ORIGINAL ARTICLE

CYTOPROTECTIVE AND ANTIINFLAMATORY ACTIVITY EVALUATION OF SOME PELARGONIUM EXTRACTS

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Abstract

The current study includes the correlation between the chemical composition and the biological activity of four *Pelargonium* species. The plant material is represented by the leaves of *Pelargonium hispidum* (*P.h*), *P. grandiflorum* (*P.g*), *P. radens* (*P.r*) and *P. zonale* (*P.z*). The total phenolic content was determinate on the methanolic extract of each species using the Folin–Ciocâlteu reagent. The quantitative determination of poliphenols for methanol extracts categorises the species by ascending scale, as follows: *P.g* < *P.r* < *P.h* < *P.z*. The UHPLC assay was used for identification and semiquantitative analysis of certain polyphenolic compounds. The membrane stabilization assay on red blood cells was used to offer information about the possible anti-inflammatory effect of the studied compounds. For all tested samples we observed that the methanolic extracts have a protective activity on the erythrocyte membrane, but mostly the methanolic extract of *P. radens* (EC_{50}= 99,98 ± 0,22 µg/mL). Another important aspect that we evaluated was the cell viability on a cell line – C2C12 – isolated from the muscle of mice. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cellular viability assay was used to determine the toxicity of methanolic extracts against the myocytes. The results from the viability assay suggested that for the methanolic extracts of *P.r* and *P.g* at the concentration of 19.5 µg/mL, the EC_{50} was 92 ± 0.52 µg/mL for *P.r* and for *P.g*, the EC_{50} was 89 ± 0.37 µg/mL. Even though the methanolic extract of *P.zonale* presented the highest concentration of polyphenols, the viability assay showed that it had a slightly cytotoxic effect, probably because of the interactions that occurred between the membrane enzymes and the MTT. Considering all the results, *Pelargonium radens* appears to be the most promising species, followed by *Pelargonium zonale*.

Rezumat

Studiul de faţă include date cu privire la corelarea dintre compoziţia chimică şi activitatea biologică a patru specii de *Pelargonium*. Materialul vegetal este reprezentat de frunzele de *Pelargonium hispidum* (*P.h*), *P. grandiflorum* (*P.g*), *P. radens* (*P.r*) şi *P. zonale* (*P.z*). Conţinutul total în polifenoli a fost determinat pentru extractele metanolice ale fiecărei specii utilizând reactivul Folin-Ciocâlteu. Determinarea cantitativă a derivaţilor polifenolici aşează într-o scară crescătoare speciile, astfel: *P.g* < *P.r* < *P.h* < *P.z*. Analiza UHPLC a fost utilizată pentru identificarea şi analiza cantitativă a unor componente polifenolice. Testul de stabilizare a membranei eritrocitare a fost utilizat pentru a furniza informaţii pentru o posibilă activitate antiinflamatoare a probelor studiate. Toate extractele testate au prezentat activitate protectoare asupra membranei eritrocitare, însă extractul metanolic de *P. radens* cel mai mult (EC_{50} = 99,98 ± 0,22 µg/mL). Un alt aspect important a fost evaluarea viabilităţii celulare – C2C12 – izolate de la nivelul musculaturii şoarecilor. Testul de viabilitate celulară cu bromura de 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazol (MTT) a fost utilizat în scopul determinării toxicităţii extractelor metanolice asupra miocitelor. Rezultatele testului au sugerat că extractele metanolice de *P.r* şi *P.g*, la concentraţia de 19,5 µg/mL, prezintă valoare EC_{50} de 92 ± 0,52 µg/mL pentru *P.r* şi pentru *P.g* de 89 ± 0,37 µg/mL. Cu toate că extractul metanolic de *P. zonale* prezintă cea mai ridicată concentraţie în polifenoli totali, viabilitatea celulară a înregistrat un uşor efect citotoxic, probabil datorită interacţiunilor ce apar între enzimele membranare şi MTT. Corelând toate rezultatele, *Pelargonium radens* s-a dovedit a fi specia care prezintă cel mai mult interes din punct de vedere al investigaţiilor ulterioare, dar şi *Pelargonium zonale* în scopul determinării mecanismului real implicat în viabilitatea celulară.

Keywords: cytoprotective effect, *Pelargonium*, antiinflamatory, cell line C2C12

Introduction

Pelargonium species can be found in the history of modern medicine from the time of British Major Stevens, who, in 1897, launched the Stevens' Consumption Cure drug which treated tuberculosis [9]. The experience he had gained in South Africa was the basis for the introduction of umckaloabo

preparations (decoction of the roots of *Pelargonium sidoides*), although the first clinical trial was conducted in 1920 on 800 patients. The promising results obtained by Doctor Adrien Sechehaye were published in 1930 and established the first antituberculostatic treatment before synthetic cytostatics started to be used. In this sense, the purpose of this paper is to highlight new sources related to this species of *Pelargonium* (as this one is less accessible in our areas), which would constitute important therapeutic resources. With this purpose in mind, we focused in our research on one indigenous species (*Pelargonium zonale*) and three species acclimatized in our country (*Pelargonium hispidum*, *Pelargonium grandiflorum* and *Pelargonium radens*) of the *Pelargonium* genus, *Geraniaceae* Family; these plant products are rich in active principles.

In research, the definite proof of the biological activity of different extract types involves tests that are based on molecular targets and various mechanisms. Such assays are relevant only for certain compounds depending on their chemical properties which allow them to bind and interfered specific targets on the cell membrane. Therefore, the cell viability test is a key indicator in orientating the present study. At the moment there are various technics that can prove the influence of a chemical substance or vegetal extract on the cell viability. Each assay has its advantages or disadvantages, some can be quick or can have a laborious protocol witch can be intricate. Thus, we used assays that are attainable and conclusive.

Materials and Methods

Plant material

The subject of the current research is two *Pelargonium* species*: hispidum* and *zonale*. Specimens were obtained from the Botanical Garden "Anastasie Fătu", Iaşi, Romania. The plants were kept in similar growth conditions to provide minimum environmental impact.

Preparation of the alcoholic extract

The vegetal extracts were obtained from the dried leaves which were grounded prior to the extraction. 2 g of each sample were extracted three times with methanol at 85ºC, on a thermostated water bath, and were brought to a level of 100 mL in a volumetric flask. The vegetable extracts were dried at 40ºC. We used the dried extract for the assays described below.

Determination of total phenols content

Each sample was mixed with 1 mL of Folin-Ciocâlteu reagent, allowed to stand for 5 min at 25ºC before adding the rest of the reagents. For 120 min, the samples were kept in the dark, before measuring the absorbance at 750 nm. Gallic acid was used as standard. The results were expressed as gallic acid equivalents (GAE) (mg/100 g dry extract) [4, 8].

Stability of the human blood cells assay

Heparinized blood was centrifuged at 2500 rpm for 10 min. The RBCs were separated from plasma and buffy coat and washed three times with 0.9% NaCl. The samples underwent the same preparation steps as described by Dzeletovic S. *et al*. [5] diclofenac was used as a standard.

HPLC Analysis

HPLC was performed with a Thermo UltiMate3000 gradient chromatograph controlled by Chromeleon interface, an autosampler, an Accucore XL C18 column (150 x 4, 6 x 4) and diode array detector (DAD). The method we used was presented in a previous article [6].

Cell viability assay

The evaluation of cell viability by MTT assay is based on the reducing capacity of the vegetal extracts on the MTT reagent. The yellow solution of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was reduced to purple formazan in the presence of living cells. The absorbance of the solution after solubilisation in DMSO was determined spectrophotometrically at 550 nm. The cell line was replicated and incubated for 48 h along with the investigated extracts. The procedure consisted in the technique described by Gaiddonn *et al*. [7]. The presented data is the average of four consecutive determinations.

Results and Discussion

The source, code name, extractible and the drug extract ratio (DER) values for the methanolic extracts are given in the following table. Also, total content of polyphenols is included in Table I.

Table I

The content of the active ingredients of the *Pelargonium* species

Code	Source	Extractible	DER*	Total polyphenolic content (mg gallic acid/100g dry extract)
P.h	*P. hispidum*	0.3155 g	6.3391	2475.92
P.g	*P. grandiflorum*	0.2745 g	7.2859	1279.62
P.r	*P. radens*	0.2546 g	7.8554	2278.71
P.z	*P. zonale*	0.4149 g	4.8204	5694.125

*DER = plant product mass: obtained extract mass

The best plant product proved to be *Pelargonium zonale* which yielded the largest quantity of extract, whereas, the leaves of the *Pelargonium radens* species yielded the lowest quantity of extractible. At the same time, the richest sources were *Pelargonium zonale* with approx. 5694 mg gallic acid/100g dry extract and *Pelargonium hispidum* respectively; *P. radens* contained almost the same amount of polyphenols. Comparing the rest of the data for *P. grandiflorum*, one might state that there is no direct correlation between the amount extracted (extractible) from the plant and the total phenolic content.

The most important compounds identified in the investigated extracts by UHPLC techniques are presented in Table II. Standards were used for reference to properly identify the compounds.

Table II

Compounds identified and quantified in *Pelargonium sp*. methanolic extracts

Compound (µg/mg dry extract)	Source			
	***Pelargonium hispidum* (*P.h*)**	***Pelargonium grandiflorum* (*P.g*)**	***Pelargonium radens* (*P.r*)**	***Pelargonium zonale* (*P.z*)**
catechin	8.717	4.332	1.169	10.930
epicatechin	1.422	0.785	0.568	2.144
cyanidol	-	-	-	2.138
cyanidol derivatives	-	-	-	33.413
delphinidin-3-o rutinoside	-	-	-	23.398
quercetin-3-arabinoside	3.996	0.516	-	8.001
quercetin	1.007		0.154	4.460
luteolin	0.407	0.092	0.918	0.071
kaempferol	0.4199	0.113	0.246	0.133
rutinoside	-	10.760	5.126	-
apigenin	-	0.758	2.246	-
rosmarinic acid	-	2.202	-	-
caffeic acid	-	-	-	0.459
chlorogenic acid	-	0.489	4.451	-
cinnamic acid	0.0519	-	-	-

The identified compounds were catechins, flavones, polyphenolic acids and anthocyanidins, secondary metabolites that are also mentioned by the scientific literature for this Genre. Anthocyanidins and catechins were present especially in *Pelargonium zonale* extract, fact sustained by the presence of a dark purple-brown horseshoe-shape mark on the leaves of this species. Moreover, recent studies have proven that the topical administration of leaf extract or juice obtained from this species has a haemostatic effect [14]. Most probably, such compounds and tannins would be the active principles that act as protein precipitating agents.

Inflammation is a pathological process whose evolution involves the presence of enzymes, proinflammatory chemical mediators, extravasation of fluids, cell migration, tissue damage, but also tissue recovery processes. The spectrophotometric method is based on the *in vitro* evaluation in a hypotonic medium of the erythrocyte membrane stability in the presence of substances with protective effects. The hypotonic medium aggression causes the lysis of erythrocytes with haemoglobin release. The results obtained in the assessment of the capacity of plant extracts to increase the stability of the erythrocyte membrane and indirectly to predict the anti-inflammatory effect are represented in Table III.

Table III

IC_{50} (mg/mL) values of the capacity of *Pelargonium sp*. methanol extracts to stabilize the erythrocyte membrane

Conc. (mg/mL)	Source – % inhibition ± standard deviation				
	P.h	***P.g***	***P.r***	***P.z***	**Diclofenac**
0.0390625	99.26 ± 0.03	98.39 ± 0.05	99.26 ± 0.41	99.38 ± 0.12	98.87 ± 0.13
0.078125	99.28 ± 0.20	98.31 ± 0.41	99.11 ± 0.17	97.56 ± 0.33	98.78 ± 0.08
0.15625	99.67 ± 0.47	98.57 ± 0.55	99.07 ± 0.24	99.19 ± 0.33	98.77 ± 0.91
0.3125	99.81 ± 0.22	98.72 ± 0.51	99.61 ± 0.62	99.32 ± 0.33	98.88 ± 0.04
0.625	99.99 ± 0.22	99.56 ± 0.14	99.21 ± 0.54	99.47 ± 0.67	98.71 ± 0.31
1.25	99.98 ± 0.74	99.46 ± 0.64	98.09 ± 0.58	99.59 ± 0.25	98.65 ± 0.34
2.5	99.56 ± 0.08	98.71 ± 0.11	99.73 ± 0.47	97.98 ± 0.64	98.78 ± 0.34
5	97.61 ± 0.20	98.15 ± 0.33	96.66 ± 0.14	71.05 ± 0.22	98.79 ± 0.37

Following the results previously obtained for the *in vitro* assays and correlating them with the literature data, we conducted a series of cell line tests to complete the picture of biological actions. At the same time, after the assessment of the methanolic extracts from the point of view of their antioxidant,

antiinflammatory activity, we chose the C2C12 cell line and the viability assay. The cell line was isolated from the muscle of mice - C2C12.
The viability assay is performed by incubating the substrate with the live cells when the generated signal is correlated to the number of the cells which have retained their viability [15]. By their apoptosis, a certain amount of substrate remains unchanged, resulting in spectrophotometrically detectable colour differences. The graphical representation of the percentage of cell viability for C2C12 depending on the concentration of the methanol extracts used is presented in the Figure 1.

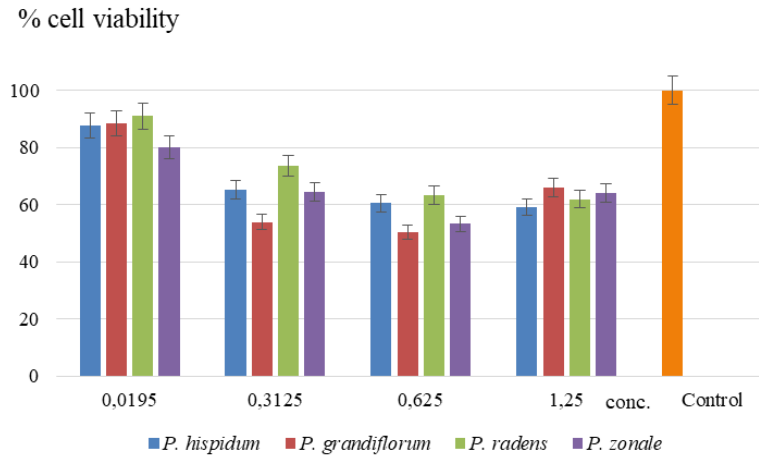

Figure 1.
Graphical representation of the percentage of cell viability for C2C12 depending on the concentration of the methanol extracts used

The analysis of the obtained results indicated a dose-dependent decline in cell viability. Thus, the methanolic extracts of *P. radens* and *P. grandiflorum* showed a viability percentage of over 90% at a concentration of 0.0195 mg/mL compared to *P. hispidum* and *P. zonale* samples at which the same dose induces the percent decreases by 10%.
The reduction of MTT is an assay that gives information on the percentage of cells that retain their viability, which is not identical to the detection of the cellular proliferation [11]. Apoptosis no longer allows the transformation of MTT so that the coloured compound (formazan) can be considered a marker of cell viability [2]. It has been postulated that the electron transfer MTT reduction reaction would involve the participation of NADH or compounds with similar potential [12]. On the other hand, various researchers believe that this transformation is catalysed by mitochondrial enzymes [1]. At this moment we can only assume that the investigated extracts might interact through such mechanisms, but further data is needed for a clear conclusion.
It was shown that O-methylated flavones had effect on glucose uptake and the underlying molecular mechanism was investigated using C2C12 myotubes. These compounds increased glucose uptake in C2C12 myotubes, regardless of the absence or presence of insulin. The GLUT4 expression on the plasma membrane was increased after the tricin treatment in the absence of insulin [13]. The treatment with compounds that include O-methylated flavones activated the insulin-dependent cell signalling pathway, including the activation of insulin receptor substrate-1 (IRS1), phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and AKT substrate. These results suggest that methylated flavones have great potential to be used as a functional agent for glycemic control [10].
The protective effect of the extracts studied in this study is closely linked to their chemical composition. Even though the flavonoids are in lower concentration than the polyphenols, it was shown in other studies that their activity can be improved by introducing it in cyclodextrines [3]. The compounds directly responsible for this type of biological activity are polyphenols; they are generally those with antioxidant properties due to their reductive capacity.

Conclusions

The protective effect of the extracts studied is closely linked to their chemical composition. The compounds directly responsible for this type of biological activity are polyphenols; they are generally those with antioxidant properties due to their reductive capacity. Nevertheless, further *in vivo* studies are necessary to assess the full biological potential of the most active extract obtained from *Pelargonium zonale*.

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