ORIGINAL PAPER

EVALUATION OF THE ANTIOXIDANT, CYTOTOXIC AND ANTITUMORAL ACTIVITIES OF A POLYPHENOLIC EXTRACT OF *ROBINIA PSEUDOACACIA* L. FLOWERS

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Abstract. *A polyphenolic extract of Robinia pseudoacacia L. flowers was prepared using a hydro-ethanolic extraction phase. The chemical analysis of the extract consisted in the assessment of the total polyphenolic content (Folin-Ciocalteu method) and the flavonoid fingerprint of the extract determined by high-performance thin-layer chromatography (HPTLC). Two methods ferric reducing antioxidant power (FRAP) and radical scavenging activity with DPPH were used to measure the antioxidant activity of the extract. The cytotoxic and antitumor effects were evaluated on two cell lines: palatal mesenchymal stem cells (pMSCs) and epithelial cells derived from human cervical adenocarcinoma (HeLa (ATTC® CCL-2™)). The chemical content of Robinia pseudoacacia L. flowers extract reveals the presence of apigenin-7-glucoside and rutin. Results indicated high antioxidant activity with both methods (FRAP and DPPH). No cytotoxic effects of the extract were observed on the pMSCs cell line. The extract induced significant necrosis and apoptosis of the HeLa cells, proving an antitumor effect in vitro. The data confirm the antioxidant and antitumor effects of the polyphenols and the therapeutic potential of Robinia pseudoacaia flowers extract. In the last years, the therapeutic effect of the compounds from Robinia pseudoacacia flowers is less discussed; even the traditional medicine mentions them as helpful medicinal means. The present paper points out the correlation between Robinia pseudoacacia flowers polyphenolic compounds and their chemical and biological effects.*

Keywords: *Robinia pseudoacacia L; polyphenols; antioxidant activity; antitumor activity.*

1. INTRODUCTION

Robinia pseudoacacia L. (acacia) is a plant species from the family of *Fabaceae* that is native to the North-eastern United States yet distributed in all regions of the earth [1].

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Its flowers are used in traditional medicine for their diuretic, spasmolytic, sedative and cholagogue effects, for the amelioration of the kidneys and biliary duct inflammation [2].
Many clinical studies suggest that flavonoids have a potential for the prevention and treatment of several diseases. Some evidence supporting the therapeutic potential of flavonoids come from the study of plants used in traditional medicine, which has shown that flavonoids are common bioactive constituents of these plants [3]. Flavonoids have been reported to possess many useful properties, including anti-inflammatory activity, enzyme inhibition, antimicrobial activity, anti-cancer, anti-allergic activity, antioxidant activity, vascular activity, and cytotoxic antitumor activity [4].

The investigation of the chemical composition pointed out that the flowers contain flavonoids (robinin (kaempferol 3,7-di-O-glycoside), ruthoside, apigenin, myricetin and luteolin), as well as other phenolic compounds (gallic, chlorogenic, ferulic, caffeic acid [5-8]. The antioxidant activity of the vegetal extracts may represent valuable information, as it may partly explain their antitumoural effect. Currently, there are numberless data in the literature regarding the antioxidant activity of vegetal compounds, which are generally correlated to their anti-aging and antitumoural action [9-14].

Considering the uses of the acacia flowers in traditional medicine, which can be correlated with their chemical composition, the present paper aims to determine the antioxidant activity of acacia flowers polyphenolic extract, as well as to identify certain flavonoid compounds known as being part of the plant product constituents through high-performance thin-layer chromatography (HPTLC) [15].

High-performance thin-layer chromatography is known as an effective tool for quality evaluation of medicinal plants due to its simplicity, reduced costs and requirements, for being successfully used to develop the chromatographic fingerprint for medicinal plants [16-18].

To estimate the antioxidant activity of the extract, two *in vitro* methods such as ferric reducing antioxidant power (FRAP) and radical scavenging activity with DPPH has been used. *In vitro* screening for antioxidant activity and high antioxidant content remains a common practice in many natural product laboratories where their ease, speed and reproducibility are valuable traits in the screening of numerous samples. The assays used must be selected specifically to best represent the overall antioxidant activity of a plant, or a certain product, encompassing different antioxidant mechanisms, compound polarity, rate of reaction, pH also. The choosen methods (both single electron transfer (SET)-based assays) best reflects the molecular mechanisms by which the polyphenolic compounds acts, measuring their reducing capacity. It is unrealistic to expect a single assay to be able to determine the total antioxidant activity of a sample. The literature recommends always using two or even three lab-protocols for the assessment [19]. The effects on the culture cells targeted the cytotoxic and antitumoral activity. The product displayed an increased antioxidant activity, lack of cytotoxic effects, as well as antitumoral activity. The chemical composition of the extract was well correlated with its chemical and biological activities determined *in vitro*.

2. MATERIALS AND METHODS

2.1. MATERIALS

All phenolic standards and solvents used in the present work were HPLC-grade (purity >99%). Methanol, ethanol, acetonitrile and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Aldrich (Steinheim, Germany). 2,4,6-Tripyridyl-s-triazine (TPTZ) and Folin–Ciocalteu reagent were obtained from Merck (Darmstadt, Germany). The phenolic

standard containing gallic acid (GA), rosmarinic acid, chlorogenic acid, apigenin-7-glucoside, rutin and luteolin-7-glucoside were purchased from Sigma Aldrich (Steinheim, Germany). Development solution: 1% NP (2- aminoethyl diphenylborinate) solution in methanol; 5% PEG (polyethylene glycol) in methanol.

The acacia flowers (*Robiniae pseudoacacia* L. *flos*) were collected from Siriu forest, county of Buzău, in the summer of 2018; they were air-dried, weighted, and stored in sealed containers outside.

2.2. METHODS

2.2.1. Extract preparation

The polyphenolic extract from the *Robiniae pseudoacacia flos* was prepared as follows: dried flowers were suspended in 50% ethanol for 72 hours under agitation, at room temperature. The ratio between the plant product and the extractive solution volume was 10 g product/ 100 mL extractive phase. Subsequently, the alcohol was removed with the help of a Royeyov IKA RV10 rotoevaporator, at 50 ºC, 200 mBarr.

2.2.2. Determination of total phenolic content (TPC)

The analysis was conducted using the spectrophotometric methods. The total phenolic content of the extract was determined using the Folin-Ciocalteu reagent according to the European Pharmacopoea method [20]. The results were expressed as mg equivalent of gallic acid (GAE) per mL extract.

2.2.3. Antioxidant activity

The Ferric reducing antioxidant power (FRAP) assay was performed according to previous reports by Benzie [21]. The FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ in 40 mM HCl (1 mL), 2.5 mL of 300 mM acetate buffer (pH = 3.6), and 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$. The FRAP reagent was added to 1 mL of sample, and the mixture was shaken for 1 minute. The reaction was carried out at room temperature, according to the protocol. A reagent blank was prepared by adding 1 mL of water instead of extract. Absorbance readings of the samples and the reagent blank were taken after 4 min at 593 nm using a UV-visible spectrophotometer Jasco V-630 (Abel Jasco, Germany). The antioxidant activity was calculated from the calibration curve made with TROLOX ($y = 704.62x$) with a range of 33.5–670 µM Trolox/L and good linearity ($R^2 = 0.9997$).

The DPPH radical scavenging activity of the *Robiniae pseudoacacia flos* extract was determined according to the method reported by Brand-Williams, with minor changes [22]. The sample (0.1 mL) was added to 2.9 mL of DPPH solution. The absorbance was measured at 517 nm after the solution had been allowed to stand in the dark for 60 min. The TROLOX calibration curve was plotted as a function of the percentage of DPPH radical scavenging activity. The results have been expressed as µM equiv Trolox/mL. All experiments were performed in triplicate. All data were presented as mean±standard deviation (SD).

2.2.4. The flavonoid fingerprint of the extract by HPTLC

The HPTLC system consisted of a CAMAG Linomat-5 automatic sample applicator

and CAMAG TLC scanner provided with CATS software (version: 1.2). The stationary phase was composed of pre-coated silica gel 60 F254 HPTLC plates (20 cm x 10 cm). Samples were applied on the plates as 7 mm wide bands via Linomat-5 automatic sample applicator equipped with a 100 l Hamilton syringe. The mobile phase was constituted of ethyl acetate-acetic acid-formic acid-water 100:11:11:27 (v/v/v/v). Linear ascending mode of development up to 80 mm, was implemented at room temperature (22 ± 2 ◦C) and 33% relative humidity in an ADC2 developer Camag glass chamber (20 cm × 10 cm) previously saturated with mobile phase vapour for 20 min. After development, the plates were dried at 105◦C for 10 min and derivatised in NP-PEG reagents [23]. The fingerprints were evaluated at 366nm in fluorescence mode with WinCats and VideoScan software. Reference compounds for HPTLC analysis are presented in **Table 1**.

Table 1. **Reference compounds**

Reference	Reference substance	Concentration [mg/mL]
SR1	Chlorogenic acid	0.5
	Apigenin 7-glucoside	0.5
SR2	Rutin	0.2
	Luteolin 7-glucoside	0.2
	Rosmarinic acid	0.5
SR3	Chlorogenic acid	0.3
	Hyperoside	0.2

2.2.5. Estimation of cytotoxic and antitumor effects of the Robiniae pseudoacacia L. extract

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 [24].

2.2.6. Cytotoxic assay

For the cytotoxic assay, two cells' lines were used: fully characterized palatal mesenchymal stem cells isolated from human oral cavity (pMSCs) [25], and epithelial cell line derived from human cervical adenocarcinoma (HeLa ATTC® CCL-2™). Before use, the cell lines were maintained in standard culture conditions. In order to obtain cell suspensions, the cells were treated with 0.25% trypsin-EDTA (Sigma-Aldrich) for 5 minutes.

The viability of the pMSCs and HeLa cells treated with acacia flower 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, called formazan. The intensity of the chromogen reaction depends on the number of viable cells and their proliferation capacity.

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 (5; 2.5; 1.5; 1.0; 0.5 µL) of watery acacia flower extract. 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 analyzed by measurement of optical density at 450 nm with a BioTek Synergy 2 microplate reader. Since optical density is directly correlated with mithochondiral activiy, its values were compared between treated cells and untreated control in order observes the differences regarding viability and cell proliferation capacity between groups.

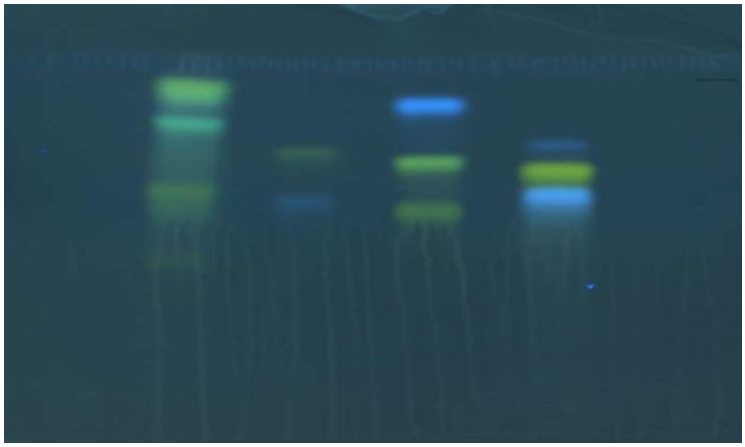
2.2.7. Apoptosis assay

The BT Pharmigen FITC Annexin V Apoptosis detection kit was used to determine the apoptosis. The cells were coloured with Annexin V and propidium iodide. (PI), according to the kit instructions, while the fluorescent coloration intensity was read with a BD FACS Canto II flow cytometer, with the 488 nm wavelength argon laser. Results were interpreted using the FACS Diva6.1 software. Thus, fluorescence intensity was presented in dot plots, each being divided into 4 quadrants. Cells that did not stain with neither of the fluorescent dyes appeared in quadrant 3 (Q3-viable cells). Cells that only stained with Annexin appeared in quadrant 1 (Q1-apoptotic cells), while cells that stained with both Annexin and PI appeared in quadrant 2 (Q2-late apoptotic cells). Cells in quadrant 4 (Q4) only stained with PI and therefore are necrotic cells. A total of 10.000 cells were analyzed and included in each dot plot.

3. RESULTS AND DISCUSSION

3.1. RESULTS

The qualitative analysis of flavonoid compounds for the ethanolic flower extract of *Robiniae pseudoacacia flos* was conducted using the high-performance thin-layer chromatography (HPTLC) method. Fig. 1 displays a typical TLC plate showing separation of standards and sample. The identity of the spots of flavonoids in the sample solutions was confirmed by comparing their R_F values in the sample with the reference standards (Table 2).



R1 SR1 SR2 SR3

Figure 1. HPTLC fingerprint of some *Robiniae pseudoacacia flos*, after derivatisation with NP-PEG, 366nm.

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

Sample	Compound	R_F	Colour
SR1	Chlorogenic acid Apigenin-7-glucozida	0.59 0.74	dark blue spot light green spot
SR2	Rutin Luteolin 7-glucoside Rosmarinic acid	0.57 0.70 0.87	yellow- greenish spot yellow- greenish spot dark blue spot
SR3	Chlorogenic acid Hyperoside	0.61 0.68	light blue spot yellow spot
R1= extract of *Robiniae pseudoacacia flos*	Rutin Apigenin 7-glucoside -	0.62 0.82 0.92	yellow- greenish spot light green spot yellow- greenish spot

R_F values are averages from three replicate measurements; R_F is the ratio of the distance the spot moved above the origin to the distance the solvent front moved above the origin. Results of the total polyphenolic content and antioxidant activity of *Robinia pseudoacacia flos* polyphenolic extract are shown in Table 3.

Table 3. Antioxidant activity of the polyphenolic extract of *Robiniae pseudoacacia flos* measured by two in vitro methods

Total phenolic content (TPC) mg GAE/mL	FRAP µM equiv Trolox/mL	DPPH µM equiv Trolox/mL
0.72±0.02	$0.8 \cdot 10^{-3}$ ±0.01	0.141±0.02

Cytotoxic assay

The pMCSs and HeLa cells were treated with 5 different concentrations (5; 2.5; 1.5; 1.0; 0.5 µL) of *Robinia pseudoacacia* extract. After 24 h of treatment, no difference in optical density (OD) and therefore cell proliferation capacity was seen between control and treated cells. Also, no significant differences were identified by ANOVA (GraphPad Prism6) (Figs. 2 and 3).

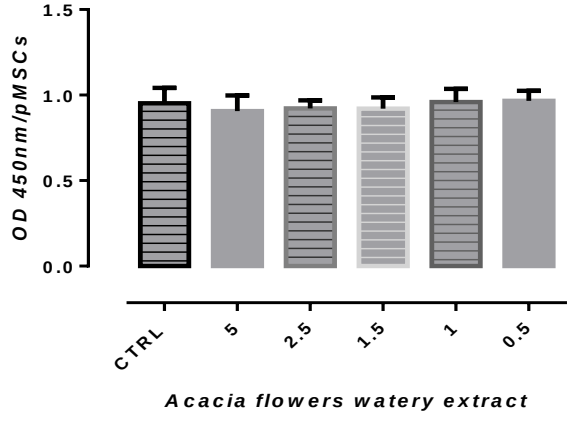


Figure 2. Effects of *Robinia pseudoacacia flos* extract on pMSCs.

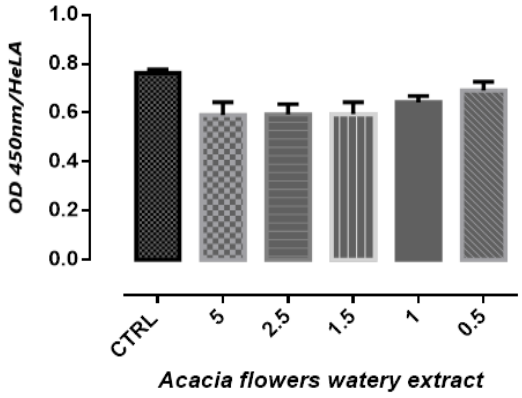


Figure 3. Effects of *Robinia pseudoacacia flos* extract on HeLa cells.

Cytometric apoptosis

The acacia flowers extract induces the apoptosis of treated tumour cells, in a significant percentage as compared to the control sample. In the case of HeLa cells, a certain effect of apoptosis induction by the *Robiniae pseudoacacia flos* can be observed (Table 4 and

Fig. 4). Table 4 presents the percentage of viable, apoptotic, late apoptotic, and necrotic cells identified by flow cytometry, after Annexin/PI stainig, obtained from the FACS Diva 6.1 software. Viable cells are shown on the dot plots in Q1, apoptotic cells in Q3, late apoptotic in Q2 and necrotic cells in Q4 (see Fig. 4).

Table 4. Apoptosis and necrosis of HeLa cells

Sample	Viable [%]	Apoptosis [%]	Late apoptosis [%]	Necrosis [%]
Control	93.5	2.4	1.5	2.6
Robinia pseudoacacia flos 0.5 µL	82.2	5.5	5.1	7.1
Robinia pseudoacacia flos 1 µL	79.8	6.2	7.7	6.4
Robinia pseudoacacia flos 1.5 µL	78.7	9	6.8	5.6
Robinia pseudoacacia flos 2.5 µL	77.3	6.8	7.2	8.7
Robinia pseudoacacia flos 5 µL	78.2	7.6	7.6	6.5

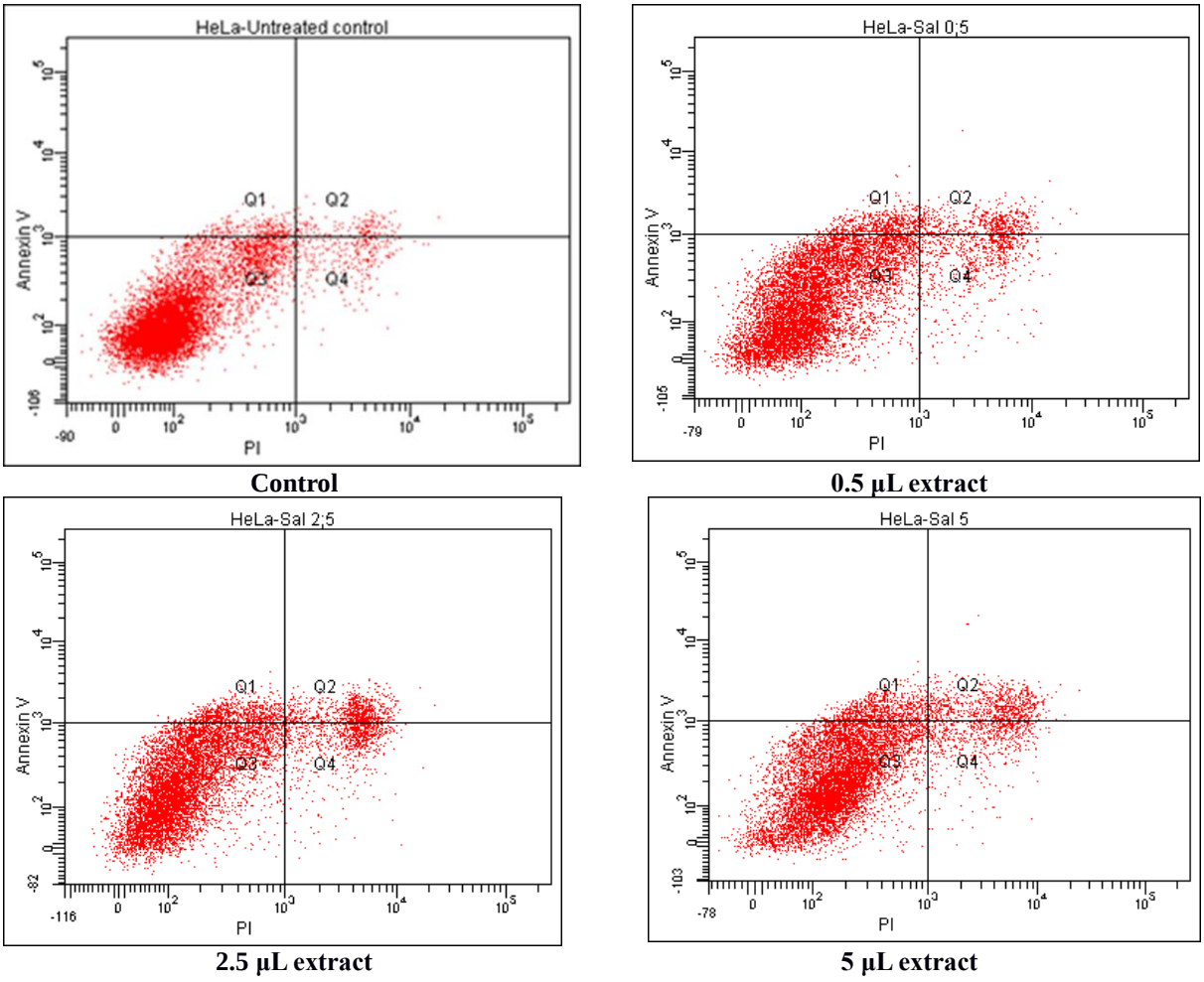


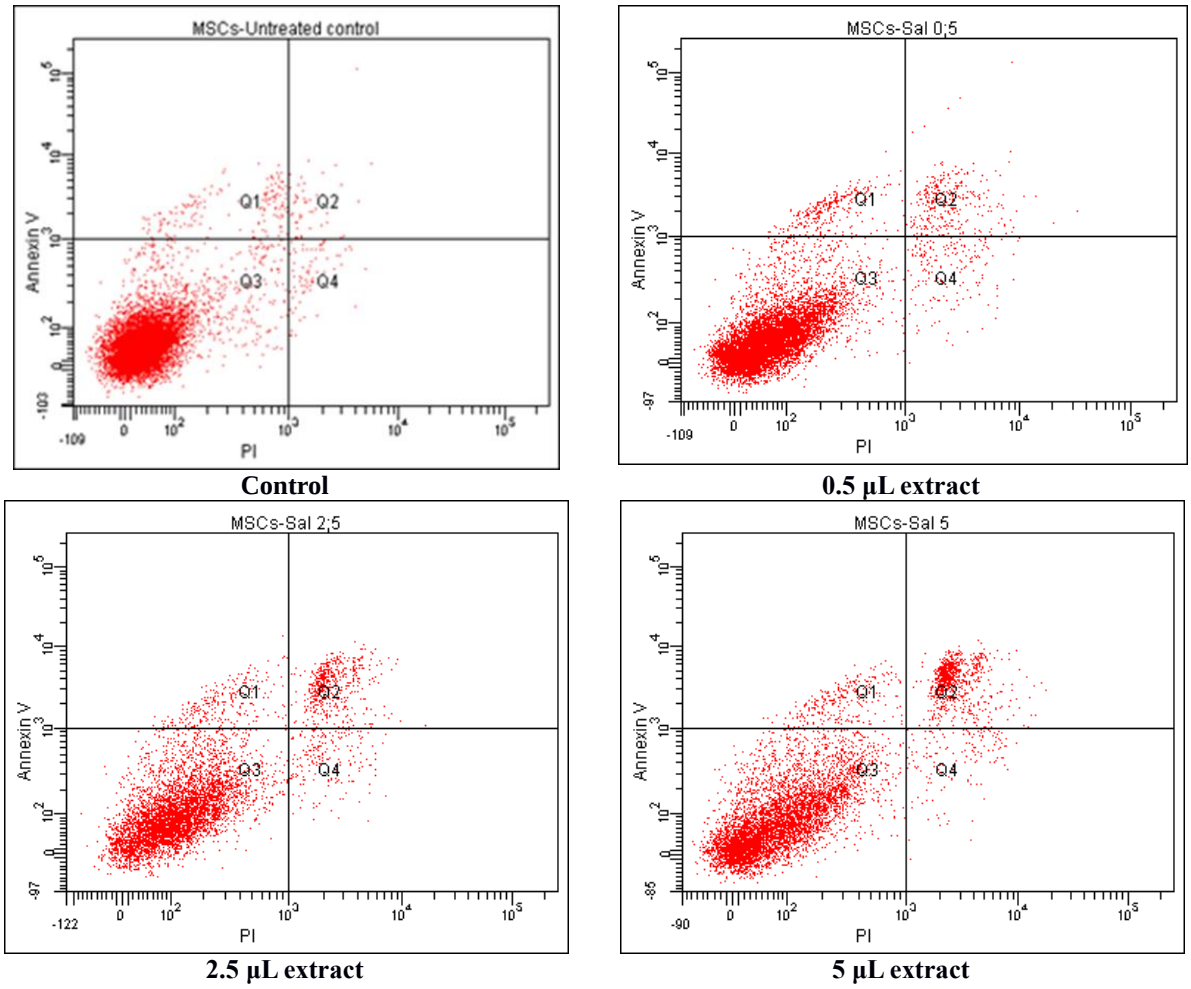
Figure 4. Apoptosis of HeLa cell line shown by flow cytometry (Q3=viable cells, Q1=apoptotic cells, Q2=late apoptotic cells, Q4=necrotic cells)

After the treatment of palatal mesenchymal cells with different concentrations of the extract, the following results were obtained concerning the induction of apoptosis and cellular necrosis (Table 5 and Fig. 5). Table 5 presents the percentage of viable, apoptotic, late apoptotic, and necrotic cells identified by flow cytometry, after Annexin/PI stainig, obtained from the FACS Diva 6.1 software. Viable cells are shown on the dot plots in Q1, apoptotic cells in Q3, late apoptotic in Q2 and necrotic cells in Q4 (see Fig. 4). When comparing the

results obtained for tumoral cells versus normal palatal mesenchymal stem cells, we noticed that the Robina pseudoacaccia flower extract has a selective apoptotic effect, as the tumoral cells were more affected than the normal cells in what cellular viability was concerned.

Table 5. Apoptosis and necrosis of palatal mesenchymal stem cells

Sample	Viable [%]	Apoptosis [%]	Late apoptosis [%]	Necrosis [%]
Control	97.3	1.6	0.5	0.7
Robinia pseudoacacia flos 0.5 µL	91.4	3.1	3.4	2.1
Robinia pseudoacacia flos 1 µL	89.3	3	4.4	3.3
Robinia pseudoacacia flos 1.5 µL	85.5	3.6	8.2	2.7
Robinia pseudoacacia flos 2.5 µL	84.2	4.1	8.5	3.2
Robinia pseudoacacia flos 5 µL	82.2	3.9	11.7	2.3



Control **0.5 µL extract**

2.5 µL extract **5 µL extract**

Figure 5. Apoptosis of palatal mesenchymal stem cells shown by flow cytometry (Q3=viable cells, Q1=apoptotic cells, Q2=late apoptotic cells, Q4=necrotic cells).

3.2. DISCUSSION

The high-performance thin-layer chromatography result shows in the extract the presence of two flavonoid compounds contained in the used standards: apigenin-7-glucoside (R_F = 0.82, the presence of yellow-green spot) and rutin (R_F = 0.62, the colour of spot is light green). At the same time, the chromatogram shows the presence of an undetectable compound

(the colour of the spot is greenish, while R_F are a little higher). The results obtained in this study showed that the flowers are rich in phenolic compounds and have significant antioxidant activity. The determination of the total polyphenolic content with Folin-Ciocalteu reactive indicated a high percentage of polyphenolic compounds in the extract (32.16 %). It appears that both methods reveal an increased antioxidant activity. The molecules identified in the composition of analysed extract have known antioxidant properties, which is according to the results we obtained [7]. The differences in the amount of the antioxidant activity of extract assessed through the two methods are related to the differences in the measuring principles, reaction kinetics on which each method is based and different reaction medium. The DPPH assay measured a change in the stable radical DPPH by the electron-donating ability of the sample [26]. The FRAP value measures the reduction of the ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) by donor electrons in the sample [27].

As shown in Fig. 2, no cytotoxic effect in palatal stem cells was recorded. In HeLa cells, a minor anti-proliferative potential can be noticed associated with minor morphological modifications, upon the first concentrations (5µL and 2.5 µL) as compared to the control cultures. The rest of the tested concentrations indicated a proliferation degree like the control cultures.

The results show an *in vitro* antitumoral action exercised by the *Robinia pseudoacacia flos* extract over the HeLa line, by the induction of cells apoptosis and necrosis as well as by the decrease of their viability in the culture, revealed through the MTT test, as reported [28]. The plant extract also exercises the antitumoral action by binding to microtubes, inhibiting the topoisomerases, binding to the DNA, stopping the cell cycle and apoptosis. Certainly, one of the mechanisms by which the *Robinia pseudoacacia flos* extract determined the inhibiting of the in vitro development of Hela cervical cancer line cells was through the induction of apoptosis, as observed following the flow cytometry test.

The study also reveals that the polyphenolic extract had no cytotoxic effect over the palatal mesenchymal stem cells, also confirmed by the MTT test. Taking into consideration that these cells are known as displaying increased sensitivity to toxic action of different chemical compounds, this investigation demonstrated that the use of *Robinia pseudoacacia flos* extract does not present a toxicity risk over the normal cells of the body.

4. CONCLUSION

The *Robinia pseudoacacia* flowers polyphenolic extract contains a significant percentage of polyphenolic compounds and presents an increased antioxidant activity. Among the polyphenolic molecules, the apigenin-7-glucoside and rutin have been identified through HPTLC. Tests on cell cultures demonstrated that the analysed extract has no cytotoxic effect on palatal mesenchymal stem cells in the culture and induces the cells necrosis and apoptosis in a significant percentage on HeLa cells line, thus having an *in vitro* antitumoral effect. The results are according to the recent data in the literature and entitle us to continue the study on the polyphenolic compounds contained in the acacia flowers for applications in both therapeutic and dermato-cosmetic fields.

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