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Topical application of *Calendula officinalis* (L.): Formulation and evaluation of hydrophilic cream with antioxidant activity

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The aim of the study was the formulation of suitable medicinal form for the topical application of *Calendula officinalis* L. Dry Calendula extract was used as an active compound. This extract was proved to be an effective scavenger of H₂O₂ radicals in *in vitro* studies with the mitochondria of rat cardiac muscles. Several compositions of the cream base were evaluated and the hydrophilic cream containing complex emulsifier was chosen as the delivery system. Subsequently, Calendula extract was incorporated, and the concentration of extract which provided significant antioxidant effect ($p < 0.05$), has been determined. Antioxidant activity of the cream with Calendula extract was due to the content of carotenoids, polyphenols and flavonoids. Cream with the best properties (0.9% of Calendula extract) contained 0.73 ± 0.04 mg/100 g of total carotenoids expressed as β -carotene. This cream was then examined microscopically, and stability studies including evaluation of organoleptic properties, microbiologic quality, and determination of variations in total carotenoid content during the storage were made. Achieved results suggest that developed cream with Calendula extract poses the good-quality emulsion system warranting the stability of carotenoids, and thus the therapeutic, namely antioxidant activity of preparation.

Key words: *Calendula officinalis*, dry extract, antioxidant activity, hydrophilic cream, preparation, evaluation.

INTRODUCTION

Herbal therapy is becoming increasingly popular among patients and physicians – many herbal preparations are used for various ailments including those of the skin (Bedi and Shenefelt, 2002; Reuter et al., 2010). The traditional practice of topically treating dermatologic conditions with plant-derived medicines predates the cultures of ancient Egypt and remains vital today in the industrialized

countries (Brown and Dattner, 1998). Herbal treatments that have been used for centuries are now being studied scientifically.

Oxygen-centered free radicals known as reactive oxygen species (ROS) play different roles *in vivo*. Some are positive. However, ROS may be very damaging. This oxidative damage is considered to play a causative role in aging and several degenerative diseases associated with it, which often manifest adversely in the skin (Masaki, 2010; Pietta, 2000). Although, human body is naturally equipped with endogenous defense systems

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and their efficiency is incomplete (Rabišková et al., 2009). In conjunction with physiopathological situations (UV radiation, smoking, air pollutants, inflammation etc.), in which ROS are produced, the negative effects increase, and thus the supplementation with antioxidants for diminishing the cumulative effects of oxidative damage is necessary (Barja, 2002; Pietta, 2000). Antioxidants naturally occurring in plants may be employed topically or systemically for minimizing the harmful effects and in preventing and treating pathological and physiopathological conditions related to oxidative stress (Cimino and Saija, 2005; Fonseca et al., 2010; Masaki, 2010; Rabišková et al., 2009; Vaillant et al., 2000). *Calendula officinalis* L. (Asteraceae), also known as pot marigold or common marigold, is the herb of ancient medicinal repute with use in traditional and homeopathic medicine (Četković et al., 2004). Marigold grows as a wild and common garden plant throughout Europe and North America. The dried ligulate florets, the dried composite flowers (*Calendulae flos*) and the dried aerial parts (*Calendulae herba*) of *C. officinalis* collected during the flowering period are used in medicinal products (EMEA, 1999). Pharmacological studies have confirmed that *C. officinalis* exhibit a broad range of biological effects, such as antimicrobial (Attard and Cuschieri, 2009; Radioza and Iurchak, 2007; Roopashree et al., 2008), anti-inflammatory (Amoian et al., 2010; Preethi et al., 2009; Ukiya et al., 2006), immunomodulatory (Attard and Cuschieri, 2009;), antioxidant (Četković et al., 2004; Fonseca et al., 2010), wound healing (Leach, 2008; Preethi and Kuttan, 2009), antiviral (Kalvatchev, 1997) and anti-tumoral (Ukiya et al., 2006). Some of these effects are very interesting for possible future development. Constituents of *C. flos* responsible for mentioned and some other effects include flavonoids, triterpene saponins, free and esterified triterpene alcohols, carotenoids, polysaccharides, sterols and volatile oil with sesquiterpenes (EMEA, 1999; CIREP, 2001; Muley et al., 2009).

This is why was marigold widely used in traditional herbal preparations, and is still the subject of scientific investigations. The main use of preparations from *C. officinalis* is topical. From this point of view constituents responsible for the antioxidant effect are especially important. ROS causes lipid peroxidation of the skin top layer (stratum corneum) which reduces the natural barrier function. Topical antioxidants may protect the skin from oxidative stress, cutaneous photodamage (Morganti et al., 2002; Pinnell, 2003), help correct a variety of skin conditions (Madey and Pinnell, 2001), and are related with anti-aging (Darvin et al., 2006; Vaillant et al., 2000).

Among the marigold constituents with antioxidant properties belong mainly flavonoids and carotenoids (Četković et al., 2004; Pintea et al., 2002). Some carotenoids (e.g. lutein, lycopene, zeaxanthin) are found naturally in human skin (Darvin, 2007). Recent clinical trials have underscored the efficacy of lutein and zeaxanthin (Palombo et al., 2007). These xanthophyll

carotenoids were administered either orally, topically or both. The results obtained indicate that administration of lutein and zeaxanthin provides high degree of antioxidant protection.

Darvin et al. (2008) demonstrated that a correlation exist evidently between the appearance of the skin with regard to furrows and wrinkles, and the level of carotenoid lycopene in the skin. However, the human body cannot synthesize carotenoids itself, thus they need to be obtained systemically or through topical application. Surprisingly, the systemic application of synthetic or extracted antioxidants sometimes showed positive, but sometimes also negative effects on the human organism (Biesalski and Obermueller-Jevic, 2001). The negative results obtained by the systemic application of antioxidant substances seem to be caused by the application of only a single-component at relatively high concentration during medical treatment (Lowe et al., 1999). The topical application of antioxidants is less critical, because of the small penetration depth of formulations into the skin and the absence of direct contact with living cells (Darvin, 2007).

The literature shows that antioxidant substances of the living organism always act as a „protection chain“, that is, different antioxidant substances possess a synergic effect and protect each other from direct destruction in the reactions of neutralization of the free radicals and other reactive species (Eichler et al., 2002; Wrona et al., 2003).

Consequently, application of mixture of antioxidant substances at low concentrations seems to be more effective for the protection of skin. Medicinal plants, partly *C. officinalis*, provide such material. Topical application of medicinal plants requires the suitable medical form. Thus the aim of our investigation was the formulation of such medical form, concretely, preparation and evaluation of hydrophilic cream with Calendula extract.

MATERIALS AND METHODS

Chemicals and excipients

The chemicals and samples were available commercially and were used as received: gallic acid, Folin-Ciocalteu reagent (Fluka), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercetin (Sigma). All other chemicals used were of analytical grade. Pharmaceutical excipients used for preparation of cream such as mineral oil (*Paraffinum liquidum*), white petrolatum (*Vaselinum album*), stearic acid (*Acidum stearicum*), glycerol monostearate (*Glyceroli monostearas*), carbomer 934P (*Carbomerum*), triethanolamin (*Trolaminum*) and purified water (*Aqua purificata*) were of Ph. Eur. quality. Dry Calendula extract 5:1 (*Extractum Calendulae*) was received from Naturex (France).

Assessment of H₂O₂ production in Calendula extract water solution

Generation of reactive oxygen species (ROS) by mitochondria was estimated as the release of H₂O₂ fluorimetrically (Thermo Scientific fluorometer) using Amplex Red assay for H₂O₂. Mitochondria

Table 1. Composition of the cream bases and creams with Calendula extract.

Part	Compound	Formulation (% weight)			
		Cream base			
		F1	F2	F3	F4
A	<i>Vaselinum album</i>	12	12	12	12
	<i>Paraffinum liquidum</i>	12	12	12	12
	<i>Glyceroli monostearas</i>	-	4.3	-	4.3
	<i>Acidum stearicum</i>	7.3	5	7.3	5
B	Trolaminum	2	2	2.5	2.5
	<i>Aqua purificata</i>	30	30	30	30
C	Carbomerum	0.2	0.2	0.2	0.2
	<i>Aqua purificata</i>	36.5	34.5	36	34
D	Calendula extract	-	-	-	-
	<i>Aqua purificata</i>				

(0.25 mg/ml) were incubated at 37°C in the standard medium (20 mM imidazole, 20 mM taurine, 0.5 mM dithiothreitol, 1.6 mM MgCl₂, 100 mM MES, 3 mM KH₂PO₄, 3 mM CaK₂EGTA, and 7.1 mM K₂EGTA), which was supplemented with pyruvate and malate (6 mM each) plus antimycin A (1 µM) – control sample, and with 0.13, 0.26 or 0.39 mg/ml of Calendula extract – treatment. After incubation, Amplex Red (10 µM) and horseradish peroxidase (1 U/ml) were added, and fluorescence (excitation at 544 nm, emission at 590 nm) was measured. Amplex Red fluorescence response was calibrated by adding known amounts of H₂O₂.

Preparation of cream base and cream with Calendula extract

The composition of the cream bases and the creams with Calendula extract is shown in Table 1. Parts A and B were heated separately to 70°C. Part C compound carbomer was previously spread in a thin layer on the water surface, kept for 8 h until it was dissolved, and subsequently heated to 70°C. Part A was added to Part B with stirring, and Part C was added afterwards. The mixture was continuously stirred until the temperature of 35°C was reached. Then Part D was added (if the cream with Calendula extract was prepared), mixed properly, and the cream was cooled with stirring to room temperature. Prepared creams were storage at the room temperature (25 ± 2°C) in the tightly closed plastic containers protected from light.

Preparation of cream extracts for determination of total polyphenols, flavones and flavonols

Creams were extracted 6 times by obtaining 0.50 g of the product and 10 ml of the extractant, a 96% (V/V) ethanol and isopropanol mixture at the ratio of 8:2 (V/V). The resulting extracts were mixed, and the extractant was added to reach 100 ml volume. Three such extracts were produced from the cream base and from each cream.

Total polyphenol content

Total polyphenol content was determined using colorimetric method. 2.0 ml of the prepared extract was oxidized using Folin-Ciocalteu reagent (400 µl), and sodium carbonate solution (75 g/l) was then added to the reaction mixture to reach a 10.0 ml volume. After 2 h, the suspension was centrifuged for 10 min at 5000 rpm,

and absorption was measured at a 760 nm wavelength. The amount was calculated using the gallic acid calibration curve. The results were expressed as gallic acid equivalent (GAE) mg per 100 ml of the sample (extract).

Total flavone and flavonol content

Flavones and flavonols contents were analyzed by the colorimetric method. 9.8 ml of the prepared extract was mixed with a 10% solution of aluminum chloride (200 µl). After 30 min, absorption was measured at a 425 nm wavelength. The amount was calculated using quercetin calibration curve. The results were expressed as the quercetin equivalent (QE) mg per 100 ml of the sample (extract).

DPPH reduction

The free radical scavenging ability was investigated using stable 2,2-diphenyl-1-picrylhydrazyl phosphate (DPPH) radical. The prepared extract (400 µl) was replenished up to 2.0 ml with 0.1 mM of DPPH methanol solution, and absorption was measured after 30 min at a 517 nm wavelength. DPPH reduction was calculated when taking into account the absorption of the control investigation, and the observed activity was compared to quercetin calibration curve. The results were expressed as quercetin antioxidant activity equivalent (QE) µmol per 100 ml of the solution (extract).

Differential centrifugation

The resistance of the formulated creams to centrifugation was tested in a 20 ml-graduated cylinder at 10,000 rpm for 1 - 20 min at different temperature conditions, such as 4, 25, and 45°C, using a centrifuge Ependorf 5810-R (Sigma, Germany) as described by Cockton and Wynn (1952).

Microscopic examination of the cream

The particle size of the internal phase was measured using an optical microscope (Lambda, Ltd.), which was fitted with a camera and computer software (LECO Corp., Michigan, USA) for image

analysis transmitted on the monitor. The microscopic samples were prepared by spreading a very thin layer of the cream on the specimen slide and pressing it well with a cover slip. Three preparations were made by taking small amounts of the cream from different places. The preparations were observed under passing light. At first preparations were inspected under 100-fold magnification, and measurements were performed under 400-fold magnification. In each of the three preparations, 100 particles were measured. In addition to that, photographs of the preparations were made during microscopy. On the basis of measurements the degree of dispersity was calculated by computer software.

Total carotenoid content

6.0 g of the cream was placed into a small flask, where 20 ml of petrol ether was added, and the resulting mixture was agitated for 5 min. Subsequently, the sample was filtered into a 25 ml measuring flask and diluted up to the mark. The extinction value of the resulting yellow-colored solution was measured spectrophotometrically at a 440 nm wavelength in a 1 cm-thick cuvette. The reference solution was petrol ether. At the same time the extinction value in a standard sample of potassium bichromate was measured. Standard potassium bichromate solution contained 0.0900 g of potassium bichromate in 250 ml of purified water. 1 ml of this solution corresponds to 0.00208 mg of β -carotene. Total carotenoid content was expressed as β -carotene (mg/100 g of cream), and calculated using the following formula:

$$X = \frac{0.00208 \times A \times 25 \times 100}{A1 \times a} \quad (1)$$

Where,

A – Extinction value of the studied solution

A1 – Extinction value of the standard solution

a – Amount of the cream, g

Organoleptic evaluation

The organoleptic features of the cream were examined at the same temperature ($25 \pm 2^\circ\text{C}$), lighting and humidity (60% RH) conditions to assess variations in appearance, phase separation, colour, or smell. Samples were evaluated after 1, 7, 14, 30 days of preparation, then each month for 12 months after preparation.

Chemical stability

The chemical stability of the creams with Calendula extract was evaluated on the basis of changes in total carotenoid content during the storage at the temperature of $25 \pm 2^\circ\text{C}$. Spectrophotometrical measurements (as mentioned above) were made in time intervals of 3 months, that is, 3, 6, 9 and 12 months after preparation.

Microbiological quality and stability

The cream with 0.9% of Calendula extract was evaluated immediately after preparation and after elapse of one year of storage at the temperature of $25 \pm 2^\circ\text{C}$. Microbiological examination was made in accordance with requirements of European Pharmacopoeia for non-sterile products (Eur., 2009a; b; c).

Statistics

Data are presented as means \pm S.D. Nonparametric methods were applied for making inferences about the data. Differences between mean values in dependent groups were tested using Wilcoxon matched pairs test. Differences between mean values in independent groups were tested using nonparametric Kruskal-Wallis test with Dunns post-hoc evaluation. $p < 0.05$ was taken as the level of significance. Statistical analysis was performed by using the software package Statistical 1999, 5.5 StatSoft Inc., USA.

RESULTS

At the beginning of the experiment we tried to determine the concentration of Calendula extract that would have acceptable antioxidant effect. Investigations were performed by applying fluorimetry with the use of mitochondria of rat cardiac muscles. All concentrations of Calendula extract had the influence on H_2O_2 production in mitochondria (Figure 1) – the antioxidant activity increased with an increasing amount of extract, but this activity in case of the lowest concentration of extract was not statistically significant.

Formulation of the cream with Calendula extract started from the cream base. Four compositions (F1 – F4) varying in the type and amount of emulsifier were prepared (Table 1). Compositions containing only one emulsifier (F1 and F3) – triethanolamin stearate (type o/w – oil in water) formed *in situ* from stearic acid and triethanolamin – were not stable. Addition of glycerol monostearate (type w/o – water in oil) resulted in formation of the suitable emulsion system (F2 and F4), where the composition F4 was the most stable, and was chosen as the base for incorporation of three concentrations of Calendula extract.

The cream base F4 and creams with Calendula extract were evaluated chemically – total carotenoid content, total polyphenol content, total flavone and flavonol content and DPPH reduction were determined (Table 2). In case of the cream with 0.3% of Calendula extract was content of the active constituents low and antioxidant activity weak – the values did not differ from these of the cream base. Measured values of the active compounds in the cream with 0.9% of Calendula extract differed statistically significant ($p < 0.05$), therefore, this cream was chosen for further microscopic evaluation and stability testing.

Conventional emulsion preparations differ as polydisperse, that is, they always contain a distribution of droplet sizes (McClements, 1999). Therefore, the knowledge of the average size of the particles and the width of the distribution is often sufficient. The number of droplets (particles of internal phase) in most emulsion preparations is extremely large, and so their size can be considered to vary continuously from some minimum value to some maximum value. The particles size of our preparations varied from 0.132 - 7.352 μm with mean diameter of 1.13 μm (cream base F4) or 0.13 - 8.48 μm

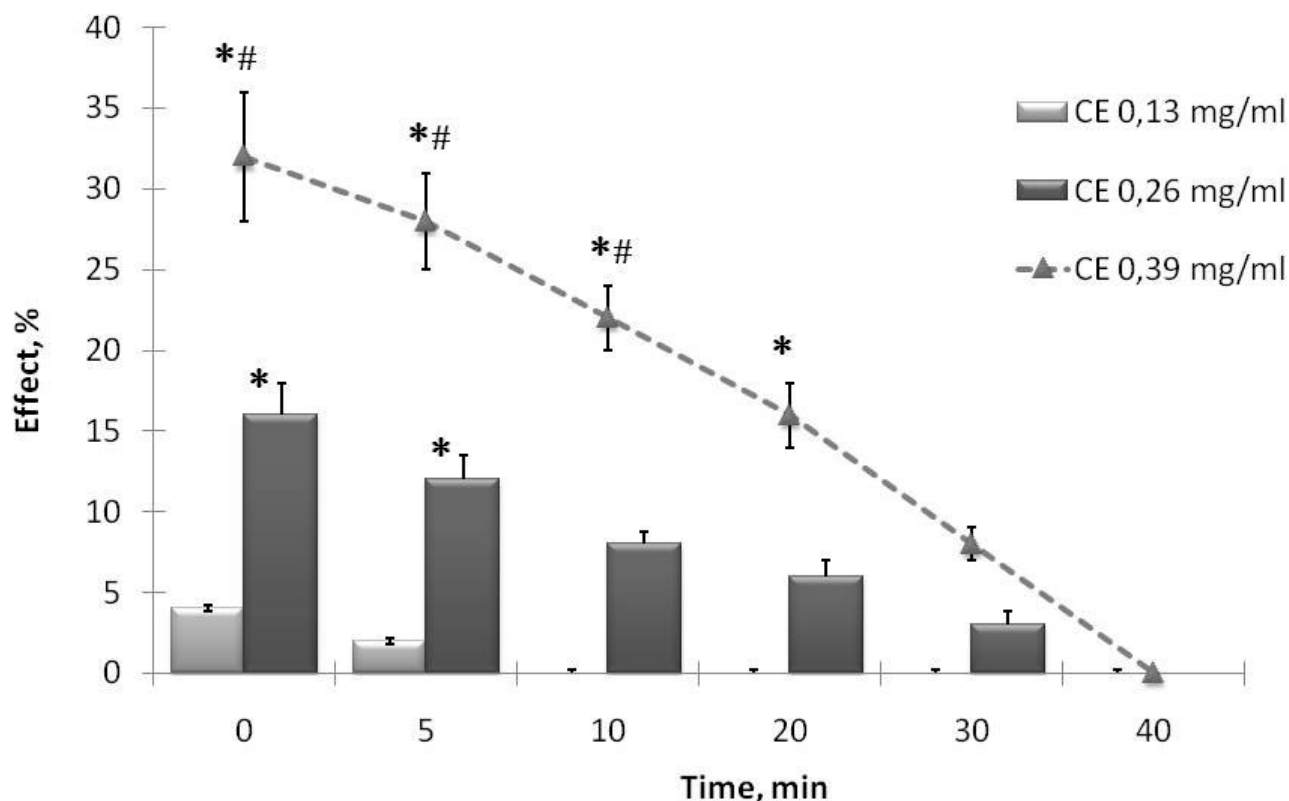


Figure 1. The effect of different concentrations of Calendula extract (CE) on the free radical generation in mitochondria. * $p < 0.05$ vs the control, # $p < 0.05$ vs CE 0.13 mg/ml, CE 0.0.26 mg/ml, Supplements were added in the following order: mitochondria (0.25 mg/ml) incubated in the standard medium, 6 mM pyruvate+6 mM malate; 1 μ M antimycin A, Calendula extract (0.13, 0.26 or 0.39 mg/ml), 10 μ M Amplex Red and 1 U/ml horseradish peroxidase.

Table 2. Content of the main active constituents and antioxidant activity of the creams with Calendula extract.

Concentration of Calendula extract in the cream (%)	Total carotenoid content (mg/100 g)	Total polyphenol content (GAE mg/100 ml) ^a	Total flavone and flavonol content (QE mg/100 ml) ^b	DPPH reduction (QE μ mol/100 ml) ^c
0.3	0.26 \pm 0.01	< 50	< 1	< 50
0.6	0.49 \pm 0.03	64 \pm 4	3.4 \pm 0.32	102 \pm 7*
0.9	0.73 \pm 0.04	92 \pm 3*#	4.2 \pm 0.21*#	163 \pm 9*#
Cream base F-4	-	< 50	< 1	< 50

^a data expressed as gallic acid equivalent (GAE) mg per 100 ml of the cream extract (0.5 g of the cream), ^b data expressed as quercetin equivalent (QE) mg per 100 ml of the cream extract (0.5 g of the cream), ^c data expressed as quercetin equivalent (QE) micromoles per 100 ml of the cream extract (0.5 g of the cream), * $p < 0.05$ vs cream base; # $p < 0.05$ vs cream with 0.3 and 0.6% of Calendula extract. Values are means \pm SD of triplicate determinations.

with mean diameter of 1.105 μ m (cream with 0.9% of Calendula extract). Just these results predict the good quality of prepared creams.

Organoleptic evaluation of creams was maintained 12 months. Immediately after preparation all formulations were homogenous. The cream base was white and glossy. The addition of the Calendula extract to the preparations resulted in the formulations with characteristic appearance, color and smell. The cream with 0.9% of Calendula extract remained during the

storage without the changes in color, surface appearance or phase separation. Determination of total carotenoid content during the storage demonstrated, that prepared creams were chemically stable – deviation amplitude in all measurements (comparison of total carotenoid content immediately after preparation with contents during storage) was within the range of 5%.

Microbial quality of the cream with Calendula extract was satisfactory and met the requirements of European Pharmacopoeia (Eur., 2009a) – neither immediately after

preparation or after 12 months of storage contained creams any *Pseudomonas aeruginosa* and *Staphylococcus aureus* and not more than 10^4 micro-organisms (aerobic bacteria plus fungi) per 1 g of cream were found.

DISCUSSION

The cells of the skin, similarly to the cells of the heart or another organ, contain very large numbers of mitochondria that produce energy during the respiration process (Rojas-Urdaneta and Poleo-Romero, 2007). The main physiological function of mitochondria is production of ATP but it includes also the control of cell survival and death (Bras et al., 2005; Pedersen, 2009). The activation of the respiration and energy production of mitochondria processes strengthens the protective function of the skin, decreases transepidermal moisture loss, and improves skin regeneration (Konig et al., 2006). Therefore, the investigations based on affecting of mitochondrial functions are of great importance. In their experiment, the fluorimetric measurements demonstrated (Figure 1) that 0.26 and 0.39 mg/ml of Calendula extract significantly decreased H_2O_2 production in mitochondria – extract was proved to be an effective scavenger of H_2O_2 radicals in *in vitro* studies. The highest amount of radicals was scavenged by Calendula extract (CE) on the first minute. Later on, the antioxidant activity of extract was decreasing, which may be related to the aging of the mitochondria (Burns and Richter, 2008). There are scientific findings indicating that the metabolic activity of mitochondria and the capacity of ATP synthesis decrease with time (Barja, 2002; Prahil et al., 2008). Obtained results were applied for determination of suitable concentration of Calendula extract in hydrophilic cream.

Hydrophilic creams were chosen for incorporation of Calendula extract because of the range of positive properties. As the emulsions o/w with a hydrophilic external phase, they are miscible with water and skin secretions and thus are easily removed from skin or clothing (Betageri and Prabhu, 2002). They are not occlusive because they leave little residue on the skin. Properly designed o/w creams are elegant systems, pleasing in both appearance and feel post application (Flynn, 2002). They are good for most topical purposes and are quite acceptable from a cosmetic viewpoint. Choice of the cream type was influenced by the results of Getie et al. (2002) that hydrophilic cream provides the highest release of flavonoids in comparison with amphiphilic or lipophilic one. Hydrophilic cream was also used for incorporation of green tea polyphenols with the aim of prevention of UVB-induced oxidation of lipids and proteins in mouse skin (Vayalil et al., 2003). Data obtained from this *in vivo* study suggest that topical application of such preparation provides greater photoprotective effect than peroral solution, and could be useful in human skin care. So, hydrophilic cream may

serve as an optimal delivery system for such plant preparations containing polyphenols and flavonoids as Calendula extract is.

At the beginning of the study, different cream bases (F1 – F4) were prepared (Table 1). Taking in consideration, that the product was believed to be the type of o/w emulsion, was cream stabilized with only emulsifier o/w (triethanolamin stearate forming *in situ* during the process of preparation) (F1 and F3) or with complex emulsifier: primary – triethanolamin stearate (o/w) and secondary – glycerol monostearate (w/o) contained in lipophilic phase (F2 and F4). Test with coloring agents (Sudan III, methylene blue) and electrical conductivity test confirmed the development of hydrocream. The cream was additionally stabilized by increasing viscosity of external phase emulsion with carbomer gel formed *in situ* by neutralization of carbomer with triethanolamin during preparation. The stability of prepared bases was evaluated by centrifugation for 1 - 20 min at 4°C. The formulations that were resistant were selected for further evaluation at higher centrifugation temperatures of 25 and 45°C.

Cream bases with only one emulsifier were markedly unstable – separation of aqueous and oil phases was observed after 3 (F1) or 6 (F3) minutes of centrifugation at the lowest temperature. Formulation F2 with complex emulsifier was more stable – separation of phases occurred after 8 (4°C) or 4 min (25°C). The most resistant to centrifugation was cream base F4, which remained stable during 20 min at all of temperatures (4, 25 and 45°C). Observed different resistance of the formulated creams to centrifugation accords with the data from literature that from the stability standpoint, the addition of an emulsifying agent is critical to formulation of emulsion, and that hydrophilic creams with complex emulsifier have the better quality than such stabilized with o/w type emulsifier only (Betageri and Prabhu, 2002; Marquell et al., 2006). The better stability of cream base F4 may probably be related with the greater amount of triethanolamin (2.5 vs 2.0% in F2). Triethanolamin in our formulations had double task – formation of the primary emulsifier (triethanolamin stearate) and neutralization of polyacrylic acid (carbomer), thus forming the gel and increasing viscosity of external phase of the cream. Increased viscosity of the continuous (external) phase enhances stability of emulsion preparation by retarding the movement of droplets (particles of internal phase) (McClements, 1999). Concentration of 2% was evidently too low for both two tasks, hence was base F2 less quality. The centrifugation test, if it is carried out during the formulation process of semisolid emulsion preparations, provides fast information about probable behavior of these preparations during storage. In our previous study (Bernatoniene et al., 2010) creams resistant to centrifugation at the most exacting conditions remained stable for all the time of investigation.

Determination of the active compounds in the creams

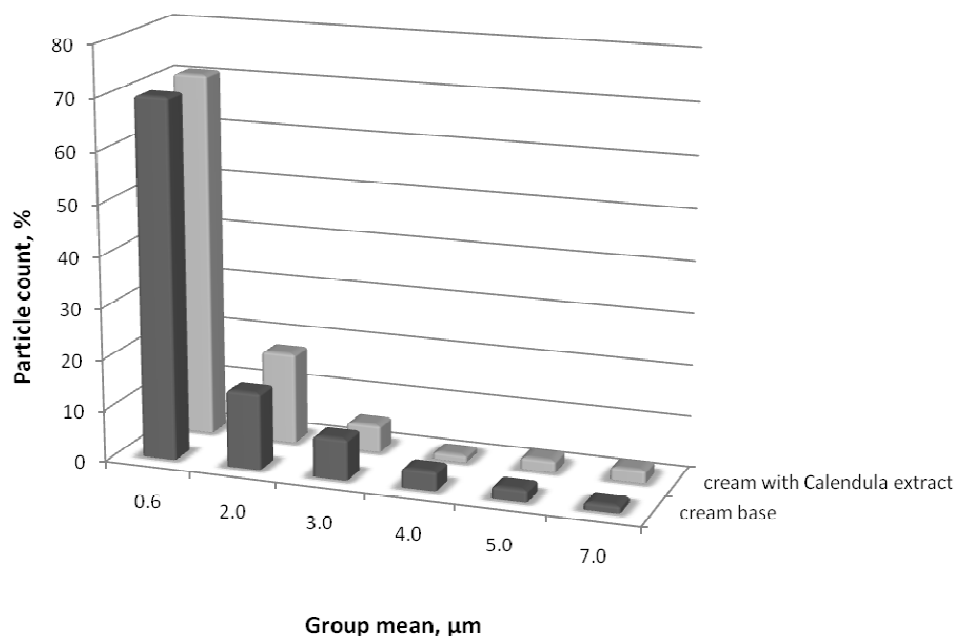


Figure 2. A comparison of the internal phase particle size in the cream base and cream with Calendula extract.

with different concentrations of Calendula extract (Table 2) give evidence that the cream with the highest concentration of extract (0.9%) contained enough constituents to be able to scavenge free radicals – DPPH reduction was statistically significant ($p < 0.05$). Observed antioxidant activity was due to the content of carotenoids, polyphenols and flavones and flavonols (a group of phenolic compounds), which correspond with numerous scientific reports (Četković et al., 2004; Darvin, 2007; Masteikova et al., 2009; Pieta, 2000). Some of these compounds are able to permeate through the stratum corneum (which is the main barrier against the penetration of exogenous substances through the skin) and, so, to penetrate into deeper skin layers (Bonina et al., 1996; Darvin, 2007; Kim, 2007). Consequently, preparations with plant extracts rich in the above active constituents, such as Calendula extract in their experiment, may provide the wide range of therapeutic, protective or preventive effects (Freedman, 2009; Fuchs et al., 2005; Hadfield et al., 2008; Pommier et al., 2004; Roveroni-Favaretto et al., 2009), and thus the development of topical formulations based on *C. officinalis* is justified.

Particle (droplet) size and particle size distribution (degree of dispersity) are very important characteristics of the emulsion system, because they indicate quality and stability of the preparation as well as the effectiveness of manufacturing process and the influence of the emulsifier on the quality of the emulsion (Marquell-Oliveira et al., 2007; McClements, 1999). Stokes's law states that the velocity at which a droplet moves is proportional to the

square of its radius. The stability of an emulsion can therefore be enhanced by reducing the size of the droplets. In general, both small oil droplets (lipophilic particle in o/w cream) and high viscosity contribute to the stability of the emulsion (Huang et al., 2001). Mean particle size of the preparations was below $2 \mu\text{m}$, what itself means the suitable quality of the prepared creams. However, when presenting the particle size data, it is convenient to divide this size range into a number of discrete size classes and the number of droplets that fall into each class (McClements, 1999). In this case, all measured particles were divided into 6 classes (Figure 2). As the graph illustrates, the major part of the cream particles (about 70%) fell into the first class with group mean of $0.60 \mu\text{m}$, which confirms a high quality of the prepared hydrocreams.

The results of a comparison of the cream base (F4) with the cream containing 0.9% of Calendula extract suggest that both of them were of the similar quality – the difference of droplet number in the class between these creams was minimum (Figure 2), that is, in the cream base there were less particles up to $1 \mu\text{m}$ than in cream with Calendula extract (69.7 vs 71%), and the total number of particles up to $3 \mu\text{m}$ was slightly less (92.7 vs 94%). These small differences, however, may be due to the subjective character of the measurement. Microscopic images of the cream with Calendula extract are presented in photographs (Figure 3). When the preparation was magnified 100-fold, a very homogenous, uniform picture was seen. At a 400-fold magnification, the majority of particles were very small, a few larger



Figure 3. Microscopic image of the cream with Calendula extract under 100-fold (A) and 400-fold (B) magnification.

particles, and no particles greater than 10 µm were found. The degree of dispersity (particle size distribution) was 1.64×10^4 and $1.33 \times 10^4 \text{ cm}^{-1}$, respectively. The obtained results show that both the cream base and cream with Calendula extract belong to stable emulsion systems.

Carotenoids belong to the main constituents responsible for the therapeutic activity of *C. officinalis*. Unfortunately, the same structural attributes of carotenoids that are thought to impart health benefits also make these compounds highly susceptible to oxidation and degradation (Boon et al., 2010). The stability of carotenoids is dependent on various factors such as nature and composition of the product, processing treatments, packaging and storage conditions (Dutta et al., 2005). Thus, the variation of the carotenoid content in

the cream with Calendula extract during the storage may be very significant quantitative indicator of the cream chemical stability. Total carotenoid content in our cream recorded during the storage just a little decrease – from $0.73 \pm 0.04 \text{ mg/100 g}$ of the cream immediately after preparation to $0.70 \pm 0.01 \text{ mg/100 g}$ at the end of the observation (12 months after preparation). This decrease is under 5% meaning the suitable stability of carotenoids in the prepared cream. Such stability may be due to the composition of cream base (Table 1), in which the lipophilic phase of the cream (Part A) contained white petrolatum, mineral oil and stearic acid, which fell into the group of saturated compounds. The hypothesis establishes the study of Bezbradica et al. (2005), where the stability of carotenoids in marigold oil extracts were studied with conclusion that preparations with oils that

contain only saturated compounds (e.g. paraffin oil) show the highest stability of carotenoids. The importance of auxiliary substances in quality of topical semisolid preparations emphasized also Semkina et al. (2005).

Microbial quality of the cream determined at the end of observation (12 months after preparation), which met the requirements of European Pharmacopoeia, proved that the antimicrobial efficiency of Calendula extract in concentration of 0.9% was sufficient to preserve the preparation against micro-organism contamination.

Conclusion

As the result of the experiment suitable medical form for the topical application of *C. officinalis* was developed. Performed examinations of prepared hydrophilic cream with Calendula extract proved the significant antioxidant activity and suitable chemical and microbial stability. As the preparation containing the mixture of antioxidant substances in relatively low concentrations, this cream may be sufficient for the regular topical application as the effective long term protection of the skin against ROS caused damage.

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