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Production of Melissa Officinalis (Lemon Balm) Extracts Applying Multiple Methods and Investigation of its Encapsulation Using Chitosan

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1. Abstract

In the present study, Melissa officinalis (lemon balm) extract production was investigated using various extraction methods including maceration, soxhlet, ultrasound-assisted, enzyme-assisted and microwave-assisted extraction. Effectiveness of the employed methods was deliberated through determining the total phenolic content, antioxidant activity and extraction efficiency of solids content. Accordingly, Melissa officinalis extract with total phenolic content of 311.94 mg gallic acid/g of plant, antioxidant activity of 36 mg DPPH/g of plant as well as solids extraction efficiency of 22.81% were resulted confirming a beneficial herbal medicine. Besides, synthesis of chitosan nanoparticles loaded with Melissa officinalis was carried out by an ionization method. The effect of Melissa officinalis loading concentration on both the particle size and particle size distribution of chitosan nanoparticles was evaluated. Examining different concentrations of Melissa officinalis (1.0, 3.0 and 5.0 mg /mL), chitosan nanoparticles of 24, 118 and 145 nm were eventually obtained.

2. 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 the 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 addition, 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 (MO) is highly appreciated due to its high therapeutic properties together with possessing multiple anti-oxidant compounds. This plant originates from the mint family and is a massively grown annual plant which 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] the surface of which is rough and vegan. Melissa officinalis has revealed promising characteristics such as anti-oxidant, soothing, stimulating, anti-parasitic, anti-contractile, anti-tumor, sedative and hypnotic effects [4]. An anti-inflammatory drug normally contains proteins which can inhibit protein biosynthesis in cancer cells. The biological activity of such medicines is related to their essential oils which despite having been studied broadly, their volatile compounds require further evaluation. 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 treatment of sleep disorders [5]. The main compounds in this plant include gallic acid, chlorogenic acid, caffeic acid, rosmarinic acid, ellagic acid, isoquercetin, quercetin, routine and camphorol. Also, 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. [8] 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 treatment of some human cancer cells to a great extent. Moreover, Akhondzadeh et al. [4] applied MO extract to cure patients with mild to moderate Alzheimer's, in 2003. Their results revealed that MO extract could lead to a noticeable effect on the patients' cognitive function while attenuating their inconvenience and stress.

Employing various techniques, it is possible to extract the bioactive compounds from all medicinal herbs including Melissa officinalis. These methods are divided into two categories: conventional and unconventional extraction methods. Soxhlet, maceration and extraction by hydro distillation are among the main conventional routes. However, microwave-assisted, ultrasound-assisted, enzyme-assisted, pulsed electric field-assisted, pressurized liquid as well as super-critical fluid extraction methods fall into the unconventional extraction procedures [9]. Since many herbal and even chemical medicines virtues may be eliminated or changed during the production process or in the body on the way to the target organ, drug delivery systems have been much focused as they can overcome the abovementioned challenges, considerably. 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 the vital issues; they minimize the unwanted side effects and through accumulation of therapeutic compounds at the target site lower doses of the drug is consequently required [10].

One of the most effective substances providing viable drug delivery is chitosan. It has recently been used in several areas, especially in pharmaceutical industries due to its simple preparation technique together with the ability of loading macromolecules, thus facilitating their transportation against mucosal factors [11]. 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 [12].

In this research, a number of extraction methods were examined for producing Melissa officinalis extracts and they were evaluated through calculation of total phenolic content, antioxidant activity and extraction efficiency of solids content. The extracts were loaded in Chitosan nanoparticles as possible future drug delivery systems and their concentrations were altered for evaluating the size changes.

3. Materials and Methods

3.1. Materials and Equipment

Melissa officinalis was purchased from a local store in Babol, Iran. Chitosan and sodium tripolyphosphate were obtained from Merck. Ethanol, folin ciocalteu, DPPH , Cellulase, Pectinase and other substances were all supplied by Sigma Aldrich.

3.2. Preparation and Extraction of Melissa Officinalis Extracts

Thoroughly rinsed with water, MO leaves were dried in an oven at 40°C for one week. Afterwards, 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 was then 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 [13].

- In the maceration method, 1g of dried plant powder was poured into a beaker, followed by addition of 20 mL of the solvent. The beaker's surface was completely covered with Para film to prevent solvent evaporation and was subsequently incubated in an incubator shaker for 24 hours at ambient temperature. Finally, the extract was filtered using What man filter No. 1 [14].
- In the soxhlet extraction method, 12.5 g of dried plant powder was poured into a thumb which is a thick filter paper and the extraction soxhlet chamber was placed on a flask containing 250 mL of solvent at 80° C. After the operation was completed, the extract was filtered using What man filter No. 1 [15].
- In the enzyme-assisted extraction method, 1g of dried plant powder was combined with the commercial enzymes of cellulase and pectinase at the ratio of 5% w/w of plant, to prepare samples with 100% cellulase, 100% pectinase, 50% cellulase +50% pectinase as well as a control sample without enzyme. Using 5% acetic acid, pH of 3.5-4 was set for the solvent, 20 mL of which was added to the as-prepared samples. That was followed by a two-hour incubation step at 40° C to avoid solvent evaporation. Finally, the container's surface was covered and the extract was filtered with the aid of What man filter No. 1 [16].
- In the microwave-assisted extraction method, an Erlenmeyer flask was employed for appropriate mixing of 1g dried plant powder with 20 mL of the extraction solvent. The achieved sample was placed in a microwave oven using 450 watts of power and the extraction process was performed for 1, 3 and 5 minutes in order to control the solvent temperature. The samples were removed from the microwave every 20 seconds and placed in an ice-water bath. At the end, the extract was filtered using What man filter No .1 [17].
- In the ultrasound-assisted extraction method, after combining 1g of dried plant with 20 mL of the extraction solvent in a small Erlenmeyer flask, sonication of the samples placed in an ice bath, was conducted by a 7 mm ultra-sonication probe with 70% power. The extraction process was carried out at different durations of 10, 15, 20 and 30 minutes and the extracts were filtered using What man filter No .1 [18]. This method was also implemented applying an ultrasound bath using the as-prepared mixture in Erlenmeyer flask placed inside an ultrasonic bath with 120 watts

of power. To avoid solvent evaporation, the bath temperature was set at 0° C and the final steps were performed the same with the previous procedure.

The operation conditions for the extraction methods are summarized in (Table 1).

Table 1: Conditions for the extraction methods: maceration, soxhlet, ultrasound, enzyme and microwave-assisted extractions.

Extraction Method	maceration	soxhlet	microwave	enzymatic	ultrasound
Time	24 h	24 h	1, 3 and 5 min	2 h	10, 15, 20 and 30 min
Temperature	25 °C	-	0 °C	40 °C	0 °C
Plant weight (g)	1	12.5	1	1	1
Solvent (Ethanol %)	70	70	70	70	70
Solvent volume (mL)	20	250	20	20	20

3.3. Extraction Efficiency Evaluation

2.3.1. Determination of Total Phenolic Content (TPC)

Total phenolic content was determined using Folin–Ciocalteu method. Accordingly, 0.5 mL of the extracts obtained from the abovementioned extraction methods and 2.5 mL of 0.2 N Folin–Ciocalteu reagent were poured into a test tube. After 5 minutes, 2 mL of a 75 g/L sodium carbonate was added to the solution, followed by incubation at room temperature for 2 hours. The solution absorption was measured at 760 nm by a UV-VIS spectrophotometer and considering different gallic acid concentrations of 0 to 0.1 g/L, a calibration curve was prepared. Therefore, the results of total phenolic content were expressed as mg of gallic acid per g of dried extract (mg GA/ g extract) [19].

3.3.2. Determination of the Antioxidant Activity (AA)

Regarding one of the most common methods for measuring the antioxidant activity of plant specimens, 0.05 mL of the extracts previously obtained from Melissa officinalis was poured into a test tube to which, 1.95 mL of a 0.025 g/L DPPH was added. The samples were preserved in a dark place for 30 minutes and the solution absorption was measured at 515 nm, afterwards [20]. Moreover, the inhibition percentage of the free radicals was calculated as follows (Eq.1) :

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

Where $A_{control}$ and A_{sample} are the (…) for each test sample and the control sample, respectively.

3.3.3. Determination of Total Solid Content (TSC) Efficiency

To determine the TSC efficiency, 1 mL of the extracts previously obtained was weighed, poured into a glass and placed in an oven at 40° C. After 48 hours, the contents of the glass were weighed again [21]. The total extraction percentage was calculated according to Eq.2:

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

Where $m_{extract}$ is the remaining solid mass after removing from the oven, m_{raw} material stands for the plant mass used in the extraction and V_t denotes 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 [16].

3.4. Preparation of Nanoparticles

Chitosan nanoparticles were prepared by ionic gelation using tripolyphosphate as crosslinking agent. Such that, the extraction solution obtained from dissolving 1mg of the extract powder in 1mL 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 to the resulted mixture which was then stirred for another 15 minutes at 700 rpm. The obtained nanoparticles were separated through centrifugation at 13500 rpm for 30 minutes at 4° C. Then, they were collected and dried in the freeze dryer at -50° C for 24 hours and stored at -4° C for further use [22].

4. Results and Discussion

4.1. Extraction Methods Efficiencies

As explained in the previous sections, in order to compare the different utilized extraction methods including maceration, soxhlet, ultrasound-assisted, enzyme-assisted and microwave-assisted extractions and to select the most efficient one for extracting the effective compounds from MO, three distinct parameters of Total Phenolic Contents (TPC) (Figure 1), Antioxidant Activity (AA) (Figure 2) and Total Solid extraction efficiency (TSC) (Figure 3) were ascertained. The obtained results are depicted in the following diagrams considering the best condition in all methods;

Among all the extraction methods, the 3-minute microwave-assisted extraction test yielded the highest levels of TPC, AA and TSC within a significantly decreased extraction time, however with 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 of time by microwave extractions. This is consistent with the results reported in 2003 by Pan [24] who investigated tea polyphenols and caffeine extractions from green tea leaves using a microwave-assisted extraction method. The enzyme-assisted extraction method using pectinase was recognized as the second appropriate procedure which outperformed using pure cellulase. 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 shorter extraction periods of 10 to 20 minutes. This agrees well with the results obtained by Fu et

al. [23] investigating the extraction of luteolin and apigenin from pigeonpea using an enzymatic extraction method. Furthermore, the soxhlet method offered the lowest efficiency in all the three factors due to the long duration of extraction as well as the high operating temperature leading to degradation of the plant effective compounds.

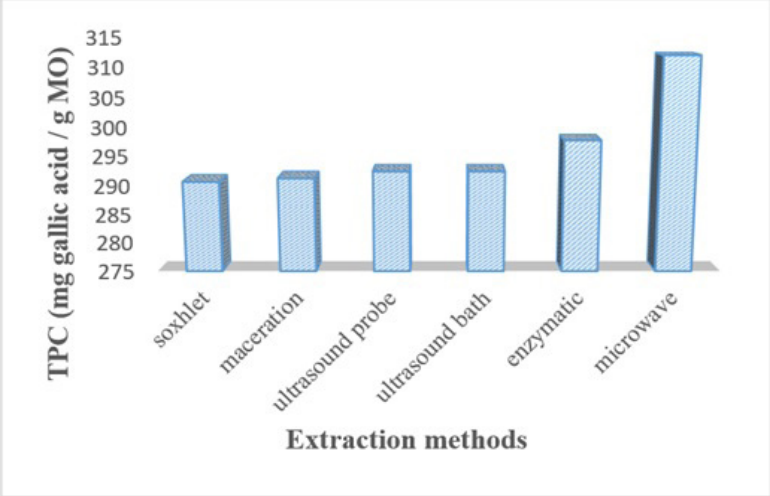

Figure 1: The effects of using different extraction methods on TPC (mg gallic acid/ g MO)

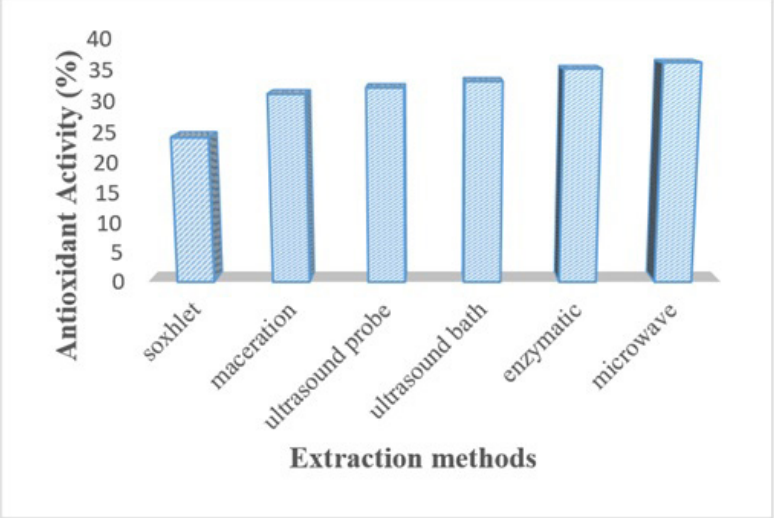

Figure 2: The effects of using different extraction methods on AA (%).

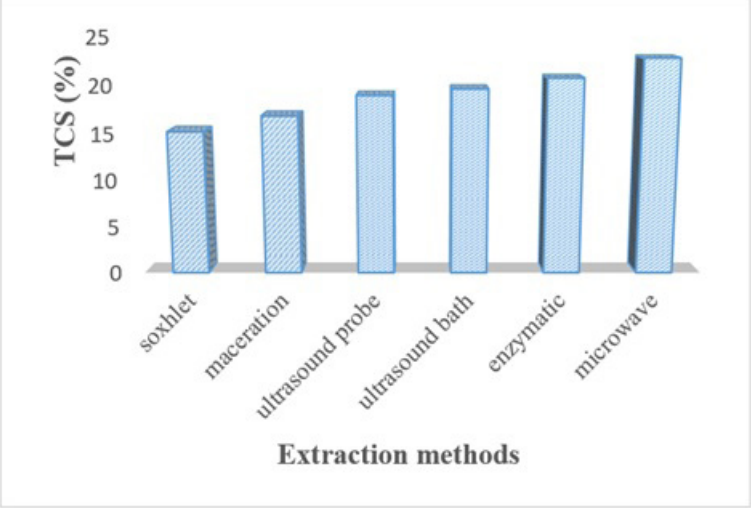

Figure 3: The effects of using different extraction methods on TSC (%)

4.2. Characterizations

4.2.1. Nanoparticles Morphology

The morphology of nanoparticles was determined using SEM and is presented in (Figure 4). According to the image, particles with a completely spherical shape were obtained. As could be observed, some larger particles are formed as well in a number of regions which could be ascribed to their high surface energy and also the probable particles collision during the drying steps.

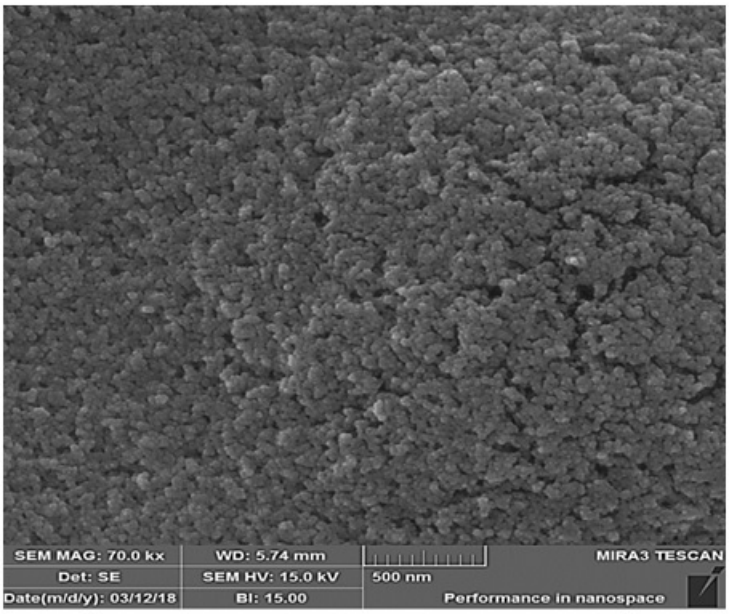

Figure 4: SEM image of the produced chitosan/extract nanoparticles

4.2.2. Nanoparticles Size

The nanoparticles size is of particular importance concerning drug delivery systems. The size distribution of the synthesized nanoparticles was measured by Zeta Seizer as presented in (Table 2). As could be realized, nanoparticles of 24, 118 and 145 nm were obtained. Different Melissa officinalis concentrations of 0.1, 0.3 and 0.5 mg/mL were examined for evaluating how the extract concentration may influence the nanoparticle size. As depicted in Table 2 and (Figures 5 to 7), the size of nanoparticles is strongly affected by the extract concentration used in the production procedure. In other words, a small change in the extract concentration (from 0.1 to 0.5) 0 g/L) leads to a remarkable effect on the nanoparticles size (from 24-145 nm); so that the higher the concentration, the larger the nanoparticles.

Table 2: Effects 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

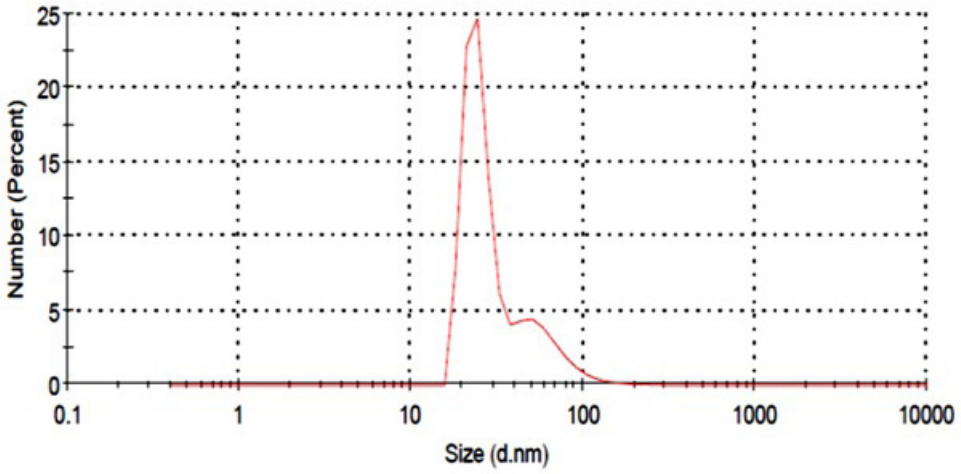

Figure 5: Particle size distribution of lemon balm nanoparticles with initial concentration of 0.1 g/L.

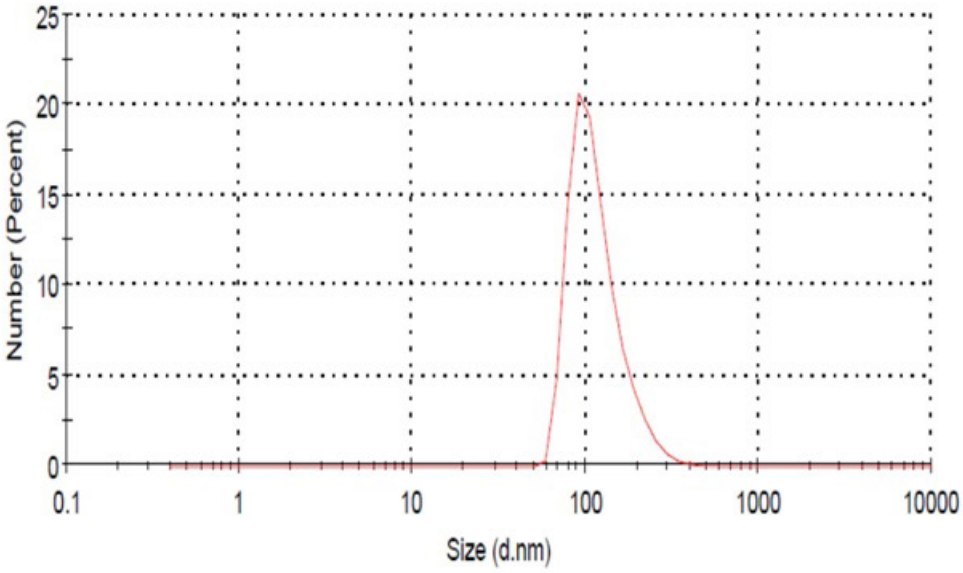

Figure 6: Particle size distribution of lemon balm nanoparticles with initial concentration of 0.3 g/L.

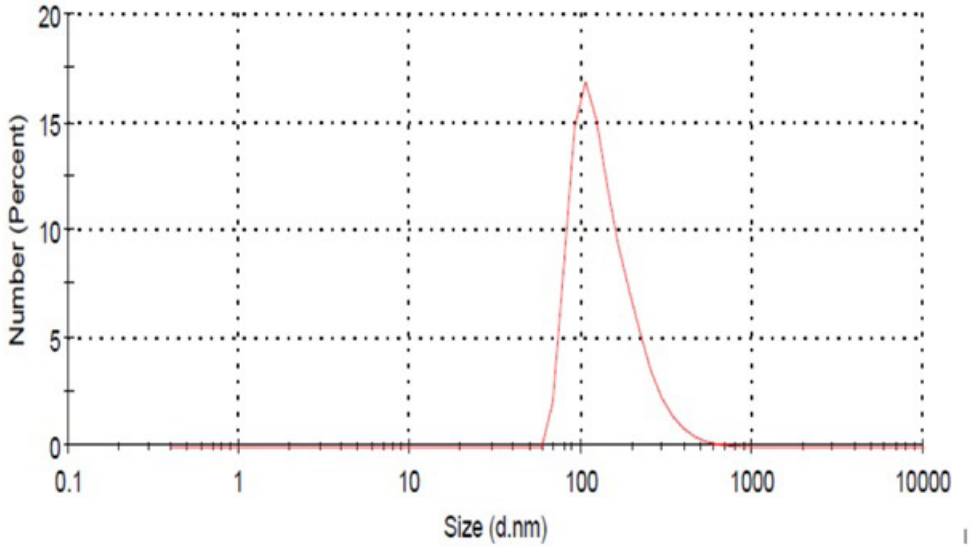

Figure 7: Particle size distribution of lemon balm nanoparticles with initial concentration of 0.5 g/L.

5. Conclusion

Different extraction methods were assessed for Melissa officinalis (lemon balm) extract production through measuring three parameters of 1) total phenolic content, 2) antioxidant activity and 3) total solid content. Among all the methods applied including maceration, soxhlet, ultrasound-assisted, enzyme-assisted and microwave-assisted extractions, the highest efficiency was achieved for the microwave-assisted extraction method presenting the highest values for all parameters. While some extraction methods require a huge amount of solvent and a long extraction time, microwave-assisted approach could yield favorable results in only 3 minutes using a small volume of solvent. In addition, this method can be considered as a green technique, since it minimizes the environmental degradations as well as usage of chemical solvents. 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 of size on the extract initial concentration.

6. Acknowledgement

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