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RESEARCH ARTICLE

The nanoemulsion-based nanogel of *Artemisia dracunculus* essential oil with proper activity against *Leishmania tropica and Leishmania major*

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ABSTRACT

The most common form of leishmaniasis is cutaneous leishmaniasis, and it is distributed in around 100 countries. Preparing essential oil-based nanoformulations with the leishmanicidal effect is a promising strategy for the development of new drugs. In this study, the leishmanicidal effects of essential oils of *Artemisia dracunculus, Zataria multiflora,* and *Zingiber officinale* against promastigotes of *Leishmania tropica* and *Leishmania major* were first evaluated. The nanoemulsion of the most potent essential oil, *A. dracunculus*, was then prepared; particle size was 7.86 ± 4 nm, and particle size distribution (SPAN) was 0.96 ± 0.1. By addition (1.5% w/v) of carbomer 940, nanoemulsion transformed into the nanogel dosage form. The leishmanicidal property of the nanogel was significantly better than that of non-formulated *A. dracunculus* essential oil. Interestingly, the promastigotes' viabilities at a 160 µg/mL concentration were decreased to ~ 0%. The prepared green nanoformulation could be used as supplementary drugs in cutaneous leishmaniasis.

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INTRODUCTION

The most common form of leishmaniasis is cutaneous leishmaniasis; it is distributed in around 100 countries worldwide [1]. It is caused by obligate intracellular protozoa of the genus of *Leishmania* [2, 3]. *Leishmania major* and *Leishmania tropica* in the old world are responsible for the cutaneous leishmaniasis in rural and urban areas, respectively [4, 5].

Pentavalent antimonials, miltefosine, and amphotericin B are recommended drugs to treat cutaneous leishmaniasis [6, 7]. However, due to their limitations, including toxicity and lack of proper efficacy, the development of new drugs has become crucial [8]. Plants and their metabolites are excellent sources for finding green substances with leishmanicidal effect [9]. For example, essential oils (EO)s of cinnamon, thyme, and oregano showed proper leishmanicidal effect against different species of promastigotes of the *Leishmania* genus [10, 11]. However, the effectiveness of EOs can be lost by evaporation or degradation by oxidation and UV light [12]. Therefore, they should be formulated.

Pastes, ointments, and creams have been widely used for topical drug delivery. These formulations are very sticky; their usage thus is challenging. [13, 14]. The use of nanoemulsion for topical drug

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delivery has recently attracted more attention. They possess many advantages, such as higher skin permeation and retention and long storage time [15, 16]. Nanoemulsion-based nanogels are another dosage form for topical drug delivery with advantages of nanoemulsion and improved stability and facilitated usage. They are extensively employed in cosmetics and pharmaceutical preparations [17, 18].

In this study, the leishmanicidal properties of the three medicinally important EOs, including *Artemisia dracunculus* (ADEO), *Zataria multiflor*a (ZMEO), and *Zingiber officinale* (ZOEO) against promastigotes of *L. major* and *L. tropica* were investigated. Then, by preparing nanoemulsion-based nanogel of ADEO (more active than others), we tried to improve the effectiveness.

MATERIALS AND METHODS

Materials

ADEO and ZMEO were obtained from Zardband Pharmaceuticals Co (Iran). ZOEO was provided by Green Plant of Life Co. (Iran). *L. major (*MHOM/IR/75/ER*)* and *L. tropica (*MHOM/SU/74/K27*)* was provided by Pasteur Institute of Iran supplied. Tween 20 (Polysorbate 20), NaOH (Sodium hydroxide), and MTT powder (3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide) was purchased from Merck Chemicals (Germany). Penicillin-Streptomycin, RPMI cell culture media, DMSO (Dimethylsulfoxide) were bought from Shellmax Co. (China). FBS (Fetal bovine serum) and Carbomer 940 were bought from Gibco Co. (USA) and SDFCL Co. (India).

Investigation of leishmanicidal activity of essential oils

Leishmanicidal properties of the EOs were investigated using MTT assay in 48-well plates. The required dilution serial of each EO was prepared by two-fold successive dilutions of a stock solution. The stock solutions 5120 µg/mL were prepared using an aqueous PBS (containing 0.5% DMSO). The promastigotes of *L. major* and *L. tropica* (625000/mL) at the logarithmic phase were used for the leishmanicidal bioassays. They cultured in RPMI complete medium (FBS 10% and Penicillin-Streptomycin 1%).

First, 400 µL/well of each promastigote and serial dilution (400 µL/well) were added to a 48-well plate and incubated for 24 h incubation at 25 °C. After that, 50 µL/well of MTT solution was added and incubated for another 4 h. After that, 200 µL of DMSO was added to each well for dissolving formazan crystals. Finally, the optical density (A) of wells was read at 570 nm using a plate reader (Synergy HTX Multi-Mode Reader, USA), and the viability was calculated using equation 1.

In each of the three repetitions, control and blank groups were considered (n = 3). The wells in control groups were filled similar to the sample groups; only 400 µL of PBS was used instead of EO serial dilution. Also, blank wells loaded with the same amounts of RPMI complete medium and PBS (400:400 µL).

$$Viability(\%) = (A sample - A blank / A control - A blank) \times 100 \quad (1)$$

GC-MS analysis

Ingredients of ADEO were only identified because it showed better activity than other examined EO. For chemical composition, analysis of GC-MS was used as described in our previous study [19].

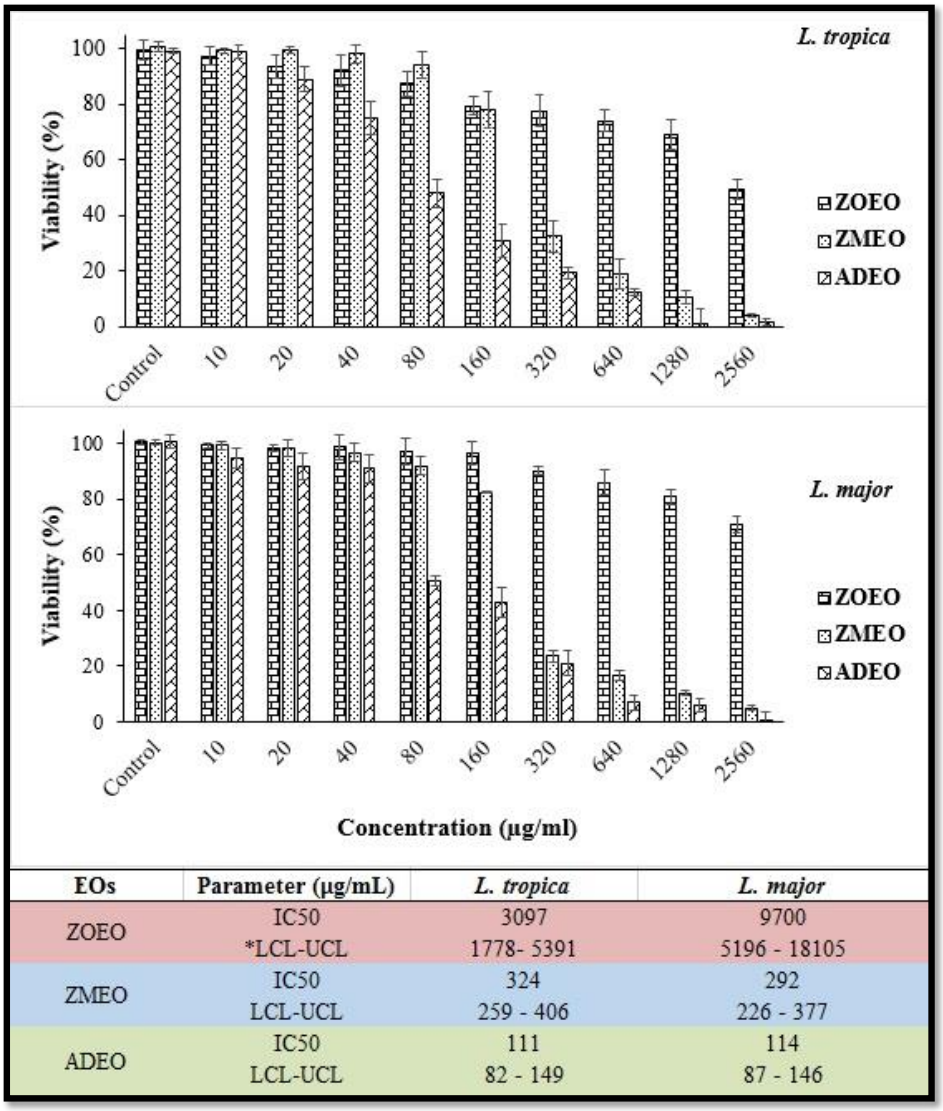
The procedure of preparation of nanoemulsion

For the preparation of nanoemulsion, ADEO (50 µL) and tween 20 (100-1000 µL) were blended for 10 min at 500 rpm to prepare a homogenous mixture. Distilled water was then added dropwise to the oily phase to reach 5000 µL. The mixture was stirred at 2000 rpm for another 30 min to form nanoemulsion. A nanoemulsion with the smallest particle size and acceptable particle size distribution (SPAN), i.e., < 1, was finally selected as the optimum nanoemulsion.

The particle size SPAN of prepared samples was investigated using dynamic light scattering (DLS, K-ONE.LTD, Korea) at 25 °C. SPAN was calculated using equation $SPAN = D90 - D10 / D50$, where D is the diameter of the particles. D10, D50, and D90 are the percentile of particles that have a diameter lower than these values.

The procedure of preparation of nanogel

The optimum nanoemulsion was selected for the preparation of nanoemulsion-based nanogel. First, carbomer 940 (1.5% w/v) as the gelling agent was dispersed into the nanoemulsion under a mild magnetic stirring (120 rpm, overnight). The pH was then raised from 4 to ~ 7 by adding NaOH solution (25% w/v) for completing the gelation process. A blank gel was also prepared in the same process and ingredients, only without ADEO.



EOs	Parameter (µg/mL)	*L. tropica*	*L. major*
ZOEO	IC50	3097	9700
	*LCL-UCL	1778- 5391	5196 - 18105
ZMEO	IC50	324	292
	LCL-UCL	259 - 406	226 - 377
ADEO	IC50	111	114
	LCL-UCL	82 - 149	87 - 146

*Lower and Upper Confidence Limits

Fig. 1. Leishmanicidal effects of the EOs

The stability of the prepared nanogel was 6-month monitored at two temperatures (4°C and ambient temperature). Besides, the viscosity of the nanogel was investigated using a rheometer machine at 25°C (Anton Paar rheometer, model MCR-302, Austria).

Evaluation of the leishmanicidal properties of the nanogel

The nanogel and blank gel's leishmanicidal activity were investigated using the MTT assay as follows. 400 µL/well of each promastigote and 400 µL/well of PBS was first added well. After that, 6.4 (± 5% w) and 12.8 (± 5% w) mg of the samples (nanogel and blank gel) was added to wells. The process continued as described in section 2.2. By adding such mentioned amounts of the nanogel, the concentration of ADEO eventually was fixed at 80 and 160 µg/mL.

Statistical analysis

For determining the half-maximal inhibitory concentration (IC50) of each EO against promastigotes of *L. tropica* and *L. major*, CalcuSyn software (Free version, BIOSOFT, UK) was used. IC50s of EOs were compared together using one-

way ANOVA analysis. Also, the leishmanicidal effects of ADEO and the nanogel were compared using an independent sample t-test. The analyses were performed using SPSS software (v. 21, IBM, USA) with confidence intervals of 95%.

RESULTS

Leishmanicidal effects of the EOs

The leishmanicidal effects of the EOs are given in Fig. 1. Effectiveness of ADEO with IC50 of 111 and 114 µg/mL against *L. tropica* and *L major*, respectively, significantly better than ZOEO and ZMEO (one-way ANOVA, sig < 0.05). The obtained IC50s for *L. tropica* and *L. major* after treatment with ADEO had no significant difference (Independent sample t-test, sig > 0.05). Furthermore, at a concentration of 80 µg/mL, their viability was reduced to 50%. Therefore, this point was selected to investigate the effect of nanoformulating ADEO into a nanogel dosage form.

Ingredients of the ADEO

The five major constituents of ADEO included p-allyanisole, cis-ocimene, beta-cimene Y, limonene, and 3-methoxycinnam aldehyde with portions of 67.623, 8.691, 7.577, 4.338, and 1.490%, respectively.

Prepared nanoemulsion-based nanogel

Ten formulations were prepared for obtaining the proper nanoemulsion with small particle size and narrow particle size distribution (SPAN < 1). Their constituents and ingredients are listed in Table 1. Only F8 (0.96 ± 0.01) and F9 (0.95 ± 0.01) had acceptable SPAN among the prepared sample. However, the particle size of F8 (7.86 ± 4 nm) was significantly lower than F9 (245 ± 13 nm); therefore, it was selected as the optimum nanoemulsion.

ADEO nanogel was prepared by the addition of carbomer 1.5% w/v to the optimum nanoemulsion. Figures of the optimum nanoemulsion and the nanogel are depicted in Fig. 2A. Besides, DLS analysis of the optimum nanoemulsion is shown in Fig. 2B.

The viscosity of the nanogel follows non-Newtonian fluids that viscosity decreases with increasing shear rate. Interestingly, viscosity changes at different shear rates follow the Carreau-Yasuda model (see Fig. 2C). Furthermore, no phase separation, sedimentation, and creaming were seen in the nanogel after 6-month storage at 4°C and ambient temperature, confirming its proper stability.

The leishmanicidal properties of the nanogel

From Fig. 3, the blank gel reduced the viability of *L. tropica* and *L. major* to 87 ± 4% and 93 ± 3%, respectively. However, the leishmanicidal effect of ADEO (80 µg/mL) was significantly better than blank gel with the viability of around 50% against both promastigotes (Independent sample t-test, sig < 0.05). Leishmanicidal effect of the

Table 1. Characteristics of the prepared nanoemulsions of ADEO

No.	Ingredients (µL)			Size analyses	
	ADEO	Tween 20	Water	Particle size	SPAN
F1	50	100	4850	54.9	8.39
F2	50	200	4750	195	1.37
F3	50	300	4650	314	1.11
F4	50	400	4550	13.6	35.27
F5	50	500	4450	8.24	1.33
F6	50	600	4350	7.62	1.01
F7	50	700	4250	7.98	1.15
F8	50	800	4150	7.86	0.96
F9	50	900	4050	245	0.95
F10	50	1000	3950	7.29	3.97

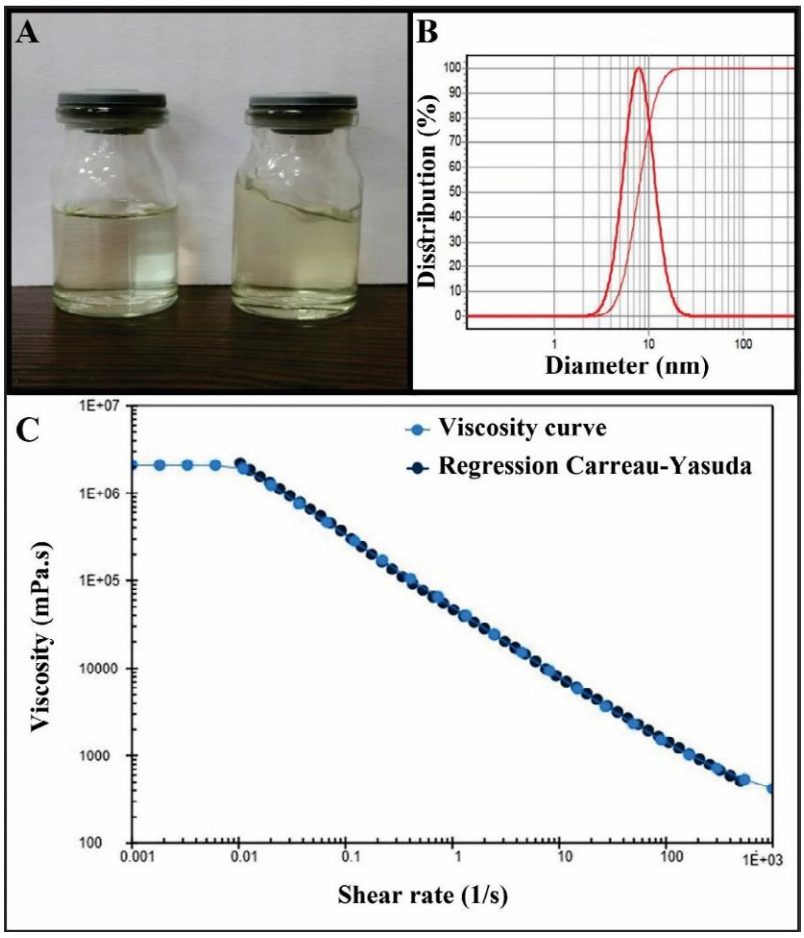


Fig. 2. Images of the optimum nanoemulsion and nanogel (A), DLS analysis of the selected nanoemulsion (B), Viscosity curve of the nanogel

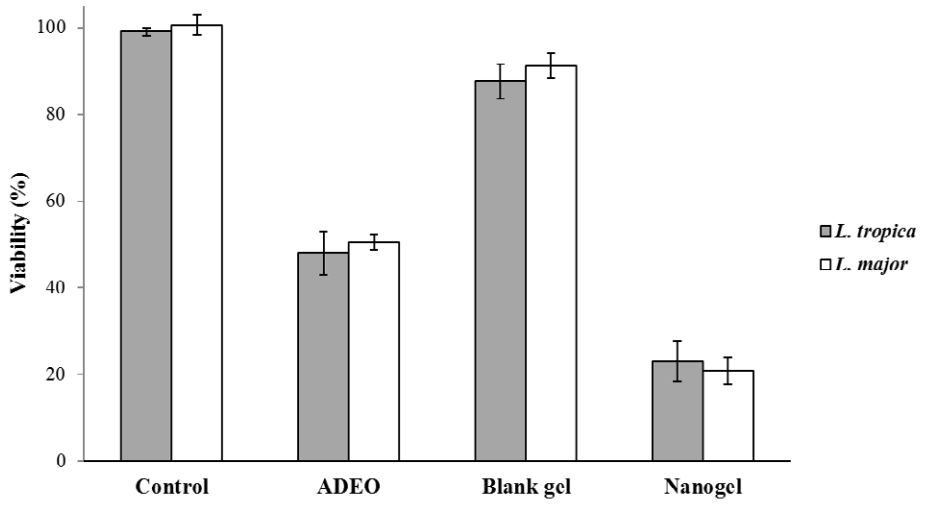


Fig. 3. Comparison of leishmanicidal activities of ADEO (80 µg/mL), nanogel (having ADEO 80 µg/mL), and blank gel

nanogel, having ADEO 80 µg/mL, significantly better than non-formulated ADEO (Independent sample t-test, sig < 0.05); viability of *L. tropica* and *L. major* were reduced to 23 ± 4% and 21 ± 3%, respectively. Interestingly, using the nanogel at a higher concentration (having ADEO 160 µg/mL), 100% efficiency was observed; viabilities of the promastigotes were reduced to 0% (Data not given).

DISCUSSIONS

From the literature, leishmanicidal activities (IC50) of some EOs against *L. tropica* have been reported. For instance *Zataria multiflora* (89.30 µg/mL), *Thymus capitellatus* (35.00 µg/mL) and *Nigella sativa* (9.30 µg/mL) [20-22]. Besides, EOs of *Citrus limon*, *Cymbopogon citratus*, and *Lavandula angustifolia* possess leishmanicidal effect against *L. major* with IC50s of 231.40, 38.00, and 110.00 µg/mL, respectively [23-25]. Considering the results, ADEO showed acceptable efficiency against the mentioned promastigotes.

Among the developed nanoformulation for topical drug delivery (nanoemulsion, liposomes, niosome, and polymeric nanoparticles), the preparation of nanoemulsions is more straightforward than others and does not require advanced equipment [16, 26]. However, nanoemulsions with low viscosity are not proper for topical applications; thickening or gelling agents were applied to increase their viscosity. Nanoemulsions are transformed into nanogel by adding a type of gelling agents such as xanthan gum, ethylcellulose, sodium carboxymethylcellulose, hydroxypropyl methylcellulose, and carbopol [27, 28].

Some reports have been found on the preparation of nanoemulsion-based nanogel of EOs or chemical drugs. For example, Quercetin's nanogel (an anti-rheumatic drug) was prepared by adding carbopol 940 (1.0% w/v) into primary nanoemulsion having a particle size of 130 nm. The drug's therapeutic effectiveness was improved by increasing skin permeability and enhancing its physicochemical stability [29]. Furthermore, by formulating *Rosmarinus officinal* EO into nanoemulsion, IC50 against *L. major* was decreased from 260 to 80 µg/mL [30]. In brief, by preparing nanoemulsion-based nanogel, at least three advantages are achievable; improvement of leishmanicidal effect, controlling EO volatility, and facilitating topical usage.

CONCLUSIONS

Leishmanicidal properties of three medicinally important EOs were investigated. Nanoemulsion-based nanogel of the most potent EO, ADEO, was then prepared. After treating the promastigotes of *L. tropica* and *L. major* with the nanogel (160 µg/mL), their viabilities were reduced to ~ 0%.

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CONFLICT OF INTEREST

No authors declared a conflict of interest.

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