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Galium verum L. petroleum ether extract – antitumor potential on human melanoma cells

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Abstract. The important role of plants in the prevention and treatment of several ailments has been known since ancient times. Plants are a rich source of biocompounds with known therapeutic benefits. At the moment, natural products are a hope for certain diseases that modern medicine fails to cure. Cancerous pathologies were and still are a disease category in which the most effective treatment with the fewest adverse effects is constantly being sought. Malignant melanoma is the skin cancer that has produced the highest number of deaths and is intensively studied. *Galium verum* L. is a plant known for its traditional uses, for this reason, it is currently being studied in the therapy of several diseases, including cancer. Our study aimed to phytochemically characterize the petroleum ether extract and to evaluate its safety and antitumor potential on HaCaT and A375 cell lines, respectively. The FT-IR assay revealed that the GvPE extract comprises functional groups of lipophilic compounds and phenolic compounds, four compounds being qualitatively identified: rutin, isoquercitrin, quercetol, and chlorogenic acid through LC-MS analysis, in addition, a medium antioxidant capacity was observed. The results obtained on HaCaT showed that GvPE does not significantly reduce cell viability, and up to the tested doses it does not produce a relevant cytotoxic effect. In the case of skin cancer cells, the concentration of 55 μg/mL revealed a viability percentage of up to 55%, the cells becoming round and detaching from the plaque. Finally, the nuclear evaluation exhibited that the GvPE extract has an apoptotic-like effect, with the production of nuclear fragmentation and chromatin condensation.

***Keywords*:** *Galium verum* L., FT-IR, phytochemical screening, anticancer potential, *in vitro* assays.

1. Introduction

Traditional medicine has used plants for therapeutic purposes since ancient times, they being an important source of bioactive ingredients. Plants are the oldest form of health care due to the large reserve of phytocompounds that over time have demonstrated minor adverse reactions compared to synthetic medication [1-4].

Galium species are some of the plants intensively used in ethnomedicine in a wide variety of therapeutic conditions, comprising approximately 660 species in the world, of which over a third are distributed in Europe, and in Romania about 38 species. *Galium verum* L., also known as lady's bedstraw, is an invasive, perennial, herbaceous plant that grows from 60-120 cm. It has dark green leaves, placed in a spiral shape, with bristles on the lower surface, and the flowers are yellow, small, and arranged in dense inflorescences [5, 6].

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Over time, the phytocompound-rich composition of these plants has been shown. The main compounds identified belong to several classes such as phenolic acids, flavonoids, iridoid glycosides, terpenes, and fatty compounds, but also aldehydes, anthraquinones, and small amounts of saponins, tannins, essential oils, and vitamin C were also observed [3, 5, 7-10].

Different studies have exposed the effect of extracts of *Galium* sp. *Galium verum* L. is known for its use in the treatment of epilepsy, and gout, but also for its diuretic, choleretic, sedative, and antimicrobial effects [11]. Thus, scientific data reported that *G. verum* L. extract has a strong inhibitory effect on several strains of *Candida*, as well as on *Listeria monocytogenes*, and in addition, revealed an important antihemolytic effect correlated with the antioxidant activity which is due to the inhibition of peroxidation [6, 12-15]. However, detailed studies on the composition and therapeutic uses of this plant are not as numerous compared to other plant species. Therefore, scientific research on *Galium verum* L. has been intensified.

Several research have investigated the apoptotic effects of this plant on cancer cell lines. Furthermore, the cytotoxic effect of *Galium verum* L. extracts on the larynx [16], neck and head [17], colon [18, 19], liver [19], and breast [20] cancer cells was highlighted. Currently, research in this branch is still a challenge for the scientific world, cancer being a pathology that aggressively attacks humanity.

Melanoma is a malignant tumor responsible for the largest number of skin cancer deaths worldwide. Malignant melanoma is a type of cancer that develops from the uncontrolled proliferation of melanocytes [21]. The fight against this pathology is still a challenge for the medical world due to its heterogeneity and especially the high toxicity given by standard therapy. Therefore, it is desirable to discover and use new effective phytocomplexes in the treatment of this type of cancer with as few adverse effects as possible, and a good alternative can be represented by plants. Plant extracts have been proven to be effective in killing and inhibiting many types of cancer cells, both in *in vitro* assays and *in vivo* models [22, 23].

Our study aimed at the phytochemical assessment of the petroleum ether extract of *Galium verum* L. (GvPE), referring to the identification of functional groups of organic molecules by FT-IR, the detection of polyphenolic compounds by LC-MS analysis, as well as the evaluation of antioxidant activity and, likewise, to investigate the potential antitumor effect of the extract on the malignant melanoma line - A375 and, in parallel, to observe the effect on the human healthy keratinocyte cell line - HaCaT.

2. Experimental

2.1. Chemicals and reagents

For the obtaining of the extract from the *Galium verum* L. herba, multiple solvents were utilized, ethanol 95% was acquired from Girelli Alcool SRL (Milano MI, Italy) and distilled water from Invitrogen (MA, USA), while petroleum ether was bought off from Sigma Aldrich (Steinheim, Germany).

For the LC-MS evaluation were utilized the following standard substances: quercetol, quercitrin, isoquercitrin, chlorogenic acid, 4-O-caffeoylquinic acid, and rutin, quercetin, acquired from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid and methanol were procured from Merck (Darmstadt, Germany) and the ultrapure deionized water was furnished by a MiliQ® Water Purification System (Merck Millipore, Darmstadt, Germany).

To evaluate the antioxidant potential, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used from Sigma Aldrich (Steinheim, Germany); moreover, to compare the results, vitamin C (ascorbic acid) was utilized as a standard substance (Lach-Ner Company (Prague, Czech Republic)). All chemicals utilized in this study were of analytical purity.

To assess the *in vitro* effect were used various reagents like high glucose Dulbecco's Modified Eagle's Medium (DMEM), cellular supplement fetal calf serum (FCS), and antibiotics penicillin/streptomycin (Pen/Strep) provided by PAN-Biotech GmbH (Aidenbach, Germany). Phosphate-buffered saline (PBS), trypsin-EDTA solution, and the *in vitro* solvent dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (Darmstadt, Germany), MTT viability kit (3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide) from Roche Holding (Basel, Switzerland), and, respectively, Hoechst 33342 staining dye solution from Thermo Fisher Scientific, Inc., Waltham, MA, United States.

2.2. Cell lines

The *in vitro* evaluations were accomplished on human keratinocyte cell line - HaCaT, (CVCL_0038, CLS, Eppelheim, Germany) and human malignant melanoma cell line - A375 (CRL-1619™, ATCC®, Manassas, Virginia, USA).

2.3. Extraction procedure

In the current study, it was utilized dried aerial part of the *Galium verum* L., acquired from AdNatura Company (S.C. ADSERV S.R.L) kept at room temperature.

The extraction technique initially consisted of crushing 25 g of dried plant and mixing it with 150 mL of 95% ethanol. After 24 h at 22±2 °C, the ethanolic extract was ultrasonicated for 30 min (ultrasonic water bath), filtrated, and then concentrated with a vacuum rotary evaporator at 25 °C and 60 mbar. From the 25 g of initial aerial part material, 10 g of the *Galium verum* L. herba were weighed, over which 150 mL of distilled water and 200 mL of petroleum ether were added and then were sonicated. After a period of 24 hours, the petroleum ether and aqueous phases were separated using the separatory funnel. Finally, the petroleum ether phase was concentrated with a rotary evaporator at a pressure of 500 mbar and a temperature of 25 °C and maintained at 4 °C. After all, it derived the petroleum ether extract of *Galium verum* L. (GvPE), the extract of interest from this study. For phytochemical tests, GvPE was diluted in 95% ethanol respectively in 0.5% DMSO for the biological analysis, up to the final stock concentration of 1 mg/mL.

2.4. Phytochemical evaluation

2.4.1. Fourier transform infrared spectroscopy (FT-IR) investigation. The FT-IR spectroscopy was utilized to identify the chemical structure of molecules and to determine the functional groups of the compounds present in the extract, by matching the obtained absorption bands with those in the library. A spectral region between 4000-400 cm^{-1}, a temperature of 22±2 °C, and a resolution of 4 cm^{-1} were used [24].

2.4.2. Liquid Chromatography - Mass Spectrometry (LC/MS) analysis. The detection of polyphenolic compounds was determined by LC/MS, using the Agilent Technologies 1100 HPLC Series System (Agilent, Santa Clara, CA, USA). The system was furnished with an autosampler, degasser, binary pump, column thermostat, and UV detector, which was coupled with an Agilent 1100 mass spectrometer. A reverse-phase analytical column at 48 °C was utilized for separation. The detection of the compounds in the petroleum ether extract of *Galium verum* L. was carried out in UV mode, as well as in MS mode. For the detection of polyphenolic acids, it was used a wavelength of 330 nm for 17 minutes, respectively 370 nm for 38 minutes for flavonoids. A mobile phase, with a binary gradient of methanol and acetic acid 0.1% (v/v), was utilized for the identification, as exemplified in one of our previous articles [25]. The limit of quantification for compounds was 0.1 μg/mL.

2.5. Antioxidant capacity

The antioxidant activity of the GvPE was determined using the DPPH assay, which measures free radical scavenging capacity. The results were represented as EC_{50} - which represents the concentration needed to obtain an antioxidant effect of 50%.

In short, an ethyl alcohol solution of DPPH was formulated, and then 2.7 mL DPPH solution was added together with a volume of each sample (6 concentrations) into a quartz cuvette of size 10x10 mm. Absorbances were read using a UviLine 9400 spectrophotometer (SI Analytics, Mainz, Germany), every 20 minutes at 517 nm wavelength. Moreover, 0.4 mg/mL vitamin C in ethanol was utilized as etalon for the comparison. To determine the DPPH free radical inhibition percentage, the calculation formula represented in one of our previous studies was used [26].

2.6. Anticancer potential

2.6.1. Cell culture. Cells were cultured in DMEM supplemented with 1% admixture Pen/Strep and 10% FCS. The cells were kept in specific atmospheric conditions, in a Steri-Cycle i160 CO_2 incubator (Thermo Fisher Scientific, USA), at 5% CO_2 and 37°C. Cell lines were grown in 75 cm^2 flasks and were regularly passaged every 3 days, using trypsin-EDTA 0.25%, after reaching 80–90% confluence. Cell counting was conducted with Countess™ II Automated Cell Counter, using Trypan blue solution. The assays were executed under standard conditions, temperature 37 °C and humidified atmosphere CO_2 5% by using MSC Advantage Class II Biological Safety Cabinet, (Thermo Fisher Scientific, USA). The cells were tested with five concentrations (15-55 μg/mL) of GvPE for accomplishment of analyses.

2.6.2. Cell viability assay. The petroleum ether extract of *Galium verum* L. was evaluated regarding the cytotoxicity on the two cell lines (HaCaT and A375) by using the MTT viability method. The MTT test is a colorimetric analysis for measuring cell metabolic effects.

Briefly, HaCaT and A375 were seeded onto 96-well plates (10^4 cells/well). At appropriate confluence, the cell medium was removed and then stimulated with various concentrations of petroleum ether extract (15-55 μg/mL) for 24 hours. The control was represented by untreated cells. Cells were evaluated by the addition of 10 μL of MTT 1 solution for 3 hours. The mitochondrial reductase precipitated MTT as blue crystals, which were diluted in lysis solution (100 μL) from the MTT assay kit. The reduced MTT was finally measured spectrophotometrically at 570 nm using a Cytation 5 device (BioTek Instruments Inc., Winooski, VT, USA), as described by Gag et al. [27]. Experiments were executed in triplicate. The percentage of cell viability was calculated as follows: cell viability $(\%) = 100 - [(A_0 - A_s)/A_0 \times 100]$, where A_0 = absorbance of control sample and A_s = absorbance of tested samples.

2.6.3. Cell morphology and confluence assessment. To verify the activity of GvPE on the morphology and confluence of HaCaT and A375 cell lines, a microscopic evaluation was carried out. Cells were investigated under bright field illumination using Cytation 1 (BioTek Instruments Inc., Winooski, VT, USA). The images were examined with Gen5 microplate data collection and analysis software (BioTek Instruments Inc., Winooski, VT, USA).

2.6.4. Nuclear staining analysis. The toxicity of 55 μg/mL of GvPE at nuclear level was examined by application of the Hoechst 33342 staining test. In brief, cells were grown in 12-well plates (10^5 cells/well) and stimulated with the GvPE for 24 h. After 24 hours, the medium was removed, and the Hoechst solution (1:2000 in PBS) was added and incubated for 10 minutes at 22±2 °C, in the dark; then, the Hoechst reagent was washed with phosphate-buffered saline. Finally, the pictures were captured using Cytation 1 (BioTek Instruments Inc., Winooski, VT, United States) and analyzed utilizing Gen5™ Software (BioTek Instruments Inc., Winooski, VT, United States).

2.7. Statistical analysis

The results are displayed as mean ± SD (standard deviation), the differences were compared by applying the one-way ANOVA assay followed by Dunett's multiple comparisons post-test. For *in vitro* the utilized software was GraphPad Prism version 9.4.0 for Windows (GraphPad Software, San Diego, CA, United States). The statistically significant differences between data are scarred with * (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$). OriginLab 2020b software (OriginLab Corporation - Data Analysis and Graphing Software, Szeged, Hungary) was utilized to interpret the statistical results regarding the antioxidant activity and FT-IR analysis of the extract.

3. Results and discussion

3.1. Phytochemical examination

3.1.1. FT-IR spectroscopy. Figure 1 outlines the spectral profiles of the lady's bedstraw petroleum ether extract, from 3427 to 547 cm^{-1}.

The results of the FT-IR evaluation are presented in Table 1.

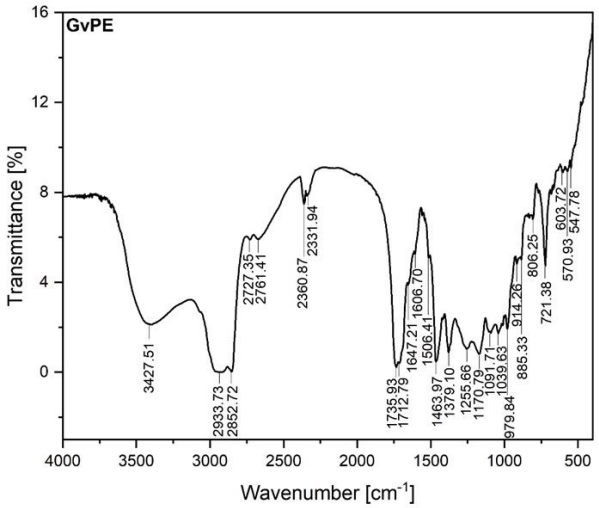

Figure 1. FT-IR spectrum of *G. verum* L. petroleum ether extract.

Table 1. Peak values, functional groups, and bonds of GvPE extract registered in the spectrum

Wavenumber (cm^{-1})	Functional groups	Bond
GvPE extract	**GvPE extract**	**GvPE extract**
3427.51	Alcohol	OH stretching (H-intermolecular bonded)
2933.73; 2852.72; 2727.35; 2761.41	Alkane / Alcohol (acid)	C-H stretching / OH stretching (intramolecular bonded)
2360.87; 2311.94	Carbon dioxide	O = C = O stretching
1735.93; 1712.79	Aldehyde	C=O stretching
1647.21; 1606.70	Alkene	C=C stretching
1506.41; 1463.97	Benzene	C=C stretching
1379.10	Alkane	C-H bending
1255.66	Acid	C-O stretching
1170.79	Ether	C-O stretching
1091.71	Alcohol / Ether	C-O stretching
1039.63	Nitrile	C-N stretching
979.84	Alkane (disubstituted (trans)) / Alkene	C=C bending / =C-H bending
914.26	Alkene	=C-H bending
885.33	Alkane (1,2-disubstituted)	C=C bending
806.25	Alkane (1,4-disubstituted) / Halo compounds	C=C bending / C-Cl stretching
721.38	Alkane (1,2-disubstituted) / Halo compounds	C=C bending / C-Cl stretching
603.72	Halo compounds	C-Cl stretching
570.93; 547.78	Halo compounds	C-Br stretching

The spectrum shows characteristic peaks. FT-IR evaluation recorded intense absorption bands at approximately 2933, 2852, and 1730 cm^{-1} but also between 1000 and 1400 cm^{-1}. The peak of 2933.72 cm^{-1}, with the –CH, $–CH_2$, and $–CH_3$ stretching vibrations, can be obtained from sugars and carbohydrates [28]. Also, the peaks approximately at 2933, 1712, 1606, 1463, 1379, 1091, and 721 cm^{-1} can be attributed to organic and phenolic compounds [29].

Based on the C-H bonds we can consider the presence of fats but also of starch due to bands between 2800 and 3500 cm^{-1} (C–H stretching and O-H stretching region) [30], this may be due to the solvent used, petroleum ether, which has a strong capacity to extract lipophilic bioactive compounds [31].

A previous study indicated that the 1735 cm^{-1} peak can be assigned to the C=O ester functional group, and the 1039.63 cm^{-1} wavenumber to the nitrile group, indicating the presence of chlorophylls [32]. The C–H and C=C ring-related vibrations can show the presence of aromatic rings in the structure of the chemical compound. The stretching of the C=C–C aromatic bond appears in the region of 1506.41 - 1463.97 cm^{-1}.

The region with the frequency of 500–1400 cm^{-1} in the IR spectrum is called the fingerprint region, the pattern of absorbance bands is characteristic of the compound as a whole. Furthermore, it was observed that the region between 540 and 720 cm^{-1} is specific to the stretching vibration of halo compounds (C-Cl and C-Br stretching bond).

3.1.2. LC/MS analysis. Table 2 illustrates the polyphenolic compounds of the lady's bedstraw petroleum ether extract, derived from LC-MS analysis.

Table 2. Polyphenolic content of the *G. verum* L. petroleum ether extract by LC-MS method

Compound	UV detected	MS qualitatively detected	Concentration (µg/mL)
Chlorogenic acid	No	Yes	-
Isoquercitrin	No	Yes	-
Rutin	No	Yes	-
Quercetol	No	Yes	-

Following the LC-MS examination, we wanted to determine the main polyphenolic compounds from the extract, knowing the presence of these phytocompounds in the vast majority of plants, and even in *Galium* sp. [4, 11, 25], but especially because of their therapeutic importance [33, 34]. *Galium verum* herba was subjected to extraction in several stages, in this study the phase of interest was that of petroleum ether. The obtained results showed us that 3 flavonoids were qualitatively identified in the GvPE extract, such as rutin, quercetol, and isoquercitrin, and a polyphenolic acid, chlorogenic acid, on the same trajectory as the literature data, but they were not quantified, being below the detection limit of 0.1 µg/mL,

For the petroleum ether extract, it is understandable that could not quantify hydrophilic chemical compounds such as polyphenolic compounds, because the solvent used, petroleum ether, is a non-polar solvent that is mainly used for the extraction of fats, oil, and unsaponifiable matter [31, 35]. Essential fatty acids are important biocompounds with a key role in the synthesis of cell membranes, such as prostaglandins, which regulate various biological functions: blood pressure, blood coagulation, heart rate, and fertility. Moreover, it has been shown that they have a significant role in regulating the immune system by controlling inflammation and eliminating infection and a potential anti-tumor effect by stopping the initiation of the cell cycle of various cancer cells [36-38].

Moreover, the FT-IR analysis provided us with information about the presence of fats in our extract by detecting the peaks and correlating them with the specific functional groups of lipophilic compounds, such as chlorophyll. Chlorophylls are compounds of therapeutic interest, that demonstrated antioxidant activity and anti-proliferative effects in pancreatic cancer cell lines (PaTu-8902, BxPC-3, MiaPaCa-2) in a dose-dependent manner (10-125 µmol/L) [39, 40].

Therefore, we can affirm that the solvent used for extraction has a considerable influence on the process of extracting, based on its interactions with the extracted bioactive compounds, moreover, the extraction method used, the applied parameters, and the geographical area of the plant can also influence the extraction of plant compounds [35, 41].

3.2. Antioxidant activity

Figure 2 presents the time-dependent antioxidant capacity of GvPE extract performed at various concentrations (1 – 0.05 mg/mL) compared with the ascorbic acid ethanolic solution (AA) at a concentration of 0.4 mg/mL.

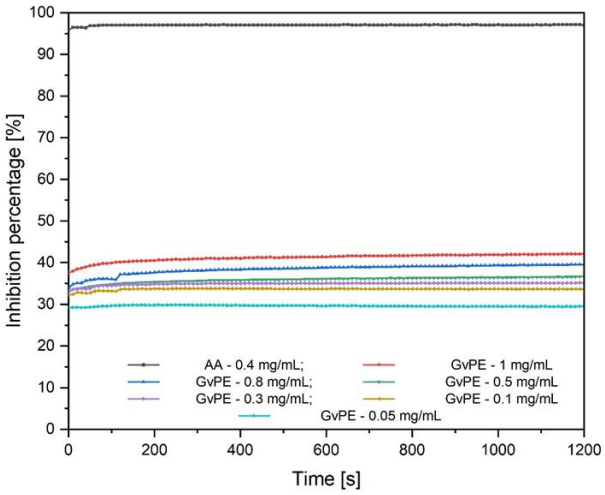

Figure 2. Time-dependent antioxidant activity of the petroleum ether extract of *Galium verum* L.

Table 3 reveals the inhibition percentage (IP) (%) of the concentrations tested of GvPE extract, as an average of three measurements ± standard deviation (SD). Further, by using these values, the EC_{50} of the GvPE fraction was calculated (EC_{50} = 10.99±5.83 mg/mL; R^2 = 0.95128).

Table 3. The inhibition percentage of GvPE extract at six concentrations tested

Examined extract concentration (mg/mL)	Inhibition percentage - IP (%)		EC_{50} (mg/mL)
	Vitamin C (standard)	GvPE	GvPE
1	97.08±0.04	42.10 +/- 0.04	10.99±5.83
0.8		39.54 +/- 0.03	
0.5		36.64 +/- 0.04	
0.3		35.14 +/- 0.04	
0.1		33.66 +/- 0.04	
0.05		29.49 +/- 0.04	

The tested samples of GvPE extract show moderate antioxidant activity, between 30% and approximately 42%. The concentrations of 0.5 mg/mL and 0.3 mg/mL of the extract showed a similar inhibition percentage, with small differences between the values. Moreover, the antioxidant activity of the samples of different concentrations obtained from the petroleum ether extract of *Galium verum* L. is concentration-dependent. In summary, it is observed that all the GvPE extract concentrations consume DPPH free radicals in the first 150 seconds, after which the reaction reaches equilibrium.

Previous studies have also demonstrated the antioxidant capacity of lady's bedstraw extract. The study led by Friščić et al. revealed for *G. verum* L. 80% methanol extract an EC_{50} of 30.72 µg/mL [42]. Other results were reported by Vlase and his team, who highlighted an EC_{50} of approximately 105 µg/mL for the alcoholic extract of *G. verum* L. (70%) [6]. Furthermore, another study reported significant half-maximal inhibitory concentrations for the methanolic extract range from 3.10 µg/mL to 8.04 µg/mL [43]. According to Danila et al., the aqueous extracts of *G. verum* L. exhibited important antioxidant activity but were stronger for the methanolic extracts [44].

The data from the literature show some differences compared to those obtained in our study. Once again, we can observe the influence of the solvent used in the extraction process, but even the volume of solvent and ratio of vegetable material: solvent, the particle size of the raw material, the extraction temperature, and duration can have a significant influence. Moreover, these differences can be attributed to the geographical area of the plant, and even the concentration and type of reagent used to determine the antioxidant activity [45].

3.3. Antitumor potential

3.3.1. Cell viability test. The first *in vitro* experiments determined the effect on the cell viability of the GvPE extract on the healthy keratinocyte cell line - HaCaT and in parallel on the malignant melanoma line - A375 by the MTT assay, to assess the cytotoxic ability of the petroleum ether extract.

Figure 3 shows the viability percentages of the five GvPE extract concentrations on the HaCaT line after 24 hours of exposure.

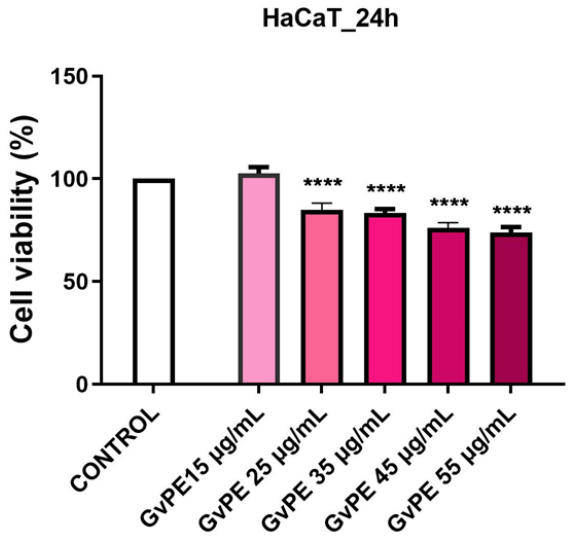

Figure 3. *In vitro* cell viability examination of the GvPE in HaCaT cells after 24 h of treatment by conducting the MTT test. Five concentrations were used for this analysis - 15, 25, 35, 45, and 50 µg/mL. Results are represented as viability percentages (%) normalized to control and are stated as mean values ±SD of three experiments effected in triplicate. The statistical differences between the untreated group and the treated group were confirmed by applying the one-way ANOVA test followed by Dunnett's multiple comparisons post-test ($^{****}p < 0.0001$).

At the lowest concentration, a slight stimulation of cell proliferation can be seen (102.5%). From concentrations of 25, and 35 µg/mL, a decrease in viable cells of approximately 85% can be observed, reaching the highest concentration to present a percentage of viability of approximately 77%.

According to the obtained data, we can consider that up to a concentration of 55 µg/mL the GvPE extract is safe to apply on the healthy line of human keratinocytes.

Regarding the action on the cancer line, it was reported that the first two tested concentrations of GvPE extract (15 and 25 µg/mL) produced an increase in cell viability in percentage up to 105-110%. As the dose increased, a significant decrease in cell viability was observed up to 67.3%, respectively 55.4% for the 45 and 55 µg/mL concentrations, as can seen in Figure 4.

Following the results obtained, we can state that the GvPE extract has a cytotoxic effect on the A375 tumor cells, a dose-dependent effect.

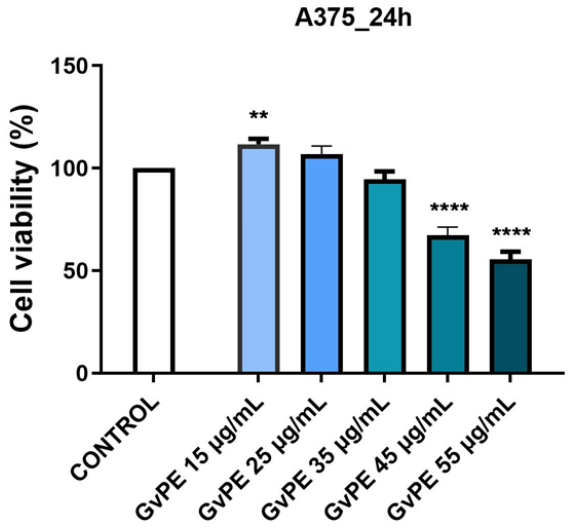

Figure 4. *In vitro* cell viability examination of the GvPE in A375 cells after 24 h of treatment by conducting the MTT test. Five concentrations were used for this analysis - 15, 25, 35, 45, and 50 µg/mL. Results are represented as viability percentages (%) normalized to control and are stated as mean values ±SD of three experiments effected in triplicate. The statistical differences between the untreated group and the treated group were confirmed by applying the one-way ANOVA test followed by Dunnett's multiple comparisons post-test ($^{**}p < 0.01$; $^{****}p < 0.0001$).

3.3.2. Cell morphology and confluence examination. Next, the effect of the GvPE extract was evaluated microscopically. To visualize the morphological changes and the confluence of the two cell lines following the application of the extract for 24 hours, a bright-field microscopic evaluation was carried out.

There being no considerable differences between the five applied concentrations, for the microscopic evaluation we used the most representative ones.

At the dose of 15 µg/mL, no changes are seen in the cell's morphology or the confluence compared to the control cells. However, at the concentration of 55 µg/mL, a slight decrease in confluence is observed with the appearance of round cells, detached from the plate, but in a small proportion (Figure 5).

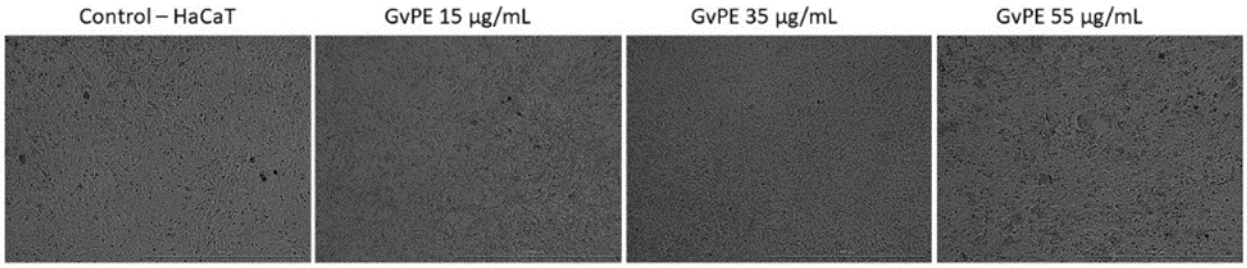

Figure 5. Microscopical presentation of cellular morphology and confluence of keratinocytes following a 24-hour treatment with the GvPE extract (15, 35, 55 µg/mL). The scale bars represent 100 µm.

On the malignant melanoma line, the GvPE extract produced significant changes observed with the help of microscopy. Thus, at the concentrations of 35 and 55 µg/mL, respectively, the extract induced a visible decrease in cell confluency. In addition, it was observed that more than 40% of the cells detached from the plate and changed their shape, becoming round and small (Figure 6).

These data are consistent with data obtained by MTT analysis.

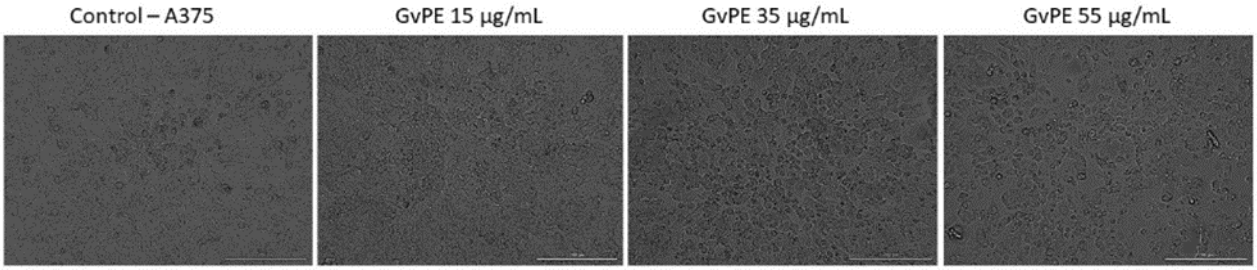

Figure 6. Microscopical presentation of cellular morphology and confluence of melanoma cells following a 24-hour treatment with the GvPE extract (15, 35, 55 µg/mL). The scale bars represent 100 µm.

3.3.3. Nuclear morphology analysis. At the nuclear level, the effect on melanoma cells of the highest extract concentration was assessed. Figure 7 shows the nuclear staining of A375 cells after a 24-hour application of 55 µg/mL of GvPE.

Following the Hoechst staining test, the untreated cells had a regular nucleus with no visible changes. At the opposite pole, at the concentration of 55 µg/mL, nuclear fragmentation, and chromatin condensation were observed, which are considered apoptotic-like signs.

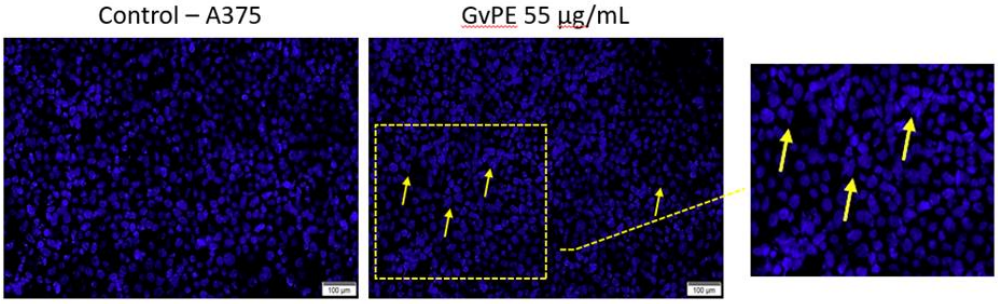

Figure 7. Nuclear evaluation of A375 cells following a 24-hour stimulation with the GvPE extract at 55 µg/mL. The scale bars present 100 µm.

The results obtained by us are in agreement with those reported up to this point in the specialized literature.

A previous study showed that the petroleum ether fraction of *Galium verum* L. possesses a cytotoxic effect on colon (HT29) and liver (HepG2) cancer cell lines. The two cell lines were exposed to 6 concentrations (3.125-100 µg/mL) of the extract. It was observed that all tested concentrations significantly decreased the viability of HT29 cells, while in the case of HepG2 cells, only the dose of 3.125 decreased cellular viability compared to control cells. In addition, concentrations of 50 and 100 µg/mL stimulated the proliferation of HepG2 cells [19]. In the same direction as our findings, the group led by Pashapour et al., reported that the alcoholic extract of *G. verum* L. induces apoptosis through the Bax-dependent pathway in colon tumor cells and increases intracellular ROS levels [18].

Schmidt and co-workers revealed that the aqueous extract of *G. verum* L. at a dose of 33.3 µl/mL has anti-invasive properties (on the extracellular matrix substrate invasion) in drug-sensitive and drug-resistant laryngeal cancer cell lines [16]. Another research group verified the inhibitory effect of the methanolic extract of *Galium aparine* on breast cancer lines, MCF-7 and MDA-MB-231. The study concluded that the methanolic extract

decreased the viability of both types of breast tumor cells in a concentration- and time-dependent manner. After a 72 h stimulation, at the concentrations of 300, 500, and 800 µg/mL, inhibition percentages were obtained for MDA-MB-231 cells of 62, 43, and 30%, respectively for MCF-7 cells of 75, 40, and 20%. Moreover, it was found that treatment with the methanolic extract altered the secretion of angiogenic cytokines in both cancer lines; thus decreasing the secretion of vascular endothelial growth factor (VEGF), tissue factor (TF), and neuregulin-1 (NRG1-β1) [46].

4. Conclusions

The present study captures a characterization of the phytochemical profile and biological activity of the petroleum ether extract of *Galium verum* L. Following the experiments, in the GvPE extract we qualitatively identified phenolic compounds, such as rutin, isoquercitrin, quercetol, and chlorogenic acid. Moreover, the FT-IR spectrum outlined functional groups that can be attributed to lipophilic compounds, biocompounds that can be present in the extract due to the non-polar solvent used, which we know has the ability to extract this type of phytocompounds. The GvPE extract proved to possess medium antioxidant activity and a significant antitumor potential on the A375 cell line. At the level of healthy cells - HaCaT, no cytotoxic effect was observed up to the tested concentrations. The GvPE extract at a dose of 55 µg/mL decreased the viability of A375 cells to a percentage of 55%. In parallel, changes in cell morphology were observed, with the appearance of small round and shiny cells and, in addition, signs of programmed cell death, fragmentation nuclear and chromatin condensation.

Galium verum L. extract can be considered as a potential candidate in malignant melanoma therapy, but more complex studies are needed to understand its mechanism of action

Conflict of interest

The authors declare that there is no conflict of interest regarding this research article.

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