. Quicker heating for the extraction; reduced thermal gradients, reduced process time, less solvent usage, and higher yields are some advantages of this method. The bioactive compounds can be extracted more rapidly and have a better recovery than conventional extraction processes [13]. Recently, microwave-assisted extraction was developed for a variety of bioactive compounds from fruits, vegetables, and medicinal plants [14]. Since many herbals and even chemical medicines features may be eliminated or damaged in the body on the way to the target organ, it is necessary to be kept at first and then transfer according to drug delivery systems to lead special drugs to the related site. Controlled drug delivery systems have demonstrated several superiorities over traditional ones due to the direct drug transmission to the reaction site leaving a profound effect on vital issues [15]. The encapsulation process provides a protective barrier around the biocontrol agent so that harmful external factors such as pH, humidity, and ultraviolet radiation do not damage its action. Encapsulation of bioactive agents has been developed in recent years as a new potential tool for ecological and sustainable plant production. Encapsulation in biopolymer matrices has been recognized as an effective method for the controlled release of a bioactive agent used for plant protection [16].

Chitosan is one of the most effective substances for providing viable drug delivery. It is one of the

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most important biopolymers that is widely used in biological and medical sciences especially for the encapsulation of essential oils and extracts due to its biocompatibility and pharmaceutical industries [17]. Chitosan was known as a safe material and approved as a food additive by the food and drug administration [18]. Barrera-Ruiz et al. reported chitosan nanoparticles loaded with cinnamon, thyme, and Schinus molle essential oils that were effective against some foodborne pathogens [19]. As a cationic hetero polymer obtained from Chitin (a natural polysaccharide in schizophrenic shrimp, mushrooms, vegetables, and yeast), Chitosan is a random copolymer of (1, 1) D-glucosamine and N-acetyl D-glucosamine [20]. Alginate, chitosan, and starch are biodegradable and biocompatible polysaccharides that are safe for humans, and widely used for different branches of science especially agriculture [21]. Various factors, including the physio-chemical properties of coating and core materials and their application, are effective in choosing the right encapsulation process [22]. Drug-incorporating microspheres can be generated *via* different approaches based on: emulsion solvent evaporation, emulsion cross-linking, spray drying technique, emulsion-solvent diffusion, multiple emulsions, ionic gelation, and self-assembly techniques including layer-by-layer formation. The ionic gelation technique is widely used to get nano-sphere particles in the encapsulation process [23]. In this research, some extraction methods were carried out to produce *MO* extracts and evaluated through the calculation of total phenolic content, antioxidant activity, and extraction efficiency of solids contents. Chitosan nanoparticles loaded with *MO* extract were synthesized by ionic gelation method as a biodegradable polymer to show the possible study of encapsulation of the extract and eco-friendly technique for more studies. Particle sizes were compared together according to various concentrations of the applied *MO* extracts. Microwave-assisted extraction was the best extraction method and encapsulation of the *MO* extract was carried out well by chitosan, as an eco-friendly technique.

EXPERIMENTAL

Materials And Methods

Materials

Melissa officinalis was purchased from a local store in Babol, Iran. Chitosan and sodium tripolyphosphate were obtained from Merck. Ethanol, folin ciocalteu, 2, 2-diphenyl, 1- picryl hydrazyl (DPPH), Cellulase, Pectinase, and other substances were all supplied by Sigma-Aldrich products.

Apparatus

The equipment used in this study includes analytical balance manufactured by A&D company, magnetic stirrer model R-50 (Italy), water bath manufactured by Memmert model WB22 (Germany), sonicator model QTD1730 (Korea), Centrifuge manufactured by Hermle company (Germany) and UV/Visible spectrometer model 6305 manufactured by Jenway (UK) was used to determine total phenolic content according to the standard curve for Gallic acid. Vacuum freeze dryer model FDE-350 (Korea) was applied for the separation of the synthesized nanoparticles. Surface morphological information of the encapsulated nanoparticles was obtained by the FE-SEM model MIRA3TESCAN-XMU. The mean particle sizes of the capsulated nanoparticles were measured by the Dynamic Light Scattering (DLS) technique. Ultrasonic-assisted extraction (UAE) system (400W, 24kHz) model UP400S (Germany) with probe diameter 7 mm, Microwave-assisted extraction (MAE) 450W Samsung model CQ-4250 (South Korea) and Ultrasonic bath model vgt 1730 QTD (120W, 40kHz) were used for extraction.

Preparation of Melissa officinalis extracts

Thoroughly rinsed with water, *MO* leaves were dried in an oven at 40°C for one week. Afterward, the dried leaves were milled and the obtained powder was passed through a sieve No.40 with a pore size of 0.425 mm and then was stored in a sealed container protected from moisture. Extraction of *MO* was performed using five different methods of maceration, soxhlet, ultrasound-assisted, enzyme-assisted, and microwave-assisted extractions. All the extracts were prepared with 70% ethanol as the solvent and a solid-to-solvent ratio of 1:20 [24]. In the maceration method, 1 g of dried plant powder was poured into a beaker, followed by the addition of 20 mL of the solvent. The beaker's surface was completely covered with Parafilm to prevent solvent evaporation and was subsequently incubated in an incubator shaker for 24 hours at ambient temperature. Finally, the extract was filtered using Whatman filter No. 1 [25]. In the soxhlet extraction method, 12.5 g of dried plant powder was poured into a thimble porous and the extraction soxhlet chamber was placed on a flask containing

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250 mL of solvent at 80 °C. After the soxhlet process, the extract was filtered using Whatman filter No. 1 [26]. In the enzyme-assisted extraction method, 1 g of dried plant powder was combined with the commercial enzymes of cellulase and pectinase at the ratio of 5% w/w of a plant to prepare samples with 100% cellulase, 100% pectinase, cellulase-pectinase (1:1) as well as a control sample without enzyme. pH levels of the solvent were set to 3.5-4 using 5% acetic acid. 20 mL of this solution was added to the prepared samples and kept in the incubator at 40 °C for 2h. Finally, the extract was filtered via filter paper Whatman No. 1 [27]. In the microwave-assisted extraction method, 1g of dried plant powder with 20 mL of the extraction solvent was put in a conical flask. The mixture was placed in a microwave oven of 450 watts and the extraction process was performed for 1, 3, and 5 minutes, separately. The temperature of the solution was controlled by an ice-water bath. Finally, the extract was filtered *via* filter paper Whatman No. 1 [28]. In the ultrasound-assisted extraction method, 1g of dried plant with 20 mL of the extract was put in a conical flask and sonication was placed by a 7 mm ultra-sonication probe in ice water. The extraction process was carried out at different durations of 10, 15, 20, and 30 minutes and the extracts were filtered using filter paper Whatman No .1 [29]. Extraction was also implemented by applying an ultrasonic 120 watts in an ice-bath 0°C. Hydroalcoholic 70% extraction was carried out from dried powder of *MO* in all of the extraction methods. Characterizations of the extraction methods are summarized in Table 1.

Evaluation of the extracts

Determination of total phenolic content

Total phenolic content (TPC) was determined using Folin–Ciocalteu method. Accordingly, 0.5 mL of the extracts with 2.5 mL of 0.2 N Folin–Ciocalteu reagent were poured into a test tube. After 5 minutes, 2 mL of sodium carbonate solution (75 g/L) was added to the mixture and put in an incubator at room temperature for 2 hours. Then, solution absorption was measured at 760 nm by a UV-VIS spectrophotometer. Absorbance was evaluated by the calibration curve which has been already prepared according to different gallic acid concentrations. Therefore, the results of total phenolic content were expressed as mg of gallic acid per g of dried extract (mg GA/ g extract) [30].

Determination of antioxidant activity

According to one of the most common methods for the measurement of the antioxidant activity (AA), 0.05 mL of the extracts of *MO* was poured into a test tube containing 1.95 mL solution of DPPH (0.025 g/L). The samples were kept in a dark place for 30 minutes and then the absorption of the solution was measured at 515 nm [31]. Moreover, the inhibition percentage of the free radicals was calculated as the equation (1) mentioned below:

$$\%AA = 100 - \left(\left(\frac{A_{control-} A_{sample}}{A_{control}} \right) \times 100 \right) \quad (1)$$

Where A control and A sample are the absorbance of the sample and control, respectively.

Total solid content efficiency

In order to determine the TSC efficiency, 1 mL of the extracts was weighed in a watch glass and placed in an oven at 40 °C. After 48 hours, the contents of the glass were weighed again [32]. The total extraction percentage was calculated according to this equation (2):

$$\%TSC = \left(\frac{m_{extract} \times V_t}{m_{raw\ material}} \right) \times 100 \quad (2)$$

Where $m_{extract}$ is the remains of the solid mass after the solvent evaporation, $m_{raw\ material}$ refers to the weight of 1mL extract and Vt represents the volume of extract obtained through the extraction. Subsequently, the solvent was concentrated using a rotary evaporator at 40 °C and dried in a freeze dryer at -50 °C for 24 hours [27].

Table 1. Characterization of the extraction methods

Extraction Method	Time	Temperature	Plant weight	Solvent volume
Maceration	24 h	25 °C	1g	20 mL
Soxhlet	24 h	-	12.5g	250 mL
Microwave	1, 3 and 5 min	0 °C	1g	20 mL
Enzymatic	24 h	40 °C	1g	20 mL
Ultrasound	10, 15, 20 and 30min	0 °C	1g	20 mL

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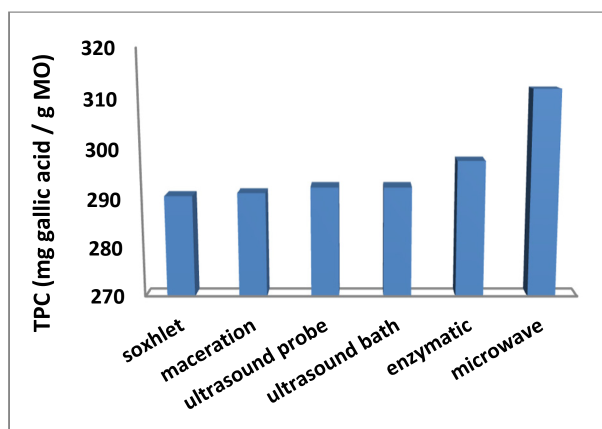

Fig. 1. Evaluation of various extraction methods on TPC

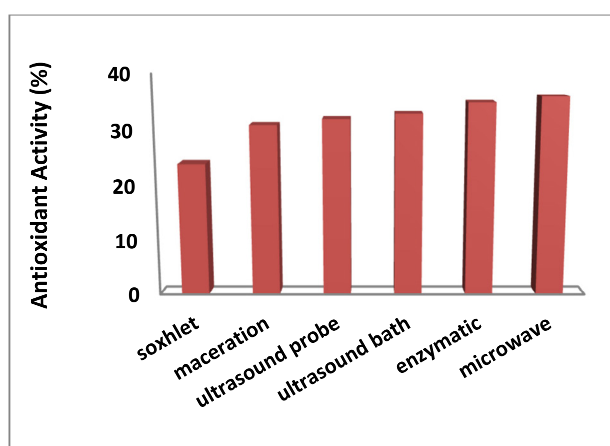

Fig. 2. Evaluation of different extraction methods on AA

Preparation of Nanoparticles

Chitosan nanoparticles were prepared by ionic gelation using tripolyphosphate as a crosslinking agent. Such that, the extraction solution obtained from dissolving 1 mg of the extract powder in 1 mL of ethanol solution (70 %), was added to 10 mL of chitosan solution and stirred for 10 minutes. Next, 3.3 mL of sodium tripolyphosphate solution was added drop wisely to the mixture which was then stirred for 15 minutes at 700 rpm. The obtained nanoparticles were kept at 4 °C for 24hrs and then separated through centrifugation at 13500 rpm for 30 minutes. later on, was dried in the freeze dryer at -50 ° C for 24 hours and stored at 4°C for further use [33].

RESULTS AND DISCUSSION

Extraction methods efficiencies

In order to compare the different utilized extraction methods including maceration, soxhlet, ultrasound-assisted, enzyme-assisted, and microwave-assisted extractions, three distinct parameters of total phenolic contents (Fig. 1), antioxidant activity (Fig. 2) and total solid extraction efficiency (Fig. 3) were ascertained. Results indicated that among all of the extraction methods in this study, the 3-minute microwave-assisted extraction had the highest levels of TPC, AA, and TSC.

However, by increasing the duration to 5 minutes, the extraction efficiency declined which could be attributed to the probable removal of phenolic and antioxidant compounds over longer periods by microwave extractions. This is consistent with the results reported in 2003 by Pan who investigated tea polyphenols and caffeine extractions from green tea leaves using a microwave-assisted extraction method [34].

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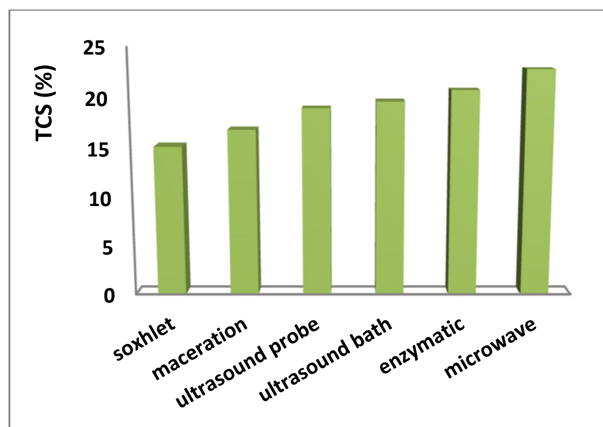

Fig. 3. Evaluation of different extraction methods on TSC

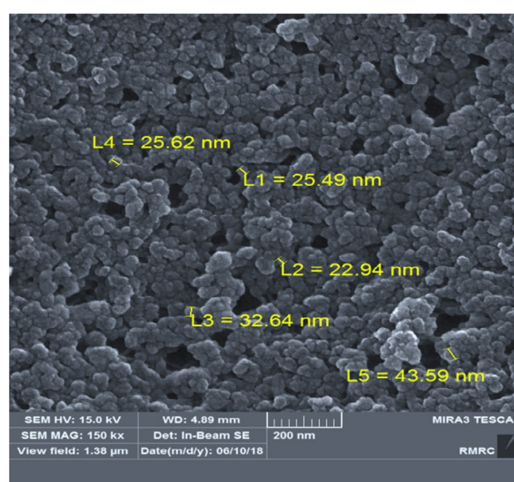

Fig. 4. FE-SEM imaging on the surface of the particles for 0.1mg /mL concentrtion of the extract

The enzyme-assisted extraction method using pectinase was recognized as the second appropriate procedure which outperformed using pure cellulose. In addition, the weakest performance applying this method was observed for the mixture of cellulase and pectinase. Although no significant difference in the results was obtained using the two ultrasound-assisted extraction methods during 30 minutes, the probe ultra-sonication process revealed more acceptable results in the extraction periods of 10 to 20 minutes. This agrees well with the results obtained by Fu et al. investigating the extraction of luteolin and apigenin from pigeon peas using an enzymatic extraction method [35]. Furthermore, the soxhlet method offered the lowest efficiency in all three factors due to the long duration of extraction as well as the high operating temperature leading to the degradation of the effective plant compounds.

Characterizations

Morphology Study

Imaging on the surface of the nanocapsule particles for 1.0 mg /mL concentration of the extract was carried out using an FE-SEM device as shown in Fig.4. Uniformity and almost spherical shape of the particles were shown in this image. Since the nanoparticles were not dispersed well before the FESEM process, in the micrograph it was observed that they were agglomerates with heterogeneous sizes that differ from the sizes reported using the DLS technique. Of course, the conception of the nanoparticle size by the DLS technique is more correct.

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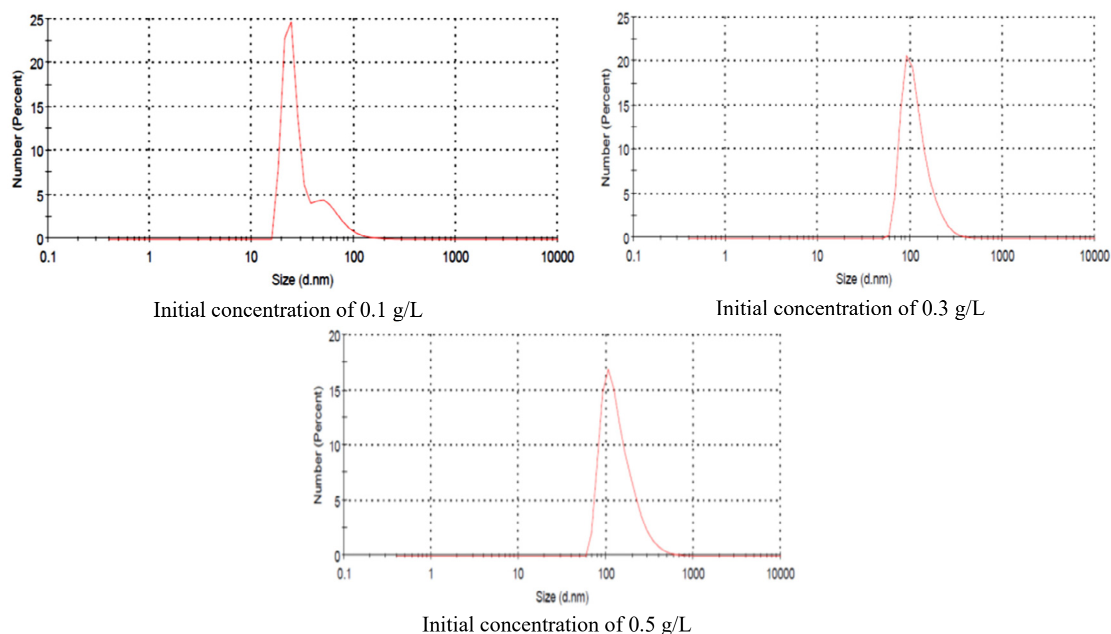
Table 2. Effect of the extract concentrations on the nanoparticle size.

Melissa officinalis concentration (g/L)	Nanoparticles size (nm)
0.1	24
0.3	118
0.5	144



Initial concentration of 0.1 g/L

Initial concentration of 0.3 g/L

Initial concentration of 0.5 g/L

Fig. 5. Particle size distribution of lemon balm nanoparticles with different extract concentrations

Micro and nanospheres could be easily administered through a syringe needle, and carry several pharmaceutical types, like drugs, bioactive compounds, vaccines, antibiotics, or hormones. In addition, nanospheres have an important additional feature: they can entrap and protect cells, therefore serving also as a platform for cell applications. The components of the micro and nanospheres can mimic the 3D matrix found in the native cell environment [36].

Nanoparticles size

The nanoparticle's size is of particular importance concerning drug delivery systems. The size distribution of the synthesized nanoparticles was measured through the DLS technique according to Zeta Seizer as presented in Table 2. Different concentrations of the extract 0.1, 0.3, and 0.5 mg/mL were examined for evaluating how the concentration of the extract may influence the nanoparticle size. As could be realized, nanoparticles of 24, 118, and 145 nm were obtained for 0.1, 0.3, and 0.5 mg/mL concentrations, respectively. As depicted in Fig. 5, the size of nanoparticles is strongly affected by the used concentration in the production procedure. In other words, a small variation in the concentration of the extract (from 0.1 to 0.5 g/L) leads to a remarkable effect on the size of the nanoparticles (from 24-145 nm).

CONCLUSION

An existing lot of bioactive compounds in plant extracts caused that, so the researchers try to keep them and attend to increase the efficacy of these natural products. Capsulation of plant extracts could be the answer to such complications. In this study, *MO* extracts *via* various methods were carried out and evaluated by measuring three parameters of 1)TPC, 2)antioxidant activity, and 3) TSC which indicated that the best extraction was a

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microwave-assisted approach. *MO* extracts as a case study were encapsulated in chitosan nanoparticles. The obtained particle sizes were closely 75 nm. This study demonstrated that the nanoencapsulation of herbal extracts containing antioxidant bioactive compounds could be introduced as a good candidate to transfer the bioactive compounds safely to another side of the body or protect them. Since it minimizes environmental degradation as well as the usage of chemical solvents, this method can be considered a green technique. Also, to preserve the plant properties for a longer period, the extract was encapsulated with chitosan through an ionic gelation method. The nanoparticles size analysis expressed a strong dependence on particle size with a concentration of *MO* extract.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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