The Annals of the University Dunarea de Jos of Galati
Fascicle VI – Food Technology (2023), 47(2), 172-189

ORIGINAL RESEARCH PAPER

PHYTOCHEMICAL AND BIOLOGICAL SCREENING OF AQUEOUS *GALIUM VERUM* L. EXTRACT

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Received on 27 November 2023
Revised on 14 December 2023

Abstract

Plants are an important source of active compounds with numerous therapeutic benefits. Nowadays, plant extracts have attracted an increased attention as candidates in the prevention and treatment of various diseases, including cancer. *Galium verum* L. is considered an effective remedy due to its extensive traditional use, thus the investigation of its anticancer potential has gained momentum. Malignant melanoma is an aggressive type of cancer with an increased incidence. Therefore, the purpose of this paper was to analyze the cytotoxic effect of GvH_2O extract on A375 cells. Furthermore, the safety of the extract was evaluated on HaCaT cells and the results showed that the GvH_2O does not exert a cytotoxic action on keratinocytes but stimulates their proliferation at 15 - 55 µg/mL. In addition, the GvH_2O extract was characterized from a phytochemical point of view. The results showed that GvH_2O contains polyphenolic compounds (rutin and chlorogenic acid),

https://doi.org/10.35219/foodtechnology.2023.2.11

possesses antioxidant activity and can be effective on Gram-positive bacteria as well as on *E. coli*. GvH_2O extract showed a cytotoxic effect on melanoma cells, causing changes in confluence and morphology and revealing nuclear characteristics specific to apoptosis. Finally, *Galium verum* L. may be a possible candidate for skin cancer therapy, but additional studies are needed.

Keywords: *Galium verum* L., antioxidant activity, DPPH test, antimicrobial activity, cytotoxicity, melanoma

Introduction

Nature, through plants, has always been and still is a valuable source of active principles with important therapeutic benefits for human health. Nowadays, plant extracts have come to the fore due to their content in bioactive compounds such as polyphenols, which, being of natural origin, are considered perfect candidates as they can replace synthetic compounds that in some cases can be toxic, presenting numerous adverse effects (Stepanyan *et al*., 2023).

Although there is evidence about the therapeutic actions of plants (Al-Snafi, 2017; Al-Snafi, 2018a), detailed research regarding their biological properties is needed to be used in the prevention and treatment of various diseases, including cancerous pathologies. Among them, we find the *Galium* species that are considered effective and safe remedies due to their extensive traditional use (Turcov *et al*., 2022).

The genus *Galium* belongs to the *Rubiaceae* family, it includes over 650 species of herbaceous, perennial, or annual plants, spread over the world (Europe, Africa, Asia, and North America) (Hanganu *et al*., 2018). However, studies focused on the full knowledge of these plants, and their uses are still limited compared to other plant families.

In Romania, the genus *Galium* is represented by approximately 38 species, among which the best known is *G. verum* L. (lady's bedstraw); next to *G. mollugo* L. (hedge bedstraw); *G. aparine* L. (cleavers or stickyweed); *G. odoratum* L. (syn. *Asperula odorata*, woodruff) (Turcov *et al*., 2022). *G. verum* L. is a perennial plant, approximately 50-70 cm tall, with rhizomes and an erect stem, densely puberulent. The species has sessile leaves arranged in a spiral, with hermaphroditic flowers, disposed in terminal panicles and golden yellow corolla (Al-Snafi, 2018b).

Galium species present a rich and complex content of phytoconstituents found in the aerial part, in flowers and leaves, the main compounds identified belong to phenolic acids, flavonoids, iridoid glycosides, saponins, essential oils, and vitamin C. *G. verum* has the highest composition of polyphenols (Shynkovenko *et al*., 2018; Tava *et al*., 2020). The pharmacological properties of *Galium* species are well-known, and recommendations regarding the administration of the plant in internal or external diseases are available (Turcov *et al*., 2022). In traditional medicine, *G. verum* is used for its depurative, diuretic, laxative, antirheumatic, and sedative action (Mocan *et al*., 2019). Several studies have shown that *Galium* extracts possess antimicrobial activity (Khan *et al*., 2022) and cardioprotective effects (Bradic *et al*., 2020).

In recent years, emphasis has been placed on the anti-cancer action of *Galium* extracts. Atmaca *et al*. (2016) showed that *G. aparine* methanolic extract is cytotoxic on MCF-7 and MDA-MB-231 breast cancer cell lines in a concentration and time-dependent manner, and does not have a toxic effect on MCF-10A breast epithelial cells. Furthermore, the group led by Aslantürk *et al*. (2017) demonstrated that methanolic extract and ethyl acetate fraction of *Galium aparine* have notable apoptotic activity on human MCF-7 breast cancer and also on Caco-2 colon cancer cells. Schmidt *et al*. (2014a) reported that *G. verum* aqueous extract inhibits the motility of head and neck cancer cell lines (HLaC78 and FADU).

Investigations on the *in vitro* effect of the *G. verum* L. extract on human melanoma cells are needed, because there are no available studies in the literature up to this point on the antitumor action of this plants on this specific type of cancer. Malignant melanoma is considered a rare pathology, being the main cause of death worldwide, so it requires in-depth investigations (Naik, 2021).

Therefore, the aim of the current study was to outline the phytochemical screening of the aqueous extract obtained from the aerial part of the *G. verum* L. species, including the antioxidant capacity together with the biological evaluation - antimicrobial activity, and the *in vitro* anticancer effect, on healthy human keratinocyte (HaCaT), and human skin cancer lines (A375).

Materials and methods

Reagents and bacterial strains

The solvents used to obtain the last phase (aqueous phase) from the *G. verum* plant material, were ethanol 95% (v/v) and distilled water, purchased from Girelli Alcool SRL (Milan MI, Italy), respectively Invitrogen (MA, USA), while petroleum ether, diethyl ether, ethyl acetate and butanol were procured from Sigma Aldrich (Steinheim, Germany).

For the antioxidant capacity investigation, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used, acquired from Sigma Aldrich (Steinheim, Germany), and as standard, it was used the ascorbic acid, purchased from Lach-Ner Company (Prague, Czech Republic). All chemicals used were of high analytical purity.

The standards used for the LC-MS analysis were: rutin, quercetin, quercetol, quercitrin, isoquercitrin, chlorogenic acid, and 4-O-caffeoylquinic acid, procured from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and methyl alcohol were acquired from Merck (Darmstadt, Germany). A MiliQ Milli-Q® Integral Water Purification System (Merck Millipore, Darmstadt, Germany) insured the ultrapure deionized water.

For the *in vitro* testing, a specific culture medium, high glucose Dulbecco's Modified Eagle's Medium (DMEM), was used along with cell culture supplement - fetal calf serum (FCS) and penicillin/streptomycin (Pen/Strep - 10,000 IU/mL) procured from PAN-Biotech GmbH (Aidenbach, Germany). Other reagents used were: trypsin-EDTA solution, phosphate buffer saline (PBS), dimethyl sulfoxide (DMSO) acquired from Sigma-Aldrich (Darmstadt, Germany) and MTT viability kit (3-(4,5-

dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Roche Holding (Basel, Switzerland).

The antimicrobial activity examination was performed with bacterial strains procured from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). The four chosen species are representative of the human pathogenic bacteria, namely: *Staphylococcus aureus* - ATCC 25923, *Streptococcus pyogenes* - ATCC 19615, *Escherichia coli* - ATCC 25922, and *Pseudomonas aeruginosa* - ATCC 27853. All tested bacteria were isolated on Columbia agar with sheep blood 5% (Thermo Scientific, Waltham, Massachusetts, USA).

Cell culture

The *in vitro* analysis were carried out on immortal keratinocyte cell line (HaCaT, CLS, CVCL_0038) purchased from the Cell Lines Service (Eppelheim, Germany) and on human malignant melanoma cell line (A375, ATCC® CRL-1619™) procured from the ATCC (Manassas, Virginia, USA). The cells were cultured in a specific culture medium: DMEM supplemented with 10% FCS and 1% Pen/Strep. The tests were conducted under standard conditions at37 °C, in a humidified atmosphere with CO2 5%. The cells were stimulated with five concentrations (15-55 µg/mL) of *G. verum* L. aqueous extract.

Plant material and extraction method

The dried aerial part (herba) of the *G. verum* L., was acquired from a local store named AdNatura (S.C. ADSERV S.R.L, Timisoara, Romania, batch no. 11/2022) kept at a temperature of 22±2 °C and ground before extraction.

The extraction method was performed as follows: 200 g of dried and ground plant was mixed with 1000 mL of 95% ethanol. After 24 h of extraction at22±2 °C, the ethanolic extract was sonicated for another 30 min, using an ultrasonic water bath (Elma S120 Elmasonic) and filtered through a Whatman filter paper grade 4. To remove the ethanol, a vacuum rotary evaporator set at 25 °C and 60 mbar was used. The extraction of the bioactive compounds from the aerial part of *G. verum* L. was carried out in several stages. From the 200 g of plant product subjected to ethanolic extraction (phase one), after the filtration stage, 10 g of solid residue were weighed, over which 150 mL of distilled water was added and then 200 mL of petroleum ether. After 24 h, the petroleum ether phase was separated using a separator funnel, and further, was concentrated in a rotary evaporator (500 mbar and 30°C), thus obtaining the second extract phase. Over the remaining solid aqueous residue, 200 ml of different solvents were added one after the other, repeating the same process described above, obtaining sequentially phases from the extract: diethyl ether phase (concentrated at 750 mbar and 30°C), ethyl acetate phase (concentrated 170 mbar and 30°C), and butanol phase (concentrated at 50 mbar and 30°C). The last phase, the aqueous phase was concentrated at 30 mbar and 30°C, thus was obtained the extract investigated in the current study, GvH2O, which was maintained in a refrigerator at 4°C, until further evaluation. The schematic description of the extract preparation procedure can be seen in Figure 1.

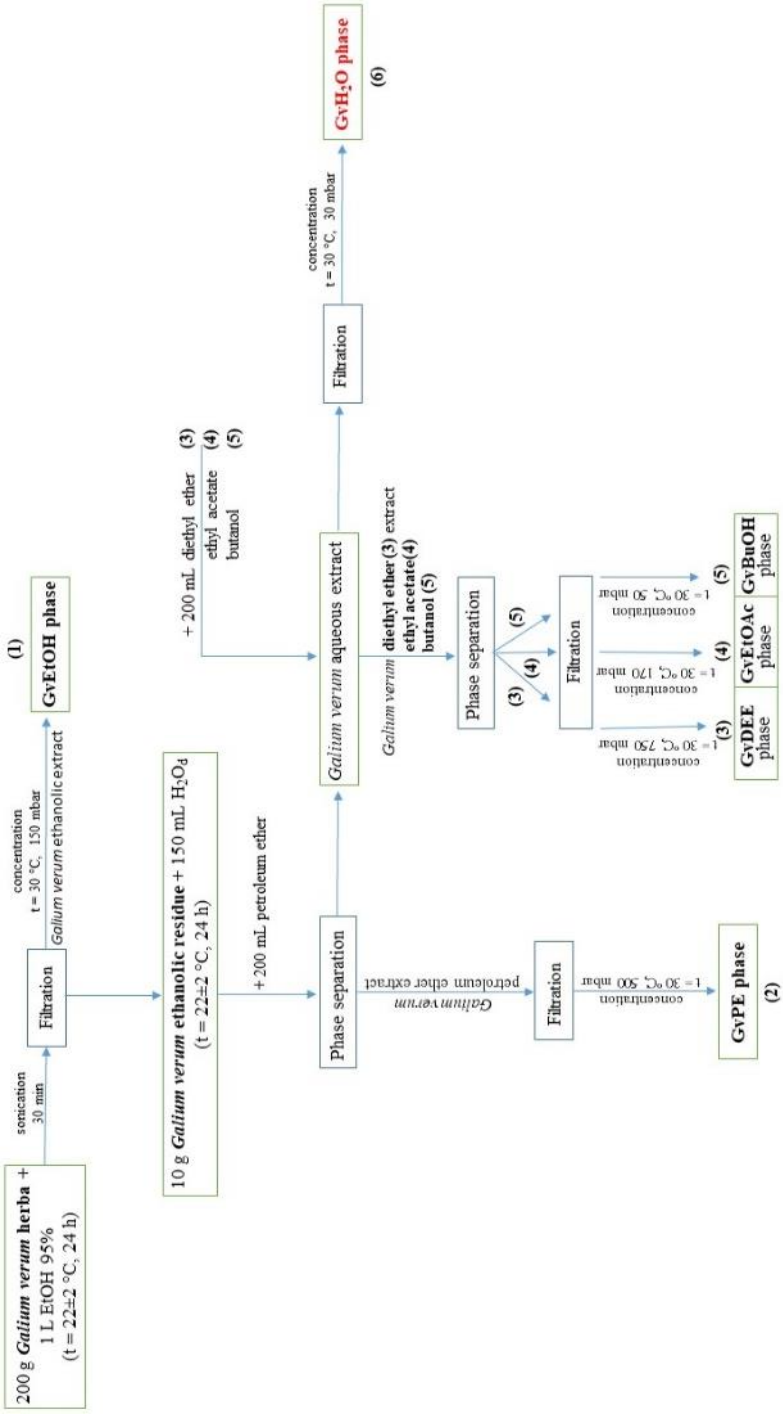

Figure 1. Schematic protocol for the preparation of *Galium verum* L. extracts.

For the phytochemical screening, antioxidant capacity evaluation, and biological assays, a stock solution of 1 mg/mL of *G. verum* aqueous extract was obtained.

Phytochemical evaluation

Fourier transform infrared spectroscopy (FT-IR) investigation

By employing FT-IR, the presence of organic molecules from the aqueous extract of *G. verum* L., was identified. FT-IR technique is a qualitative analysis that enable the identification of the organic functional groups of the polyphenolic compounds present in the extract, based on the match between the absorption bands obtained and those registered in the library. The working conditions included a temperature of 22±2 °C, a spectral region between 4000-400 cm^{-1} using KBr granules, and a resolution of 4 cm^{-1}.

Liquid Chromatography-Mass Spectrometry (LC/MS) analysis

The identification of polyphenolic compounds was performed by LC/MS, using the Agilent Technologies 1100 HPLC Series system (Agilent, Santa Clara, CA, USA). The chromatographic system was equipped with a degasser, binary gradient pump, column thermostat, autosampler, and UV detector and coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap SL). A reverse-phase analytical column (Zorbax SB-C18 100 × 3.0 mm i.d., 3.5 µm particles) at a temperature of 48 °C was used for separation. The detection of the compounds in the aqueous extract of *G. verum* L. was performed in UV mode, as well as in MS mode. For the detection of polyphenolic acids, the wavelength was set at 330 nm for 17 min, respectively 370 nm for 38 min, for the detection of flavonoids and their aglycones. The MS system was set up using an electrospray ion source in negative mode (nebulizer 60 psi (nitrogen), dry gas nitrogen at 12 L/min, and dry gas temperature 360 °C). A mobile phase, with a binary gradient consisting of methanol and acetic acid 0.1% (v/v), was used for the analysis. The first elution (5% methanol), was carried out for 35 min at a flow rate of 1 $mL \cdot min^{-1}$, started with a binary linear gradient, and ended at 42% methanol; then isocratic elution started with 42% methanol that took 3 min, followed by column re-equilibration with 5% methanol which took 7 min.

To identify the phytocompounds, the MS spectra of the polyphenols found in the extract were compared with the MS spectra of the standard solutions introduced in a mass spectra library. Following the matching of the bands, the polyphenolic compounds were identified based on the differences between their molecular mass and the MS spectra obtained following the qualitative analysis. The limit of quantification and detection for each compound was 0.1 µg/mL. Furthermore, the detection limit was calculated as the minimum concentration producing a peak reproduction with a signal-to-noise ratio greater than three. Chromatographic data were processed with ChemStation (vA09.03) and Data Analysis (v5.3) software from Agilent (Santa Clara, CA, USA). To determine the concentration of polyphenols in the extract, the calibration curves of the corresponding standard substances were used for a five-point plot in the range of 0.1-50 µg/mL, with good linearity (R^2 = 0.999), and the results were highlighted as µg of polyphenolic compound/mL of *G. verum* L. aqueous extract.

Antioxidant capacity

The antioxidant capacity of the aqueous extract of *G. verum* L. was performed using the DPPH free radical scavenging test. The results were expressed as an EC_{50} value, a value that refers to half of the maximum inhibitory concentration of the antioxidants contained in the extract, necessary to eliminate 50% of DPPH free radicals. First, 5 concentrations from the stock solution of the GvH_2O extract of 1 mg/mL were prepared: 0.8 mg/mL; 0.5 mg/mL; 0.3 mg/mL; 0.1 mg/mL, and 0.05 mg/mL. Then, the ethanolic solution of 0.1 mM of DPPH free radical was prepared, as well as the ethanolic solution of the standard (ascorbic acid) – 0.4 mg/mL. The resulted solutions, as well as the aqueous extracts of *G. verum* L., were kept in the refrigerator throughout the analysis. Further, extract was added to a quartz cuvette along with 2.7 mL of ethanolic DPPH solution. The absorbance values were read every 5 seconds, for 20 min using a UviLine 9400 spectrophotometer (SI Analytics, Mainz, Germany), at a wavelength of 517 nm. An ethanolic solution of ascorbic acid (0.4 mg/mL) was used for comparison. To calculate the DPPH free radical inhibition percentage, the equation described in the study reported by Sipos *et al.* (2021) was applied.

In vitro antimicrobial effects

The antimicrobial activity of the aqueous extract of *G. verum* L. was performed by determining the minimum bactericidal concentration (MBC) and the minimum inhibitory concentration (MIC). The broth dilution test was conducted according to the specifications given by the Clinical Laboratory and Standard Institute (CLSI), described in detail in a previous study (Weinstein et al., 2018). The standardized 0.5 McFarland bacterial inoculum was diluted in 0.85% sodium chloride to obtain $5x10^5$ colony-forming units/mL (CFU). After that, the extract and bacterial suspension were added to Mueller Hinton broth (Thermo Scientific, Massachusetts, USA), supplemented with blood and β-Nicotinamide adenine dinucleotide (β-NAD) for *Streptococcus pyogenes*, obtaining dilutions with concentrations of 3.75, 7.5, 15 and 30 mg/mL. After 24 h of incubation at 35 °C, the MIC value was interpreted as the lowest concentration without visible growth. The MBC is considered the lowest concentration that killed 99.9% of the bacteria and was established by sub-cultivating 1 µL of suspension from the test tube on Columbia agar with 5% sheep blood. The assays were conducted in triplicate for each strain tested.

Anticancer potential

Cell viability assessment

The aqueous extract of the species *G. verum* L. was tested for its cytotoxic effect on the human melanoma cell line - A375, and the safety effect on healthy keratinocytes – HaCaT.

The MTT test was used to highlight the action of the extract on cell viability. In short, the method consisted of the following steps: A375 and HaCaT cells were seeded in 96-well plates (10^4 cells/well in 100 µL medium/well), and were allowed approximately 24 h to attach and form the appropriate confluence and then to be stimulated with 5 increasing concentrations of aqueous extract (15, 25, 35, 45 and

55 µg/mL) for 24 h. After this time interval, 10 µL of MTT 1 reagent was added to each well and incubated for 3 h. Mitochondrial reductase from viable cells cleaved yellow tetrazole MTT, and formed dark blue formazan crystals, which were dissolved by adding 100 µl/well of lysis buffer for 30 min. Finally, the absorbance was measured spectrophotometrically at 570 nm with a Cytation 5 device (BioTek Instruments Inc., Winooski, VT, USA). The control was represented by untreated cells. Experiments were performed in triplicate.

Cell morphology and confluence evaluation

To emphasize the effect of the aqueous extract of *G. verum* L. on the HaCaT and A375 cell lines, their confluence and morphology were microscopically explored after 24 h of stimulation. Cells were bright-field imaged using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA) and analyzed with Gen5 microplate data collection and analysis software (BioTek Instruments Inc., Winooski, VT, USA).

Nuclear staining evaluation

To complete the activity of the aqueous extract of *G. verum* L., the Hoechst 33342 test was achieved, to define the cell death type. Therefore, A375 cells were cultured at an initial density of $1x10^5$ cells/well and treated with 15 and 55 µg/mL of aqueous extract of *G. verum* L. for 24 h. Then, the culture medium was removed and 500 µL/well of Hoechst staining solution diluted in PBS (1:2000) was added, for 10 min in the dark at 22±2 °C. Finally, the wells were washed three times with PBS and photographed. Images were caught using the fluorescence inverted microscope Olympus IX73 (Olympus, Tokyo, Japan).

Statistical analysis

The results of experiments are expressed as mean ± standard deviation (SD). For the *in vitro* biological studies, the statistical differences between samples were determined with the One-way ANOVA test followed by Dunnett's multiple post-hoc test comparisons, by using GraphPad Prism software version 9.4.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com). OriginLab 2020b software (Origin Lab - Data Analysis and Graphing Software, Szeged, Hungary) was employed to process the statistical data derived from the antioxidant capacity and FT-IR investigations of *G. verum* L. aqueous extract.

Results and discussion

Phytochemical screening

FT-IR investigation

The molecular fingerprint of the *G. verum* L. aqueous extract generated from the signal of each molecule or chemical structure at a specific wavenumber is described in Figure 2.

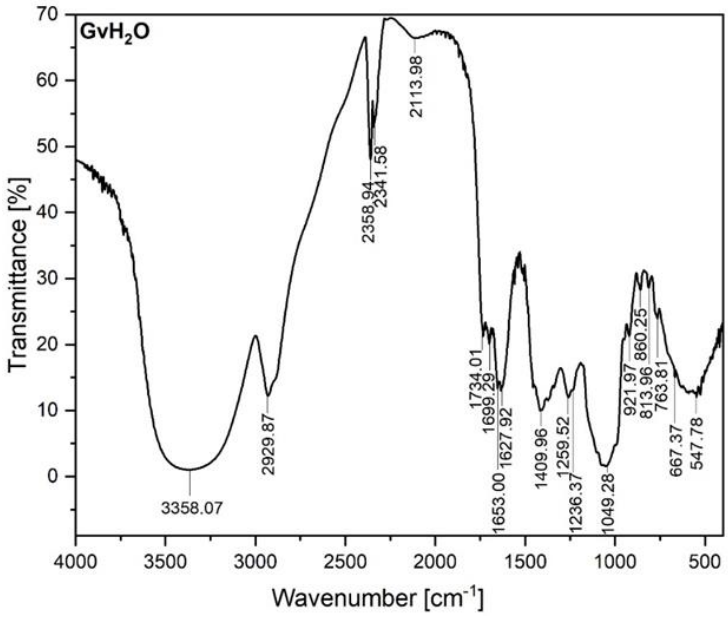

Figure 2. FT-IR spectrum of aqueous *Galium verum* L. extract.

FT-IR analysis recorded strong absorption bands at approximately 3350, 2350 and 1050 cm^{-1}. The first important, strong and well-defined band is located at 3358.07 cm^{-1}, which can be attributed to the O-H stretching vibration (with H atom intermolecular bonded) present in water or flavones. The band located at around 2929.87 cm^{-1} in the extract can be attributed to O-H stretching (with H atom intramolecular bonded) present in acidic functional groups (phenolic acid) or to the saturated aliphatic C-H stretching functional groups from alkanes. One can observe at 2113.98 cm^{-1} the C≡C stretching vibrational bond from the alkyne functional group. The bands recorded between 1734.01 cm^{-1} and 1627.92 cm^{-1} can be attributed to the C=C functional groups, present in alkenes (cyclic or di/tetra-substituted), but could also be attributed to the C=O stretching vibration functional groups from conjugated acids or aldehydes, as well as to δ-lactames functional groups. The absorption peaks of average intensity recorded in the 1200-1400 cm^{-1} range, highlighted the presence of the C-O functional group, most likely from aromatic esters as well as alkyl aryl ethers but also can highlight the presence of O-H bending functional group from carboxylic acids. The strong band defined at 1049.28 cm^{-1} is attributed to CO-O-CO stretching vibrations present in anhydrides, and the weak bands recorded between 750-920 cm^{-1} are attributed to the C-H bending vibrations (1,2,4- and 1,2,3-trisubstituted) present in alkenes as well as to C-Cl stretching functional groups from halo compounds. The region between 540 and 750 cm^{-1} is specific to the out-of-plane stretching vibration of halo compounds (C-Cl and C-Br stretching functional groups).

FT-IR is a widely used technique for the determination of antioxidants in many plants, such as herbs, fruits, cereals, oils, coffee, and honey (Cozzolino, 2015).

FT - IR is usually performed for the determination of flavonoids from different plants (Wulandari et al., 2016). In the current study, FT-IR identified that the extract comprises a strong out-of-plane C-H bending vibration for the substituted benzene ring, suggesting the presence of phenols and flavonoids. Moreover, the functional groups of alkanes, alcohols, and phenols present are considered the major groups of compounds with biological activity (Corcoran *et al.*, 2012).

The most important functional groups were recorded at 3358.07 cm^{-1}, which are attributed to hydroxyl groups from alcohols, phenols, and carboxylic acids. This signal most often reveals the presence of flavones, such as rutin. The band recorded at 2929.87 cm^{-1} indicates O-H functional groups in phenolic acids, such as chlorogenic and 4-O caffeoylquinic acids, but at the same time may correspond to CH3 vibrations (Szymczycha-Madeja *et al.*, 2013). Previous studies have stated that 1734 cm^{-1} and 1653 cm^{-1} peaks can be attributed to the C=O ester functional group, which indicates the presence of carotenoids, chlorophyll, and proteins (Mezzomo and Ferreira, 2016; Li *et al.*, 2018).

Liquid Chromatography-Mass Spectrometry (LC-MS)

Table 1 shows the polyphenolic content of the *G. verum* L. aqueous extract, derived from LC-MS analysis.

Table 1. Polyphenolic content of the *Galium verum* L. aqueous extract by LC-MS.

Compound name	UV identified	MS qualitatively identified	Concentration [µg/mL]
Chlorogenic acid	Yes	Yes	2.063
4-O caffeoylquinic acid	No	Yes	-
Rutin	Yes	Yes	0.560

LC-MS analysis highlighted the presence of three phenolic compounds in the aqueous extract of *G. verum* L. Chlorogenic acid was the most abundant compound in the extract, rutin was identified in a smaller quantity, slightly over 0.5 µg/mL, while 4-A caffeoylquinic acid was identified by UV, but below the limit of quantification.

Taking into account that the vegetable product was subjected to a series of extractions with different solvents, we noted that the concentration of the compounds with biological activity in the final aqueous phase was low. The polyphenolic content obtained is in agreement with what was reported in previous studies on the *G. verum* L. species. Previous studies indicate the proportion of the compounds, and additionally report the identified compounds from the flavonoid class such as quercetin, kaempferol, diosmetin; polyphenols, such as neochlorogenic acid, dicaffeoylquinic acid, but also iridoids: asperuloside (Danila *et al.*, 2011; Laanet *et al.*, 2023).

The identification by FTIR spectrophotometry of these absorption bands supported the findings from the phytochemical screening, which identified the presence of phenolic acids and flavonoids.

Antioxidant activity

Figure 3 depicts the antioxidant capacity of the *G. verum* L. aqueous extract, evaluated at 6 different concentrations (1 mg/mL – 0.05 mg/mL), in comparison with the ascorbic acid ethanolic solution (AA). It can be seen that all the prepared samples have quite good antioxidant capacity (between 30 and 50%). Moreover, it can be observed that samples with concentrations of 0.8 mg/mL and 0.5 mg/mL revealed almost identical values in terms of inhibition percentage. The EC_{50} of *G. verum* L. aqueous extract was 2.58±1.61 mg/mL (R^2 = 0.94174).

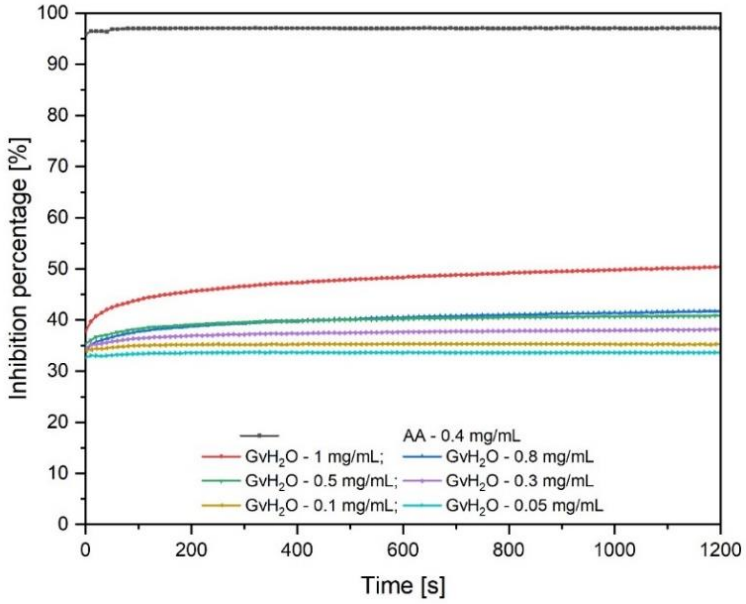

Figure 3. Time-dependent antioxidant capacity of the aqueous extract of *Galium verum* L. as compared with the standard (ascorbic acid ethanolic solution).

Except for the stock solution of GvH_2O extract (1 mg/mL), all the samples tested consumed the DPPH free radical within the first 200 seconds (~3 min) then the reaction kinetics reached equilibrium.

Previous studies that have investigated the antioxidant activity of *G. verum* L. extracts showed a similar trend to the results obtained in the current study when water was used as a solvent. Anyway, a different trend was observed when other solvents were used. For example, the aqueous extracts of *G. verum* L. showed a significant antioxidant action, but the methanolic extracts exerted a more effective peroxidation inhibition activity, although in some cases a relatively lower content of phenolic compounds was observed compared to the aqueous extracts (Mavi *et al*.,

2004; Danila *et al*., 2011). Lakić *et al*. (2010) showed that the methanolic extracts had an IC_{50} varying from 3.10 μg/mL to 8.04 μg/mL, depending on the geographical area where the plant was collected. Moreover, Vlase *et al*. (2014) reported that the DPPH free radical scavenging activity recorded for the 70% ethanolic extract of *G. verum* L. was 105.43 μg/mL.

The differences observed in this study and those previously reported could be due to a variety of factors, such as the geographical area, the type and volume of solvent used for extraction, the method and extraction parameters used, but mostly, they may be the result of the different concentration of phytocompounds present. It is known that the phenolic compounds contribute significantly to the antioxidant capacity of medicinal plants, due to their high potential to neutralize free radicals (Lapornik *et al*., 2005; Machado *et al*., 2013; Plaskova and Mlcek, 2023).

Antimicrobial analysis

The antimicrobial activity of GvH_2O extract against Gram-positive and Gram-negative bacilli strains is described in Table 2. The antibacterial action was quantified by micro-dilution test, and the determination of MIC (mg/mL) and MBC (mg/mL) was conducted.

Table 2. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) values.

Test compounds	Microbial strains	MIC [mg/mL]	MBC [mg/mL]
GvH_2O	*Streptococcus pyogenes (Gram +)*	30	30
	Staphylococcus aureus (Gram +)	30	30
	Escherichia coli (Gram -)	30	30
	Pseudomonas aeruginosa (Gram -)	NA	NA

NA – no activity (absent antimicrobial activity)

The results showed that the aqueous extract has antimicrobial activity on the two Gram-positive bacterial strains studied and, furthermore, on the *Escherichia coli* strain, but it does not have an antimicrobial effect on the *Pseudomonas aeruginosa* bacterium.

Our study demonstrated that the aqueous extract is effective against both Gram-positive and Gram-negative (*Escherichia coli*) bacilli strains, having bactericidal action, as it is reproduced in the literature (Tava *et al*., 2020). The lack of activity on the *Pseudomonas aeruginosa* strain can be explained by the fact that Gram-negative strains are more resistant due to the complex structure of the bacterial wall. In addition, the *Pseudomonas aeruginosa* strain is a strain resistant to numerous antibiotics, such as β-lactams or aminoglycosides.

However, some data in the literature showed that, by employing the right solvent in the extraction process, the *G. verum* L. plant material could have effect on *Pseudomonas aeruginosa* bacterium. Vasilevna *et al*. (2016) stated that the

chloroform extract of *G. verum* L. showed antimicrobial activity on the Gram-negative strain of *Pseudomonas aeruginosa*; the effect may be due to the solvent used.

Anticancer potential

Viability test

Initially, the aqueous extract was tested regarding the activity on cell viability on the HaCaT cell line, respectively A375 by means of the MTT colorimetric test.

Figure 4A shows the effect of the five tested concentrations of the GvH_2O extract for 24 h on human keratinocytes - HaCaT. A pronounced effect of cell proliferation was observed. The lowest applied dose induced an increase in cell viability of 119.5% followed by 114.9% in the case of the 25 µg/mL concentration, while the highest dose of 55 µg/mL produced a slight decrease in the cell viability of 99.3%. We can state that the aqueous extract of *G. verum* L. did not affect the healthy skin cells, it did not have a cytotoxic effect.

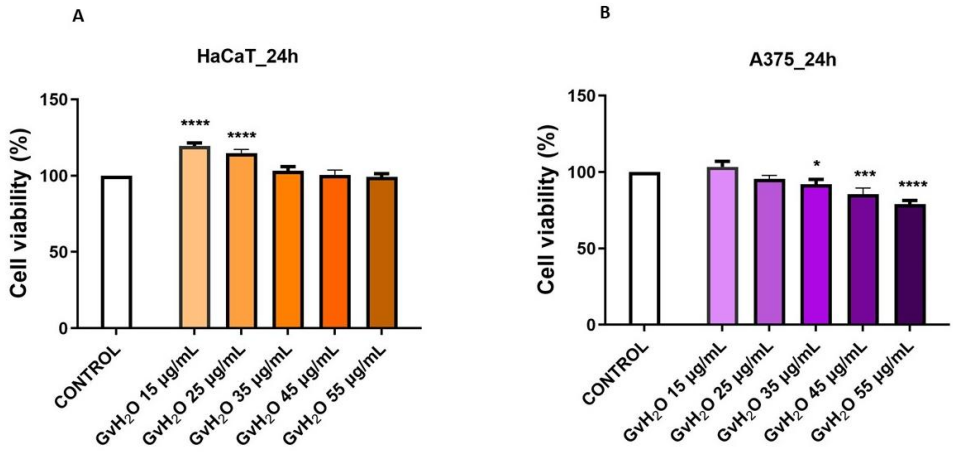

Figure 4. Viability percentages of HaCaT (A) and A375 (B) cells after 24 h of exposure to aqueous extract of *Galium verum* L. Statistical differences between the control cells and the treated cells were examined by applying the one-way ANOVA analysis followed by Dunett's multiple comparisons post-test (*$p < 0.05$; *** $p < 0.001$; ****$p < 0.0001$).

Figure 4B reveals the effect of the *G. verum* L. aqueous extract on the tumor line - A375. With the increase in concentration, a decrease in cell viability was measured. The lowest dose produced a slight proliferation with a viability rate of 103.4%, while the lowest percentage of viability was below 80% due to the dose of 55 µg/mL.

Considering the results obtained in the present study, we can state that the *G. verum* L. aqueous extract did not affect the human keratinocytes, being observed a strong proliferation of cells. Regarding the tumor cells, a decrease in their viability was evident with the increase of the applied doses, thus being able to conclude that this aqueous extract can have a cytotoxic effect on human malignant melanoma cells.

Cell morphology and confluence

In order to visualize the effect of the extract on the two cell lines, their morphology and confluence were microscopically evaluated after 24 h of stimulation (Figures 5A and 5B). Due to the fact that no significant changes in viability were observed between the middle concentrations tested, we decided to further evaluate and highlight the morphological aspect at three, the most suggestive concentrations.

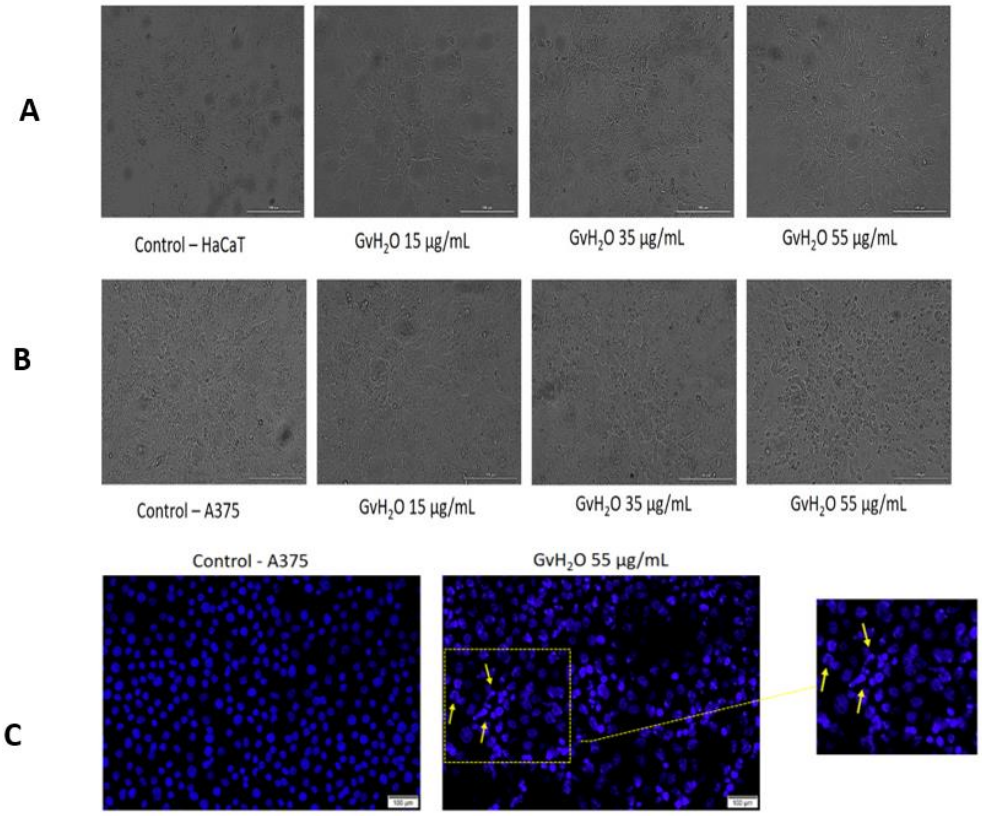

Figure 5. Morphology and confluence of HaCaT (A) and A375 (B) cells; and A375 nuclei stained with Hoechst 33342 dye (C) after a 24 h treatment with GvH_2O extract. The scale bars indicate 100 µm.

In the case of healthy HaCaTcells, for all tested concentrations, no changes in morphology were observed, but only a gradual decrease in cell confluence (15 > 55 µg/mL). The stimulated cells did not show different characters in respect to the untreated cells.

While at the level of tumor cells, the highest concentration of the GvH_2O extract demonstrated a decrease in the confluence of the cells and more of a change in their morphology, more than 20% of the cells were detached from the plaque, and from their slightly elongated shape they have become round.

Nuclear morphology evaluation

To complement the antitumor activity of the GvH_2O extract, the effect on the nuclei of A375 cells was followed. Nuclear morphology was revealed by Hoechst 33342 staining.

The control cell revealed a uniform, regular appearance of the nuclei without morphological changes, while the cells treated with the dose of 55 µg/mL of GvH_2O extract expose a condensation of the chromatin, a fragmentation of the nuclei, and a shiny aspect of them that seems to be apoptosis (Figure 5C).

The results of the *in vitro* activity of *G. verum* L. aqueous extract are consistent with data found in the literature. The group led by Schmidt *et al*. (2014b), reported that the aqueous extract of *G. verum* L. inhibited the growth of chemosensitive laryngeal carcinoma cell lines (Hep-2 and HLaC79) but also of chemoresistant laryngeal carcinoma cell lines with overexpression of glycoprotein P. (Hep2-Tax, HLaC79-Tax) at the concentrations of 50 and 100 µl/ml, however, the extract did not affect the formation of angiogenesis. Also, the aqueous extract of *G. verum* L. showed a toxic effect on the head and neck cancer cell lines (HLaC78 and FADU), while the mucosal keratinocytes showed a low sensitivity against high concentrations of the extract (100 µL/mL) after 48 h of exposure. Inhibition of invasion was more pronounced in the invasive cell line HLaC78. In primary mucosal keratinocytes, the extract protects DNA against benz[a]pyrene, one of the DNA toxic agents in cigarette smoke (Schmidt *et al*., 2014a).

Another study reported that the methanolic extract of *G. verum* L. can have an antitumor action against breast cancer cells (MCF-7 and MDA-MB-231), without affecting normal breast epithelial cells (MCF-10A). The extract was cytotoxic to breast cancer cell lines in a concentration- and time-dependent manner and, in addition, showed G1 block after 72 h of treatment. Furthermore, flow cytometry analyses revealed that apoptosis was induced in MDA-MB-231 cells, while necrosis was induced in MCF-7 cells (Atmaca *et al*., 2016).

Our research group tested several concentrations (15-50 µg/mL) of the GvH_2O extract, which produced a stimulation of human keratinocytes, without presenting a cytotoxic effect. In another study, it was demonstrated that the methanolic extract of *G. verum* L. also increased the survival rate of the healthy cell line of human fibroblasts - AGO, at the same applied doses as in our study. However, at a concentration of 400 µg/mL, the viability of fibroblasts was reduced, by increasing apoptosis (Pashapour *et al*., 2022).

Following the data obtained, *G. verum* L. plant material draws attention to be studied in more depth in terms of its anticancer potential, as future studies are needed regarding the action on human melanoma cells.

Conclusions

The present study included an evaluation of the phytochemical and biological profile of the aqueous extract of *Galium verum* L. The results showed that, following the evaluation of several concentrations, the GvH_2O extract possesses antioxidant

activity and contains polyphenols which were quantified through LC-MS analysis, specifically, rutin and chlorogenic acid. The aqueous extract proved to be effective on various pathogens, especially on Gram-positive bacterial strains but also on gram-negative strains (*Escherichia coli*). Regarding the anticancer effect *in vitro*, the results showed that GvH_2O extract does not affect human keratinocytes, rather it stimulates their proliferation, and it can exhibit a dose-dependent cytotoxic effect on malignant melanoma cells - A375, with signs of apoptosis.

Acknowledgments

The *in vitro* evaluations were performed within the Research Center for Physico-Chemical and Toxicological Analysis of the "Victor Babes", University of Medicine and Pharmacy, Timisoara.

References

Al-Snafi, A.E. 2017. Phytochemical constituents and medicinal properties of *Digitalis lanata* and *Digitalis purpurea* - A review. *Indo American Journal of Pharmaceutical Sciences*, **4**(2), 225-234.

Al-Snafi, A.E. 2018-a. Medical importance of *Juniperus communis* - A review. *Indo American Journal of Pharmaceutical Sciences*, **5**(3), 1979-1792.

Al-Snafi, A.E. 2018-b. *Galium verum*–A review. *Indo American Journal of Pharmaceutical Sciences*, **5**, 2142–2149.

Aslantürk, Ö.S., Çelik, T.A., Karabey, B., Karabey, F. 2017. Apoptotic activities of ethyl acetate and methanol extracts of *Galium aparine* L. *Journal of Pharmaceutical Research International*, **15**(6), 1–16.

Atmaca, H., Bozkurt, E., Cittan, M., Dilek Tepe H. 2016. Effects of *Galium aparine* extract on the cell viability, cell cycle and cell death in breast cancer cell lines. *Journal of Ethnopharmacology*, **20**(186), 305-310.

Bradic, J., Jeremic, N., Petkovic, A., Jeremic, J., Zivkovic, V., Srejovic, I., Sretenovic, J., Matic, S., Jakovljevic, V., Tomovic, M. 2020. Cardioprotective effects of *Galium verum* L. extract against myocardial ischemia-reperfusion injury. *Archives of Physiology and Biochemistry*, **126**(5), 408-415.

Corcoran, M.P., McKay, D.L., Blumberg, J.B. 2012. Flavonoid basics: Chemistry, sources, mechanisms of action, and safety. *Journal of Nutrition in Gerontology and Geriatrics*, **31**(3), 176-189.

Cozzolino, D. 2015. Infrared spectroscopy as a versatile analytical tool for the quantitative determination of antioxidants in agricultural products, foods and plants. *Antioxidants (Basel)*, **4**(3), 482-97.

Danila, A.O., Gatea, F., Radu, G.L. 2011. Polyphenol composition and antioxidant activity of selected medicinal herbs. *Chemistry of Natural Compounds*, **47**, 22–26.

Hanganu, D., Burtescu, R.F., Petrescu, S., Pripon Furtuna, F.R., Chise, E., Turcus, V., Benedec, D., Oniga, I., Olah, N.K. 2018. *Galium* species–Polyphenolic content and their antioxidant potential. *Hop and Medicinal Plants*, **26**(1–2), 84–93.

Khan, S.A., Khan, H., Ahmad, S., Rehman, F.U., Khan, A.A., Khan, M.A. 2022. GCMS characterization and biological potential of the seeds and aerial part of *Galium tricorne* Stokes. *Brazilian Journal of Biology*, **20**(84), e256920.

Laanet, P.R., Saar-Reismaa, P., Jõul, P., Bragina, O., Vaher, M. 2023. Phytochemical screening and antioxidant activity of selected estonian *Galium* species. *Molecules*, **28**(6), 2867.

Lakić, N.S., Mimica-Dukić, N.M., Isak, J.M., Božin, B.N. 2010. Antioxidant properties of *Galium verum* L. (Rubiaceae) extracts. *Central European Journal of Biology*, **5**, 331–337.

Lapornik, B., Prošek, M., Wondra, A.G. 2005. Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering*, **71**(2), 214-222.

Li, X., Zhou, R., Xu, K., Xu, J., Jin, J., Fang, H., He, Y. 2018. Rapid determination of chlorophyll and pheophytin in green tea using Fourier transform infrared spectroscopy. *Molecules*, **23**(5), 1010.

Machado, M.T.C., Mello, B.C.B.S., Hubinger, M.D. 2013. Study of alcoholic and aqueous extraction of pequi (*Caryocar brasiliense* Camb.) natural antioxidants and extracts concentration by nanofiltration. *Journal of Food Engineering*, **117**(4), 450-457.

Mavi, A., Terzi, Z., Ozgen, U., Yildirim, A., Coşkun, M. 2004. Antioxidant properties of some medicinal plants: *Prangos ferulacea* (Apiaceae), *Sedum sempervivoides* (Crassulaceae), *Malva neglecta* (Malvaceae), *Cruciata taurica* (Rubiaceae), *Rosa pimpinellifolia* (Rosaceae), *Galium verum* subsp. *verum* (Rubiaceae), *Urtica dioica* (Urticaceae). *Biological and pharmaceutical bulletin*, **27**(5), 702–705.

Mezzomo, N., Ferreira, S.R.S. 2016. Carotenoids functionality, sources, and processing by supercritical technology: A review. *Journal of Chemistry*, **2016**, 1-16.

Mocan, A., Diuzheva, A., Bădărău, S., Moldovan, C., Andruch, V., Carradori, S., Campestre, C., Tartaglia, A., De Simone, M., Vodnar, D., Tiecco, M., Germani, R., Crișan, G., Locatelli, M. 2019. Liquid phase and microwave-assisted extractions for multicomponent phenolic pattern determination of five Romanian *Galium* species coupled with bioassays. *Molecules*, **24**(7), 1226.

Naik, P.P. 2021. Cutaneous malignant melanoma: A review of early diagnosis and management. *World Journal of Clinical Oncology*, **12**(1), 7–19.

Pashapour, S., Heshmati, M., Mousavi, Z., Esmaeili, S. 2022 Effect of whole methanolic extract of *Galium verum* on AGO cell line. *Toxicology Communications*, **4**(2), 10.

Plaskova, A., Mlcek, J. 2023. New insights of the application of water or ethanol-water plant extract rich in active compounds in food. *Frontiers in Nutrition*, **10**, 1118761.

Schmidt, M., Polednik, C., Roller, J., Hagen, R. 2014-a. *Galium verum* aqueous extract strongly inhibits the motility of head and neck cancer cell lines and protects mucosal keatinocytes against toxic DNA damage. *Oncology Reports*, **32**(3), 1296-1302.

Schmidt, M., Scholz, C.J., Gavril, G.L., Otto, C., Polednik. C., Roller, J., Hagen, R. 2014-b. Effect of *Galium verum* aqueous extract on growth, motility and gene expression in drug-sensitive and –resistant laryngeal carcinoma cell lines. *International Journal of Oncology*, **44**, 745–760.

Shynkovenko, I.L., Ilyna, V.T., Kovalyova, M.A., Goryacha, V.O., Golembiovska, I.O., Koshovyi, M.O. 2018. Saponins of the extracts of *Galium aparine* and *Galium verum*. *Synthesis and Analysis of Biologically Active Substances*, **4**, 16–23.

Sipos, S., Moacă, E.A., Pavel, I.Z., Avram, Ş., Creţu, O.M., Coricovac, D., Racoviceanu, R.M., Ghiulai, R., Pană, R.D., Şoica, C.M., Borcan, F., Dehelean, C.A., Crăiniceanu, Z. 2021. Melissa officinalis L. aqueous extract exerts antioxidant and antiangiogenic effects and improves physiological skin parameters. *Molecules*, **26**(8), 2369.

Stepanyan, L.A., Sargsyan, T.H., Israyelyan, M.H., Karapetyan, K.J., Tsaturyan, A.H. 2023. Study of the chemical composition, antibacterial and antioxidant activity of various extracts of the aerial part of *Leonurus cardiaca*. *Farmacia*, **71**, 5.

Szymczycha-Madeja, A., Welna, M., Zyrnicki, W. 2013. Multi-element analysis, bioavailability and fractionation of herbal tea products. *Journal of the Brazilian Chemical Society*, **24**, 777–787.

Tava, A., Biazzi, E., Ronga, D., Avato, P. 2020. Idetification of the volatile components of *Galium verum* L. and *Cruciata leavipes* Opiz from the Western Italian Alps. *Molecules*, **25**(10), 2333.

Turcov, D., Barna, A.S., Trifan, A., Blaga, A.C., Tanasă, A.M., Suteu, D. 2022. Antioxidants from *Galium verum* as ingredients for the design of new dermatocosmetic products. *Plants (Basel)*, **11**(19), 2454.

Vasilevna, I.T., Volodymyrivna, G.O., Leonidivna, T.E., Aleksandrovna, K.I., Mihaylovna, K.A. 2016. Antimicrobial activity of the genus *Galium* L. *Pharmacognosy Communications*, **6**(1), 42-47.

Vlase, L., Mocan, A., Hanganu, D., Benedec, D., Gheldiu, A., Crișan, G. 2014. Comparative study of polyphenolic content, antioxidant and antimicrobial activity of four *Galium* species (Rubiaceae). *Digest Journal of Nanomaterials and Biostructures*, **9**(3), 1085-1094.

Weinstein, M.P., Patel, J.B., Burnham, C.A., Campeau, S., Conville, P.S., Doern, C., Eliopoulos, G.M., Galas, M.F., Humphries, R.M., Jenkins, S.G., Kircher, S.M., Lewis, J.S., et al.. 2018. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Clinical and Laboratory Standards Institute*, **11**th ed, Wayne, PA, USA.

Wulandari, L., Retnaningtyas, Y., Lukman, N., Lukman, H. 2016. Analysis of flavonoid in medicinal plant extract using infrared spectroscopy and chemometrics. *Journal of Analytical Methods in Chemistry*, 4696803.