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Development and *in vitro* Characterization of Nanoemulsion and Nanoemulsion-based Gel Formulations Containing *Heracleum persicum* Ethanol Extract

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ABSTRACT

Objective: We aimed to prepare the ethanol extract (Hp-Et) of the aerial parts of *Heracleum persicum* (Hp) and to determine its antioxidant capacity. We also aimed to develop nanoemulsion (NE) and NE-based gel (NEG) formulations containing this extract for topical application to the skin for wound healing and to characterize these formulations *in vitro*.

Methods: After the preparation of Hp-Et, its antioxidant capacity was determined by FRAP, CUPRAC, and DPPH methods. Then, blank NE (B-NE) and the extract-containing NE (Hp-Et-NE) formulations were developed and *in vitro* characterized [morphological analysis; centrifuge test; the determination of droplet size (DtS), polydispersity index (PDI) and zeta potential; viscosity and pH measurements; FT-IR analysis]. Additionally, B-NEG and Hp-Et-NEG were prepared and *in vitro* characterized [viscosity and pH measurements; FT-IR analysis].

Results: Droplet size and zeta potential values of NE formulations were around 200 nm and -30 mV, respectively. PDI values were less than 0.4. The pH values for NE and NEG formulations were in the range of 4.63±0.01-5.73±0.01. The NE and NEG formulations showed Newtonian and pseudoplastic behaviors, respectively.

Conclusion: Hp-Et-NEG exhibits the desired pseudoplastic behavior for topical application to the skin.

Keywords: Ethanol extract, *Heracleum persicum*, *in vitro* characterization, nanoemulsion, nanoemulsion-based gel

INTRODUCTION

Nanoemulsions (NEs), which consist of an oil phase, aqueous phase, and emulsifier/s and have a generally accepted droplet size (DtS) range of 20-200 nm, are also known as mini-emulsions and submicron emulsions and have long-term physical stability. NEs' DtS affects their stability and optical and rheological properties. It also affects the absorption/penetration of active ingredients.[1,2] Although NEs have various advantages, including increasing the solubility of low water-soluble active ingredients and improving their bioavailability, they also have disadvantages, including low retention time and spreadability due to their low viscosity. Therefore, by using a suitable gelling agent (such as Carbopol, chitosan, sodium carboxymethylcellulose), the NE is converted into a NE-based gel (nanoemulgel, NEG) and turned into a system that can be easily applied to the skin topically.[3] In recent years, studies have been carried out to prepare NE-based gel formulations for wound healing. Morsy et al.[4] prepared a NE-based gel formulation containing atorvastatin for topical application for wound healing and reported that the prepared formulation provided significant wound healing. In another study, they reported that the NE-based gel formulation containing curcumin prepared for wound healing played an important role in increasing the skin penetration and wound-healing activity of curcumin.[5]

Heracleum persicum (Hp), belongs to the family Apiaceae, is a perennial plant and widely used as a flavoring, carminative, digestive, and spice. It is a native plant in Türkiye (registered in the upper Euphrates, upper Murat-Van, Hakkari, and Adana sections).[6–8]

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Hp has various biological effects, including antimicrobial, anti-inflammatory, analgesic and antioxidant properties. In traditional medicine, this plant contains bioactive compounds such as flavonoids, furanocoumarins, triterpenes, terpenoids, anethole, and alkaloids. Moreover, it has been used for the treatment of urinary, respiratory, neurological, rheumatological, and gastrointestinal disorders.[6,7] Synergistic treatments with anti-inflammatory, antibacterial, and antioxidant, properties should be developed to promote wound healing.[9] According to traditional Persian medicine, Hp is among the plants known to have wound-healing properties. However, the wound-healing properties of Hp have not been investigated in modern research.[10]

In our study, we aimed to prepare the ethanol extract of the aerial parts of Hp (Hp-Et) and to determine its antioxidant capacity by "ferric reducing antioxidant power (FRAP)", "cupric reducing antioxidant capacity (CUPRAC)", and "DPPH radical scavenging" assays. We also aimed to develop NE and NEG formulations containing this extract for topical application to the skin for wound healing and to characterize these formulations *in vitro*.

METHODS

Materials

St. John's wort oil, Labrafac PG, Lipoid S100, Protasan UP G213 and Kolliphor RH 40 were obtained from Pharmaoils (Türkiye), Gattefossé (France), Lipoid GmbH (Germany), Novamatrix (Norway), and Sigma (USA), respectively. Ethanol, $CuCl_2.2H_2O$, diphenylpicrylhydrazyl (DPPH), tripyridyltriazine (TPTZ), neocuproine (Nc), ammonium acetate, and $FeCl_3$ were obtained from Tekkim (Türkiye), Merck Millipore (Germany), and Sigma-Aldrich (USA), respectively.

Hp-Et's Preparation and Its Antioxidant Capacity Determination

After pulverizing the dried aerial parts of Hp with a laboratory blender, we added 250 mL of ethanol to powder (10 g) and mixed it at room temperature on a magnetic stirrer (600 rpm; 24 h). After the filtration, ethanol was removed by a rotary evaporator (40 °C, 90 rpm). FRAP, CUPRAC, and DPPH radical scavenging activity assays were used to determine its antioxidant capacity.

In the FRAP assay, first, FRAP reagent was prepared freshly [0.3 N acetate buffer (pH 3.6), 10 mM TPTZ solution (in 40 mM HCl) and 20 mM $FeCl_3$ solution; 10:1:1 (v/v/v)].[11] Then, 10 µL of the sample (Trolox standard solution or Hp-Et) and 200 µL of FRAP reagent were added into the wells of the 96-well plate and incubated for 30 min at room temperature, protected from light. At the end of the period, absorbance was measured at 593 nm.[11–13]

In the CUPRAC assay, 10 mM $CuCl_2.2H_2O$ solution, 7.5 mM Nc solution and ammonium acetate (pH 7.0) buffer were prepared .[14] Then, 66 µL of the sample (Trolox standard solution or Hp-Et) was added into each well of 96-well plates containing $CuCl_2.2H_2O$ solution, Nc solution, and acetate buffer. After incubation (room temperature, protected from light; 30 min), absorbance was read at 450 nm.

In the DPPH radical scavenging assay, 70 µL of freshly prepared DPPH solution and 210 µL of the sample (Trolox standard solution or Hp-Et) were added into each well of 96-well plates. After 30 min incubation at room temperature, protected from light, absorbance measurements were carried out at 515 nm.[15] Then, the percent DPPH inhibition was calculated.[16]

NE and NEG Formulations' Preparation

A mixture of St. John's Wort oil and Lipoid S100 in a hot water bath (70 °C) was prepared and then mixed on a magnetic stirrer until it reached room temperature (750 rpm). Hp-Et (10 mg) was dissolved in Labrafac PG. Then, it was added to the oil phase and mixed it on a magnetic stirrer (750 rpm; 5 min). The aqueous phase (Kolliphor RH 40 and ultrapure water) was added to the prepared oil phase under magnetic stirring (750 rpm). Hp-Et-NE formulation was prepared by first applying high-speed mixing (Ultraturrax; 27500 rpm, 6 min) and then ultrasonication (65% power, 12 min) to reduce the DtS of the prepared coarse emulsion.

The B-NE formulation was prepared using the same procedure without adding Hp-Et. Furthermore, Protasan UP G213 (1%) was added to the NE formulations and mixed overnight on a magnetic stirrer (600 rpm) to prepare the B-NEG and Hp-Et-NEG formulations.

Characterization of NE and NEG Formulations

Centrifuge test

NE formulation (5 g) was centrifuged (15 min; 3500 rpm) to assess whether phase separation occurred.

Determination of DtS, polydispersity index (PDI), zeta potential, and pH values of formulations

The Zetasizer Nano ZSP ("Malvern Instruments Ltd., UK") was used to determine the DtS, PDI, and zeta potential values of 200-fold diluted NE formulations.

Additionally, pH measurements of NE and NEG formulations were performed using a pH meter ("Thermo Scientific, Orion 3 StarTM, USA").

FT-IR Analysis

FT-IR spectra of Hp-Et, B-NE, B-NEG, Hp-Et-NE, and Hp-Et-NEG were taken in the range of 4000-400 cm^{-1}.

Rheological analysis

Viscosity measurements of B-NE, B-NEG, Hp-Et-NE, and Hp-Et-NEG were carried out at room temperature using a Brookfield viscometer (RV DV2T; USA).

Statistical analysis

The "Independent t-test" ("SPSS Statistics Version 22.0 software; SPSS Inc., USA") was used to compare the obtained results. The results are shown as mean±standard deviation (SD) (P<.05: the difference was considered significant).

RESULTS AND DISCUSSION

FRAP, CUPRAC, and DPPH assays were used to determine the antioxidant capacity of Hp-Et. Trolox solution in ethanol was used as a standard for all three assays. Figure 1 shows the obtained *Trolox standard curves* and equations (in the concentration range of 1-100 µg/mL for FRAP and CUPRAC assays and 1-10 µg/mL for DPPH assay). We presented the total antioxidant capacity values of Hp-Et in Table 1.

In a study, antioxidant capacities of Hp Desf., *Chaerophyllum macropodum* Boiss., and *Prangos ferulacea* (L.) Lindl. were determined using DPPH assay. 50% inhibitory concentration values for *P. ferulacea, C. macropodum* and Hp were 0.242 mg/mL, 0.623 mg/mL and 0.438 mg/mL, respectively.[17]

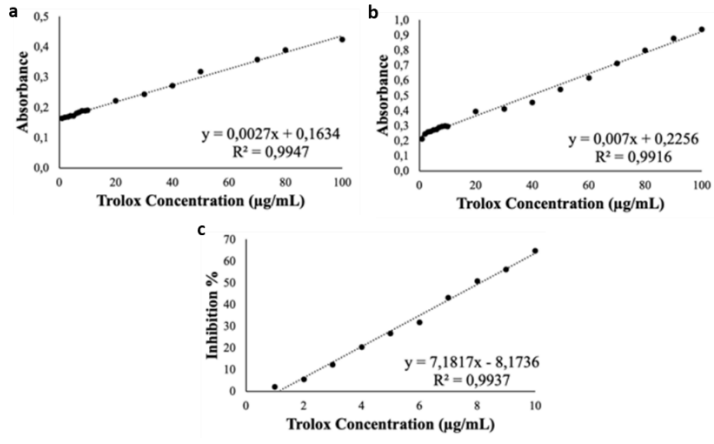

Figure 1. *Trolox standard curves* and equations for FRAP (a), CUPRAC (b) and DPPH (c) assays.

Table 1. Total antioxidant capacity values for Hp-Et (n=3; Mean±SD)

	Trolox-equivalent antioxidant capacity (µg/mL)		
	FRAP	CUPRAC	DPPH
Hp-Et (500 µg/mL)	18.44±0.11	24.25±0.58	12.60±0.08

After the centrifuge test, there was no creaming or phase separation in the B-NE and Hp-Et-NE formulations. Table 2 shows the DtS, PDI, and zeta potential values determined for B-NE and Hp-Et-NE formulations. DtS, PDI, and zeta potential affect the physical stability of NEs. The increased surface area (due to nano-sized droplets) also affects the skin penetration of active compounds.[18–20] The Brownian motions of nano-sized droplets are significant for the physical stability of NEs.[18,19]

The DtSs of the NE formulations prepared in our study were around 200 nm (Table 2). There was no statistically significant difference between the DtSs of the B-NE and Hp-Et-NE formulations (P>.05). The PDI values found for the B-NE and Hp-Et-NE formulations were less than 0.4 (Table 2). The PDI is usually less than 0.05 for highly monodisperse standards, while it is more than 0.7 for systems with broad size distribution. In this case, acceptable PDI values are in the range of 0.05-0.7, depending on the sample type.[21]

The zeta potential values of the NE formulations prepared in our study are around -30 mV (Table 2). There was no significant difference between the zeta potential values of the B-NE and Hp-Et-NE formulations (P>.05). For emulsions, zeta potential values of +30 mV and above and -30 mV and below are considered appropriate for good physical stability.[20] The TEM image of the Hp-Et-NE formulation is given in Figure 2. Nano-sized and nearly spherical droplets were obtained.

Table 2. The DtS, PDI and zeta potential values determined for B-NE and Hp-Et-NE formulations (Mean±SD; n=9)

Formulation	DtS (nm)	PDI	Zeta Potential (mV)
B-NE	204.01±11.40	0.368±0.027	-33.14±0.96
Hp-Et-NE	210.86±8.55	0.370±0.031	-33.44±1.25

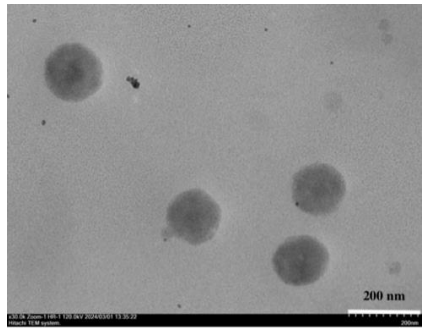

Figure 2. The TEM image of the Hp-Et-NE formulation

In addition, the pH values measured for NE and NEG formulations are given in Table 3. Human skin pH is generally acidic but can vary widely between pH 4.0 and 7.0.[22] In our study, the pH values for NE and NEG formulations range from 4.63 to 5.73 (Table 3). The difference between the pH values of B-NE and Hp-Et-NE formulations was significant (P<.05). Similarly, the difference between the pH values of B-NEG and Hp-Et-NEG formulations was also statistically significant (P<.05). It was observed that a slight decrease in the pH of the formulations occurred in the presence of the extract (Table 3). The pH of Hp-Et-NE and Hp-Et-NEG formulations was within the acceptable range for topical application to the skin.

FT-IR spectra of Hp-Et, NE, and NEG formulations are presented in Figures 3a and 3b. A broad band (at around 3300 cm^{-1}) associated with O-H groups was observed in the FT-IR spectrum of Hp-Et. In addition, we observed C-H stretching vibration at 2923 cm^{-1}, C=C and C=O stretching vibrations at 1510 cm^{-1}-1735 cm^{-1}, C–O stretching vibrations at 1035-1246 cm^{-1} and C–H bending vibrations at 1455 cm^{-1} (Figures 3a and 3b). These results are consistent with the literature.[23]

The B-NE and Hp-Et-NE formulations and similarly the B-NEG and Hp-Et-NEG formulations have similar spectra. The peaks belonging to Hp-Et were not observed in the FT-IR spectra of Hp-Et-NE and Hp-Et-NEG.

Table 3. NE and NEG formulations' pH values (Mean±SD; n=3)

Formulation	pH
B-NE	4.84±0.02
Hp-Et-NE	4.63±0.01
B-NEG	5.73±0.01
Hp-Et-NEG	5.46±0.01

The rheograms of NE and NEG formulations are shown in Figures 4a and 4b. In addition, the viscosity and "n" (flow behavior index) values determined for these formulations are given in Table 4. Viscosity affects the features such as stability, skin feel and penetration, and spreadability.[24,25] In our study, the viscosity values of B-NE and Hp-Et-NE formulations were around 2 cP (P>.05, Table 4). The viscosity values of B-NEG and Hp-Et-NEG formulations were determined as 78.19 cP and 75.97 cP, respectively (P>.05, Table 4). In addition, the "n" values were around 1 for NE formulations (n=1 Newtonian behavior[26]) and less than 1 for NEG formulations (pseudoplastic behavior[26]).

Therefore, the NE-based gel formulations which was prepared have suitable rheological properties (pseudoplastic behavior) for topical application.

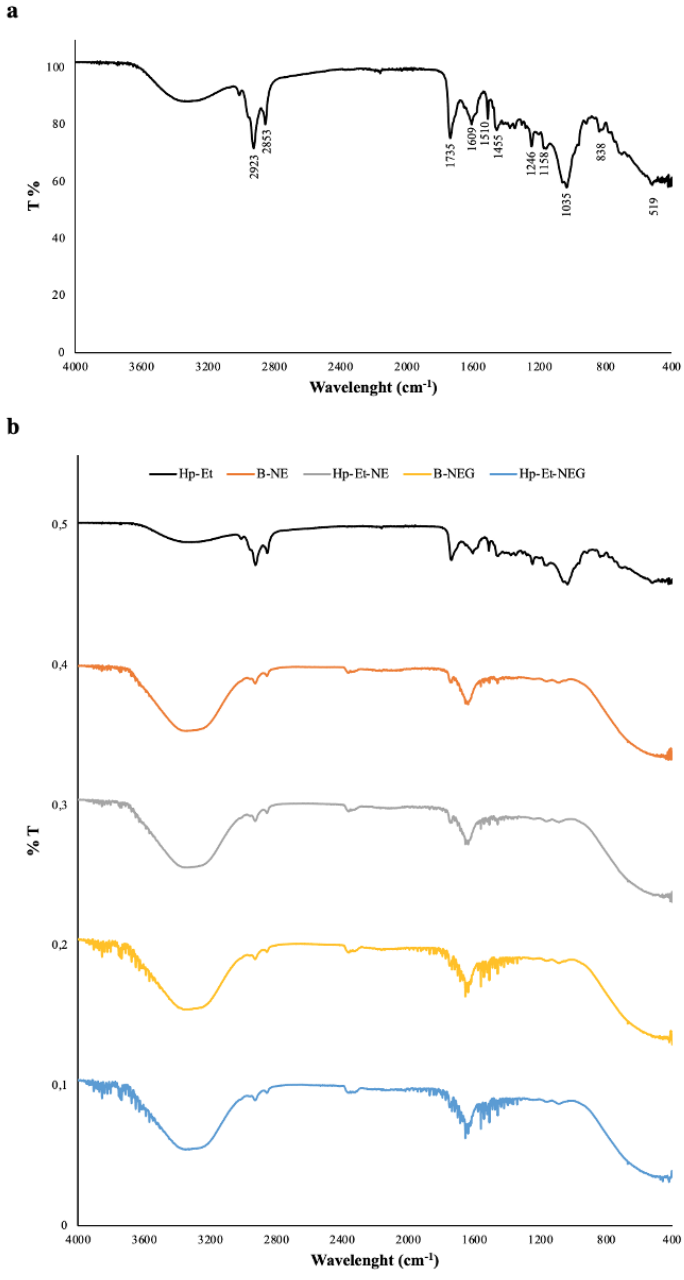

Figure 3. FT-IR spectra of Hp-Et (a and b), NE and NEG formulations (b)

Table 4. The viscosity and "n" (flow behavior index) values of the prepared formulations (Mean±SD; n=3)

Formulation	Shear rate (s^{-1})	Viscosity (cP)	n
B-NE	1500	2.14±0.05	1.012
Hp-Et-NE		2.20±0.01	1.043
B-NEG	300	78.19±1.59	0.865
Hp-Et-NEG		75.97±1.36	0.873

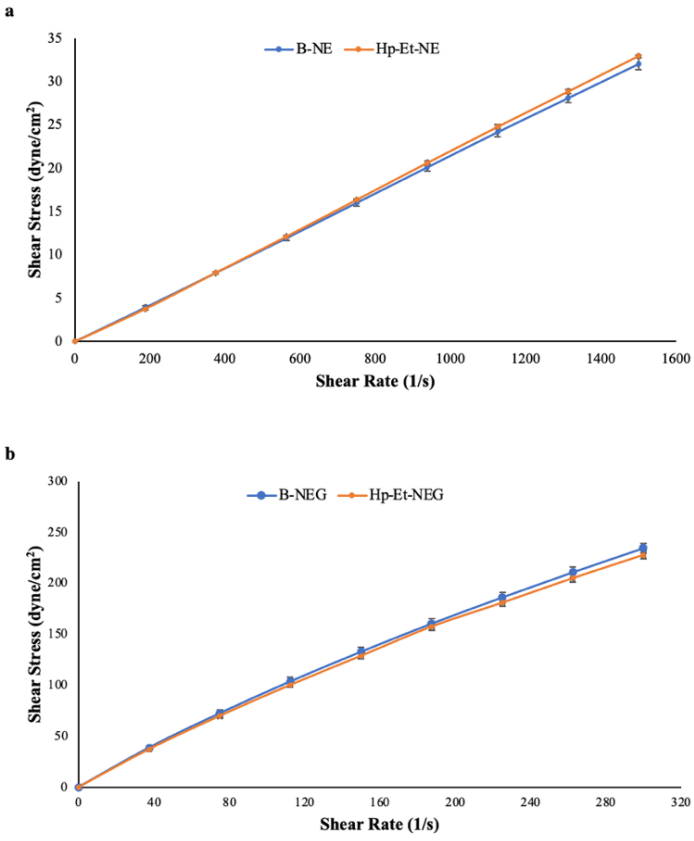

Figure 4. The rheograms of B-NE, Hp-Et-NE (a), B-NEG and Hp-Et-NEG (b) formulations (Mean±SD; n=3)

CONCLUSION

In our study, NE and NEG formulations containing Hp-Et were prepared for topical use for wound healing, and *in vitro* characterization studies were carried out. Hp-Et-NEG formulations had suitable pH and rheological properties (pseudoplastic behavior) for topical application to the skin.

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Conflict of Interest: The authors have no conflicts of interest to declare. Meltem Çetin, who is featured in this article, is also on the editorial board of the journal. This situation is considered as a relationship that may create a conflict of interest. In order to ensure an impartial and transparent refereeing process, the process was carried out without assigning an assistant editor and without transferring the author's editorial position to the referees. In addition, in order to prevent conflicts of interest, all stages of this process were managed in accordance with the journal's ethical rules and international ethical guidelines such as COPE and ICMJE.

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