Journal of Research in Pharmacy

Research Article

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Formulation of a natural nanosystem based on β-cyclodextrin/arginine/xanthan to increase antifungal activity of *Salvia officinalis* essential oil from Algeria (Bejaïa, Kalaa n'Ath Abas)

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Received: 8 September 2021 / Revised: 27 January 2022 / Accepted: 28 January 2022

Abstract: The aim of this work is to study for the first time the chemical composition of *Salvia officinalis* essential oil from Algerian region of Kabylia (Kalaa n'Ath Abas) and to evaluate its antifungal activity. The originality of this work consists in the development of a new nanosystem based on natural excipients (β-cyclodextrin, xanthan gum and arginine) to increase its antifungal activity. The yield of *Salvia officinalis* essential oil was 2.07%, it is mainly composed of α-Thujone (23.21%), 1,8-Cineole (14.17%) and Camphor (11.02%). Nanoemulsion based on β-cyxlodextrins has an average diameter of 219.1±5.2 nm, a zeta potential of 36.2±4.8 mV and a viscosity of 0.87±0.03 Pa.s. This essential oil showed a good antifungal activity against all tested strains (*Candida albicans*, *Microsporum canis* and *Trichophyton equinum*). Moreover, a significant increase of this activity was noted after essential oil nano-emulsification. The easy penetration of nanodroplets composing the prepared system through fungal cell wall and their high solubility in fungal cell cytoplasm permit to increase antifungal activity of studied essential oil.

KEYWORDS: *Salvia officinalis*, essential oil, chemical composition, antifungal activity, cyclodextrins, fungicidal effect enhancement.

1. INTRODUCTION

Medicinal plants contain an invaluable number of molecules with therapeutic effects capable of treating and preventing many existing diseases. Many of these plants are daily used in our food in the preparation of herbal teas [1], drinks [2] or gourmet meals. Among these plants we find sage (*Salvia officinalis*).

Salvia officinalis is a native mediterranean plant belonging to the Lamiaceae family, it is cultivated all over the world because of its important economic interest due to its wide use in food industries, cosmetics and pharmaceuticals. Sage is considered as an aromatic plant because its leaves contain an essential oil [3], the latter is mainly composed of 1,8-cineole, α-thujone and Camphor [3–8], other compounds may also be present

Nait Bachir Y, Sahraoui N, Medjkane M, Hadj Ziane A. Formulation of a Natural Nanosystem based on β-cyclodextrin/ Arginine/ Xanthan to Increase Antifungal Activity of Salvia officinalis Essential Oil from Algeria (Bejaïa, Kalaa n'Ath Abas). J Res Pharm. 2022; 26(3): 581-597.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.156

with large amounts such as β-thujone [9,10], a-humulene [11] or borneol [12]. Several biological activities of this essential oil have been studied, mainly antimicrobial activity [7,9,13,14], antifungal activity [15–21], antioxidant activity [22], antimutagenic activity [6] and anti-inflammatory activity [8].

Cyclodextrins are natural polysaccharides considered as caged molecules composed of a hydrophilic surface and a hydrophobic cavity, their cavities can encapsulate hydrophobic molecules and form inclusion complexes with them [23]. The reason behind the interest in hydrophobic molecules encapsulation in the cavities of cyclodextrins is to increase their physicochemical stability, their resistance to environmental conditions, and mainly to increase of their biological activity [23,24]. Indeed, the formation of the inclusion complex increases the solubility of encapsulated molecule to become more soluble in aqueous biological media. Hence, increasing the therapeutic effects of encapsulated molecules [25].

The aim of this work is to study the effect of *Salvia officinalis* essential oil encapsulation in β-cyclodextrin on its antifungal activity. The work will be carried out in three main steps: the first step is extraction of *Salvia officinalis* essential oil and determination of its chemical composition by gas chromatography coupled with mass spectrometry, the second step is formulation of a nanoemulsion based on β-cyclodextrin-essential oil inclusion complex and its characterization by laser particle size, zetometry and viscosimetry, the third and final step is the study of antifungal activity of *Salvia officinalis* essential oil before and after its formulation by two methods (the disc method and the determination of MIC/MFC) on three pathogenic germs: *Microsporum canis, Trichophyton equinum and Candida albicans.*

2. RESULTS

2.1. Extraction and chemical composition of essential oil

The extraction yield is 2.07%. Table 1 shows the chemical composition of *Salvia officinalis* essential oil. This analysis allowed the identification and quantification of 48 compounds that correspond to 97.27% of the obtained oil. The main compounds of this essential oil are: α-thujone (23.21%), 1,8-cineole (14.17%) and camphor (11.02%).

Table 1: Chemical composition of *Salvia officinalis* essential oil.

N°	Compounds[a]	%	IR[b]
1	Tricyclene	0.71	927
2	α-Thujene	0.94	935
3	α-Pinene	4.67	939
4	Camphene	7.01	954
5	β-Pinene	5.03	979
6	β-Myrcene	2.59	991
7	α-Phellandrene	2.01	1003

http://dx.doi.org/10.29228/jrp.156

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8	δ-3-Carene	t	1011
9	Limonene	1.6	1029
10	**1.8-Cineol**	**14.17**	**1022**
11	(Z)-β-Ocimene	0.46	1037
12	(E)-β-Ocimene	0.05	1049
13	γ-Terpinene	3.04	1060
14	p-mentha-3,8-diene	t	1072
15	2-Nonanone	0.49	1079
16	α-Terpinolene	0.23	1089
17	**α-Thujone**	**23.21**	**1101**
18	β-Thujone	8.62	1112
19	**Camphor**	**11.02**	**1121**
20	Borneol	0.47	1196
21	Bornyl acetate	0.69	1285
22	2-Undecanone	0.63	1293
23	α-Cubebene	t	1340
24	γ-Pyronene	0.04	1345
25	Ylangene	0.09	1371
26	α-Copaene	0.01	1377
27	β-Elemene	0.57	1391

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28	Benzyl isovalerate	0.02	1395
29	β-Caryophyllene	1.42	1421
30	Aromadendrene	t	1441
31	α-Amorphene	0.51	1442
32	Geranyl acetone	0.18	1455
33	α-Humulene	1.04	1457
34	Germacrene-D	0.09	1480
35	Valencene	0.59	1482
36	α-Muurolene	0.62	1499
37	(E.E)-α-Farnesene	0.86	1507
38	δ-Cadinene	0.19	1523
39	Cadina-1.4-diene	0.25	1532
40	α-Cadinene	0.23	1539
41	α-Calacorene	0.3	1546
42	Nerolidol	0.21	1561
43	Caryophyllene oxide	0.54	1583
44	T-Muurolol	0.62	1642
45	β-Eudesmol	0.34	1651
46	α-Cadinol	0.78	1654
47	Benzyl Benzoate	t	1753

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48	Hexahydrofarnesyl acetone	0.13	1844
	Total of identified compounds	**97.27**	
	Unidentified	2.73	

[a]: Retention index in relation to the n-alkane serie (C8-C24) calculated on non-polar capillary column type HP5MS.

[b]: Nomenclature of identified compounds in essential oil.

2.2. Preparation and characterization of nanoemulsion

The prepared nanoemulsion has a clear appearance and an aromatic odour similar to that of Salvia officinalis. Characteristics of the prepared nanoemulsion are given in Table 2.

Table 2: Characteristics of nanoemulsion.

Physicochemical characteristics	Values
Essential oil content	19.82 ±0.16 %
Average diameter	219.1 ±5.2 nm
Polydispersity index	0.52±0.08
Zeta potential	36.2 ±4.8 mV
Viscosity	0.87 ± 0.03 Pa. s

Figure 1 (A) represents the particle size distribution of nanoemulsion obtained by DLS analysis, particle size study showed that the nanoemulsion has a single particle size, an average diameter of 219.1 ±5.2 nm and a polydispersity index of 0.52 ±0.08.

Figure 1 (B) represents the intensity of the electric cloud on nanodroplets surface contained in the formulation that was measured by zeta meter.

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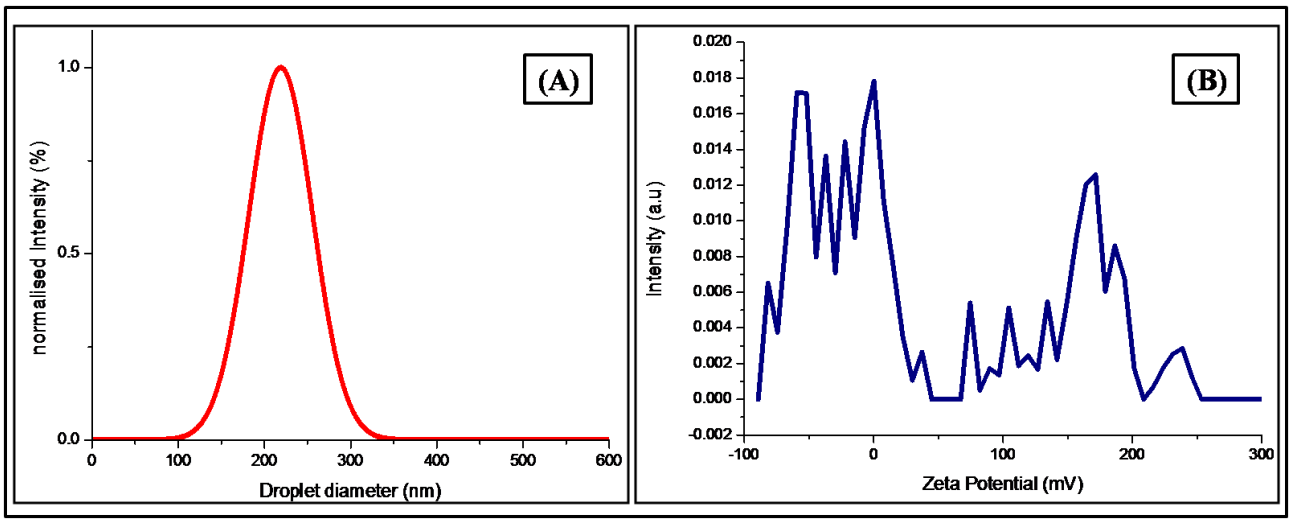

Figure 1: Granulometric distribution (A) and Zeta potential (B) of prepared nanoemulsion.

Figure 2 shows a microphotograph of the nanoemulsion obtained by transmission electron microscopy. The nanodroplets observed under microscope have a variety of geometric shapes (oval, polygonal, etc.), the edges of droplets are irregular, but their dispersion in the continuous phase is uniform. The size of nanodroplets obtained is variable but remains around 200 nm.

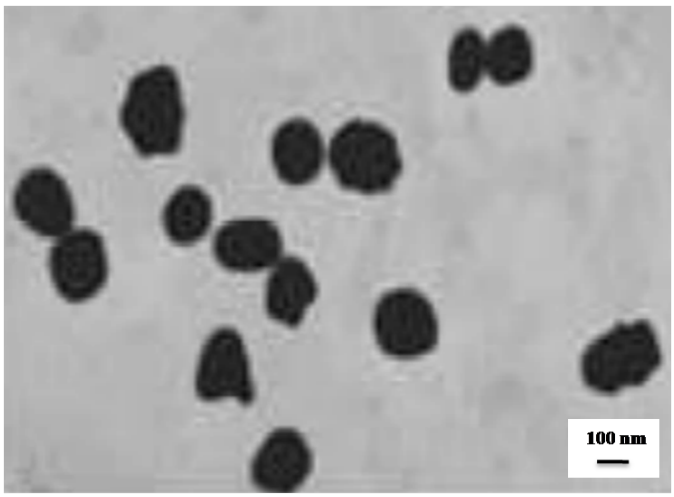

Figure 2: TEM microphotography of prepared nanoemulsion.

2.3. Evaluation of antifungal activity

Antifungal activity was evaluated using two different methods; the diffusion method on agar from discs and the determination of minimum inhibitory concentration and minimum fungicide concentration (MIC/MFC).

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In this step, we compared essential oil, nanoemulsion and placebo (nanoemulsion without essential oil was replaced by physiological water) the purpose of using placebo is to verify that other components of the formulation have no effect on the fungal strains used.

Antifungal activity was performed on three different strain. Figure 3 represents the results of antifungal activity on *Candida albicans* strain. Figure 3 (A) shows a photograph of petrie dish showing the inhibition zones for essential oil, nanoemulsion and placebo against fungal strain *Candida albicans*. Figure 3 (B) shows a photograph of agar tubes showing MIC for nanoemulsion against fungal strain *Candida albicans*.

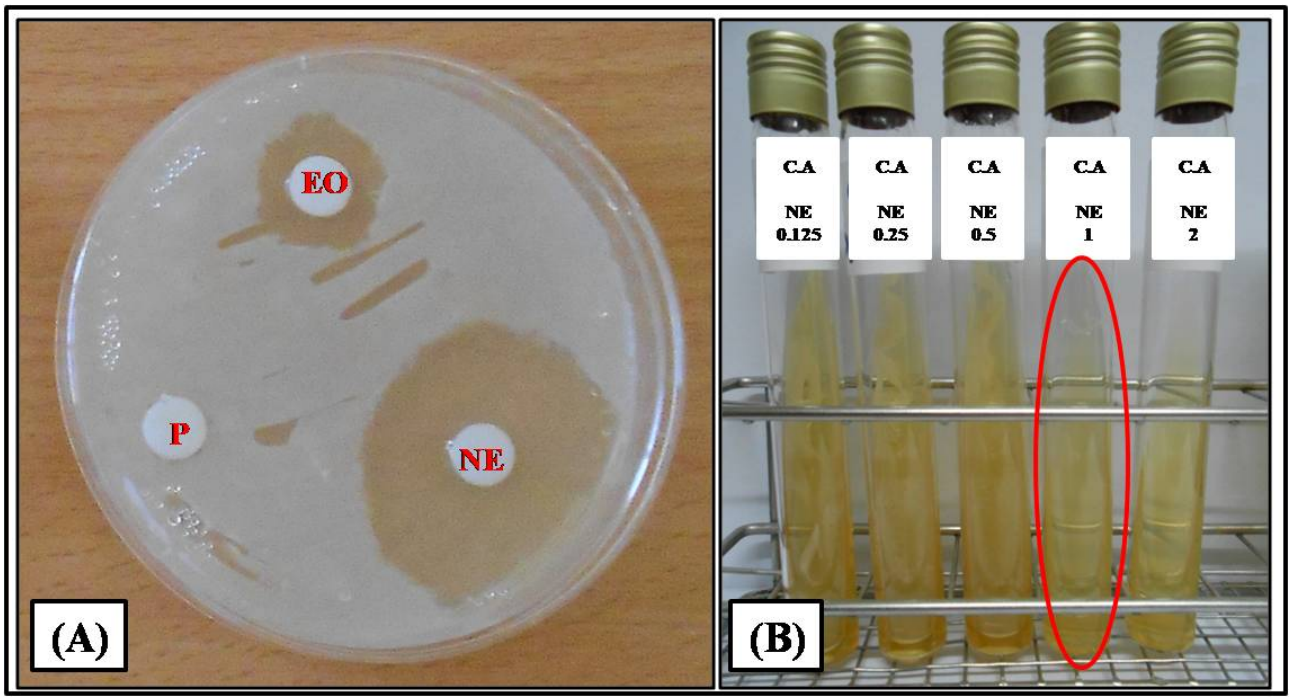

Figure 3: Results of antifingic activity. (A) photograph of petrie dish showing the inhibition zones for essential oil, nanoemulsion and placebo against fungal strain *Candida albicans*. (B) photograph of agar tubes showing MIC for nanoemulsion against fungal strain *Candida albicans*. (NE) nanoemulsion, (EO) essential oil and (P) placebo.

2.3.1. Diffusion method on agar from discs

The results of antifungal activity by agar diffusion method using the 9mm discs are given in Table 3.

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Table 3: Antifungal activity of *Salvia officinalis* essential oil and nanoemulsion using agar diffusion method from discs.

Microorganisms	Diameters of the inhibition zone (mm)			
	Placebo	Essential oil	Nanoemulsion	Effect of the formulation
M. canis	0±0.0	43±0.0(*)	68±1.4(*,#)	**+58.14%**
T. equinum	0±0.0	29±2.1(*)	37±1.4(*,#)	**+27.59%**
C. albicans	0±0.0	19±0.0(*)	31±3.5(*,#)	**+63.16%**

* : Statistically highly significant difference between placebo and other groups ($p < 0.001$).

: Statistically significant difference between essential oil and nanoemulsion ($p < 0.005$).

All tested strains were susceptible to *Salvia officinalis* essential oil. Generally, antifungal activity of essential oil increases after its formulation, for studied concentration (10µl of essential oil) the diameters of inhibitions increase significantly ($p < 0.005$) and this for: M. canis from 43±0.0mm to 68±1.4mm, T. equinum from 29±2.1mm to 37±1.4mm and C. albicans from 19±0.0mm to 31±3.5mm.

2.3.2. Determination of minimum inhibitory concentration and minimum fungicide concentration (MIC/MFC)

The results of antifungal activity (MIC/MFC) of *Salvia officinalis* essential oil and nanoemulsion are given in Table 4. We observe that MIC and MFC decrease after nanoemulsification of essential oil with the exception of *Salvia officinalis* essential oil MIC which doesn't change and remains constant at the value of 0.125µl/ml after its nanoemulsification.

Table 4: Minimum inhibitory concentrations and minimum fungicide concentrations (MIC/MFC) of *Salvia officinalis* essential oil and nanoemulsion.

Microorganisms	MIC (µl/ml)			MFC (µl/ml)		
	Essential oil	Nanoemulsion	Effect of the formulation	Essential oil	Nanoemulsion	Effect of the formulation
M. canis	0.125	0.125	**0%**	0.5	0.125	**-75%**
T. equinum	0.5	0.25	**-50%**	1	0.5	**-50%**
C. albicans	2	1	**-50%**	1	0.5	**-50%**

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3. DISCUSSION

The study of *Salvia officinalis* essential oil chemical composition in Algeria is limited to three studies carried out in: central Algeria (Algiers [3]), Algeria's east (Batna [26]) and El-Kala [27], while in the other regions it was never studied. In this study, we revealed for the first time sage essential oil chemical composition of the Kabylian region (Kalaa n'Ath Abas), the majority compounds of this essential oil are α-thujone, 1,8-cineole and Camphor. The results obtained in our work agree with those obtained in Algeria [3,26,27], Serbia [6], Montenegro [7] and Albania [8]. However, some studies show other majority compounds additional to those already cited as α-Humulene in Libya [11], Borneol in Brazil [12], β-Thujone in Egypt [9] and France [10].

The preparation of inclusion complex between β-cyclodextrin and various molecules composing the essential oil by freeze-drying method results in obtaining a solid powder. Contact of the latter with air can allow evaporation of encapsulated volatile compounds, that's why we have developed a nanoemulsion based on these inclusion complexes. Indeed, the dispersion of inclusion complexes in water increases their stability because the essential oil is a hydrophobic compound. In addition, adding electrolytes like arginine and xanthan gum increases the polarity and ionic power of aqueous solution (zeta potential of nanoemulsion is 36.2mV) which promotes the formation of inclusion complexes [28,29].

Essential oil nanodroplets formed in the system are in the order of 219.1±5.2 nm in diameter, dispersion stabilization of these droplets is ensured by xanthan gum that increases the system viscosity which is in the order of 0.87± 0.03 Pa.s (high viscosity avoids the phenomenon of droplet sedimentation [30,31]) and by arginine which increases zeta potential of droplets (36.2 ±4.8 mV), this increase will engender an increase of repulsion forces between droplets and avoids the phenomenon of coalescence [30].

The result obtained by laser particle size could be confirmed by observation with transmission electron microscopy. Indeed, according to the images obtained by TEM, the size of nanodroplets obtained is variable but remains around 200 nm.

Unlike conventional nanoemulsions stabilized by soluble surfactants, nanodroplets of this nanoemulsion aren't spherical but exhibit a wide variability of non-spherical forms with irregular edges, this result is due to the nature of stabilizing system used. Indeed, the nanodroplets are composed of a cluster of complex inclusions based on cyclodextrins and essential oil stabilized by xanthan gum.

Antifungal activity evaluation of *Salvia officinalis* essential oil has been extensively studied for the strains of *Candida albicans* [16,21] and *Microsporum canis* [16,17,21] but evaluation of this activity on *Trichophyton equinum* is not included in the bibliography. The previously mentioned studies show large variations between the different results obtained from antifungal activity of *Salvia officinalis* essential oil on *Candida albicans* and *Microsporum canis*, this variation is due to two main factors, the first is variation of different strains resistances used and the second is due to chemical composition of essential oils used. Indeed, oils with high levels of α-thujone (the majority compound of *Salvia officinalis* essential oil) and β-thujone showed stronger antifungal and anti-elastase activities [32]. Our essential oil showed good antifungal activity against all strains tested. In addition, a significant ($p<0.005$) increase in this activity was noted after nanoemulsification of the essential oil.

Antimicrobial activity of essential oils is due to the nature of their phenolic compounds which intervene in the denaturing of microorganisms' cell membrane, in inhibition of their toxins and in the destruction of their genetic material [33].

The increase in antifungal activity of this essential oil after its incorporation in the innovative nanosystem can be explained by two mechanisms:

The first mechanism is mainly due to the formulation of essential oil nanodroplets contained in the nanoemulsion, droplets having an average diameter of 219.1±5.2 nm penetrate more easily through the cell membrane of microorganisms (proposed mechanism is noted as "Mechanism 1" in Figure 4).

The second mechanism is due to the use of cyclodextrins as cage molecules, inclusion complexes formed between β-cyclodextrin and essential oil are highly soluble in aqueous media such as fungal cells cytoplasm.

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Hence, optimization of essential oil action on different cell organelles contained in the cytoplasm (proposed mechanism is noted "mechanism 2" in Figure 4).

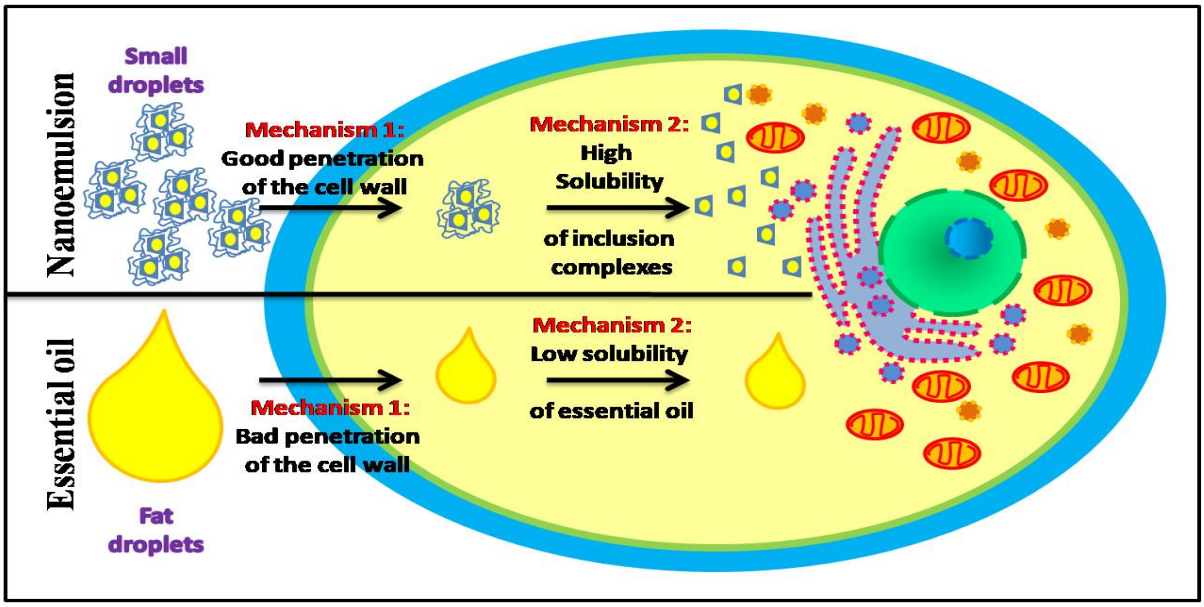

Figure 4: Mechanism of the increase in antifungal activity of *salvia officinalis* essential oil after its nanoemulsification.

4. CONCLUSION

The extraction yield of *Salvia officinalis* essential oil from Algeria is 2.07%. The main compounds of this essential oil are α-thujone (23.21%), 1,8-cineole (14.17%) and Camphor (11.02%).

The nanoemulsion containing 20% of essential oil, 20% of β-cyclodextrin, 5% of arginine and 1% of xanthan gum presents a very good physicochemical characteristic. The average diameter of the nanoemulsion is 219.1 ±5.2 nm, its polydispersity index is 0.52±0.08, its zeta potential is 36.2 ±4.8 mV and its viscosity is 0.87 ± 0.03 Pa.s).

The antifungal activity evaluation of sage essential oil on dermatophytes was shown. A natural nanosystem containing this essential oil was successfully carried out.

The need to recharge is what drives consumers back to nature, that's why we have developed this system based on natural excipients (β-cyclodextrin, xanthan gum and arginine) in order to increase the antifungal activity of sage essential oil.

This system can be considered as a basis for future works aiming to increase the pharmacological activities of different therapeutic substances.

5. MATERIALS AND METHODS

5.1. Materials

5.1.1. Plant material

The leaves of *Salvia officinalis* were collected at the flowering stage (Friday, May 22, 2015) in the village of Kalaa n'Ath Abas, Béjaïa city, at 186km from Algiers, Algeria. The geographical position of harvesting place

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is shown in Figure 5 (Geographic coordinates: 36° 17′ 47′′ North, 4° 34′ 51′′ East). After harvest, the plant is washed and dried in the shade for a week.

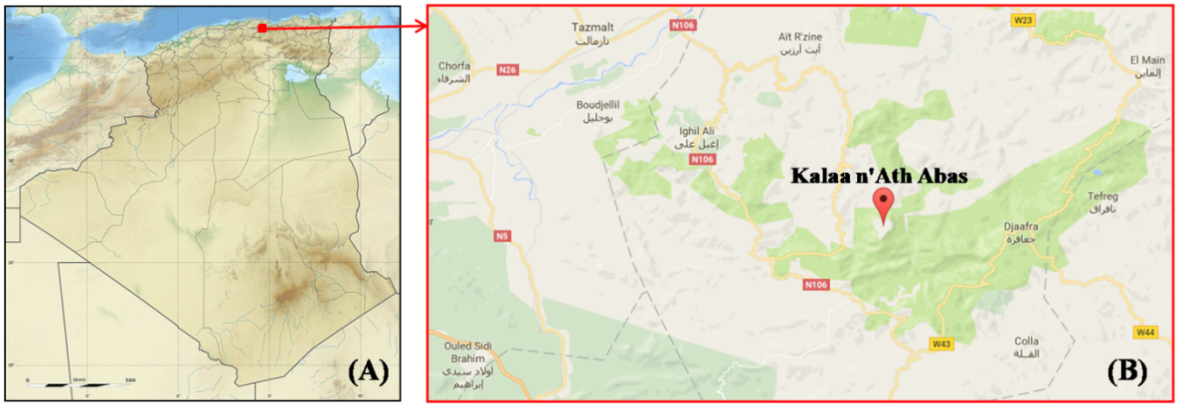

Figure 5: Geographical position of Kalaa n'Ath Abas on the map of Algeria (A), exact location of Kalaa n'Ath Abas and its surroundings (B).

5.1.2. Chemical products

β-cyclodextrin (97% purity) and L-arginine (98% purity) are purchased from Sigma-Aldrich. Xanthan gum is offered by Saidal Pharmaceutical Laboratory. Distilled water and all other products used are of analytical grade.

5.2. Extraction of essential oil

The extraction of *Salvia officinalis* essential oil was carried out by hydrodistillation using the Clevenger apparatus. The protocol used is described by the European Pharmacopoeia [34], 150g of the dry plant is introduced into a 2l flask containing 1.5l of distilled water; After 3 hours of extraction, the essential oil is recovered and then weighed. The extraction yield is calculated using the following equation:

$$\text{Yield} = \frac{\text{mass of the extracted essential oil}}{\text{mass of the plant used}}$$

5.3. Chemical composition of essential oil

The chemical composition of the essential oil is determined using a gas chromatography device coupled to a Hewlett-Packard 6890 series GC systems (Agilent Technologies) mass spectrophotometer (GC/MS) coupled to a quadruple mass spectrophotometer HP 5973. The system used is equipped with an HP5MS capillary column (5% phenyl methylsiloxane, 30m x 0.25mm, 0.25µm film thickness). The analytical conditions are: injector temperature 250°C, isothermal temperature 60°C, 8min at 250°C, 30min at 2°C/min; Helium was used as vector gas (He, 0.5ml/min, split 1/20); Ionization voltage 70eV and scan range 35-500uma. The identification of compounds is based on the comparison of their retention times with identified standards and computer database using the retention times of an alkanes series [35,36].

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5.4. Preparation of nanoemulsion

The nanosystem is prepared in three steps. The new proposed formulation process of nanoemulsion is shown in Figure 6.

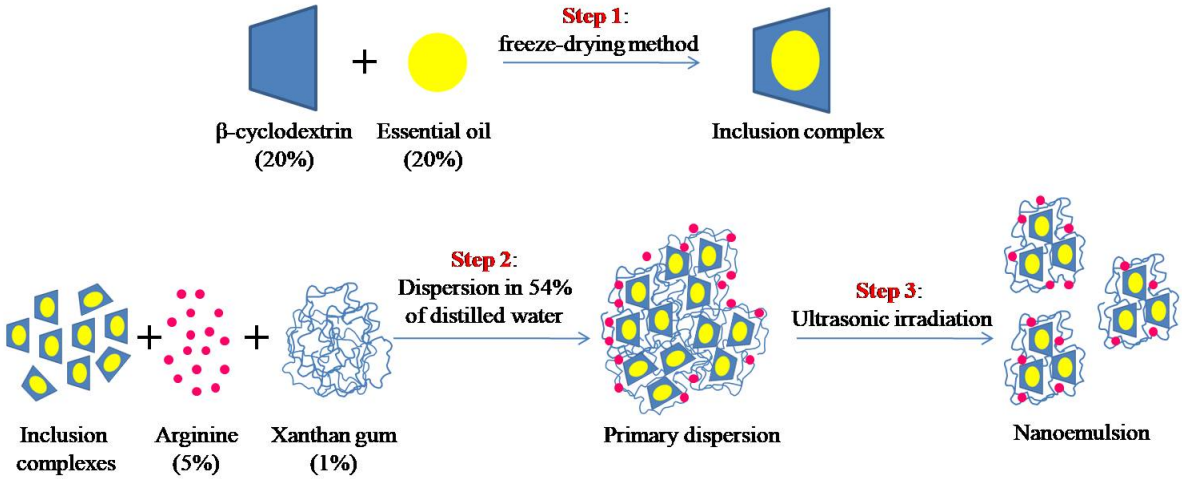

Figure 6: The new proposed formulation process of nanoemulsion.

Preparation of inclusion complex (step 1): the inclusion complex is prepared by freeze-drying method described by [23,37] with some modifications. Briefly, the essential oil is mixed with an aqueous solution of β-cyclodextrin (10% w/w) at a mass ratio of 1:1. After 24 hours of stirring at room temperature (25°C), the system is freeze-dried in a Cryodos® type device for 48 hours at a temperature of -50°C.

Formulation of primary dispersion (step 2): the previously prepared solid inclusion complexes are mixed directly with an aqueous solution containing arginine and xanthan gum using an Ultra Turrax stirrer (T25 IKA Labortechnik, Germany) at 24000 RPM for 5 minutes.

Nanoemulsion formulation (step 3): the pre-prepared primary emulsion undergoes sonication using a type sonizer (Ultrasonics, USA) with a high frequency of 20kHz and an output power of 750W. The ultrasonic amplitude used is 40% during an irradiation time of 60 seconds, a sonication probe with a diameter of 13mm was used. Table 5 shows the composition of prepared nanoemulsion.

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Table 5: Composition of the nanoemulsion.

Compounds	Quantities
Essential oil	20%
β-cyclodextrin	20%
Arginine	5%
Xanthan gum	1%
Distilled water	54%

5.5. Characterization of nanoemulsion

5.5.1. Determination of essential oil content in nanoemulsion

Extraction of total essential oil from nanoformulation was realized by hexane [29]. 1ml of nanoemulsion, 20 ml of distilled water and 10 ml of hexane was added in glass tubes. The prepared systems were sonicated during 20 min at 60°C using ultrasonic bath and the organic phase was separated from the aqueous phase. SEO content in nanoemulsion was determined by spectroscopic quantification of *Salvia officinalis* essential oil in the organic phase extracted previously using Agilent 8453 UV-Vis spectrophotometer at 256 nm. The measurements are triplicated and all experiments were carried out at 25°C. *Salvia officinalis* essential oil content in nanoemulsion was calculated as follows:

$$\text{Essential oil content (\%)} = \frac{\text{Essential oil content in 1ml of nanoemulsion}}{0.2} \times 100$$

5.5.2. Determination of average diameter

The average diameter is determined using a light-ray diffraction granulometer of type SZ-100 Nanopartica. The sample is analysed after 1/100 dilution using a crystal vessel, refractive index used for analysis is 1,466. The analysis was tripled and performed at 25°C.

5.5.3. Zeta potential measurement

A zetameter type SZ-100 Nanopartica has been used. The sample is analysed after 1/100 dilution. Analysis was tripled and performed at 25°C.

5.5.4. Viscosity measurement

The viscosity of the nanoemulsion was measured using a Brook Field viscosity meter (For LV-II viscosity range), applied shear was set to 170 s^{-1}. The analysis was tripled and performed at 25°C.

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5.5.5. TEM observation

In the preparation of microscopic grid, drop of emulsion (negatively stained with phosphotungstic acid) was placed on copper grid and was dried in vacuum for 6 h. The surface morphology of the nanoemulsion was observed by a transmission electron microscopy using Zeiss LEO 900 transmission electron microscope.

5.6. Evaluation of antifungal activity

5.6.1. Fungal strains used

Three microbial strains were selected for this study: *Microsporum canis, Trichophyton equinum and Candida albicans.*

5.6.2 Preparation of inoculums

A suspension of fungal cells in sterile physiological water is prepared from fresh cultures of strains used. The cellular concentrations of inocula are evaluated by the turbidity method and are expressed by measurement of optical density (OD at 620nm) using a UV-Visible spectrophotometer of type Beckman DU 520. An OD of 2-3 corresponds to 107-108 germs/ml.

5.6.3. The diffusion method on agar from discs

In petrie dishes containing 15ml of prepared sabouraud culture medium, the strain tested is inoculated with a swab soaked by the suspension of fungal cells. Then, three 9mm discs are deposited in the center of each third of the container. The first disc is soaked with 10 µl of essential oil, the second is soaked with an equivalent amount of nanoemulsion (50µl of nanoemulsion containing 20% of essential oil), the third disc is soaked with 50µl of nanoemulsion placebo (essential oil is replaced by physiological water). Prepared cans are incubated at 25°C for 48 hours [38]. After incubation, the diameters of inhibition zones are measured using a slide stand.

5.6.4. Determination of minimum inhibitory concentration and minimum fungicide concentration (MIC/MFC)

The method used is solid dilution described by [39] with several modifications. Briefly, the sabouraud culture medium is heated to 95°C in a water bath, the first agar containing 1µl of essential oil per 1ml of agar (mother suspension at 2µl/ml) is prepared. Then, a serie of dilutions is carried out (1, 0.5, 0.25, 2.0125 and 0.063µl/ml). A gallery of 6 test tubes containing 10ml of each solution (from 2µl/ml to 0.063µl/ml) is cooled on an inclined surface to obtain an inclined solid medium. Each dilution gallery is inoculated with previously prepared inoculum suspension and incubated directly at 25°C for 48 hours. MIC is the lowest concentration at which the germ didn't grow.

In order to determine the MFC, all the previously studied tubes that did not show flare-ups are transplanted on inclined tubes containing a sabouraud agar (which does not contain an active ingredient), The MIC is the lowest concentration at which the germ didn't grow after 48 hours of incubation.

5.7. Statistical analysis

Statistical analysis of all results was performed using IBM SPSS Statistics (Statistical Package for the Social Sciences) version 20.0 for Windows. A t-test was used to test equality of the mean differences at a 0.05 significance level.

http://dx.doi.org/10.29228/jrp.156

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Conflict of interest statement: The author declared no conflict of interest.

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