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CHARACTERIZATION IN TERMS OF PHYTOCHEMICAL CONTENT AND MEDICINAL POTENTIAL OF THE *STELLARIA MEDIA* PLANT EXTRACT

Florina Miere (Groza)[1], Mariana Ganea[2], Andrei George Teodorescu[3*], Tunde Horvath[2], Alina Hanga-Farcas[1], Nagy Csaba[4], Marcel Zdinca[1], Mihaela Zdinca[1], Luciana Dobjanschi[1]

1. ***Department of Preclinical Disciplines, Faculty of Medicine and Pharmacy, University of Oradea, 410087, Oradea, Romania.***
2. ***Department of Pharmacy, Faculty of Medicine and Pharmacy, University of Oradea, 410087, Oradea, Romania.***
3. ***Department of Morphological Disciplines, Faculty of Medicine and Pharmacy, University of Oradea, 410087, Oradea, Romania.***
4. ***Department of Pharmacy, University of Medicine and Pharmacy, Science and Technology of Targu Mures, Targu Mures, 540142, Romania.***

All authors contributed equally to this work.

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ABSTRACT

In this paper, the *Stellaria media* plant, which is part of the *Caryophyllaceae* family, is highlighted from the point of view of its chemical composition through qualitative and quantitative methods such as Folin-Ciocalteu and HPLC-DAD_ESI for highlighting the total phenols. The biomedical properties were also tested, such as the antioxidant capacity through the DPPH and TEAC methods, the antimicrobial capacity compared to different antibiotics on *E. coli* and *S. aureus*, but also the cicatrizing capacity *in vitro* on normal human dermal fibroblast-type cells of the plant extract under two forms of application (extract 1 mg/ml respectively its liposomal form) compared to the positive control (liposomes without extract included and 50 mg/ml alanine) and negative control (untreated *in vitro* simulated wound). To be able to apply the liposomal formula with included extract on NHDF cells, liposomes were formulated using cholesterol and phosphatidylserine in a ratio of 2:3 by the lipid film hydration method (with phosphate buffer pH 7.6). The liposomal formula was characterized by microscopy, DLS analysis, and Extract Inclusion Efficiency (EE%). Last but not least, the samples applied to highlight the cicatrizing effect were tested by the MTS method regarding their toxicity on fibroblast cells.

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Introduction

Nowadays, the approach to various pathologies to treat them is directed towards traditional medicine, based on extracts from medicinal plants, which are considered without notable side effects, unlike conventional treatments [1-5]. This work brings the *Stellaria media* plant to the fore [6]. This is popularly called chickweed and is part of the *Caryophyllaceae* family along with other plants such as *Stellaria alsine*, *Stellaria pallida*, *Stellaria dichotoma*, *Stellaria nemorum*, *Stellaria holostea* [7, 8]. Until now, this *Stellaria* species is considered to be beneficial in the form of ethanolic, methanolic, and aqueous extracts or even consumed in the daily diet in various conditions such as lung diseases (asthma, bronchitis, bronchiolitis, lung cancer), cancer, skin diseases (burns, cuts, scratches having a healing effect) [9-11].

Also, the extract of the *S. media* plant is known in traditional medicine for its antipyretic, anthelmintic, antioxidant,

Corresponding Author: Andrei George Teodorescu; Department of Morphological Disciplines, Faculty of Medicine and Pharmacy, University of Oradea, 410087, Oradea, Romania. E-mail: teodorescu_andrei_george@yahoo.com.

antimicrobial, and antiobesity effects [12, 13]. *S. media* is a perennial plant that grows in cold or temperate regions and is widely distributed in Europe [8]. In the specialized literature, the chemical composition of the extract of this plant is described very succinctly, so in this work, the main phytochemical elements of the extract and their properties are highlighted by methods such as HPLC-DAD-ESI, respectively, antioxidant, antimicrobial, and chlorophyll content determination methods. Biological activities *in vitro* are also tested, such as antioxidant, antimicrobial, and wound healing activity. Regarding the testing of the healing effect, it is highlighted using the scratch method, *in vitro* on normal human dermal fibroblasts (NHDF) both for the extract applied as such and for its liposomal form.

To obtain the liposomal form, the lipid film hydration method was used and the liposomes with included extract were characterized in terms of shape, appearance, size, electrical charge, and encapsulation efficiency. **Figure 1** represents the graphic abstract of our work.

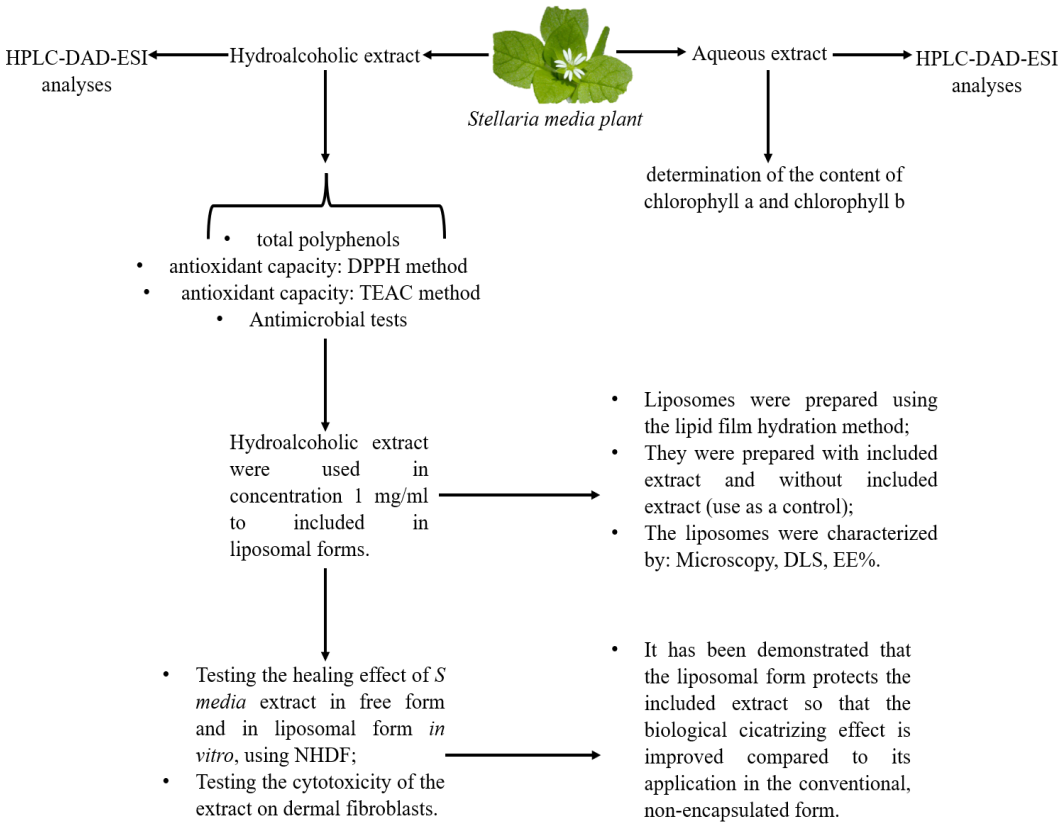


Figure 1. Graphical abstract: The analyzes used to characterize the *S. media* plant extract from the point of view of chemical composition and biomedical capacity.

Materials and Methods

Harvesting and Identifying the Chickweed Plant

The harvest of the plant, popularly called the chickweed, was carried out in the spring of 2022 in May, at the moment of flowering. The harvest took place in Bihor county, Oradea city, Romania. The plant was harvested in its entirety and was identified with the help of the botanical illustrated book by following its characteristic aspects such as the stems of the articulated type with existing nodes, the leaves of the sessile type, existing on the stem of a row of tector bristles, the flower is colored white, in the shape of a star, being made up of a corolla with 5 dialipetal type petals and the calyx has 5 sepals covered by tector hairs, both (petals and sepals) being arranged in the shape of a star, hence the Latin name of the plant (*Stellaria media*) [14].

Determination of the Chlorophyll a and Chlorophyll b Content

To determine the content of chlorophyll a and chlorophyll b used Purkiewicz *et al*., 2021 protocol with some modifications: 100 mg of fresh vegetable product was weighed, over which 3 mL of ethanol was added in a mortar. These were triturated until the green coloration of the ethanol was observed after which the contents were emptied into a test tube. The test tube was centrifuged for 10 minutes at 5000 rpm. The supernatant was decanted and subjected to spectrophotometric determinations [15]. The spectrophotometer used is Shimadzu mini UV-VIS, observing the absorbance at λ=663nm and λ=645nm [15]. Concentrations of chlorophyll a and b were expressed in mg/g fresh weight.

Obtaining the Aqueous and Hydroalcoholic Extract of the Chickweed Plant

The *S. media* plant was harvested and dried at room temperature to constant weight and was subjected to two methods of extraction of active compounds to highlight which extraction method is more favorable for the evaluation of polyphenol content [8].

Briefly, 60 grams of the dry plant for each extraction method were crushed and 70% ethanol was added to them in a ratio of 1:20 (w/v) respectively distilled water in the same volume to obtain the aqueous extract. The mixture was kept in the dark under continuous stirring for 24 hours, then filtered [16, 17].

In the case of the hydroalcoholic extract, the ethanol was removed using a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4000) at 45 °C and 90 rpm. The plant extracts were stored at −80 °C overnight and then lyophilized (Christ Alpha 1–2 Ldplus lyophilizer) [6, 18]. The lyophilisates obtained were used for the identification and quantification of polyphenols by the HPLC method.

Characterization of the Chemical Composition of Plant Extracts from a Qualitative and Quantitative Point of View Through HPLC Analysis

To identify and quantify the phenolic compounds present in the hydroalcoholic extract and the water extract of the *S. media* plant, the HPLC-DAD-ESI method by Huang *et al*., 2019 described below with some modification was used:

From the aqueous or hydroalcoholic lyophilized extract, the samples to be characterized as follows were prepared: 0.5 g of lyophilized is mixed with 5 ml of 70% alcohol, this mixture is kept in the refrigerator for 24 hours and then centrifuged at 10,000 rpm for 10 minutes and T = 240ºC in the Eppendorf AG 5804 centrifuge. The supernatant was filtered through a 0.45 µm Chromafil Xtra nylon filter and 20 µl was injected into the HPLC system. The lyophilized sample of the plant from which the aqueous extract was made is prepared in the same way [19].

Chromatographic Conditions

Agilent 1200 HPLC system equipped with a quaternary pump, solvent degasser, autosampler, photodiode UV-Vis detector (DAD) coupled with Agilent model 6110 single quadrupole mass detector (MS) (Agilent Technologies, CA, USA) was used. The separation of the compounds was carried out on a Kinetex XB C18 column, dimensions 4.6x 150 mm, with 5 µm particles (Phenomenex, USA), using the mobile phases (A) water + 0.1% acetic acid and (B) acetonitrile + 0.1% acetic acid in the gradient below, for 30 minutes, at a temperature of 250ºC, with a flow rate of 0.5 ml/min [20].

Gradient (expressed in % B): 0 min, 5% B; 0-2 min, 5% B; 2-18 min, 5%-40% B; 18-20 min, 40%-90% B; 20-24 min, 90% B; 24-25 min, 90%-5% B; 25-30 min, 5% B. Spectral values were recorded in the 200-600 nm range for all peaks. The chromatograms were recorded at the wavelength λ = 280 and 340 nm. For MS, full scan ESI positive ionization mode was used under the following working conditions: capillary voltage 3000 V, temperature 3500 C, nitrogen flow 7 l/min, and m/z 120-1200. Data acquisition and interpretation of results were done using Agilent ChemStation software.

For the quantification of phenolic compounds, calibration curves were made by injecting 5 different concentrations of standard substances dissolved in methanol: 1-Gallic acid (R^2 = 0.9978), LOD = 0.35 µg/ml, LOQ = 1.05 µg/ml; 2-Luteolin (R^2 = 0.9972), LOD = 0.11 µg/ml, LOQ = 1.24 µg/ml.

The equations of the curves were used for quantitative calculation for each phenolic compound as follows: hydroxybenzoic acid was calculated as gallic acid equivalent and flavones as luteolin equivalent. The phenolic compounds were identified by comparing the retention time, UV-Vis absorption, and mass spectra with those of the standard compounds and with data from the specialized literature [21-23]. The quantification of phenolic compounds was done by using the calibration curve with gallic acid for hydroxybenzoic acid, respectively with luteolin for flavones [21, 22, 24].

Determination of the Total Phenol Content, Antioxidant Capacity, and Testing of the Antimicrobial Activity of the Hydroalcoholic Extract

Total Polyphenols Determined by the Folin-Ciocâlteu Method

To determine the total content of phenols, the Folin-Ciocâlteu method was used, this method has the following principle: the oxidation-reduction capacity of phenolic compounds is highlighted by the reaction that takes place with the Folin-Ciocâlteu reagent and the resulting compounds are colorimetric at a wavelength of λ = 750 n.m. Briefly, 0.1 ml of 1 mg/ml solution was mixed with 1.7 ml of distilled water and 0.2 ml of Folin-Ciocâlteu reagent. Then, 1 ml of 7.5% Na_2C0_3 solution was added, and the samples were homogenized using the vortex and kept in the dark for 2 h. The results obtained are expressed in milligrams of gallic acid equivalent (GAE)/gram of dry vegetable product (dw) [14].

Determination of the Antioxidant Capacity of the S. Media Plant Extract by the DPPH Method

The plant extract was tested for antioxidant capacity using the DPPH method according to Miere (Groza) *et al*. 2019 [25]. Briefly, 0.1 mL of 1 mg/mL extract was homogenized with 2.6 mL of methanolic DPPH solution (80 µM). The sample was kept in the dark for 60 min and the absorbance was measured at 517 nm. Antioxidant capacity expressed in % DPPH radical scavenging was calculated according to equation (1):

$$\% \text{ Radical Scavenging Activity (RSA)} = [(A0 - A1) / A0] \times 100 \quad (1)$$

Where A0- was the absorbance of DPPH free radical solution in methanol

A1- the absorbance of the sample.

Determination of the Antioxidant Capacity of the S. Media Plant Extract by the TEAC Method

The TEAC method (Trolox equivalent antioxidant capacity) is based on the ability of antioxidants, in this case, phenolic compounds, to reduce the life of a cationic radical (ABTS+), which absorbs at 734 nm [26]. After preparing the specific reagents of the method, 100 µl of the extract is homogenized by vortexing with 2.9 ml of ABTS solution, and the antioxidant capacity is monitored spectrophotometrically at 734 nm [25]. Results were expressed in µmolTE/g dw.

Testing the Antimicrobial Activity of the Plant Extract by the Diffusion Method

To test the antimicrobial activity of the plant extract in a concentration of 1 mg/ml, plates with a Mueller-Hinton-type medium with a layer thickness of 80 mm were used [27]. The antimicrobial activity of the extract was tested on gram-positive bacterial strains (*Staphylococcus aureus*) and gram-negative (*Escherichia coli*) the results being compared with those obtained for different antibiotics such as Ciprofloxacin 5µg, Penicillin 10U, Oxacillin 30µg [28]. The extract was applied in a stock concentration of 1 mg/ml and after 24 hours at 37 ºC, the diameter of the inhibition zone was measured [29]. The analysis was performed in triplicate.

Formulation of Lipid Vesicles with Included Extract Using the Lipid Film Hydration Method and Characterization of the Obtained Liposomes

The lipid film hydration method is a common method of formulating giant-sized lipid vesicles, based on the formulation of two phases: a lipid and an aqueous phase, which are then strongly homogenized, spontaneously forming liposomes [30].

In the present case, the lipid phase is made up of phosphatidylserine-type phospholipids combined with cholesterol and the ratio between them is 3:2 (w/w) [31]. For the formulation of the lipid phase, dichloromethane and methanol were used as solvents in a ratio of 3:2 (v/v) and after homogenization, this mixture of solvents was removed with a rotary evaporator, creating a lipid film on the walls of the vessel from which the solvent was evaporated. For the formulation of the aqueous phase, the phosphate buffer of pH 7.6 was used, in which the extract was homogenized to incorporate the extract into liposomes, with a final concentration of 1 mg/ml.

After obtaining both phases, they are brought together and after washing the lipid film with the phosphate buffer solution in which the plant extract is found, this mixture is subjected to sonication for 1 hour and then centrifugation for 3 min at 4050 rpm. The supernatant is collected and then centrifuged again for 30 min at 10,000 rpm.

Liposomes without included extract are made in the same way, with the difference that the extract of interest is not solubilized in the phosphate buffer [32].

After these steps, the formulated liposomes are characterized in terms of shape using a phase contrast microscope with a 40x objective (OlympusCX40 (Tokyo, Japan)) and the images are captured with a Hitachi CCD camera [14].

Through DLS analysis, the size of the liposomes and the electrical surface charge are highlighted and the EE% is also tested using the Folin Ciocalteu method to determine the total polyphenols from the S. media plant extract before and after encapsulation by applying the formula (2) [21].

$$EE\%=LTPh/TPh \times 100 \quad (2)$$

Where: LTPh represents the total phenols expressed as gallic acid equivalent (mg GAE/mL) of the *S. media* extract encapsulated in the liposomes.

TPh represents total phenols content expressed as gallic acid equivalent (mg GAE/g)
extract before inclusion in the liposomal formulation.

Testing the Healing Effect of the Chickweed Plant Extract in Liposomal Form Compared to the Extract Applied in Free Form by the Scratch Method on Normal Human Dermal Fibroblasts (NHDF)

After formulating the liposomes with included extract, they were tested *in vitro* for the healing effect by the scratch method. NHDF or normal human dermal fibroblasts and a specific culture kit (Fibroblast Growth Medium-2 BulletKit) were purchased from Lonza Pharma & Biotech (Basel, Switzerland). The culture medium was Dulbecco's modified Eagle's medium containing 10% FBS (fetal bovine serum), gentamicin 50 mg/mL, amphotericin 50 mg/mL, and recombinant fibroblast growth factor hFG (CC4065) 1 mg/mL.

To test the cicatrizing effect of the *S. media* plant, the 1 mg/ml concentration extract was used (SM), respectively the same concentration in liposomal form (LIPOSM). Allantoin 50 mg/ml (ALA), the liposome without included extract (LIPO) was used as a positive control and the untreated wound (CT) was used as a control [31, 33].

Cell viability testing was also followed, respectively testing by the MTS method of the cytotoxicity of the extract and its liposomal form.

Cell Viability Assay

Cell viability was performed using the trypan blue exclusion assay [34]. NHDF cells were seeded in 24-well plates at 1×10^4 cells/well and maintained at 37◦C with 5% CO_2 for 24 hours and then the cells were treated with the plant extract in free and liposomal form for 24 hours. Cells were trypsinized (trypsin/EDTA solution (0.25 mg/mL), LONZA), neutralized using TNS-Trypsin Neutralizing Solution, (LONZA), and centrifuged (1000 rpm/7 min). The formed pellet was suspended in a cell medium and cell viability was determined using an EVE Automated Cell Counter (NanoEnTek Inc., Seoul, Republic of Korea). Cell viability was calculated according to formula (3) and the results were expressed as a percentage of the cell viability of the treated cells compared to the control. The measurements were carried out in triplicate, and data are represented as mean ± standard deviation (SD) [12].

Cell viability (%) = (number of living cells/number of total cells) × 100 (3)

MTS Assay

To perform the MTS assay, cells were seeded in sterile 98-well plates at a density of $2x10^4$/mL by adding 60 µL of suspension cell growth medium. After 24 hours, the samples were applied in a volume of 50 µL. Proliferation was determined at 36 hours [35].

Briefly, 10 µL of MTS reagent containing (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl) 2H tetrazolium) and PMS (phenazine) in -a 20:1 ratio has been added. After 2 hours, the formazan dye was quantified by measuring absorbance at 492 nm using a microplate reader (Stat Fax 2100, Palm City, USA), while the reference wavelength was 630 nm. Results were expressed as a percentage of cell viability compared to the control [21].

Scratch Assay

The scratch method was used to investigate the wound healing potential of both the extract at the concentration of 1 mg/ml and liposomes loaded with the extract at the same concentration.

For this method, 24-well plates were seeded at a cell density of $4 \times 10^4/cm^2$. After 48 hours, scratching was done vertically in each well with a sterile pipette tip. Detached cells were removed by washing with 200 µL of HEPES buffer and subsequently samples were plated in triplicate well plates.

Wound healing treatments were monitored at different time points: T0h, T24h, and T36h. Wound closure rating per area (%) was calculated for each sample at each time point by applying the formula (4) [36].

Area (%) = [Average area of wound_sample (μm^2) at time t / Average area of wound_sample (μm^2) at time T0] × 100 (4)

Results and Discussion

Following the analyzes carried out regarding the content of chlorophyll a, and chlorophyll b, respectively the determination of the total polyphenols and the antioxidant capacity by two distinct methods, the results presented in **Table 1** were obtained.

Thus, it can be seen that the fresh plant contains chlorophyll an in amounts of 20.5 mg/g fw and chlorophyll b in amounts of 5.98 mg/g fw, which highlights a high level of these pigments giving the plant strong antioxidant properties [15]. These aspects are highlighted by the highlighted antioxidant capacity values (76.58% by the DPPH method) respectively 1.5871 µmolTE/g dw by the TEAC method, which is comparable to the values found in the literature.

For example, Oladeji *et al*., 2020 highlighted a DPPH radical inhibition percentage of 62.75%, and Miere *et al*., 2022 obtained a value of 77.55% [6, 8]. **Table 1** also shows the value of the total polyphenols highlighted by the Folin-Ciocalteu method, this being comparable to the values reported in the specialized literature and also comparable to the total polyphenols quantified by HPLC-DAD-ESI analysis (22.422 mg/g dw).

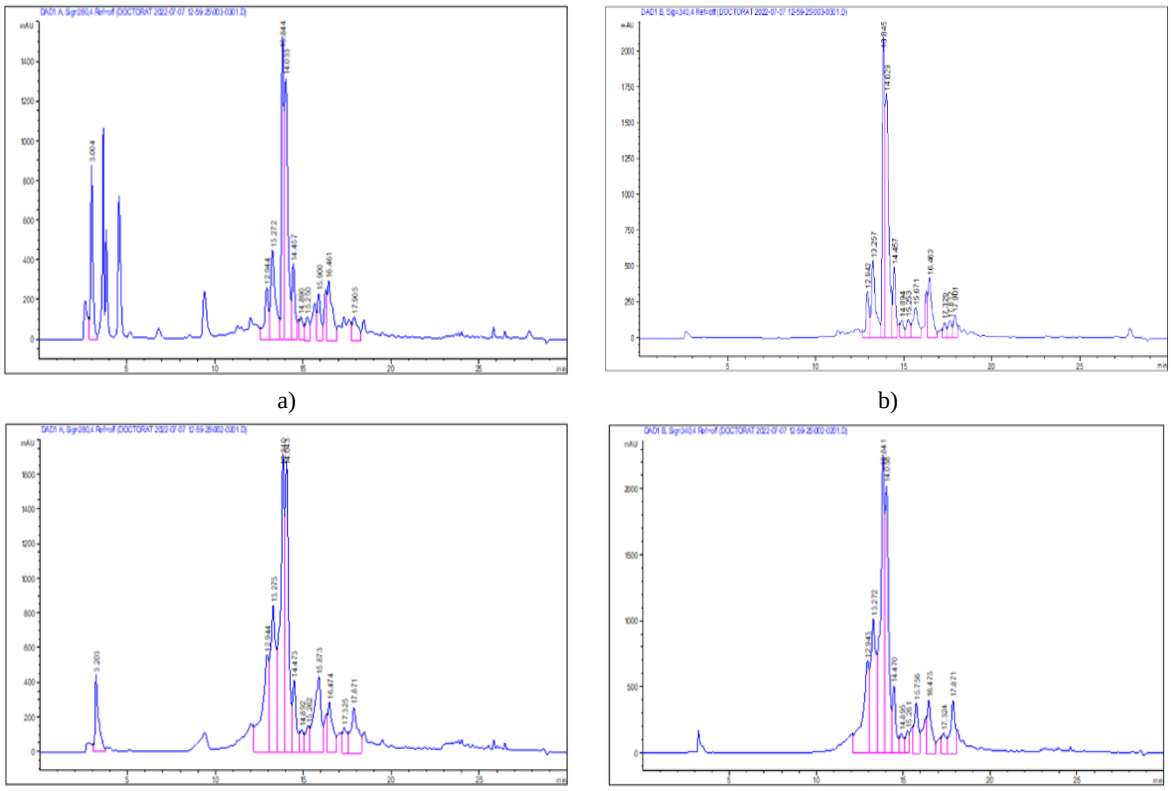
Table 1. The results of testing the antioxidant, antimicrobial activity and the total content of chlorophyll a, chlorophyll b and total polyphenols.

Chlorophyll a mg/g fw	**Chlorophyll b mg/g fw**	**Total polyphenol mg GAE/g dw**	**DPPH %**	**TEAC µmolTE/g dw**
20.5±1.2214	5.98±1.102	21.45±2.5897	76.58±2.3347	1.5871±0.5874
Antimicrobial test				
Samples	***S. aureus***		***E. coli***	
Diameter ± SD (mm)				
S. media 1 mg/ml	**15.2540±1.258**		**12.0254±1.2458**	
Ciprofloxacin 5 µg	26.33±0.1133		31.65±1.2498	
Penicillin 10U	32.65±0.3245		-	
Oxacillin 30 µg	31.25±0.5733		22.16±2.5470	

The antimicrobial activity was also tested for the hydroalcoholic extract of the S. media plant at a concentration of 1 mg/ml, the values obtained being read compared to the diameter of inhibition created by different antibiotics **(Table 1)**. It can be stated that the antimicrobial properties of the extract are present in both gram-positive and gram-negative bacteria, specifying that this activity is more intense in the positive bacteria (*S. aureus*).

According to other authors, the extract of the S. media plant shows its antimicrobial character also on other pathogenic germs such as *S. typhi, P. aureginosa, K. pneumonia and B.cereus, K. pneumoniae, S. marcescens and P. aeruginosa* [6, 8].

To identify the most efficient extraction method for the phenolic compounds present in the S. media plant, the HPLC-DAD-ESI method was used and the chromatograms for the two types of extracts at two different wavelengths are presented in **Figure 2**.



a) b) c) d)

Figure 2. The chromatograms obtained by the HPLC-DAD-ESI method for the hydroalcoholic and aqueous extract of the *S. media* plant. a) chromatogram obtained at a wavelength of 280 nm for the aqueous extract of the *S. media* plant, b) chromatogram obtained at a wavelength of 340 nm for the aqueous extract of the *S. media* plant, c) chromatogram obtained at a wavelength of 280 nm for the hydroalcoholic extract of the *S. media* plant, d) the chromatogram obtained at a wavelength of 340 nm for the aqueous extract of the *S. media* plant.

Following the interpretation of the chromatograms presented in **Figure 2**, the main phenolic compounds present in both types of extracts of the S. media plant were identified, and these are presented and quantified in **Table 2**.

Table 2. Identification and quantification of phenolic compounds in the *S. media* sample, quantity expressed in mg/g dry sample.

Peak No.	R_t (min)	UV λ_{max} (nm)	$[M+H]^+$ (m/z)	Compound	Subclass	*S. media* hydroalcoholic extract	*S. media* aqueous extract
1	3.21	265	155	Dihydroxybenzoic acid	Hydroxybenzoic acid	1.760	2.292
2	12.94	340.268	595	Chrysoeriol-apiosyl-glucoside	Flavone	2.655	0.556
3	13.27	340.265	595	Luteolin-rutinoside	Flavone	3.137	1.305
4	13.84	350.270	565	Apigenin-apiosyl-glucoside	Flavone	5.284	2.749
5	14.04	350.270	595	Apigenin-diglucoside	Flavone	4.291	3.559
6	14.47	350.270	727.5	Apigenin-apiosyl-glucosyl-glucoside	Flavone	1.048	0.791
7	14.89	340.265	433	Luteolin-rhamnoside	Flavone	0.335	0.233
8	15.26	340.265	535	Luteolin-malonyl-glucoside	Flavone	0.361	0.264

9	15.67	350.270	433	Apigenin-glucoside	Flavone	0.933	0.647
10	16.47	340.265	463	Luteolin-glucuronide	Flavone	1.552	1.116
11	17.87	350.270	447	Apigenin-glucuronide	Flavone	1.065	0.297
				Total Phenolics		**22.422**	**13.809**

Through HPLC-DAD-ESI analysis, apigenin was identified in conjugated forms with different sugar residues: Apigenin-apiosyl-glucosides (4), Apigenin-diglucosides (5), Apigenin-apiosyl-glucosyl-glucosides (6), Apigenin-glucosides (9), Apigenin-glucuronides (11). Luteolin derivatives such as: Luteolin-rutinoside (3), Luteolin-rhamnoside (7), Luteolin-malonyl-glucoside (8), Luteolin-glucuronide (10) were also identified. All these representatives are part of the class of flavones and represent 92.15% of the total phenolic compounds identified and quantified in the hydroalcoholic extract. In this type of extract, the hydroxybenzoic acid compound, namely Dihydroxybenzoic acid (1), was also identified and quantified.

The same compounds were identified and quantified for the aqueous extract of the S. media plant, but it can be seen in **Table 2** that the hydroalcoholic extraction is more beneficial, extracting a total of phenols of 22,422 mg/g dw compared to 13,809 mg/g dw (the aqueous extract of *S. media* plant).

Apigenin is a flavonoid-type compound found in the *S. media* plant, is responsible for the antiobesity action of the plant but also for reducing the risk of ovarian cancer. *In vivo* studies have also shown beneficial effects for apigenin in conditions such as diabetes, amnesia, Alzheimer's, insomnia, and depression [8].

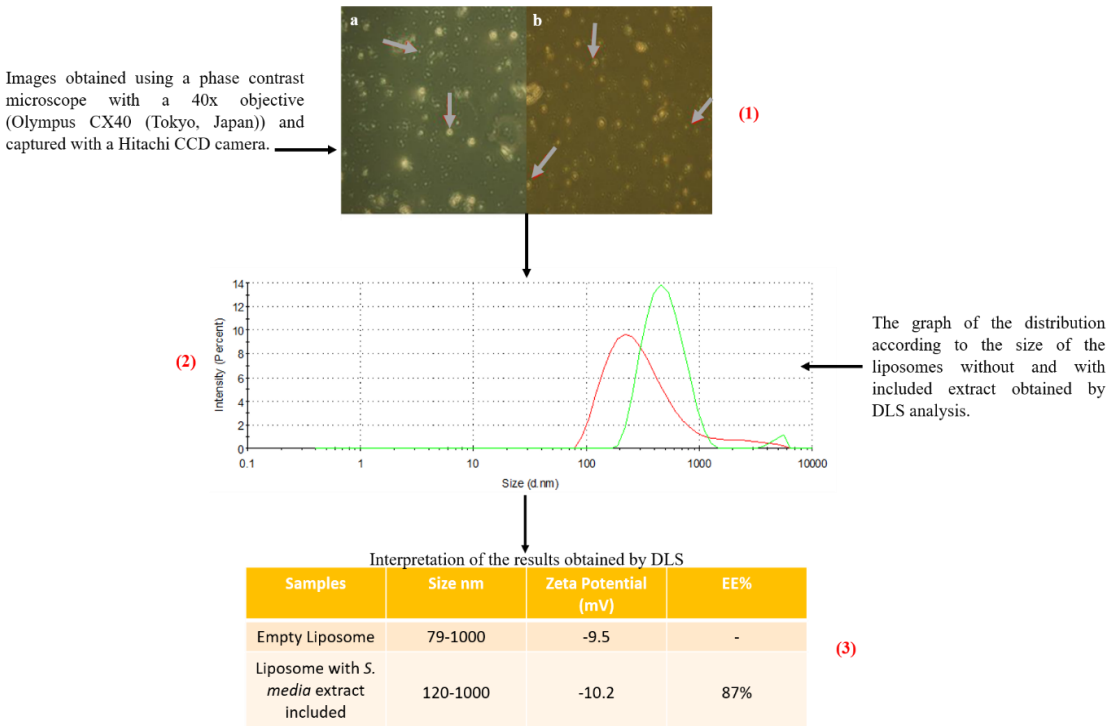
Compounds such as vicenin-2, isoquercetin, genistein, caffeic acid, ferulic acid, vanillic acid, and chlorogenic acid were also identified in the chemical composition of the *S. media* plant extract [37].

Other authors identified in the *S. media* plant extract the compounds gypsogenin and oleanolic acid (compounds with an important antioxidant but also anticancer role) and the authors Arora *et al*., 2014 discovered three new metabolites of the plant (2, 4, 5, 7 tetramethyloctane, 2,2,4-trimethyloctan-3-one, 6-methyl heptyl-30-hydroxy-20 methylpropanoate) [38].

Taking into account the phytochemical composition rich in flavonoids of the hydroalcoholic extract obtained from the *S. media* plant, its antioxidant and antimicrobial capacity, and the high content of chlorophyll pigments, we wanted in the continuation of the study to test the cicatrizing biological activity of the extract both in free form and in encapsulated, liposomal form.

To formulate the liposomes, cholesterol was used according to the working method, having the role of increasing the permeability of the cell membrane (of fibroblasts in this study) and the phospholipid phosphatidylserine.

After formulating the liposomes by the lipid film hydration method, a quick and inexpensive method, they were characterized according to **Figure 3**.



Samples	Size nm	Zeta Potential (mV)	EE%
Empty Liposome	79-1000	-9.5	-
Liposome with *S. media* extract included	120-1000	-10.2	87%

Figure 3. The results of the characterization of liposomes with and without *S. media* extract are included. (1) represents the first stage of the characterization of formulated liposomes. a-microscopic image of liposomes without *S. media* extract included, a b-microscopic image of liposomes with *S. media* extract included. The white arrows indicate liposomes with a characteristic round shape; (2) represents the second stage of characterization of the formulated liposomes, the DLS analysis graph, the red curve represents the liposomes without *S. media* extract included and the green curve represents the liposomes with *S. media* extract included; (3) represents the summation of the DLS analysis and EE% test results.

Figure 3 shows the results obtained through the microscopic analysis of the liposomes with and without *S. media* extract included (1), observing the round, spherical shape of the liposomes and their homogeneous dispersion in the microscopic field. This analysis practically confirms the spontaneous formulation of liposomes at the moment of the union of the lipidic phase and the hydrophilic phase (according to the aspects presented in the material and methods chapter). Through DLS analysis, the size range of liposomes with or without *S. media* extracts included was identified.

According to **Figure 3** (3), the size of the formulated liposomes is between 79-1000 nm for liposomes without included extract (75% of the total liposomes being up to 500 nm in size) and for liposomes with *S. media* extract included the size is between 120 -1000 nm (66% of the total liposomes being up to 500 nm in size). Regardless of the type of liposomes (with or without extract included), the measured dimensions are characteristic of giant-type liposomes.

It was also of interest to measure the surface electric charge of the liposomes formed, this being negative in both situations (-9.5 for liposomes without included extract and -10.2 for liposomes with *S. media* extract included) which denotes an increased stability of the liposomal formula and a low tendency of aggregation and agglomeration of nanoparticles.

EE% was calculated following the protocol described in the Material and methods chapter and the value obtained after applying the formula that takes into account the total phenols is a favorable one compared to other percentages highlighted in the literature, namely 87%.

After the characterization of the liposomal formula (LIPOSM), it was tested for its biological healing character compared to the non-incorporated extract (SM) and the positive control represented by alanine 50 mg/ml (ALA), the liposomes without included extract (LIPO) respectively the negative control (Untreated wound -CT) **(Figure 4)**.

Samples	Viability %	MTS assay after 24h	Area (%) T12h	Area (%) T36h
CT	93.74±6.253	92.548±2.158	25.587±1.548	70.579±2.333
ALA	95.478±5.214	95.568±3.178	27.1540±1.124	81.547±2.548
LIPO	92.5487±2.587	91.581±5.118	26.4871±2.154	75.587±2.5471
LIPOSM	95.5571±2.489	94.8111±1.115	30.987±2.1541	86.884±2.5478
SM	93.964±5.487	93.448±4.245	27.4123±0.487	81.2221±2.689

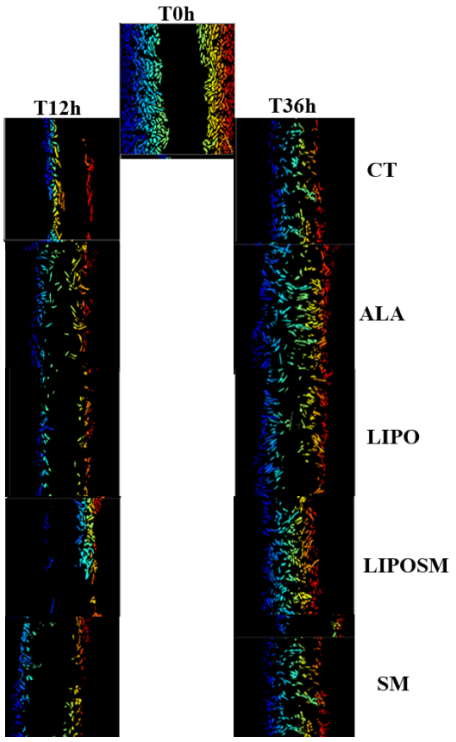


Figure 4. The results regarding the *in vitro* testing on human dermal fibroblasts of the viability (%), cytotoxicity (MTS assay), and healing effect (scratch assay) of the *S. media* plant extract in free form (SM 1 mg/ml) and liposomal form (LIPOSM) compared with the positive (LIPO, ALA) and negative (CT) control.

Determining cell viability is a mandatory test to be performed when establishing the cell culture. The higher the cell viability, the faster the moment of confluence is reached and the scratch test can be performed. According to the results obtained, cell viability, in this case, is between 92.5487% and 95.5571%, which denotes an increased activity of NHDF cells in the cell plates in which they were seeded. This viability can be greatly influenced by the samples to be tested so it is still necessary to test their cytotoxicity as a function of time.

The cytotoxicity of the samples on the cell culture was tested by the MTS method and compared with the CT sample. The MTS test was performed at time T0 of applying the samples and later at T24 hours, obtaining cell viability between 91.581% and 95.568%, which indicates that the applied particles are not toxic to the cell culture.

Considering these preliminary tests, the scarring effect of the liposomal form of the *S. media* extract (LIPOSM) and the free extract 1 mg/ml (SM) was tested by the scratch method from time T0h to time T36h **(Figure 4)**.

It can be seen that the SM sample after 36h shows a percentage of closure of the simulated in vitro wound area comparable to the ALA control sample and higher than that of the CT and LIPO samples (used as positive control).

At the same time, the liposomal formula shows at the same time interval (T36h) a percentage of wound area closure (86.884%) more than the SM sample (81.2221%), which indicates that the created liposomal formulation is more advantageous, increasing

the bioavailability of the included extract, favoring the passage through the membrane of the fibroblast type cell and due to the protection offered to the included extract [21].

Conclusion

Following the tests carried out, it can be concluded that the aqueous or hydroalcoholic extract of the *S. media* plant is rich in phenols, although the type of extraction is of particular importance. The hydroalcoholic extraction of these compounds is more beneficial (obtaining a higher total of phenols than in the case of the aqueous extract) so that in the future the tests regarding the antioxidant capacity, antimicrobial and healing activity were carried out on the type extract hydroalcoholic.
Through methods such as DPPH and TEAC, the antioxidant capacity of the *S. media* plant extract was demonstrated, which is also due to the high content of chlorophyll pigments.
The antimicrobial activity was also tested and demonstrated against gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria compared to antibiotics such as Ciprofloxacin 5µg, Penicillin 10U, and Oxacillin 30µg. It was evident that there is a higher sensitivity of gram-positive bacteria for the applied extract.
Also, within the work, giant-type liposomes were successfully formulated with dimensions that mostly (66%) do not exceed 500 nm and with a high extract encapsulation efficiency (87%).
The testing of the healing capacity through the scratch method is also highlighted in this work with success in terms of demonstrating the importance of applying the extract in the encapsulated form to improve the biological effect compared to the non-encapsulated extract.

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