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UVB PROTECTIVE, ANTI-AGING, AND ANTI-INFLAMMATORY PROPERTIES OF AQUEOUS EXTRACT OF WALNUT (*JUGLANS REGIA* L.) SEEDS

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Abstract: Walnut (*Juglans regia* L., fam. Juglandaceae) fruit is found to be very rich in phenolic compounds and thus to show a wide spectrum of biological activities like antioxidant, anti-inflammatory, or antitumor properties. Ethanol or methanol are preferentially used for the preparation of walnut extracts for cosmetic applications. However, it is commonly known that alcohol causes dehydration and redness of the skin. The aim of this work was to prepare an aqueous extract of undamaged walnut seeds rich in pellicles and to evaluate its antioxidant, anti-apoptotic, anti-inflammatory, and anti-aging properties *in vitro*. Conducted research clearly demonstrated that simple water extraction of undamaged kernels with pellicles allows to obtain rich in phenolic compounds (36-38 mg per g of lyophilisate) walnut extract, which protects fibroblasts and keratinocytes against oxidative stress induced by UVB dose of 35 mJ/cm^2 (5-6-h exposure on a sunny day), protects keratinocytes against UVB-induced apoptotic death, limits the development of the inflammation in epidermis, and also possesses ability to inhibit collagenase and elastase. Thus, obtained aqueous walnut extract (at a relatively low concentration of 5 µg/mL) is found to be a promising compound of sunscreen and anti-aging cosmetic formulations.

Keywords: phenolic compounds; collagenase; UVB radiation; reactive oxygen species; antioxidant; cell culture

Oxygen free radicals or reactive oxygen species (ROSs) are generally produced during various cellular metabolic processes. Pham-Huy et al. (1) summarized that endogenous free radicals are formed due to inflammation, infection, cancer, immune cell activation, mental stress, ischemia, and aging. Production of exogenous ROSs by cells is a result of alcohol consumption, air and water pollution, cigarette smoke, heavy metals poisoning, certain drugs, industrial solvents, smoked meat oil or fat and radiation (1). At low and moderate concentrations, ROSs are known to play a beneficial role in e.g. defense against infectious agents or in cellular signaling system. However, their overproduction results in oxidative stress leading to significant damage of cell structures, including DNA, proteins, lipids, and membranes (2). Illnesses caused by excessive oxidative stress include cancer, autoimmune disorders, cataract, rheumatoid arthritis, cardiovascular and neurodegenerative diseases, and hypertension. Aging is also accelerated by oxidative stress (1, 2).

Therefore, there is an increasing attention paid to both, factors affecting formation of free radicals as well as their role in human health damage and accelerated aging. Sun-emitted ultraviolet (UV) radiation reaching earth surface is composed of 98% lowest-energy UVA wavelengths (320-400 nm), which are not absorbed by stratospheric ozone layer. UVB rays (290-320 nm), lower-energy wavelengths, constitute 2% of UV radiation on earth surface and are mostly absorbed by stratospheric ozone layer (3). UVB rays induce generation of reactive oxygen species by cells and are considered to be the major factor responsible for sunburns leading to photoaging of skin, photocarcinogenesis, and immunosuppression (3-5). Recently observed reduction of stratospheric ozone level may increase the

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risk of UVB-induced harmful effects (6). Although the skin is protected against UV light by melanin, known for its UV-absorbing properties, UVA and UVB radiations may negatively affect several biomolecules present in the cells (7). In consequence, free radicals and other active intermediates are generated, including hydroxyl radicals and singlet oxygen. UVA can reach the dermis and affect physiological functions of fibroblasts (8) whereas UVB rays are mainly absorbed by the epidermis, inducing apoptosis of human keratinocytes, but also reach upper dermis harming fibroblasts (3, 9). Nevertheless, it is commonly known that UVA radiation is dangerous only upon a prolonged skin exposition to this UV range. Thus, as a logical consequence, the concept of antioxidants as a weapon against UVB radiation and free radicals formation emerged in healthcare, cosmetics fabrication, and food preservation technology. Synthetic antioxidants could be used for this purpose. However, they typically block only narrow region of UV spectrum and are potentially cytotoxic. Thus, special attention was attracted by antioxidant substances and extracts derived from natural sources (10, 11). Among these sources, walnut (*Juglans regia* L., fam. Juglandaceae) deserves to be mentioned.

Walnut fruit is reported as one of the richest in phenolic compounds among edible plants. As a consequence, walnut extracts made of leaves, husks, kernels or shells were proved to show a wide spectrum of biological activities, which are beneficial for human health in many aspects. For example, walnut-derived polyphenols exhibit the capacity for removal of free radicals. Some of them reveal even more potent antioxidant activity than L-ascorbic acid (12). Walnut extract containing flavonoids, ellagic acid, and gallic acid was also demonstrated to inhibit oxidation of human plasma and LDL *in vitro* (13). Moreover, juglone, one of the main phenolic compounds in walnut, was reported to show potential antitumor effect via induction of apoptosis of tumor cells (14). It was also suggested that some juglone derivatives may exhibit antitubercular activity (15). Apart from antioxidant activity, walnut extracts reveal also significant antimicrobial, antifungal, and antiviral capacity (16-19). Ethanolic extracts of *J. regia* leaves were demonstrated to exhibit antinociceptive and anti-inflammatory activity (20, 21). Whereas, aqueous extract of defatted seed cake of walnut kernels was found not only to mildly inhibit inflammation, UVB-induced apoptosis, and oxidative stress but also to slightly inhibit collagenase and elastase activities, suggesting its potential utility in cosmetic anti-aging applications (22). To our best knowledge, this is the sole report on anti-collagenase and anti-elastase activity of walnut seed extract.

Evaluation of the composition of methanolic walnut seed extracts revealed that approximately 20-fold more phenolic compounds (phenolic acids, syringaldehyde, juglone) is located in pellicle than in kernel (23). It can be explained by the fact that mentioned phenolic compounds play a significant role in the natural protection of lipid-rich kernel against oxidation (24). Walnut pellicles should be therefore considered as especially promising source of substances potentially active in prevention against UV light-induced skin aging and photodamage. As far, ethanol or methanol were preferentially used as solvents for extraction of phenolic compounds from walnut seeds. However, it is commonly known that alcohol causes dehydration and redness of the skin. Aqueous plant extracts used as a source of active substances are therefore more likely to protect and rejuvenate skin without the side effects mentioned.

Thus, the aim of this work was to prepare an aqueous extract of undamaged walnut seeds rich in pellicles and to evaluate its antioxidant, anti-apoptotic, anti-inflammatory, and anti-aging properties in *in vitro* conditions. Conducted research allowed us to reliably characterize prepared aqueous walnut extract and to assess its potential as a compound of cosmetic compositions intended to be applied to sensitive and hyper reactive skin that needs to be protected against environmental stress, especially from UV radiation.

EXPERIMENTAL

Preparation of the extract

Before the extraction, collected walnuts (*Juglans regia* L.) were dried for 3 days at 37°C and then whole seeds (kernels covered with pellicles) were shelled manually. Walnut extract was obtained via extraction of 40 g of undamaged kernels (with pellicles) with 100 mL deionized water at 25°C, either for 12 h or for 24 h, on the rotator (5 rpm). The resulting extract was filtrated using soft Whatman membrane, centrifuged at 8000 rpm (Sigma 3-16K) for 30 min, freezed and lyophilized (LYO GT2-Basic). Extraction yield was expressed as % of lyophilisate mass compared to the total mass of walnut seeds used for extraction. Conditions for preparation of an aqueous extract of walnut seeds were described in details in Polish patent pending ("Method for preparation of the extract of walnut seeds, composition containing the extract and application of the extract"; P.417505).

Evaluation of protein and phenolic content in the extract

Total protein content in the obtained extract was evaluated using ready-to-use Bio-Rad Bradford Reagent (Bio-Rad). Protein concentration was determined based on a calibration curve prepared using bovine serum albumin (Sigma-Aldrich Chemicals) as a standard. The test was performed according to manufacturer instruction. The Absorbance of the reaction mixture was measured at 595 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Scientific).

The total content of the phenolic compounds in the extract was determined by the test with diazosulfanilamide (known as DASA test). Briefly, 0.5 mL sample (native or subjected to protein removal via precipitation with 5% TCA) was mixed with 0.1 mL 1% sulphanilamide (Merck) in 10% acetic acid (Avantor Performance Materials), 0.1 mL 5% $NaNO_2$ and 1 mL 20% Na_2CO_3 (Avantor Performance Materials). The absorbance of the reaction mixture was immediately measured at 500 nm using Genesys 10S UV-Vis spectrophotometer. Total phenolic content was calculated based on the calibration curve prepared using vanillic acid (Sigma-Aldrich Chemicals) as a standard.

Cell lines and primary culture isolation

Cell culture experiments were conducted with the use of established cell lines purchased from ATCC (American Type Culture Collection): normal human skin fibroblasts (BJ), normal human epidermal keratinocytes transformed with HPV-16 (HEK001) as well as with the use of primary culture of human skin fibroblasts (HSFs). HSFs were isolated from the skin biopsy of healthy female volunteer aged 24-years. The research was conducted in compliance with the tenets of the Declaration of Helsinki for experiments involving human tissues. Written informed consent was obtained from the volunteer from whom the skin biopsy was taken.

The skin biopsy measuring 4 × 4 mm was washed with phosphate buffered saline (PBS, Sigma-Aldrich Chemicals), cut into few pieces using a sterile scalpel, and placed in a well of a 12-well plate. Tissue pieces were cultured in a complete culture medium (renewed every 3 days) until the cells formed a monolayer. Successful isolation of skin fibroblasts was confirmed via fluorescent staining of F-actin and vimentin filaments as described earlier (25). Vimentin filaments serve as a reliable marker of fibroblasts since they are highly expressed in mesenchymal cells. F-actin filaments are present in the cytoskeleton of cells of all origins. Stained HSFs were analyzed under fluorescence laser scanning microscope (Olympus Fluoview IV81 equipped with FV1000 laser scanner). All cells were actin-positive and vimentin-positive what confirms that they were actually fibroblasts. HSFs at passage 2 or 3 were used in the experiments.

Fibroblasts (HSFs and BJ cells) were cultured in EMEM medium (ATCC-LGC Standards) supplemented with 10% foetal bovine serum (FBS, PanBiotech) and antibiotics: penicillin (100 U/mL), streptomycin (100 µg/mL) obtained from Sigma-Aldrich Chemicals. HEK001 cells were cultured in Keratinocyte-Serum Free medium (GIBCO) supplemented with 5 ng/mL recombinant human epidermal growth factor (rhEGF), 2 mM L-glutamine (GIBCO), and antibiotics: penicillin (100 U/mL), streptomycin (100 µg/mL). All types of cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Evaluation of cytotoxicity of walnut extract

Screening cytotoxicity test was conducted using normal human fibroblast cell line (BJ). Lyophilisate of walnut seed extract was dissolved in complete culture medium to prepare a stock solution and then diluted to the following tested concentrations: 2.5, 5, 10, 20, 40, 80, 160, 320, 640, and 1280 µg/mL. Cells were seeded in 96-well plate at a concentration of 1×10^4 cells/well and exposed to the tested extracts or fresh culture medium (untreated control = 100% viability) for 24 h and 48 h. Cell viability was assessed by WST-8 assay using Cell Counting kit-8 purchased from Sigma-Aldrich Chemicals. The test was carried out according to the manufacturer protocol and repeated in 3 separate experiments (n = 3) performed in octuplicate. Viability (%) of cell was expressed as the percentage of OD values obtained with the untreated control cells.

Based on the WST-8 test results, non-toxic concentrations of walnut extract were selected and their non-cytotoxicity was confirmed using the primary culture of fibroblasts (HSFs) and BJ cells via live/dead fluorescent staining. HSFs and BJ fibroblasts were seeded in 96-well plate as described above and exposed to the selected concentrations (2.5 and 5 µg/mL) of walnut extract or fresh culture medium (untreated control = 100% viability) for 24 h. Then, double fluorescent staining was performed using calcein-AM dye (green fluorescence of viable cells) and propidium iodide (red fluorescence of nuclei of dead cells), which were components of Live/Dead Double Staining Kit (Sigma-Aldrich Chemicals). The staining was performed

according to the manufacturer protocol. Stained fibroblasts were observed under fluorescence laser scanning microscope.

Antioxidant activity of walnut extract

Walnut extract activity against oxidative stress induced by UVB radiation was assessed by measurement of reactive oxygen species (ROSs) production by cells using 2',7'-dichlorofluorescein diacetate dye (DCF-DA, Sigma-Aldrich Chemicals). Keratinocytes (HEK001) and fibroblasts (BJ and HSF) were seeded in black, clear bottom 96-well plates at a concentration of 1.5×10^4 cells/well in the case of HEK001 cells or 1×10^4 cells/well in the case of fibroblasts. Before UVB radiation, cells were incubated for 24 h in the media containing previously selected concentrations of walnut extract (2.5 and 5 μg/mL) or in fresh culture media (untreated control = positive control of oxidative stress). Then, the culture media were removed and cells were loaded with 100 μL of 25 μM DCF-DA solution prepared in appropriate culture media for 1 h at 37°C in the dark. Afterward, DCF-DA solution was discarded, the cells were washed with PBS and irradiated in 100 μL of PBS with the UVB dose of 35 mJ/cm^2 as determined using UV Light Meter UV-340 (Lutron Electronic), which enables UVA and UVB measurements in the spectrum from 290 nm to 390 nm. UVB irradiation was delivered using broadband UVB (290-315 nm) lamp (Philips PL-S 9W/12/2P). For comparison to the human fair skin, 35 mJ/cm^2 dose is equivalent to 5-6 h of exposure on the beach on a sunny day (26). The non-irradiated control cells were protected from UVB with aluminum foil (negative control of oxidative stress). After UVB radiation, cells were incubated for 2 h in Hanks' balanced salt solution (HBSS, Sigma-Aldrich Chemicals) supplemented with 10% foetal bovine serum and then the antioxidant activity of walnut extract was assessed by a fluorometric method. Activated by cellular ROSs, green fluorescent DCF was detected using Bio Tek Synergy H4 Hybrid Microplate Reader with the excitation wavelength at 485 nm and emission wavelength at 528 nm (area-scan readings were recorded). The test was repeated in 3 separate experiments ($n = 3$) and performed in quadruplicate. The results were expressed as the percentage of OD values obtained with the non-irradiated cells (negative control of oxidative stress).

Anti-apoptotic activity of walnut extract

Since UVB rays are known to induce apoptosis of main keratinocytes (3, 9), the experiment was conducted using only HEK001 cells. Keratinocytes were seeded in black, clear bottom 96-well plate as described above (Antioxidant activity of walnut extract section). The cells were preincubated for 24 h in the medium containing previously selected concentrations of walnut extract (2.5 and 5 μg/mL) or in fresh culture medium (untreated control = positive control of UVB-induced apoptosis), and then exposed in PBS buffer to UVB dose of 35 mJ/cm^2. The non-irradiated control cells were protected from UVB with aluminum foil (negative control of UVB-induced apoptosis). After UVB radiation, cells were incubated for 2 h in complete culture medium and then anti-apoptotic activity was evaluated using Annexin V-Cy3 Apoptosis Detection Kit (Sigma-Aldrich Chemicals). The assay was performed according to manufacturer protocol. The kit contains red fluorescent dye (annexin V-Cy3) that bounds to apoptotic cells and green fluorescent dye (6-CFDA) that is activated by cellular esterases and reveals fluorescence only in viable cells. This combination allows to differentiate among apoptotic cells (annexin V positive and 6-CFDA positive cells), necrotic cells (annexin V positive and 6-CFDA negative cells), and viable cells (annexin V negative and 6-CFDA positive cells). Stained cells were observed under fluorescence laser scanning microscope. The test was performed in quadruplicate (in 4 wells). For each well, images were taken from 4 randomly selected fields of view giving a total of 16 images per tested variant. Cells undergoing apoptosis (annexin V positive, 6-CFDA positive) were counted out of total at least 1500 cells.

Anti-inflammatory activity of walnut extract

Keratinocytes were seeded in 96-well plate at a concentration of 1.5×10^4 cells/well and stimulated with the mixture of 10 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* and 1 μg/mL human INF-γ (Sigma-Aldrich Chemicals) for 24 h in the absence of extract (untreated control = positive control of inflammatory reaction) and in the presence of selected concentrations of walnut extract (2.5 and 5 μg/mL). Unstimulated cells maintained in fresh culture medium served as a negative control of inflammation. Proinflammatory cytokine production (IL-6 and TNF-α) was determined using human-specific ELISA tests (Sigma-Aldrich Chemicals). The tests were performed in quadruplicate according to the manufacturer protocol.

Evaluation of inhibition of collagenase and elastase activity

Effect of walnut seed extract (obtained upon 12- and 24 h extraction) on bacterial collagenase

activity was performed using *Clostridium histolyticum* collagenase (Sigma-Aldrich Chemicals). FALGPA (Sigma-Aldrich Chemicals) dissolved in 50 mM Tricine buffer of pH 7.5 with 400 mM NaCl (Sigma-Aldrich Chemicals) and 10 mM $CaCl_2$ (Avantor Performance Materials) was used as a substrate. Prior to the measurement, walnut extract (10 µL) was preincubated with 0.2 mg/mL enzyme solution (50 µL) for 10 min. at 37°C. Then, the reaction was set up by the addition of the 0.8 mg/mL substrate (40 µL). The enzyme activity was measured in kinetic mode for 10 min. at 345 nm using Bio Tek Synergy H4 Hybrid Microplate Reader. The following final concentrations of walnut extract were tested: 2.5 and 5 µg/mL. Mixtures of enzyme and substrate solutions without walnut extract served as control (100% initial activity).

Effect of walnut seed extract (obtained upon 24 h extraction) on the activity of human enzymes was performed for recombinant human MMP-1 collagenase (901-MP) and recombinant human MMP-12 elastase (917-MP) (R&D Systems). For both enzymes, the reaction was performed according to the procedure suggested by the manufacturer using MCA-Lys-Pro-Leu-Gly-Leu-DPA-Ala-Arg-NH_2 peptide (R&D Systems) as a substrate. Before measurement, MMP-1 collagenase was activated by 1 mM *p*-aminophenylmercuric acetate (Sigma-Aldrich Chemicals) in assay buffer of pH 7.5 (50 mM Tris, 10 mM $CaCl_2$, 0.05% Brij-35 from Sigma-

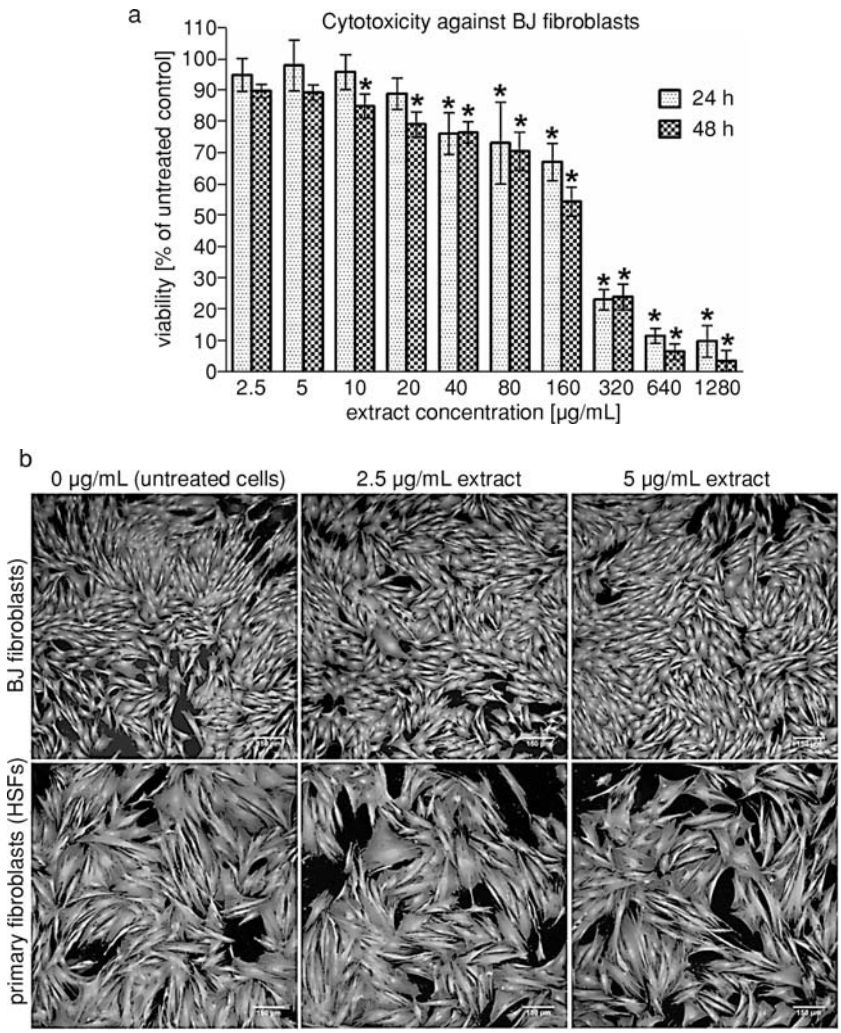

Figure 1. Cytotoxicity of obtained walnut extract: (a) screening cytotoxicity evaluation with the use of WST-8 assay and BJ fibroblasts; *significantly different results ($p < 0.05$) compared to untreated control cells (100% viability) according to the unpaired t-test; (b) confirmation of the lack of cytotoxicity of the selected concentrations of walnut extract by live/dead fluorescent staining of extract-treated BJ cells and HSFs; viable fibroblasts reveal fluorescence of cytoplasm (cell morphology may be seen) whereas dead cells show fluorescence of only nuclei

Aldrich Chemicals, 150 mM NaCl from Avantor Performance Materials) for 2 h at 37°C and then diluted to 1 ng/mL in assay buffer. MMP-12 elastase was activated by incubation in assay buffer for 30 h at 37°C and then diluted to 0.4 ng/mL using assay buffer. Prior to the measurement, walnut extract (10 µL) was preincubated with diluted enzyme solutions (40 µL) for 10 min. at 37°C. Then, the reaction was set up by the addition of the 20 µM substrate prepared in assay buffer (50 µL). The enzyme activity was measured in kinetic mode for 5 min. with the excitation and emission wavelengths at 320 nm and 405 nm, respectively, using Bio Tek Synergy H4 Hybrid Microplate Reader. The following final concentrations of walnut extract were tested: 2.5, 5, 10, 25, 50, 100, 200, 500 and 1000 µg/mL. Mixtures of enzyme and substrate solutions without walnut extract served as control (100% initial activity).

Statistical analysis

Obtained results were analyzed using GraphPad Prism 5, Version 5.03 Software. The unpaired t-test was applied to assess statistical differences ($p < 0.05$) between two groups (tested samples versus control).

RESULTS

Preparation of the extract

Extraction of walnut seeds rich in pellicles using water as an extraction medium was found to be extraction time-related. The mass of lyophilized extract obtained upon 12 h and 24 h extraction was equal to 2.15% and 2.66% of initial material mass, respectively. Analysis of total protein and phenolic content revealed that resultant extract was rich in phenolic compounds. Walnut seed extract obtained after 12 h of aqueous extraction contained: 200.9 ± 5.2 mg protein and 38.4 ± 0.2 mg phenolic compounds per g of lyophilized extract. Whereas extract obtained after 24 h of aqueous extraction contained: 174.5 ± 10.3 mg protein and 36 ± 1.7 mg phenolic compounds per g of lyophilized extract. No differences in the content of phenolic compounds between deproteinized and native extracts were found, indicating that proteins occurring in the extract did not show the ability to strongly bind to phenolic compounds.

Evaluation of cytotoxicity of walnut extract

WST-8 assay showed that upon 24 h exposure to walnut extract, BJ fibroblasts maintained high viability (above 95%) compared to the control untreated cells only in the case of the following extract concentrations: 2.5, 5, and 10 µg/mL (Fig. 1a). However, prolonged incubation (48 h) with walnut extract revealed that extract concentration of 10 µg/mL significantly ($p = 0.0419$) reduced cell viability to 85%. Extract concentrations in the range from 10 to 80 µg/ml showed a slight cytotoxic effect against human fibroblasts, whereas extract concentrations above 160 µg/mL significantly reduced cell viability exhibiting high cytotoxic effect (Fig. 1a). Thus, only extract concentrations of 2.5 and 5 µg/mL were selected for further tests as non-cytotoxic.

Live/dead fluorescent staining confirmed lack of the cytotoxicity of the selected concentrations (2.5 and 5 µg/mL) of walnut extract. Upon 24 h exposure to 2.5 and 5 µg/mL walnut extract, no dead cells (fluorescence of only cell nuclei) were

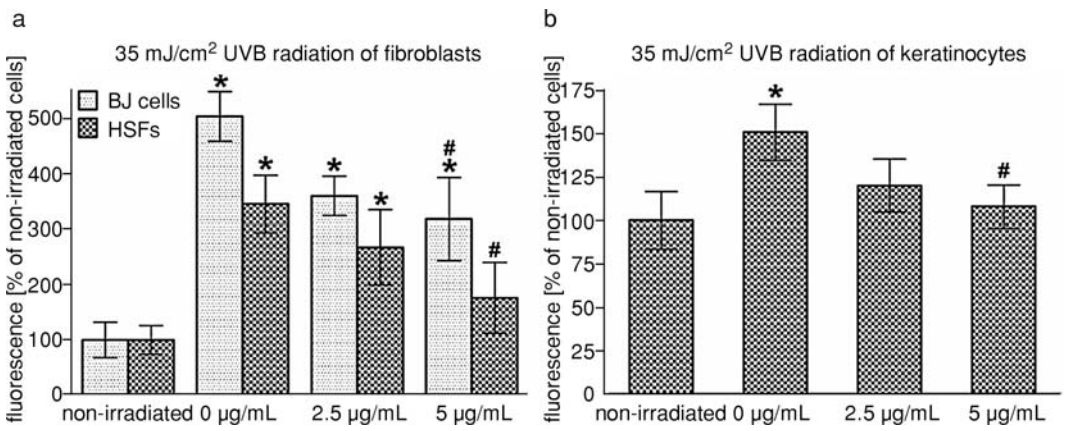

Figure 2. Antioxidant activity of walnut extract: (a) ROS production by fibroblasts (BJ cells and HSFs) and (b) keratinocytes (HEK001 cells) preincubated with walnut extract and exposed to UVB dose of 35mJ/cm² (5-6 h on a sunny day); *significantly different results ($p < 0.05$) compared to non-irradiated cells (negative control of oxidative stress), #significantly different results ($p < 0.05$) compared to untreated (0 µg/mL) cells (positive control of oxidative stress), according to the unpaired t-test

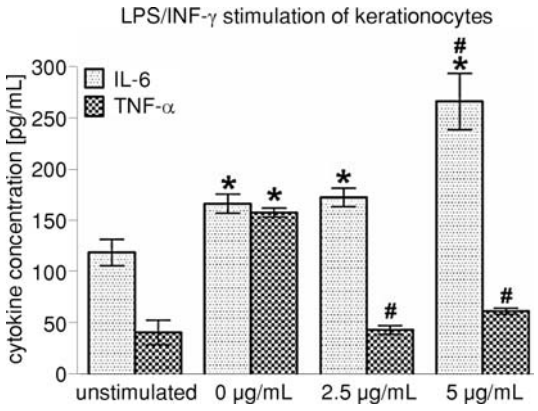

Figure 3. Anti-inflammatory activity of walnut extract: proinflammatory cytokine production by keratinocytes (HEK001 cells) cultured in the presence of the extract and stimulated with the mixture of LPS/ INF-γ; *significantly different results ($p < 0.05$) compared to unstimulated cells (negative control of inflammation), #significantly different results ($p < 0.05$) compared to untreated (0 μg/mL) cells (positive control of inflammation), according to the unpaired t-test

observed in the culture of both BJ cells and HSFs (Fig. 1b). Moreover, extract-treated cells showed unchanged, normal, typical morphology, which was the same like morphology of untreated control cells.

Antioxidant activity of walnut extract

Fibroblasts (BJ cells, HSFs) and keratinocytes preincubated for 24 h with tested extracts and exposed to the UVB dose of 35 mJ/cm^2 revealed statistically significant ($p < 0.05$) decrease in ROS production compared to the untreated control cells (positive control of oxidative stress) in a dose-dependent manner (Fig. 2). Although only slight differences in antioxidant activity between 2.5 μg/mL and 5 μg/ml concentrations of the extract were observed, statistically significant results were obtained only for 5 μg/mL extract. BJ fibroblasts preincubated with 5 μg/ml walnut extract showed approx. 1.5-fold lower ROS generation ($p = 0.0416$) compared to the positive control, whereas HSFs preincubated with 5 μg/mL extract revealed almost 2-fold lower ROS production ($p = 0.0463$) compared to the irradiated extract-untreated cells (Fig. 2a). Importantly, HEK001 keratinocytes preincubated with 5 μg/mL extract produced similar amount of ROSs to that generated by non-irradiated cells (and significantly lower, $p = 0.022$, compared to positive control of oxidative stress) indicating that walnut extract at concentration of 5 μg/mL provides nearly 100% protection of epidermis against UVB-induced oxidative stress (Fig. 2b).

Anti-apoptotic activity of walnut extract

It was demonstrated that walnut extract at a concentration of 2.5 μg/mL did not reveal protective effect against UVB-induced apoptosis of keratinocytes (Table 1). Nevertheless, amount of apoptotic cells in HEK001 culture preincubated with 5 μg/mL extract and exposed to the UVB dose of 35 mJ/cm^2 was only slightly higher than the amount of apoptotic cells in non-irradiated culture (negative control of UVB-induced apoptosis). Thus, it was confirmed that walnut extract at a concentration of 5 μg/mL provides high protection of epidermis against UVB radiation.

Table 1. Anti-apoptotic activity of walnut extract expressed as a percentage of apoptotic keratinocytes upon exposure to UVB dose of 35 mJ/cm^2 (5-6 h on a sunny day).

Type of the sample	% of apoptotic cells
Non-irradiated cells	5.1
UVB 0 μg/mL	15.9
UVB 2.5 μg/mL	14.5
UVB 5 μg/mL	7.8

Anti-inflammatory activity of walnut extract

Results obtained with ELISA tests demonstrated the walnut extract increased IL-6 production by human keratinocytes compared to the untreated cells stimulated with LPS/INF (positive control of inflammation) in a dose-dependent manner (Fig. 3). However, statistically significant result ($p = 0.0394$) was obtained only for 5 μg/mL extract. Interestingly, at the same time walnut extract significantly ($p = 0.0013$ for 2.5 μg/mL extract and $p = 0.0014$ for 5 μg/mL extract) reduced TNF-α production compared to the positive control of inflammation. In the case of HEK001 cells preincubated with 2.5 μg/mL extract, amount of released TNF-α (44 pg/mL) was close to the amount produced by unstimulated cells (42 pg/mL), which served as negative control of inflammation.

Inhibition of collagenase and elastase activity

Non-toxic concentrations of the extract (2 and 5 μg/mL) exhibited a very strong inhibiting effect on *Clostridium histolyticum* collagenase. Extract obtained after 12 h of aqueous extraction inhibited collagenase by 96% ($p < 0.0001$) and 89% ($p < 0.0001$) at concentrations of 5 μg/mL and 2.5 μg/mL, respectively (Fig. 4a). Whereas extract obtained after 24 h of aqueous extraction inhibited collagenase by 79% ($p < 0.0001$) and 60% ($p < 0.0001$) at concentrations of 5 μg/mL and 2.5 μg/mL, respectively. Thus, although walnut mixture prepared after 24 h of the extraction was obtained with a slightly higher yield, the one obtained after 12 h of the extraction was found to be a stronger collagenase inhibitor.

Efficacy of walnut seed extract as a skin-protective and anti-aging agent was confirmed using recombinant human collagenase (MMP-1) and elastase (MMP-12). The results were expected to provide the useful information on the extract application as a compound of anti-aging cosmetic formulas, on an industrial scale. For that reason, lyophilisate obtained after 24 h of aqueous extraction was selected because of the higher extraction yield. It was found that both human collagenase and elastase activities were inhibited by the extract in a dose-dependent manner (Fig. 4b). Selected during cell culture experiments non-toxic concentrations of the extract: 5 µg/mL and 2.5 µg/mL inhibited MMP-1 by 35% ($p < 0.0001$) and 25% ($p < 0.0001$), respectively. Whereas, total inhibition of collagenase was observed at a concentration of the extract equal to 500 µg/mL. Enzyme-inhibiting effect of the extract on human elastase was higher than on collagenase. Extract inhibited elastase by 50% ($p < 0.0001$) and 45% ($p < 0.0001$) at concentrations of 5 µg/mL and 2.5 µg/mL, respectively. Total inhibition of elastase was recorded for 100 µg/mL extract (Fig. 4b).

DISCUSSION

The yield process for the preparation of active natural extracts for practical applications should be reasonably high to ensure cost-effectiveness. Yield preparation of walnut extract, ranging between 2.15% and 2.66% of initial material mass, seems not to be particularly high. However, it should be noted that water is not likely to extract fatty materials. In our method, whole undamaged seeds with almost untouched pellicles were used as raw plant material. Thus, greasy kernels probably did not undergo extraction at all. Our intention was to extract mainly pellicles, which are rich in phenolic compounds, and we used whole seeds to make the process easier. Walnut pellicles were estimated to cover only 5% of the seed weight (23). Thus, approximately 43-50% of total pellicle mass was extracted in our experiments. It is worth to emphasize that in our pilot studies, separated pellicles were extracted and the resultant extract revealed similar activity to extract prepared from the whole seed. Thus, taking into account the potential application of the extract on an industrial scale, whole seeds were extracted to skip the time-consuming step of separation of the pellicles.

Phenolic compounds, which are present in walnut (*Juglans regia* L.), are known to possess relevant antioxidant activity. In this study, it was demonstrated that water extraction of whole seeds with pellicles allows to obtaining an aqueous walnut extract, which is rich in phenolic compounds (36-38 mg per g of lyophilized extract). Considering the values of walnut seed extraction yields (2.15% for 12 h process and 2.66% for 24 h process) and average pellicle content in whole walnut seed (5%), the content of phenolic compounds in walnut pellicles was determined to be 1655 mg and 1917 mg per 100 g of pellicles upon 12 h and 24 h extraction, respectively. These results are in good agreement with data obtained by Colaric et al. (23) who demonstrated that phenolic compounds content in walnut pellicles was in the range from 1000 to 2500 mg per 100 g of pellicles dependent on the cultivars of *Juglans regia* L. It is worth emphasizing that in our study we used water for extraction of undamaged kernels with pellicles and we obtained similar phenolic content like Colaric et al. who applied methanol for pellicles extraction. Due to high phenolic content, resultant aqueous walnut extract revealed significant antioxidant activity and ability to protect the cells from UVB-induced oxidative stress and apoptotic death (Fig. 2, Table 1). Importantly, walnut extract showeda higher protective effect against UVB-induced damage of keratinocytes than fibroblasts. Since UVB rays are mainly absorbed by the epidermis, which is composed in 95% of keratinocytes (3), it may be concluded that obtained extract appears to be a very promising compound of sunscreen formulas to protect epidermis against UVB-induced photodamage and photoaging.

According to available literature, antioxidant potential of walnut extract highly depends on the solvent used for extract preparation. Anjum et al. (27) demonstrated that grounded walnut (*Juglans regia* L.) extracted with the acetone revealed significantly higher antioxidant activity than methanol- and water-extracted samples. Whereas, Yang et al. (28) reported that solvent used for the extraction of walnut (*Juglans regia* L.) shells had a significant effect on total flavonoids content and antioxidant activity of the resultant extract. Among all tested extracts (water, methanol, ethanol, chloroform, n-butanol, ethyl acetate), aqueous extract revealed the lowest antioxidant activity and the methanolic extract showed the greatest total antioxidant activity. Similarly, Zhang et al. (29) determined that ethyl acetate and n-butanol extracts from walnut kernel showed higher free radical-scavenging activity than water or petroleum ether fractions. Although most researchers reported low antioxidant activity of aqueous walnut extract, prepared in this study extract of whole seeds with pellicles showed relatively high ability to protect the cells against oxidative stress. Moreover, the economic benefit of this

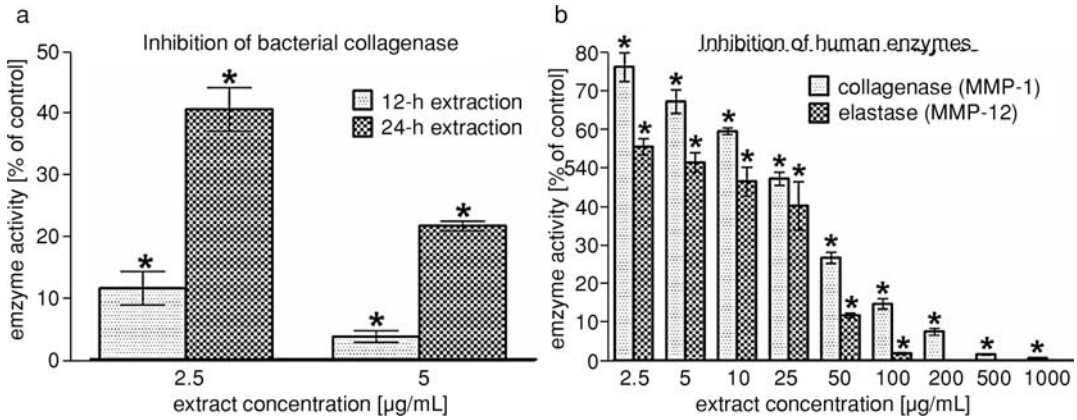

Figure 4. Anti-aging activity of walnut extract: (a) inhibition of *Clostridium histolyticum* collagenase activity by the walnut seed extract obtained upon 12- and 24 h extraction; (b) inhibition of MMP-1 (human collagenase) and MMP-12 (human elastase) by walnut seed extract obtained upon 24 h extraction; *significantly different results ($p < 0.05$) compared to the control (100% initial activity) according to the unpaired t-test

extraction method should be taken into account – after the extraction, walnut seeds can be dried and used as a raw material for walnut oil pressing.

Prolonged exposure to UV light may cause sunburns and UVB-induced inflammatory reaction. In order to evaluate whether obtained walnut extract possesses the ability to limit the development of the inflammatory reaction, keratinocytes were cultured in the presence of the extract and stimulated with the mixture of LPS and INF-γ to induce proinflammatory cytokine production. It was demonstrated that walnut extract caused an increase in IL-6 production and in the same time decrease in TNF-α release by the cells (Fig. 3). Although IL-6 is known to be proinflammatory agent, this cytokine was also proved to play crucial anti-inflammatory role in local and systemic acute inflammatory responses by controlling the level of proinflammatory cytokines like IL-1 and TNF-α (30, 31). Thus, it may be suggested that walnut extract slightly increased IL-6 production by keratinocytes, which acted as an anti-inflammatory cytokine and caused inhibition of TNF-α release.

In the cosmetic industry, natural extracts obtained from various plants are widely used as anti-aging compounds in a multitude of formulations. Anti-collagenase and anti-elastase activity of obtained walnut extract were found to be much higher (Fig. 4) than the one recorded for the aqueous extract of walnut (*Juglans regia* L.) seed cake described in US patent. Mentioned walnut seed cake extract inhibited collagenase only by 6.4-17.2% when used at high concentrations (0.5-10 mg/mL) (22). These concentrations were much higher than active concentrations (2.5 and 5 µg/mL) of walnut extract described in this work. Importantly, among 150 methanolic extracts from medicinal plants, which were tested at 100 µg/mL concentration, only one (from the seeds of *Areca catechu*) exhibited over 50% inhibiting activity against elastase (32). Whereas aqueous white tea extract was reported to exhibit almost 90% collagenase- and elastase-inhibiting activity, but at concentration of 25 µg/mL (33) which is 5-fold higher than active concentration of obtained in this study walnut extract. In the same report, extracts from green tea, lavender, milk thistle, and witch hazel were demonstrated to inhibit collagenase by 15-30% and elastase by 0-50%, but also at relatively high concentration of 25 µg/mL. This observation confirms that obtained in this study extract of undamaged walnut seeds is very promising potential anti-wrinkle agent, which may be used in anti-aging cosmetic formulations.

CONCLUSION

Presented research demonstrated that simple water extraction of undamaged kernels with pellicles allows to obtaining rich in phenolic compounds walnut extract, which reveals significant antioxidant, anti-apoptotic, anti-inflammatory, and anti-aging properties. Based on obtained results it may be concluded that the extract of 5 µg/mL shows very promising potential as a compound of cosmetic compositions intended to be applied to sensitive skin requiring protection against environmental stress like UVB radiation or to mature type skin requiring anti-aging treatment. The extract of 5 µg/mL was proved to protect cells in the dermis and epidermis against oxidative stress induced by UVB dose of

35 mJ/cm^2 (5-6-h exposure on a sunny day), to protect cells in the epidermis against UVB-induced apoptotic death, to limit the development of the inflammation in epidermis via inhibition of TNF-α release, and to possess anti-aging properties via inhibition of collagenase and elastase. Thus, walnut extract at a relatively low concentration of 5 μg/mL is a promising compound of sunscreen and anti-aging cosmetic formulations.

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