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IN VIVO STUDIES AND STABILITY STUDY OF *CLADOPHORA GLOMERATA* EXTRACT AS A COSMETIC ACTIVE INGREDIENT

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Abstract: Marine algae are widely used as cosmetics raw materials. Likewise, freshwater alga *Cladophora glomerata* may be a good source of fatty acids and others bioactive agents. The aims of this study was to find out if the addition of the extract from the freshwater *C. glomerata* affects the stability of prepared cosmetic emulsions and to investigate *in vivo* effects of the extract in cosmetic formulations on hydration and elasticity of human skin. Extract from the freshwater *C. glomerata* was obtained using supercritical fluid extraction (SFE). Two forms of O/W emulsions were prepared: placebo and emulsion containing 0.5% of *Cladophora* SFE extract. The stability of obtained emulsions was investigated by using Turbiscan Lab Expert. Emulsions were applied by volunteers daily. Corneometer was used to evaluate skin hydration and cutometer to examine skin elasticity. Measurements were conducted at reference point (week 0) and after 1st, 2nd, 3rd and 4th week of application. The addition of *Cladophora* extract insignificantly affected stability of the emulsion. The extract from *C. glomerata* in the emulsion influenced the improvement of both skin hydration and its elasticity. Thus, freshwater *C. glomerata* extract prepared *via* SFE method may be considered as an effective cosmetic raw material used as a moisturizing and firming agent.

Keywords: freshwater green macroalgae, supercritical fluid extraction, corneometer, cutometer

Algae are well known and commonly used cosmetics raw materials containing a wide range of biologically active compounds. *Cladophora glomerata* is a filamentous green macroalga occurring both in marine and in freshwater habitat. Most of research concern marine species of the genus *Cladophora* and indicate the occurrence of such bioactive phytochemicals as: unsaturated and saturated fatty acids (1, 2), sterols, terpenoids (3), amino acids, proteins (4), phenolic compounds (5) and others. However, also a few studies have been reported concerning the content of bioactive substances in freshwater *C. glomerata (L.)* (Kütz), e.g.: fatty acids (FAs) (6), sterols, terpenes, carbohydrates, glycosides (7), sulfated polysaccharides (8), amino acids (6), phenolic compounds, carotenoids (9) and volatiles (10). Hence, different biological activities, such as: antioxidant (9), antibacterial, antifungal (7) and antiprotozoal (10), have been described for the freshwater genus.

Due to the occurrence of various bioactive agents and their beneficial effects, algae extracts are one of the most popular natural cosmetic ingredients on the market (11). The extraction process involves the separation of a substance from a matrix based on diffusion using appropriate solvents. Therefore, it allows the isolation of active metabolites from algae. In recent years, supercritical fluid extraction (SFE) with CO_2 as a green solvent has been frequently used for isolation of bioactive compounds from algae (9, 12, 13). SFE allows for the fast extraction rate and high yield of receiving bioactives with a low consumption of organic solvents, as well as it is a suitable method for obtaining thermally labile substances (14). Thanks to SFE, extracts with higher amounts of bioactive agents, and thereby much superior cosmetic activity can be obtained in a shorter time compared to conventional extraction methods.

Because of a relatively high amount of fatty acids in freshwater *C. glomerata* species (6, 7), extracts from this algae could be useful as cosmetics ingredient. Fatty acids contained in the extracts may act as emollients, which protect skin against the loss

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of water, as well as function as antiinflammatory, antiallergic and antioxidant agents. Moreover, fatty acids may indirectly affect the stimulation of the skin hydration and elasticity (15, 16). In our previous studies, we isolated sulfated polysaccharides from freshwater *C. glomerata* (8). Polysaccharides are able to uptake water in skin, hence, they are used as moisturizing agents in cosmetology (15, 17). We also found that *C. glomerata* extract obtained by SFE method is a rich source of carotenoids and phenolic compounds (9). These bioactive metabolites possess antioxidant properties (9, 15). To sum up, the use of SFE may allow to obtain algae extracts rich in different bioactive agents utilized as valuable cosmetics ingredients with beneficial effects on skin: moisturizing and anti-aging.

The goal of this work was the preparation of oil-in-water (O/W) emulsion with freshwater *C. glomerata* extract made *via* supercritical fluid extraction (SFE) as a novel cosmetic active ingredient. First of all, the stability study was carried out to find out if the addition of the SFE extract from the freshwater *C. glomerata* affected the stability of the emulsion. The main aim of this study was to investigate *in vivo* effects of the extract in cosmetic emulsion formulations on hydration and elasticity of the skin.

EXPERIMENTAL

Algae material and preparation of SFE extract

Freshwater *C. glomerata* was collected in the first week of July 2013 from Oporzyn Lake (N52°55', E17°9') located in the north part of the Greater Poland Voivodeship, (Wielkopolska region, Poland). Algae were collected manually from the middle of their mats, from the surface of the lake water. The biomass was harvested using boats applying strip, a cable or a special rake. After the collection, fresh algal biomass was immediately weighed, washed and purified. Afterwards, the material was dried in a drying chamber with forced air circulation (FD by Binder) at 35°C until a water content in dry matter reached level of less than 15%, and then, the dry biomass was powdered using a laboratory mill with grinding tank.

The extractions of powdered algal material was processed in the Fertilizer Research Institute in Puławy (Poland), as already described by Messyasz et al. (6). SFE was carried out using pure CO_2 as a solvent and the multi-purpose pilot unit was used at three different pressures: 300, 500 and 700 bar, at the temperature of 45°C. The second extraction was performed at 700 bar and 45°C. The samples were taken every 30 min. The obtained extract was used for emulsion preparation and the application test for the skin hydration and elasticity.

Preparation of emulsion formulations

Oil-in-water (O/W) type emulsions: placebo (Emulsion 1) without the extract and emulsion containing 0.5% of *Cladophora* SFE extract (Emulsion 2) were prepared. The composition of the formulations is given in Table 1. The O/W emulsions were prepared by separately heating and stirring the water and oily phases to the same temperature of 60°C, followed by homogenization for 4 min at 1500 rpm (IKA Ultra-Turrax T 18 digital). After achieving temperature of 30°C the preservative (benzyl alcohol), lemongrass oil and the extract were added to the emulsions and mixed.

Stability study

The stability of prepared cosmetic emulsions (Emulsion 1 and Emulsion 2) was investigated using Turbiscan Lab Expert (FORMULACTION). This device can operate in either scanning or single position mode. In scanning mode, the optical head cam-

Table 1. Composition of emulsion formulations (% w/w).

Ingredient (INCI name)	Emulsion 1	Emulsion 2
Aqua (Water)	59.4	58.9
Helianthus annuus (sunflower) seed oil	23	23
Glycerine	10	10
Methyl glucose sesquistearate	4	4
Cetyl alcohol	3	3
Cladophora glomerata extract	-	0.5
Benzyl alcohol	0.2	0.2
Cymbopogon schoenanthus (lemongrass) oil	0.2	0.2
Xanthan gum	0.2	0.2

era scans the entire height of the sample (55 mm), collecting the transmitted and backscattered light every 40 μm. This is the most universal measurement that enables the detection of the particle migration of tested emulsions. The light source in this device is a light emitting diode that emits near infrared light with a wavelength of 880 nm. Curves obtained from the measurement represent the intensity of the transmission or backscattering reference standard in percent of the amount of measuring cell function (in millimeters). To analyze the stability of a transparent cosmetic formulations, transmittance phenomenon (T) is used, whereas for opaque samples - backscattering phenomenon (BS). The sample was analyzed after placing emulsions in a cylindrical measurement cell. Studies were carried out for a period of 70 days from the date of the formulations preparation. After the analysis, graph presenting the dependence of sample backscattering intensity (BS) from the sample on the height of cell was obtained (no-reference mode). In order to trace the changes occurring in the sample, results are analyzed in reference mode, where the results are compared to the first measurement. The second parameter, that was used to monitor the coalescence kinetics in the samples *versus* aging time, is the Turbiscan Stability Index (TSI). TSI sums all the variations detected in the sample (size and / or concentration).

Application test

As many as 20 persons belonging to different age profiles took part in the application tests as volunteers. They were randomized into two groups with 10 persons each. The first group consisted of volunteers at the age of 20-30 (20+), whereas the second group – at the age range of 40-50 (40+). Tests were carried out on the inner side of the left arm. Emulsion 1 and Emulsion 2 were applied in designated areas of the arm next to each other once daily (evenings) for a period of 4 weeks. Measurements were conducted at reference point (week 0, before the application of emulsions) and after 1st, 2nd, 3rd and 4th week of application. Also the negative control was done, where skin not treated with any emulsion was examined week by week. All measurements were performed in controlled conditions at a temperature of 21–23°C and with an average relative humidity of 35.8%. The application test included corneometer and cutometer analysis, which are described in details below. Measurements were replicated 10 times for corneometer analysis and 4 times for cutometer. The percent of changes in skin hydration and elasticity was calculated for each volunteer individually after every week of the experiment towards the reference point (week 0) as in the equation below. Then, the average value of % change for every group of volunteers was calculated for each parameter weekly.

$$\% \text{ Change} = [(A - B) / B] \times 100$$

where A – individual value of any parameter after the 1st, 2nd, 3rd and 4th week of the experiment, B – the value of the parameter at the week 0

Corneometer analysis

The level of the hydration of skin SC (s*tratum corneum)* can be examined by corneometer, which is a diagnostic device used in laboratory tests prior and in the production of cosmetics. It allows to determine the water content of the SC and the moisturizing cosmetic action. With a help of corneometer the capacitive resistance of the SC is determined, which is a derivative of the degree of hydration. During the test, the skin moisture measurement is performed using the Corneometer CM 825 (Courage + Khazaka Electronic GmbH) connected to the central unit MPA5, coupled with the computer. The capacitive method is used, because depending on the level of hydration of the epidermis, capacitance changes are indicated by a measuring unit. On the surface of the probe head metal tracks are placed. They are separated from the skin by a glass plate in order to prevent from current conduction. Scattered electric field, penetrating the skin during the measurement, determines its insulation. The depth of penetration of the scattered electric field is small enough to measure only moisture of the outer layers of skin. The measurement is made by placing a vertical probe head in the measured area of skin. The probe device starts the measurement when pressed against the head gentle to the skin surface. Several measurements must be performed, each time selecting a slightly different portion of the surface in a certain area of skin. Values obtained by corneometer are presented in conventional units [CU]. If the obtained results are < 30 this means that skin is very dry, if they are between 30-45 units then skin is dry/normal, and if the values are > 45 then skin is well hydrated.

Cutometer analysis

Skin elasticity is defined as the biomechanical property involving the inability to reversible deformation, which is significantly associated with the flexibility of suitable skin fibers. For the measurement of skin firmness and elasticity, Cutometer MPA 580 (Courage + Khazaka Electronic GmbH) connected to the central unit MPA5, coupled with the computer, that allows to obtain reproducible meas-

urement results, is used. The measuring principle is to make a small negative pressure and suck a small area of the skin to the interior of the probe, where the measurement of skin elasticity takes place. The measurement is done using an optical system consisting of a transmitter and a light receptor, as well as the two prisms, which by appropriate alignment with each other, send the light beam from the source to the receptor. The intensity of the light beam depends on the depth to which the skin can be sucked into the interior of the probe. To interpret results obtained with cutometer the parameter R_2 have to be taken into account. This parameter defines the deformability of the skin. The more the value is close to 1 (100%) the more flexible the skin is.

RESULTS

Stability study

The Turbiscan Lab Expert apparatus can detect different types of emulsion instability, including the phenomenon of flocculation, coalescence, sedimentation and creaming of the emulsion and the observation of their progress in time. In our study, backscattering phenomenon (opaque samples) was used. Results are presented as a graph (Fig. 1) showing the variations of the delta backscattering intensity as a function of time (ΔBS(t)). In order to follow the particle size increase, the variation of the delta backscattering in the middle of the sample is plotted. Figure 1 shows that the addition of an algae extract (Emulsion no. 2) leads to a decrease of the particle size (increase of the backscattering level). The results suggest that the formulations undergo flocculation which is reversible instability involving the formation of agglomerates in the internal phase droplets without confluence and growth of their surface. The obtained delta BS value ranges from ~1 to ~2 and it is the highest for Emulsion 2 containing *Cladophora* extract (2.04). This value indicates a lower stability of Emulsion 2 in comparison to Emulsion 1, but it falls within the normal range (up to 10). ΔBS parameter for the cosmetic base (Emulsion 1) is 0.92, which proves that it is more stable than Emulsion 2.

Another parameter, that allows to monitor the coalescence kinetics in the samples *versus* ageing time, is the Turbiscan Stability Index (TSI). TSI sums all variations detected in the sample (size and / or concentration). At a given ageing time, the higher is the TSI, the worse is stability of the sample. This parameter for both cosmetic formulations was examined and the results are shown in Figure 2. The value of the TSI for cosmetic base (Emulsion 1) was 0.9, what proves its highest stability. However, the examined parameter values for Emulsion 2 containing *Cladophora* extract are twice higher than the cosmetic base, what corresponds to its slight instability over time and the of effect of the addition of algae extract on destabilization of the emulsion.

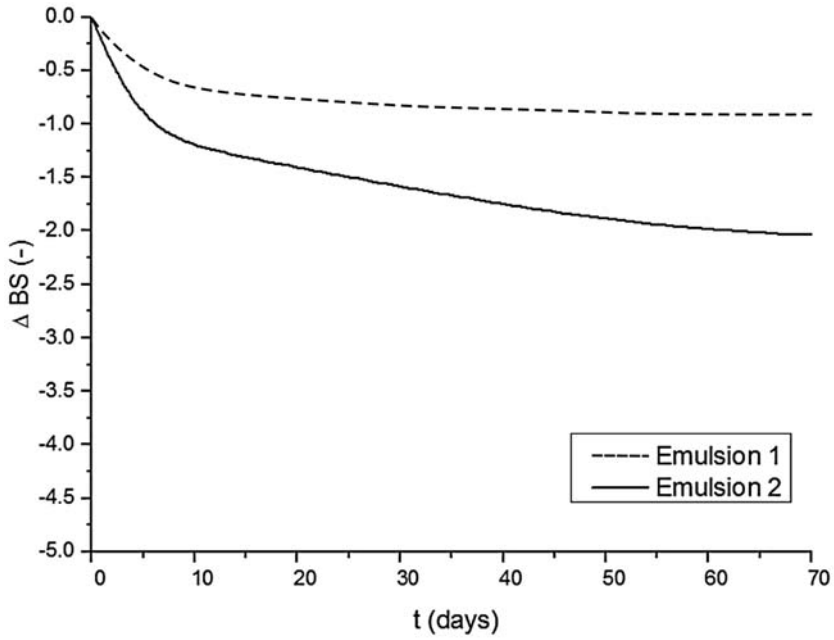

Figure 1. Variation of ΔBS parameter as a function of time for Emulsions 1 and 2

Table 2. Results of skin hydration measurements with corneometer CM 825. Values presented are the mean of CU ± SD.

Group	Emulsion	0 week	1 week	2 week	3 week	4 week
20+	Negative control (untreated skin)	33.55 ± 6.31	33.45 ± 4.89	33.42 ± 5.52	33.32 ± 6.70	33.25 ± 5.15
	Emulsion 1	-	39.06 ± 1.91	40.70 ± 2.25	40.30 ± 2.88	40.88 ± 2.11
	Emulsion 2	-	40.61 ± 2.68	43.02 ± 1.48	43.31 ± 1.15	48.66 ± 2.58
40+	Negative control (untreated skin)	28.97 ± 6.57	28.82 ± 5.05	28.68 ± 5.59	28.68 ± 5.70	28.62 ± 6.24
	Emulsion 1	-	35.10 ± 2.13	35.50 ± 2.84	36.66 ± 3.37	36.82 ± 4.69
	Emulsion 2	-	37.51 ± 5.53	38.40 ± 3.66	39.58 ± 4.29	41.22 ± 4.46

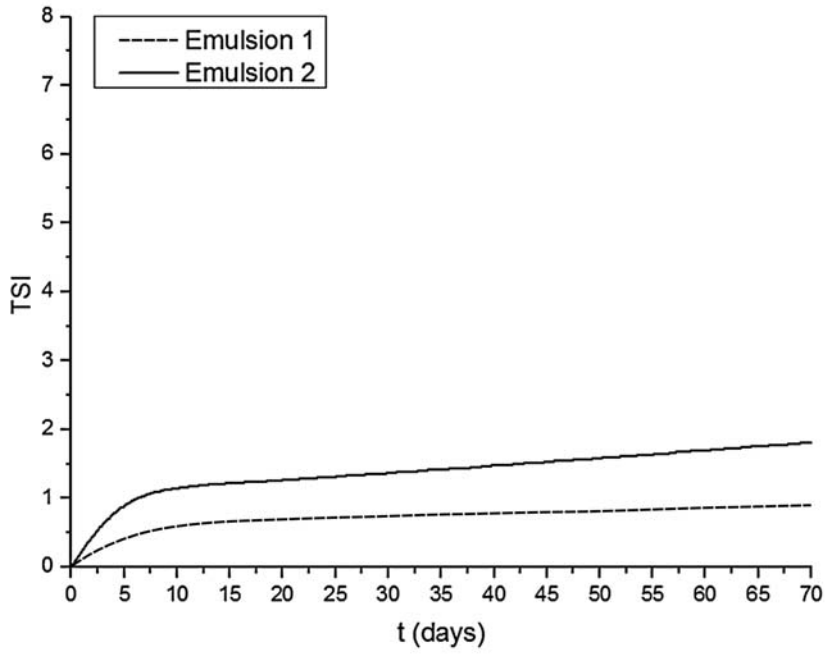

Figure 2. Dependence of TSI parameter on time for Emulsion 1 and 2

Corneometer analysis

The results for corneometer analysis are shown in Table 2 as well as in Figures 3 and 4 as a percentage change in the skin hydration after every week of the application of tested emulsions. First of all, it was observed that the addition of *Cladophora* extract to the emulsion affected the increase of skin hydration. In the case of the group of 20+ the increase in skin hydration after the application of Emulsion 2 containing the extract was not significant compared to Emulsion 1 (placebo) after the first three weeks (Table 2, Fig. 3). However, after each next week the difference in the increase between tested creams was much higher, up to the last (the 4th) week of the application, where 45% increase in skin hydration for Emulsion 2 and 22% for Emulsion 1 was noted. The highest value of skin hydration was obtained after the last week after the application of the cream containing the tested extract, which was 48.66 for the group of 20+. Thus, the result indicated that the average skin hydration was improved to the level of well moisturized skin for the emulsion with *Cladophora glomerata* extract.

In the group of 40+ the trend in the percentage increase in skin hydration was similar (Fig. 4) as in the previous group. After the each week of the application test we noticed approximately 10% better increase in skin hydration for the Emulsion 2 than for Emulsion 1 within this group. Similarly to the group 20+ the highest increase for the cream with the extract compared to the placebo was

observed after the 4th week – 42% for the Emulsion 2 and 27% for Emulsion 1. The highest skin hydration value observed for this group was 41.22 after 4 weeks of Emulsion 2 application, which refers to dry/normal skin. However, the result is very satisfactory in comparison to the reference point (28.97), where the average skin condition was weak (dry skin).

Cutometer analysis

The second part of the application test was cutometer analysis. The results of R_2 parameter are

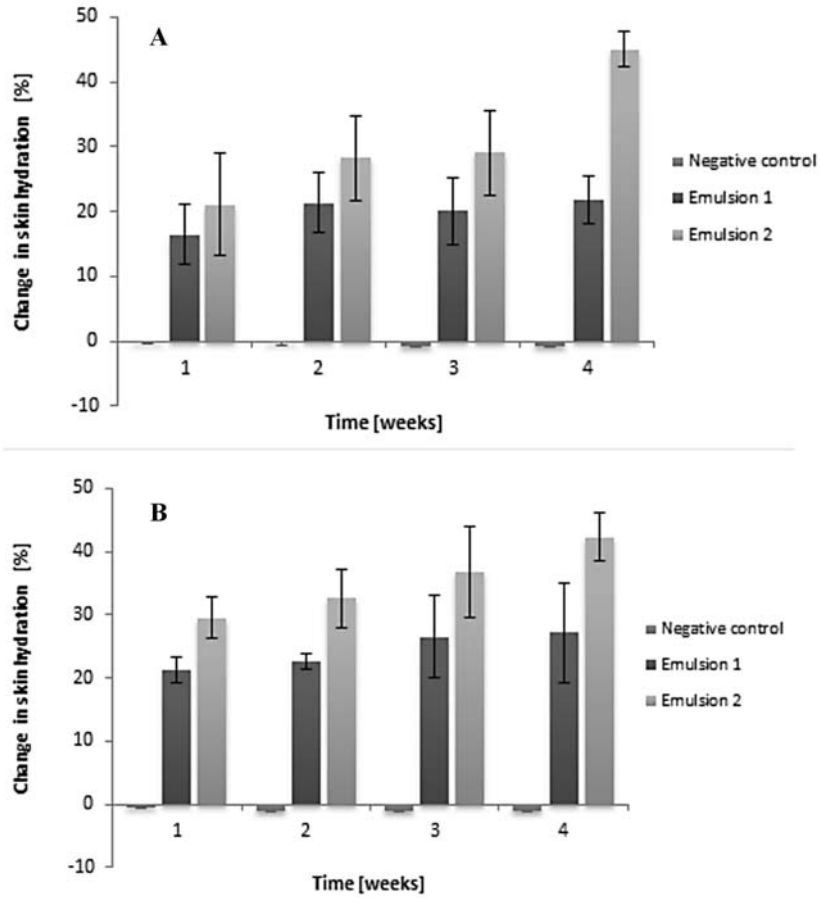

Figure 3. Percentage of changes in skin hydration after the application of Emulsion 1 and Emulsion 2. **A** - in the group of age of 20+. **B** - in the group of age of 40+. Percentage is calculated relative to the initial values as in Table 2

Table 3. Results of skin elasticity measurements with cutometer MPA 580. Values presented are the mean of R_2 parameter ± SD.

Group	Emulsion	0 week	1 week	2 week	3 week	4 week
20+	Negative control (untreated skin)	0.8552 ± 0.0622	0.8522 ± 0.0367	0.8555 ± 0.0276	0.8542 ± 0.0329	0.8565 ± 0.0456
	Emulsion 1	-	0.8760 ± 0.0240	0.8830 ± 0.0226	0.8784 ± 0.0229	0.8973 ± 0.0336
	Emulsion 2	-	0.9154 ± 0.0433	0.9412 ± 0.0198	0.9433 ± 0.0191	0.9602 ± 0.0114
40+	Negative control (untreated skin)	0.7576 ± 0.0573	0.7574 ± 0.0423	0.7568 ± 0.0498	0.7569 ± 0.0507	0.7561 ± 0.0586
	Emulsion 1	-	0.7698 ± 0.0414	0.7686 ± 0.0764	0.7814 ± 0.0656	0.7870 ± 0.0524
	Emulsion 2	-	0.7895 ± 0.0589	0.8032 ± 0.0693	0.8212 ± 0.0486	0.8378 ± 0.0312

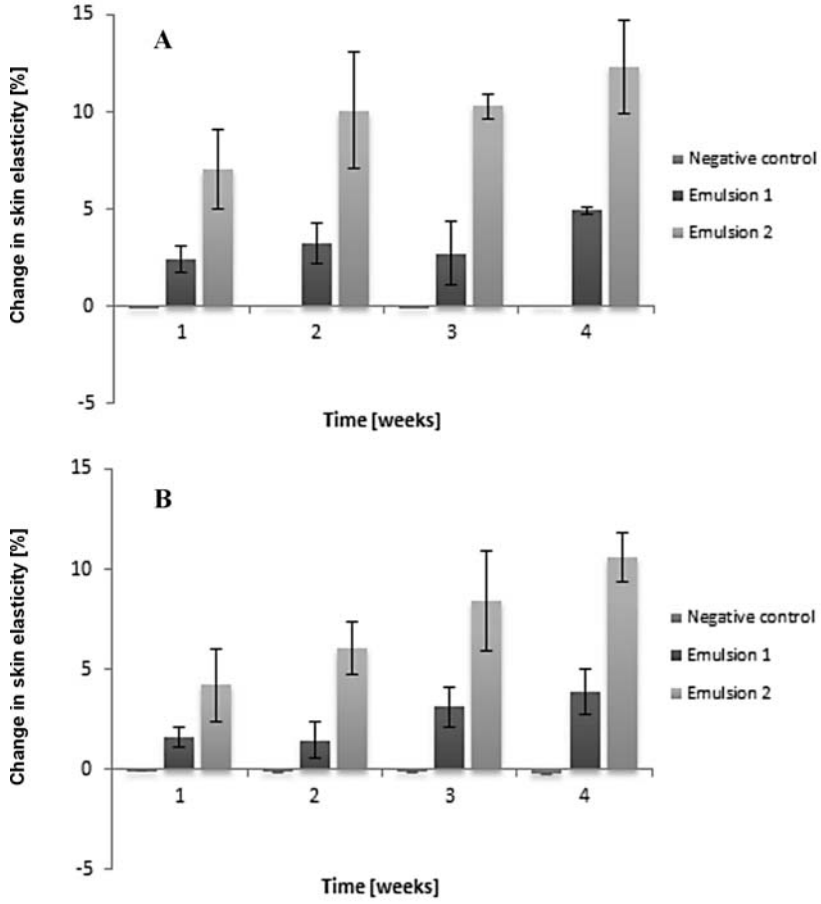

Figure 4. Percentage of changes in skin elasticity after the application of Emulsion 1 and Emulsion 2. **A** - in the group of age of 20+. **B** - in the group of age of 40+. Percentage is calculated relative to the initial values as in Table 3

presented in Table 3. Percentage changes in skin elasticity after the application of tested emulsions are shown in Figures 5 and 6. In both groups of volunteers, an increase in skin elasticity in the case of cosmetic base (Emulsion 1) and cream with *Cladophora* extract (Emulsion 2) was observed. In each case the increase was higher after the application of Emulsion 2 when compared with a base cream. Within the group of 20+ there were obtained better results in the increase of skin elasticity using Emulsion 2 (up to 12.28% after the 4th week) (Figure 5) than in the group of 40+ (up to 10.59% after the same week). Also the values of R_2 parameter were higher for this group starting from 0.8556 at the reference point up to 0.9602 after 4 weeks of Emulsion 2 application. The value 0.9602 is very high (close to the highest possible value – 1), which means a considerable improvement of skin elasticity for the group 20+ after a regular application of the cream with *Cladophora* extract.

As far as the group of 40+ is concerned, a much smaller increase in skin elasticity was noticed both after the application of Emulsion 1 and Emulsion 2 compared to the group of 20+. After the 4th week of Emulsion 2 application there was observed an increase in skin elasticity up to 0.8378, which refers to 10.59% increase in the parameter, while in the case of Emulsion 1 – up to 0.7870 (3.88%). Obtained values of R_2 were lower for the group 40+ than for the group 20+. The average value at the week 0 for the negative control was 0.7575, whereas for Emulsion 2 after the week 4 – 0.8378. Lower efficiency of applied creams to skin elasticity improvement in the group of 40+ might be due to a weaker abilities of older skin to regeneration.

DISCUSSION AND CONCLUSION

Freshwater algae are not popular research objects as a cosmetics ingredients. Products contain-

ing marine algae dominate on the cosmetic market (11). On the other hand, freshwater *C. glomerata* emerge as a rich source of such bioactive agents as: fatty acids (6, 7), carotenoids, phenolic compounds (9), sulfated polysaccharides (8), sterols, terpenes, carbohydrates, glycosides (7), resulting in its antioxidant (9), antibacterial and antifungal properties (7). Using CO_2 as a solvent in SFE method it is possible to obtain extract from *Cladophora* with high amounts of non-polar bioactive agents, such as fatty acids (6) and carotenoids (9), which may affect as moisturizing, firming and antioxidant ingredients.

The emulsion containing algal extract was slightly less stable than the base emulsion. In order to increase the stability of Emulsion no. 2, a higher amount of thickening agent, i.e. xanthan gum or emollient (methyl glucose sesquistearate) should be added to this formulation. To sum up, results of stability study show that the addition of *Cladophora* extract to the cosmetic emulsion did not affect significantly the emulsion destabilization.

Results obtained for corneometer analysis show that the addition of SFE extract from *C. glomerata* to the emulsion influenced the improvement of skin hydration. Better results were observed for the group of 20+ where the increase in skin hydration was more significant after use of Emulsion 2 than Emulsion 1 after the last week of the experiment (45% improvement in skin hydration). The differences between skin hydration parameters in the group 20+ and 40+ may result from better ability of young skin to regeneration, keeping the adequate level of skin hydration and its superior absorption of the active ingredients from cosmetics. In comparison to the placebo, the application of the cream with *Cladophora* extract resulted in higher moisturizing effect after each week in both groups of volunteers. On the other hand, high increase in skin hydration was also observed after the use of the placebo. It may result from others moisturizing components of the cream base, such as sunflower seed oil and glycerine.

However, in every case the improvement in skin hydration was higher after the use of the emulsion containing the extract. The SC water retention is a crucial factor in keeping the skin supple and flexible and influences skin permeability to molecules. So far, no original research concerning moisturizing properties of the freshwater *C. glomerata* has been published. However, the presence of carbohydrates, specifically sulfated polysaccharides (8), which are humectants (hygroscopic substances able to bind water), may affect moisturizing properties of the alga. On the other hand, the freshwater *C. glomerata* was found as a good source of both saturated and unsaturated fatty acids (FAs) (6), what indicated its antioxidant, antibacterial and antifungal activities (7). In our studies in the case of SFE of *C. glomerata* supercritical CO_2 as a solvent was used which is non-polar. Due to the usage of this solvent it was possible to obtain the extract from *Cladophora* rich in non-polar substances, such as fatty acids. Because of the hydrophobicity of fatty acids and their important role in creating of hydrolipid layer of the skin they act in cosmetics as emollients. Thus, FAs are able to protect skin against the loss of water and in this way they provide the adequate level of skin hydration and prevent its drying (15, 17). This effect was observed in our results of corneometer analysis – the presence of the SFE extract from *Cladophora* in cosmetic emulsion resulted in an increase in skin hydration. In fact, FAs smooth the skin by filling spaces between skin flakes and adding a complementary occlusive activity, which contributes to SC hydration (18).

In contrast to skin hydration, resulted up to 45% increase in the parameter after using Emulsion 2 in the group of 20+, the improvement of skin elasticity was significantly lower (up to 12.28% increase in the same emulsion and group). However, even the little increase in skin elasticity may improve its appearance and condition. It was also observed that the differences in the increase of skin elasticity were greater than for the skin hydration. Hence, these results clearly indicate the firming properties of *C. glomerata* extract. Skin elasticity decreases with age because of harmful external factors, such as free radicals and UV radiation, which cause skin damage resulting in wrinkles. Carotenoids and phenolic compounds contained in extract from *C. glomerata* might affect as anti-aging compounds due to their antioxidant properties (9). Moreover, the observed improvement in skin elasticity could be the effect of fatty acids action as emollients. If the skin is protected from excessive water loss then it is better moisturized, and consequently – soft and flexible (15, 17).

In freshwater macroalga, which comes from the natural habitat, the issues of purity and safety must be addressed. In our previous work, we proved that the alga did not contain harmful quantities of toxic metals (6). Furthermore, it was demonstrated in toxicological studies that freshwater *C. glomerata* is safe for humans (19).

In summary, the stability study proved that the addition of the extract did not affect significantly the destabilization of the emulsion. Moreover, *in vivo* studies using corneometer and cutometer demon-

strated the influence of freshwater *C. glomerata* extract on skin hydration and elasticity improvement in two age groups of 20+ and 40+. Emulsion 2 containing *Cladophora* extract exhibited higher moisturizing than firming properties. However, skin elasticity improvement was more evident for Emulsion 2 than emulsion without algae addition. Thus, the freshwater alga *C. glomerata* can be an effective cosmetic raw material used in a novel moisturizing and firming formulations.

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