Pol. J. Environ. Stud. Vol. 32, No. 3 (2023), 2043-2051
DOI: *10.15244/pjoes/157546* ONLINE PUBLICATION DATE: 2023-02-14

Original Research

Antioxidant Activity, UV-screen, Cytotoxic and Antitumoral Activities of a Polyphenolic Extract of *Helichrysum arenarium* (L.) Moench Flowers

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Received: 15 October 2022
Accepted: 16 December 2022

Abstract

Helichrysum arenarium (L) Moench (sandy everlasting) is a valuable plant for therapy, having multiple applications in traditional medicine, applications which are confirmed nowadays by science. The main polyphenols found in sandy everlasting flowers were shown to have antioxidant and UV-screen effects.

In order to expand our knowledge concerning the synergistic therapeutic effects of the polyphenols from *Helichrysum arenarium* (L) flowers, an extract was prepared using 50% ethanol as extraction phase, followed by the removing of ethanol using a Royeyov IKA RV10 rotary evaporator. The chemical analysis of the extract consisted in the assessment of total polyphenol content (Folin-Ciocalteu) and flavonoid fingerprint (high-performance thin-layer chromatography -HPTLC).

For the polyphenolic extract there were assessed the antioxidant activity (measured by two methods: ferric reducing antioxidant power (FRAP) and radical scavenging activity with DPPH)) and sun protection factor (SPF) (assessed using an in vitro spectrometric method).

Furthermore, the cytotoxic and antitumor effects were evaluated on two cell lines: pMSCs (palatal mesenchymal stem cells) and HeLa (ATTC® CCL-2™) cell line (epithelial cells derived from

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human cervical adenocarcinoma). The extract contains apigenin-7-glucoside, luteolin-7-glucoside and rosmarinic acid. The extract shows a significant UV-screen protection effect, particularly for UVB radiations. The extract has not cytotoxic effects on palatal mesenchymal stem cells. The cytometric apoptosis assay on HeLa cell line reveals the *in vitro* antitumor effect of the extract. The results highlights both antioxidant and antitumor activity as well as the UV-screen effect of the polyphenols from *Helichrysum arenarium* flowers. All these data not only confirm the high antioxidant properties of the polyphenols contained in the sandy everlasting flowers, but also open new ways of applications for therapy of this plant.

Keywords: *Helichrysum arenarium*, polyphenols, antioxidant activity, antitumor activity

Introduction

Helichrysum arenarium (L.) *Moench* (sandy everlasting) is a perennial herbaceous plant belonging to *Asteraceae* family [1]. It is native in Middle East, Balkans and Central European countries as well as in China.

Due to its successful use in traditional medicine (hepatoprotective, detoxication agent, for the treatment of the hepatic and gallbladder disorders, gastric atony, chronic cholecystitis, hepatitis, pancreatitis, gastro-intestinal disorders, intestinal parasites), the monography of *Helichrysi arenarii flos* vegetal product was included in World Health Organisation, Physician's desk reference for herbal medicine and German pharmaceutical Codex [2]. In addition, in some regions, it is used as diuretic [3].

The literature identified 39 molecules belonging to the flavonoid class in the composition of *Helichrysum arenarium* flowers, this class of compounds being characteristic for the *Helichrysum arenarii flos* [4, 5] plant product. Of these, the most abundant is chalcone isosalipurposide, a compound that gives the golden yellow colour to the involucres and is responsible for the hepatoprotective effects of the plant product [6]. High amounts of kaempferol, quercetin, luteolin and apigenin glycosides have also been identified [4, 7, 8].

The significant content in polyphenolic compounds induces a significant antioxidant activity of the vegetal product, as shown the literature data [9, 10].

In addition, the high amount of flavonoids suggests an increased UV protection activity of *Helichrysum arenarium* extracts, fact also noticed by Jarzycka at al. (2013) [11].

During experiments, we were able to prove the increased antioxidant activity, as well as the *in vitro* antitumor effect of polyphenolic extracts of *Robinia pseudoacacia* flowers [12].

Starting from this information, the present paper aims to bring to light the correlation between the chemical content and biological effects of the flavonoidic extract of *Helichrysum arenarium* flowers. The extract obtained from the plant product was chemically characterised by determining the total polyphenolic content and by identifying some flavone compounds known to be part of the plant product composition (fingerprint method) using a chromatographic method (HPTLC). This technique is known as an effective tool for the quality assessing of plant extracts. It is relatively simple, not expensive and has reduced requirements. For that reason it has been successfully used to develop chromatographic fingerprints for medicinal plant extracts [13, 14].

Subequently, the antioxidant activity and UV screen effect of the flavonoidic extract of *Helichrysum arenarium* flowers were quantified using *in vitro* methods. The effect on the cells in culture was targeted on its cytotoxic action and its antitumor action. The studied product demonstrated high antioxidant activity, significant UV protective action, especially in the UVB range, lack of cytotoxic effects and antitumour action. The content in polyphenolic compounds of the extract fits well with its antioxidant activity and UV-screen effect. The results we obtained lead us to continue the study of its biological properties in order to evaluate its potential for applications in dermatocosmetology. Thus, the main objectives of the study were:

Characterisation of antioxidant action and UV-screen effect of the polyphenolic extract of *Helichrysum arenarium* flowers, correlated with the main molecules in its composition;

Study of its effects on cells in cultures, as a first step in testing the biological effects of the sand everlasting flowers extract, which could have applications in therapy and/or cosmetology.

Experimental

All chemicals and reagents used in this study were obtained from Sigma Aldrich (Steinheim, Germany) and Merck (Darmstadt, Germany).

Plant Material

Helichysum arenarium plants were botanically identified by macroscopic and microscopic examination and subsequently collected from wild flora areas in the Dobrogea region, Romania. The flowers were processed in a dry place away from light, until they had less than 4% drying loss. The obtained plant product (*Helichysum arenarium flos*) was observed using an optical

microscope, magnification 40X on an Epifluorescent Microscope Fluoro 2 with Bel Photonics DV-1300 digital camera, BEL Engineering S.R.L., Monza, Italy.

Extraction Procedure

The 50% ethanol extract from *Helichysum arenarium* dried flowers (*Helichysum arenarium flos*) was prepared by suspending the dried flowers in the extractive phase in a ratio of 10 g product/100 mL extractive phase at 25ºC. The vegetal product was left to extraction for 10 hours under continuous stirring. Subsequently the alcohol was removed using a Royeyov IKA RV10 rotary evaporator (50ºC, 200 mBarr).

Determination of Total Phenolic Content (TPC)

The analysis was conducted using the spectrophotometric methods. The total phenolic content was determined according to the European Pharmacopoeia method, the values were expressed as mg GAE/mL [15].

Antioxidant Activity

The antioxidant activity of the polyphenolic extract was evaluated by the spectrometric technique using a UV-visible spectrophotometer Jasco V-630 (Able Jasco, Germany).

The Ferric reducing antioxidant power (FRAP) assay was performed according to method presented previous by Benzie [16] with slight modifications [17].

The DPPH radical scavenging activity of the *Helichysum arenarium* extract was performed according to the method reported by Brand-Williams [18], with slight modifications [17]. The results have been expressed as µM equiv Trolox/mL. All measurements were performed in triplicate and the results were presented as mean ± sd.

The Flavonoid Fingerprint of the Extract by HPTLC

An HPTLC system composed of CAMAG Linomat-5 automatic sample applicator and a CAMAG TLC scanner equipped with CATS software (version: 1.2) was used to indicate the presence of flavonoids. The mobile phase consisted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v); as stationary phase pre-coated silica gel 60 F254 HPTLC plates were used (20 cm x 10 cm, Merck, Darmstadt, Germany). The sample application technique and the development mode were carried out according to the method described by Bratu et al., [12]. After development, the plates were dried at 105ºC for 10 min and derivatised in NP-PEG reagents [19]. The fingerprints were evaluated at 366 nm in fluorescence mode with WinCats and VideoScan software. Standard compounds for HPTLC analysis are: S1 (Chlorogenic acid and Apigenin 7-glucoside of 0.5 mg/mL) and S2 (Rutin, Luteolin 7-glucoside of 0.2 mg/mL and Rosmarinic acid of 0.5 mg/mL).

Sun Protection Factor (SPF)

The Sun Protection Factor (SPF) was determined by spectrometric method in vitro, proposed by Dutra et al. 2004 [20], using a UV-vis spectrophotometer (Jasco V-630). 0.1 g of sample was weighed, transferred to a 25 mL volumetric flask, diluted to volume with ethanol, followed by ultrasonication for 5 min and then filtered through cotton, rejecting the ten first mL. A 1.0 mL aliquot was transferred to 10 mL volumetric flask and diluted to volume with ethanol. Subsequently, a 2.0 mL aliquot was transferred to a 10 mL volumetric flask and the volume integrated with ethanol. The absorption data were obtained in the range of 290 to 320, using 1 cm quartz cell, and ethanol as a blank, for every 5 nm. The SPF values were determined using the Spectra Manager programme.

Estimation of Cytotoxic and Antitumor Effects

Its potential is to highlight the mitochondrial and metabolic activity of the cells and, implicitly the cellular proliferation index. MTT is reduced in the mitochondria of metabolically active cells by succinate dehydrogenase to yield a water-insoluble purple formazan crystal [21].

Cytotoxic and apoptosis effects were determined according to the method described by Lazarević et al., 2019 [22] and Bratu et al., 2021 [12]. Thus, for the cytotoxic assay, two cells' lines were used: fully characterised palatal mesenchymal stem cells isolated from human oral cavity (pMSCs), and epithelial cell line derived from human cervical adenocarcinoma (HeLa ATTC® CCL-2™). The viability of the pMSCs and HeLa cells treated with Helichrysi arenarii extract was assessed using MTT assay (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich). MTT is a tetrazolium salt transformed by mitochondrial reductases of metabolically active cells, to a dark blue product, named formazan.

For the MTT assay, $1x10^4$ cells/well was seeded on 96 well plates in 200 µL complete culture medium: DMEM (Sigma-Aldrich) supplemented with 10% bovine foetal serum, 1% antibiotic-antimycotic, 1% glutamine, 1% NEA (non-essential amino-acids). After 24 h, the cells were treated with 5 different concentrations of *Helichysum arenarium flos* extract (5; 2.5; 1.5; 1.0; 0.5 µL). Control samples were represented by untreated cells. All treatments and controls were performed in triplicate. To assess the proliferation capacity of treated and untreated cells, the cultures were incubated with 150 µL MTT solution (1 mg/mL) in PBS (Phosphate Buffered Saline, Sigma). The cultures were incubated for 3 h at 37ºC. The MTT reagent was removed and 150 mL/well of DMSO (dimethyl sulfoxide) was added. The plates were analysed by measuring the optical

density at 450 nm with a BioTek Synergy 2 microplate reader. Since optical density is directly correlated with mitochondrial activity, its values were compared in treated cells and untreated control in order to observe the differences regarding viability and cell proliferation capacity between groups.

The BT Pharmigen FITC Annexin V Apoptosis detection kit was used to determine the apoptosis. The fluorescent intensity was read with a BD FACS Canto II flow cytometer, with the 488 nm wavelength argon laser. Results were interpreted using the FACS Diva 6.1 software. A total of 10,000 cells were analyzed, and the fluorescence intensity was presented in dot plots, each being divided into 4 quadrants: quadrant 3 (viable cells Q3) does not show fluorescence; quadrant 1 (Q1-apoptotic cells) stained only with annexin; quadrant 2 (late Q2-apoptotic cells) stained with both annexin and PI, and quadrant 4 (Q4) stained only with PI are necrotic cells.

Results and Discussion

Identification of the plant by microscopic examination

The microscopic examination of the *Helichysum arenarium* powder preparation allowed the observation of the following elements: stomata, fibres, parenchyma fragments, pollen grains, single and multicellular perithecia (Fig. 1).

Table 1. The content of total polyphenols (TPC) and the antioxidant activity (AA) of *Helichrysum arenarium* flowers extract.

Following the results obtained, it can be seen that *Helichrysum arenarium* flowers extract is rich in phenolic compounds (67.70% compounds in the extract) and have a significant antioxidant activity.

It can be noticed that both methods reveal an increased antioxidant activity, fact also confirmed by the results of other authors [11], who measured the antioxidant activities of aqueous and lyophilised extracts of *H. arenarium* flowers by different methods (DPPH method, chemiluminometric method, reducing power properties). The molecules identified in the composition of the analysed extract have recognised antioxidant properties, which is according to our results) [23].

The differences in the antioxidant activity of the extract evaluated by the two methods are due to differences in the measurement principles, the reaction kinetics on which each method is based and the different reaction medium conditions. The DPPH assay measures a change in the stable radical DPPH by the electron donating ability of the sample. The FRAP value measures the reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donor electrons in the sample [24].

High-performance thin-layer chromatography is known as a technique applied in routine analyses, due to its advantages of reliability in the quantification of analytes at micro and even nanogram levels with low cost [13]. In this paper the qualitative analysis of flavonoid compounds for the ethanolic extract of *Helichysum arenarium* was conducted using a chromatographic (HPTLC) method. The identity of the flavonoids in the extracts was achieved by comparing their R_F (the distance the spot moved above the origin to the distance the solvent front moved above the origin) values from the sample (S) with the reference compounds, S1 and S2 (Table 2).

Chromatographic analysis shows the presence in the extract of four important flavonoid compounds commonly found in plant products: Chlorogenic acid (R_F = 0.60), Luteolin 7-glucoside (R_F = 0.69), Apigenin-7-glucoside (R_F = 0.76) and Rosmarinic acid (R_F = 0.85) [3, 7, 25].

From the above images, the presence of rutin (R_F 0.58) cannot be accurately determined since in *Helichrysum arenarium* extract, at this R_F value an overlap of substances is observed, leading to a broadening of the separate band.

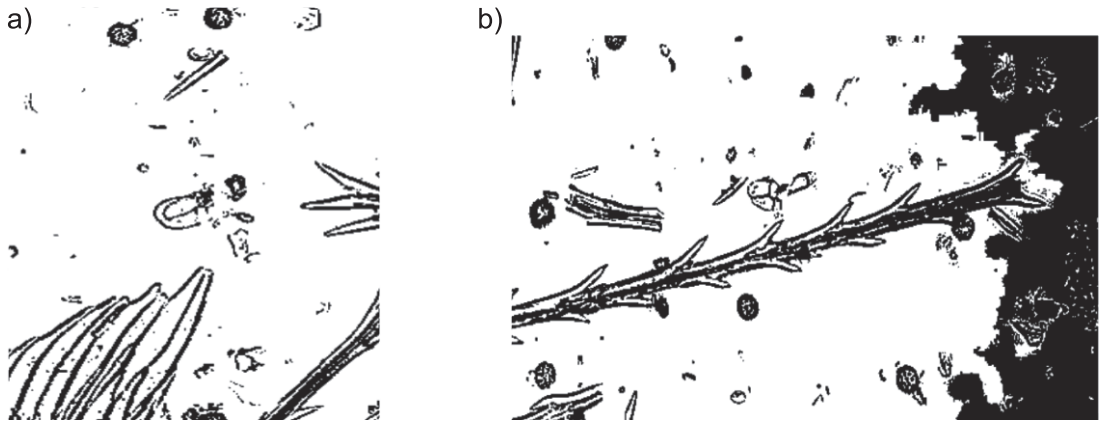

Fig. 1. Microscopic examination of *Helichrysum arenarium* powder preparation: a) Glandular hair, b) Pappus fragment and pollen grains with echinulate exine.

Table1. Total phenolic content and antioxidant activity of *Helichrysi arenarium* extract.

Total phenolic content (TPC) mg GAE/mL	FRAP µM equiv Trolox/mL	DPPH µM equiv Trolox/mL
1.42±0.12	0.15±0.01	38.8±4.02

Table 2. R_F values and colours of standards and sample.

Sample	Compound	R_F	Colour
S1	Chlorogenic acid	0.60	Dark blue spot
	Apigenin-7-glucozida	0.74	Light green spot
S2	Rutin	0.58	Yellow spot
	Luteolin 7-glucoside	0.72	Yellow- greenish spot
	Rosmarinic acid	0.88	Dark blue spot
S = extract of *Helichrysi arenarii*	Chlorogenic acid	0.60	Dark blue spot
	Luteolin 7-glucoside	0.69	Yellow spot
	Apigenin-7-glucozida	0.76	Green spot
	Rosmarinic acid	0.85	Dark blue spot

The UV Radiation Screen Activity

Solar UV radiation is a potent mutagen and epidemiological and molecular studies classify it as a leading cause of skin cancer. Approximately 5% of solar radiation energy is emitted in the UV range and is divided into three categories according to wavelength: short wavelength UVC (200-280 nm), medium wavelength UVB (280-320 nm) and long wavelength UVA (320-400 nm). UVC radiation is extremely damaging to the skin as it induces genotoxic stress.

The analysed polyphenolic extract showed protection activity against UV solar radiation, particularly for UVB (SPF = 6.9857) and UVC (SPF = 3.2541).

Literature data agree with our results, with apigenin having anti-UVA and UVB photoprotective effects tested on human keratocytes [26-28].

The literature data, supported by the results presented in this paper, suggest that plant extracts containing apigenin can be used as sunscreen ingredients in innovative cosmetic products. It is also of interest that the extract under analysis confers protection in UV areas that have the most harmful effect on the skin and on living cells in general.

Cytotoxic Assay

For the evaluation of the cytotoxic effect of *Helichrysum arenarium* aqueous extract, palatal mesenchymal stem cells (pMSCs) were treated with 5 different dilutions (5µl; 2.5 µl; 1.5 µl; 1.0 µl; 0.5 µl). After 24 h of treatment there were no morphological changes and no changes in the degree of proliferation compared to untreated cells. The results obtained by MTT and interpreted with GraphPad Prisma 6 software can be seen in Fig. 2.

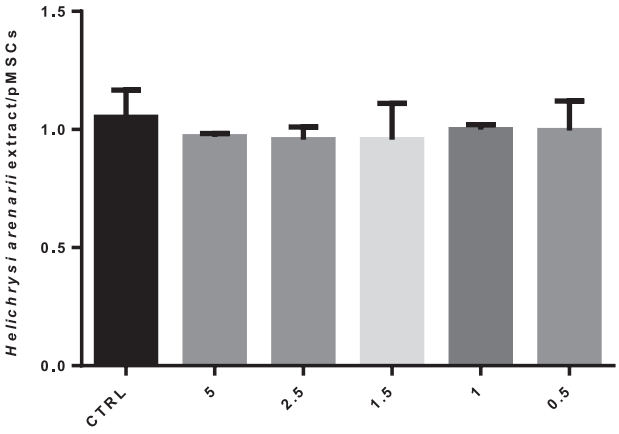

Fig. 2. Effects of *Helichrysum arenarium* extract on pMSCs.

In the case of HeLa cells, a minor antiproliferative potential associated with minor morphological changes can be observed at the concentrations of 5 µl and 2.5 µl to control samples. The remaining concentrations tested showed a similar degree of proliferation as the control cultures. The results obtained at MTT and interpreted with Prism 6 software can be seen in Fig. 3.

Cytometric Apoptosis

Helichysum arenarium flos extract had the ability to induce both necrosis and apoptosis of treated tumour cells at a significant rate compared to the untreated control. This effect was observed to be more significant the higher the concentration of the extract was, as

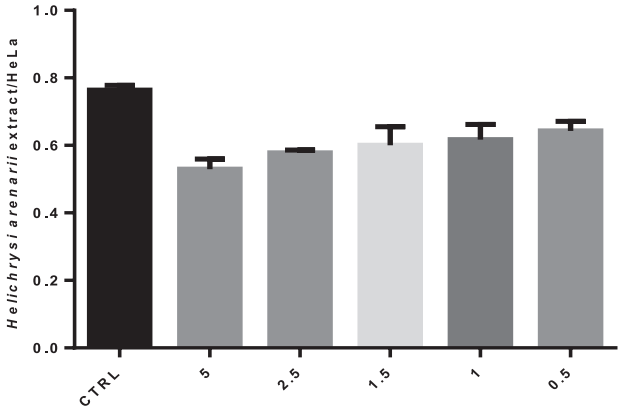

Fig. 3. Effects of *Helichrysum arenarium* extract on HeLa cells.

shown in Table 3 and Fig. 4. Apoptosis of the HeLa cell line indicates the presence of viable cells (Q3), apoptotic cells (Q1), Q2 = late apoptotic cells (Q2), and necrotic cells (Q4).

In the case of pMSCs, after treatments with extract in different amounts, the following results were obtained regarding the induction of apoptosis and cell necrosis (Table 3 and Fig. 5).

These results demonstrate that *Helichrysum arenarium flos* extract has a non-selective action in terms of inducing apoptosis and cell necrosis, this effect being observed in both tumour and palatal mesenchymal stem cells.

Table 3. Apoptosis and necrosis of palatal mesenchymal stem cells (pMSCs) and HeLa cells.

	Viable, [%]		Apoptosis, [%]		Late apoptosis, [%]		Necrosis, [%]	
	pMSCs	HeLa cells	pMSCs	HeLa cells	pMSCs	HeLa cells	pMSCs	HeLa cells
Control	97.3	93.5	1.6	2.4	0.5	1.5	0.7	2.6
Helichrysi. arenarium flos 0.5 µl	82.7	79.7	3.7	3	10.7	1.7	2.9	15.6
Helichrysi. arenarium flos 1 µl	77.9	78	7.7	4.6	9.1	5.7	5.3	11.6
Helichrysi. arenarium flos 1.5 µl	74.9	71.3	6.2	8.9	14.1	11.9	4.8	8
Helichrysi. arenarium flos 2.5 µl	70.9	72.2	11	7.2	13	10.2	5.1	10.4
Helichrysi. arenarium flos 5 µl	63.1	62	4.8	6.4	19.9	15.9	12.2	15.7

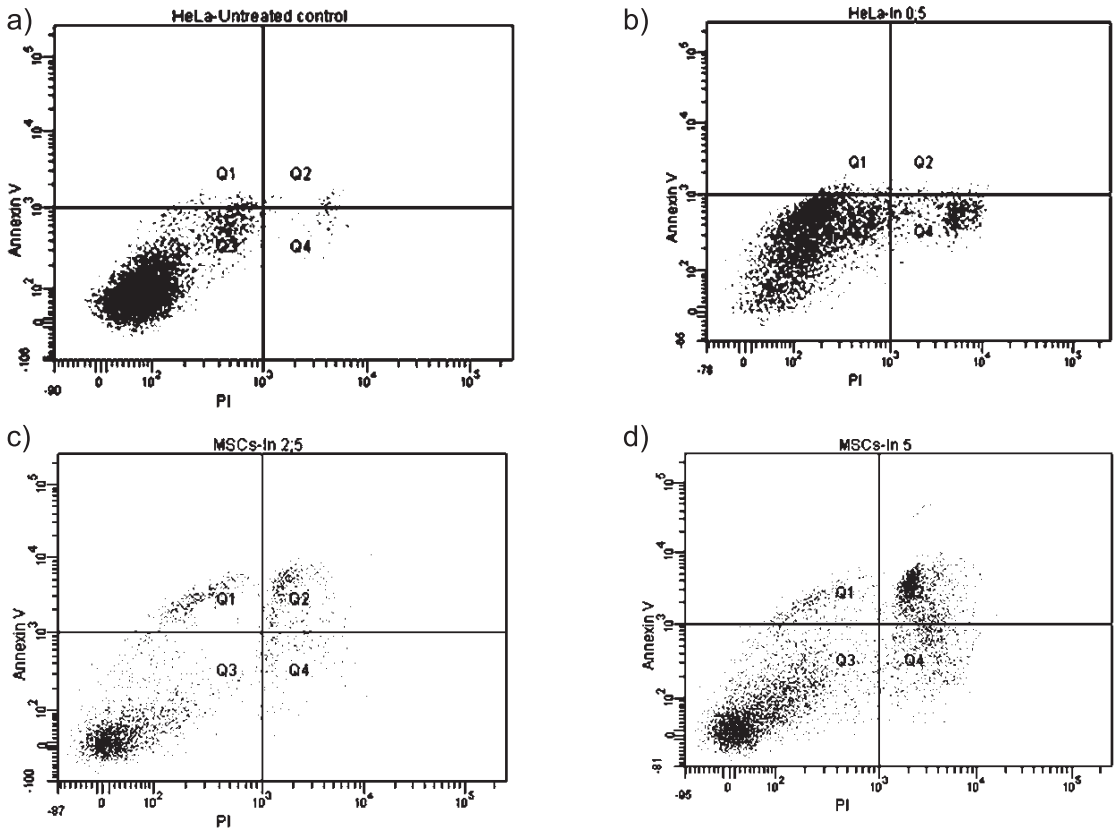

Fig. 4. Apoptosis of HeLa cells: a) Control, b) 0.5 µL extract, c) 2.5 µL extract, d) 5 µL extract.

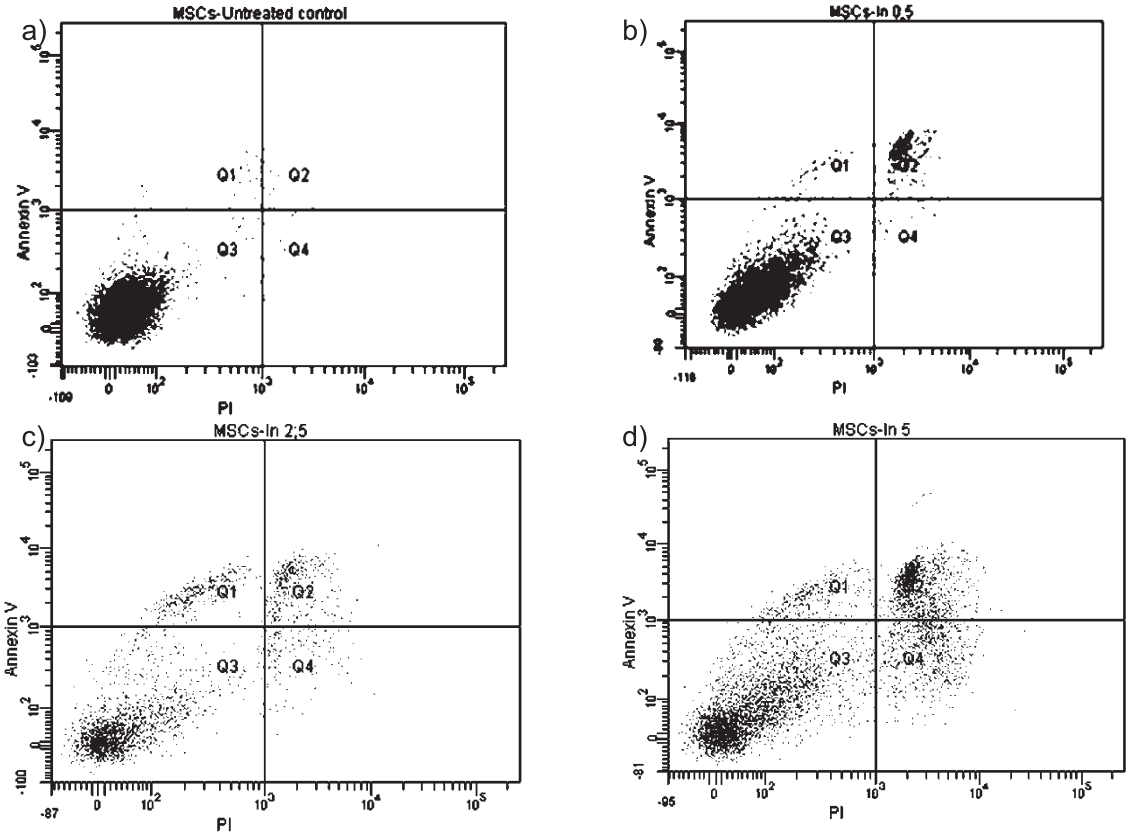

Fig. 5. Apoptosis of pMSCs: a) Control, b) 0.5 µL extract, c) 2.5 µL extract, d) 5 µL extract.

The antitumor action of *Helichrysum arenarium* plant has also been demonstrated by other researchers, who conducted research on sarcoma 45, [29]. In contrast, Lazarevic et al. (2019), failed to demonstrate an inhibitory effect of *Helichrysum arenarium* essential oil on oral squamous cell carcinoma and SCC-25 cell line [22].

Our results demonstrate the in vitro antitumor action of *Helichrysum arenarium* extract on the HeLa line by inducing apoptosis and necrosis of the cells and by decreasing their viability in culture as revealed by the MTT assay. As previously shown Gulfishan et al., 2018) [30], plant extracts exert their antitumor action at the cellular level by binding to microtubules, inhibiting topoisomerase, DNA binding, cell cycle arrest and apoptosis. Clearly, one of the mechanisms by which *Helichrysum arenarium* extract inhibited the in vitro development of HeLa cervical cancer line cells was through the induction of apoptosis, as observed by flow cytometry assay.

The plant is also well known in traditional medicine and is used as a cholagogue, choleretic, diuretic, mild spasmolytic, hepatoprotective agent and for detoxification [1]. Thus, the beneficial effects on the body are obvious, and our study also demonstrated that the polyphenolic extract did not have a cytotoxic effect on palatal mesenchymal stem cells, with the MTT assay confirming this. Considering that these cells are known to have an increased sensitivity to the toxic action of various chemical compounds, we consider that the use of *Helichrysum arenarium* polyphenolic extract does not pose a risk of toxicity to normal cells of the body.

Conclusions

The polyphenolic extract from the flowers of the *Helichrysum arenarium* contains a high number of polyphenols and shows antioxidant activity and UV shield effect, mainly in UVB and UVC domains.

Tests on cells cultures demonstrated that the analysed extract has no cytotoxic effect on palatal mesenchymal stem cells in culture and induced in a significant proportion the cells necrosis and apoptosis in HeLa cells line, thus having an *in vitro* antitumoral effect.

All results we obtained agree with the recent data in literature and entitle us to continue our study on *Helichrysum arenarium* plant and isolated compounds in it for applications in therapeutic and dermatocosmetic field.

Acknowledgments

This work was supported by the Department of Pharmaceutical Sciences, University of Constantza.

Conflict of Interest

The authors declare no conflict of interest.

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