THE EVALUATION OF NORMO-GLYCEMIC AND CYTO-REGENERATIVE EFFECTS OF *PELARGONIUM SPECIES* EXTRACTS

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Abstract

Alpha-amylase and alpha-glucosidase are enzymes that modulate the chain breaking process of alpha-linked polysaccharides with an impact on promoting postprandial hyperglycaemia. Pancreatic alpha-amylase is an enzyme that catalyses the hydrolysis of starch and glycogen, yielding glucose and maltose. Alpha-glucosidase is an enzyme located in the brush border of the small intestine that acts upon alpha $(1 \rightarrow 4)$ glycosidic bonds. Alpha-glucosidase catalyses the hydrolysis of starch and disaccharides to glucose. The inhibition of these two enzymes is an important step in the management of postprandial hyperglycaemia. This research includes data about the anti-inflammatory effect and the rebounding capacity of cells in the presence of two extracts obtained from *Pelargonium* species. The results showed that the hydroalcoholic extract of *P. grandiflorum* had an inhibitory capacity of $55.67 \pm 0.06\%$ at 0.625 mg/mL for alpha-amylase activity and $66.6 \pm 0.31\%$ at 0.078 mg/mL for alpha-glucosidase activity. On the other hand, *P. grandiflorum* extract showed an improvement in the inflammatory process by about 50% and *P. hispidum* methanolic extract showed an anti-oedematous effect of 45% compared to the control.

Rezumat

Alfa-amilaza și alfa-glucozidaza sunt enzime ce catalizează hidroliza legăturilor alfa-polizaharidice, având un impact major asupra hiperglicemiei postprandiale. Alfa-amilaza pancreatică catalizează hidroliza amidonului și glicogenului la glucoză și maltoză. Alfa-glucozidaza este localizată la nivelul marginii în perie a inestinului subțire și acționează asupra legăturilor glicozidice α $(1 \rightarrow 4)$. Alfa-glucozidaza catalizează hidroliza amidonului și dizaharidelor la glucoză. Inhibiția acestor enzime este foarte importantă pentru controlul hiperglicemiei postprandiale. Prezentul studiu cuprinde date referitoare la efectele antiinflamator și de refacere a capacității de aderență celulară în prezența a două extracte din specii de *Pelargonium*. Rezultatele indică faptul că extractul hidroalcoolic de *P. grandiflorum* are o capacitate de inhibiție a alfa-amilazei de $55,67 \pm 0,06\%$ la 0,625 mg/mL și de $66,6 \pm 0,31\%$ a alfa-glucozidazei la 0,078 mg/mL. De asemenea, extractul de *P. grandiflorum* a prezentat un efect de limitare a procesului inflamator cu 50% și extractul de *P. hispidum* a prezentat un efect antiedematos de 45% față de controlul.

Keywords: *Pelargonium*, alpha-amylase, alpha-glucosidase, TPA-induced oedema

Introduction

People have always resorted to natural resources, which were an important food source as well as solution for finding prophylaxis and treatment. Although there have been periods when different synthetic drugs were considered irreplaceable and eclipsed plant sources, once with the world development and the appearance of illnesses related to the modern civilization, obliged the scientific world once again to turn its attention to herbal resources, hoping to find valuable solutions for modern therapy [29].

Therefore, our attention is focused on easily accessible species on which there is little information in the literature from a chemical and biological point of view, and we started from the information available in the traditional medicine.

Currently, diabetes is a major pathology all around the world that implicates dysfunctions in glucose metabolism [2]. Either the glucose cannot be used by the cells in order to normalise the blood level if

there is not enough insulin or the receptors for insulin not recognise it. The manifestations of insulin-resistant glucose metabolism include reduced glucose transport and phosphorylation and reduced rates of glycogen synthesis [10], whereas abnormal fatty acid metabolism entails increased accumulation of triglycerides and other lipid structures as well as dysregulation of lipid oxidation during fasting and insulin-stimulated conditions.

Diabetes is a leading cause of death in most developed countries and there is substantial evidence that it is epidemic in many developing and newly industrialized nations. Complications from diabetes, such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness are resulting in increasing disability, reduced life expectancy, and enormous health costs for virtually every society [12].

Chronic hyperglycaemia, the predominant metabolic state of diabetes, can exacerbate defective glucose disposal by interfering with insulin action in insulin-target tissues, such as skeletal muscle [6, 7, 28]. The vegetal extracts are rich in polyphenolic compounds that have also an antioxidant capacity, therefore the combination between normalisation of the glucose blood level and the antioxidant capacity is revolutionary for diabetic patients [22]. It is well known that these patients present many complications because of the production of reactive oxygen species and of inflammatory conditions [29].

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. 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 [16].

Our previous studies indicated the antioxidant properties of extracts from *Pelargonium species* on *in vitro* tests [19]. Side effects of current antidiabetic drugs and negative consequences of postprandial hyperglycaemia stimulated the researchers to find new drugs for type 2 diabetes, able to delay or to reduce glucose absorption [18].

Due to the side effects of some antidiabetic drugs, many present studies try to identify functional foods and plant based medicines able to influence metabolic processes and to be used in the prevention and cure of diabetes and obesity. Hence, the attractive targets like *in vitro* inhibition of alpha-glucosidase and alpha-amylase are currently studied. Previously, several *in vitro* studies have been performed yielding potential α-glucosidase inhibitors from various food components and plants like cranberry, *Cuscuta reflexa*, pepper, soy bean extracts, etc., and alpha-amylase inhibitors from cheese, oregano, cranberry extract, Fenugreek and Balanite, but never on *Pelargonium species* extracts [3, 5, 27]. Natural alpha-glucosidase and alpha-amylase inhibitors from plant sources offer an attractive strategy for the control of postprandial hyperglycaemia [1, 4, 14, 21].

Inhibition of alpha-glucosidase and alpha-amylase, enzymes involved in the digestion of carbohydrates, can significantly decrease the postprandial increasing of blood glucose after a mixed carbohydrate diet and therefore can be an important strategy in the management of postprandial blood glucose level in type 2 diabetic patients and borderline patients [13, 23].

Plant extracts have long been used in ethno-medicine to treat diabetes and are currently accepted as an alternative for diabetic therapy or to complete this. However, for many plant extracts, there is no clear understanding of the mechanism of action. The *in vitro* alpha-glucosidase inhibitory activity may not always correlate with the *in vivo* one [1, 32]. There are no previous reports of any *in vitro* alpha-glucosidase and alpha-amylase inhibitory activity of *Pelargonium species*, so in our study we tried to identify these properties and the ability of extracts to control the inflammatory process.

In order to evaluate the cyto-regenerative effect of the extracts, we did the Scratch assay that involves the assessment of the migrating capacity of the cells and the rebounding capacity of the cells in the presence of the vegetal extracts.

Materials and Methods

Reagents, cell line and animals
Ethanol (Chimopar SA, Romania), methanol (SC Chimreactiv, Romania), dimethyl sulfoxide - DMSO (Merck, Germany), monopotassium phosphate (Merck, Germany), dipotassium phosphate (Merck, Germany), 3,5-dinitrosalicylic acid (DNS) (Sigma-Aldrich, Germany), p-nitrophenyl-D-glucopyranoside (Sigma-Aldrich, Germany), sodium chloride (Merck, Germany), alpha-glucosidase (Sigma-Aldrich, Germany), alpha-amylase (Sigma-Aldrich, Germany), acarbose CRS (European Pharmacopoeia Reference Standard, EDQM Strasbourg, France), indomethacin MK 4% (Fiterman Pharma SRL, Romania), tetradecanoylphorbol-13-acetate - TPA (Sigma-Aldrich, Germany), Dulbecco´s modified Eagle Medium with high glucose and foetal bovine serum (FBS) (Sigma-Aldrich, Germany), penicillin/streptomycin (Sigma-Aldrich, Germany), HaCaT cell line (was offered by the University of Debrecen, Hungary), SKH-1 mice (Charles River Laboratories, Hungary).

Plant material. The subjects of the current research are two *Pelargonium* species: *hispidum* (P1) and *grandiflorum* (P2). Specimens were obtained from the "Anastasie Fătu" Botanical Garden, Iași, Romania. The plants were kept in similar growth conditions to provide a minimum environmental impact.

Preparing of the hydro-alcoholic/methanolic extracts

2 g of dried and powdered leaves were extracted with 2 x 50 mL ethanol-water 1:1, respectively 2 x 50 mL methanol, on water bath, at reflux, for 45 minutes. The extracts are filtered and washed to complete the obtained volume to 100 mL using the same solvent. After cooling, the extracts were dried using a rotary evaporator. Dried extracts were solved in dimethyl sulfoxide (DMSO) in order to obtain solutions with concentrations between 0.039 and 5 mg/mL [8].

Alpha-amylase inhibition test

The method is based on enzyme inhibition, so the transformation of starch to reducing oligosaccharides that react with 3,5-dinitrosalicylic acid is blocked. The extracts in different concentrations and 20 mM phosphate buffer pH 6.9, containing porcine alpha-amylase (0.5 mg/mL) were incubated at 25°C for 10 min. 0.5% starch solution in 20 mM phosphate buffer pH 6.9, was added and the mixture was incubated at 25°C for 10 min. The reaction was stopped with 96 mM 3,5-dinitrosalicylic acid (DNS) colour reagent. After boiling and cooling the mixture, the absorbance was measured at 540 nm. The percent of alpha-amylase inhibition was calculated as follows: $(1-B/A) \times 100$, where A is the absorbance of control and B is the absorbance of samples containing extracts. The concentration of the extract required to inhibit the activity of the enzyme by 50%, IC50 was calculated by regression analysis. Experiments were performed in triplicate [15].

Alpha-glucosidase inhibition test

The method is based on the inhibition of alpha-glucosidase that catalyses the hydrolysis of p-nitro-phenyl-D-glucopyranoside to p-nitrophenol. The extracts in different concentrations, were made up with equal volumes of DMSO and distilled water. Sample solutions mixed with alpha-glucosidase (0.1 U/mL) in 100 mM phosphate buffer, pH 7.0 were incubated for 5 min. After the incubation, p-nitrophenyl-alpha-d-glucopyranoside was added and incubated again. The absorbance of the final solution was measured at 405 nm. The percent of alpha-glucosidase inhibition was calculated as follows: $(1-B/A) \times 100$, where A is the absorbance of control and B is the absorbance of samples containing extracts. The inhibitory concentration of the extract required to inhibit the activity of the enzyme by 50%, IC50 was calculated by regression analysis. Experiments were performed in triplicate. Acarbose was used as positive control [4].

TPA-Induced oedema test

The anti-oedematous/anti-inflammatory effect of the investigated samples was demonstrated on a TPA-induced animal model. We used 15 mice, SKH-1 females without hair, 3 mice for each group. SKH-1 female mice were kept under standard conditions (24ºC, 55% relative humidity). There were five groups: Control group - not treated, TPA group - treated with TPA, Indomethacin group - treated with TPA and indomethacin - topical administration, Group 1 – TPA+P1M (*P. hispidum* methanolic extract 5 mg/mL) and Group 2 – TPA+P2M (*P. grandiflorum* methanolic extract 5 mg/mL). The protocol was approved by the Bioethics Committee of "Victor Babeş" University of Medicine and Pharmacy, Timişoara, Romania. After 24 h from the topical administration we measured the inflammatory parameters (ear base size, length, and mass) [11, 20].

The scratch assay

The evaluation of cell division capacity by using the scratch technique is based on *in vitro* assessment of the ability of plant extracts to stimulate the process of restoring intercellular bonds that were destroyed mechanically. For this test we used keratinocyte cell line (HaCat) that were cultivated in 12 wells plates and then treated with DMSO (control), 0.0195 mg/mL and 1.25 mg/mL methanolic extract of P1 and P2 [11].

Results and Discussion

The *Pelargonium species* were studied less from this therapeutic point of view and this research will contribute to the biological characterization of this group of plants. The methanolic extracts of *Pelargonium species* showed a great antioxidant activity by assessing the free-radical scavenging capacity (DPPH radical, ABTS cation), also ferrous ion-chelating capacity, and super-oxide anion radical scavenging capacity [9]. Moreover, the antibacterial, antifungal and anti-inflammatory activity were studied on these types of extracts and showed good results [11]. This is the reason of choosing the methanolic extracts for the anti-inflammatory assay.

The results of the UHPLC analysis showed that *P. hispidum* was richer in cyanidol, flavonols and its derivatives, whereas *P. grandiflorum* contains more catechins [33].

Our data were in accordance with the literature related to the types of compounds usually found in *Pelargonium species*, but unlike other authors we found only relatively small amounts of tannins in both samples (less than 0.5 mg/g), these results have been published before [19].

The values obtained for the alpha-amylase and alpha-glucosidase inhibition assays are showed in Table I and Table II.

Table I

The results of the alpha-amylase inhibition test

| Source – % inhibition ± standard deviation								
Sample conc. (mg/mL)	0.039	0.078	0.156	0.3125	0.625	1.25	2.5	5
P1E	30.30 ± 0.26	42.73 ± 0.20	48.12 ± 0.16	50 ± 0.02	53.64 ± 0.23	56.67 ± 0.17	59.22 ± 0.10	73.41 ± 0.17
P1M	40.09 ± 0.21	47.45 ± 0.28	51.81 ± 0.06	56.59 ± 0.28	57.64 ± 0.17	75.48 ± 0.29	88.52 ± 0.14	91.74 ± 0.07
P2E	48.63 ± 0.31	66.60 ± 0.31	76.35 ± 0.11	82.63 ± 0.29	88.63 ± 0.27	91.36 ± 0.11	93.05 ± 0.05	94.26 ± 0.07
P2M	38.43 ± 0.21	42.62 ± 0.21	46.71 ± 021	50.57 ± 0.17	54.39 ± 0.09	62.59 ± 0.31	68.58 ± 0.32	80.54 ± 0.14
Acarbose	34.55 ± 0.04	44.87 ± 0.08	55.68 ± 0.05	60.87 ± 0.12	67.58 ± 0.04	75.44 ± 0.03	82.77 ± 0.04	95.47 ± 0.11

*P1E – hydro-alcoholic extract of *Pelargonium hispidum*, P1M – methanolic extract of *Pelargonium hispidum*,

*P2E – hydro-alcoholic extract of *Pelargonium grandiflorum*, P2M – methanolic extract of *Pelargonium grandiflorum*.

Table II

The results of the alpha-glucosidase inhibition test

Source – % inhibition ± standard deviation								
Sample conc. (mg/mL)	0.039	0.078	0.156	0.3125	0.625	1.25	2.5	5
P1E	17.49 ± 0.17	21.64 ± 0.23	29.55 ± 0.27	38.71 ± 0.22	45.34 ± 0.05	50.56 ± 0.08	65.83 ± 0.03	69.38 ± 0.03
P1M	20.33 ± 0.12	29.92 ± 0.05	35.68 ± 0.23	48.40 ± 0.08	51.27 ± 0.06	59.64 ± 0.17	64.36 ± 0.13	75.83 ± 0.04
P2E	35.35 ± 0.21	40.21 ± 0.11	48.32 ± 0.03	52.35 ± 0.05	61.45 ± 0.15	70.52 ± 0.02	81.81 ± 0.02	90.18 ± 0.03
P2M	19.84 ± 0.08	25.52 ± 0.04	39.51 ± 0.03	47.27 ± 0.06	55.67 ± 0.06	67.25 ± 0.07	78.21 ± 0.07	83.37 ± 0.11
Acarbose	20.47 ± 0.03	39.56 ± 0.02	48.99 ± 0.01	57.54 ± 0.05	67.78 ± 0.04	70.99 ± 0.07	82.47 ± 0.01	93.65 ± 0.04

*P1E – hydro-alcoholic extract of *Pelargonium hispidum*, P1M – methanolic extract of *Pelargonium hispidum*,

*P2E – hydro-alcoholic extract of *Pelargonium grandiflorum*, P2M – methanolic extract of *Pelargonium grandiflorum*.
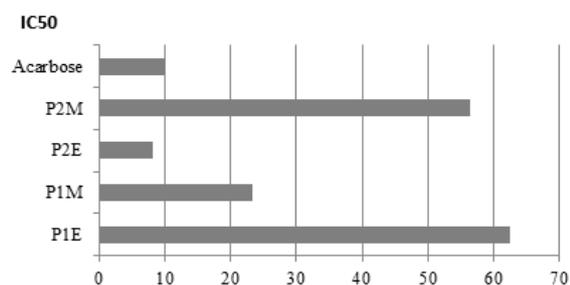
Figure 1.

The results of the IC50 (µg/mL) in the alpha-amylase inhibition test
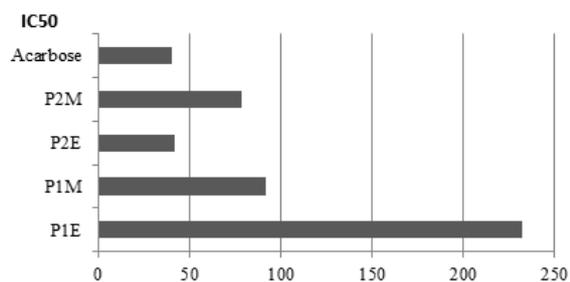
Figure 2.

The results of the IC50 (µg/mL) in the alpha-glucosidase inhibition test

On the alpha-amylase inhibition assay the best activity was showed by the methanolic extract of *Pelargonium hispidum* (P1M) and also the hydro-alcoholic extract of *Pelargonium grandiflorum* (P2E). On the other hand, at the alpha-glucosidase inhibition assay the methanolic extract of *P. grandiflorum* (P2M) had an

IC50 = 78.29 ± 0.02 µg/mL compared with acarbose that had an IC50 = 40.25 ± 0.05 µg/mL (Figures 1 and 2).

Following the results we obtained on these assays, we could observe that the IC50 on the alpha-amylase inhibition is lower than the alpha-glucosidase inhibition because alpha-amylase has larger substrate specificity and could transform more compounds than the alpha-glucosidase that transform just glucose. Also the best activity was showed by the hydroalcoholic extract of the *P. grandiflorum* (P2E) with an IC50 = 41.68 ± 0.1 µg/mL.

Vegetal extracts with mild enzyme inhibitory effects have some advantages compared to acarbose that could induce digestive side effects such as flatulence, diarrhoea, abdominal distension and bloating [25]. These effects are the consequences of carbohydrates transformation by intestinal bacteria on the large intestine [26].

The type of polyphenols is important for biological properties of extracts, so, catechol catechins were twice more active than the pyrogallol catechins on the inhibition of α-amylase and α-glucosidase [17]. According to Tadera *et al*., the inhibitory activity of flavonols against α-amylase and α-glucosidase is correlated to the chemical structure of the compounds. It was shown that the luteolin, myricetin and quercetin were potent inhibitors. This capacity is depended by the position and the number of hydroxyl groups on the rings in the polyphenols structure [34]. Inhibitory activity against alpha-amylase is more important for

bound phenolic extract such as hydro-alcoholic extract than free phenolic extract such as methanolic extract [1]. For our samples, just *P. grandiflorum* hydro-alcoholic extract was more active than methanolic extract against alpha-amylase, and it is dependent on the complex composition of these extracts.

According to the results obtained from previous assays and using the information obtained in the UHPLC analysis [19], we decided to test only the methanolic extracts in the TPA-induced oedema assay and the scratch test on the cell line.

The values obtained on the *TPA-induced oedema* were determined after a single topical application of TPA. The maximum expression of oedema was observed 24 hours after the administration of TPA with important differences for the groups treated with the investigated *Pelargonium* extracts. For the same dose (5 mg/mL) applied topically, the most intense oedema reduction effect was present in the group 2 treated with the methanolic extract of *P. grandiflorum*, in which inflammation compared to the negative control (TPA group) decreased by about 50%, and, compared to the positive control (treated with indomethacin-TPA+IND group), decreased by about 30%. *P. hispidum* methanolic extract (group 1) showed an anti-oedematous effect of 45% compared to the negative control. The parameters we obtained in this assay are presented in Table III, after measuring the base (mm) of the ear, the length (mm) and the weight (g).

Table III

The results of the TPA-induced oedema test

Group	EAR PARAMETERS		
	BASE (mm)	LENGTH (mm)	WEIGHT (g)
Control	5.46 ± 0.023	14.50 ± 0.03	0.05 ± 0.016
TPA group	8.33 ± 0.023	16.80 ± 0.03	0.29 ± 0.016
TPA+indomethacin group	7.69 ± 0.023 [a]	16.05 ± 0.03 [a]	0.20 ± 0.016 [a]
Group 1	6.37 ± 0.023 [a]	16.11 ± 0.03 [a]	0.18 ± 0.016 [a]
Group 2	6.60 ± 0.023 [a]	16.80 ± 0.03	0.16 ± 0.016 [a]

[a] extremely statistically significant ($p < 0.0001$) – treated groups *vs*. TPA group

According to the results obtained in the process of restoring intercellular bonds it was clear that the P1M and P2M, after 24 hours of cell treatment, the anti-migrating process is low, which proves the fact that it has no cytotoxic effect on cells, especially at 0.0195 mg/mL (Figure 3). These results are promising and lead us to the information that the extracts can be used in rebounding tissues.
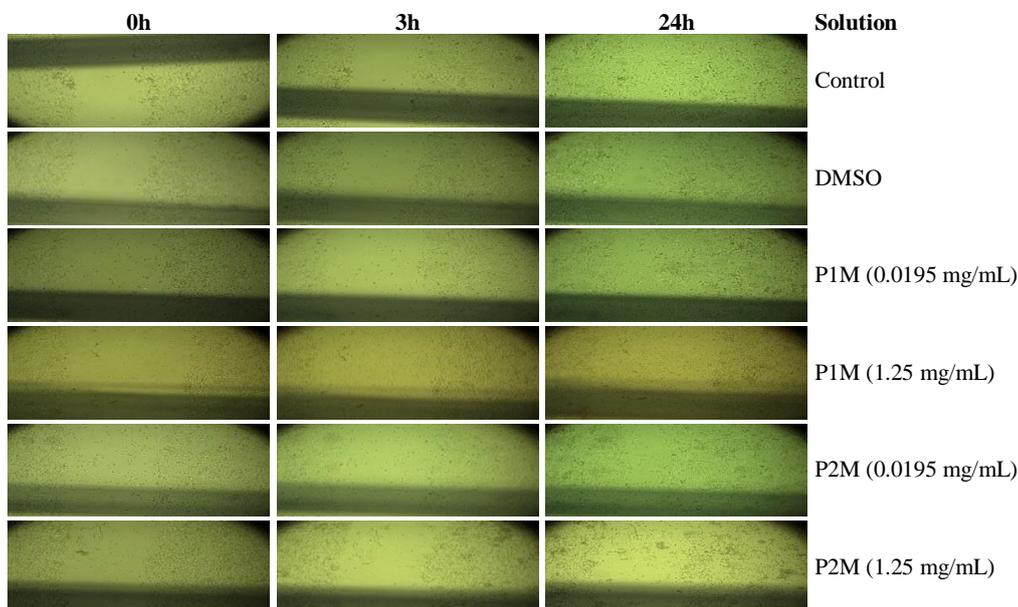
Figure 3.

The anti-migrating effect of methanol extracts on HaCaT cells

The antioxidant activity and the ability of polyphenols to inhibit enzymes involved in the synthesis of pro-inflammatory eicosanoids, cytokines or chemokines explain the anti-inflammatory properties of these [36]. On the other hand, Mueller and collaborators showed that the anti-inflammatory activity of some plant extracts with rich content in quercetin, resveratrol, kaempferol reduced the pro-inflammatory interleukin (IL)-6 [24]. TPA-oedema induced test and our previously

antioxidant tests reveal the anti-inflammatory properties of extracts with higher polyphenols content.

Oxidative stress with overproduction of reactive oxygen species could induce or promote tissue injuries and finally will affect tissue regeneration. *In vivo*, vegetal extracts increase the activity of endogenous antioxidant enzymes. The vegetal extracts with high concentrations in polyphenols have the biggest effect on tissue regeneration [35, 36].

These results can be correlated with ours and sustains the fact that the ethanolic extract of *P. grandiflorum* presents a higher activity on the inhibitory assays due to its composition and higher extractability in flavonols compounds, also their important effect in anti-inflammatory mechanism and tissue regeneration. Flavonols such as myricetin and quercetin possess antioxidant and anti-inflammatory properties and have the ability to protect keratinocytes and endothelial cells. Also, polyphenols increase the biosynthesis of collagen and elastin, stabilize collagen, and inhibit enzymes involved in their degradation. The hydro-alcoholic extracts improve collagen cross-linking and improve the properties of dentin depending on the time of exposure. These properties sustain the use of polyphenols rich vegetal extracts in tissue engineering for tissue regeneration, bone regeneration, and to improve the mechanical and chemical properties of biomaterials used in medicine [31].

Conclusions

Polyphenols protect cells against the damages caused by reactive oxygen species as a product of energy metabolism. It is well known that diabetes is linked with oxidative stress in the body. The poly-phenol-rich extracts can be a potential source of anti-diabetic agents for the postprandial hyperglycaemia control and diabetic damages arising from oxidative stress [25]. As far as we got with the determination and correlating the results it is clear that the extracts of the *Pelargonium species* that we tested have promising utility in the diabetes therapy.

The tests carried out on healthy cell line, HaCaT, demonstrated that the rebounding capacity of such cells is dependent on the concentration of extract used. Thus, it appears that the extractive fraction in which flavonoids are predominant has a more important migrating effect.

The target pathologies are numerous, with focus on inflammatory conditions, such as rheumatism, rheumatoid arthritis, as well as many others for which the allopathic solution proposes classes of selective and/or non-selective steroidal and/or non-steroidal anti-inflammatory drugs, but which come with numerous side effects that lead to the treatment discontinuation. Thus, there are a number of premises that require research regarding the exploitation of natural resources with obvious therapeutic value.

Conflict of interest

The authors declare no conflict of interest.

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