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Do *Aloe vera*, *Bryophyllum pinnatum*, *Callisia fragrans*, and *Pelargonium graveolens*, used in folk medicine to treat skin diseases, exhibit antioxidant activity?

Anna Horecka[1,A-D,F] ✉, Anna Olszewska[2,A-B,F], Sebastian Mertowski[3,E], Jerzy Kuliński[4,D], Anna Hordyjewska[1,A-D,F]

[1] *Department of Medical Chemistry, Medical University, Lublin, Poland*
[2] *Department of Human Physiology, Medical University, Lublin, Poland*
[3] *Department of Experimental Immunology, Medical University, Lublin, Poland*
[4] *Institute of Rural Health, Lublin, Poland*
A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

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Abstract

Introduction and Objective. *Aloe vera, Bryophyllum pinnatum, Callisia fragrans and Pelargonium graveolens* are medicinal plants traditionally used in folk medicine to treat a variety of diseases. The aim of the study is to evaluate the antioxidant properties of *Callisia fragrans,* and to the best of the authors' knowledge is the first such study on the topic.

Materials and Method. The focus of the study was on the antioxidant activities of plant ethanol extracts in an *in vitro* model of the mouse L929 fibroblastic cell line, in which oxidative stress was induced by hydrogen peroxide (H_2O_2). Extracts were obtained from biennial plants? Fibroblasts were first stimulated with 100 μM H_2O_2 for 3 h and subsequently incubated with various concentrations of plant extracts: 3.5 g/ml (extract 1); 1.75 g/ml (extract 2); and 0.875 g/ml (extract 3) for 24 h.

Results. A significant decrease in ferric reducing antioxidant power (FRAP) and malondialdehyde (MDA) were observed in cell lysates treated with *Aloe vera, Bryophyllum pinnatum* and *Pelargonium graveolens* extracts. The concentration of carbonyl groups was statistically lower in cell lysates treated with *Aloe vera* and *Pelargonium graveolens* extracts. Significant increase in protein carbonyl groups concentration was observed in cell lysates treated with *Callisia fragrans,* but no significant changes were noticed in MDA and FRAP concentrations.

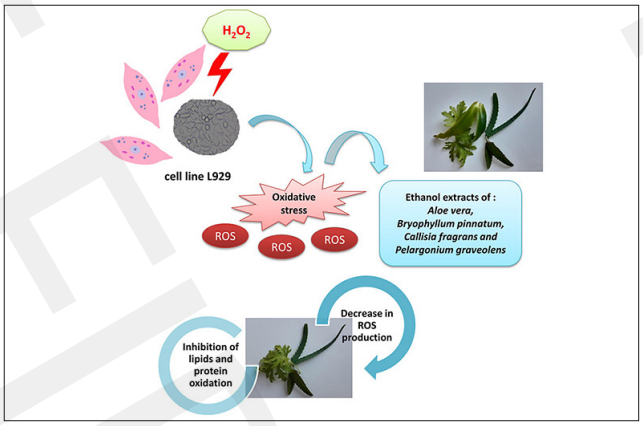
Conclusions. The results obtained confirmed antioxidant activities of *Aloe vera*, *Bryophyllum pinnatum* and *Pelargonium graveolens,* which may contribute to their protective effects in cutaneous diseases. They also have the ability to protect fibroblasts in skin by a decrease in ROS production, and by the inhibition of lipid and protein oxidation. Therefore, they can be used in dermatology for the treatment of cutaneous diseases in the form of external skin care products. *Callisia fragrans,* however, showed the opposite trend, indicating a possible pro-oxidant behaviour requiring further investigation.

Key words

medicinal plants, *Aloe vera, Pelargonium graveolens, Bryophyllum pinnatum, Callisia fragrans*, oxidative stress in skin

INTRODUCTION

Aloe vera, Bryophyllum pinnatum, Callisia fragrans and Pelargonium graveolens are medicinal plants traditionally used in folk medicine for the treatment of various diseases, such as: hypertension, rheumatoid arthritis, wound healing [1, 2], diabetes mellitus [2], ulcers [3], skin diseases, herpes [4], anti-tumour [5, 6] and influenza activity [7]. About 75% of the African population rely on natural medicine and the named plants are used as herbal remedies for various disorders [2]. The most known medicinal plant – *Aloe vera* – belongs to the Asphodelaceae family and grows naturally in tropical climates. It contains about 75 biologically active substances, including anthraquinones that inhibit cyclooxygenase activity, as well as naphthoquinones, flavonoids, saponins, sterols, amino acids and antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase and vitamins A, C and E. Its anti-inflammatory, antineoplastic, antifungal



Graphical abstract

✉ Address for correspondence: Anna Horecka, Department of Medical Chemistry, Medical University, Lublin, Poland
E-mail: anna.horecka@umlub.pl
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and antimicrobial properties have been shown to accelerate wound healing and stimulate collagen formation [8, 9]. *Bryophyllum pinnatum,* which originates from Madagascar, belongs to the Crassulaceae family and contains biologically active constituents, such as flavonoids, alkaloids, lipids, and kaempferol rhamnoside. It was reported to be used in the treatment of asthma, diabetes, hypertension, diarrhoea and the healing of ulcers [10]. *Pelargonium graveolens,* known as geranium, belongs to the Geraniaceae family and originates from South Africa. It possesses anti-allergic, anti-inflammatory, diuretic and anti-diabetic properties due to presence of flavonoids, kaempferol 3,7-di-O-gluciside, quercetins, rutin, quercetin 3-O-pentoside, kaempferol 3-O-gluciside, and kaempferol 3-O-rhamnoside-gluciside, and essential oils, e.g. β-citronellol, citronellyl formate, and geraniol [11]. *Calissia fragrans* belongs to the Commelinaceae family and grows wild in Mexico. It contains active flavonoids, fospholipids and amino acids, and has been used especially in Eastern Europe for the treatment of skin diseases and joint disorders. To date, limited research has been conducted on the properties of the *Calissia fragrans,* and only its anti-herpetic [4] and anti-hypertensive [12] activities have been confirmed.

Oxidative stress in skin is mainly caused by ultraviolet (UV) radiation, exposure to electromagnetic fields, irradiation by X-rays and γ-rays, pollutant chemicals (xenobiotics, aromatic amins), nutrition, drugs, cosmetics, alcohol intake, tobacco smoke and stress, which cause inflammation, and consequently lead to skin diseases and aging [13].

There are several studies which report the positive effects of medicinal plants on human health [14]. Glucomannan, gibberellin acid and polysaccharides found in the juice of *Aloe vera,* stimulate the production of collagen. In turn, emodin and aloin inhibit the activity of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2) and nitric oxide, and reduce the level of interleukin (IL)-8, tumour necrosis factor- alpha (TNF-α), IL-6, IL-1β [15]. Aloesin is shown to be comparable to hydrocortisone in *in vitro* studies and is able to reduce oedema in the mouse model [16]. *Aloe vera* has been shown to affect the activity of matrix metalloproteinases- 9 (MMP-9) [17].

Plant essential oils derived from *Pelargonium graveolens* (geranium) have antifungal potential and have been used as a topical antiseptic [18]. They also possess immunomodulatory and anti-inflammatory abilities – supressing nitric oxide and PGE2 [3, 19]. A study by Simoes et al. [20] showed that essential oils derived from *Pelargonium graveolens* also demonstrate increased estrogenicity.

Medicinal plans, therefore, are promising sources of active ingredients which can be used in the treatment of many diseases.

OBJECTIVE

The aim of this study is to determine the antioxidant properties of *Aloe vera*, *Bryophyllum pinnatum Callisia fragrans* and *Pelargonium graveolens* ethanol extracts in an *in vitro* model of the mouse L929 fibroblastic cell line, where oxidative stress – the main cause of skin damage – was induced by hydrogen peroxide (H_2O_2). The study was conducted to support the clinical application of plant extracts.

To the best of the authors' knowledge, this is the first study to evaluate the antioxidant properties of *Callisia fragrans,* and presents a novel approach to evaluating oxidative stress.

MATERIALS AND METHOD

Preparation of extracts. *Aloe vera, Bryophyllum pinnatum, Callisia fragrans* and *Pelargonium graveolens* were identified and confirmed by Wirginia Kukuła-Koch from Department of Pharmacognosy at the Medical University of Lublin in eastern Poland. Plant extracts were obtained from biennial plants, the leaves of which (14 g) were macerated in 96% ethanol (100 ml), sonicated, centrifuged, filtered through a 0.22 μm membrane filter, and evaporated (from 100 ml to 2 ml to obtain a final concentration of 7 g/ml). The extracts were stored at 4 °C until further use. Just before usage, the concentrated extracts were dissolved in Phosphate Buffered Salin (PBS) 2, 4 and 8 times. The concentration of the obtained solutions were 3.5 g/ml (extract 1); 1.75 g/ml (extract 2); and 0.875 g/ml (extract 3).

Cell culture. The experiment was conducted on reference cell line L929 (cell line origin – mouse C3H/An connective tissue), obtained from ATCC (specification – NCTC clone 929 [L cell, L-929, derivative of Strain L] (ATCC® CCL-1™). Cell cultures were grown in Modified Eagle Medium (MEM, Pan-Biotech); supplemented with 5% foetal bovine serum (Pan-Biotech), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B at 37 °C (Pan-Biotech) in a humidified atmosphere comprising 5% CO_2 in the air. To establish the proper concentration of hydrogen peroxide, the cells were incubated in triplicate with various H_2O_2 concentrations (50, 100, 150, 200 μM) to determine the maximal H_2O_2 dose that neither changes the viability of the cells nor affects the cells via oxidative stress. The H_2O_2 dose selected for further experiments was set at 100 μM (Fig. 1).

After growing for 24 h, the cells were stimulated with 100 μM H_2O_2 for 3 h to induce oxidative stress, and subsequently incubated for 24 h. with 3 different concentrations of plant extracts. All measurements were performed in triplicate. A solvent control was included in the experimental design.

Cell viability assay. Cell viability was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche), according to the manufacturer's manual. First, the yellow tetrazolium salt (MTT) was metabolized by cells to purple formazan crystals. Fibroblasts were seeded on 96-well microplates at the density of $1x10^4$ cells/well for 24 h. The next day, the culture medium was removed and the cells were exposed to different concentration of plant extracts and H_2O_2 – tested in triplicates. Next, 20 μl of MTT solution (5 mg/ml) was added to each well and incubated for 4 h. The formazan grains typical for viable an cells were solubilized with 200 μl of DMSO. Absorbance was measured at 570 nm using an Epoch UV–Visible Spectrophotometer (Biotek, USA). The percentage of cell viability was calculated and compared to control, which has 100% viability.

Cell lysate. After 24 h of incubation, the post-culture fluid was removed, the cells were washed with 1 ml of ice-cold PBS and scraped-off. Next, 0.5 ml of cells were placed in a tube

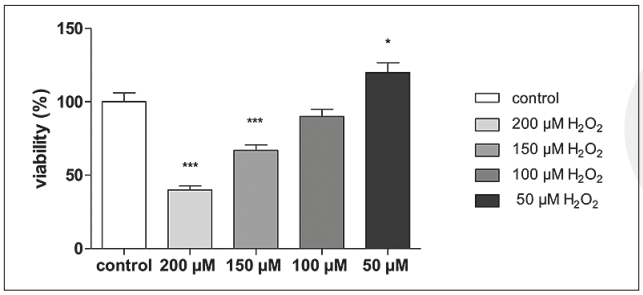

Figure 1. Viability (%) of L929 fibroblasts cells incubated for 24 h with various H2O2 concentrations (50, 100, 150, 200 µM). Results are expressed as the percentages of surviving cells over control cells.
Data are means (SD); ***p<0.001; *p<0.05

and 1 ml of cold lysis buffer (5 mM potassium phosphate pH = 7.8 containing 0.9 % sodium chloride and 0.1 % glucose) was added. Tubes were placed in an ultrasound machine for 20 min. and subsequently centrifuged at 4 °C for 15 min at 3,000 rpm. The supernatant (cell lysate) was stored at -80 °C until the analysis.

Oxidative stress parameters

FRAP. The total antioxidant capacity of cell lysates was determined by ferric reducing antioxidant power (FRAP), according to the Benzie and Strain method [21]. The standard curve was prepared with $FeSO_4$ at concentrations 0 – 1,000 µmol/l. A working reagent was prepared by an mixing aqueous solution of $FeCl_3$ (20 mmol/l), acetate buffer of pH 3.6, TPZT (2,4,6-tri-pyridyl-s-triazine) (10 mmol/l) in 40 mmol/l HCl (ratio 10:1:1). Standards and samples (10 µl) were mixed with 20 µl of de-ionized water and 200 µl of the working reagent on a 96-well plate. The colour intensity was measured at 593 nm after 30 min incubation at 37 °C in the dark. Results were calculated per protein content of cell lysates and expressed in µmol/mg of protein.

MDA. The level of lipid peroxidation in cell lysates was measured according to Ledwozyw et al. [22]. First, the cell lysates were deproteinised by adding 400 µl of trichloroacetic acid solution (TCA) in 0.6 M HCl to 80 µl of sample. The mixture was incubated for 15 min at room temperature. After that time, 240 µl of thiobarbituric acid (TBA) was added which was prepared by dissolving 500 mg of TBA in 6 ml of 1M NaOH, and then diluting with 69 ml of water. Next, it was heated in a boiling water bath for 30 min and cool at room temperature. Then, 640 µl of n-butanol was added to the tubes, which were sealed and shaken vigorously for 10 min to extract the malondialdehyde (MDA) complex from TBA to the butanol phase. The next step was to centrifuge the tubes (1,500 rpm for 10 min) in order to separate the phases. 200 µl of the butanol phase was then added to each well of the 96-well plate. Absorbance was measured at a wavelength of 532 nm using n-butanol as a blank. The concentration of MDA was calculated from a calibration curve (range 1–10 µM). A standard was prepared by hydrolysis of 16.4 µl of 1,1,3,3-tetraethoxypropane (TEP) stock solution in 50 ml of 0.2 mM hydrochloric acid to obtain concentration of 10 mM. The final concentration was expressed in nmol/mg protein.

Protein carbonyl groups. The level of protein oxidation in cell lysates was measured on the basis of the method proposed by Mesquita et al. [23], based on the reaction between the 2,4-dinitrophenylhydrazine (DNPH) and carbonyl groups. Working reagent (10 mM DNPH in 0.5 M H_3PO_4) was prepared by diluting 2.5 ml of phosphoric acid (V) in 50 ml of water and adding 9 mg dinitrophenylhydrazine. Next, 100 µl of this reagent was added to each well of the 96-well plate. 100 µl of sample and 50 µl of 6M NaOH per well was then added. Next, the plate was incubated in the dark at room temperature for 10 min. Absorbance was measured at 450 nm. The concentration of the carbonyl groups was calculated based on the molar absorptivity for the hydrazone derivatives of carbonyl groups. The concentration was expressed in nmol/mg protein.

Protein content. BCA Protein Assay (Thermo Scientific Pierce, USA) was used to measure total protein concentration in cell lysates. All results were calculated on mg of protein and expressed in nmol/mg of protein for the concentrations of MDA and carbonyl groups, and in µmol/mg of protein for the concentration of FRAP.

Statistical analysis. Statistical analysis was performed by GraphPad Prism 5.0 software (San Diego, CA, USA) using one-way ANOVA followed by Tukey's test. Shapiro-Wilk test was used to verify conformity of variables distribution to hypothetical normal distribution. Values were considered statistically significant when $p < 0.05$. Data were expressed as the mean + standard deviations (SD).

RESULTS AND DISCUSSION

Incubation of the cells with H_2O_2 induced oxidative stress, resulting in MDA, FRAP, as well as increase in the carbonyl groups (Fig. 2A, 2B, 2C). Statistically significant increases in oxidative stress parameters were found after incubation with medicinal plant extracts. Compared to the group of cells treated only with H_2O_2, the FRAP and MDA concentrations were statistically lower in cell lysates treated with *Aloe vera* (AV), *Bryophyllum pinnatum* (BP), and *Pelargonium graveolens* (BP) extracts. The effect was observed in a dose-dependent manner.

A significant decrease was observed in FRAP concentration (AV1=0.395 µM/mg of protein, AV2=0.894 µM/mg of protein, p<0.001; BP1=0.996 µM/mg of protein, p<0.001; BP2=1.843 µM/mg of protein, p<0.01; PG1=1.087 µM/mg of protein, p<0.001 vs 100 µM H_2O_2 =3.696 µM/mg of protein) and MDA concentration (AV1=7.165 nM/mg of protein, AV2=9.722 nM/mg of protein, AV3=12.864 nM/mg of protein, BP1=17.030 nM/mg of protein, BP2=18.947 nM/mg of protein, PG1=12.04 nM/mg of protein, PG2=22.642 nM/mg of protein, p<0.001 vs 100 µM H_2O_2 =37.987 nM/mg of protein) (Fig. 2A, 2C).

The concentration of protein carbonyl groups was statistically lower in cell lysates treated with *Aloe vera* and *Pelargonium graveolens* extracts (AV1= 0.098 nM/mg of protein, p<0.01; AV2=0.127 nM/mg of protein, p<0.05; PG1=0.127 nM/mg of protein, p<0.05 vs 100 µM H_2O_2 = 0.325 nM/mg of protein) (Fig. 2B). The effect was the strongest for the highest concentration of plant extracts.

The present study did not reveal any statistical difference in the concentration of protein carbonyl groups in *Bryophyllum pinnatum* extract, compared to the group treated only with 100µM H_2O_2. Interesting results were observed for the

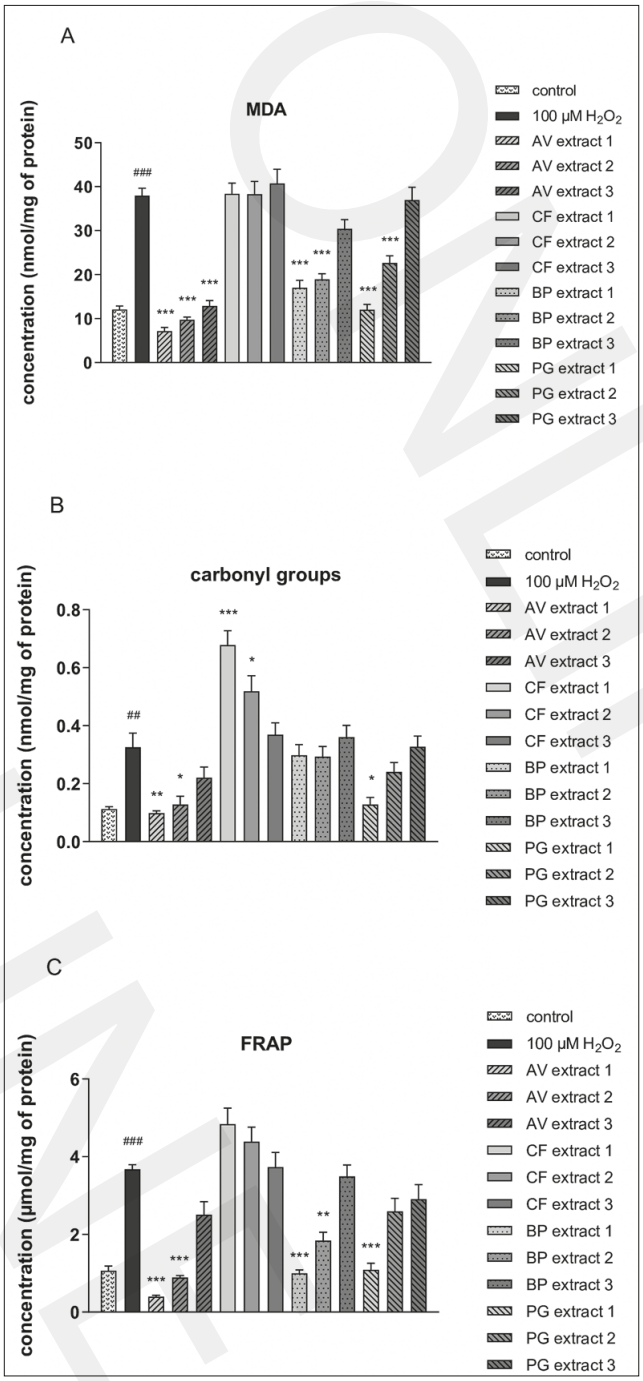

Figure 2. Changes in MDA (2A), carbonyl groups (2B) and FRAP (2C) concentrations in cell lysates after incubation with medicinal plant extracts, compared to control and H202 group. L929 fibroblasts cells were stimulated with 100 μM H2O2 for 3 h to induce oxidative stress, and subsequently incubated for 24 h with 3 different concentrations of plant extracts.
AV – Aloe vera extract (1, 2, 3); BP – Bryophyllum pinnatum extract (1, 2, 3), CF – Callisia fragrans extract (1, 2, 3); PG – Pelargonium graveolens extract (1, 2, 3). Data are means (SD), ***indicates p<0.001; ** indicates p<0.01; * indicates p<0.05 when compared to H202 group. ANOVA followed by Tukey's test as *post-hoc*. ## indicates p<0.01, ### indicates p<0.001 when compare to control, t-test

Callisia fragrans (CF) extract. An increase in protein carbonyl groups clearly indicated oxidative damage, while a higher FRAP concentration at the highest concentration indicated a pro-oxidant or redox-disrupting effect (CF1=0.678 nM/mg of protein; p<0.001; CF2=0.519 nM/mg of protein; p<0.05 vs 100 μM H_2O_2=0.325 nM/mg of protein) (Fig. 2B). The concentration of 100μM hydrogen peroxide did not induce apoptosis and did not change viability of the cells (Fig. 1). A strong protective effect of plant extracts on H_2O_2 induced cells was observed. *Aloe vera, Bryophyllum pinnatum,* and *Pelargonium graveolens* improvd proliferation of the cells (Fig. 3).

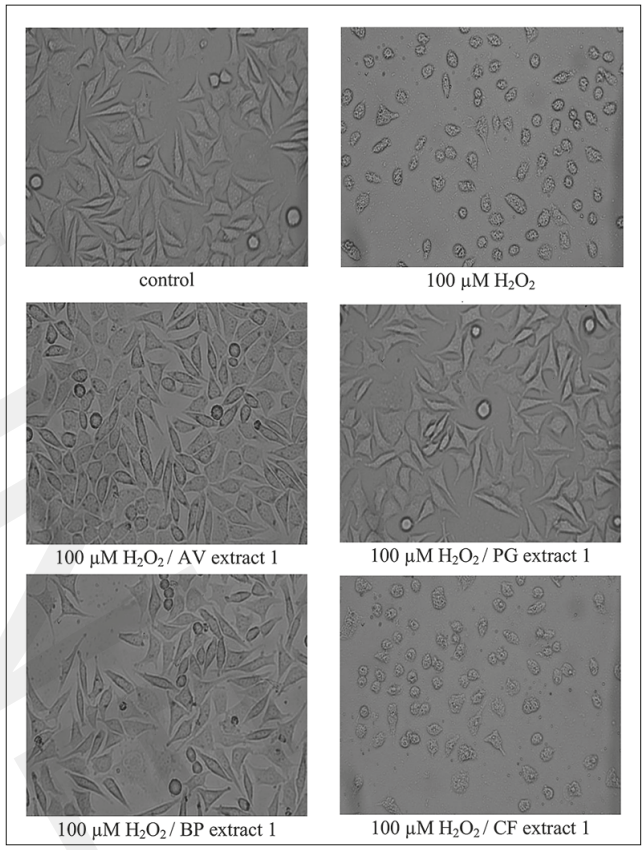

Figure. 3. Microscopy images of L929 fibroblasts cells after incubation with medicinal plants and H202, compared to control (magnification 400x).
AV – Aloe vera extract 1; BP – Bryophyllum pinnatum extract 1; CF – Callisia fragrans extract 1; PG – Pelargonium graveolens extract 1

Several reports have been shown that oxidative stress plays a major role in pathologies of the skin, such as atopic dermatitis, urtricaria, psoriaris, skin carcinogenesis, impairment in wound healing, as well as allergic and inflammatory dermatologic diseases [24, 25]. Oxidative stress is caused by reactive oxygen species that normally are in balance in the skin tissue due to the antioxidant system [23]. Skin is rich in polyunsaturated fatty acids, and can be easily oxidized by reactive oxygen species (ROS). Increased ROS production causes oxidative damage of lipid, protein and DNA, resulting in the degradation of collagen fibres and connective tissue of the dermis, which consequently lead to cell apoptosis [22]. It has been reported that the majority of antioxidants are present in the epidermis, and that their level increases in aging. Water soluble vitamin C is one of the antioxidants which play a crucial role as a co-factor of collagen synthesis. Unfortunately, vitamin C is not synthetized and should be supplemented with food. The reduction of collagen, elastic fibres, and hyaluronic acid observed with aging cause wrinkles [26].

The current study focused on the antioxidant activities of *Aloe vera*, *Bryophyllum pinnatum Callisia fragrans* and *Pelargonium graveolens* in mouse fibroblasts damaged by

oxidative stress caused by H_2O_2. Fibroblasts are the cells of dermis which secrete collagen and can be a source of extra-cellular matrix components [27]. The results obtained confirmed antioxidant activities of *Aloe vera*, *Bryophyllum pinnatum* and *Pelargonium graveolens* which may contribute to their protective effects in cutaneous diseases.

Callisia fragrans, commonly used in folk medicine in Eastern Europe, has scarcely been studied previously, with only one study confirming antiviral properties. Ethanol extract decreased the infection of herpes simplex virus 1 and 2 (HSV-1 and HSV-2) on Vero cells, whereas the aquatic extract inhibited the varicella zoster virus (VZV) [4]. The phenomenon of *Callisia fragrans* is that although it has never been tested, it continues to be used in folk medicine as a remedy for many diseases. Research by the authors of the current study have not confirmed any antioxidant activities, but some pro-oxidant activities have been found. The concentration of protein carbonyl groups increased, which means that oxidative stress is much more intensive after treatment with the plant. However, other markers of oxidative stress, such as the indicator of lipid peroxidation – MDA and the indicator of antioxidant power -FRAP does not change so pro-oxidative activity cannot be confirmed.

The observed effect of *Callisia fragrans* extract may be related to redox-active phytochemicals, such as polyphenols and flavonoids, that can act as antioxidants. However, under certain conditions they may undergo redox cycling, generating reactive intermediates and exhibiting pro-oxidant behaviour. This dual activity often depended on their concentration and the presence of transition metals, such as iron or copper, which can catalyze Fenton-type reactions and enhance the formation of reactive oxygen species. Therefore, the balance between antioxidant and pro-oxidant effects in the *Callisia fragrans* extract could be influenced by both its polyphenolic composition and trace metal content [28].

The antioxidant and anti-diarrheal activity of the methanol extract of *Bryophyllum pinnatum* has been confirmed previously [10]. Methanol and ethyl acetate extracts have been confirmed to have antimicrobial and antioxidant properties [29], and it was also noted that the aqueous extract of *Bryophyllum pinnatum* inhibits production of prostaglandins and polypeptide kinins. These results suggest that it also has antinociceptive properties [2]. According to reports, antioxidant therapy improved oxidative stress-related wound healing [27].

CONCLUSIONS

Aloe vera, Bryophyllum pinnatum, and *Pelargonium graveolens* ethanol extracts are able to protect fibroblasts in skin by decreasing ROS production and the inhibition of lipid and protein oxidation. Therefore, the plant extracts can be used in dermatology in the form of external skin care products for the treatment of cutaneous diseases, and reduce oxidative stress. However, in this first study to evaluate the antioxidant activity of *Callisia fragrans,* no such activity was observed. In contrast, *Aloe vera*, *Bryophyllum pinnatum* and *Pelargonium graveolens* did exhibit antioxidant effects, while *Callisia fragrans* showed a contrasting trend, indicating a possible pro-oxidant property that should be explored in future studies.

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