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In vitro release and stability assessment of *Origanum compactum* liposomal dispersion with antifungal activity

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Abstract
Origanum compactum, an endemic Moroccan medicinal herb, possesses many different activities such as antibacterial, antifungal, antioxidant and anticancer. The aim of this study was to investigate the stability and antifungal activity of liposomal dispersion with this essential oil. Liposomal dispersion stability was evaluated by testing the vesicle size, polydispersity index and zeta potential. It was also examined the *in vitro* release of thymol and carvacrol from liposomal dispersion.
The major components of this essential oil were carvacrol (58.4%), thymol (12.5%) and γ-terpinene (10.7%). *Origanum compactum* essential oil showed a strong antifungal activity, and the inhibition zones ranged from 24 to 45 mm. After 210 minutes, 80.88% thymol and 16.67% carvacrol were released. Stability assessment was performed for three months and the liposomal dispersion showed a good stability.

Keywords: *Origanum compactum*, liposomes, stability, *in vitro* release, zeta potential

1. Introduction
Origanum compactum (*O. compactum*) Lamiaceae, an endemic Moroccan medicinal herb, has been used in the treatment of many diseases especially for the treatment of bacterial and fungal infections due to the strong antimicrobial action of the main components (thymol and carvacrol) [1, 2, 3, 4, 5]. Disadvantages of essential oils are poor water solubility, instability and poor delivery to target cells. Incorporation of essential oils into liposomes is a suitable solution to improve these problems and improve the stability of essential oils [6]. Incorporation provides better efficacy, reduce their irritant effect, and leads to prolonged action of the preparation itself. Liposomes are suitable drug carriers because they can incorporate hydrophilic, hydrophobic and amphipathic drug substances, they are quite similar to cell membranes [7] and can be made in various sizes, ranging from several nanometers to micrometers. Physicochemical properties of liposomes and zeta potential are very important for stability assessment. The main reasons for liposome instability are their possibility of hydrolysis, peroxidation reactions and ability of aggregation [8].
One of the most important parameter for assessing the stability of liposome preparations is zeta potential (surface charge) [9]. Particles with zeta potentials > +30 mV or < −30 mV are normally considered stable [10]. Polydispersity index (PdI) is a parameter that indicates the quality or homogeneity of the dispersion [11]. It ranges from 0 (monodisperse) to 0.5 (relatively wide distribution) [12]. The aim of this study was to investigate the stability of liposomal dispersion with *O. compactum* essential oil and to examine the antifungal activity of the essential oil against *Candida albicans* which can cause infections in various conditions such as pregnancy or diabetes or after taking antibiotics.

2. Materials and Methods
2.1. Materials
O. compactum (Pranarom International; Ghislenghien, Belgique) essential oil was used in this study and it was obtained by hydrodestillation at a low pressure.
For preparing the liposomal dispersion Phosal IP 40 (Lipoid, Germany) was used. It is a combination of phospholipids natural origin (soja lecithin): phosphatidylcholine + lysophosphatidylcholine, non-polar lipids, ethanol and tocopherols.

2.2. Chemical composition of *O. compactum* essential oil

GC/MS analysis of the *O. compactum* essential oil was performed on Agilent Technologies 7890B gas chromatograph, coupled with inert, selective 5977A mass detector. The essential oil was dissolved in diethyl ether and 1µL of the prepared solution was injected in split/splitless inlet set at 250 °C in 50:1 split mode and helium was used as the carrier gas.

The identification of compounds was based on the comparison of experimentally obtained retention indices (RI^{exp}) as well as on the comparison of EI mass spectra of essential oil components with data from Willey 6, NIST11 and RTLPEST 3 mass spectra libraries.

2.3. Antifungal activity of *O. compactum* essential oil

Antifungal activity of essential oil was performed on standard *Candida albicans* ATCC 10231 and 20 clinical strains isolated from vaginal and cervical swabs, from patients at the University Clinical Center Tuzla (BIH). Antifungal activity was performed using agar diffusion method according to CLSI guidelines [13] with some modifications [14]. Plates with Sabouraud Dextrose Agar (SDA) were inoculated with fungal suspensions. On each plate was added 50 µL of essential oil (6 mm in diameter) and than incubated at 37 °C for 24 h. The sizes of the inhibition zones were measured after incubation.

2.4. Preparation of liposomal dispersion with *O. compactum* essential oil

Liposomal dispersion was made by using high pressure homogenizer (Emulsiflex-C3, Avestin, Canada) at 500 bar in five cycles, and pre-mixing of Phosal IP 40 and *O. compactum* essential oil with Aqua ad injectabilia [15].

2.5. Stability assesment based on vesicle size, polydispersity index and zeta potential

The characterization studies included testing of vesicle size, polydispersity index (PdI) and zeta potential. These analysis were carried out by using Zetasizer (Nano series) ZS 90, Malvern Instruments [15, 16]. For zeta potential measurings, samples were diluted with 0.9% NaCl (conductivity at 50 µS/cm). The stability study included an examination of these parameters over the next three months. Liposomal dispersion was stored at 4 ± 1 ºC.

2.6. Measurement of electrical conductivity and pH of liposomal dispersion

Electrical conductivity was measured with a CDM 230 conductometer (Radiometer, Copenhagen, Denmark). Measurement of the electrical conductivity of liposome dispersion was performed by direct immersion of a conductometer probe in the test sample. The probe was calibrated with 0.01 M KCl solution at room temperature before operation.

The pH value was measured using a pH meter (BOECO Germany pH meter BT - 600). pH measurements of liposomal dispersion were performed by direct immersion of the appropriate pH meter electrode in the test sample. Prior to commissioning, the device was calibrated in the prescribed manner using standard pH 4.0 and 7.0 buffers.

2.7. *In vitro* release of carvacrol and thymol from liposomal dispersion*:*

10 mL of liposomal dispersion (internal phase) is defective in a membrane which, after closure, was immersed in 40 mL of phosphate buffer (pH = 4.5) (outer phase) and mixed on a magnetic stirrer at 300 min^{-1} rpm. One mL of phase compound dissolution was sampled at the following time intervals: 0, 30, 90, 150 and 210 minutes and each was filtered on a membrane filter 0.45 µm. 1 mL of pure buffer was returned each time after sampling. Each sample and filtered volume of the outer phase solution was extracted with 1 mL of ether and the ether layer recorded on GC-MS according to the previously described method.

3. Results and Discussion

3.1. GC-MS analisys of *O. compactum* essential oil

Carvacrol (58.4%), thymol (12.5%) and γ-terpinene (10.7%) were found to be the major components of the oil.

Research by Khadija [17] *et al*. showed that the contets of the four main components of oregano oil varied drastically from sample to sample: carvacrol (0.7-73.2%), thymol (tr.-31.7%), γ-terpinene (10.7-30.1%) and p-cymene (5.3-24.7%). Also research by Santamarina [18] showed that the main compounds in oregano essential oil were carvacrol (43.26%), thymol (21.64%), p-cymene (13.95%) and γ-terpinene (11.28%).

Table 1: Chemical composition of *O. compactum* essential oil

No.	t_{ret}, min	Compound	RI^{exp}	RI^{lit}	Content, %
1	7.15	α-Thujene	925	924	0.3
2	7.37	α-Pinene	933	932	0.5
3	7.83	Camphene	948	946	0.1
4	8.64	1-Octen-3-ol	975	974	0.2
5	8.70	β-Pinene	977	974	0.1
6	8.91	3-Octanone	983	979	0.1
7	9.08	Myrcene	989	988	1.1
8	9.59	α-Phellandrene	1005	1002	0.1
9	9.81	δ-3-Carene	1010	1008	0.1
10	10.03	α-Terpinene	1016	1014	1.2
11	10.34	o-Cymene	1024	1022	7.6
12	10.47	Limonene	1028	1024	0.3
13	10.50	β-Phellandrene	1029	1025	tr
14	10.58	1,8-Cineole	1031	1026	0.2
15	11.14	(E)-β-Ocimene	1045	1044	0.1
16	11.64	γ-Terpinene	1059	1054	10.7
17	12.75	Terpinolene	1088	1086	0.2
18	12.77	p-Cymenene	1089	1089	tr
19	13.17	Linalool	1099	1095	1.5
20	15.07	Camphor	1145	1141	0.2
21	16.44	Terpinen-4-ol	1177	1174	0.6

22	16.99	α-Terpineol	1190	1186	0.1
23	17.40	trans-Dihydrocarvone	1200	1200	tr
24	19.21	Carvacrol, methyl ether	1243	1241	0.2
25	21.36	Thymol	1294	1289	12.5
26	21.99	Carvacrol	1305	1298	58.4
27	26.65	(E)-Caryophyllene	1422	1417	1.5
28	27.99	α-Humulene	1455	1452	0.1
29	30.70	δ-Cadinene	1524	1522	0.1
30	33.01	Caryophyllene oxide	1585	1582	0.4
Total identified (%)					98,5

3.2. Results of antifungal activity of *O.compactum* essential oil

O. compactum essential oil showed a strong antifungal activity with inhibition zones ranged from 24 to 45 mm. Inhibition zones were higher in clinical strains than the standard strain.

Table 2: Antifungal activity of *O. compactum* essential oil

Name of organism	Code/number of isolate	*O. compactum* ZI (mm) Mean ± SD
C. albicans	ATCC 10231	25.0 ± 1.00
C. albicans	19830	25.6 ± 0.57
C. albicans	19586	26.6 ± 0.57
C. albicans	20960	28.7 ± 0.60
C. albicans	19968	28.9 ± 0.11
C. albicans	19960	24.0 ± 1.00
C. albicans	19709	27.3 ± 0.55
C. albicans	38149	23.6 ± 1.03
C. albicans	45205	31.1 ± 0.76
C. albicans	38333	30.5 ± 0.50
C. albicans	45208	25.0 ± 1.00
C. albicans	45278	32.3 ± 1.52
C. albicans	19249	36.5 ± 0.50
C. albicans	18303	40.3 ± 0.55
C. albicans	19252	27.1 ± 0.89
C. albicans	19112	45.0 ± 1.00
C. albicans	19103	35.3 ± 0.55
C. albicans	10839	25.6 ± 0.57
C. albicans	10295	27.3 ± 0.51
C. albicans	19274	38.3 ± 0.60

*SD – standard deviation; ZI – zone of inhibition

The antifungal activity of the oregano essential oil has been confirmed also by other authors [19]. Liolios [20] and contributors tested the essential oil of *Origanum dictamnus* (wild and cultivated), carvacrol and thymol on *Candida albicans* and obtained zones of inhibition were 9 to 12 mm.

3.3. Results of characterization studies of liposome (size, polydispersity index and zeta potential)

The results of characterization studies for liposomal dispersion are shown in Table 3.

Table 3: Characterization studies of liposomal dispersion

Liposomal dispersion age	Size (nm)	Polydispersity index (PdI)	Zeta potential (mV)
1 month	262.4	0.231	-55.60
2 months	298.1	0.241	-54.4
3 months	307.1	0.250	-52.8

The liposomes with size ≤ 300 nm are mostly used in preparation of dermal products [21], and this liposomal dispersion can be used for this purpose. Even after three months, the size of the liposomes and PdI did not changed much**,** and the values of the zeta potential remained approximately the same.

Polydispersity index (PdI) is an important parameter for liposomes. The values of PdI were lower than 0.3, so liposomal dispersion can be considered homogeneous [22].

Particles with a potential of +30 mV and more, or -30 mV or lower are considered stable [23] and zeta potential of our liposomal dispersion remained above -50 mV.

A slight increase in liposome size and a slight decrease in the zeta potential are due to the aggregation that happens over time. The results presented in the Table 4 prove that the liposomal dispersion remained stable within three months.

3.4. Measurement of electrical conductivity and pH of liposomal dispersion

Conductometry is used to determine the nature of dispersions and emulsions and to monitor their stability over time. The value of electrical conductivity of liposomal dispersion was 198.5 µS/cm. The conductivity result obtained in this study shows that the liposome dispersion had the aqueous phase as continuous, i.e. it did not show phase inversion.

The pH of the liposome dispersion was 5.64.

3.5. Results of *in vitro* release of thymol and carvacrol from liposomal dispersion

An *in vitro* assay of the release rate of thymol and carvacrol was performed using the dialysis bag technique, where liposomal dispersion with incorporated essential oil represents the donor phase and the pure buffer medium surrounding the dialysis bags represents the acceptor phase [24, 25]. The percentage (peak areas) of released thymol and carvacrol from liposomal dispersion were determined at predefined time intervals using GC analysis, and the resulting release profiles are shown in Figure 1.

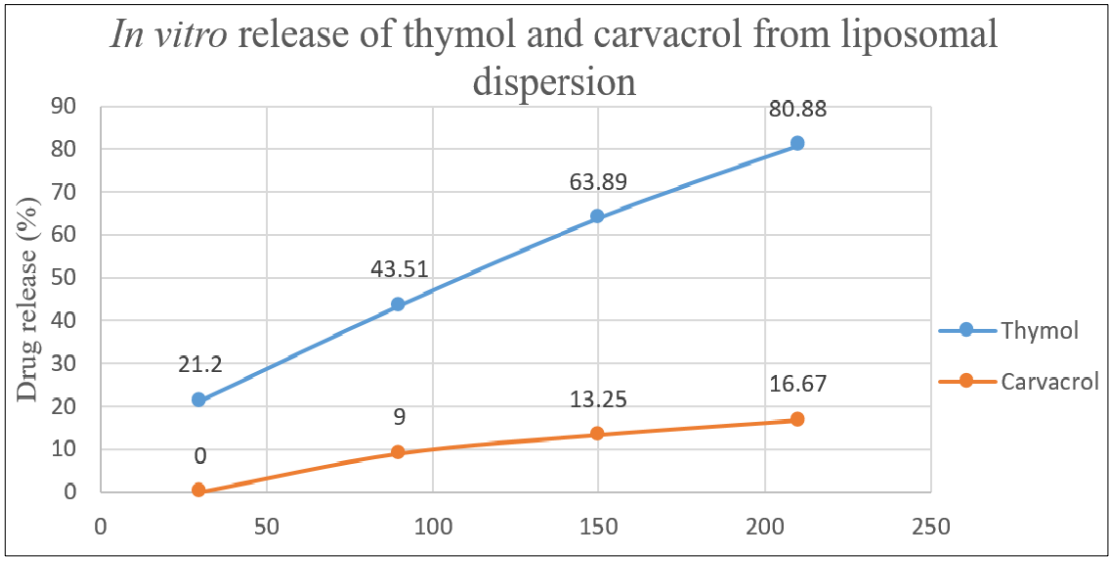

Fig 1: *In vitro* release of thymol and carvacrol from *O. compactum* liposomal dispersion

It is observed that thymol is released in greater quantity than carvacrol. Monitoring the *in vitro* release of liposome substances is difficult due to the small size of the liposomes and the slow release, especially when the lipophilic substance is released into the hydrophilic test medium. 80.88% thymol and 16.67% carvacrol were released in 210 minutes.

Although carvacrol is the most abundant component in essential oil, its release is lower than release of thymol. The reason may be the higher affinity of carvacrol for liposomes and the formation of certain intermolecular interactions [26].

4. Conclusion

Liposomal dispersion with Phosal IP 40 and *O. compactum* essential oil showed a good quality, suitable for further use in the preparation of dermal products with antifungal action. The zeta potential values indicated that the liposomal dispersion remained stable over the test period.

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