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Original Article

Investigating Characterizations and Antifungal Effects of Solid Lipid Nanoparticles (SLNs) Loaded with Essential Oil of *Citrus Aurantifolia* on Isolated Malassezia Strains

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Abstract

Introduction: Solid Lipid Nanoparticles (SLNs) were introduced as a different carrier system from emulsions, liposomes, and polymer nanoparticles. Physico-chemical characteristics and stability of drugs that are loaded on SLNs, depends on the properties of drugs and components used in it.

Materials and Methods: In this study, qualitative assessment was performed by GS/MS and UV-VIS to identify compounds in the essential oil of *Citrus Aurantifolia*. In a part of the research, the type of lipid phase and surfactant that was used to prepare the formulations of nanoparticles containing essential oil. Also, particle size, PDI, Zeta potential, Entrapment efficiency were analyzed. The best formulation of Lim-SLNs was released based on the physicochemical properties and finally, the antifungal activity of Citrus Aurantifolia Skin Essential Oil (CASEO) loaded in SLN compared with Clotrimazole and CASEO was investigated using MIC test against Malassezia fungus.

Results: The results of the GC/MS showed that the main component of CASEO included Limonene, α-Terpinene, Trenlene and α-Terpineal. Also, the best formulation of LIM-SLN14 with glycerol monostearate lipid phase had more release than the LIM-SLN10 formulation of stearic acid lipid phase. CASEO loaded in lipid lymphatic nanoparticles at 0.008% tuberosity of CASEO inhibited 90% of *malassezia* furfur. Our results confirmed that CASEO with a guide of SLNs has a good antifungal effect.

Conclusion: These findings are important because frequent usage of antifungal drugs leads to adverse effects; therefore, the antifungal substances extracted from plants are new, more effective, and act as specific solutions for the development of anti-malassezia drugs.

Keywords: SLNs, Citrus aurantifolia, Malassezia

1. Introduction

Tinea or petiasis versicolor is a fungal infection on the surface of the skin caused by various *Malassezia* species [1]. *Malassezia* species are part of the yeast flora of human and other Warm-blooded animals. Lipolytic activity, the accumulation of lipid in the wall, production of hyphae and production of tryptophan dependent pigments are among the most important pathogens of *Malassezia*. The disease affects the surface of the stratum corneum and is characterized by scaling and hyperpigmentation of the affected area discontinuously or continuously [2].

The affected areas are mainly the upper trunk, proximal parts and face. The most important external factors in the occurrence of this disease are high temperature and humidity, and the most important internal factors in the occurrence of this disease are greasy skin, sweating, hereditary factors, corticosteroids and immunodeficiency [3, 4].

Increasing the dose of common drugs and subsequently increasing the bad effects on drugs has led to the greatest attention today to drugs of natural origin such as medicinal plants with less side effects. *Citrus aurantifolia* is from Rutaceae`s family, and its extraction has a profound effect on the health of skin cells, including preventing the appearance of cellular changes in the direction of cancer, as well as delaying the aging of cells. *Citrus Aurantifolia* Skin Essential Oil (CASEO) has anti-parasitic properties on various creams. The pleasant smell of lemon`s essential oil is related to the presence of citral. This essential oil has a significant antibacterial effect on bacteria belonging to the genus *Bacillus*, *staphylococcus* aureus, *Escherichia coli* of *salmonella*, *mycobacterium tuberculosis* species. It has also a wide range of antimicrobial activity against fungi and yeasts. CASEO has a good antiseptic properties to disinfect contaminated water. The effects of this essential oil on a number of bacteria, including penicillin-resistant *staphylococcus aureus*, have already been shown to have anti-inflammatory and anti-viral effects, especially against HSV-1 and HSV-2. As the main component of this essential oil, limonene prevents the growth and reproduction of HIV [5-8].

Solid Lipid Nanoparticles (SLNs) is a new generation of nanosystems used to transmit drugs. This system consists of solid and liquid fats, which, due to its special structure, makes it possible to load different drugs in this nona carrier [3]. The fats used in the structure of these nanoparticles are very suitable for skin rejuvenation and have no toxicity and are very suitable for use in the treatment of skin diseases. The small size of these nanoparticles when used in dermatology is not in direct contact with stratum corneum, which increases the content of skin moisture due to the formation of a thin layer on the skin surface, and then the drug, loaded in these nanoparticles is transferred to the subcutaneous layers. These carriers have been used in many studies in the field of inhaled oral and topical inhaled drug injections [9].

Proper selection of fats, surfactants, their components and their amount can affect particle size, long-term stability during storage, drug loading, and release behaviors, which means that each drug requires a specific formula for SLNs. Problems such as drug loading restrictions, irregular drug release and drug disposal, thus the provision and maintenance of SLNs have limited for their use [10, 11].

In this study, the components in the essential oil of the *citurs aurantifolia* skin were identified by GS/MS. UV-Vis spectroscopy was used to draw a calibration curve; also, for producing formulation for different SLNs, ultrasonic method were used. Pparticle size, Poly Dispersity Index (PDI), Zeta Potential (ZP) and Entrapment Efficiency (EE%) were checked out. The best formulation of nanoparticles was obtained based on the physicochemical properties of the released test, and finally the antifungal activity of CASEO was evaluated in comparison with Clotrimazole and essential oil using MIC test against *Malassezia* fungus.

2. Materials and Methods

2.1 GC/MS Analysis of Citrus Aurantifolia Fruits

For this research, *citrus aurantifolia* fruits were prepared from orchards under the supervision of Jihad-e Agriculture Jiroft (Jiroft city is the capital of Jiroft County, Kerman Province, Iran), and then the initial washing was done, using distilled water twice, without chemical detergent and the

essential oil was prepared by clevanger Apparatus`s skin. The ingredients in CASEO were identified by GC/MS (Agilent Technologies, USA).

2.2 UV-Vis Spectroscopy

To obtain Maximum absorption (λ_{max}), human uptake by ethanol alcohol 99.8% of the initial stock was prepared (100); then, various concentrations including 0.05, 0.1, 0.15, 0.2, 0.25 (ppm) dilution were prepared. Afterwards, uptake optical density (OD) in the range of 200-400 nm was taken by UV-Vis (SHIMADZU, Japan) and the absorption curve, line equation, linearity coefficient and its standard deviation were examined.

2.3 How to Prepare SLNs Containing CASEO

Production of SLNs was done with ultrasonic Homogenizer, for this purpose from lipid phase (Stearic acid, and Glycerol Monostearate) and watery phase (Tween and deionized water)was used and to start the lipid phase 10ºC was heated more than the melting point the lipophilic essential oil was combined with the lipid phase and homogenized. Then the watery phase was added to the lipid phase and the coagulation phase was performed. For the next step, the sonication was performed for 10 minutes (10 seconds steps) at 400 Hz and in the ice water bath. The resulting emulation was stored at refrigerator temperature.

2.4 Surfactant

In this study, two surfactants Tween 80 (hydrophilic), Span 60 and Span 80 (lipophilic) were used. Depending on the concentration of lipid in different formulations, one to five percent was used. The ratio of Tween to Span was 1:2, and surfactant was 75%. After selecting the optinal formulation, the effect of surfactant type of particle size and essential oil loading rate in new particles was evaluated.

Table 1. Formulation CASEO (Lim: Limon Essential oil (CASEO); GMS; Glycerol Monostearate; SA: Stearic Acid; T 80: Tween 80; SP 60: Span 60; SP 80: Span 80**)**

Formula	Lim	SA	GMS	T 80	SP 60	SP 80	Water
Lim-SLN9	0.1	0.4	-	0.5	0.25		98.75
Lim-SLN10	0.1	0.4	-	0.5		0.25	98.75
Lim-SLN11	0.2	0.4	-	0.5	0.25		98.75
Lim-SLN12	0.2	0.4	-	0.5		0.25	98.75
Lim-SLN1	0.3	0.4	-	0.5	0.25	-	98.75
Lim-SLN2	0.3	0.4	-	0.5	-	0.25	98.75
Lim-SLN5	0.4	0.4	-	0.5	0.25		98.75
Lim-SLN6	0.4	0.4	-	0..5		0.25	98.75
Lim-SLN13	0.1	-	0.4	0.5	0.25		98.75
Lim-SLN14	0.1	-	0.4	0.5		0.25	98.75
Lim-SLN7	0.2	-	0.4	0.5	0.25		98.75
Lim-SLN8	0.2	-	0.4	0.5		0.25	98.75
Lim-SLN3	0.3	-	0.4	0.5	0.25	-	98.75
Lim-SLN4	0.3	-	0.4	0.5	-	0.25	98.75
Lim-SLN15	0.4	-	0.4	0.5	0.25		98.75
Lim-SLN16	0.4	-	0.4	0.5		0.25	98.75

2.5 Physical and Chemical Characteristics Determination of Particle Size, Scattering Index and Zeta Potential

Zetasizer Nano ZS (Malvern UK) device was used to measure particle size, PDI, and zeta potential (ZP). For this purpose, the samples were poured in a special cell and examined at a constant angle of 90 degrees at 25ºC degrees temperature.

2.6 Determining the Percentage of Essential Oil

The percentage of CASEO loaded was completed according to the following formula. The amount of unloaded free

essential oil was assessed using the centrifugation process. For this purpose, the samples were poured into Eppendorf 2 ml refrigerated centrifuge for 2 minutes at 18000 g in 4°C temperature. The supernatant was then removed from the deposited nanoparticles and filtered with a 45 nm and its absorption was read as spectrophotometers at 267 nm. EE% amount was calculated using the following formula. m_e: the amount of drug confined; m_0: primary drug level.

$$EE(\%) = \frac{me}{m0} \times 100$$

2.7 pH Measurement

The formulation was measured by a digital pH meter (SANA SL-901, Iran) at 25 °C.

2.8 Investigation of SLNs Morphology by TEM

SLNs containing one to ten essential oils were diluted, using deionized water. Then, the sample was placed on a copper grid to form a thin layer. Then two drops of Phosphotungstic Acid (PTA) 2% were added. After a minute, the extra filters were removed from the copper grade by a paper filter to create a thin layer for imaging. The sample was then allowed to dry at 40°C. Then the morphology of the nanoparticles was observed by TEM (PHILIPS CM30 300Kv) in different magnifications.

2.9 Determining the Amount of Essential Oil Release

To calculate the release of essential oil from nanoparticles, the membrane diffusion technique was performed. An analysis bag measuring 12000 daltons was prepared for 24 hours before the test in deionized water. In order to observe the effect of PH of the release environment on the release rate of essential oil, Lim-SLN14 and Lim-SLN10 formulations were released in two environments with different release conditions. The initial release environment was simulated according to the skin conditions, so that Bafrastant was prepared with PH: 5.1 and the test temperature was kept at 5 degrees. The second release environment was considered with plasma condition as it was prepared with phosphate buffer with pH: 7.4 at 37°C ±0.5 temperature. Then to observe the lipid effect used in the release of the Lim-SLN14 formulation, which was released from GMS fat phase in the phosphate buffer environment, the release rate was determined by Lim-SLN 10 formulation, the lipid phase of which was stearic acid and tested in the same condition.

2.10 Lim-SLN Release Method

The dissolution device was used to perform the amount of essential oil release by Lim-SLNs. In this section, the temperature was 37ºC, RPM:60 and 5 cc of the synthesized solution containing the essential oil were inside each of the dialysis bags. Then they were checked at 0.5,1,2,4,6,24 hours. After copulation, the samples were diluted to a ratio of 1:1 and the absorption was recorded by spectrophotometry. The formula is used to calculate the actual concentration.

$$\mathbf{Ct_n = c_n + \frac{v}{V} \sum_{t=1}^{n-1} (Ct_t)}$$

- **St_n:** real concentration at any sampling time
- **C_n:** concentration at any time obtained from the calibration curve (appearance concentration)
- **V:** the total volume of the receiving phase, which in our study was equal to 50 cc
- **v:** the volume harvested during sampling (equal to 4 cc)
- **Ct_t:** the actual concentration calculated in the previous sampling

The release rate of CASEO loaded in SLNs was studied in In-vitro and phosphate buffer at pH:7.4, pH: 5.1 measurements of CASEO release rate up to 24 hours was performed for both selected formulations, using dialysis bags.

2.11 Micro-Dilution Test to Determine MIC

In the last section, 100 ml was added to the wells of 96 well Microtiter plates. Ten 100 µl of herbal essential oil with a concentration of 1 µg/ml was added to each of the fungal plates in the first row. Dilution was performed. One percent of Clitromazole was used for positive control. After the addition of the fungal suspension for 24 hours, incubation was performed at 37ºC. Respiratory activity was determined by changing the color from blue to pink after adding 20 µl of purest blue to the well and incubated at 37ºC for 30 minutes.

3. Results

According to GC/MS analysis, 28 combinations were identified in CASEO, shown in table 2. The main consistent essential oils are Limonene (49.07 %), Ternolene (14.78%) α-Terpinene (11.29%), α-Terpineol (7.44 %), and 1,8– Cineole (4.12 %).

Table 2. Identify the percentage of components in the essential of citrus aurantifolia by GC/MS.

No.	Compounds	RI[a]	GC area (%)
1	2-Methyl-3-buten-2-ol	624	**0.05**
2	(Z)-Salvene	847	**0.17**
3	Ethyl isovalerate	849	**0.11**
4	Tricyclene	921	**0.17**
5	α-Pinene	932	**0.58**
6	Camphene	946	**2.53**
7	Thuja-2,4(10)-diene	953	**0.52**
8	Sabinene	969	**0.48**
9	β-Pinene	974	**1.12**
10	Yomogi alcohol	999	**0.82**
11	Limonene	1019	**49.07**
12	p-Cymene	1020	**1.32**
13	1,8-Cineole	1026	**4.12**
14	Santolina alcohol	1034	**0.41**
15	cis-Arbusculone	1046	**0.19**
16	α-Terpinene	1049	**11.29**
17	γ-Terpinene	1054	**0.23**
18	Ternolene	1078	**14.78**
19	Artemisia alcohol	1080	**0.32**
20	Chrysanthenone	1124	**0.37**
21	trans-p-Menth-2-en-1-ol	1136	**0.35**
22	Sabina ketone	1154	**0.32**
23	Pinocarvone	1160	**0.11**
24	α-Terpineol	1171	**7.44**
25	n-Decanol	1266	**0.18**
26	Bornyl acetate	1284	**0.59**
27	trans-Sabinyl acetate	1289	**0.35**
28	Carvacrol	1298	**0.23**
Total			**98.22**

(Retention Index: RI)

3.1 UV-Vis Spectroscopy

According to the spectra obtained from the spectrophotometer, the maximum absorption of CASEO is achieved at a wavelength of 267 nm. The concentrations used, the mean absorption and the standard deviation for the calibration curve are given in table 3. Figure 1 shows the calibration curve. According to the line equation and the correction coefficient obtained, the linearity of the essential oil absorption of the wavelength of 267 was determined.

Table 3. Concentration of CASEO, their proportional absorbance, average absorbance and standard deviation for drawing standard curves

Concentration CASEO (mg/ml)	A_1	A_2	A_3	A_{verage}	SD
0.05	0.264	0.363	0.294	0.306	**0.0507**
0.1	0.572	0.758	0.646	0.658	**0.0936**
0.15	0.919	1.152	1.018	1.023	**0.1169**
0.2	1.244	1.503	1.49	1.379	**0.1459**
0.25	1.608	1.893	1.75	1.75	**0.1425**

A: Absorbance SD: Standard Deviation

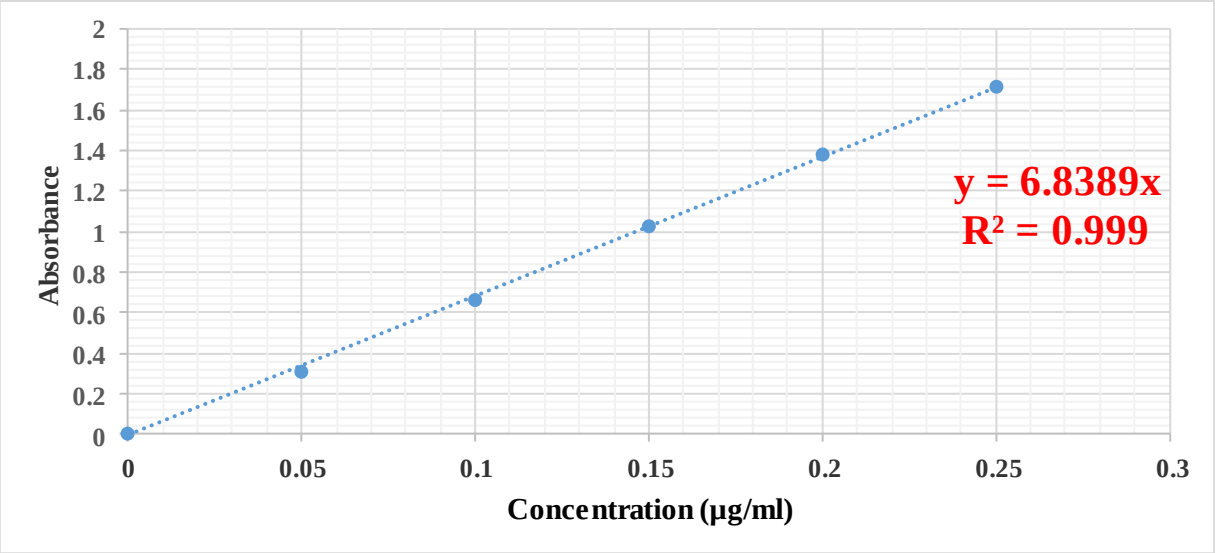

Figure 1: The standard curve of CASEO in 267 nm wavelength.

3.2 Physicochemical Properties

3.2.1 Particle Size

Figure 2 shows the results of the SLNs particle size test containing CASEO with different Lim-SLNs formulations based on the lipid phase. As can be seen, there is no change in the concentration of the loaded essential oil from 0.1% to 0.2% in relation to the particle size of the prepared nanoparticles; however, when the concentrator increases to 0.3% to 0.4%, the particle size of SLNs containing CASEO increases. The highest particle size was observed in Lim-SLNs formulation, with Stearic acid as a lipid phase, Span 60 as a surfactant and concentration 0.4% of citrus aurantifolia skin essential oil. The smallest particle size was observed in Lim-SLN14 formulation in which Glycerol Monostearate was used as the lipid phase, Span 80 as a surfactant and 0.1% concentration Limonene was used. As can be seen in the diagram above, when Span 80 is used in the formulation, the particle size of the nanoparticles decreases compared to Span 60. On the other hand, the change in lipid phase from stearic acid to Glycerol Monostearate led to a reduction in the particle size of the lipid nanoparticles containing sour lemon essential oil.

3.2.2 Poly Dispersity Index (PDI)

Figure 2 shows the results of the scattering index containing CASEO with different formulations based on the lipid phase. The numerical scattering index is between zero and one, and the closer to one, the more scattered the particles, which is not desirable. In these formulations, the highest dispersion index of nanoparticles was 0.546 reported. Therefore, all formulations had an acceptable dispersion index.

3.2.3 Zeta-Potential (ZP)

Figure 2 shows the results of SLNs zeta potential analysis containing CASEO with different formulations based on lipid phase. As can be seen in the diagram, most of the prepared formulations had a zeta potential

in the range of -7 to -16 mv, indicating an acceptable range for zeta potential. The highest potential was observed in Lim-SLN16 formulations and the lowest zeta potential were observed in Lim-SLN8 and Lim-SLN16 formulations. As can be seen in the diagram, the application of Stearic acid lipid phase caused more zeta potential compared to Glycerol Monostearate.

3.3 Entrapment Efficiency (EE %)

The results of a loading test containing CASEO with different formulations based on the lipid phase are shown in figure 2. As can be seen, most of the prepared formulations had very good essential oil loading efficiency and the highest amount of essential oil loading was observed in Lim-SLN11 formulations. However, Lim-SLN4 formulation with Glycerol Monostearate, surfactant 80 and Tween 80 lipid phases had the lowest essential oil loaded. According to the result, Lim-SLN14 and Lim-SLN10 were selected as the best formulations.

The formulations made had pH 5.6-6.4 and the pH is within the acceptable range of topical use.

3.4 Lim-SLN Morphology Study

The TEM images are shown in figure 3. These images showed that the prepared nanoparticles were spherical.

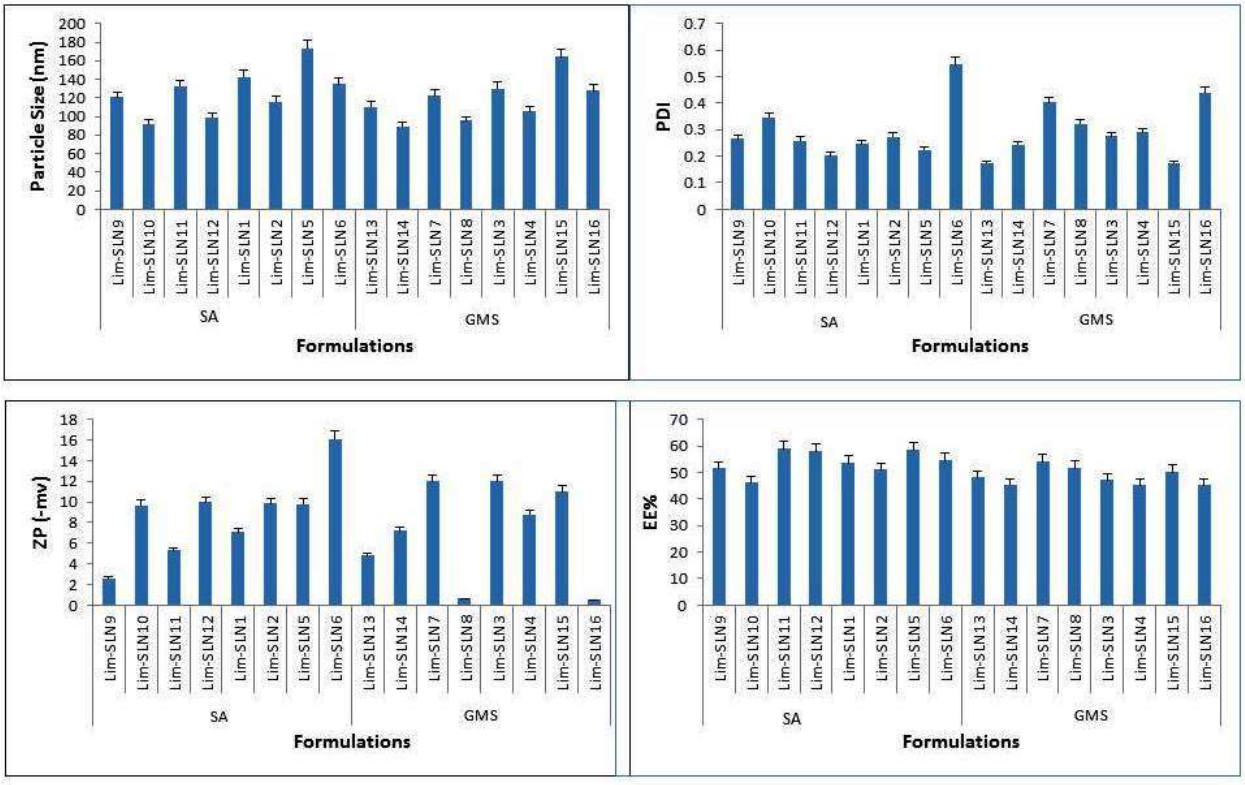

Figure 2. Particle size of different formulations prepared according to lipid phase Measurement of pH of Lim-SLNs formulations

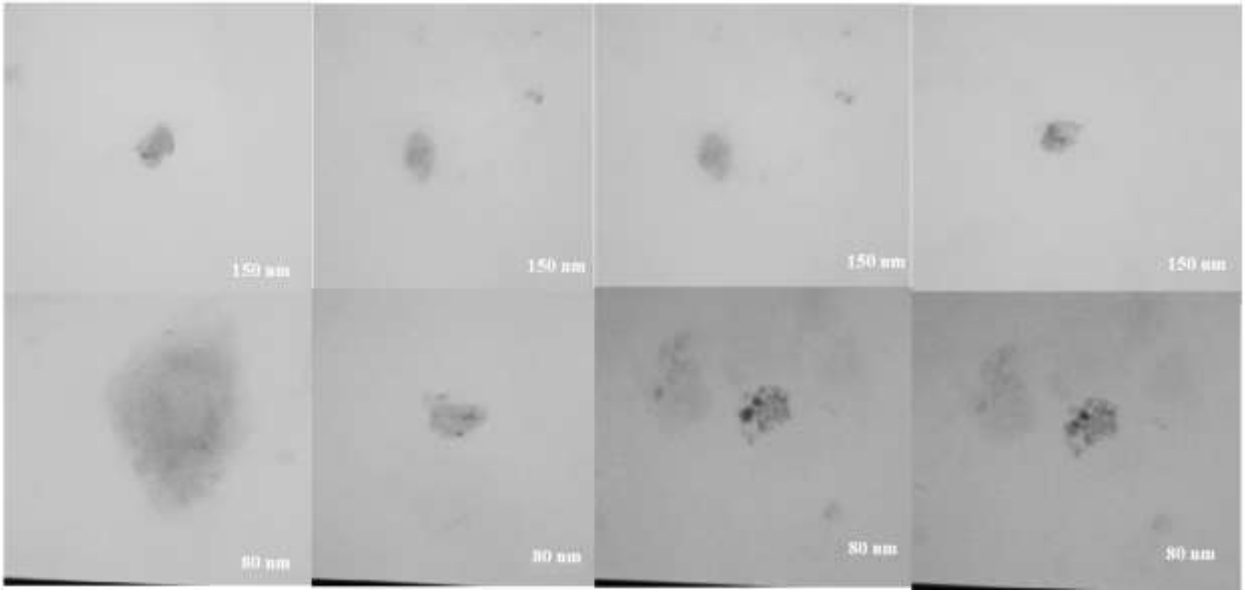

Figure 3. Transition Electron Microscope (TEM) images of it containing sour lemon skin essential oil.

3.5 Evaluation of Citrus Skin Essential Oil Release on In-Vitro Conditions

To obtain the release of essential oil, Lim-SLN14 and Lim-SLN10 formulations were used which was optimal in terms of particle size and loading term. Table 3 shows the results of this test, their means and standard deviations. According to the analysis of the results and the graph of the two drawings, it was observed that the formulation of Lim-SLN14 with lipid phase of Glycerol Monostearate has been released more than the formulation of Lim-SLN10 with lipid phase of SA. The formulation containing the fat phase on the first two hours released 38% of the essential oil and in 24 hours 83% of the essential oil were released. However in Lim-SLN10 formulation in the first two hours, 39% and in 24 hours, 80% of the essential oil was released. T_{25} (the time that was required to release 25% of essential) for GMS–SLNs and SA–SLNs formulations were approximately one hour and T_{50} (the time that was required for release of 50% of essential) for both formulations were approximately 4 hours. It was found that the release of essential oil is less than the formulations ($P < 0.05$); therefore, the lipid nanoparticles increased the release of essential compared to pure essential. The results showed that pH did not have a significant effect on the essential oil of of CASEO release, loaded in SLNs.

Table 4. Percentage of cumulative release at different time points

pH:7.1							
Formulation	**Time (hr)**						
	0	**0.5**	**1**	**2**	**4**	**6**	**24**
Control	0	8.12±0.31	22.56±0.35	34.26±0.07	46.31±0.57	60.02±1.41	72.87±1.20
SA-SLNs	0	8.70±0.1	25.31±0.42	38.71±1.84	51.11±1.41	64.67±1.34	80.55±0.85
GMS-SLNs	0	8.51±0.35	24.27±0.49	38.01±0.87	51.95±2.49	67.25±4.67	83.05±6.85

pH:5.1							
Formulation	**Time (hr)**						
	0	**0.5**	**1**	**2**	**4**	**6**	**24**
Control	0	8.78±0.18	21.13±0.12	35.15±0.06	47.20±0.31	61.5±2.45	71.25±1.03
SA-SLNs	0	8.16±0.22	26.01±0.31	39.12±1.22	52.01±0.98	65.01±1.56	86.15±0.06
GMS-SLNs	0	8.24±0.12	24.12±0.31	38.89±0.48	52.12±0.22	66.83±3.28	82.44±4.22

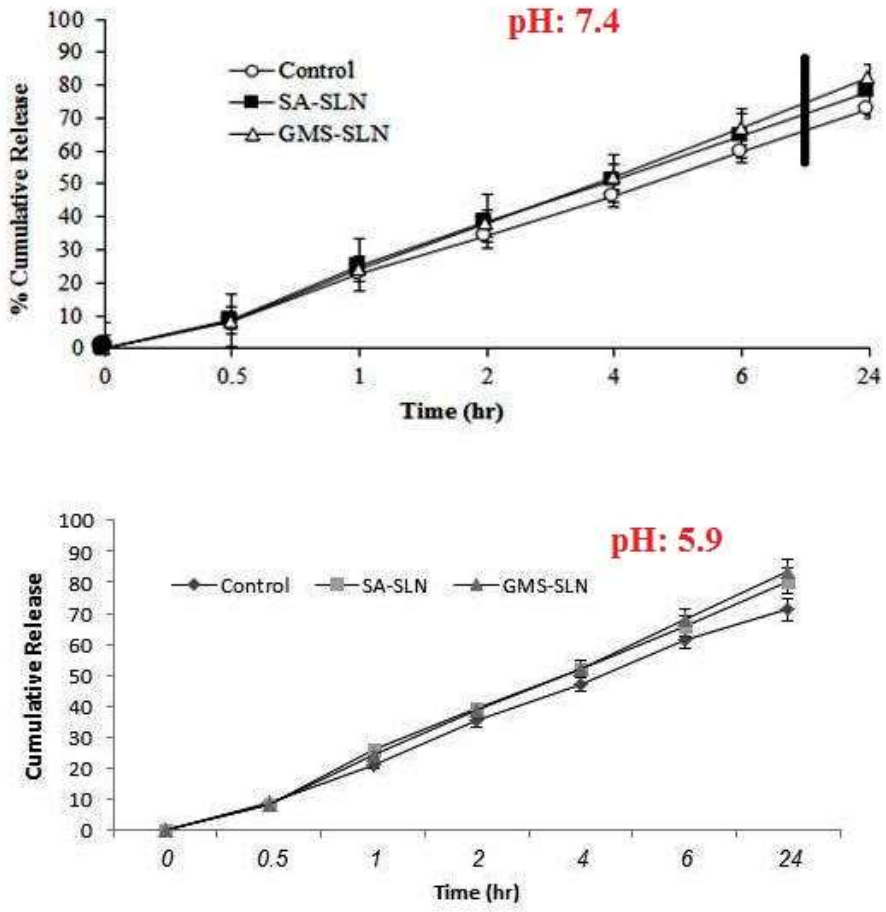

Figure 4. comparison of control formulations, SA-SLNs, GMS-SLNs in pH: 5.9 and pH: 7.4

Determination the minimum concentration of essential oil surrounding the skin a *Malassazia furfur* the results of the current study showed that the essential oil of the Citrus aurantifolia skin of the site loaded in Lim-GMS-SLN10 in the concentration of 0.008% of lemon essential oil caused the inhibition of 9% of the *Malassezia Furfur*. The minimum inhibitory concentration was 0.8 % for Clitromazole and 0.0625 % for lemongrass essential oil. These results indicate the very high effect of SLNs containing CASEO on *Malassezia* fungus, which in lower concentrations compared to CASEO and Clitromazole drug has inhibited this fungus in extracorporeal conditions. The results are shown in table 4. Hence, CASEO has good antifungal activity.

Table 5. CASEO antifungal activity against Malassezia furfur, using MIC test.

Isolates	Lim (%)		Lim & SA-SLNs%		Lim & GMS-SLNs %	
	MIC	MIC90	MIC	MIC90	MIC	MIC90
No1	0.125		1		0.008	
No2	0.0625		0.25		0.004	
No3	0.0625	0.0625	0.5	0.8	0.004	0.008
No4	0.0625		0.5		0.008	
No5	0.125		0.25		0.008	

Lim: Limon Essential oil (CASEO); GMS; Glycerol Monostearate; SA: Stearic Acid;

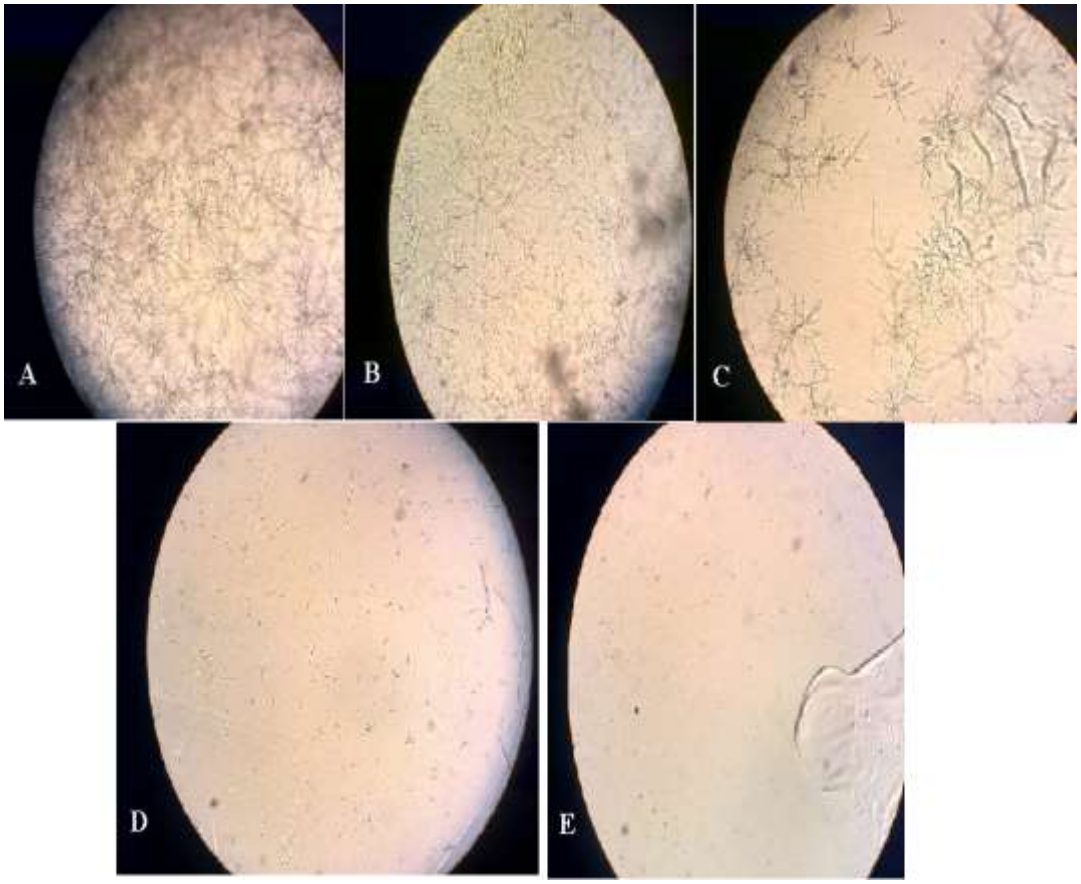

Figure 5. Antifungal activity of Citrus aurantifolia essential oil against Malassezia fungus using MIC test concentrations of CASEO increases. The concentration of the drug is increasing from A to E (A:0.05, B: 0.1, C:0.15, D:0.2 and E:0.25), and at higher concentrations (E and D) it is clear that Mallasezia fungus is inhibited.

4. Discussion

The results of the current study indicated that the particle size decreases with increasing surfactant concentration than essential oil and lipid concentration. Creating smaller nanoparticles due to higher surfactant concentrations can be attributed to reduced surface tension between the aqueous phase and the lipid phase. The results of the current study are confirmed in the study conducted in this field. The results of Abdelbary and et al study showed a decrease in particle size with increasing surfactant concentration. Previous studies have also shown that increasing the concentration of surfactants can be considered as a barrier to the surfactant of nanoparticles that prevents the binding of nanoparticles to each other and increases the stability of formations [12]. Nanoparticles made with Span 80 had fewer particle size than nanoparticles made with Span 60. This result is in line with the results of a study by Pinto et al, who said that span 80 is more saturated with high levels of unsaturated fatty acids than Span 60 with high levels of saturated fatty acids [13].

The results of the current study showed by changing the type of fat phase from Glycerol Monostearate to Stearic acid, the particle size and viscosity of the formulations increased. The structure of Stearic acid consists of 18 Carbon chain, thus has a higher melting point than Glycerol Monostearates. In addition, it has been reported that Glycerol Monostearate not only plays a role as a fat phase in the preparation of solid lipid nanoparticles but also as a surfactant in intensifying the process of emulsion formation. Therefore, it can be said that glycerol mono-stearate has the capacity to create smaller nanoparticles. This conclusion has been reported in

Abdelbary et al, research [14]. Motawea et al, reported particle size reduction by changing the type of lipid from competitor to pressure, and used it to lower the melting point of the fat and attribute it to less viscosity [15]. In the present study, the smallest particle size is obtained when using glycerol mono-stearate lipid and span 80 surfactants, which is in line with a result of the above studies.

The results of the present study showed that increasing the concentration of CASEO from 1% to 2% does not cause significant changes in the particle size of nanoparticles. This result is consistent with studies conducted in this field. In the study by Gomes et al, it was reported that nanoparticles lipid carriers containing finasteride and minoxidil did not significantly change the size of nanoparticle size [16]. However, with increasing the concentration of essential oil to 4%, the particle size has increased. It has also been reported that the drug has no effect on particle size, but when higher concentration is used, it can cause an increase in size [13, 14]. Therefore, as our study showed, the application of two percent concentration of Citrus aurantifolia skin essential oil had not affected the particle size but if more essential oil concentrations are used, it can most likely lead to an increase in the size of the nanoparticles.

Factors affecting drug loading include the type and amount of lipid phase, the solubility of the drug in the lipid phase, the type and amount of surfactant used, and the active ingredient [17]. In the present study, it was observed that the formulations prepared with Span 60 surfactant had a higher loading percentage compared to the formulations prepared with Span 80. Downloading this can be attributed to the solid structure of Span 60 due to cooling in the refrigerator temperature during the formulation of nanoparticles but as mentioned, Span 80 had a lot of unsaturated fatty acids in its structure and this makes it liquid and therefore does not have the same capacity as Span 60 [18].

The results of the current study showed that the rate of release of CASEO from nanoparticles was higher than single essential oil. This is attributed to the larger particle size of the active ingredient compared to the nanoparticle formulation as well as the higher level of nanoparticles, and these factors are more effective in dissolving and releasing more essential oils [19]. In the first four hours, about 40% of the essential oil was released and then the release process was done. The initial release indicates the loading of the essential oil at the nanoparticle level, and the release control indicates the loading of the essential oil at the center of the lipid matrix of the nanoparticles [20]. The results of this part of the study showed that nanoparticles with Glycerol Monostearate lipid phase had faster release of essential oil compared to stearic acid lipid phase. Nanoparticles made with glycerol stearate lipid phase were smaller in particle size. Decreasing the particle size and subsequently increasing the level created are both factors that can increase the amount of the release.

The anti-mallesezia activity of CASEO observed in this study can be attributed to the components of Limonene, α-Terpineol and 1.8-Cineole in CASEO. Limonene was half of the essential oil components in the most important component of CASEO. Limonene antifungal activity has been observed in other studies [21]. Limonene belongs to the Triterpenes, the largest natural product involved in a variety of functions such as defense against pathogens and predators, as well as a signal to harmless organisms as pollinators for plants [22]. One of the anti-fungal mechanism of Limonene has been reported to induce apoptosis, and it has been shown that Limonene affects the signaling pathway and cell membrane [23]. In this way, it prevents the formation of fungal hypha, which is an important pathogenic cause of fungs [24].

Inhibition of hypha formation which is induced by limonene at low concentrations, could be responsible for inhibiting morphogenesis. Limonene also inhibits the growth of yeast fungi by damaging cell walls and plasma membranes. Therefore, the antifungal effects observed in the present study can be attributed to the combination of Limonene in the essential oil of this plant. Studies found that 1.8-Cineole had a lower inhibitory and fungal concentration than Nystatin. Also, anti-aflatoxin activity has been shown this component. The mechanism of antifungal activity of 1.8-Cineole has not been reported yet. Therefore CASEO has antifungal activity against *Malassezia* [25-27].

However, many ingredients in essential oil such as Gamma-terpinene and Terpineol in addition to Limonene have antifungal activity and can affect the antifungal activity of the fruit. This has been shown in many studies and the results of the current study are in line with the researches conducted in this filed [28, 29].

5. Conclusion

The inhibitory activity of CASEO loaded in lipid particles was similar to that of Clitromazole 8%. This finding is important because repeated usage of antifungal drugs leads to adverse effects such as hepatitis and skin adhesions. Therefore the antifungal substances extracted from plants are of great importance for the development of new, effective and specific anti-material drugs. In fact, this antifungal activity of sour lemon essential oil can be attributed to the presence of components such as Limonene, Gamma-Tripeonle and Tipinollen. However, other ingredients in the lemon essential oil can affect the antifungal activity. The antagonistic effects of the compounds in the essential oil of this plant should also be considered.

Conflict of interest

The authors declare no conflict of interest.

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