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RESEARCH ARTICLE

Antimicrobial potential of Mentha Spp. essential oils as raw and loaded solid lipid nanoparticles against dental caries

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ABSTRACT:

Objective: This study evaluates the antimicrobial potential of *Mentha* spp. (*M.spicata* L.,*M. xpiperita* L. and *M. pulegium* L.) essential oils as raw and loaded solid lipid nanoparticles (SLNs)against dental caries. **Design:** Essentials oils extraction from fresh aerial parts of *Mentha* spp. was carried out using hydro distillation technique. Solid lipid nanoparticles of *Mentha* essential oil were prepared by w/o/w type double emulsification method. The antimicrobial activity of both pure *Mentha* spp. essential oils and *Mentha* spp. SLNs was determined against bacteria presented in saliva collected from 12 patients using agar diffusion assay. **Results:** *Mentha* spp. essential oils loaded solid lipid nanoparticles (MSLNs) were spherical shaped with sizes ranged from 111 to 202 nm and with PDI from 0.43 to 0.76, EE% between 85 and 88, and ZP of −11.8 to -40 mV. Antimicrobial results showed that MSLNs exhibited higher *in vitro* antimicrobial activity than pure *Mentha* spp. essential oil. Particularly, with an inhibition zone of 20 mm. These both MSLNs were even more active than the reference compound novobiocin. **Conclusion:** Our findings demonstrate that *Mentha* spp. essential oils as a nanostructure increase the efficiency of these natural products as antibacterial agents against caries.

KEYWORDS: *Mentha* spp. essential oils; solid lipid nanostructure (SLNs); dental caries; antibacterial, zeta potential, encapsulation efficiency.

1. INTRODUCTION:

Essential oils (EO) are concentrated natural extracts derived from a plant source to be excellent sources of bioactive compounds with antioxidant and antimicrobial properties. (Wiwattanarattanabut, K.; Choonharuangdej, S.; Srithavaj, (2017). The popularity of essential oils has blossomed throughout recent years. The art of processing pure essential oils often requires many pounds of the plant source to obtain a very small amount of the essential oil. This form of holistic medicine has been around since the ancient ages and becoming more popular as Americans are looking for holistic healing versus the chemical alternatives. Production of essential

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oils, one of two processes are used either extraction or distillation and it depends on the source it is derived from (Song, J.K.; Bae, J.M. (2016). The issue of antibiotic-resistant bacteria becoming more common, it is increasingly more important to find an alternative or additive product; Essential oils, unlike antibiotics, do not appear to have resistant properties or loose efficacy over time. (Curk, F.; Ollitrault, F.; Garcia-Lor, A. 2016). Dental caries is one of the most common dental infectious diseases (Asikainen et al. 1993). Dental caries is due to bacteria which produce acids that cause a breakdown of teeth (Aas et al. 2008). Mentha spp. is well known genus used by the human throughout history. The major applications include products for oral care, chewing gum, liquors and fragrances. Its potential depends on its multiple properties as carminative, antioxidant, antifungal or antimicrobial. There was also described as potential nutraceutical and functional food for prevention and treatment diseases (Gruľová D, De Martino L, Mancini E, Salamon I, De Feo V. (2015). The antimicrobial activity of EO is especially focused on the treatment of strains resistant to conventional antibiotics (Eloff JN. (1998). Extensive studies evidence the remarkable biological activity of essential oils as antimicrobial agents such as those obtained from *Mentha* spp. (Asikainen et al. 1993; São Pedro et al. 2013; Bouyahya et al. 2017). *Mentha pulegium* and *M. xpiperita* essential oils have shown potent antibacterial activity against *Staphylococcus aureus* (Bouyahya et al. 2017) and against *E.coli* (da Silva Ramos et al. 2017). This activity is mainly due to their secondary metabolites oxygenated monoterpenes such as limonene, menthol, menthone, pulegone, piperitone oxide, β-pinene and 1,8-cineole (Bouyahya et al. 2017; da Silva Ramos et al. 2017).The results of the study of Elmastaş, Dermirtas, Isildak, and Aboul-Enein (2006) indicated that also s-carvone, a main constituent of M. spicata essential oil, possesses high antioxidant activity compared to α-tocopherol. The water-soluble extracts from the Mentha species (*M. aquatica, M. haplocalyx, M. dalmatica, M. verticillata, M. spicata,* and *M. piperita*) demonstrated varying degrees of efficacy in antioxidant assays, with the *M. piperita* extract being better than the other extracts. As Dorman, Koşar, Kahlos, Holm, and Hiltunen (2003) determined, the level of antioxidant activity was strongly associated with the phenolic content. Ahmad, Fazal, Ahmad, and Abbasi (2012) observed that methanolic extracts of Mentha species possess antioxidant capacity in the sequence: *Mentha longifolia* followed by *M. officinalis* and *M. piperita*, respectively. Fialová, Tekeĺová, Mrlianová, and Grančai (2008) compared antioxidant activity of several mints (*M. spicata, M. piperita, M. longifolia*) harvested in two harvest times. Chlorhexidine (CHX), one of the most effective drugs administered for periodontal treatment, presents collateral effects including toxicity when used for prolonged periods; An inhibition zone test demonstrated that both bacteria were sensitive to the EO; XTT analysis and CFU counts confirmed that 10-fold-diluted EO determined a statistically significant ($p < 0.05$) reduction in bacteria count and viability towards both biofilm and planktonic forms in a comparable manner to those obtained with CHX.. In conclusion, EO exhibited bactericidal activity similar to CHX, but a superior cytocompatibility, making it a promising antiseptic alternative to CHX. (Azzimonti, Cochis, Beyrouthy ME, Iriti M, Uberti, Sorrentino, Landini MM, Rimondini Varoni EM (2015). The composition of the Mentha essential oil directly affects the effectiveness of its antimicrobial activity, which have displayed differences in its constituents depending on the growing area. The chemistry of mentha oil is very complex and highly variable. Analysis by gas chromatography–mass spectrometry (GC-MS) revealed that the prominent components are menthol, isomenthone, limonene, iso-menthanol, menthol acetate, carvone, β-pinene, α-pinene, 1,8-cineole, α-terpineol, isopulegol, pulegone, piperiton, piperitone oxide, and β-phellandrene. Riachi L.G., De Maria C.A.B. (2015). Both the in vivo and in vitro antimicrobial power of these components on *Streptococcus mutans* and *Streptococcus pyogenes* have been evaluated, showing outstanding activity. Peixoto I.T.A., Furlanetti V.F., Anibal P.C., Duarte M.C.T., Höfling J.F. (2009). In addition, the oil exhibited bactericidal effects against *S. aureus, Staphylococcus epidermidis, B. cereus,* and *E. coli* (Bakkali F., Averbeck S., Averbeck D., Idaomar M. 2008). The MIC of the *M. piperita* essential oil was determined against various bacterial strains and varied from 1.13 to 2.25mg/mL; the MIC of Gram-positive bacteria (*B. subtilis* and *S. aureus*) was lower than that of Gram-negative bacteria (*E. coli, P. aeruginosa,* and *Pseudomonas fluorescens*). (Ebrahimzadeh M.A., Nabavi S.M., Nabavi S.F. 2010).

A significantly large part of current literature on the encapsulation of EOs deals with micrometric size capsules, which are used for the protection of the active compounds against environmental factors (e.g., oxygen, light, moisture, and pH), to decrease oil volatility and to transform the oil into a powder. Encapsulation in nonmetric particles is an alternative for overcoming these problems but additionally, due to the subcellular size, may increase the cellular absorption mechanisms and increasing bioefficacy (Malmsten M, Bysell H, Hansson P. (2010). Particle size, shape, and surface properties of the nanoparticles play a vital role in the uptake of nanosized delivery systems across the mucosal membrane. The nanocarriers with particle size of 50–300 nm, positive zeta potential, and hydrophobic surface were found to have preferential uptake as compared to their counterparts (Oishi M, Miyagawa N, Sakaru T, Nagasaki Y.) (2007). Menthol was successfully loaded

into NLCs in the amorphous structure in the ratio of 1:10 Menthol: lipid by dissolving in the oil phase. Menthol-loaded NLCs were around 100nm with narrow size distribution (PDI 0.2). The antimicrobial efficiency of the encapsulated menthol was tested on four different microorganisms, demonstrating that MIC and MBC values in the case of NLCs were lower than the menthol emulsion. It can be concluded that essential oils could be used as antibacterial supplement and food preserving agents. (K. Sumalatha, A. Srinivasa Rao, P. Latha 2014).

The present study aims to prepare and characterize different Mentha spp. essential oils uploaded in solid lipid nanoparticles (MSLNs) and to evaluate their different antibacterial activity in patients who suffer from dental caries.

2. MATERIALS AND METHODS:

2.1. Plant species:

Fresh aerial parts of *Mentha spicata* L., *Mentha piperita* L. and *Mentha pulegium* L. were collected in May 2018 from mountains of Saint Katherine in Sinai (Egypt). All samples were identified in the Eco-station, Department of Pharmacognosy, Faculty of Pharmacy, October University for Modern Sciences and Arts, Giza (Egypt). Voucher specimens were deposited at the Herbarium of the Eco-station of this cited department.

2.2. Patient samples:

Both pure *Mentha* spp. essential oils and *Mentha* spp. essential oils-loaded SLNs were tested against bacteria presented in saliva collected from 12 patients (five males and seven females) suffering from caries. These patients were aged 40−50 years and they were enrolled in the clinic of Restorative Dentistry Department, Faculty of Dentistry, October University for Modern Sciences and Arts (MSA University), Giza, Egypt. The severity of dental caries was measured according to DMFT (decayed, missing, filled tooth) index (Katge et al. 2015; Idrees et al. 2017). Patients status were described as moderate stage. For collection of samples, patients with caries gummed a normal bit of chewable gum for 7 minutes. One ml of saliva was collected by syringe and then delivered to lab in ice.

The inclusion criteria were that none of patients have suffered from any systemic diseases and they had used no antibiotics in the previous 4 weeks. All patients signed a consent approval.

2.3. Preparation of essential oils:

About 100g of fresh aerial parts from each sample of *Mentha* species were placed in 400ml water and subjected to hydro distillation for 2 h using a Clevenger – Type 5 apparatus (British Pharmacopoeia, 1980). Anhydrous sodium sulphate was added to eliminate water traces. Essential oils were stored at 4°C.

2.4. Preparation of Mentha species essential oils-loaded SLNs:

Solid lipid nanoparticles of *Mentha* essential oil from different species were prepared by w/o/w type double emulsification method (Table 1). *Mentha* spp. essential oil was dispersed in aqueous mixture of methanol (75% v/v) and lecithin and cholesterol were dissolved in dimethylsulphoxide (DMSO). The essential oil was then added slowly to lipid mixture and homogenized for 15 min at 15000 rpm in ultra - probe sonicator to produce white cloudy primary emulsion. The resultant primary emulsion was poured into 2% w/v of polyvinyl alcohol (PVA) solution and homogenized for additional 10 min at 15000rpm. The resultant w/o/w type emulsion was stored at room temperature. The solvent was evaporated in a rotary evaporator at 45°C. The stable emulsion was freeze dried at -20°Cunder reduced pressure to get dried powder of solid lipid nanoparticles (Vijayan et al., 2013).

Table 1. Composition for formulations of different Mentha species essential oils-loaded solid lipid nanoparticles (SLNs).

Materials	*F1 Mentha spicata*	*F2 Mentha x piperita*	*F3 Mentha pulegium*
Mentha essential oil (ml)	1	1	1
Lecithin (mg)	10	10	10
Cholesterol (mg)	30	30	30
Dimethyl sulphoxide (DMSO) (ml)	10	10	10
Compritol 888 (mg)	10	10	10
Tween 80(ml)	2	2	2
2% w/v of polyvinyl alcohol (ml)	2	2	2
H_2O (ml)	30	30	30

2.5. Characterization of Mentha species essential oils-loaded SLNs:

2.5.1. Transmission electron microscopy (TEM):

The size of the nanoparticles was studied using a JEOL 1010 (JEOL Ltd, Tokyo, Japan) transmission electron microscopy. One drop of nanoparticle dispersion was placed on the grid, dried for 3 to 5 minutes, and drained on the filter paper. The grid was further dried by keeping it in the petri plate; then it was loaded in the TEM, and areas were scanned for observation of nanoparticles. Pictures were taken under the electron microscope (Amer et al. 2016).

2.5.2. Scanning electron microscopy (SEM):

The surface morphology of *Mentha* spp. SLNs was observed by SEM. A small amount of MSLN was taken in metal stub. The stub was coated with conductive gold

by Hitachi 1010 ion sputter and observed under Hitachi 3000 N Scanning electron microscope (JSM 5610 LV SEM, JEOL, Japan) chamber. The image was scanned at an acceleration voltage of 20 kV with a chamber pressure of 0.8mmHg (Gomes et al. 2011).

2.5.3. Particle size analysis, polydispersity index and Zeta potential:

MSLNs dispersions were characterized for average particle size (z-average size) using Laser diffraction (Malvern Mastersizer 2000 SM, Malvern Instruments Corp) with beam length 2.40mm, range lens of 300 RF mm, and at 14.4% obscuration. Polydispersity (PDI) describes the degree of non-uniformity of a size distribution of particles. This index is dimensionless and scaled such that values smaller than 0.05 are mainly seen with highly monodisperse standards. PDI values bigger than 0.7 indicate that the sample has a very broad particle size distribution and it is probably not suitable to be analyzed by the dynamic light scattering. PDI is basically a representation of the distribution of size populations within a given sample (Zhang et al. 2010). Surface charge was determined by measuring the electrophoretic mobility of nanoparticles using Malvern Mastersizer (Hosseini et al. 2013).

2.5.4. Entrapment efficiency:

The entrapment efficiency was calculated by using 10ml of solid lipid nanoparticles dissolved in 20mL of ethyl alcohol and the solution was centrifuged at 12,000rpm. The supernatant fluid was collected and passed through membrane filter. The quantity of oil in the solution was measured by ultra violet spectroscopy at 254nm (Anandrao et al. 1999) using the following formula:

Drug entrapment (%) = Quantity of oil in nanoparticle/ Mass of oil in the formulation×100.

2.6. Antimicrobial activity:

The antimicrobial activity of pure *Mentha* spp. essential oils and *Mentha* spp. SLNs were determined using agar diffusion assay (Kirby Bauer Diffusion Susceptibility). Plates contained trypticase Soya agar (TSA) media; as they are general-purpose, nonselective media providing enough nutrients to allow for a wide variety of microorganisms to grow and inoculated with 100µl saliva samples were prepared for detection of aerobic bacteria in saliva. Three holes were done in each TSA media using cork borer (10mm diameter). 100µl aliquots of each of the three *Mentha* spp. essential oil mentioned previously were transferred in each hole. Plates were then incubated at 37°C for 48 hours for caries forming bacteria. The antibacterial activity was evaluated by measuring the diameter of growth inhibition zone.

2.7. Statistical analysis:

Data were statistically analyzed using SPSS version 16.0 software (SPSS Inc., Chicago, Illinois, USA). One-way analysis of variance (ANOVA) was used to analyze data of *Mentha* species. The post-hoc Bon-ferroni test for multiple comparisons was done to compare the mean difference among *Mentha* spp. and Novobiocin antibiotic. Nonparametric Mann-Whitney U test was used to compare between pure EO and EO SNLs of each *Mentha* species. Data were presented as mean ± SD. A *P* value of less than 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION:

3.1. Transmission electron microscopy (TEM).:

Mentha SLNs had near smooth, spherical shape with no evident sign of aggregation (Figure 1).

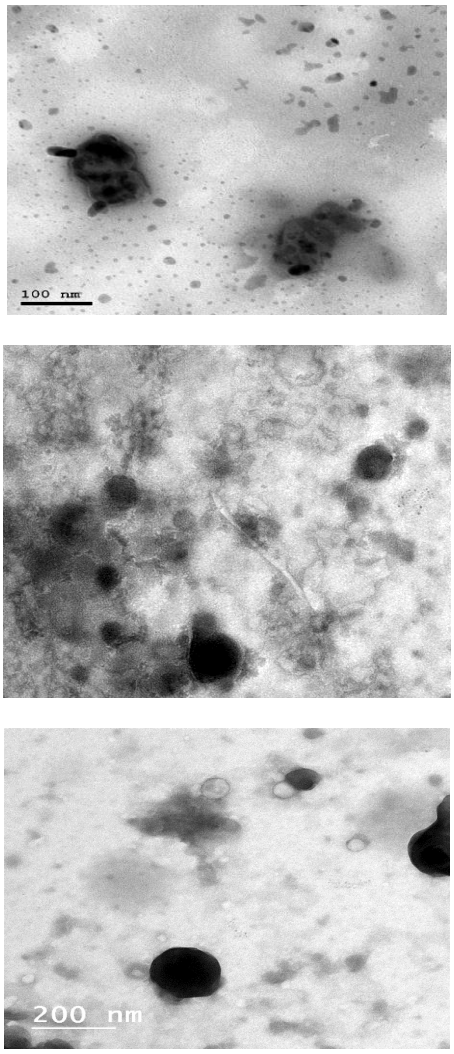

Figure 1. Transmission electron microscope (TEM) images of *Mentha* spp. essential oils-loaded solid lipid nanoparticles (SLNs).

3.2 Scanning Electron Microscope (SEM):

These results were confirmed by Scanning Electron Microscope (SEM) as shown in Figure 2. Smooth and spherical shape of the particles. Also, particles generally agglomerated owing to carriers ' lipid nature, surfactant presence, and sample preparation before SEM analysis.

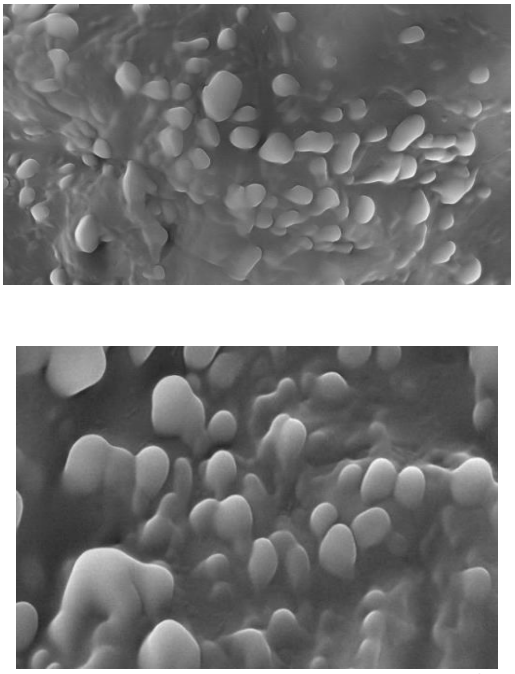

Figure 2. Scanning Electron Microscope (SEM) images of *Mentha* spp. essential oils-loaded solid lipid nanoparticles (SLNs).

3.3 Particle size, polydispersity index (PDI) and zeta potential analysis:

Particle size measurement was done to ensure that particles of the SLNs are of the nanometer range. we observed that all prepared SLNs were in the nano-sized range (average particle size values ranged from MSLNs ranged from 111±0.26nm to 202±0.42nm (n=3). Moreover, all formulations had low values of polydispersity (0.43±0.12 - 0.76±0.32) (n=3) which shows particle size uniformity. Particularly, mean particle size for *M. spicata*, *M. xpiperita* and *M. pulegium* were 100nm with PDI 0.52%, 136nm with PDI 0.43 and 202 with PDI 0.76%, respectively, as shown in Table 2 and Figure (3). Regarding zeta potential, values were -11.8 mv± 0.13 for *M. spicata*, -40 mv±0.23 for *M. xpiperita* and -39.7 mv±0.43 for *M. pulegium* (Table 2). Measuring zeta potential allows for predictions of colloidal dispersion storage stability. Particle aggregation is less likely to occur for charged particles with high zeta potential due to electric repulsion. The encapsulation efficiency percentage (EE%) of MSLNs was in the range between 85-88% as shown in Table 2.

Table 2. Particle size, PDI, zeta potential and entrapment efficiency (EE) of the formulations of different Mentha species essential oils-loaded solid lipid nanoparticles (SLNs).

	Particle size (nm)	PDI (%)	Zeta potential (mv)	EE (%)
F1 (*Mentha spicata***)**	111 ± 0.26	0.52 ± 0.11	-11.8± 0.13	88 ± 0.12
F2 (*Mentha x piperita***)**	136 ± 0.32	0.43 ± 0.12	-40± 0.23	85 ± 0.11
F3 (*Mentha. Pulegium***)**	202 ± 0.42	0.76 ± 0.32	-26.7± 0.43	88 ± 0.45

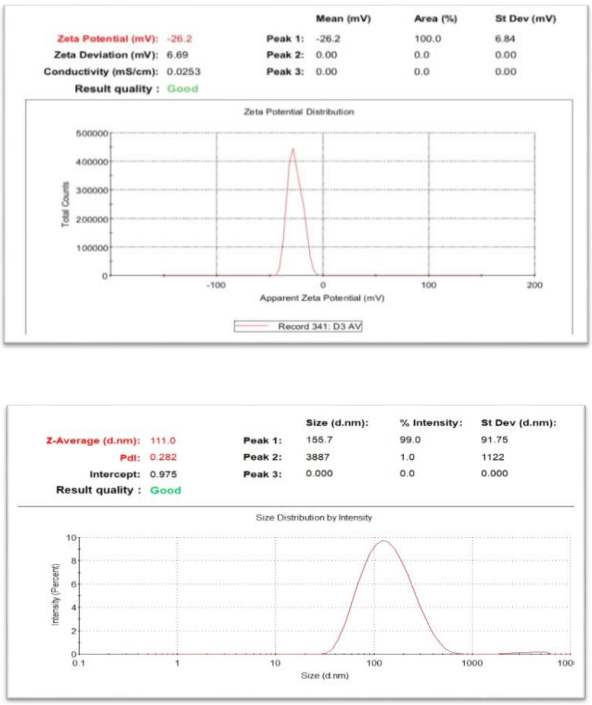

Figure (3): Zeta potential and particle size of Mentha species solid lipid nanostructure.

3.4 Antibacterial activity of Mentha spp. essential oil-loaded solid lipid nanoparticles (MSLNs) compared to pure essential oils:

As shown in Table 3, twelve saliva samples from moderate caries suffering patients were exposed to both pure essential oils and essential oils loaded SLNs of *Mentha viridis*, *Mentha pulegium* and *Mentha piperita*; aerobic bacteria were sensitive to both *M. xpiperita* SLNs and *M. spicata* SLNs with the same zone of inhibition mean (20 mm). Moreover, significant statistical differences in antibacterial activity were found between pure *M. spicata* EO and its SLNs as well as pure *M. xpiperita* EO and its EO SLNs. Both *M. spicata* SLNs and *M. xpiperita* SLNs were even significantly more active than Novobiocin antibiotic. EO SLNs of

Mentha piperita are the best result and have significant difference (0.034, 0.034 respectively) compared to Novobiocin antibiotic.

Table 3. (A, B, C, D)Antimicrobial activity of Mentha spp. essential oil pure and loaded solid lipid nanoparticles (MSLNs) against different bacteria causing caries.

(A)

	Mentha spicata	
	Pure EO	**EO SLNs**
Number sensitive samples	3	7
Zone of Inhibition (mean ± S.D.)	15 mm ± 1	20 mm ± 1.16[a,b]

(B)

	Mentha pulegium	
	Pure EO	**EO SLNs**
Number sensitive samples	zero	3
Zone of Inhibition (mean ± S.D.)	zero	17 mm ± 0 [a]

(C)

	Mentha x piperita	
	Pure EO	**EO SLNs**
Number sensitive samples	4	7
Zone of Inhibition (mean ± S.D.)	16.3 mm ± 0.96	20 mm ± 1.16[a,b]

(D)

	Novobiocin antibiotic (30 mg)
Number sensitive samples	6
Zone of Inhibition (mean ± S.D.)	18.7 mm ± 1.2

(a) Significant difference at P < 0.05 compared to its pure EO; (b) Significant difference at P < 0.05 compared to its Novobiocin antibiotic.
Pure EO: pure essential oil
EOSLNs: Essential oil solid lipid nanostructure lipid carriers.

EO SLNs of *Mentha pulegium* showed significant difference (0.37) compared to Novobiocin antibiotic, with mean ±S.D. inhibition zone equal to 17mm±0. This results were almost similar to that obtained by gram negative bacteria tested with Khosravi Zanjani et al., (2015) (17.45mm by disk diffusion method). No zone of inhibition (resistance EO SLNs) was observed with EO of *Mentha pulegium*. Hussain et al. (2010) estimated components of *M. piperita* essential oils collected during summer and winter. They found that they are menthone (28.13% and 25.54%), menthyl acetate (9.51% and 9.68%), limonene (7.58% and 7.73%) and isomenthone (4.04% and 7.63%), respectively. Piperitenone oxide (60.10% and 64.60%), piperitenone (6.37% and 1.97%) and germacrene D (5.13% and 5.97%). From these results they documented that a significant variation in the content of most of the chemical components and biological activities of collected plants were noticed in different times of the year. They added that seasonal variation exercised notable effects on the antimicrobial activity of Mentha essential oils. Their results contrary with different researches; Aridogan et al.,(2002), Iscan etal., (2002); Yadegarinia etal. (2006). Solórzano-Santos and Miranda-Novales (2012) suggested that essential oils effectiveness as antimicrobial agents is due to the ability of the short extension of their carbon chains to react with lipid cell membranes leading to disturbance in permeability and consequently bacterial death. Moreover, essential oils effect may also lead to either inhibition of certain proteins and intercalation into DNA due to penetration into the bacterial cell. Furthermore, essential oils effectiveness compare to commercially available antibiotics may be due to phyto-synergic interactions among its constituents. This interaction mechanism cannot be yet understood but this could be related to a significant antimicrobial resistance reduction (Allaker and Memarzadeh, 2014). On the other hand, delay and sustain of drug release using a nano-carriers formulations may improve treatment effectiveness and reduce toxicity. In addition, the ability of nanoparticles to control biofilms formation within oral cavity, as a function of their biocidal, anti-adhesive and delivery capabilities, increase its importance in dental diseases treatment (Maderuelo et al., 2011). Moreover, variations in chemical composition may also explain differences in *Mentha* spp. species activity. Previous works have attributed antimicrobial activity to monoterpene hydrocarbons. The compounds carvone, 1,8-cineole, limonene, camphor, β-caryophyllene and oxygenated monoterpenes are the main fraction of *M. spicata* essential oil (Kokkini et al., 1995). The major compounds identified in *M. pulegium* essential oils were pulegone, cineole and piperitenone (Kanakis et al., 2012). Finally, Hussain et al. (2010) determined that the major components of *M. xpiperita*essential oils were menthone, menthyl acetate, limonene and isomenthone. According to Khosravi Zanjani et al., (2015) who agreed with Ait-Ouazzou et al., (2012); Kanakis et al., (2012) that the chemical composition of Mentha pulegium essential oil which was pulegone (19.89%), cineole (19.38%) and piperitenone (15.14%) can differ in the concentration of phenolic compounds according to geographic condition of the plant. Mahboubi and Haghi, (2008) and Hajlaoui et al., (2009) suggested that Mentha pulegium essential oil shows a strong antimicrobial activity against microorganisms, especially gram-positive bacteria and Ait-Ouazzou et al., (2012) justified Mentha pulegium essential oil as a strong bacteriostatic activity against all strains. Delay and sustain of the drug release at specific rate after administration have an advantage in effective treatment, and can also reduce toxicity, Maderuelo etal., (2011). This advantages was provided through using a nano-carriers formulations for the oil due to nanoscale particles dimensions, i.e. new routes of administration can be considered, Pothakamury and Barbosa-Canovas (1995) In addition, the ability of nanoparticles to control

the formation of biofilms within the oral cavity, as a function of their biocidal, anti-adhesive and delivery capabilities, increase its importance in treatment of dental diseases.

4. CONCLUSION:

Mentha spp. essential oil are promising agents to maintain and promote healthcare based on its antimicrobial properties. Nanoencapsulation of *Mentha* spp. essential oils in solid lipid nanoparticles (MSLNs) represent an excellent strategy for overcoming all essential oil limitations, lowering their dose and increasing long-term safety of these constituents. The present study has demonstrated that *M. spicata* SLNs and *M. xpiperita* SLNs were more active against bacteria causing caries than novobiocin antibiotic and their pure essential oils.

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