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# A LIPOSOMAL CONTROLLED DELIVERY SYSTEM FOR *PLANTAGO MAJOR* L. EXTRACTS AND THEIR BIOLOGICAL ACTIVITY FOR WOUND HEALING

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**ABSTRACT**

The objectives of this study are to determine the effective features of water and aqueous-ethanol extracts of *Plantago major* L. (Pm-d$H_2O$ and Pm-EtOH, respectively) in wound healing, such as antimicrobial, antioxidant potential, and fibroblast proliferation. Furthermore, it was targeted to obtain liposome structures loaded with Pm-extract for controlled release, characterize the obtained liposomes, and determine their release profiles. Antimicrobial activity (inhibition zone and minimum inhibition concentration), total antioxidant and oxidant (TAS, TOS, and OSI) potentials, total phenolic content (TPC), and total flavonoid content (TFC) of Pm extracts were tested. The radical scavenging activity of Pm extracts was quantified by the DPPH assay. Antioxidant capabilities of Pm extracts were determined with ABTS, CUPRAC, and FRAP. In vitro cytotoxicity tests of Pm extracts were examined on dermal fibroblast cells with MTT assay. Liposome structures loaded with Pm extracts were obtained using the ethanol injection method. Plant extract-loaded liposomes were characterized, and in vitro release kinetics were revealed by dialysis technique.

Pm-EtOH extract inhibited the growth of microorganisms at lower concentrations than Pm-d$H_2O$ extract. According to TAS, TOS, and OSI values, it was concluded that there was a moderate antioxidant effect in Pm extracts in general. TPC and TFC of the Pm-EtOH extract are higher than the Pm-d$H_2O$ extract. Inhibition percentages of Pm-EtOH extract are higher than Pm-d$H_2O$ extracts against DPPH free radicals. The ABTS, CUPRAC, and FRAP antioxidant capacities of the Pm-EtOH extract are higher than that of the Pm-d$H_2O$ extract. Furthermore, according to dynamic light scattering and microscopy images, these plant extracts loaded liposomes had a high entrapment efficiency, a wide size range, and good stability.

Pm extracts showed high cell viability and no cytotoxic effect on HDF cells. Liposome structures loaded with Pm extracts are fine alternatives for effective and controlled release to the wound site due to high entrapment efficiency, a wide size range, and good stability.

**Keywords:** Antimicrobial activity, Antioxidant activity, Fibroblast proliferation, Liposome production, Liposome characterization, *Plantago major* L. extracts

## INTRODUCTION

Medicinal plants, whose important effects have been known since antiquity, are widely utilized as a natural treatment for several diseases, involving inflammation, in treating skin-related diseases, and in the healing of wounds ***(Farcas et al., 2020)***. Recently, attention to the therapeutic potential of medicinal plants has increased owing to their low toxicity and fewer side effects compared to synthetic compounds. A genus including over 200 species, *Plantago* herbs are recognized worldwide as medicinal plants. *Plantago* species commonly utilized in modern medicine: *P. asiatica*, *P. lanceolata*, *P. media*, *P. major*, *P. indica*, *P. sempervirens*, *P. ovata*, and generally these genera are indicated by a wide variety of constituent phytochemicals, however, the most general is iridoid glycosides, flavonoids, hydroxycinnamic acids, alkaloids, xylose, terpenoids, terpenes and saponins (leaves), polysaccharides, vitamins, unsaturated fatty acids, and galacturonic acid (mucilage seeds) ***(Fierescu et al., 2021)***. Part of the family Plantaginaceae, *P. major* L. has been demonstrated to be a strong antioxidant, antimicrobial, and anti-inflammatory and has immune-modulating properties and functions to treat all types of wounds to treat burns, stop bleeding, and speed up the healing process ***(Abd-Razik et al., 2012)***. *P. major* L. includes biologically active compounds as well as polysaccharides, flavonoids, terpenoids, iridoid glycosides, lipids, caffeic acid derivatives, alkaloids, and some organic acids ***(Jamilah et al., 2012)***. Indeed, the utilization of plants or derived phytochemicals to treat various diseases has been used since the earliest times. It is believed that approximately 25% of all medications administered globally are derived from plants. Nevertheless, the use of herbal therapies causes issues as well as reduced solubility and restricted absorption and bioavailability. Furthermore, bioactive chemicals are frequently subjected to in vivo hydrolysis, oxidation, photolysis, and low stability during processing and storage, necessitating the development of stabilization platforms ***(Baranauskaite et al., 2018)***. Such constraints have been circumvented by colloidal delivery techniques such as nanoemulsions, nanogels, and nanoparticles (***Chen et al., 2021***; ***Liu et al., 2021***). Nonetheless, the application of these colloidal delivery techniques in certain areas may be restricted due to the increasing requirements for desired biocompatibility. In addition, research on liposome-based delivery systems has increased due to their cellular delivery, lipid bilayer structure, and also excellent biocompatibility. Liposomes are biocompatible vesicular systems that can be prepared from lipids with tunable physicochemical properties and also provide slow release at the target site over long periods. One of the foremost significant preferences of liposomes are suitable candidates for encapsulation and delivery of plant extracts as they can contain both lipophilic and hydrophilic complexes ***(Sogut et al., 2021)***. Furthermore, the obtainment of the feasible in vitro/in vivo effect depends on the characteristic properties of the liposomal formula, i.e. encapsulated drug dosage concentration, size distribution, polydispersity index, or zeta potential ***(Danaei et al., 2018)***. The target of this study was to provide an original perspective on the extraction of P. major L. by liposomal-controlled delivery systems. In this context, firstly water and aqueous-ethanol extracts were acquired from the fresh leaves of the plant collected from Ankara-Turkey by ultrasonic extraction method. The effective properties of both extracts in wound healing, such as antimicrobial and antioxidant potential, were revealed by various methods. In addition, the cytotoxic effects of these extracts on human dermal fibroblast cells were assessed. These extracts, whose biological properties and biocompatibility were determined, were loaded into liposome compositions by ethanol injection technique to provide controlled release in the wound area. The surface morphologies, zeta potential, and size distributions of the obtained liposome structures were characterized, and their release kinetics and express encapsulation efficiencies were evaluated.

## MATERIAL AND METHODS

### General experimental procedures and chemicals

The extracts were evaporated utilizing a rotary evaporator (Heidolph, Darmstadt-Germany) and freeze-dried plant extract powder with freeze-drier (LABCONCO,

Kansas-ABD). *In vitro* cytotoxicity test absorbance values were determined with a microplate reader (Gen5 BioTek, Epoch-USA). The obtained liposomes were ultrasonicated with an ultrasonic processor. (SONIC VC505, Thermo Fisher Scientific, Porto Salvo). The surface morphologies of liposomes were observed through transmission electron microscopy (JEOLJEM 1220) and the zeta potential was determined by Zeta-Potential and Mobility Meter (MALVERN Nano ZS90). The polydispersity index and mean size of liposomes were evaluated by using dynamic light scattering (DLS) (MALVERN CGS-3). The biological activity assays were read in a multifunctional microplate reader (Thermo Scientific™ Multiskan Go™, USA). The chemical reagents and standards were supplied by Sigma-Aldrich® and Merck, Germany. Biological reagents were supplied by Sigma-Aldrich®, Germany.

**Plant material**

The fresh leaves of *P. major* L. (Pm) were collected from A4 Ankara: Hacettepe University Sıhhiye Campus, from 39.933021, 32.864095 region (Turkey) in June 2021 authenticated by Prof. Dr. İffet İrem Çankaya (Hacettepe University, Faculty of Pharmacy, Department of Pharmaceutical Botanics, Sıhhiye, Ankara). Voucher specimens were stored in the Herbarium of Hacettepe University (HUEF21015).

**Preparation of plant extracts**

The fresh leaves of *P. major* L. were dried, and the leaves were powdered. Pm-d$H_2O$ and Pm-EtOH extracts were obtained by slightly modifying the ultrasonic extraction method. ***(Latiff et al., 2021).***

**Antimicrobial susceptibility test**

***Test microorganisms***

These tests were carried out utilizing the reference strains containing *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, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 12600 and *Candida albicans* ATCC 10231

***Antimicrobial activity***

Antimicrobial activity was evaluated by disc diffusion test as specified by the Clinical and Laboratory Standards Institute ***(CLSI, 2016).*** Inhibition zone diameters (mm) were calculated and then compared with the control group (positive control: Penicillin G (10 mg/mL), Amikacin (30 mg/mL) and Fluconazole (10 mg/mL) negative control: DMSO+d$H_2O$).

***Determination of minimum inhibitor concentration (MIC)***

The minimal drug concentration that inhibits the growth of a microorganism is called the MIC value. Microdilution assay as described by the Clinical and Laboratory Standards Institute (CLSI, 2006) was utilized to define the MIC value of Pm extracts leading to inhibition of test microorganisms [(positive control: Penicillin G (10 mg/mL), Amikacin (30 mg/mL) and Fluconazole (10 mg/mL) negative control: DMSO 50% d$H_2O$ (v/v)]

**Determination of total antioxidant and oxidant (TAS, TOS, and OSI) potentials**

Total oxidant status (TOS), Total antioxidant status (TAS), and oxidative stress index (OSI) of Pm extracts were determined by Rel Assay Diagnostic kits. ***(Erel, 2004; Erel, 2005)***. Trolox and hydrogen peroxide standards were consumed as references for TAS and TOS analyzes, respectively. The following equation was applied while determining the oxidative stress index [OSI (Arbitrary Unit = AU)] value ***(Erel, 2005).***

$$OSI(AU) = \frac{TOS,\ \mu mol\ H_2O_2\ equiv./L}{TAS,\ mmol\ Trolox\ equiv./L \times 10}$$

**Total flavonoid content (TFC) and Total phenolic content (TPC)**

The TPC of Pm extracts was defined using the Folin-Ciocalteu technique with minor modifications (Gamez-Meze et al., 1999) and gallic acid as standard ($R^2$=0.9974). The findings were described as mg of Gallic acid equivalent per gr of extract (mg GAE/g extract). All measurements were carried out in triplicate.
The TFC of Pm extracts was defined by the $AlCl_3$ colorimetric procedure (Chang et al., 2002) using quercetin as standard ($R^2$=0.9967). The findings were stated as milligram quercetin equivalent per gram of dry extract (mg QAE/g extract). All measurements were performed in triplicate.

**DPPH radical scavenging activity assay**

The radical scavenging activity of Pm extracts was determined by slightly modifying the in vitro DPPH assay defined by Brand-Williams et al. (1995).
DPPH-methanol mixture, sample-methanol mixture, and Quercetin were used as control, blank, and positive control, respectively. Percent inhibition was determined utilizing the equation given below. The DPPH free radical scavenging activity of the Pm extracts was defined by the percentage inhibition through the Quercetin standard curve ($R^2$=0.9787). The half-maximum inhibitory concentration (IC50) of the extract was defined by plotting radical scavenging activity versus concentration.

$$Inhibition(\%) = \frac{Absorbance\ of\ the\ control - Absorbance\ of\ the\ sample}{Absorbance\ of\ the\ control} \times 100$$

**Antioxidant capacity by ABTS, CUPRAC, and FRAP assays**

The ABTS radical cation scavenging capacity of Pm extracts was tested with minor changes ***(Re et al.,1999)***. ABTS radical cation scavenging activity was determined in terms of Trolox equivalent antioxidant capacity (TEAC) on the standard curve obtained utilizing Trolox as a reference ($R^2$=0.9941).
The cupric ion-reducing antioxidant capacity **(**CUPRAC) of Pm extracts was carried out with minor changes ***(Apak et al., 2004)***. The findings were stated as gallic acid equivalent (mg GAE /g extract) utilizing the gallic acid standard curve ($R^2$=0.9979). The ferric-reducing antioxidant power **(**FRAP) of Pm extracts was determined ***(Oyaizu et al., 1986)***. The results were determined as Quercetin equivalent (mg QAE/g extract) using the Quercetin standard curve ($R^2$=0.9927).

***In vitro*** **cell viability assay**

In vitro cell viability tests of Pm extracts were analyzed by MTT assay in Human Dermal Fibroblast Cells (PCS-201-012) for 24, 48, and 72 hours ***(Kumar et al., 2018)***. Untreated cells were investigated as a control and considered 100% viable. All studies were performed in triplicate and in vitro assay data were statistically analyzed utilizing GraphPad Prism 9 with one-way ANOVA and Tukey's multiple comparison tests. Data are shown as means with 95% confidence intervals. P values less than 0.05 are considered to be statistically significant.

**Preparation of Pm extract-loaded liposomes.**

Pm extract-loaded liposomes were manufactured by ethanol injection technique ***(Khoshraftar et al., 2020)***. 30 mL EtOH (96%) and 1 g phosphatidylcholine were stirred continually at 50 °C for 1 h until the lecithin was fully resolved. A rotary evaporator was operated to evaporate the solvent under low pressure. Pm-d$H_2O$ and Pm-EtOH extracts (1.5 g) were dissolved in 36 mL PBS at ambient temperature and incorporated into the sample. The liposomes were then sonicated in ultrasonics at 40% amplitude for 5 min and the PBS and liposomes were separated after centrifugation of the sample. The samples were kept in the refrigerator overnight and then centrifuged at 6000 rpm for 10 min. The samples were dried in an oven at 50 °C for 24 hours.

**Characterization of Pm extract-loaded liposomes.**

The surface morphology of liposomes loaded with Pm extracts was monitored by cryo-TEM and the zeta potential of liposomal plant extracts was detected by Zeta-Potential and Mobility Meter (25 °C, 90° angle). Furthermore, the polydispersity index and average size were analyzed by dynamic light scattering (DLS) technique.

**Entrapment efficiency (EE%) of Pm extract-loaded liposomes.**

The evaluation of the entrapment efficiency (%EE) was performed by a slightly modified centrifugation technique ***(Khoshraftar et al., 2020)***. Pm extracts were initially detected by UV-Vis spectrophotometry at 275 nm, the peak wavelength at which Pm extracts have the highest adsorption. To establish a calibration curve, the absorbance versus concentration of different concentrations of Pm extracts solubilized in dH2O was prepared. EE (%) was calculated by quantifying free Pm extracts in the supernatant following liposome centrifugation. Samples were centrifuged at 4000 rpm for 30 minutes at 4°C. UV/VIS spectrophotometer at 275 nm was utilized to measure the concentration of free Pm extracts in the supernatant. The EE % of Pm extracts was determined using the following formula: EE (%) = ($AD_a$ / $AD_b$) x 100%, where $AD_a$ is the amount of Pm extracts in the liposome acquired after centrifugation. $AD_b$ is the amount of Pm extracts present in the liposomes before centrifugation.

***In vitro*** **release kinetics (%) of Pm extract-loaded liposomes.**

*In vitro* release kinetics of Pm extracts and loaded liposomes were performed using a modified dialysis method ***(Khoshraftar et al., 2020)***. The analysis was performed in 100 mL PBS (pH = 7.4) at 37 °C. Liposomes loaded with Pm extracts were placed in a cellulose dialysis bag (MW cut-off 12,000). The dialysis bag was then set in an isolated medium and stirred at the required settings. 1 mL of the released

medium was harvested and analyzed at a wavelength of 275 nm. The dialysis properties of free Pm extracts mixed with PBS were studied using the same method. Free Pm extracts in the supernatant at each preset period were utilized to determine the release rate of Pm extracts from liposomes. In vitro release (%) = [(total amount of Pm extracts - residue of Pm extracts)/total amount of Pm extracts]x100.

## RESULTS AND DISCUSSION

### Antimicrobial activity of Pm extracts

The concentration-dependent antimicrobial activities of Pm extracts against pathogen microbes are shown in Table 1. Both types of extracts especially showed an effective antimicrobial activity against *S. aureus* ATCC 25923, *K. pneumoniae* NRRLB 4420, *P. aeruginosa* ATCC 11778, *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 12600 and *C. albicans* ATCC 10231. The maximum inhibition zones against *P. aeruginosa* ATCC 11778 of Pm-d$H_2O$ and Pm-EtOH extracts at 300 mg/mL concentration were observed at 17±0.60 mm and 16±0.45 mm, respectively. In another study, when the antimicrobial activity of methanol extract (1000 mg/mL) obtained from *P. major* L. leaves against Gram-positive and negative bacteria were investigated, inhibition zones of *Lactobacillus* sp. and *P. aeruginosa* were measured as 25±1.3 mm and 24±0.9 mm, respectively ***(Abd-Razik et al., 2012)***. The disc diffusion method was used to investigate the antimicrobial activity of various extracts (ethyl acetate, petroleum ether, and aqueous fractions) from *P. major* L. leaves, and the ethyl acetate fraction was the most effective against Gram-positive and negative bacterial strains. High inhibition zones of 16.7±1.0 mm and 14.3±0.6 mm were shown on *S. aureus* and *B. cereus*, respectively. Against *P. aeruginosa* and *Acinetobacter bowie*, moderate ones of 13.3±0.6 and 11.3±0.6 mm were achieved ***(Karima et al., 2015)***.

MIC values of Pm extracts are shown in Table 2. MIC values of Pm-d$H_2O$ extract at 37.5 mg/mL were evaluated against *S. aureus* ATCC 25923 and *S. aureus* ATCC 6538 and MIC value was defined as 18.75 mg/mL for *S. aureus* ATCC 12600. Also, MIC values of Pm-EtOH extract were evaluated at 37.5 mg/mL against *K. pneumoniae* NRRLB 4420, *E. coli* ATCC 35218, and *P. aeruginosa* ATCC 27853.

**Table 1** Antimicrobial activity of Pm extracts

| Microorganisms | Inhibition Zone (mm±STD) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Pm-d$H_2O$** (mg/mL) | | | **Pm-EtOH** (mg/mL) | | | **$PC_1$** | **$PC_2$** | **NC** |
| | 100 | 200 | 300 | 100 | 200 | 300 | | | |
| *L. monocytogenes* ATCC 1911 | - | - | - | - | - | - | 23.0±0.50 | 14.0±0.50 | - |
| *S. aureus* ATCC 25923 | 9.0±0.45 | 14.0±0.20 | 16.0±0.65 | 12.0±0.35 | 15.0±0.20 | 16.0±0.40 | 30.0±0.50 | 12.0±0.25 | - |
| *K. pneumoniea* NRRLB 4420 | 10.0±0.30 | 15.0±0.45 | 15.0±0.25 | 12.0±0.50 | 15.0±065 | 16.0±0.35 | 29.0±0.50 | 15.0±0.35 | - |
| *P. aeruginosa* ATCC 11778 | 10.0±0.55 | 14.0±0.30 | 17.0±0.60 | 12.0±0.45 | 16.0±0.15 | 16.0±0.45 | 30.0±0.25 | 15.0±0.33 | - |
| *E. faecalis* ATCC 51289 | - | - | - | - | - | - | 23.0±0.25 | 15.0±0.45 | - |
| *E. coli* ATCC 35218 | - | - | - | 9.0±0.50 | 11.0±0.50 | 11.0±0.25 | 32.0±0.50 | 12.0±0.50 | - |
| *B. subtilis* NRS-744 | - | 10.0±0.45 | 13.0±0.30 | 10.0±0.50 | 12.0±0.40 | 13.0±0.20 | 31.0±0.55 | 20.0±0.50 | - |
| *S. aureus* ATCC 6538 | 12.0±0.55 | 15.0±0.55 | 15.0±0.35 | 13.0±0.45 | 14.0±0.45 | 15.0±0.75 | 43.0±0.35 | 16.0±0.70 | - |
| *E. coli* ATCC 25922 | - | - | - | 9.0±0.60 | 13.0±0.25 | 13.0±0.55 | 30.0±0.70 | 16.0±0.50 | - |
| *P. aeruginosa* ATCC 27853 | 11.0±0.60 | 16.0±0.70 | 17.0±0.20 | 11.0±0.20 | 13.0±0.55 | 15.0±0.40 | 22.0±0.35 | 14.0±0.55 | - |
| *S. aureus* ATCC 12600 | 10.0±0.35 | 13.0±0.25 | 14.0±0.55 | 12.0±0.35 | 13.0±0.50 | 14.0±0.40 | 43.0±0.55 | 15.0±0.60 | - |
| *C. albicans* ATCC 10231 | 9.0±0.30 | 12.0±0.20 | 12.0±0.50 | - | 11.0±0.50 | 13.0±0.60 | 30.0±0.75 | - | - |

for *C. albicans* ATCC 10231 $PC_1$: Fluconazole (10 mg/mL), NC: DMSO+$H_2O$;
for other test microorganisms $PC_1$: Penicillin G (10 mg/mL), $PC_2$: Amikacin (30 mg/mL) NC: DMSO+$H_2O$

**Table 2** MIC values of Pm extracts

| Microorganisms | MIC (mg/mL) | |
|---|---|---|
| | **Pm-d$H_2O$** | **Pm-EtOH** |
| *L. monocytogenes* ATCC 1911 | - | - |
| *S. aureus* ATCC 25923 | 37.5 | 18.75 |
| *K. pneumoniea* NRRLB 4420 | - | 37.5 |
| *P. aeruginosa* ATCC 11778 | - | 18.75 |
| *E. faecalis* ATCC 51289 | - | - |
| *E. coli* ATCC 35218 | - | 37.5 |
| *B. subtilis* NRS-744 | - | - |
| *S. aureus* ATCC 6538 | 37.5 | 18.75 |
| *E. coli* ATCC 25922 | - | 75.0 |
| *P. aeruginosa* ATCC 27853 | - | 37.5 |
| *S. aureus* ATCC 12600 | 18.75 | 18.75 |
| *C. albicans* ATCC 10231 | - | - |

For *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 11778, *S. aureus* ATCC 6538 and *S. aureus* ATCC 12600, MIC values were 18.75 mg/mL. Generally, it was observed that Pm-EtOH extract inhibited the growth of microorganisms at lower concentrations than Pm-d$H_2O$ extract. While both extract types show no inhibitory effect against *C. albicans* 10231 in the concentration range studied, they have an inhibitory effect against *S. aureus* ATCC 25923, *S. aureus* ATCC 6538, and *S. aureus* ATCC 12600. Most of the acute and chronic wound infections included consortia of both oxygen-consuming and anaerobic microbiota. Generally, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Escherichia coli*, *Corynebacterium* spp., and *Enterococcus* spp. are the most common bacterial species at the wound site ***(Bessa et al., 2015)***. The one of major results of the present study was that Pm extracts exhibited antimicrobial activity against the overwhelming species at the wound region but no antifungal activity against fungi; these findings are comparable to the results detailed by Abd-Razik et al. (2012), attributed the antimicrobial activity to some polar or non-polar compounds of low molecular weight rather than in the plant extract.

### Total antioxidant and oxidant (TAS, TOS, and OSI) potentials of Pm extracts

Antioxidant and oxidant (TAS, TOS, and OSI) potentials of Pm extracts are given in Table 3. When TAS, TOS, and OSI values were evaluated, it was determined that there was a moderate antioxidant effect in Pm extracts in general. The oxidative stress index (OSI) in Pm extracts was found to be quite low. In a study, the antioxidant and oxidant potential of the ethanol extract obtained from the aerial parts of the *Thymbra spicata* plant was evaluated. As a result of the research carried out, the TAS value of the *Thymbra spicata* extract was determined as 8.399±0.102, the TOS value as 6.530±0.115, and the OSI value as 0.078±0.001. As a consequence, the following points have been identified, *Thymbra spicata* has high antioxidant potential ***(Mohammed et al., 2000)***. In another research, TAS, TOS, and OSI values of the water extract of *Gundellia tournefortii* L. seeds were determined. The TAS value of *Gundellia tournefortii* L. seeds in the extract was determined as 6.831±0.489, TOS value of 3.712±0.584, and OSI value of 0.054±0.463 ***(Saraç et al., 2019)***.

**Table 3** TAS, TOS, and OSI values of Pm extracts

| Extracts | Total Antioxidant Status (TAS) (mmol Trolox /L) | Total Oxidant Status (TOS) (μmol $H_2O_2$ /L) | Oxidative Stress Index (OSI) |
|---|---|---|---|
| Pm-d$H_2O$ | 7.597±0.012 | 5.603±0.003 | 0.074 |
| Pm-EtOH | 9.383±0.115 | 3.274±0.021 | 0.035 |

**TPC and TFC of Pm extracts**

Table 4 shows the TPC and TFC of Pm extracts. TPC of Pm-EtOH extract (60.837±0.211mg GAE/g extract) is higher than Pm-d$H_2O$ extract (30.301±0.870 mg GAE/g extract). *Plantago* sp. has generally high viscosity mucilage plant and contains components in polysaccharide structure ***(Noshad et al., 2021)***. With this content, the water solubility of the plant is lower and the phenolic components of the plant dissolve better, especially in alcohol-based environments (Abate et al., 2017). It was determined that better dissolution of phenolic components in aqueous ethanol extracts. Similarly, the TFC of Pm-EtOH extract (79.439±0.123 mg QAE/g extract) is higher than Pm-d$H_2O$ extract (51.210±0.327 mg QAE/g extract). These values were higher than the values of 344.70±0.021 mg GAE/100 g extract and 45.23±0.034 mg QAE/100 g extract reported for *P. lanceolate* 90% MeOH extract (Abate et al., 2017). In a study, TPC and TFC values of *P. major* seed mucilage extract were determined 76.79 ± 1.6 mg GAE/g extract and 97.80 ± 2.0 mg QAE/g extract, respectively ***(Alizadeh-Behbahani et al., 2017)***.

**Table 4** TPC and TFC of Pm extracts

| Extracts | **TPC** (mg GAE/g extract) | **TFC** (mg QAE/g extract) |
|---|---|---|
| Pm-d$H_2O$ | 30.301±0.870 | 51.210±0.327 |
| Pm-EtOH | 60.837±0.211 | 79.439±0.123 |

**DPPH radical scavenging activity**

The DPPH stable free radical method is a straightforward, quick, and sensible technique for defining the antioxidant activity of chemicals, and bioactive components ***(Koleva et al., 2002)***. Inhibition percentages of Pm extracts against DPPH radical are shown in Figure 1. Pm extracts reduced the stable radical and demonstrated scavenging activity to varying degrees, depending on the extract concentration. Inhibition percentages of Pm-d$H_2O$ and Pm-EtOH extracts against DPPH free radical at 800 μg/mL concentration were 46.173±1.830% and 89.970±1.422% and $IC_{50}$ values were determined as 1025.98 μg/mL and 473.86 μg/mL, respectively. These values were higher than the values (between 31.0 to 39.0% and 38.9 to 43.0%) reported for DPPH radical scavenging capacities of methanolic and ethanolic extracts of *Plantago ovata* seeds (***Shah et al., 2020)***. In other research, the DPPH radical scavenging capacity of *P. lanceolata* leaf extracts (90% methanol) was reported as 90.6% ***(Abate et al., 2017)***.

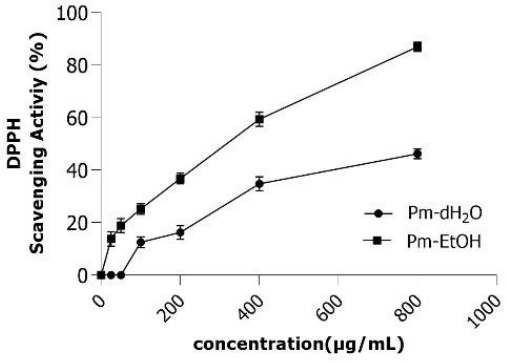


**Figure 1** DPPH scavenging activity of Pm extracts

**Antioxidant capacity of Pm extracts by ABTS, CUPRAC, and FRAP**

Several researchers investigated that natural antioxidants could be antioxidant supplements ***(Fransen et al., 2012; Rezaeian et al., 2015)***. Therefore, it is important to examine antioxidant capacity from different perspectives. The antioxidant activity of Pm extracts was determined by several *in vitro* methods. Antioxidant capacities of Pm extracts acquired with ABTS, CUPRAC, and FRAP are given in Table 5.

**Table 5** Antioxidant capacities of Pm extracts with ABTS, CUPRAC, and FRAP

| Extracts | ABTS (mg Trolox/g extract) | CUPRAC (mg GAE/g extract) | FRAP (mg QAE/g extract) |
|---|---|---|---|
| Pm- d$H_2O$ | 26.189±0.210 | 24.066±0.750 | 27.474±1.760 |
| Pm-EtOH | 50.506±0.236 | 85.950±0.991 | 51.690±1.471 |

In the ABTS radical cation scavenging assay, Pm-EtOH extract had the best antioxidant activity, with values of 50.506±0.236 mg Trolox equivalent/g extract, 85.950±0.750 mg gallic acid equivalent/g extract in CUPRAC assay and 51.690±1.471 mg quercetin equivalent/g extract in FRAP assay. Furthermore, the same extract was found to have the highest TFC and TPC values; 79.439±0.123 mg quercetin equivalent/g extract and 60.837±0.211 gallic acid equivalent/g extract (Table 4), respectively. The antioxidant activity of Pm-EtOH extract was found to be higher than that of Pm-d$H_2O$ extract by *in vitro* antioxidant activity determination methods. In addition, the total phenolic content of the Pm-EtOH extract was also higher than the Pm-d$H_2O$ extract. As in many previous researches on the assessment of antioxidant activity, a correlation between antioxidant capacity and phenolic concentration was observed ***(Chang et al., 2020; Sanna et al., 2022)***. There are many extraction techniques to recover antioxidants from plants. An antioxidant action does not depend on the extraction strategy but moreover on the solvent utilized for extraction. Antioxidant compounds dissolve in certain solvents according to their chemical properties and polarity. ***(Turkmen et al., 2006).*** Polar solvents are often preferred for the recovery of polyphenols from plant matrices. Aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate are used. Ethanol is recognized as an effective solvent for polyphenol extraction and is also safer for human consumption ***(Dai and Mumper et al., 2010)***. In previous research, the CUPRAC of *P. major* EtOH extract was determined as 33.6±0.19 μM Trolox equivalent/g extract and FRAP of the same plant extract was reported as 97.66±2.37 μM Trolox equivalent/g extract ***(Lukova et al., 2018)***, also the ABTS radical cation scavenging activity of *P. media* EtOH extract was measured as 275 μg Trolox equivalent/g extract (***Farcas et al., 2020)***.

***In vitro*** **cell viability assessment of Pm extracts**

Cell viability of Pm extracts to human dermal fibroblast cell line (PCS-201-012) was evaluated over a wide period (24, 48 and 72 hours) and the results are given in Figures 2a and 2b. Cell viability of Pm-dH2O extract was higher than the control group at all concentrations for 24 and 48 hours. At 72 hours, cell viability was higher than the control group at all concentrations except the highest concentration of 1000 μg/mL. When the cell viability results of the Pm-EtOH extract were evaluated, cell viability was higher than the control group at all concentrations except 1000 μg/mL at the 24$^{th}$ hour. Cell viability was higher than the control group at all concentrations except 500 and 1000 mg/mL at the 48$^{th}$ hour and all concentrations except 250, 500, and 1000 μg/mL at the 72$^{nd}$ hour. Current studies have mostly focused on the antiproliferative effect of this plant extract in cancer cell lines has been evaluated ***(Kartini et al., 2017; Poor et al., 2017; Rezadoost et al., 2019)***. In a study, *in vitro* fibroblast growth stimulation of *P. lanceolata* aqueous, ethanolic 96%, aqueous-glycerine, and aqueous-glycol extracts on human skin fibroblast (ATCC CRL-2522) cells reported that ethanolic and aqueous-glycerine extracts at concentrations below 25 mg/mL did not influence cell viability, while 50 mg/mL concentration reduced their viability by 45% and 40%, separately and glycerine extract increased *in vitro* skin fibroblast proliferation ***(Niziol-Lukaszewska et al., 2019).***

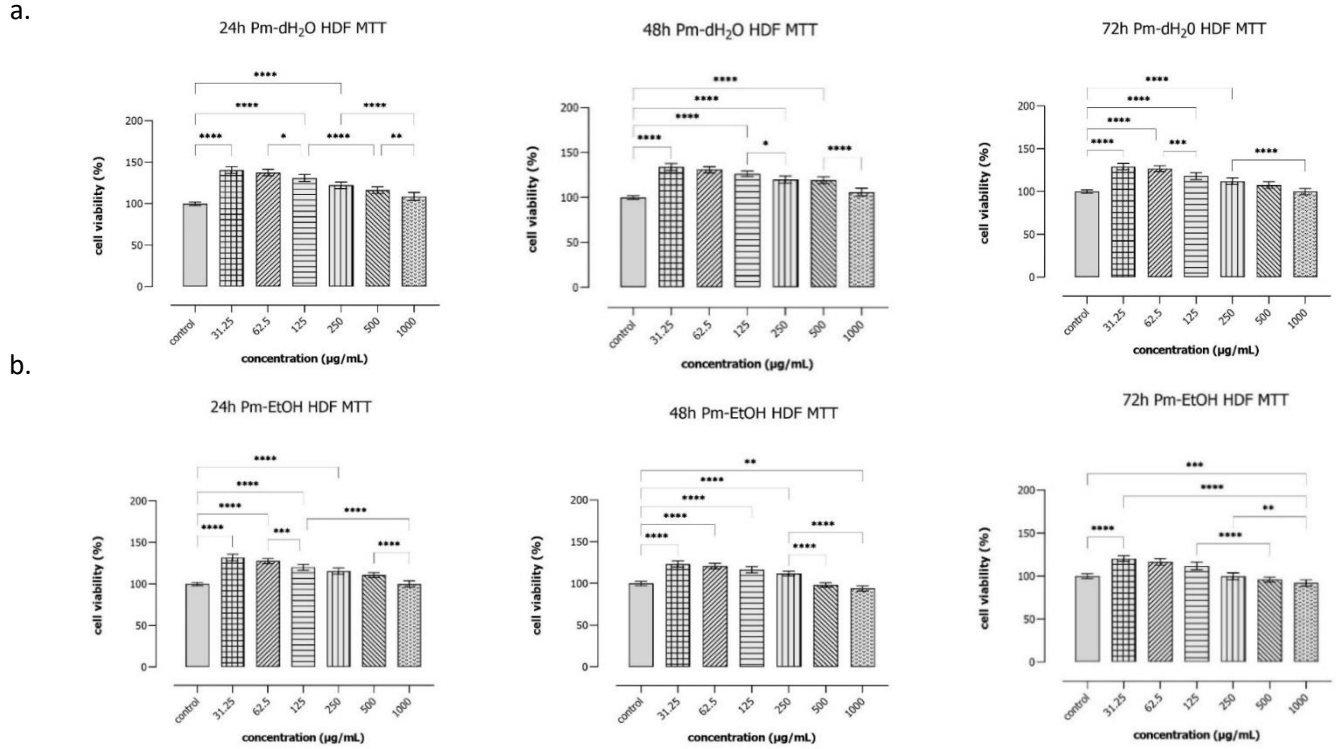


**Figure 2**. **(a)** Cell viability of Pm-dH$_2$O extract and **(b)** Pm-EtOH extract on PCS-201-012 cells (A, P value below 0,05 was considered as statistically significant)

**Characterization of Pm extract-loaded liposomes**

***Transmission electron microscopy (TEM)***

TEM images clearly show the microstructural variations and particle clusters of the samples, and TEM images of liposomes loaded with Pm extracts are presented in Figures 3a and 3b. In general, liposomes appear to have a spherical form and consist of relatively uniformly distributed smooth surfaces and widely distributed sizes.

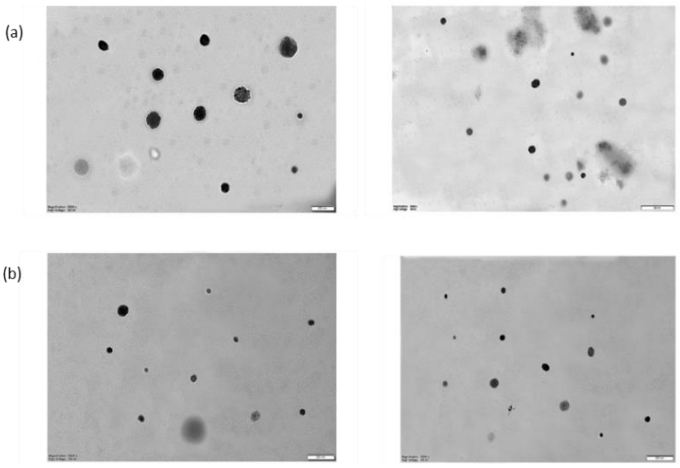


**Figure 3** TEM images of the **(a)** Pm-dH$_2$O and **(b)** Pm-EtOH extract-loaded liposomes

***Particle size distribution and ζ-potential***

Figures 4a and 4b show the dynamic light scattering (DLS) and ζ-potential analysis of the liposomes. Particle size and particle size distribution are significant factors in the physical stability of liposomes. The polydispersity index (PdI) value, in general, is close to monodisperse when between 0.1-0.25 a narrow distribution is obtained. PdI value of 0.5 above, large-diameter particles depending on the presence and aggregate formation from a broad particle size distribution can be mentioned ***(Tripathi et al., 2010; Mohammadpour et al., 2012)***. The mean size of the liposomes loaded with Pm-dH$_2$O extract was 452.4 nm and the PdI value was determined as 0.468. The mean size of the liposomes loaded with Pm-EtOH extract was 537.0 nm and the PdI value was 0.558. The PdI value of liposomes loaded with Pm-dH$_2$O extract is below 0.5. However, the PdI value of liposomes loaded with Pm-EtOH extract is a bit more than 0.5. Accordingly, the sizes of the particles also increased. Zeta potential is a parameter used to determine the dispersal stability of liposomal compositions. Characterizing the surface charge of the particles offers data about the repulsive forces between the particles and thus allows the stability of the dispersions to be estimated. Generally, zeta potential values < -30 mV and > 30 mV form stable systems as high surface charges induce repulsion and prevent aggregation (***Gharib et al., 2017; Rafiee et al., 2017)***. Pm-dH$_2$O and Pm-EtOH extract-loaded liposomes had negative zeta potential values (−15.3 mV and −14.9 mV) and a small negative surface charge, demonstrating the formation of physically stable liposomal dispersions. Liposomes with negative surface charge are especially preferred for drug or active ingredient delivery to the dermal surface ***(Gillet et al., 2011)***. Additionally, components encapsulated at negative zeta potential are protected more stably ***(Zou et al., 2014)***. Furthermore, it has been demonstrated that positively charged liposomes preserve their antibacterial and antibiofilm properties better than neutral and negatively charged liposomes of the same size ***(Drulis-Kawa et al., 2009; Dong et al., 2015)***.

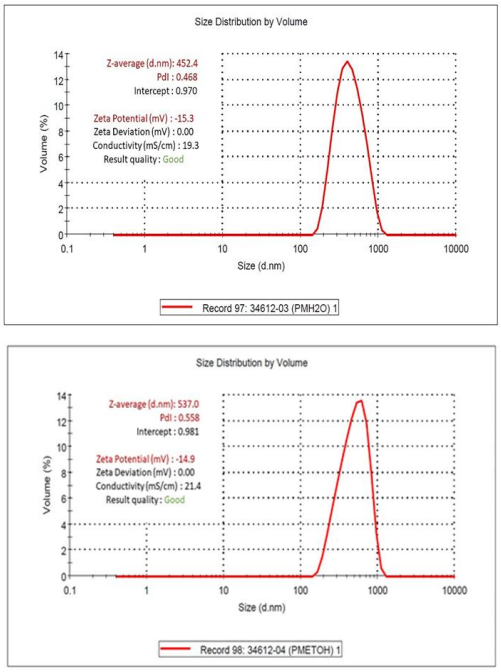


**Figure 4** Mean size, PDI value, and ζ-potential of **(a)** Pm-dH$_2$O and **(b)** Pm-EtOH extract-loaded liposomes

**Entrapment efficiency (EE%) of Pm extract-loaded liposomes.**

The entrapment efficacy of liposomes including Pm extracts was defined to be approximately 90.8±2.03% (Pm-dH2O) and 86.3±1.97% (Pm-EtOH), respectively. The obtained results show that liposome vesicles have a very high entrapment efficacy. In another study, nanoliposomes loaded with P. major seed (PMS) extract were obtained and the encapsulation effectiveness of nanoliposomes including PMS extract was described as 96% ***(Khoshraftar et al., 2020)***.

***In vitro* release kinetics (%) of Pm extract-loaded liposomes.**

The cumulative release of Pm extracts from liposomes is shown in Figure 5. After 6 h of testing, approximately 69.7% of the originally entrapped Pm-dH$_2$O extract was released from liposomes whereas 65.3% of entrapped Pm-EtOH extract was released from liposomes. The release percentages of Pm-dH$_2$O and Pm-EtOH extracts loaded liposomes were 83.4% and 77.1% after 24 h, respectively. The increased release of Pm extracts from liposomes was owing to the increased membrane permeability with time. Free Pm-dH$_2$O extract released 85.2% at the 6$^{th}$ hour and 92.1% at the 24$^{th}$ hour. Free Pm-EtOH extract had a release rate of 76.3% at the 6$^{th}$ hour and 86.6% at the 24$^{th}$ hour. The release profile of plant extracts loaded liposomes indicates that slower and controlled release systems were acquired compared to free plant extracts.

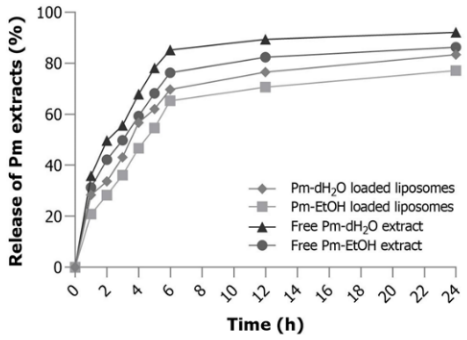


**Figure 5** The cumulative release of Pm-dH$_2$O and Pm-EtOH extracts from liposomes

## CONCLUSION

In this study, the effective properties of wound healing such as the antioxidant and antimicrobial potential of Pm-dH$_2$O and Pm-EtOH extracts were revealed. It has also been determined that these extracts show high cell viability and ensure the proliferation of human dermal fibroblast cells. Liposome structures loaded with Pm extracts were produced for effective and controlled release to the wound site. As indicated by the data presented here, liposomes were successful in encapsulating Pm extracts with high entrapment efficiency, good stability, and size distribution. Liposomes containing Pm extracts displayed a minor negative surface charge and a negative zeta potential, indicating the production of physically stable liposomal dispersions. Negatively surface-charged liposomes are beneficial for efficient dermal drug delivery. Overall, these findings suggest that plant extracts, such as Pm extracts, can be used to create functional nanocomposites with controlled release properties to aid wound healing.

**Author contributions: O.E.A:** Methodology, Data curation, Analysis, Validation, Writing – original draft preparation. **P.A.C:** Supervision, Data curation, Validation, Writing – reviewing, and editing. All of the authors approved the final manuscript.

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