Research article

Ethanol and glycerol green emulsifying solvent for the formation of a *Lavandula stoechas* essential oil/β-cyclodextrin inclusion complex: mixture design and adulticidal activity against *Culex pipiens*

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HIGHLIGHTS

- Camphor, fenchone, camphene, linalool, 1,8 cineole, borneol, and α-pinene represented the main compounds in *L. stoechas* EO.
- The mixture design between ethanol and glycerol as green emulsifiant allowed for improvement of the encapsulation efficiency.
- The FTIR, SEM, PXRD and TGA were confirmed the processes of inclusion complex.
- The encapsulation of *L. stoechas* EO by β-cyclodextrin was accompanied by the increase of thermal stability and adulticidal activity.

ARTICLE INFO

ABSTRACT

The purpose of this study is to investigate the effect of essential oil medium on the inclusion complex of *L. stoechas* EO in β-cyclodextrin, as well as to examine the impact of the encapsulating action on the adulticidal activity. In line with this, *L. stoechas* EO was hydrodistilled and determined through GC-MS. Furthermore, the optimization of EO medium was conducted using a binary mixture design of ethanol and glycerol as green emulsifier solvent. Fourier transform infrared spectroscopy, scanning electron microscopy, X-ray powder diffraction and thermogravimetric analysis were used to verify the establishment of the IC. The insecticidal effect of the created formulation was evaluated against *C. pipiens* female mosquitoes. The optimum ethanol: glycerol ratio was 0.73: 0.27, corresponding to 58.86% of encapsulation efficiency. The fumigant test showed that, after 24 h of exposure, *L. stoechas* EO exerted only 24.56 ± 1.04%, while the encapsulated oil killed 57.89% of the adult population. At the highest dose (312.5 μl/L), the encapsulated oil provided the most significant effect on adults (100% mortality after 54 h) compared to non-encapsulated oil (100% mortality after 72 h). The encapsulated form of *L. stoechas* EO constitutes a promising alternative for the control of mosquitoes that are responsible for human diseases.

1. Introduction

Lavandula stoechas (*L. stoechas*) is an endemic Mediterranean plant belonging to the family of Lamiaceae [1]. This plant often grows in Morocco at very high altitudes on calcareous soils, particularly in the north of the country [2]. According to the Ministry of Agriculture, the area cultivated by lavender in Morocco, including *L. stoechas* covers 3000 Ha with a yield that varies between 5 and 15 Q/Ha. The price of the dried plant varies between 2.5 5.5 euros/Kg and the price of a liter of essential oil varies between 180 and 400 euros. The fenchone/camphor chemotypes are the main monoterpenes identified in the *L. steochas* essential oils from many Mediterranean countries with the dominance of

eucalyptol compound (1,8-cineole) [3, 4]. The main chemotypes identified in the *L. stoechas* essential oils grown in Corsica such as fenchone (14.9%–75.5%), camphor (2.5%–56.2%) and 1,8-cineole (0.17%–8%) showed a remarkable difference [5]. Dadalioglu et al. [6] reported that the chemical composition of the aerial parts *L. stoechas* EO from Turkish, wherein camphor was (18.2%), fenchone (55.6%), and 1,8-cineole (8%). Zuzarte et al. [7] revealed that *L. stoechas* essential oil in Sardinia Island (Italy) was rich on fenchone (37.0%), camphor (27.3%), bornyl acetate (6.2%), and 1,8-cineole (6.0%). In Morocco, Fenchone (30.5%), camphor (18.2%), 1,8-cineole (8.6%), and camphene (3.5%) were identified to be the main components *L. stoechas* oils [8]. *Lavandula stoechas* EO has emerged as a bioinsecticide through the study of its insecticidal, larvicidal, pupal and repellent actions against various insects species, including mosquitoes [9]. In Morocco, studies of the insecticidal effect of essential oils of the *Lavandula* genus to control *Culex pipiens* are limited to larvicidal studies and no study on the adulticidal effect has been recorded. However, several studies evaluated the larvicidal and repellent properties of various *Lavandula* species in the control of *Culex* mosquitos [10, 11]. The larvicidal activity *L. angustifolia* and *L. dentata* essential oils against *C. pipiens* was evaluated [12]. Moreover, Baranitharan el al [13]. studied the mosquitocidal effects of *L. angustifolia* essential oil against three significant human pathogen vectors; *Aedes aegypti, Anopheles stephensi,* and *Culex quinquefasciatus.* A study by Pavela [14] revealed that 30 compounds had a specific larvicidal impact on *C. quinquefasciatus* larvae. According to this investigation, α-pinene (LD$_{50}$ = 95 μg/ml) had a particular larvicidal effect compared to other terpene compounds, in particular, 1,8-cineole, camphor, and borneol (LD$_{50}$ > 250 μg/ml). Al-Sarar et al. [15] indicated the adulticidal activities of *L. dentata* on female adults of *C. pipiens.* The LC$_{50}$ value recorded was about 0.217 μl/L, while the main components of this oil are camphor (60.39%) and fenchone (28.05%). Three insect pests attacking stored products (*Lasioderma serricorne, Rhyzopertha dominica* and *Tribolium castaneum*) were the subject of an evaluation of the fumigant toxicity of *L. stoechas* EOs by Ebadollahi et al [16]. The results of this investigation revealed that this EO had a significant insecticidal property, as illustrated by the high mortality percentages recorded after 72 h of fumigation. Essential oils have poor solubility in water, spontaneous volatility and a tendency for degradation, as well as being difficult to handle. To solve these problems, encapsulation has been conducted using multiple methods: emulsion, films, liposomes and inclusion complexes [17]. IC are one of the most commonly used strategies of encapsulation, due to being based on host-guest interaction between the inner cavity of cyclodextrin (CD) and the essential oil. Cyclodextrin are crystalline, non-hygroscopic cyclic oligosaccharides formed by six, seven and eight glucopyranose units to form α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin, respectively [18]. Cyclodextrin's spatial arrangement is as a conical cylinder, whereby the external part is hydrophilic due to primary and secondary hydroxyl and the interior section is apolar [19]. The interest in the use of cyclodextrins (CDs) is based on the roles played by these molecules in the protection of guest molecules. For this reason, CDs are currently used in cosmetics, food and pharmaceutical sectors. Among CDs, β-cyclodextrin is the most appropriate, due to its reasonable price, easy availability and for suitable encapsulation of various EO's main compounds (mono and sesquiterpenes) [20, 21].

The dilution solvent of cyclamen aldehyde (Cya) has recently been examined in the IC of Cya/β-cyclodextrin [22]. The preparation of 1, 8-cineole/hydroxypropyl-βCD has been optimized using response surface methodology (RSM) to examine the temperature, time and molar ratio in the encapsulation process [23]. Furthermore, the ratios of βCD: rosemary EO and ethanol: water have been investigated within the inclusion procedure [24]. Based on the mechanism of molecular inclusion, when water molecules in aqueous solutions occupy the apolar interior cavity of CD, substitution with EO is promoted by way of favorable energetic process [25, 26, 27].

This study is devoted to deepening the understanding of the molecular inclusion mechanism, through fostering this substitution and facilitating the interactions between EO and the inner cyclodextrin cavity by weakening the interactions among the EO molecules. Within this constraint, the effect of the *L. stoechas* EO medium was optimized for encapsulation efficiency using a mixed experiment design.

This research intended to encapsulate this EO in βCD to enhance its adulticidal activity. Thereafter, the insecticidal potential of the coated EO was experimentally tested against *C. pipiens*, which are considered a vector of important arboviruses and have been investigated as a vector of the West Nile virus [28].

2. Material and methods

2.1. Chemical material

Glycerol was purchased from laboratory reagents & fine chemicals (Mumbai, India), while β-cyclodextrin was supplied by Applichem (France, Paris). The remaining chemicals were of analytical grade.

2.2. Plant material and essential oils extraction

The areal parts (leaves, stems and flowers) of the *L. stoechas* plants were collected in June 2019 in the commune of Timezgana (34° 33′ 02.7″ N 4° 40′ 49.3″ W) in the Taounate in the north central region of Morocco, at an altitude of approximately 800 m. These species were identified by Professor Badr Satrani, botanist at the Forestry Research Center—Rabat (FRC-Rabat), Morocco. The fraction of fresh plants (100 g) was hydro-distilled for 3 h in a Clevenger-type apparatus to produce a light-yellow oil. The oil phase was dehydrated using sodium sulfate, filtered and kept refrigerated in glass vials at 4 °C.

2.3. Chromatographic analysis

To determine its chemical composition, the *L. stoechas* EO was analyzed using gas chromatography combined with mass spectrometry, and flame ionization detector (GC-MS/FID). The gas chromatographic analyses of *L. stoechas* EO were performed on a Hewlett-Packard gas chromatograph (HP 6890) equipped with a HP-5 capillary column (30 m × 0.25 mm; 0.25 μm for layer thickness), an FID detector and an injector set at 275 °C. The oven temperature was adjusted to 50 °C for 5 min and subsequently increased to 250 °C at 4 °C/min. The carrier gas employed was N$_2$ at 1.8 ml/min using a 1: 50 split ratio and a flow rate of 72.1 ml/min. The essential oil was diluted 1: 50 in methanol and the injection performed with an injected volume of 1.2 μl.

The analysis was conducted using a Hewlett-Packard gas chromatograph (HP 6890) coupled to a mass spectrometer (HP 5973) with a HP-5MS capillary column (5% PHME cross-linked siloxane) (30 m × 0.25 mm; film thickness 0.25 μm). The column temperature was adjusted to 50 °C and increased to 250 °C at 2 °C/min. The helium was a carrier gas (at 1.5 ml/min) and a split mode ratio (1: 74.7) was used with a flow rate of 112 ml/min. The parameters employed in the mass spectra were ionization voltages of 70 eV, where the ion source temperature was 230 °C.

The essential oil components were determined based on their retention indices, as compared to a similar series of normal alkanes (C$_8$–C$_{28}$), through examination of their mass spectra fragmentation patterns in accordance with those referred to the literature.

2.4. Mixture design of experiment

In order to optimize the encapsulation efficiency (EE%) using an emulsion solution of EO, an investigation into the optimal formulation was conducted using a combination of the diluent and the dispersion solvent. The chosen solvent for the dilution of EO was ethanol, which is known as a suitable co-surfactant [29], while glycerol was selected as an emulsifier [30]. In the green chemistry stage, glycerol was used on account of its many characteristics: solubility, volatility and safety [31].

A simplex-lattice design for two constituents was selected for the mixture design to find the formulation that provides the optimum EE% with the minimum number of experiments. As shown in Figure 1, the design was composed of seven experiments, including two pure components (experiment 1 and 2), the mixture 0.5/0.5 (experiment 7), this experiment has been tripled in order to determine the lack of fit of the model, axial points (experiment 5 and 6) and two mixtures 0.67/0.33 of the two components.

The postulated mathematical model was a Scheffe quadratic model, as represented in the following equation (Eq. (1)):

$$Y = b_1 X_1 + b_2 X_2 + b_{12} X_1 X_2 + \varepsilon \tag{1}$$

Where Y represents the response (EE%), b_1, b_2 and b_{12} are the coefficients of the two linear terms and the quadratic term, respectively. X_i is the proportion of components and ε is an error term.

The content of each component ranged from 0 to 1 and the sum of their components satisfied the relation (Eq. (2)) [32]:

$$\sum_{i=1}^{i=n} X_i = 1 \tag{2}$$

The analysis of variance enabled testing of the significance of the postulated models. The ratio between the mean square regression (MSR) and the mean square of residuals (MS$_r$) was calculated to find F$_{ratio(R/r)}$. The coefficient of determination R^2 was used to confirm the quality of regression [33]. Finally, the t-student test was used to determine the significance of the regression coefficients in each component [34]. All tests were performed at a 95% significance level.

The software DESIGN EXPERT version 12 (2019, Minneapolis, USA) was used for experimental design treatment and the desirability function was conducted to find the optimal combination [35]. A test point was performed to confirm the validity of the proposed model.

2.5. Encapsulation of L. stoechas EO in βCD

2.5.1. Preparation of inclusion complexes (ICs)

The IC was prepared using the co-precipitation method [36]. Briefly, 1.50 g βCD was solubilized in distilled water (50 mL) using magnetic stirring at 50 °C. Then, 200 mg of *L. stoechas* EO was added to 4 ml ethanol/glycerol at different volume ratios and dropped (1 ml/min) on the aqueous solution, with continuous stirring for 3 h. The mixture was kept overnight at 4 °C and the precipitate was subsequently filtered and dried in an oven at 50 °C for 6 h.

2.5.2. The encapsulation efficiency (EE%)

For the purpose of determining the encapsulation efficiency, the sample was dissolved in ethanol and it was immersed in an ultrasound water bath, as described by Zhang et al. [37]. To clarify the solution, the mixture was centrifuged and filtered. In line with this, the calibration curve was established using varied EO concentrations (1.0–7 µg/ml) prepared in ethanol. Additionally, the absorbance (A) was measured by spectrophotometer at $\lambda_{max} = 205$ nm and the concentration (C (µg/ml)) was deduced using Eq. (3) with a coefficient of determination at $R^2 = 0.9846$.

$$A = 1.2647\,C - 0.6303 \tag{3}$$

Lastly, the EE% was calculated using Eq. (4).

$$EE\,\% = \frac{m\,(mg)}{200} \times 100 \tag{4}$$

2.6. Physicochemical and morphological characterization

In order to confirm the formation of the IC, the characterization of IC was focused on the optimum one in the name LSEO/βCD. The spectrums of βCD, *L. stoechas* EO and LSEO/βCD were obtained using Fourier transformer infrared spectroscopy FTIR (Vertex 70 – Bruker, Shanghai, China) with a resolution of 4 cm^{-1}, 32 scans and wavelengths from 400 to 4000 cm^{-1} [38]. Scanning electron microscopy (JSM-IT500HR, Croissy-sur-Seine, France) allowed for display of the dried powdered βCD and LSEO/βCD in different magnifications. On a piece of aluminum, samples were adhered to carbon black tape, and then covered with gold. The photomicrographs were taken at an excitation voltage of 16.0 kV. The diffractograms of βCD and the IC were acquired using diffractometer system (X'Pert Pro, Kolkata, India). The generator conditions in voltage and current were 40 kV and 30 mA, respectively. The X-ray diffraction (XRD) measurements were obtained at room temperature with Cu radiation in the range from 3° (2θ) to 50° (2θ). Finally, thermal analyses of the *L. stoechas* EO, βCD and LSEO/βCD (approximately 10 mg) were performed in a thermogravimetric analyzer (LINSEIS STA PT1600, Paris, France) under nitrogen gas from room temperature up to 500 °C (10 °C/min). All analyses were conducted in the innovation city at Sidi Mohamed Ben Abdellah University (Fez, Morocco).

2.7. Mosquito rearing and fumigant toxicity of C. pipiens

2.7.1. Insects rearing

Culex pipiens mosquito larvae (Diptera: Culicidae) were recovered from a breeding site called Oued El Mehraz in an urban area of Fez city in northeast Morocco. The adult mosquitoes were guarded in mosquito net cages ($24 \times 24 \times 24$ cm^3) at 22.6 ± 2 °C and $70 \pm 5\%$ (RH) under 14:10 h (light/dark) photoperiod cycles, and they were fed on sucrose solution (10 % w/v). Females aged 2–3 days were used. The morphological recognition of *C. pipiens* was performed by the Moroccan identification key as indicated by [39].

2.7.2. Fumigant test

In the context of studying the impact of incorporation *L. stoechas* EO into βCD on the insecticidal activity of *C. pipiens* adult females, the fumigant toxicity test was performed using glass jars (1L) according Zahran et al. [40] with minor modifications. To obtain different concentrations; 19.53, 39.06, 78.125, 156.25, and 312.5 µL/L air, *L. stoechas* EO and LSEO/βCD were solubilized in dimethyl sulfoxide (DMSO) (0.01% v/v). Whatman N°1 pieces of filter paper (7 cm diameter) were impregnated with the various concentrations and they were then fixed on the lower side of the glass jars screw cap. A chemical insecticide (Deltamethrin, 0.05% w/v) was used as the positive control and DMSO as the negative control.
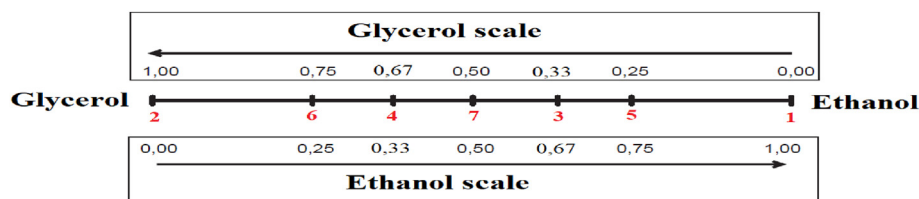

Figure 1. The experimental points of ethanol/glycerol binary design.

2.8. Statistical analyses

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 21). Mortality percentages were calculated using Abbott's formula (Eq. (5)) and presented as means ± SE from three replicates (20 mosquitoes per replicate, n = 60 per test) of each experiment. Lethal dose (LD_{50}) values were determined using Probit analysis according to Finney's mathematical methods (Finney 1952). Data from the fumigant test were examined statistically through analysis of variance (two-way ANOVA). Means were separated using Tukey's Honestly Significant Difference (HSD). All data results with $p \leq 0.05\%$ were considered statistically significant.

$$\% \text{ Mortality Corrected} = \left[\frac{\% \text{ Mortality Observed} - \% \text{ Mortality Control}}{100 - \% \text{ Mortality Control}} \right]$$
$$\times 100.$$

$$(5)$$

3. Results and discussion

3.1. Identification of the chemical components in L. stoechas EO

Lavandula stoechas EO was mainly characterized by the oxygenated monoterpenes (85.46%), with a large amount of monoterpene ketones (76.12%) and then the monoterpene alcohols (6.88%) and a little of the sesquiterpene compounds (1.73%). The EO mostly consisted of camphor (43.97%), fenchone (30.39%), camphene (4.09%), borneol (2.92%) and α-pinene (2.84%) (Figure 2 and Table 1).

3.2. Statistical validation of the postulated model

The experimental design, including the different combinations of the two studied components with the values of the observed responses, is presented in Table 2. Prior to starting the mixture design analysis, the results showed that the binary combination $0.5X_1$–$0.5X_2$, the combination $0.66X_1$–$0.33X_2$ and the combination $0.75X_1$–$0.25X_2$ had the highest EE%.

The analysis of variance (Table 3) showed that the main effect of the regression was significant, given the probability of risk significance p-value was less than 0.05 (<0.0001). As expected, the calculation of $F_{(MSR/MSr)}$ was equal to 85.63 was higher than the value of $F_{(0.05; 2.4)}$ at the 95% confidence level, which was equal to 6.94. Besides, the

Table 1. Chemical composition of *L. stoechas* EO.

Peak N°	Component	Retention time (min)	Retention Index	Percentage (%)
1	Tricyclene	5.82	927	0.25
2	α-Pinene	7. 65	939	2.84
3	Camphene	8.14	954	4.09
4	Thuja-2,4(10)-diene	8.80	957	0.05
5	3-Octanol	9.53	995	0.06
6	*p*-Cymene	10.22	1026	0.32
7	Limonene	11.36	1032	0.31
8	Eucalyptol	11.57	1035	1.42
9	γ-Terpinene	12.76	1062	0.34
10	*cis*-Linalool oxide	13.54	1077	0.41
11	Camphenilone	13.86	1086	0.08
12	Fenchone	14.25	1088	30.39
13	Linalool	14.87	1101	0.74
14	Fenchol	15.34	1121	1.12
15	α-Campholenal	15.91	1130	0.24
16	Camphor	16.94	1147	43.97
17	Pinocarvone	17.28	1168	0.41
18	Borneol	17.93	1172	2.92
19	*trans*-Linalool oxide	18.24	1177	0.05
20	Terpinen-4-ol	18.65	1181	0.22
21	p-Cymen-8-ol	18.78	1187	0.34
22	α-Terpineol	18.95	1193	0.12
23	Myrtenal	19.15	1198	0.25
24	Verbenone	19.47	1212	1.08
25	*trans*-Carveol	20.13	1221	0.21
26	Fenchyl acetate (endo)	20.51	1224	*0.05*
27	Carvone	21.26	1246	0.12
28	Bornyl acetate	23.48	1289	1.2
29	1,3-p-menthadien-7-al	24.67	1293	*0.07*
30	Geranyl butyrate	34.68	1549	0.05
31	1,10-di-epi Cubenol	37.74	1623	1.52

postulated models don't showed a lack of fit since their p-values were greater than 0.05 and their calculated $F_{LOF/PE}$ were lower than the theoretical $F_{LOF/PE}$ ($F_{0.05;1.2} = 19.24$) at 95% of confidence.
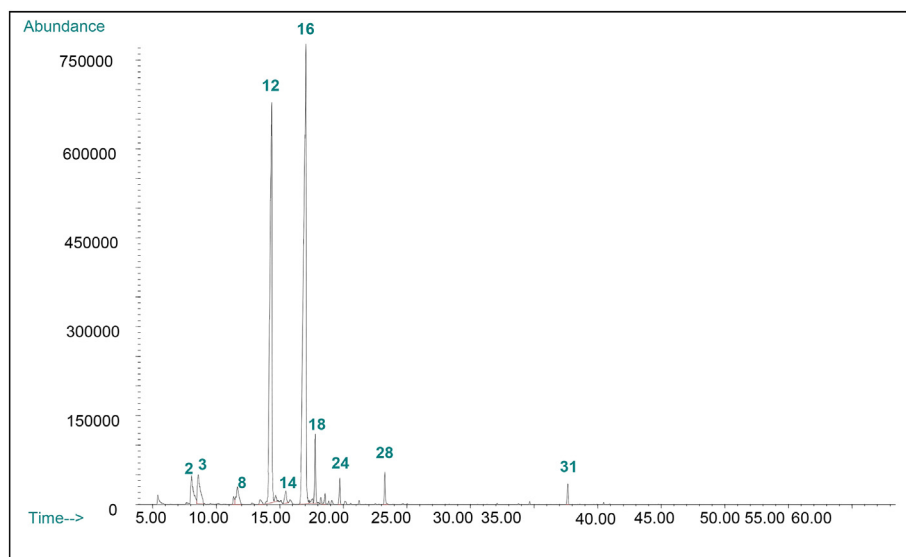

Figure 2. GC-MS chromatogram of *L. stoechas* EO.

Table 2. The encapsulation efficiency EE (%).

Run	X_1: Ethanol	X_2: Glycerol	EE (%)
1	1	0	53.9
2	0	1	30
3	0.67	0.33	58
4	0.33	0.67	47
5	0.75	0.25	57.8
6	0.25	0.75	46
7	0.5	0.5	58
8	0.5	0.5	58.7
9	0.5	0.5	57.3

Table 3. Analysis of variance for the adapted model.

Model	DF	Sum of squares	Mean square	F	p-value
Regression	2	702.43	351.22	85.63	<0.0001
Residual	6	24.61	4.10		
Lack of fit	4	23.63	5.91	12.06	0.0781
Pure error	2	0.98	0.49		
Total	8	727.04			
R^2	0.96				

The coefficient of determination was equal to 0.96; these findings are confirmed in the graph (Figure 3), which shows the curve of the predicted values according to actual values in the perfect shape of a line.

3.3. Medium effects and fitted model

The effects of the two studied components as well as the statistical values of t-student and the observed probability (p-value) are grouped in Table 4. All model coefficients represented on this table were statistically significant, with a p-value less than 0.05. Therefore, all coefficients were required to be present in the proposed model.

The mathematical model adopted was the augmented simplex-lattice design presented by the following equation (Eq. (6)):

Table 4. Estimated regression coefficients of the adopted model.

Term	Coefficient	Estimation	Standard error	t-student	p-value
Ethanol	b_1	53.82	1.86	28.94	<0.0001
Glycerol	b_2	29.23	1.86	15.72	<0.0001
Ethanol*Glycerol	b_{12}	59.25	7.03	8.43	0.0002

$$Y = 53.82\ X_1 + 29.23\ X_2 + 59.25\ X_1X_2 + \epsilon \quad (6)$$

Figure 4 represents the variation of EE% depending on the mixture of two compounds. According to this graph, the maximum encapsulation efficiency was obtained between the setting 0.5/0.5 and 0.75/0.25.

The desirability function was used in order to reach the maximum possible value for the studied response (EE%). Figure 5 indicated that the maximum value of the EE% that could be achieved by the validated model was equal to 58.86%. The achievement of this value of yield is possible, with a desirability of 99%, by ensuring a mixture of 0.73 of ethanol and 0.27 of glycerol.

From a chemical standpoint, the dilution of EO in ethanol allows for a reduction in concentration, which improves its dispersion in glycerol. As a consequence, there is a weakening of interactions between EO molecules and interactions with the inner cavity of the cyclodextrin become more accessible.

3.4. Characterization of LSEO/βCD

To confirm the inclusion process, characterization of a new peak in the FTIR spectra of LSEO/βCD with reference to *L. stoechas* EO was conducted, while simultaneously comparing between LSEO/βCD and βCD spectra (Figure 6). The FTIR spectra of the *L. stoechas* EO exhibited the vibration at 2,896 and 2,958 cm^{-1}, corresponding to a stretching vibration of C–H, and another intense peak at 1,745 cm^{-1}, attributed to the carbonyl. For the IC (LSEO/βCD) spectra, the intensity of absorption band for C–O decreased significantly. This radical reduction can be attributed to hydrogen bonding established between the *L. stoechas* EO and βCD [41].
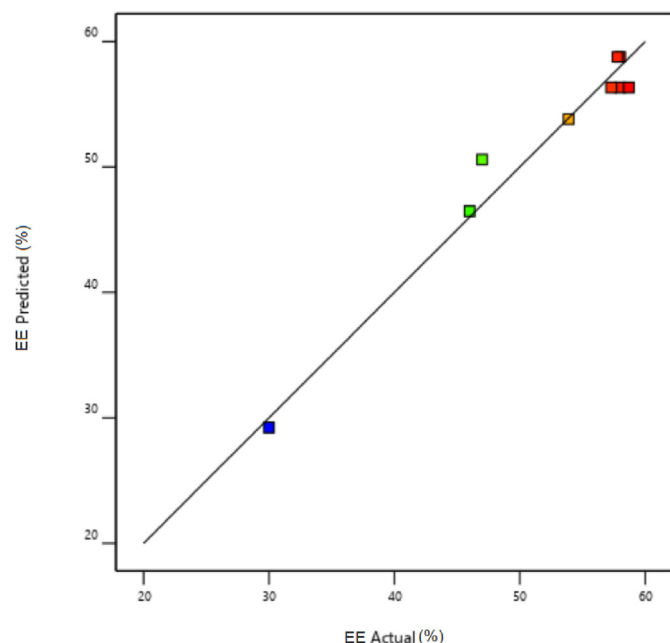
Figure 3. Curves of the predicted values according to the actual ones.
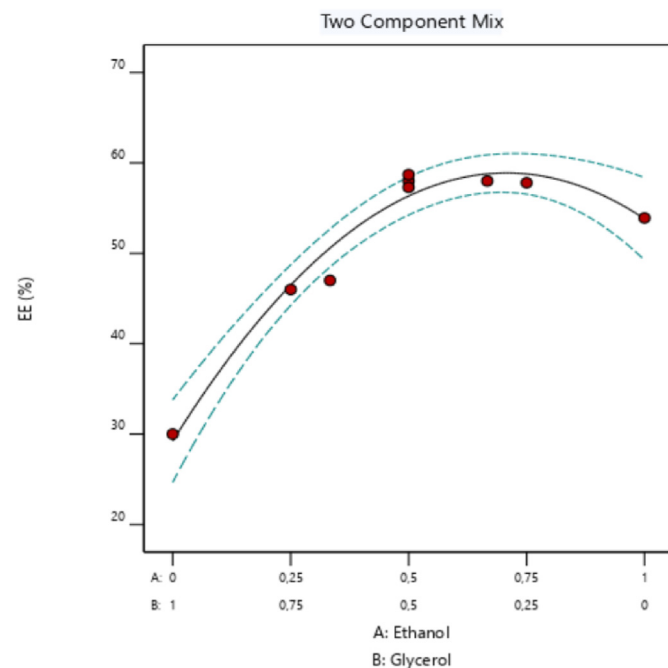
Figure 4. Binary mixture graph showing the area of maximization of the response studied as a function of the two components ethanol and glycerol rate.
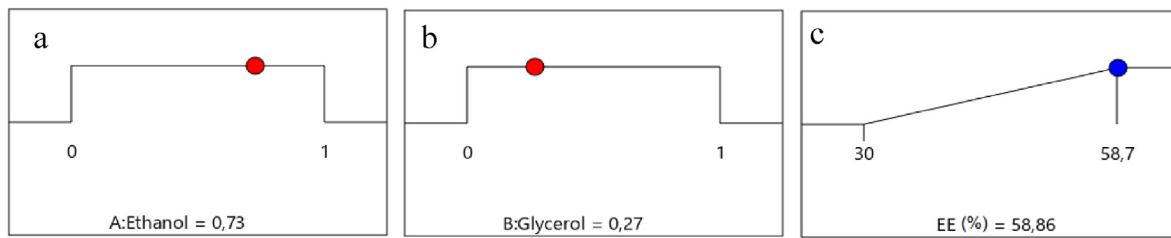
Figure 5. Desirability profile of optimal conditions to maximize encapsulation efficiency ('a' ethanol, 'b' glycerol and 'c' encapsulation efficiency).
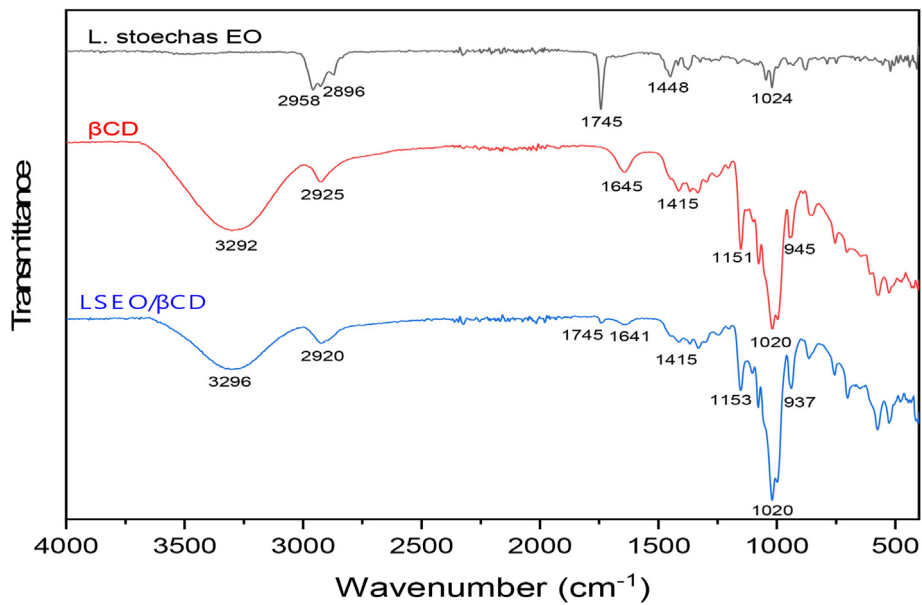

Figure 6. FTIR spectra of *L. stoechas* EO, βCD and LSEO/βCD.

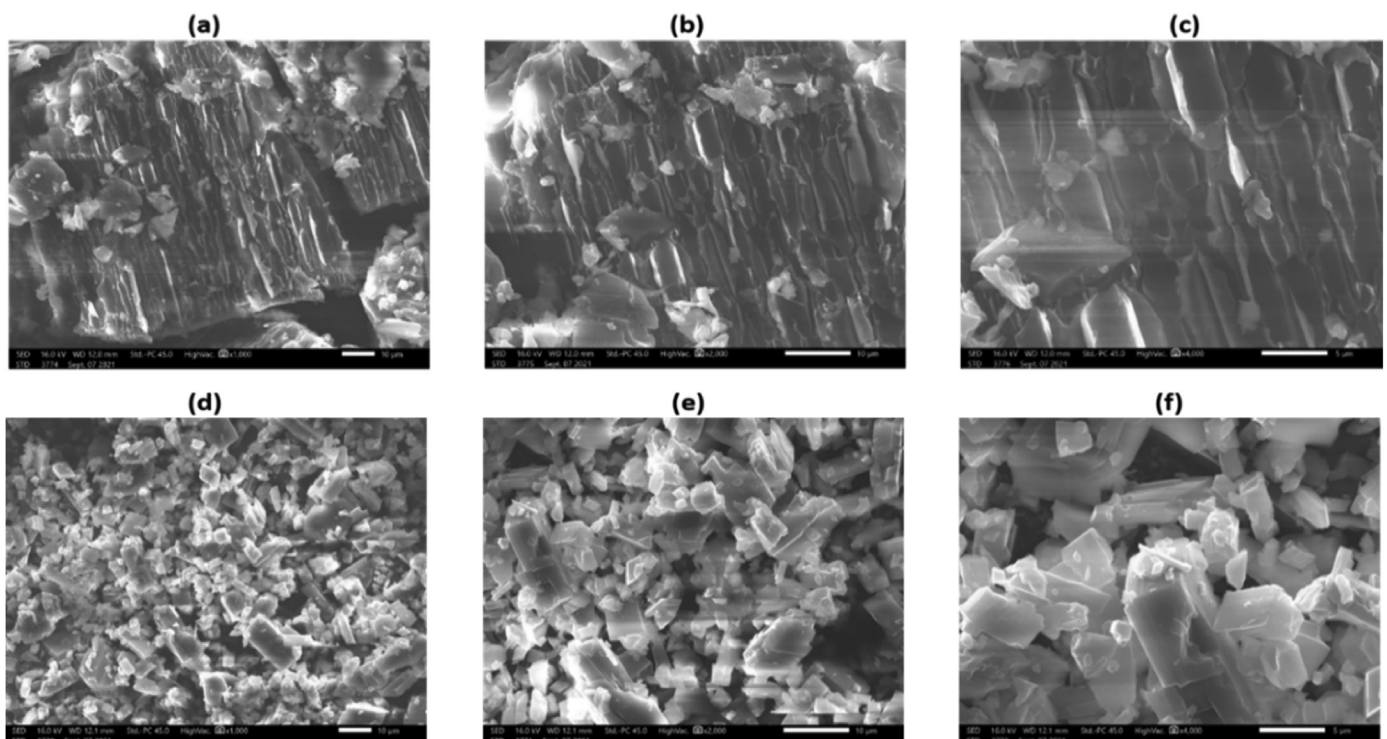**Figure 7.** Micrographs of βCD (a, b, c) and LSEO/βCD (e, d, f) in different magnifications (1000, 2000 and 4000×, respectively).

The spectrum of βCD was characterized by absorption bands at 3,292 and 2,925 cm^{-1} for the O–H and C–H stretching vibrations, respectively [42]. The spectrum of IC showed the same bands with modifications (shifted to 3,296 and 2,920 cm^{-1}) in intensity for the O–H band and in sharpness for the C–H band due to the variation of host environment. In the fingerprint region, the βCD and IC showed almost a superposition of both spectra.

SEM helped to approve the host-guest interactions by analyzing the morphological changes between the βCD and IC. Figure 7 (a, b, c) shows the photomicrographs of βCD, magnified at 1,000, 2,000 and 4,000 times respectively, which appeared as rectangular crystals with the presence of drops on the cover of the crystals. After building the IC, the morphology, as shown in Figure 7 (d, e, f), appeared as small misshapen particles and agglomerated in clusters. Similar observations were reported by [43], who elucidated adequate incorporation of LSEO in βCD.

To obtain the results of SEM, X-ray diffraction was used to determine the crystallinity nature of the βCD and IC [44]. As observed in Figure 8, the βCD showed many intensifying peaks, at 8.6°, 12.5°, 15.9°, 22.7° and 35.8°, reflecting the high degree of crystallinity, which is similar to the results depicted in the photomicrograph (Figure 7a). The IC has usually shown a more amorphous nature than βCD [45], which has been depicted by the disappearance of numerous peak characteristics of βCD and appearance at three peaks, at 7.0°, 9.9° and 17.5° (Figure 8). The modification of crystallinity between two solids signifies the evidence for the inclusion molecular procedure.

The formation of an IC could improve the stability and control the release properties of an essential oil [46], as confirmed in Figure 9. Thermograms showed that *L. stoechas* EO had one weight loss area up to 190 °C, referring to its entire volatilization. βCD had two distinct stages of weight loss, whereby the first corresponded to evaporation of water adsorbed, accompanied by a loss of 14% of the initial mass, while the second, where the degradation of βCD started from 284 °C, referred to a weight loss of 78.8%. Between these two stages, βCD was characterized by a region of thermal stability (114–284 °C). The pyrolysis process of LSEO/βCD was initiated by the evaporation of water, with a decrease of mass up to 6.8% causing some water molecules to be substituted by LSEO molecules within the βCD cavity. Compared with the TGA obtained for βCD, the thermogram of LSEO/βCD showed significant changes in the thermal regions until the decomposition (by continuous mass loss) temperature of βCD at 254 °C. The noted mass loss may be associated with the thermal decomposition of *L. stoechas* EO molecules. It is evident that the inclusion process considerably improved the thermal stability of *L. stoechas* EO.

3.5. Adulticidal activity of LSEO/βCD against C. pipiens

The insecticidal activity varied according to the type of sample tested (DMSO, Deltamethrin, *L. stoechas* EO and LSEO/βCD), concentrations and the time of the treatment, whereby the activity was found to be dose- and time-dependent.

As shown in Table 5, the adulticidal activity varied between the *L. stoechas* EO and LSEO/βCD. It appears that the encapsulated oil has an important insecticidal effect against *C. pipiens*, as the LD$_{50}$ values were determined using a Probit regression analysis, at 88 μl/L air and 22 μl/L air, respectively, after 24 h and 48 h of treatment showing suitable correlation ($R^2 = 0.96$). In contrast, for *L. stoechas* EO, the LD$_{50}$ values were only recorded after 48 h and were in order of 83.99 μl/L air ($R^2 = 0.97$). The fumigant test was statistically significant at p-value = 0.005.

Figure 10 (A, B) illustrates the mortality rate of *C. pipiens* adults over time and in the function of various concentrations. The obtained results indicate that the mortality of *C. pipiens* adults increases in the case of the encapsulated EO; it was in fact dose and time-dependant. The highest mortality rates were recorded at a concentration of 312.5 μl/L air and were estimated at 24.56 ± 1.04% and 59.89 ± 1.09% for non-encapsulated and encapsulated EOs, respectively, after 24 h of treatment. However, mortalities after 48 h of exposure were obtained at 68.42
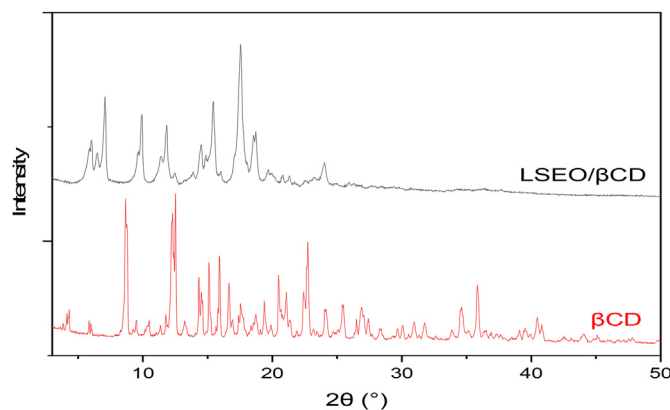

Figure 8. XRD diffractograms of βCD and LSEO/βCD.

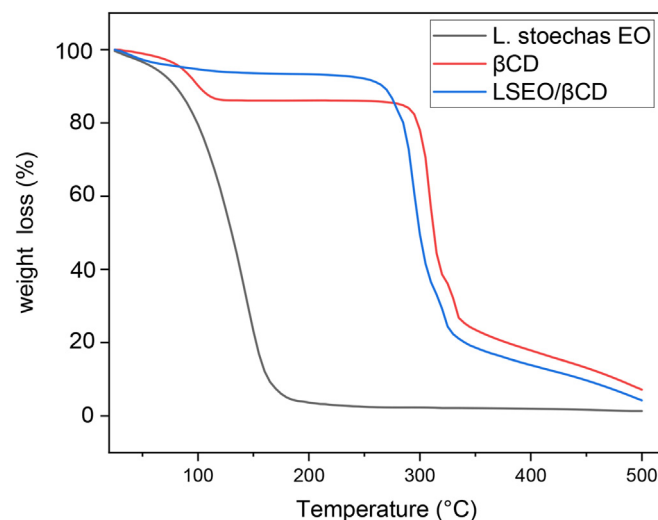**Figure 9.** TGA thermogram of *L. stoechas* EO, βCD and LSEO/βCD.

± 1.54% and 92.45 ± 0.34 for both EOs, respectively. The obtained data also showed that the LSEO/βCD caused mortality higher than 50% after 24 h, while 50% mortality for the *L. stoechas* EO needed 48 h of exposure. In comparison, the Deltamethrin showed a mortality rate of 33.33 ± 1.54% after 48 h. This mortality was almost obtained at 19.53 μl/L air for the free *L. stoechas* EO (35.08 ± 1.01% after 48 h) and at 156.25 μl/L air for the encapsulated *L. stoechas* EO (35.08 ± 1.01%) after just 12 h of exposure. This last finding revealed a slow liberation of *L. stoechas* EO when in the encapsulated form, together with a reduction of volatility and increase of stability. This finding revealed that there was a slow liberation of *L. stoechas* EO when it was used in the encapsulated form with a reduction of volatility and increase of stability.

The statistical treatment revealed that the insecticidal effect of both samples tested was considered highly significant (p < 0.0001), as well as the effect of concentrations (p < 0.0001) at p-value = 0.05 level of significance. Moreover, the interaction (sample × concentration) was also statistically significant with some variation of significance (p < 0.0001 after 24 h and p = 0.0012 after 48 h of exposure), which means that the non-encapsulated EO and the encapsulated one exerted different insecticidal effect against the mosquito adults of *Culex pipiens* at all concentration used (Table 6). Thus, the encapsulated EO proved higher toxicity compared to the free one.

The encapsulation of *L. stoechas* EO in βCD improved the insecticidal effect by increasing the mortality percentages. This insecticidal potential maybe related to the controlled release of *L. stoechas* EO during the fumigant test, which may have been due to the fact that the

Table 5. Adulticidal activity of *L. stoechas* EO and LSEO/βCD against *C. pipiens* adults after 24 h and 48 h of exposure.

| Sample	Time (h)	LD$_{50}$[a] (µl/L air)	CI$_{95}$	Slope[b]	Intercept[c]	R^2	p-value[d]
L. stoechas EO	24	>312.5					
	48	83.99	(74.83–94.49)	0.66 ± 0.10	3.73 ± 0.20	0.93	0.003
LSEO/βCD	24	88	(75.5–99.6)	0.415 ± 0.04	4.192 ± 0.08	0.96	0.002
	48	22	(16.79–30.23)	0.737 ± 0.08	4.004 ± 0.16	0.96	0.003

[a] Lethal concentration killing 50% of exposed adults population.
[b] Slope of the regression line ± Standard Error.
[c] Intercept of the regression line ± Standard Error.
[d] Not significant (p-value > 0.005).
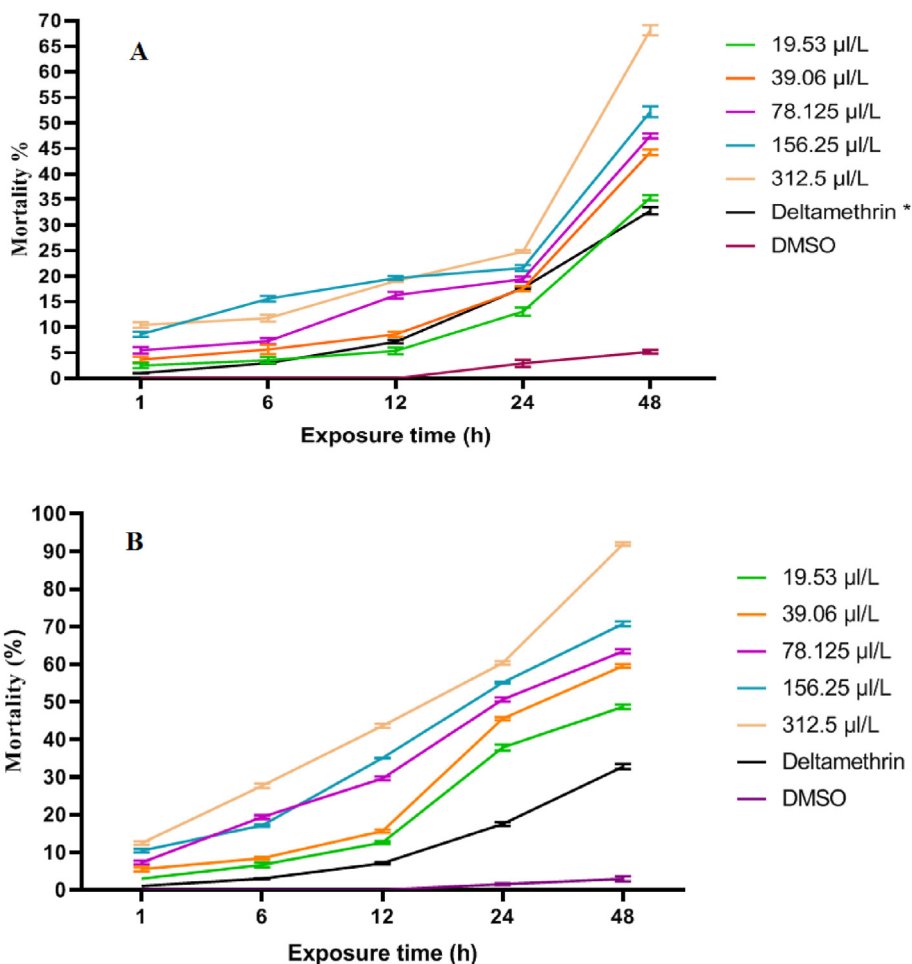
Figure 10. A) Mortality over time of *Culex pipiens* adults after exposure to *L. stoechas* EO according to different concentrations. B) Mortality over time of *Culex pipiens* adults after exposure to LSEO/βCD according to different concentrations. *: Deltamethrin as positive control and DSMO as negative control.

Table 6. Two-way analysis of variance results after fumigation assays of *L. stoechas* EO and LSEO/βCD against *Culex pipiens* adults.

Factor		F	df	p-value
Sample*	24 h	6755	1	<0.0001
	48 h	1060		<0.0001
Concentration	24 h	229.3	4	<0.0001
	48 h	421.5	4	<0.0001
Interaction	24 h	11.99	4	<0.0001
	48 h	6.907	4	p = 0.0012

F: Fisher test; df: Degree of freedom.
* *L. stoechas* EO and LSEO/βCD.

encapsulation of the EO into the β-cyclodextrin reduces its high volatility, prevents its rapid degradation and increases its action period [47], which subsequently enhances the insecticidal activity. Despite the low efficacy, LSEO also had an insecticidal effect that is probably linked to the presence of multiple compounds such as camphor, fenchone, camphene, linalool, 1,8-cineole, borneol and α-pinene, belonging mainly to the monoterpenes proportion, which are known for an insecticidal effect against various species [11]. Indeed, the *Lavandula* species, including *L. stoechas*, have proven adulticidal and repellent effect against the *Culex* genus and other insect species [15,48,49].

To better understand the boost of the insecticidal effect, it's evident to know how the encapsulated oil acts and exerts its biological activity on the mosquito. *Lavandula stoechas* EO contains a mixture of bioactive

compounds with a 93.72% fraction of monoterpenes (85.46% of the oxygenated monoterpenes and 8.26% of the monoterpene hydrocarbons). This fraction, either alone or in synergy with the sesquiterpenes fraction (1.73%), is apparently responsible for the insecticidal activity. However, these components may evaporate or be the subject of photochemical reactions [50] before reaching the target insect, which is why the encapsulation occurs. The encapsulation represents an operation in which the active molecule is encapsulated into the βCD. In doing so, the bioactive compound is protected from the external environment; its liberation depends on the external conditions and type of cyclodextrin [51].

As a result, the encapsulation process may guarantee the controlled liberation of the active compound, which additionally prevents the chemical alteration of the active agents or possible interaction between different product components [52]. Furthermore, the encapsulation advantage is not only related to the enhancement of the physicochemical stability but the improvement of bioactivity [53].

Encapsulation has attracted a broad spectrum of researchers due to the numerous advantages found in various fields. There have been a number of reports in the context of encapsulation using other methods (e.g., emulsions and using nanoparticles such as SiO_2) and investigating the insecticidal effect (larvicidal, pupal and emergence of adults) [54, 55]. However, no published studies have examined encapsulation by cyclodextrin in order to improve the insecticidal effect of EOs against mosquito adults. Some reports have studied the microencapsulation of selected monoterpenoids (linalool, S-carvone, camphor, geraniol, γ-terpinene and fenchone) on β-cyclodextrin using the co-precipitation method, subsequently proving an insecticidal effect against some stored products pests [56]. Based on this paper and the extant literature [57], the encapsulation method may improve the insecticidal effect of *L. stoechas* EO by extending the efficacy period while progressively releasing the active compounds of the EOs.

The effect of environmental temperature (15, 20, 25 and 30 °C) on the insecticidal efficiency of *Thymus vulgaris* EO against *Spodoptera littoralis* and *C. quinquefasciatus* larvae were investigated by [58]. The lethal doses were significantly dropped with increasing temperature when applied to *S. littoralis*, however, an opposite result was observed against *C. quinquefasciatus*. On this basis, we are conscious that further experiments at different temperatures will be necessary to obtain sufficient information on the insecticidal efficacy of *L. stoechas* EO and environmental temperature.

The promising results obtained in our study, provide preliminary data for elaborating a bioinsecticide based on essential oils and treated with encapsulation techniques, which have the characteristic of slow liberation, and overcome the low stability of essential oils. Furthermore, they could be utilized in integrated vector management (IVM) programs for mosquito net impregnation, in accordance with WHO recommendations for developing pesticides based on natural products.

4. Conclusions

As part of this study, the preparation of a medium containing a diluent (ethanol) and an emulsifier (green solvent glycerol), based on a mixed design, allowed for improvement of the encapsulation efficiency. This research showed promising results, as encapsulation using the outlined IC could considerably improve the insecticidal effect of the *L. stoechas* EO. The results of this study could contribute to obtaining a new efficient platform based on eco-solvents and biomolecules that allow overcoming the limitations of the currently used pesticide formulation.

However, further experiments are needed to determine the potential of encapsulated EOs as alternative and effective insecticides. Thus, to collect sufficient information on the efficacy of the adulticidal effect of the encapsulated *L. stoechas* essential oil and their major compounds, including the evaluation of the persistence of the insecticidal effect by the tarsal test.

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