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DITTRICHIA VISCOSA METHANOL EXTRACT-CONTAINING NANOEMULSION AND NANOEMULSION-BASED GEL FORMULATIONS: PREPARATION AND *IN VITRO* CHARACTERIZATION STUDIES

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Abstract: *Dittrichia viscosa* (L.) Greuter (Dv) has antimicrobial, analgesic, antioxidant, anti-inflammatory, cytotoxic, and wound-healing properties. We aimed to prepare the methanol extract (Dv-Me) of the aerial parts of Dv and to determine its antioxidant capacity by FRAP, CUPRAC, and DPPH methods. In addition, we prepared nanoemulsion (NE) and NE-based gel (NEG) formulations containing Dv-Me for topical application to the skin for wound healing and characterized these formulations *in vitro*. The antioxidant capacity of Dv-Me was determined by CUPRAC, FRAP, and DPPH methods. Then, NE formulations with/without extract (B-NE and Dv-Me-NE) were developed and *in vitro* characterized [morphological analysis; centrifuge test; viscosity and pH measurements; FT-IR analysis; the determination of zeta potential, droplet size and polydispersity index (PDI)]. Besides, B-NEG and Dv-Me-NEG were prepared and *in vitro* characterized [FT-IR analysis; viscosity and pH measurements]. The droplet size and zeta potential values of NE formulations were smaller than 185 nm and around -30 mV, respectively. PDI values were found to be less than 0.3. The pH values of Dv-Me-NE and Dv-Me-NEG were found to be 5.13±0.01 and 5.87±0.02, respectively. The NE and NEG formulations showed Newtonian and pseudoplastic behaviors, respectively. As a result, Dv-Me-NEG exhibits the desired pseudoplastic behavior for topical application to the skin.

Keywords: *Dittrichia viscosa*, *in vitro* studies, methanol extract, nanoemulsion, nanoemulsion-based gel

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1. Introduction

Dittrichia viscosa (L.) Greuter (Dv; former name: "*Inula viscosa* (L.) Aiton"), known in Turkey as 'kanser otu' or 'yapışkan andız otu', belongs to the Asteraceae family and is an herbaceous perennial plant (Brahmi-Chendouh et al., 2019; Canli Taşar, 2020; Seca et al., 2014). It is widespread on the slopes of the coastal regions of the Mediterranean (Brahmi-Chendouh et al., 2019). It has been used traditionally to treat various diseases/conditions, such as bronchitis, rheumatic pain, tuberculosis, infertility, and cancer. It also has wound-healing activity (Danino et al., 2009; Mrid et al., 2022). Dv contains various compounds, such as essential oils, sesquiterpenes, triterpenoids, lactones, flavonoids, sesquiterpene acids, and guaianolides. Therefore, it has several biological properties such as antipyretic, anti-inflammatory, antimicrobial, antidiabetic, antioxidant, and anti-scabies (Brahmi-Chendouh et al., 2019; Mrid et al., 2022; Rechek et al., 2023; Seca et al., 2014).

Excessive levels of reactive oxygen species (ROS) cause an imbalance between antioxidant defense mechanisms and cellular production of free radicals (oxidants), an increase in the inflammatory response, and wound repair inhibition. Therefore, antioxidants are significant in eliminating ROS damage during wound healing. In this sense, plant extracts containing antioxidant compounds are gaining increasing importance (Rhimi et al., 2019).

Dv has been used in Jordan and Palestine for its wound-healing effect (Khalil et al., 2007). In an in vivo study, the wound-healing effect of the aqueous extract of the aerial parts of Dv (formulated in Pluronic F127) was demonstrated by histological evaluation. They emphasized that the wound-healing effect of the extract could be due to its anti-inflammatory activity (Khalil et al., 2007). In another study, an ointment formulation was prepared containing the ethanolic extract of leaves of Dv (2.5% and 5%; w/w). They evaluated the wound healing activities of these formulations in an excision wound model in mice by applying it daily for 12 days after wound formation. They observed full re-epithelialization

and complete healing in the group treated with the 5% extract-containing ointment on day 12. They reported that antioxidants such as polyphenolic compounds support wound healing (Rhimi et al., 2019).

Nanoemulsions (NEs), which are also known as mini-emulsions and submicron emulsions, consist of an oil phase, aqueous phase, and emulsifier/s, have a generally accepted droplet size range of 20-200 nm and have long-term physical stability. NEs' droplet sizes affect their rheological properties and stability as well as active compounds' absorption/penetration (Mushtaq et al., 2023; Preeti et al., 2023). NEs, which exhibit low retention time and spreadability due to their low viscosity, are converted into an NE-based gel (NEG) using a suitable gelling agent (such as sodium carboxymethylcellulose, chitosan, Carbopol), thus making them easily applicable topically to the skin (Donthi et al., 2023). NE-based gel formulations are widely prepared for topical application for wound healing (Algahtani et al., 2021a; Morsy et al., 2019).

It was aimed to prepare the methanol extract of the aerial parts of Dv (Dv-Me) and to determine its antioxidant capacity by "cupric reducing antioxidant capacity" (CUPRAC), "ferric reducing antioxidant power" (FRAP), and "DPPH radical scavenging" assays. We also aimed to prepare and *in vitro* characterize Dv-Me-containing NE and NEG formulations for wound healing.

2. Materials and Methods

2.1. Materials

Isopropyl myristate, Lipoid S100, Labrafac PG, Kolliphor RH 40, and Protasan UP G213 were obtained from Sigma-Aldrich (Switzerland), Lipoid GmbH (Germany), Gattefossé (France), Sigma (USA), and Novamatrix (Norway), respectively. Methanol, $CuCl_2.2H_2O$, tripyridyltriazine (TPTZ), diphenylpicrylhydrazyl (DPPH), neocuproine (Nc), $FeCl_3$, and ammonium acetate were obtained from J.T. Baker (Norway), Merck Millipore (Germany), and Sigma-Aldrich (USA), respectively.

2.2. Methods

2.2.1. Preparation of Dv-Me and determination of antioxidant capacity

The dried Dv aerial parts were pulverized using a laboratory blender. Then, we added 250 mL of methanol to 10 g of powder and mixed it on a magnetic stirrer (600 rpm; at room temperature; 24 h). After the filtration, methanol was removed by a rotary evaporator (40 °C, 90 rpm). Dv-Me was stored in airtight containers at 2-8 °C, protected from light.

FRAP, DPPH radical scavenging activity, and CUPRAC assays were used to determine antioxidant capacity of Dv-Me. For the FRAP assay, the FRAP reagent was prepared freshly [0.3 N acetate buffer (pH 3.6), 20 mM $FeCl_3$ solution and 10 mM TPTZ solution (in 40 mM HCl); 10:1:1 (v/v/v)] (Spiegel et al., 2020). Then, 200 µL of FRAP reagent and 10 µL of sample (Trolox standard solution or Dv-Me) were added into the wells of the 96-well plate. After 30 min incubation (protected from light; room temperature), we read the absorbance at 593 nm (Benzie and Strain, 1996; Büyüktuncel, 2013; Spiegel et al., 2020).

For the CUPRAC assay, 10 mM $CuCl_2.2H_2O$ solution, 7.5 mM Nc solution, and ammonium acetate (pH 7.0) buffer were prepared (Apak et al., 2006). Then, $CuCl_2.2H_2O$ solution (60 µL), Nc solution (60 µL), acetate buffer (60 µL), and the sample (Trolox standard solution or Dv-Me) (66 µL) was added into each well of 96-well plates, respectively. After incubation (30 min; room temperature; protected from light), we read the absorbance at 450 nm.

For the DPPH radical scavenging assay, we added 210 µL of the sample (Trolox standard solution or Dv-Me) and 70 µL of freshly prepared DPPH solution into each well of 96-well plates. After 30 min incubation (protected from light; room temperature), the absorbance was read at 515 nm (Mekky et al., 2017). Then, we calculated the percent DPPH inhibition (Salar et al., 2015).

2.2.2. NE and NEG formulations' preparation

We prepared a mixture of Lipoid S100 (2%) and isopropyl myristate (8%) in a hot water bath (70 °C). Then, we mixed it on a magnetic stirrer (600 rpm) until it reached room temperature. We added Dv-Me (10 mg) in Labrafac PG (2%) to the oil phase and mixed on a magnetic stirrer (5 min; 600 rpm). After adding the aqueous phase consisting of Kolliphor RH 40 and ultrapure water to the oil phase under magnetic stirring (600 rpm), high-speed mixing for 7 min (Ultraturrax; 27500 rpm) and ultrasonication for 14 min (55% power) were applied to reduce the droplet size of the obtained emulsion and prepare the Dv-Me-NE formulation, respectively.

The blank NE (B-NE) formulation was prepared using the same procedure without adding Dv-Me.

To prepare NEG formulations (B-NEG or Dv-Me-NEG), Protasan UP G213 (1%) was added to the B-NE or Dv-Me-NE formulations and mixed overnight on a magnetic stirrer (600 rpm).

2.2.3. NE and NEG formulations' characterization

First, NE formulation (5 g) was centrifuged (15 min; 3500 rpm) to assess whether phase separation occurred. Then, we used the Zetasizer Nano ZSP ("Malvern Instruments Ltd., UK") to determine the zeta potential, droplet size, and PDI values of NE formulations (100-fold diluted). pH and viscosity measurements of NE and NEG formulations were carried out at room temperature with a pH meter ("Thermo Scientific, Orion 3 StarTM, USA") and a Brookfield viscometer (RV DV2T; USA), respectively. In addition, FT-IR spectra of Dv-Me, B-NE, B-NEG, Dv-Me-NE, and Dv-Me-NEG were taken in the range of 4000-400 cm^{-1}. The TEM image of NE formulation containing Dv-Me (Dv-Me-NE) was also obtained ("Hitachi HighTech HT7700, Japan").

2.3. 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) ($p<0.05$: statistically significant).

3. Results and Discussion

With the increase in chronic diseases such as peripheral vascular diseases and diabetes in the world, the frequency of acute or chronic wounds has also increased. Topical drug application provides significant advantages in wound treatment. The topical application of active ingredients for wound healing offers direct access to affected areas. NE-based gel formulation has attracted considerable attention in recent years as an effective delivery system that can facilitate the topical application of active compounds and improve the therapeutic efficacy of these compounds in wound healing (Ahmad et al., 2019; Algahtani et al., 2021b).

Dv has antimicrobial, analgesic, antioxidant, anti-inflammatory, cytotoxic, and wound healing properties. Mssillou et al. (2022) showed that the hydroalcoholic extract of Dv achieved nearly complete wound healing on the 21st day in burn-wounded rats.

In light of this information, NE and NE-based gel formulations containing Dv-Me for wound healing were prepared and characterized *in vitro* in our study. First, the methanol extract of Dv was prepared and its total antioxidant capacity was evaluated using FRAP, CUPRAC and DPPH assays. Trolox solution in methanol was used as a standard for all three assays. We presented the obtained Trolox standard curves and equations (in the concentration range of 1-10 µg/mL for DPPH assay and 1-100 µg/mL for FRAP and CUPRAC assays) in Figure 1. Table 1 shows the total antioxidant capacity values of Dv-Me.

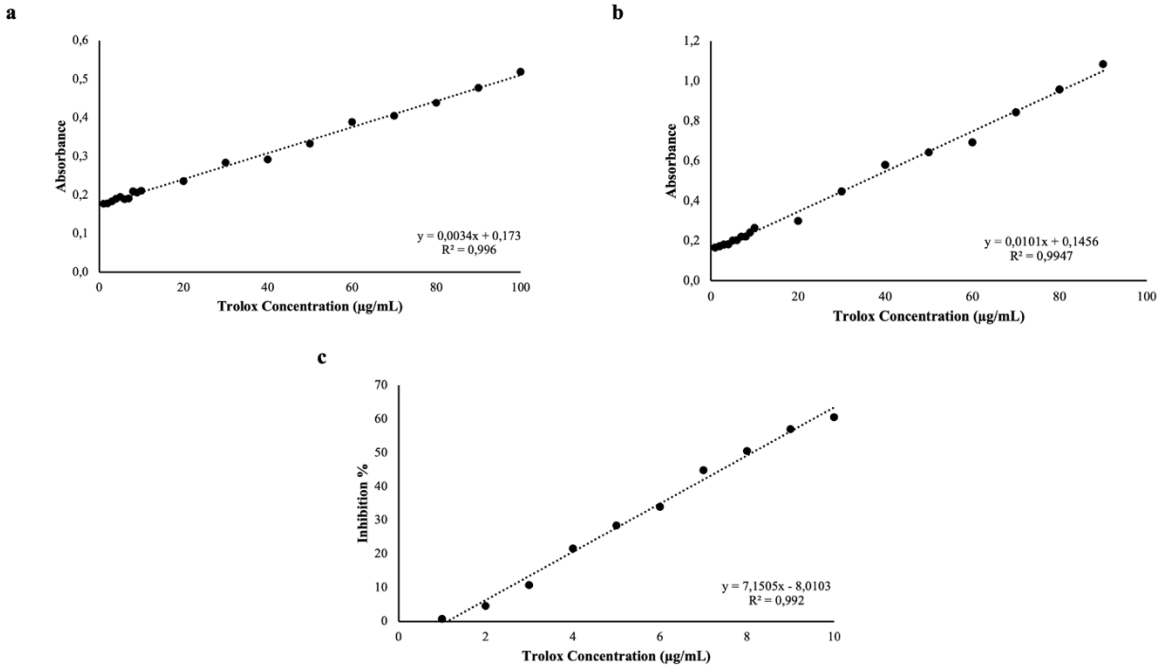

Figure 1. Trolox standard curves and equations for FRAP (a), CUPRAC (b) and DPPH (c) assays

Table 1. Dv-Me's total antioxidant capacity values (Mean±SD; n=3)

Dv-Me (500 µg/mL)	Trolox-equivalent antioxidant capacity (µg/mL)		
	FRAP	CUPRAC	DPPH
	51.58±0.32	72.22±2.17	42.65±0.36

SD= standard deviation

Then, we prepared the NE formulation containing Dv-Me and first evaluated whether there was phase separation in the NE formulations after centrifugation. There was no phase separation in the prepared NE formulations after centrifugation. The zeta potential, droplet size and PDI values are significant parameters for the physical stability of NE formulations. In addition, the increased surface area (due to nano-sized droplets) also affects the skin penetration of active compounds (Ashaolu, 2021; Solans et al., 2005; Ugur Kaplan et al., 2019). Thus, we determined these parameters. The droplet sizes of the NE formulations (B-NE and Dv-Me-NE) were smaller than 185 nm (Table 2). The droplet sizes of B-NE and Dv-Me-NE formulations were statistically different ($p<0.05$). An increase in the droplet size of NEs occurred depending on the presence of the extract. Additionally, the PDI values found for B-NE and Dv-Me-NE formulations were smaller than 0.3 (Table 2), indicating the droplet size distribution of NE formulations was in an acceptable narrow range ("Ugur Kaplan et al., 2019"). The zeta potential values of the NE formulations were around -30 mV (Table 2). There was a decrease in the zeta potential value of NE formulation in the presence of Dv-Me. The zeta potential values of B-NE and Dv-Me-NE formulations were

statistically different ($p<0.05$). The absolute zeta potential values of 30 mV and above provide good physical stability for emulsions (Mahamat Nor et al., 2017). The TEM image of the Dv-Me-NE formulation (Figure 2) showed that nano-sized and approximately spherical droplets were obtained.

Table 2. The zeta potential, droplet size, and PDI values determined for the NE formulations (Mean±SD; n=9)

Formulation	Droplet Size (nm)	PDI	Zeta Potential (mV)
B-NE	145.08±9.01	0.238±0.044	-32.10±1.36
Dv-Me-NE	182.12±8.04	0.258±0.038	-28.46±1.69

SD= standard deviation

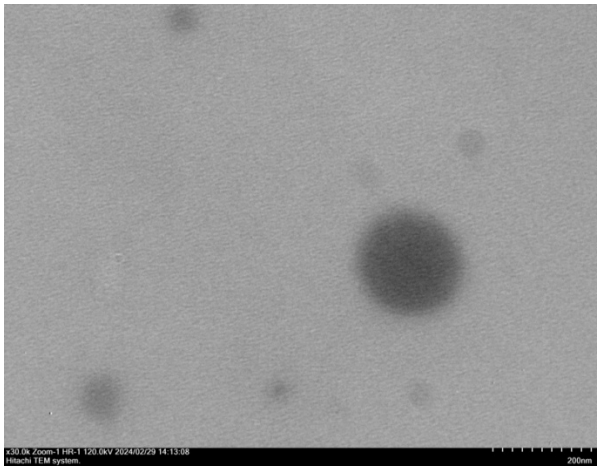

Figure 2. The TEM image of NE formulation containing Dv-Me (Dv-Me-NE).

The pH of human skin is typically acidic, but it can range from 4.0 to 7.0 (Lambers et al., 2006). The formulations with very low or very high pH values cause skin irritation. In our study, Table 3 shows that the pH values of NE and NE-based gel formulations were ranged from 5.13 to 6.10. The pH values of B-NE and Dv-Me-NE or B-NEG and Dv-Me-NEG formulations were statistically different ($p<0.05$). There was a slight decrease in the pH values of the formulations in the presence of the extract (Dv-Me). As a result, the pH values of NE and NEG formulations containing Dv-Me are suitable for topical application to the skin.

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

Formulation	pH
B-NE	5.24±0.02
Dv-Me-NE	5.13±0.01
B-NEG	6.10±0.02
Dv-Me-NEG	5.87±0.02

SD= standard deviation

FT-IR spectra of Dv-Me, NE, and NEG formulations are presented in Figures 3a and 3b. In the FT-IR spectrum of Dv-Me, there was a broad band associated with O-H groups at around 3200-3300 cm^{-1}. In addition, we observed C-H stretching vibration at 2923 cm^{-1}, C–O stretching vibration at 1155 cm^{-1}, C–H bending vibration at 1469 cm^{-1}, C=O and C=C stretching vibrations at 1692 cm^{-1} and 1623 cm^{-1}, respectively (Figures 3a and 3b). These results are consistent with the literature (Kebir et al., 2015; Kouache et al., 2022). The B-NE and Dv-Me-NE formulations and the B-NEG and Dv-Me-NEG formulations have similar spectra. The peaks belonging to Dv-Me were not observed in the FT-IR spectra of NE and NEG formulations containing the extract.

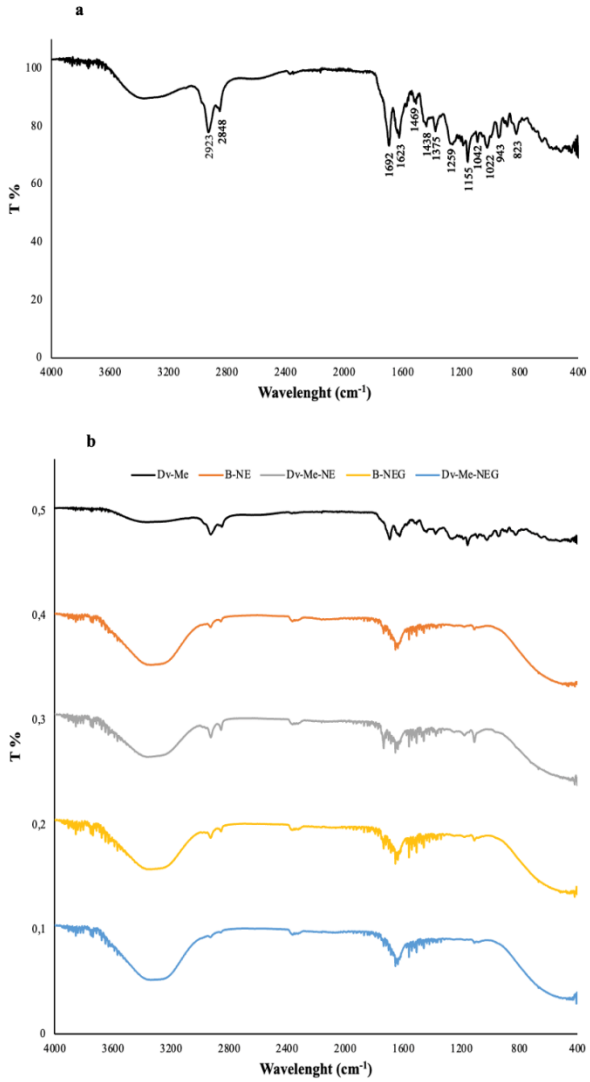

Figure 3. FT-IR spectra of Dv-Me (a and b), NE and NEG formulations (b)

The viscosity of NEs is a function of the components in the formulation (oil, water, and surfactant) and their concentrations (Lovelyn and Attama, 2011). The viscosity and "n" (flow behavior index) values determined for NE and NEG formulations are given in Table 4. The rheograms of these formulations are shown in Figures 4a and 4b. The B-NE and Dv-Me-NE

formulations' viscosity values were 2.56 cP and 2.79 cP, respectively. A slight increase in viscosity of the formulation was observed in the presence of the extract ($p<0.05$, Table 4). To overcome the obstacles of low viscosity and spreadability of NE, NE-based gel formulations are widely prepared. The viscosity values of B-NEG and Dv-Me-NEG formulations were 72.31 cP and 75.31 cP, respectively ($p>0.05$, Table 4). In addition, while the "n" value was around 1 for NE formulations, it was less than 1 for NEG formulations. n=1 represents Newtonian behavior; n<1 represents pseudoplastic flow behavior (Teixeira et al., 2017). As a result, the NE-based gel formulations have suitable rheological properties for topical application.

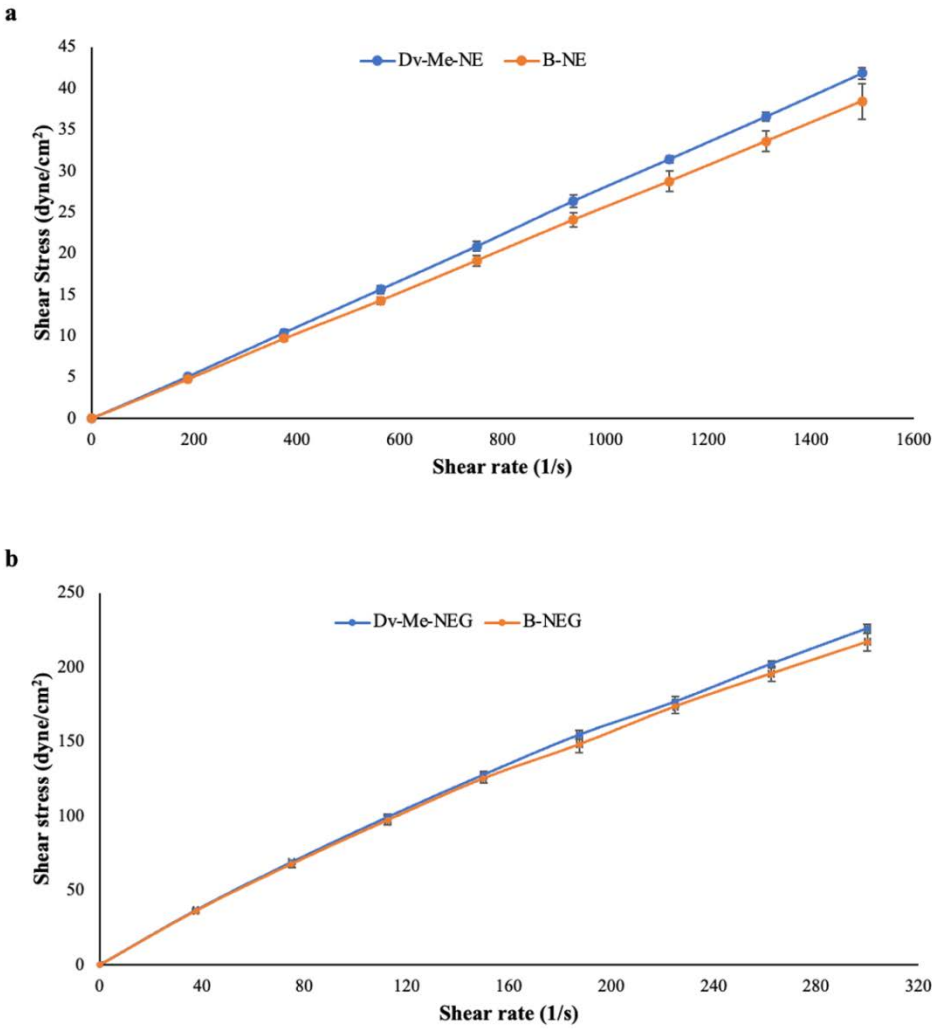

Figure 4. The rheograms of the NE formulations (a) and the NEG formulations (b) (Mean±SD; n=3)

Table 4. The viscosity and "n" values of NE and NEG formulations (Mean±SD; n=3)

Formulation	Shear rate (s^{-1})	Viscosity (cP)	n
B-NE	1500	2.56±0.03	1.003
Dv-Me-NE		2.79±0.09	1.013
B-NEG	300	72.31±1.98	0.860
Dv-Me-NEG		75.31±0.99	0.872

SD= standard deviation

4. Conclusion

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

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	F.S.	A.B.U.K.	Y.F.K.	M.C.
C	-	-	-	100
D	-	50	-	50
S	-	-	-	100
DCP	25	25	25	25
DAI	-	50	-	50
L	25	25	25	25
W	10	20	10	60
CR	-	40	-	60
SR	-	50	-	50
PM	-	-	-	100
FA	-	50	-	50

C=Concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

Ethics committee approval was not required for this study because of there was no study on animals or humans.

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