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Development of controlled delivery systems by nanoliposomes of *Hypericum perforatum* L. extracts

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ABSTRACT

Hypericum perforatum L. is a popular and widespread medicinal plant used in a wide range of therapy, including gastrointestinal diseases, heart diseases, and skin-related diseases. The rapid development of nanotechnology and its applications in pharmacology have enabled the controlled release of drugs and bioactive components. This study aimed to investigate liposomal formulation for controlled release enriched with methanol and ethanol extract of *H. perforatum*, which has antioxidant, antimicrobial, and proliferative effects. In this context, firstly, the biological activity (antimicrobial, antioxidant, and cell viability) of *H. perforatum* methanol (Hp-MeOH) and ethanol (Hp-EtOH) extracts obtained by ultrasonic extraction method was revealed. Hp-MeOH and Hp-EtOH extracts have a larger zone of inhibition against *Enterococcus faecalis* ATTC 51289 and *Pseudomonas aeruginosa* ATCC 11778, respectively than the positive control amikacin (30mg/ml). Hp-MeOH and Hp-EtOH extracts were found to have a high total antioxidant status and low total oxidant status and oxidative stress index value. Hp-MeOH and Hp-EtOH extracts have a scavenging capacity of DPPH radicals between 23-89% and 27-90%, respectively in the studied concentration range. In addition, the effect of Hp-MeOH and Hp-EtOH extracts on cell viability of dermal fibroblast cells was evaluated for 24, 48, and 72 hours and induction of proliferation of fibroblasts was observed. Highly stable liposomes were successfully developed which encapsulated 82.6 ± 3.63% and 89.8 ± 2.74% Hp-MeOH and Hp-EtOH extracts, respectively. Liposomal structures loaded with Hp-MeOH and Hp-EtOH extracts showed a more controlled and slower release than the free extract.

1. Introduction

In recent years, encapsulation of bioactive compounds and natural plant extracts using nanotechnology has been the focus of pharmaceutical, cosmetic, and food industries and researchers. Encapsulation of plant-based bioactive compounds and crude plant extract may be a useful tool to prevent their degradation and instability due to light, oxygen, and free radicals and to enhance their effective delivery (Tripathy & Srivastav, 2023). Plant extracts contain alkaloids, phenolic acids, polyphenols, proteins, and terpenoids that have important effects and functions such as antioxidant, antimicrobial, anti-inflammatory, and anticancer. Therefore, they have been used as natural therapeutic and complementary substances since ancient times (Sharma et al., 2022). In addition, bioactive plant extracts have significant advantages over synthetic components, such as fewer toxic side effects, high bioavailability, and cheap and easy availability (Parham et al., 2020). To maintain these important properties in a stable form for a long time and to act at the desired site at an effective dose in a controlled manner, plant extracts or their components are combined with biocompatible delivery systems (Rahman et al., 2020). Liposomes, one of the biocompatible and controlled release systems, are small lipid-

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based vesicles with a phospholipid bilayer. These lipid-based vesicles are enormously versatile as they can load several molecules, protect them from degradation, modify their pharmacokinetics and improve the bioavailability profile and eventually improve the therapeutic effect of the incorporated components (Luiz et al., 2023). Liposomes are mainly composed of phospholipids and these components are generally regarded as safe (GRAS) since they are present in the human body, as well as in food products that we consume (Naziris & Demetzos, 2022). Liposomes are prepared using various and combined methods. The choice of method depends on factors such as physicochemical properties, effective concentration and toxicity level, and large-scale production from a pilot plant. The initial loading of the phytochemicals to be encapsulated can be carried out during the preparation of liposomes (passive loading) or by placement across the transmembrane by the carrier (active loading). Liposome production methods include hydration techniques, sonication methods, and solvent evaporation methods (ether injection, ethanol injection, and reverse phase evaporation methods). In addition, micro fluidization and lyophilization are prominent in industrial-scale liposome production (Dua et al., 2012; Maja et al., 2020).

Widely used in folk and modern medicine, *Hypericum perforatum* L. is a perennial herb belonging to the Hypericaceae family. *H. perforatum* is associated with antidepressant, anti-inflammatory, antimicrobial, antiseptic, antioxidant, antitumoral, analgesic, antineoplastic, metabolic syndrome-improving action, and wound-healing activity. Thanks to its essential oils, flavonoids, phenolic acids, naphthodiantrons (hypericin and pseudohypericin), and phloroglucinols (hyperforin and adhyperforin), it has a traditional use in the treatment of wounds, ulcers, and burns (Ali et al., 2018; Nobakht et al., 2022).

Recently, plant extracts have come to the forefront in the development of pharmaceutical formulations to be used in the treatment of many diseases. In this context, this study aimed to produce and characterize liposome structures from *H. perforatum* extract, which has versatile effects and biological activity. Thus, carrier structures with controlled release properties have been created in which this plant, which has a wide range of uses, can be included in pharmaceutical formulations. The pharmacokinetic properties of this lipid carrier system loaded with *H. perforatum* extract can be improved or its therapeutic effects can be increased by combining it with different plant extracts.

2. Materials and methods

2.1. Materials

Dimethyl sulfoxide (DMSO) (D4540), phosphate-buffered saline (PBS) (806552), Folin-Ciocalteu's reagent (47641), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D9132), gallic acid (91215), and quercetin (Q4951) were all obtained from Sigma-Aldrich (USA). For the cell experiments, a human dermal fibroblast (HDF) cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco modified eagle medium (DMEM) (11880028), penicillin/streptomycin (15140122), and fetal bovine serum (FBS) (A3840101) was obtained from GIBCO (Grand Island, NY, USA). For the cell viability and proliferation analyses, an MTT assay kit (M5655) was purchased from Sigma-Aldrich (USA).

2.2. Plant material and extract preparation

H. perforatum (Hp) plants were collected from Afyonkarahisar: Erkmen region (Turkey) in June 2022 and authenticated by Prof. Dr. Mustafa Kargioglu. A voucher specimen (No: AKU-10964) was deposited at the Archives of Afyon Kocatepe University Herbarium. The aerial parts of *H. perforatum* were dried and powdered. The modified ultrasonic extraction method was used to prepare 50% (v/v) aqueous methanol and 50% (v/v) aqueous ethanol extracts of *H. perforatum* (Hp-MeOH and Hp-EtOH) (Latiff et al., 2021).

2.3. Evaluation of antimicrobial activity

2.3.1. Test microorganisms

The antimicrobial tests were performed using referenced strains such as *Listeria monocytogenes* ATCC 1911, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* NRRLB 4420, *Pseudomonas aeruginosa* ATCC 11778, *Enterococcus faecalis* ATCC 51289, *Escherichia coli* ATCC 35218, *Bacillus subtilis* NRS-744, *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600 and *Candida albicans* ATCC 10231.

2.3.2. Antimicrobial activity

The Clinical and Laboratory Standards Institute (CLSI) disc diffusion assay was used to test antimicrobial activity in vitro (CLSI, 2016). Inhibition zone diameters (mm) were measured and evaluated in comparison with the control group. The lowest concentration of a drug that inhibits a microorganism's apparent growth is known as the minimum inhibitory concentration (MIC) value. Microdilution assay following the methods described by the Clinical and Laboratory Standards Institute was used to determine MIC of Hp-MeOH and Hp-EtOH extracts, which leads to inhibition of the growth of the test bacteria (CLSI, 2012) and fungus (CLSI, 2002). The lowest extract concentration that induced 50% cytotoxicities (50% inhibition of microorganism growth) was determined as the MIC value. Penicillin G (10 mg/ml), amikacin (30 mg/ml), and fluconazole (10 mg/ml) were used as positive controls, and distilled water (dH_2O) was used as the negative control.

2.4. Determination of in vitro TAS, TOS, and OSI values

Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) values of the Hp-MeOH and Hp-EtOH extracts were assessed using commercially available Rel Assay Diagnostic kits (Erel, 2004, 2005). Trolox and hydrogen peroxide standards were used as references for TAS and TOS analyses, respectively. *Eq. 1* was used while calculating the oxidative stress index [OSI (Arbitrary Unit = AU)] value (Erel, 2005).

$$OSI\ (AU) = \frac{TOS\ (\mu mol\ H2O2\ equiv./l)}{TAS\ (mmol\ Trolox\ equiv./l)} x\ 10 \qquad Eq.\ 1$$

2.5. Total phenolic content (TPC)

The total phenolic content of the Hp-MeOH and Hp-EtOH extracts was determined according to the Folin–Ciocalteu method with minor changes (Gamez-Meza et al., 1999) and gallic acid as a standard. 150 µl of suitably diluted extract samples, calibration solutions, and blank solution, as well as 150 µl of Folin-Ciocalteu's reagent, were added to each test tube. The mixture was thoroughly mixed, and after 10 min, 3 ml of Na_2CO_3 solution (2% w/v, in water) was added. The mixture was stored at room temperature for 30 min. After incubation, the absorbance of the mixture was measured at 760 nm in a UV spectrophotometer. The results were calculated using the standard calibration curve of gallic acid (R^2 = 0.9971) and expressed as gallic acid equivalents (mg GAE/g).

2.6. Determination of DPPH radical scavenging capacity

The radical scavenging capacity of Hp-MeOH and Hp-EtOH extracts was measured by the DPPH assay in vitro with minor changes (Blois, 1958). Briefly, a solution of 0.135 mM DPPH radical in methanol was prepared. 100 µl of DPPH radical solution was mixed with increasing concentrations (39.06 - 5000 µg/ml) with 100 µl of plant extracts and incubated for 30 mins at room temperature in the dark. After incubation, the absorbance of the reaction mixture was measured at 517 nm. A mixture of DPPH and methanol were used as the control, a mixture of samples and methanol was used as blank, and quercetin in similar concentrations with samples was used as the positive control. Percentage (%) inhibition was calculated using *Eq. 2*. The DPPH free radical scavenging activity of the plant extracts was determined by the inhibition percentages using the quercetin standard curve (R^2 =0.9787).

$$Inhibition\ (\%) = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} x\ 100 \qquad Eq.\ 2$$

2.7. In-vitro cell viability assay

In vitro cell viability tests of Hp-MeOH and Hp-EtOH extracts obtained by ultrasonic extraction method were examined using Human Dermal Fibroblast Cell Line (PCS-201-012) at different concentrations for 24, 48, and 72 h. The viabilities of the cells were studied with an MTT assay (Kumar et al., 2018). Briefly, cells at the 70-80% confluency were trypsinized and seeded onto a 96-well plate at the cell/well density of 1 × 10^4. The medium (DMEM complete with 10% FBS and 1% penicillin-streptomycin) was then replaced with a medium containing Hp-MeOH and Hp-EtOH extracts incubated for 24, 48, and 72 hours at 37 °C in humidified air containing 5% CO_2. After the incubation period, the MTT reagent was added to wells and incubated at 37 °C for two more hours at dark in 5% CO_2. After incubation, the MTT reagent was removed from the cells, DMSO was added to each well, and the plate was shaken at low speed for 5 minutes at room temperature. Absorbance values were measured with a microplate reader at a wavelength of 570 nm and cell viability was calculated. Untreated cells were used as the control (cells in wells with no sample other than DMEM medium) and were considered 100% viable. All studies were carried out in triplicate and in vitro test data were statistically analyzed using GraphPad Prism 9 with One-Way ANOVA and Tukey's multiple comparisons tests. The data were presented as a mean with a 95% confidence interval (CI). '*p*' values less than 0.05 were deemed statistically significant.

2.8. Preparation of Hp-MeOH and Hp-EtOH extracts loaded liposomes

Hp-MeOH and Hp-EtOH extracts-loaded liposomes were fabricated using the ethanol injection method (Khoshraftar et al., 2020). 30 ml of ethanol (96%) and 1 g of phosphatidylcholine were constantly agitated at 50 °C for 1 hour until the lecithin was completely dissolved. A rotary evaporator was then used to evaporate the solvent under reduced pressure. Plant extract (1.5 g) was combined with the sample after being dissolved in 36 ml of phosphate buffer at room temperature. Then, the prepared liposomes were subjected to ultrasonication at 40% amplitude for 5 minutes in ultrasonic and after the sample was centrifuged, PBS and liposomes were separated. The samples were refrigerated overnight, followed by centrifugation at 6000 rpm for 10 min. The samples were dried in an oven dryer at 50 °C for 24 h.

2.9. Characterization of Hp-MeOH and Hp-EtOH extracts loaded liposomes

The zeta potential of liposomes was determined (25 °C, 90° angle) by Zeta-Potential and Mobility Meter. Also, the polydispersity index (PDI) and mean size of Hp-MeOH and Hp-EtOH extracts loaded liposomes were measured using the dynamic light scattering (DLS) technique.

2.10. Entrapment efficiency (EE%) of Hp-MeOH extracts loaded liposomes

Assessment of entrapment efficiency (EE%) was carried out using the modified centrifugation method (Khoshraftar et al., 2020). Hp-MeOH and Hp-EtOH extracts were initially measured at 520 nm with UV-Vis spectrophotometry, the wavelength at which the Hp-MeOH extract has the greatest adsorption. To construct a calibration curve, absorbance versus concentration of Hp-MeOH extract at different concentrations dissolved in dH_2O was prepared. The EE (%) was calculated by measuring free Hp-MeOH and Hp-EtOH extracts in the supernatant following liposome centrifugation. The samples were centrifuged for 30 minutes at 4 °C at 4000 rpm. UV/VIS spectrophotometry a 520 nm was used to determine the concentration of free Hp-MeOH and Hp-EtOH extracts in the supernatant.

The EE% of the Hp-MeOH and Hp-EtOH extracts was calculated using *Eq. 3*:

$$EE\ (\%) = \frac{AEa}{AEb} x\ 100 \qquad Eq.\ 3$$

where AEa is the amount of extract (Hp-MeOH and Hp-EtOH) in the liposome found after centrifugation, AEb is the amount of extract (Hp-MeOH and Hp-EtOH) in liposomes found before centrifugation.

2.11. In vitro release kinetics of Hp-MeOH and Hp-EtOH extracts loaded liposomes

In vitro release kinetics of free plant extracts and plant extracts loaded liposomes were carried out utilizing the modified dialysis technique (Khoshraftar et al., 2020). The analysis was carried out in 100 ml of PBS (pH = 7.4) at 37 °C. Extracts loaded liposomes were filled in a cellulose dialysis bag (MW cut-off 12.000). After, the dialysis bag was placed in an isolated environment and mixed with the specified settings. 1 ml of the released content was withdrawn at a specified time and replaced with an equal amount of fresh PBS solution. 1 ml of the release medium was gathered and measured at 520 nm wavelength. The dialysis behavior of free plant extract mixed with PBS was examined using the same method. The free plant extract in the supernatant at each preset period was used to calculate the plant extract release rate from liposomes (*Eq. 4*).

$$In\ vitro\ release\ (\%) = \frac{Total\ amount\ of\ plant\ extract - The\ residue\ of\ plant\ extract}{Total\ amount\ of\ plant\ extract} x\ 100 \qquad Eq.\ 4$$

3. Results and discussion

3.1. Antimicrobial activity of Hp-MeOH and Hp-EtOH extracts

The antimicrobial activity of Hp-MeOH and Hp-EtOH extracts was tested against pathogenic microorganisms (Tables 1 and 2). The results show a significant concentration-dependent antimicrobial activity against a wide range of pathogenic microorganisms. Hp-MeOH extract induced a larger inhibition zone against *S. aureus* ATCC 25923, *E. faecalis* ATCC 51289, *B. subtilis* NRS 744, *S. aureus*

ATCC 6538 and *C. albicans* ATCC 10231 than other pathogens. Hp-EtOH caused a larger inhibition zone against *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 11778, *E. faecalis* ATCC 51289, *E. coli* ATCC 35218, and *E. coli* ATCC 25922 than other pathogens. Among all pathogenic microorganisms tested, Hp-MeOH and Hp-EtOH extracts induced the highest inhibition zone against *E. faecalis* ATCC 51289 (21 mm ± 0.75) and *P. aeruginosa* ATCC 11778 (28 mm ± 0.90), respectively than the positive control amikacin (30mg/ml).

Table 1. Antimicrobial activity of Hp-MeOH extract (mg/ml)*

Test microorganisms	Inhibition zone (mm ± STD)					PC1	PC2	NC
	9.37	18.75	37.5	75	150			
L. monocytogenes ATCC 19115	10 ± 0.25	11 ± 0.25	12 ± 0.35	13 ± 0.25	15 ± 0.50	25 ± 0.80	15 ± 0.60	-
S. aureus ATCC 25923	11 ± 0.20	12 ± 0.25	13 ± 0.25	15 ± 0.33	17 ± 0.50	29 ± 0.90	12 ± 0.10	-
K. pneumoniae NRRLB 4420	-	8 ± 0.10	8 ± 0.25	9 ± 0.20	9 ± 0.25	28 ± 0.50	14 ± 0.40	-
P. aeruginosa ATCC 11778	11 ± 0.20	12 ± 0.25	14 ± 0.40	15 ± 0.33	16 ± 0.70	30 ± 0.80	15 ± 0.20	-
E. faecalis ATCC 51289	13 ± 0.40	15 ± 0.50	18 ± 0.65	20 ± 0.50	21 ± 0.75	24 ± 0.50	14 ± 0.20	-
E. coli ATCC 35218	9 ± 0.25	11 ± 0.50	10 ± 0.25	12 ± 0.15	13 ± 0.15	30 ± 0.50	13 ± 0.40	-
B. subtilis NRS 744	8 ± 0.25	10 ± 0.25	14 ± 0.50	18 ± 0.50	20 ± 0.50	30 ± 0.50	19 ± 0.70	-
S. aureus ATCC 6538	11 ± 0.10	12 ± 0.50	13 ± 0.50	15 ± 0.50	17 ± 0.50	32 ± 0.70	16 ± 0.50	-
E. coli ATCC 25922	8 ± 0.10	9 ± 0.25	11 ± 0.25	14 ± 0.35	15 ± 0.50	30 ± 0.80	16 ± 0.50	-
P. aeruginosa ATCC 27853	11 ± 0.20	12 ± 0.20	13 ± 0.50	14 ± 0.25	16 ± 0.25	21 ± 0.30	14 ± 0.30	-
S. aureus ATCC 12600	11 ± 0.20	12 ± 0.20	13 ± 0.25	15 ± 0.50	16 ± 0.25	38 ± 1.20	15 ± 0.20	-
C. albicans ATCC 10231	13 ± 0.20	14 ± 0.50	15 ± 0.50	16 ± 0.45	18 ± 0.50	30 ± 0.80	-	-

* for *C. albicans* ATCC 10231; PC_1: Fluconazole (10 mg/ml), NC: dH_2O; for other test microorganisms; PC_1: Penicillin G (10 mg/ml), PC_2: Amikacin (30 mg/ml), NC: dH_2O

Table 2. Antimicrobial activity of Hp-EtOH extract (mg/ml)*

Test microorganisms	Inhibition zone (mm ± STD)					PC1	PC2	NC
	9.37	18.75	37.5	75	150			
L. monocytogenes ATCC 19115	12 ± 0.20	19 ± 0.50	21 ± 1.00	23 ± 0.50	26 ± 0.50	25 ± 0.80	15 ± 0.60	-
S. aureus ATCC 25923	9 ± 0.00	15 ± 0.50	18 ± 0.50	20 ± 0.50	23 ± 1.00	29 ± 0.90	12 ± 0.10	-
K. pneumoniae NRRLB 4420	-	-	8 ± 0.00	9 ± 0.00	10 ± 0.30	28 ± 0.50	14 ± 0.40	-
P. aeruginosa ATCC 11778	10 ± 0.50	15 ± 0.40	17 ± 0.60	18 ± 0.30	28 ± 0.90	30 ± 0.80	15 ± 0.20	-
E. faecalis ATCC 51289	10 ± 0.30	17 ± 0.60	20 ± 0.50	23 ± 0.50	25 ± 0.50	24 ± 0.50	14 ± 0.20	-
E. coli ATCC 35218	-	12 ± 0.50	15 ± 0.50	17 ± 0.20	20 ± 0.30	30 ± 0.50	13 ± 0.40	-
B. subtilis NRS 744	8 ± 0.00	14 ± 0.20	15 ± 0.50	16 ± 0.50	18 ± 0.00	30 ± 0.50	19 ± 0.70	-
S. aureus ATCC 6538	10 ± 0.50	12 ± 0.40	14 ± 0.50	15 ± 0.00	17 ± 0.20	32 ± 0.70	16 ± 0.50	-
E. coli ATCC 25922	7 ± 0.00	10 ± 0.30	18 ± 0.70	20 ± 0.50	22 ± 0.20	30 ± 0.80	16 ± 0.50	-
P. aeruginosa ATCC 27853	10 ± 0.10	12 ± 0.80	13 ± 0.50	15 ± 0.50	17 ± 0.50	21 ± 0.30	14 ± 0.30	-
S. aureus ATCC 12600	9 ± 0.50	10 ± 0.00	12 ± 0.40	14 ± 0.20	19 ± 0.30	38 ± 1.20	15 ± 0.20	-
C. albicans ATCC 10231	10 ± 0.30	12 ± 0.20	13 ± 0.50	15 ± 0.50	16 ± 0.40	30 ± 0.80	-	-

* for *C. albicans* ATCC 10231; PC_1: Fluconazole (10 mg/ml), NC: dH_2O; for other test microorganisms; PC_1: Penicillin G (10 mg/ml), PC_2: Amikacin (30 mg/ml), NC: dH_2O

MIC values of Hp-MeOH and Hp-EtOH extracts against pathogenic microorganisms are given in Table 3. When the antimicrobial activity values of the active substances are 0.1 mg/ml or less, it is considered to have high antimicrobial activity, in the range of 0.1 < MIC ≤ 0.625 mg/ml, it shows moderate antimicrobial activity, and when the MIC value is more than 0.625 mg/ml, it is considered to have weak antimicrobial effects (Awouafack et al., 2013; Kuete, 2010). In general; Hp-MeOH extract has low antimicrobial activity on *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 11778, *E. faecalis* ATCC 51289, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600 and *C. albicans* ATCC 10231 (MIC < 5 mg/ml). Hp-EtOH extract has low antimicrobial activity on *L. monocytogenes* ATCC 19115, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 11778, *E. faecalis* ATCC 51289, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853, and *C. albicans* ATCC 10231 (MIC < 5mg/ml). In a study conducted by Conforti et al. (2005), *H. perforatum* methanol extract was shown to have potent antimicrobial activity on the gram-positive and negative bacteria (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212 and *E. coli* ATCC 4350) with an MIC value of 50 µg/ml. Besides, Süntar et al. (2016) determined the MIC values of *H. perforatum* ethanol extract against *S. mutans* ATCC 21752, *S. sobrinus* ATCC 6715, and *E. faecalis* ATCC 29912 as 64, 16, 32 and 32 µg/ml, respectively. Eroğlu-Özkan et al. (2019) determined the MIC values of *H. perforatum* methanol extract against *S. aureus* ATCC 6538 and *S. epidermidis* ATCC 12228 as 4.8 and 156 µg/ml, respectively, while the same extract had no antimicrobial effect on *E. coli* ATCC 8739, *K. pneumoniae* ATCC 4352, *P. aeruginosa* ATCC 1539, and *C. albicans* ATCC 10231.

Table 3. MIC values of Hp-MeOH and Hp-EtOH extracts

Microorganisms	MIC values (mg/ml)	
	Hp-MeOH	Hp-EtOH
L. monocytogenes ATCC 19115	9.37	2.34
S. aureus ATCC 25923	4.68	4.68
K. pneumoniae NRRLB 4420	37.50	37.50
P. aeruginosa ATCC 11778	4.68	4.68
E. faecalis ATCC 51289	2.34	4.68
E. coli ATCC 35218	37.50	18.75
B. subtilis NRS 744	18.75	9.37
S. aureus ATCC 6538	4.68	4.68
E. coli ATCC 25922	37.50	18.75
P. aeruginosa ATCC 27853	4.68	4.68
S. aureus ATCC 12600	4.68	9.37
C. albicans ATCC 10231	4.68	2.34

3.2. In vitro TAS, TOS, and OSI values of Hp-MeOH and Hp-EtOH extracts

The antioxidant power of plants or plant extracts is due to their ability to eliminate or transform free radicals. It can be assumed that the higher the antioxidant capacity of a plant or plant extract, the higher its therapeutic potential (Michalak, 2022; Teixeira et al., 2017). TAS, TOS, and OSI values of Hp-MeOH and Hp-EtOH extracts are given in Table 4. It was determined that the Hp-MeOH extract had high total antioxidant status (22.352 ± 0.002 mmol/l), and low total oxidant status (4.753 ± 0.001 µmol/l). In addition, the oxidative stress index (OSI), which is important in evaluating the oxidant/antioxidant ratio of Hp-MeOH extract, was observed to be very low (0.21). Hp-EtOH extract had a high total antioxidant status value (18.804 ± 0.150 mmol/l) and a low total oxidant status value

(4.523 ± 0.120 µmol/l). OSI value of Hp-EtOH was found to be 0.24. In many studies on a plant extract, the TAS value of *Mentha* sp. was observed as 3.628 mmol/l, TOS value was 4.046 µmol/l (Sevindik et al., 2017). The TAS and TOS values of *Salvia multicaulis* Vahl. were 6.434 mmol/l and 22.441 µmol/l, respectively (Pehlivan & Sevindik, 2018). Also, TAS, TOS, and OSI values of *Gundellia tournefortii* L. have been reported as 6.831 mmol/l, 3.712 ± 0.584 µmol/l, and 0.054 ± 0.463 µmol/l, respectively (Saraç et al., 2019). Compared to these studies, TAS values of Hp-MeOH and Hp-EtOH extracts were found higher than these plants. Hp-MeOH extract can be stated as a good source of antioxidants since its extracts have a high antioxidant capacity and low OSI. Hp-MeOH and Hp-EtOH extracts can be expressed as good source of antioxidants since it has a high antioxidant capacity and OSI. Therefore, it can be evaluated in eliminating the negative effects caused by free radicals.

Table 4. TAS, TOS, OSI and TFC values of Hp-MeOH and Hp-EtOH extracts

	Hp-MeOH	Hp-EtOH
TAS (mmol Trolox /l)	22.352 ± 0.002	18.804 ± 0.15
TOS (µmol H_2O_2 /l)	4.753 ± 0.001	4.523 ± 0.12
OSI	0.21	0.24
TFC (mg GAE/g)	38.00 ± 0.02	10.52 ± 0.028

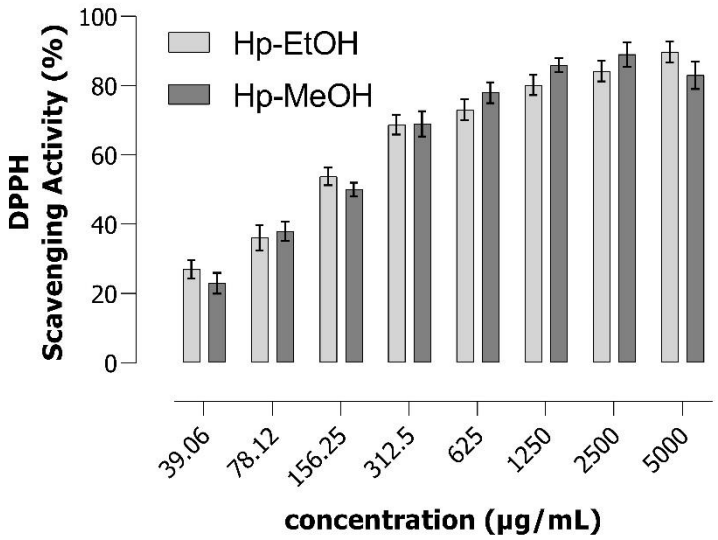

Figure 1. DPPH scavenging activity of Hp-EtOH and Hp-MeOH extracts

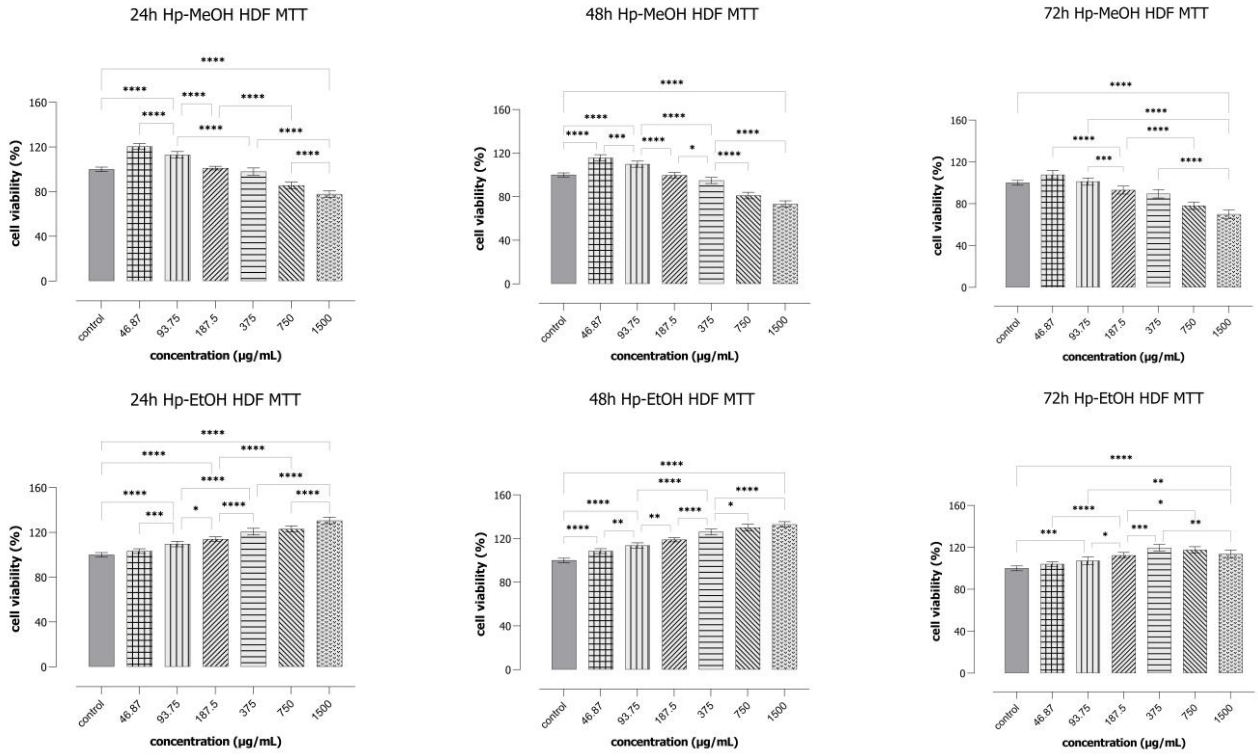

Figure 2. Cell viability (%) of Hp-MeOH and Hp-EtOH extracts (*p* value below 0.05 were considered as statistically significant)

3.3. Total phenolic content of Hp-MeOH and Hp-EtOH extracts

Phenolic compounds are important bioactive components with redox properties responsible for antioxidant activity. Hydroxyl groups in plant extracts help to scavenge free radicals (Soobrattee et al., 2005). The amount of phenolic compounds varies depending on the extraction procedure, solvents, and growing conditions of the plant and plant parts. Phenolic compounds are more soluble in polar organic solvents due to the presence of a hydroxyl group (Isidore et al., 2021). The phenolic content in the Hp-MeOH and Hp-EtOH extracts were measured using the Folin-Ciocalteu reagent and was determined as 38.00 mg GAE/g and 10.52 mg GAE/g, respectively. Comparing the studies in the literature, Sekeroglu et al. (2017) reported the total phenol content (TPC) of the powdered stem, leaves, and flower of *H. perforatum* ethanol and water extract, in which the ethanol extracts from the leaf parts of the *H. perforatum* showed the highest total phenolic content (182.93 mg GAE/g). In another study, the total polyphenolic content of fresh ethanolic extracts of *H. perforatum* was found to be between 9.33 - 50.98 mg GAE/g (Chimshirova et al., 2019). In a study by Brankiewicz et al. (2023), total phenolic content of *H. perforatum* extracts of different origins (both commercially available and laboratory-prepared from wild grown) was evaluated. Both, commercially available and laboratory-prepared *H. perforatum* ethanol extracts showed approximately 8 - 10% of phenolic content in the dry extract.

3.4. DPPH radical scavenging capacity of Hp-MeOH and Hp-EtOH extracts

There are various methods to evaluate the antioxidant capacity of natural plant extracts. The DPPH assays are widely used by many researchers for the rapid evaluation of antioxidant capacity (Pisoschi et al., 2016). The concentration-dependent DPPH scavenging capacity of Hp-MeOH and Hp-EtOH extracts is given in Figure 1. Hp-MeOH and Hp-EtOH extracts had a scavenging capacity of DPPH radicals between 23 - 89% and 27 - 90%, respectively in the studied concentration range (39.06 - 5000 µg/ml). In the study conducted by Mir et al. (2019), the capacity of *H. perforatum* methanolic extract to scavenge DPPH radicals at concentrations of 100, 200, and 300 µg/ml was determined as 35, 55 and 70%, respectively, and ascorbic acid, used as a positive control, inhibited radicals by 68% at a concentration of 300 µg/ml. Moreover, Yilmaz et al. (2021) determined that the DPPH free radical scavenging activity of *H. perforatum* dH_2O extract at 100 µg/ml was 89.3% and gallic acid and trolox used as standards inhibited DPPH radicals by 95.3% and 96.5%, respectively. It is difficult to directly compare the results of the present study with previous studies due to the use of different standards (quercetin equivalent or trolox equivalent, etc.) for the expression of DPPH scavenging capacity. On the other hand, the type of solvent and extraction conditions affect the results.

3.5. Cell viability of Hp-MeOH and Hp-EtOH extracts

The results of cell viability of HDFs upon treatment with the Hp-MeOH and Hp-EtOH extracts are given in Figure 2. When treated with 46.87, 93.75, 187.5, 750, and 1500 µg/ml of Hp-MeOH extract for 24-h, the cell viability of HDF was decreased by 120.58%, 112.99%, 101.12%, 97,95%, 85.58%, and 77.79%, respectively. A similar decrease was also evident by 115.76%, 109.7%, 99.69%, 94.86%, 81.08%, and 73.42% for each concentration at the end of 48th h. Cell viability in HDF cultures for the last time point 72nd h experiment was recorded as 107.83%, 101.24%, 93.11%, 89.58%, 78.15%, and 70.06% after 46.87, 93.75, 187.5, 750, and 1500 µg/ml of Hp-MeOH extract, respectively. In general, the viability of fibroblast cells decreased compared to the control due to the increase in the concentration of Hp-MeOH extract for 24, 48, and 72 hours. At 24th h, cell viability was higher than the control group at concentrations lower than 375 µg/ml. At 48th h, a similar trend was observed. At 72nd h, cell viability at concentrations lower than 187.5 µg/ml was higher than in the control group. Furthermore, % cell viability values at 72nd h were lower than at 24 and 48 hours due to both the decrease in nutrients to be used by the cells and the accumulation of toxic substances in the environment as a result of the metabolic activities of the cells. Dermal fibroblast cell viability of Hp-EtOH extract for 24th, 48th, and 72nd h was enhanced with increasing concentration. Hp-EtOH extract showed higher cell viability than the control group at all three-time points at all concentrations of the extract. Thus, it was determined that Hp-EtOH extract increased fibroblast cell viability and had no cytotoxic effect. The cytotoxic effect of methanolic extract of *H. olympicum* (HOM) (0.5, 1, 1.5, and 2 mg/ml) was evaluated in HDF cells for 24, 48, and 72 hours. It was reported that HOM extract can be safely used up to 2 mg/ml for HDFs without causing any cytotoxicity during the 24, 48, and 72 h of treatment periods. It was also observed that 0.5 and 1.5 mg/ml HOM treatment increased the cell viability of HDFs by 35% and 108%, respectively (Kurt-Celep et al., 2020). In a study, phloroglucinol-enriched fractions (PEF) of *Hypericum* species native to southern Brazil were evaluated for their effect on the proliferation of HaCaT cells. The *H. carinatum* and *H. polyanthemum* PEF demonstrated better results with an increase in cell proliferation (138.7% and 120.6%, respectively) (Bridi et al., 2017).

Table 5. Particle size, PDI value, and ζ-potential of Hp-MeOH and Hp-EtOH extracts-loaded liposomes

Plant extract-loaded liposomes	Average particle size (nm)	PDI value	ζ-potential (mV)
Hp-MeOH	~ 64 nm	0.59	-27.9
Hp-EtOH	~ 15 nm	0.65	-20.0

3.6. Characterization of Hp-MeOH and Hp-EtOH extracts loaded liposomes

Liposomes were used as control delivery systems for the methanol and ethanol extract obtained from the areal parts of *H. perforatum*. The mean diameter and PDI value of the liposomes were measured using the dynamic laser light scattering technique (Table 5). Extract-loaded liposomes were prepared and characterized. The mean diameter of Hp-MeOH and Hp-EtOH extracts loaded liposomes were measured at ~ 64 nm and ~ 15 nm; the PDI values of extracts were found as 0.59 and 0.65, respectively. Hp-MeOH and Hp-EtOH extracts-loaded liposomes were not homogeneously dispersed (polydispersity index > 0.50). The zeta potential is a useful tool for determining the distribution stability of liposomes. Characterizing the surface charge of the particles, provides information about the repulsive forces between them, allowing the stability of dispersions to be predicted. As a general rule, zeta potential values < -30 mV and > 30 mV form stable systems as high surface charges induce repulsion and prevent aggregation. Liposomes with a negative surface charge are beneficial for effective dermal drug delivery (Gharib et al., 2017; Gillet et al., 2011; Rafiee et al., 2017). Hp-MeOH and Hp-EtOH extracts loaded liposomes had highly negative zeta potential values of -27.9 mV and -20.0 mV, respectively (Table 5). In a study, phospholipid vesicles loaded with *H. scruglii* extract were developed and characterized. The *H. scruglii* loaded phospholipid vesicles disclosed mean diameter ranged from 120 to 160 nm, they were homogeneously dispersed (polydispersity index ≤ 0.30), and their zeta potential was highly negative (~ -45 mV). It has been reported to show suitable physicochemical properties for dermal delivery (Allaw et al., 2020). In a study by Pradeep et al. (2019),

liposome structures loaded with *H. hookerianum* extract were developed, and it was reported that highly stable liposome structures were obtained thanks to the properties of the liposomal formulation obtained with a zeta potential of -29.4 mV and an average particle size of 82.33 ± 0.41nm.

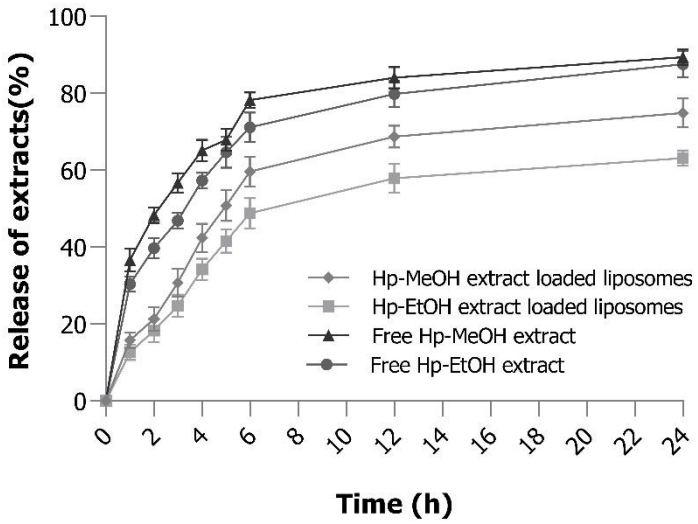

Figure 3. Release profile of Hp-MeOH and Hp-EtOH extracts from liposomes

3.7. Entrapment efficiency (EE%) of Hp-MeOH and Hp-EtOH extracts loaded liposomes

The entrapment efficiency of liposomes loaded Hp-MeOH and Hp-EtOH extracts was determined approximately as 82.6 ± 3.63% and 89.8 ± 2.74%, respectively. The amount of extract incorporated into *H. scruglii*-loaded phospholipid vesicles was found to have a very high entrapment efficiency of ~ 89% (Allaw et al., 2020). In this context, it can be said that liposomes loaded with Hp-MeOH and Hp-EtOH extracts have a high entrapment efficiency.

3.8. Release profile of Hp-MeOH and Hp-EtOH extracts loaded liposomes

During 24th h, the amount of extracts released by the liposomes and the release of extracts in the free state were evaluated and the release profile is given in Figure 3. When the release profile is evaluated, it is seen that extracts-loaded liposomes have a slower and more controlled release profile compared to free extracts. At the end of the first 6th h, 78.20% and 71.10% of the free Hp-MeOH and Hp-EtOH extracts were released, respectively while 59.6% and 48.8% of the Hp-MeOH and Hp-EtOH extracts were released from the liposome structures. At the end of 24th h, a very large amount of free Hp-MeOH and Hp-EtOH extracts were released (89.40% and 87.50%), while 74.9% and 63.1% of the Hp-MeOH and Hp-EtOH extracts loaded in liposome structures were released. When the release of phospholipid vesicles loaded with *H. scruglii* was evaluated, it was reported that a burst release reaching 20% of the initial amount was observed at the 2nd h; then, it became almost constant until the 24th h, reaching ~ 35% (Allaw et al., 2020).

4. Conclusions

The results obtained in this study revealed that *H. perforatum* methanol and ethanol extract encapsulated in liposomes is highly stable and also have the combined advantages of effective antioxidant capacity and maintenance of cell viability on dermal fibroblast cells. The results obtained indicate that liposomal plant extracts can serve as a highly useful, controlled release, and stable release vehicle for novel antioxidant components. Additional information from this study supports the use of *H. perforatum* methanol and ethanol extract as an aid in the treatment of skin diseases. In light of this study, the pharmacokinetic properties of this lipid carrier system loaded with *H. perforatum* extracts can be improved or its therapeutic effects can be increased by combining it with different plant extracts. In this way, it can be incorporated into pharmaceutical formulations for the treatment of many diseases.

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Conflict of interest

The authors confirm that there are no known conflicts of interest.

Statement of ethics

In this study, no method requiring the permission of the "Ethics Committee" was used.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary File

None.

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