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Moringa Oleifera L. Extracts as Bioactive Ingredients That Increasing Safety of Body Wash Cosmetics

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Abstract

Extracts obtained from leaves of Moringa tree (*Moringa oleifera*) are a rich source of many bioactive compounds: flavonoids, phenolic acids or carotenoids. It also contains such components as, vitamins (A, C, niacin, pantothenic acid), alkaloids, tannins or saponins. Extracts and plant substances derived from the leaves of *Moringa oleifera* L. have a strong antioxidant, toning and anti-inflammatory effect.

The work attempts to obtain a multifunctional plant extract derived from Moringa tree leaves. Obtained extracts was analyzed for their biochemical and physicochemical properties. The obtained results indicate on a strong antioxidative potential of the tested extracts. The further step was an attempt to apply the extracts in the model body wash cosmetic. The biological activity of extracts and model cosmetic formulation were assayed by *in vitro* analysis on two human cell lines: keratinocytes (HaCat,) and fibroblasts (BJ). The results showed that the tested extracts may affect on increasing of cell proliferation and reduce oxidative stress in cells. The addition of the tested extracts to the model cosmetic formulation, were contributed to the reduction of their ability to irritate the skin and improve the safety of use of the product.

Key words: *Moringa oleifera* L., antioxidant activity, cell culture, irritant potential

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Introduction

In recent years, the cosmetic industry is one of the fastest growing industries in the world. Strong competition on the cosmetic market and high consumer expectations are forcing manufacturers to look for innovative solutions in every aspect of the product life cycle. Until recently, cosmetic manufacturers created the innovative advantage of their products by incorporating new raw materials and ingredients which are less common and not used by the competition. Examples include substances such as hyaluronic acid, peptides, polysaccharides, exotic oils and plant extracts, and snail slime. With time, however, these raw materials are used by a growing number of cosmetic manufacturers, so the advantage of innovation is very quickly lost and formulators must seek new solutions. The innovativeness of cosmetics can also be generated through the form in which skin care and beauty products are offered. Increasingly, novel forms such as foams, jellies, creams or essences, are commercially available. However, after the launch of cosmetics in innovative forms the market tends to be almost flooded by products of the same type offered by various manufacturers. Consequently, the innovative advantage of such solutions is short-lived [1-5].

In the last few years, there has been a new trend on the cosmetic market, involving the formulation of innovative products on the basis of multifunctional ingredients. Substances of this type are characterized by multidirectional activity, combining biologically active properties with moisturizing effects and the ability to give cosmetics an appropriate form or improve their safety to people and the environment. The last of the properties enumerated above is particularly sought after by present-day consumers. The strong trend for “naturalness” in cosmetics has contributed to an increase in consumer awareness with regard to substances used in cosmetic production. Consumers look for products which – in addition to delivering the desired usually multifaceted activity – are safe to people and the environment, and are able to reduce adverse environmental impacts on the skin (anti-smog, anti-pollution cosmetics). Examples of such ingredients include plant extracts, which, as demonstrated by a number of studies, can be used as multifunctional cosmetic raw materials with moisturizing, soothing, anti-wrinkle and antioxidant properties, and minimize the adverse skin effects of other cosmetic product ingredients. The above characteristics are due to the complex chemical composition of plant extracts, which represent solutions of active substances derived from plants in a suitable solvent [6-13].

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In the course of research in this area, much attention has been focused on the Moringa tree (*Moringa oleifera* L.), also called the tree of life, as a source of active ingredients valuable for the cosmetic industry. Owing to the presence of a broad spectrum of bioactive compounds, the plant has powerful antioxidant, antibacterial, toning, astringent and anti-inflammatory properties [14-19]. Leaves of the Moringa tree have been found to contain flavonoids including myricetin, quercetin, kaempferol, isorhamnetin or rutin, as well as phenolic acids. Fresh leaves are, at the same time, a good source of carotenoids such as lutein, β-carotene and zeaxanthin. In addition, the Moringa tree is characterized by a high content of vitamins C and A. The active substances contained in the plant have been shown to have beneficial effects on human skin and successfully replace synthetic ingredients [20-23].

The present study was an attempt to evaluate the effect of *Moringa oleifera* leves extracts on the irritant potential of body wash gels. For the purpose of the study, a technology for obtaining extracts in the process of solvent extraction was developed. Water and mixtures of water with glycerin were used as natural extraction solvents. The extracts obtained this way were used for further analysis to determine their basic biochemical properties: the ability to neutralize free radicals, and the content of polyphenols and flavonoids. Analyzes of cytotoxicity as well as the intracellular level of reactive oxygen species were performed on *in vitro* model: keratinocytes (HaCaT,) and fibroblasts (BJ) cell lines. In addition, the model preparations with applied extracts also was analyzed on cell lines.

Material and Methods

Extract derivation method

Moringa tree leaves (*Moringa oleifera* L.) were dried with warm air at 40° C for two days. The samples obtained were later used for extraction method. Extraction of bioactive compounds from dried leaves was carried out using ultrasound-assisted extraction method. 5 g of grounded plant material and 100 mL of water with glycerine in various proportions (50/50, 60/40, 80/20) were used to obtain extract. Extraction was carried out for 10 cycles of 10 min at room temperature. Then, obtained extracts were collected and filtered through Whatman filter paper No. 10. The material was stored in the dark at 4 ° C for further analysis.

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Total Phenolic Content Determination

The total phenolic content of leaves *Moringa oleifera* L. extracts were determined spectrophotometrically by the Folin-Ciocalteu method according to the procedure reported by Singleton et al with some modifications [24]. The 300 µL of leaves extract solutions and 1500 µL of 1:10 Folin-Ciocalteau reagent were mixed and after 6 minutes in the dark, 1200 µL of sodium carbonate (7.5%) was added. After 2 h of incubation in the dark at room temperature, the absorbance at 740 nm was measured spectrophotometrically by Aquamate Helion (Thermo Scientific). The total phenolic concentration was calculated from a gallic acid (GA) calibration curve (10-100 $mg \cdot L^{-1}$). Data were expressed as gallic acid equivalents $(GA) \cdot g^{-1}$ of extract averaged from three measurements.

Total Flavonoids Content Determination

The total flavonoid content of plant extracts were evaluated using aluminium nitrate nonahydrate according to the procedure reported by Woisky and Salatino with modifications [25]. The 600 µL of plant extracts solutions and 2400 µL of mixture (80% C_2H_5OH, 10% $Al(NO_3)_3 \times 9\ H_2O$ and 1M $C_2H_3KO_2$) were mixed. After 40 min of incubation at room temperature, the absorbance at 415 nm was measured spectrophotometrically by FilterMax F5 (Aquamate Helion). The total flavonoids concentration in extracts were calculated from a quercetin hydrate (Qu) calibration curve (10-100 $mg \cdot mL^{-1}$) and expressed as quercetin equivalents $(Qu) \cdot g^{-1}$ of extract averaged from three independent measurement

DPPH Radical Scavenging Assay

Antioxidant activity of plant extract was analysed using DPPH free radical scavenging assay, according to the method described by [26]. 167 µL of 4mM ethanol solution of DPPH was mixed with 33 µL analysed samples in different concentrations (250 $\mu g\ ml^{-1}$ – 5000 $\mu g\ ml^{-1}$). The absorbance was measured at λ=516 nm in every 5 minutes for 30 minutes using UV-Vis spectrophotometer Filter Max 5 (Thermo Scientific). DPPH solution mixed with equal volume of distilled water was served as a control. The percentage of the DPPH radical scavenging were calculated using the equation:

$$\%DPPH\bullet\ scavenging = [Abs_{control} - Abs_{sample}]/Abs_{control} \times 100\%$$

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Cell culture

HaCaT (ATCC®, normal human keratinocytes and BJ fibroblasts (ATCC® CRL-2522™) was obtained from the American Type Culture Collection (Manassas, VA 20108, USA). HaCaT cells were maintained in a DMEM (Dulbecco's modified essential medium, Gibco) with L-glutamine, supplemented with 5% (vol/vol) FBS (fetal bovine serum, Gibco), and 1% (vol/vol) antibiotic (100 U·mL^{-1} Penicillin and 1000 µg·mL^{-1} Streptomycin, Gibco). Fibroblast were maintained in a MEM (Minimum Essential Medim, Gibco) contains Earle's salt and L-glutamine, supplemented with 5% (vol/vol) FBS (fetal bovine serum, Gibco), and 1% (vol/vol) antibiotic (100 U mL $^{-1}$ Penicillin and 1000 µg mL $^{-1}$ Streptomycin, Gibco). All cultured cells were kept at 37°C in a humidified atmosphere of 95% air and 5% of carbon dioxide (CO_2). When the cells reached confluence, the culture medium was removed from the flask (VWR) and cells were rinsed two times with sterile PBS (Phosphate-Buffered Saline, Gibco). The confluent layer was trypsinised using Trypsin/EDTA (Gibco) and then resuspended in fresh medium.

Cell Viability Assay

The resazurin sodium salt reduction assay, was used to assess cell viability. The assay was performed according to Ivanov et al. with some modifications [27]. Cells were placed in 96-well plates at a density of 1×10^4 cells/well with fresh medium. After 24 h of pre-culture, medium was aspirated and varying concentrations (10, 5, 3, 1%) of tested extracts were added into each well and cultured for another 24 h. The control group were non-treated cells. After time of exposure, resazurin salt solution (Sigma, R7017) was transferred into the plates for a final volume 250 µL/well and concentration of 60 µM in medium and incubated for 3 h at 37°C in darkness. The absorbance was measured at the wavelength λ=570 nm using a microplate reader FilterMax F5 (Thermo Fisher Scientific). The experiments were performed in triplicates for each extract concentration and presented as percentage of control values.

To evaluate if model cosmetic formulation (1% SCS) containing various concentrations (10, 5, 3, 1%) of *Moringa oleifera* leaves extract can affect on cells viability, the resazurin sodium salt reduction assay was used. BJ fibroblasts and keratinocytes were seeded in transparent 96- well plates at a density of 1×10^4 cells/well with fresh medium. After 24 h of pre-incubation, cells were exposed to 30 min of model cosmetic with different concentrations of Moringa tree leaves extract. The non-treated cells were a control group. After time of exposure, resazurin salt solution (Sigma, R7017) was transferred into the wells for a final volume 250 µL/well and

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concentration of 60 µM in medium and incubated for 3 h at 37°C in darkness. The absorbance was measured at the wavelength λ=570 nm using a microplate reader (FilterMax F5, Molecular Devices). The experiments were performed in triplicates for each tested substance concentration and presented as percentage of control values.

Measurement of DCF fluorescence

To measure the intracellular level of reactive oxygen species in HaCaT and fibroblasts cells, the fluorogenic dye H_2DCFDA was used. After passively diffusion into the cells, H_2DCFDA was deacetylated by intracellular esterases into the non-fluorescent compound, that upon oxidation by ROS is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF) [28]. The fluorescence was measured according to protocol previously described by [29]. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well and initially cultured before the experiment for 24 h. After this, culture medium was changed on 10 µM H_2DCFDA (Sigma) in serum-free medium (DMEM or MEM for HaCaT and fibroblasts respectively). Cells were incubated at H_2DCFDA for 45 min before treatment. After this time, an intracellular oxidative stress as well as production of ROS was induced by addition hydrogen peroxide (H_2O_2) to the cells at a final concentration of 1mM in PBS for 1h. Then medium were changed and cells were exposed into different *M. oleifera* leaves extract concentrations (10, 5, 3, 1%). The unexposed cells were control group and cells treated with 1mM hydrogen peroxide (H_2O_2) was used as a positive control. The DCF fluorescence was measured after 90 min of incubation, using a microplate reader FilterMax F5 (Thermo Fisher Scientific) at maximum excitation of 485nm and emission spectra of 530 nm.

To assay the capacity of model cosmetic formulation (1% SCS) containing various concentrations (5, 3, 1%) of *M. oleifera* leaves extract to generate intracellular level of reactive oxygen species, fluorogenic dye H2DCFDA also was used. HaCaT and BJ fibroblast were seeded in 96-well plates at a density of 1×10^4 cells per well and initially cultured for 24h. After this, culture medium was changed on 10 µM H_2DCFDA (Sigma) in serum-free medium (DMEM or MEM for HaCaT and fibroblasts respectively). Cells were incubated in H_2DCFDA for 45 min before treatment. After this, medium were changed and cells were exposed into 1% SCS with different extract concentrations. The control group were untreated cells and cells treated with 1% of Sodium Coco Sulfate (SCS) was used as a positive control. The DCF fluorescence was measured every 30 min for a total 90 min using a microplate reader FilterMax F5 (Thermo Fisher Scientific) at maximum excitation of 485nm and emission spectra of 530 nm.

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Zein test

Irritant potential of the products was measured using zein test. To 40 mL of the samples solution (10 wt%) was added 2 ± 0.05 g of zein from corn. The solutions with zein was shaken on a shaker with water bath (60 min at 35°C). The solutions were filtered on Whatman No. 1 filters and then centrifuged at 6720 g for 10 min. The nitrogen content in the solutions was determined by Kjeldahl method. 1 mL of the filtrate was mineralized in sulfuric acid (98%) containing copper sulphate penthahydrate and potassium sulphate. After mineralization the solution was transferred (with 50 mL of MiliQ water) into the flask of the Wagner–Parnas apparatus. 20 mL of sodium hydroxide (25 wt%) was added. The released ammonia was distilled with steam. Ammonia was bound by sulfuric acid (5 mL of 0.1 N H_2SO_4) in the receiver of the Wagner–Parnas apparatus. The unbound sulfuric acid was titrated with 0.1 N sodium hydroxide. Tashiro solution was used as an indicator. The zein number (ZN) was calculated from the equation:

$$ZN = (10 - V1) \times 100 \times 0.7(^{mgN}/_{100mL}),$$

where, V1 is the volume (cm^3) of sodium hydroxide used for titration of the sample. The final result was the arithmetic mean of five independent measurements.

Statistical Analysis

Each value is the mean of three replicates. Values of different parameters were expressed as the mean ± standard deviation (SD). The two-way analysis of variance (ANNOVA) and Bonferroni posttest between groups were performed at the level *P* value of <0.05 to evaluate the significance of differences between values. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., Sand Diego CA).

Results and Discussion

The major group of phytochemicals contributing to the antioxidant capacity of plant material include polyphenols, carotenoids and vitamins such as vitamin C and E. There is a several previous study, which shows that *M. oleifera* leaves are rich source of bioactive compounds with antioxidant properties. It has been reported that Moringa tree leaves have sufficient amounts of quercetin, kaempferol, β-carotene, α-tocopherol and Vitamin A [30-31].

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With regard to this findings, the present investigation was undertaken to evaluate the antioxidant capacity of aqueous/glycerin extracts of *M. oleifera* leaves. The biological activity of these extracts was also determined on *in vitro* cell line model. Afterwards, an attempt was made to develop the technology of a base cosmetic, which then was tested for its toxicity to *in vitro* cell model. The cytotoxicity of tested substances was assessed on BJ fibroblast and normal human keratinocytes (HaCaT) cell lines.

To confirm previous result, in this work an attempt was made to determine total phenolic content (TPC) and total flavonoid content (TFC). The amounts of these compounds were assayed from the calibration curves of gallic acid (y = 0.0046x+0.0452, R^2 = 0.9989), and quercetin (y = 0.0153x-0.0053, R^2=0,9996), respectively. The analysis were performed for three different dilutions (50%, 25% and 12,5%) of each extract. Obtained results shows, that the highest amount of phenols and flavonoids was characterised by 50:50 (vol/vol) aqueous/glycerin extract, while the lowest concentration of these compounds showed 80:20 (vol/vol) aqueous/glycerin extract (Figure 1). The difference between the highest and the lowest TPC value for different types of 50% extract dilution it was about 24%. Considering the TFC value, it was nearly 37%. It also was observed, that the TPC and TFC were increasing in a dose-dependent manner for all tested types of *Moringa oleifera* leaves extracts.

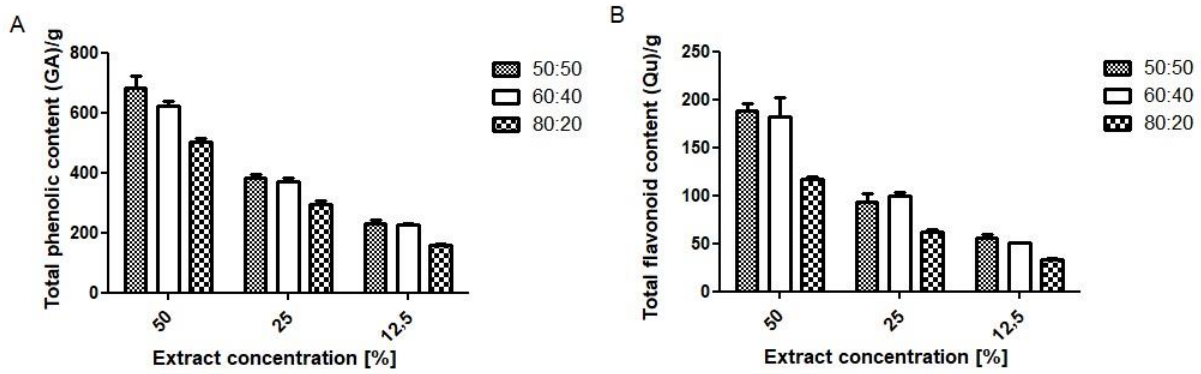

Figure 1. Total phenolic content (TPC) (A) and total flavonoid content (TFC) (B) in 50:50 (vol/vol), 60:40 (vol/vol) and 80:20 (vol/vol) aqueous/glycerin extract of *Moringa oleifera* leaves. Values are mean of three replicate determinations (n=3) ± SD. The TPC and TFC ant were calculated from the calibration curve (R^2=0.9989 and R^2=0.9996, respectively)

As it was mentioned above, the most important properties of phenolic compounds derived from plant material are their antioxidant properties. The phenols consists of a hydroxyl group and plays an essential role in the antioxidant ability by donating hydrogen and forming stable radical intermediates [32]. The mechanism of action of phenols mainly relies on neutralization of free radicals, chelation of metal ions and induction of dismutase enzymes, as well as peroxidases [33]. The next stage of our research was to evaluate ability of *M. oleifera* leaves extracts to

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scavenge free radicals. For thus, we used DPPH• reducing assay, where changes of color solution are directly linked with decreasing of absorption values and the number of formed DPPH radical [34]. To determine antioxidant properties of all tested extracts we used three different concentrations ranging from 12,5%, 25%, to 50%, the measurements were performed every five minutes over 30 min time period. Based on obtained data, it was shown, that each extract concentration has a different ability to reduce free radicals (Figure 2). The highest ability to scavenge DPPH radical was showed by 50:50 (vol/vol) aqueous/glycerin extract at the highest tested concentration (50%). After 30 min of incubation, the level of reduced DPPH• was above 56%, while in the lowest concentration (12.5%), the level of scavenged radicals were oscillated 26%. Moringa tree 60:40 (vol/vol) aqueous/glycerin leaves extract was characterized by middling antioxidant ability in comparison to other extracts. The highest reducing power of this extract was observed for 50% of concentration and it was about 41% after 30 min of incubation. Considering the 80:20 (vol/vol) aqueous/glycerin extract, it has the lowest antioxidant potential. In the highest concentration, the ability to scavenge DPPH radical was only on 35% level. Furthermore, there was observed relationship between used concentration and antioxidant potential of extracts. When the concentration was increasing, the free radical reducing power was higher. The order of free radical scavenging capacity was as follows: 50:50 (vol/vol) > 60:40 (vol/vol) > 80:20 (vol/vol) aqueous/glycerin.

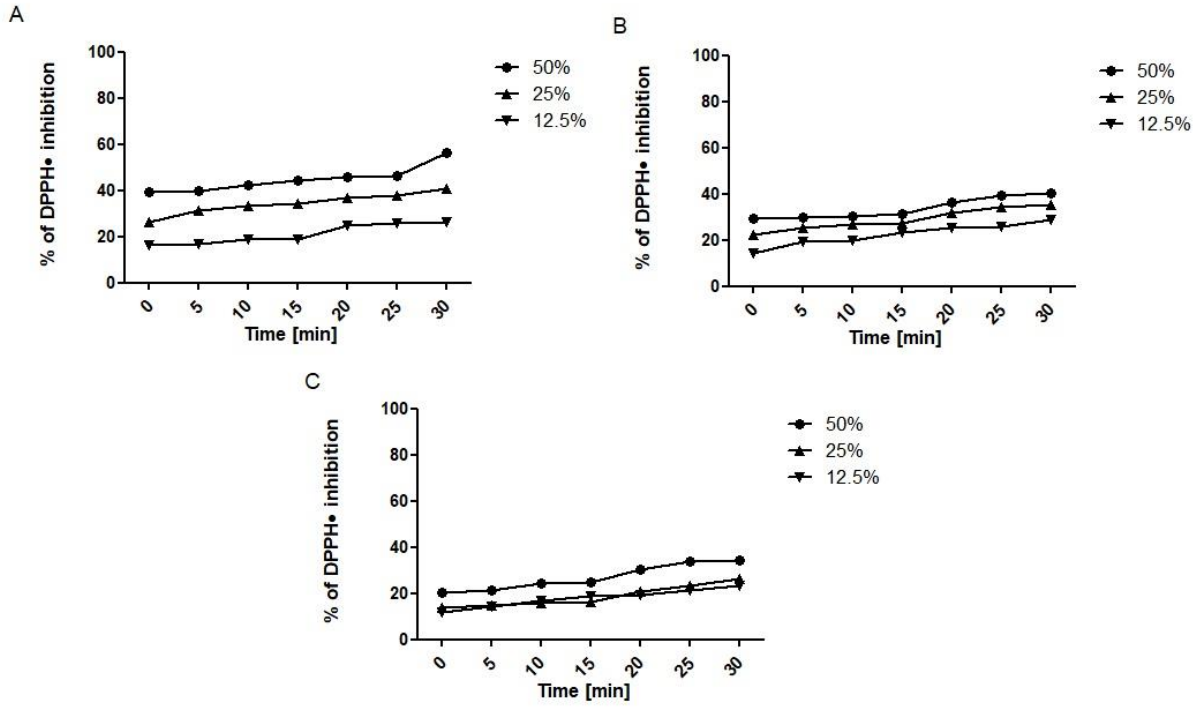

Figure 2. Kinetics of the absorbance changes in DPPH• solutions in the presence of various concentrations of 50:50 (vol/vol) (A), 60:40 (B), 80:20 (vol/vol) (C) aqueous/glycerin extract of *Moringa oleifera* leaves. Values are mean of three replicate determinations (n=3).

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Results from DPPH• scavenging assay obtained in this paper corresponds to the other research. [30] in his paper indicated on strong antioxidant activity of different parts *M. oleifera* ethanolic extracts. Using the 2-deoxyguanosine assay model, he has shown that the highest free radical scavenging activity was characterized by leaves. Remarkable antioxidant activity of drumstick leaves might be possessed due to presence of quercetin and kaempferol as well as chlorogenic acids and their derivatives, which were detected in this part of plant. In addition, [35] was hypotised that antioxidant activity of *M. oleifera* leaves extracts could be accomplished though donating protons as well as reductones, which exert activity by breaking the free radical chain.

The biological activity of leaves of *Moringa oleifera* was also investigated on cells as *in vitro* model. The cytotoxicity of tested extracts was assessed on HaCaT and BJ fibroblas cell lines using resazurin method. Both cell types were treated with various extract concentrations, ranging from 1% up to 10% in cultured medium. Obtained results indicate on cell specific effect of analyzed extracts on cell viability (Figure 3). In HaCaT cells, all tested concentrations as well as types of extracts showed stimulating effect on cell viability. The highest difference between control and treated cells was observed for all types of 1% concentration of extracts. The best ability on increasing cells viability were showed for 60:40 (vol/vol) aqueous/glycerin extracts. It also has been observed that, the cell proliferation was increasing in dose-dependent manner. In turn, in BJ fibroblast, extracts showed both, inhibitory and stimulatory effect. The highest concentrations (10% and 5%) of all tested extracts showed anti-proliferative effect. For 10% of extracts concentration, the fibroblast viability was observed on 20%, 37% and 45% level for 80:20 (v/v), 60:40(v/v) and 50:50(v/v), respectively with comparison to the control. As well as, in HaCaT the 1% of extracts concentration have shown the most positive effect on cell viability. The highest response was observed for 50:50 (v/v) aqueous/glycerin extract and it was about 157%, regards to the untreated group. The less cytotoxic potential was noted for 80:20 (v/v) *M. oleifera* leaves extract. There also was noticed, that when concentration of extract was decreased, the viability of cells was increased.

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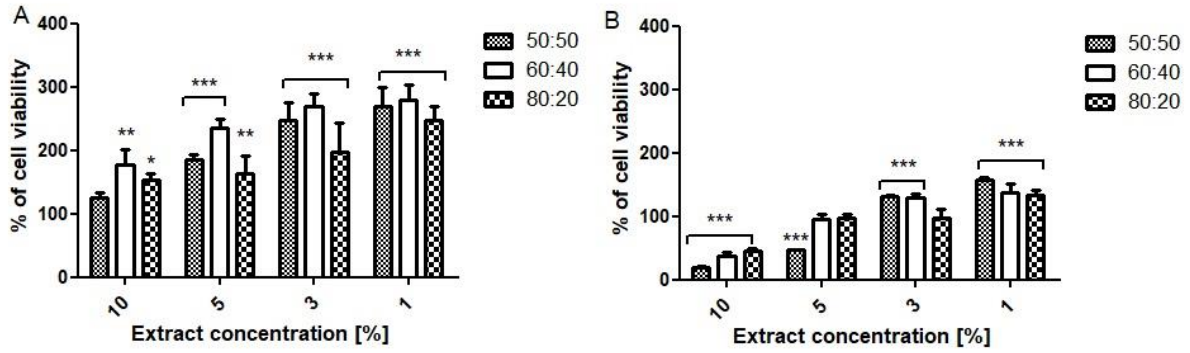

Figure 3. The effect of different concentrations of *Moringa oleifera* leaves extracts (10, 5, 3, 1 %) on resazurin salt reduction assay in cultured keratinocytes (A) and BJ fibroblast (B) after 24h of exposure. Data are the mean ± SD of three independent experiments, each of which consists of three replicates per treatment group. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ versus the control.

The equal connection in cell viability with response to increasing concentration of *M. oleifera* leaf extracts was observed by other researchers. The anti-proliferative effect was shown in KB (tumor cells), HEK-293 (human lung carcinoma) and A549 cells (human embryonic kidney cells). These papers also indicate, that anti-proliferative effect was associated with induction of apoptosis, morphological changes and DNA fragmentation [31, 36-37].

In the next stage of this experiment it has been evaluated if *M. oleifera* leaves extracts could inhibit H_2O_2 induced ROS production on *in vitro* cell models. As a substrate to determine the intracellular formation of ROS generation, H_2DCFDA assay was used. As is recommended [38], we examined whether plant extracts without cells affected the fluorescence of the H_2DCFDA. Additionally, the separated experiment showed that there were no interactions between plant extracts and H_2DCFDA substrate in DMEM or MEM medium. After preincubation cells with 1mM H_2O_2, the intracellular level of produced reactive oxygen species level robustly increased in both, HaCaT and BJ, compared to the non-treated cells. When after 90 min of cells treatment with various concentration of all types extracts, the level of ROS significantly decreased to the level oscillating to the control, group which were untreated cells (Figure 4). The high ability of extracts to reduce oxidative stress might be correlated with rich content of phenols and flavonoids. According to [36], it were directly indicated, that major bioactive substances present in leaf extracts which provides to strong inhibition of H_2O_2-induced oxidative stress are crypto-chlorogenic acid and isoquercetin.

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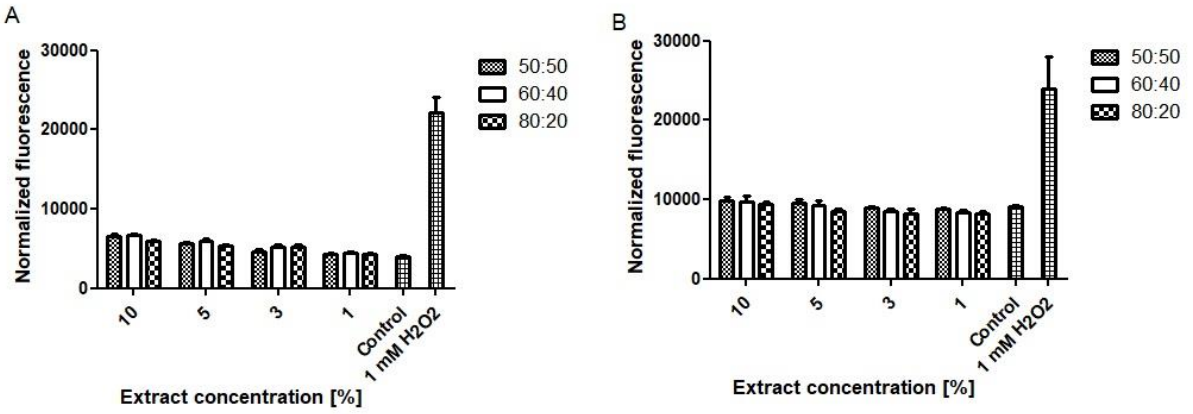

Figure 4. The effect of various concentrations of *Moringa oleifera* leaves extracts (10, 5, 3 and 1%) on the DCF fluorescence in keratinocytes (A) and BJ fibroblast (B) cells preincubated with hydrogen peroxide (H_2O_2). Medium with 1mM hydrogen peroxide (H_2O_2) was used as a positive control. The data are expressed as the mean ± SD of three independent experiments, each of which consisted of three replicates per treatment group.

Given to the antioxidant nature of tested substances, based on previous experience and available information, it was prepared a model formula of washing gel containing *M. oleifera* leaf extract. To assay if model cosmetic formulation (1% Sodium Coco Sulfate – SCS) with various concentration (5, 3 and 1%) of tested extracts can affect on cell viability it was used resazurin reduction assay. Obtained data indicate, that in HaCaT cells, tested extracts did not induce any significant decrease in cell viability in comparison to the control cells. Furthermore, it was noticed, that base (1% SCS) plus 1% or 3% of extracts can significantly increase cell metabolism. The highest cell viability growth was observed to 1% SCS+1% of 50:50 (vol/vol) aqueous/glycerin extract and it was about 30% higher than a control group. It also can be noted, that base (1% SCS) did not induce toxic effect on HaCaT cells. In turn, in BJ fibroblast cells, tested substances were exhibited an opposite effect. There was no observed either toxic as well proliferative effect on cell viability for all tested types of extracts and concentrations. Slightly increased of BJ cell metabolism was noticed for 1% SCS+5% and 1% SCS+3% of 80:20 (vol/vol) aqueous/glycerin extract (104 and 106% respectively). Percent of viable cells after treatment with 1% of SCS was little under of the control group (Figure 5). Obtained results indicated, that use of extract in cosmetic formula is not unsafe for human skin cells and additionally introduces desirable properties such as antioxidant activity.

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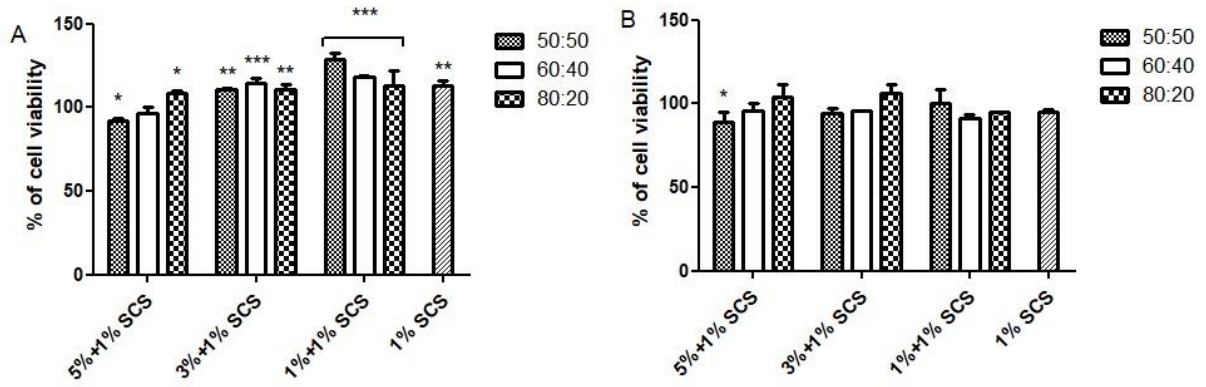

Figure 5. The effect of different model base formulation of cosmetic (1% SCS) containing various concentrations of *Moringa oleifera* leaves extracts (5, 3, 1 %) on resazurin reduction in cultured keratinocytes (A) and fibroblast (b) after 30 min of exposure. Data are the mean ± SD of three independent experiments, each of which consists of three replicates per treatment group. ***p < 0.001, **p < 0.01, *p < 0.05 versus the control.

In order to further confirm above reports, the ability of model cosmetic formulation containing *M. oleifera* leaf extract to generate intracellular ROS in keratinocytes and fibroblast was assayed. The reactive oxygen species production was measured by the use of H_2DCFDA method. As the results showed, the 60:40 (v/v) and 80:20 (v/v) aqueous/glycerin Moringa tree leaves extract did not significantly generate oxidative stress in HaCaT cells. The highest ROS production was exhibited by 1% SCS. In addition, the most intracellular ROS level increase was noticed for 1% SCS+ 5% 50:50 (v/v) extract and it was 2 fold higher than this of the non-treated group. Values for other types of extracts and concentrations oscillates about the control. There also was observed, when the extract concentration decreased, the amount of ROS in cells was also lower (Figure 6).

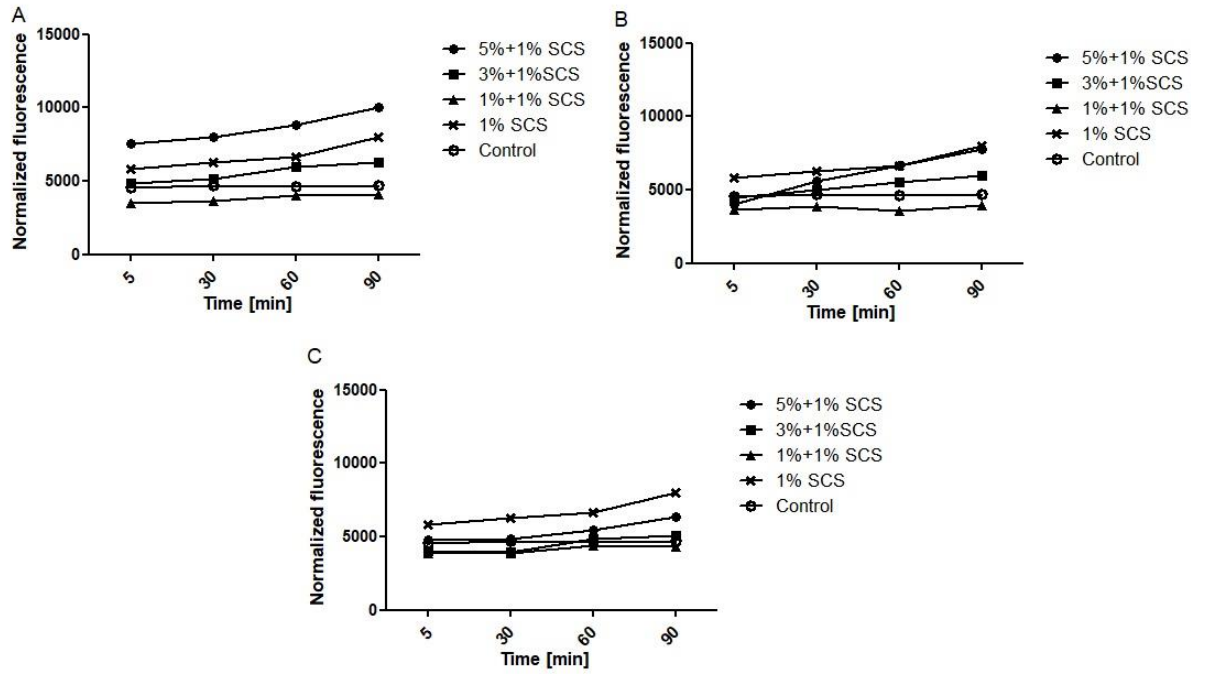

Figure 6. The effect of different model base formulation of cosmetic (1% SCS) containing various concentrations of 50:50

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(vol/vol) (A), 60:40 (B), 80:20 (vol/vol) (C) *Moringa oleifera* leaves extracts on the DCF fluorescence in normal human keratinocytes cells. Unexposed cells was used as a control. The data are expressed as the mean ± SD of three independent experiments, each of which consisted of three replicates per treatment group.

When considering BJ fibroblast results it has been showed, that the highest intracellular level of reactive oxygen production was for 1% SCS + 5% of 50:50 (v/v) extract compared to the untreated group. Values for the other concentrations of this extract was similar to the unexposed cells. For 60:40 (v/v) and 80:20 (v/v) aqueous/glycerin *M.oleifera* leaves extract did not observed any changes in induction of oxidative stress. The level of intracellular ROS was oscillate to the control group (Figure 7). Above findings correlates to the cell viability assay, and it therefore seems that all types of tested extract are a desirable plant material with remarkable antioxidant activity. These features can protect it from ageing and other diseases, where the reactive oxygen plays major role.

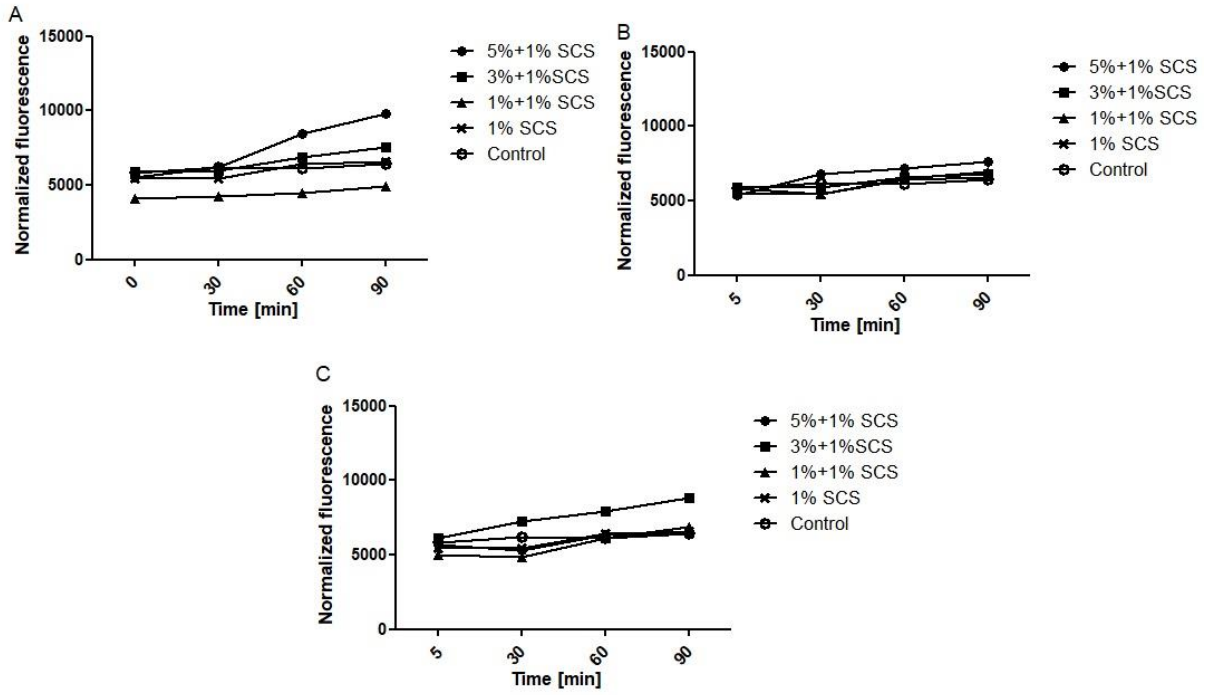

Figure 7. The effect of different model base formulation of cosmetic (1% SCS)) containing various concentrations of 50:50 (vol/vol) (A), 60:40 (B), 80:20 (vol/vol) (C) *Moringa oleifera* leaves extracts on the DCF fluorescence in fibroblast cells. Medium with 1% of Sodium Coco Sulfate (SCS) was used as a positive control. The data are expressed as the mean ± SD of three independent experiments, each of which consisted of three replicates per treatment group.

In the next step the Moringa tree leaves extracts were tested by evaluating its ability to reduce the irritant potential of Sodium Coco Sulphate (SCS), an anionic surfactant used in the formulation of body wash cosmetics. For this purpose, four samples constituting model washing systems were prepared. Each sample contained 1 wt% of SCS combined with 1, 3 and 5 wt% of the extracts prepared with different ratio of water and glycerin as extractant. The reference (baseline) sample contained 1 wt% solution of SCS without any extract. The pH of each sample

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was adjusted with 25% citric acid to a value of 5.5, the physiological pH value of the skin. The irritant potential of model body wash systems was analyzed by zein value measurements. In the surfactants solution zein protein is denatured and then is solubilized in the solution. This process simulates the behaviour of surfactants in relation to the skin proteins. The test results are presented in Fig 8.

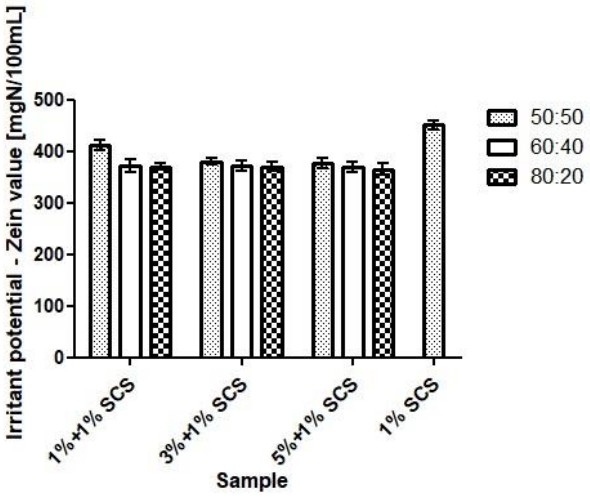

Figure 8. Irritant potential of model products containing 1.0 wt% SCS and extract from Moringa oleifera L (1%, 3%, 5%).

The test showed that the *M. oleifera leaves* extracts might improve the safety of use of the model washing system on terms of its effect on the skin. The addition of analyzed extracts to 1% SCS causes a decrease in the value of the zein number, and the same reduces skin irritation potential. The value of the measured parameter in the base sample was at 451 mgN/100 mL. According to the literature [2-4, 7-8, 10-13, 39-40], the sample should be classified as highly irritating, as the value of the zein number exceeds 400 mgN/100 mL. The skin irritation potential of the samples with addition of extracts was about 10–20% lower than the baseline. It was observed, that used extract concentrations was not significantly affect on the value of zein number, and the differences noted between the samples are within the margin of error. It was not observed significant influence of the extractant on the analysed parameter.

The decrease of the zein number following to the application of the extract to the model cosmetic formula, might be the result of presence active substances in the extracts. In aqueous solutions and at low concentrations, surfactants occur as the form of individual particles referred as a monomers [2, 4-5, 10-13]. When specific concentration, unique due to a compound is reached, referred as the critical micelle concentration (CMC), the micellar aggregates starts to appears in surfactant solutions. The irritation potential is significantly correlated with the type and concentration of the surface active agent. The highest skin irritation ability is attributed to

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anionic surfactants and surfactants present as the forms of monomers. They demonstrate the capacity to form a strong and long-lasting bonds with epidermal proteins. Following to their ability to bind with proteins, surfactants may cause denaturation and elimination (elution) proteins from the skin, which results as a cutaneous irritation. Available data indicates that the irritant potential might be reduced by bindings of monomers with various types of substances including peptides, polymers, polysaccharides and mineral salts, where all of them were found in plant extracts [2, 4-5, 10-13, 39-46]. Another factor which potentially is contributing to a reduction of irritant potential is the stabilization of micelles formed in solutions. Micelles are thermodynamically unstable aggregates, which constantly disintegrates and releases monomers into the volume phase of the solution. Stabilization of micelles in the presence of plant extracts may take place through the incorporation of such substances as proteins, polyphenols and flavonoids, as well as solvents used in the extraction process (glycerin, glycols), into their structure [2, 4-5, 10-13, 41-46].

Conclusions

The paper was an attempt to determine the properties and the applicability of extracts from *Moringa oleifera* leaves in model products. The tested extracts were characterized by a high content of phenolic compounds, flavonoids and high antioxidant potential. *In vitro* toxicity studies showed that the tested extracts in concentrations up to 5% showed a positive effect on cell proliferation and metabolism. It also has been shown that the extracts may contribute to the reduction of oxidative stress in cells. It was noted, that tested model formulation of cosmetic (1% SCS) with addition of different types of extracts in various concentrations does not affect negatively on cell metabolism. Analyzes defining the ROS level showed that model cosmetic formulation (1% SCS) with presence of tested extracts do not cause increasing formation of intracellular of reactive forms of oxygen. To summarize, conducted experiments in this paper showed, that application of *Moringa oleifera* leaves extracts to the model cosmetic formulation might contribute to reduce skin irritation and improve the safety of the product.

Conflicts of Interest

The authors declare no conflict of interest.

Literature

1. Falbe, J. *Surfactants in consumer products: Theory Technology and Application*. Springer Science & Business Media: Berlin, Germany, 2012.
2. Barel, M.; Paye, M. *Handbook of Cosmetic Science and Technology*, 4th ed.; Taylor & Francis Group: Boca Raton, FL, USA, 2014; pp. 353-365.
3. Showell, M.S. *Handbook of Detergents. Part D: Formulations*. Taylor & Francis Group: Boca Raton, FL, USA, 2006.
4. Rhein, L.; Schlossman, M. *Surfactants in Personal Care Products and Decorative Cosmetics*, 3rd ed.; Taylor & Francis Group: Boca Raton, FL, USA, 2006.
5. Broze, G. *Handbook of Detergents. Part A: Properties*; Marcel Dekker: New York, NY, USA, 1999.
6. Wasilewski, T.; Arct, J.; Pytkowska, K.; Bocho-Janiszewska, A.; Krajewski, M.; Bujak, T.; Technological and physicochemical aspects of the production of cleaning cosmetic concentrates. *Przem. Chem.* **2015,** *94*, 741-747.
7. Seweryn, A.; Wasilewski, T.; Bujak, T.; Effect of Salt on the Manufacturing and Properties of Hand Dishwashing Liquids in the Coacervate Form. *Ind. Eng. Chem. Res.*, **2016**, *55*, 1134-1141, DOI 10.1021/acs.iecr.5b04048.
8. Wasilewski, T.; Seweryn, A.; Bujak, T.; Supercritical carbon dioxide blackcurrant seed extract as an anti-irritant additive for hand dishwashing liquids. *Green Chem. Lett. Rev.* **2016,** *9*, 114-121, DOI 10.1080/17518253.2016.1180432.
9. Wasilewski, T.; Bujak, T.; Effect of the type of nonionic surfactant on the manufacture and properties of hand dishwashing liquids in the coacervate form. *Ind. Eng. Chem. Res.* **2014,** *53*, 13356-13361, DOI 10.1021/ie502163d.
10. Bujak, T.; Nizioł-Łukaszewska, Z.; Wasilewski, T.; Effect of Molecular Weight of Polymers on the Properties of Delicate Facial Foams. *Tenside Surfact. Det.* **2018,** *55*, 96-102.
11. Nizioł-Łukaszewska, Z.; Bujak, T.; Saponins as Natural Raw Materials for Increasing the Safety of Bodywash Cosmetic Use. *J. Surfact. Det.*, **2018**, *21*,12681-12695, DOI 10.1002/jsde.12168.
12. Bujak, T.; Wasilewski, T.; Nizioł-Łukaszewska, Z. Role of macromolecules in the safety of use of body wash cosmetics. Colloids Surf. B Biointerfaces **2015**, 135, 497–503, DOI 10.1016/j.colsurfb.2015.07.051.
13. Nizioł-Łukaszewska, Z.; Osika, P.; Wasilewski, T.; Bujak, T. Hydrophilic Dogwood Extracts as Materials for Reducing the Skin Irritation Potential of Body Wash Cosmetics. *Molecules*, **2017,** *22*, 320-33, DOI 10.3390/molecules22020320.
14. Anwar, F.; Latif, S.; Ashraf, M.; Gilani, A. Moringa oleifera: A food plant with multiple medicinal uses. Phytother. Res. **2007**, 21, 17– 25, DOI 10.1002/ptr.2023
15. Leone, A.; Spada, A.; Battezzati A. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of Moringa oleifera leaves: an overview. *Int J Mol Sci* **2015**, 16, 12791-12835, DOI 0.3390/ijms160612791
16. Razis, A.; Ibrahim, M.D.; Kntayya, S.B.; Health benefits of Moringa oleifera. *Asian Pac J Cancer Prev.* **2014**,15(20), 8571-8576, DOI 0.7314/APJCP.2014.15.20.8571
17. Mishra, G.; Singh, P.; Verma, R. Traditional uses, phytochemistry and pharmacological properties of Moringa oleifera plant: an overview. *Der Pharmacia Lettre*. **2011**,(2), 141-164.
18. Zhang, M.; Hettiarachchy, S.N.; Hora, R. Phytochemicals, antioxidant and antimicrobial activity of Hibiscus sabdara, Centella asiatic, Moringa oleifera and Murraya koenigii leaves. *J Med Plants Res.* **2011**, 5, 6672-6680, DOI 0.5897/JMPR11.621.
19. Makkar, H.P.; Becker, K. Nutrional value and antinutritional components of whole and ethanol extracted Moringa oleifera leaves. *Anim Feed Sci Technol.* **1996**, 63. 211-228.

20. Saini, R.; Shetty, N.; Prakash, M.; Giridhar.; P. Effect of dehydration methods on retention of carotenoids, tocopherols, ascorbic acid and antioxidant activity in Moringa oleifera leaves and preparation of a RTE product. *J Food Sci Technol.* **2014**, 51, 2176-2182, DOI 10.1007/s13197-014-1264-3.
21. Sultana, B.; Anwar, F. Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. *Food Chem*. **2008**, 108, 879-884, DOI 10.1016/j.foodchem.2007.11.053.
22. Bajpai, M.; Pande, A.; Tewari, SK.; Prakash, D. Phenolic contents and antioxidant activity of some food and medicinal plants. *Int J Food Sci Nutr*. **2005**, 56, 287-291, DOI 10.1080/09637480500146606.
23. Gopalakrishnan, L.; Doriya, K.; Kumar, D.S. Moringa oleifera: a review on nutritive importance and its medical application. *Food Science and Human Wellness*, **2016**, 5, 49-56, DOI 10.1016/j.fshw.2016.04.001.
24. Singleton, V.L.; Orthofer, R.; and Lamuela-Raventos, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Meth. Enzymol.* **1999**, 299, 152-178, DOI 10.1016/S0076-6879(99)99017-1.
25. Matejic, J.S.; Dzamic, A.M.; Mihajilov-Krstev, T.; Randjelovic, V.; Krivosej, Z.D.; Marin, P.D. Total phenolic content, flavonoid concentration, antioxidant and antimicrobial activity of extracts from three Seseli L. taxa. *Cent. Eur. J. Biol.* **2007**, 7, 1116-1122.
26. Brand-Williamis, W.; Cuvelier, M.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT Food Sci. Technol.* **1995,** 28, 25-30, DOI 10.1016/S0023-6438(95)80008-5.
27. Ivanov, D.P.; Parker, T.L.; Walker, D.A.; Alexander, C.; Ashford, M.B.; Gellert, P.R.; Garnett, M.C. Multiplexing spheroid volume, resazurin and acid phosphatase viability assays for high-throughput screening of tumour spheroids and stem cell neurospheres. *PLOS One.* **2014**, 13, 9, DOI 10.1371/journal.pone.0103817.
28. Gomes, A.; Fernandes, E.; Lima, J.L.F.C. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods.* **2005,** 65, 45-80, DOI dx.doi.org/10.1016/j.jbbm.2005.10.003.
29. Tetz, L.M.; Kamau, P.W.; Cheng, A.A.; Meeker, J.D.; Loch-Caruso, R. Troubleshooting the dichlorofluorescein assay to avoid artifacts in measurement of toxicant-stimulated cellular production of reactive oxidant species. *J Pharmacol Toxicol Methods.* **2013**, 67, 56-60, DOI 10.1016/j.vascn.2013.01.195.
30. Atawodi, S.E.; Atawodi, J.C.; Idakwo, G.A.; Pfundstein, B.; Haubner, R.; Wurtele, G.; Bartsch H.; Owen, R.W. Evaluation of the Polyphenol Content and Antioxidant Properties of Methanol Extracts of the Leaves, Stem, and Root Barks of Moringa oleifera Lam. *J Med Food.* **2010**, 13, 710–716, DOI 10.1089/jmf.2009.0057.
31. Sreelatha, S.; Jeyachitra, A.; Padma, P.R. Antiproliferation and induction of apoptosis by Moringa oleifera leaf extract on human cancer cells. *Food Chem Toxicol*. **2011**, 49,1270-1275, DOI 10.1016/j.fct.2011.03.006.
32. Fukumoto, L.R.; Mazza, G. Assessing antioxidant and prooxidant activities of phenolic compounds. *J Agric Food Chem*. **2000**, 48, 3597-604, DOI 10.1021/jf000220w.
33. Rice-Evans, C.A.; Miller, N.J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic Biol. Med*. **1996**, 20(7),933-956, DOI 10.1016/0891-5849(95)02227-9.
34. Alam, M.N.; Bristi, N.J.; Rafiquzzaman, M. Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharm J.* **2013**, 21, 143-152, DOI 10.1016/j.jsps.2012.05.002.

35. Vongsak, B.; Mangmool, S.; Gritsabapab, W. Antioxidant Activity and Induction of mRNA Expression of Antioxidant Enzymes in HEK-293 Cells of *Moringa oleifera* Leaf Extract. *Planta Med.* **2015**, 81, 1084-1089, DOI 10.1055/s-0035-1546168.
36. Vongsak, B.; Sithisarn, P.; Mangmool, S.; Thongpraditchote, S.; Wongkrajang, Y.; Gritsanapan, W. Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method. *Ind Crops Prod.* **2013**, 44, 566-571.
37. Tiloke, C.; Phulukdaree, A.; Chuturgoon, A.A. The antiproliferative effect of Moringa oleifera crude aqueous leaf extract on cancerous human alveolar epithelial cells. *BMC Complement Alternat Med*. **2013**, 13, 266, DOI 10.1186/1472-6882-13-226.
38. Szychowski, K.A.; Wójtowicz, A.K. TBBPA causes neurotoxic and the apoptotic responses in cultured mouse hippocampal neurons in vitro. *Pharmacol Rep.* **2016**, 68, 20-26, DOI 10.1016/j.pharep.2015.06.005.
39. Farn, R.J. Chemistry and Technology of Surfactants; Blackwell Publishing: Hoboken, NY, USA, 2006.
40. Rosen, M.J. Surfactants and Interfacial Phenomena, 3rd ed.; John Wiley & Sons Inc.: New York, NY, USA, 2006.
41. Moore, P.N.; Puvvada, S.; Blankschtein, D. Challenging the surfactant monomer skin penetration model: Penetration of sodium dodecyl sulfate micelles into the epidermis. *J. Cosmet. Sci.* 2003, 54, 29–46.
42. Dasilva, S.C.; Sahu, R.P.; Konger, R.L.; Perkins, S.M.; Kaplan, M.H.; Travers, J.B. Increased skin barrier disruption by sodium lauryl sulfate in mice expressing a constitutively active STAT6 in T cells. *Arch. Dermatol. Res*. 2012, 304, 65–71, DOI 10.1007/s00403-011-1168-2.
43. Dominguez, J.G.; Balaguer, F.; Parra, J.L.; Pelejero, C.M. The inhibitory effect of some amphoteric surfactants on the irritation potential of alkylsulphates. *Int. J. Cosmet. Sci.* 1981, 3, 57–68, DOI 10.1111/j.1467-2494.1981.tb00268.x
44. Faucher, J.A.; Goddard, E.D. Interaction of keratinous substrates with sodium lauryl sulfate. I. Sorption. *J. Soc. Cosmet. Chem*. 1978, 29, 323–337.
45. Hall-Manning, T.J.; Holland, G.H.; Rennie, G.; Revell, P.; Hines, J.; Barratt, M.D.; Basketter, D.A. Skin irritation potential of mixed surfactant systems. *Food Chem. Toxicol.* 1998, 36, 233–238.
46. Nielsen, G.D.; Nielsen, J.B.; Andersen, K.E.; Grandjean, P. Effects of industrial detergents on the barier function of human skin. *Int. J. Occup. Environ. Health* 2000, 6, 138–142, DOI 10.1179/oeh.2000.6.2.138