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BILBERRY ANTHOCYANINS - POSSIBLE APPLICATIONS IN SKINCARE PRODUCTS

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Abstract: *Vaccinium myrtillus* fruits are a significant source of anthocyanins and have been linked to a number of health advantages. Recent data, however, point towards the possible benefits of topical use for anthocyanins. The purpose of this study was to assess the antioxidant potential of two extracts obtained through maceration. Total anthocyanin content and HPLC-DAD qualitative analysis were completed prior to include the extract in a cream-type topical formulation. The extract with the highest anthocyanin content was incorporated in a basic O/W cream formulation and the antioxidant effect of the cream was evaluated using the DPPH radical scavenging assay. The extract's stability seven months later was also assessed. Our findings suggest that, the cream formulation offers antioxidant activity, but the activity declines by 27% when it is stored. Additionally, after seven months of storage, the formulation's color changed, probably as a result of the anthocyanins' instability.

Keywords: bilberry, antioxidant, natural cosmetics, anthocyanins, face cream formulation

1. Introduction

Vaccinium myrtillus L, or bilberry (**Fig. 1.**), belongs to the Ericaceae family, order Ericales. Fresh bilberry fruits (*Myrtilli fructus recens*), contain high concentrations of anthocyanins (Mikulic-Petkovsek et al., 2015). In addition to anthocyanins, bilberry fruits also contain 7-10% tannins (determined for dried fruits). Due to the tannin content, dried bilberry fruits (*Myrtilli fructus siccus*) are used as antiseptics and antidiarrheals in Romanian traditional medicine. The fruits also contain other polyphenolic compounds, glucids, pectins, and ascorbic acid (EMA-HMPC 2015; Gaspar et al., 2021). The physiological roles that anthocyanins play inside the plant are multiple: mediation of the response to oxidative stress through their antioxidant capacity, protection of plants from ultraviolet rays and other mechanical threats from the environment (Ma et al., 2021; Saigo et al., 2020).

The popular use of anthocyanins extracted from bilberry fruits is that of vasoprotective of capillaries, especially at the ocular level, but the extracts are also used in vascular complications induced by diabetes, as we have demonstrated in

our previous preclinical study, on diabetic Wistar rats (Ştefănescu (Braic) et al., 2018).

Anthocyanins also have a protective role in cardiovascular diseases, cancer, diabetes, etc, proved *in vitro* as well as *in vivo* in preclinical and clinical studies (Neamtu et al., 2020).

Recent years have seen consumers become more knowledgeable about the substances found in skincare products attributable to the Internet, and they frequently choose the purest, most "clean" versions with the fewest ingredients to run the lowest possible risk of skin sensitivity or allergy (Ahmed et al., 2020; Boon, 2020).

Flavonoid-containing fruits that prove photoprotective potential through various direct or indirect mechanisms have been called "*green sunscreens*" (Nunes et al., 2018). The fact that anthocyanins have antioxidant capabilities, that they can mediate the response to oxidative stress with the potential to prevent or delay the oxidation of lipids, proteins, and DNA, has led to a dizzying increase in the number of studies (Choi et al., 2016; Sarkar et al., 2014). These effects can protect the skin from the damage caused by environmental stressors such as UV radiation, pollution, and oxidative stress (Mattioli et al., 2020). Also, due to their multiple effects, anthocyanins can improve the appearance of the skin by reducing the appearance of fine lines, wrinkles, and age spots (Tsuda, 2012). However, studies focused on the effects of *Vaccinium myrtillus* anthocyanins on the skin are very limited. For this reason, the present work can contribute to the completion of knowledge regarding the behavior of the extract in a cosmetic product. Although the information that bilberries are among the best sources of anthocyanins with major health benefits is not current, research data in the dermato-cosmetic field is still scarce.

2. Materials and methods

Plant material

Vaccinium myrtillus fruits were collected in July 2020 from the spontaneous flora of Călimani Mountains, Mureș County, Romania. The fruits were immediately transferred to the laboratory. A sample of the herbal product was deposited at the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș (voucher specimen: FS-VM-19-20).

Chemicals and reagents

Cyanidin chloride and keracyanine chloride (Cyanidin-3-*O*-rutinoside chloride) were purchased from Carl Roth GmbH (Karlsruhe, Germany).

Fig. 1. *Vaccinium myrtillus*

Kuromanin chloride (Cyanidin 3-O-glucoside chloride) was purchased from PanReac (Barcelona, Spain), and delphinidin 3-O-rutinoside chloride, delphinidin chloride, 1,1-Diphenyl-2-picrylhydrazyl (DPPH•) were purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). All solvents used in determinations were of HPLC grade. Purified water was obtained using a Milli-Q system from Millipore (Bedford, MA, USA).

Extract preparation

Two types of extracts were performed. The fresh fruits were macerated for 24 hours at room temperature with a mixture of either glycerin: ethanol (1:2) → BF1 extract or with glycerin: water (3:1) → BF2 extract.

Quantitative determination of anthocyanins

Total anthocyanin content was determined according to the method described in the European Pharmacopoeia 10th edition in the monography *Bilberry fruits, fresh*, with slight modifications. Briefly, 1 mL of extract was diluted (50x) with a 0.1% HCl solution. The absorbance was read at 528 nm and the concentration was calculated using the specific absorbance of cyanidin 3-O-glucoside at this wavelength. The results were expressed as cyanidin 3-O-glucoside equivalents (CGE) / 100 g fruits (*European Pharmacopoeia*, 2019).

Identification of individual anthocyanins through HPLC-DAD

Identification of anthocyanins was performed using a modified method from the European Pharmacopoeia 10th edition (*European Pharmacopoeia*, 2019). HPLC analysis was performed on a Merck HPLC system equipped with a quaternary pump Merck Hitachi L-7100, an L-7200 autosampler Merck Hitachi and a L-7360 column thermostat. Chromatographic separation of anthocyanins was performed on an Inertsil ODS-3, 3m, 150 x 4.6 mm (GL Sciences) column. The mobile phase consisted of a mixture of A - anhydrous formic acid: water (8.5:91.5, *V/V*) and B - anhydrous formic acid: acetonitrile: methanol: water (8.5: 22.5: 22.5: 41.5, *V/V/V/V*). The following multilinear gradient was applied: 95-75% A (0-14 min), 75-35% A (14-16 min), 35-0% A (16-17 min), and 0% A (16-20 min). The flow rate was set at 1.0 mL min^{-1}, the injection volume was 10 µL, and the detection wavelength was 535 nm. Stock solutions of 0.3 mg/mL were prepared for delphinidin chloride and of 0.5 mg/mL for cyanidin 3-*O*-rutinoside, delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside, and cyanidin chloride. Prior to the injection, all samples were filtered through a 0.45 µm microporous cellulose syringe filter and transferred in HPLC vials. Identification of the peaks was based on the comparison of retention times of peaks in sample chromatogram and UV spectra with those of the standards.

DPPH· radical scavenging assay

Antioxidant activity was evaluated using a spectrophotometric method (Nisca et al., 2021). Briefly, 2.5 mL DPPH solution was mixed with different concentrations of the extract. The samples were mixed and allowed to stand at room temperature, in the dark, for 30 minutes. The absorbance was read at 517 nm. The inhibitory concentration (IC%) was calculated using the following formula: inhib%= [(A0-A1)/A0]*100. Ascorbic acid was used as the positive control. The concentration that inhibits 50% of the DPPH activity (IC_{50}) was calculated by plotting the inhibitory concentration versus the concentration on the *x*-axis (Laczkó-Zöld et al., 2018).

Cream base formulation

An oil in water (O/W) common cream base was prepared using *Prunus amygdalus* dulcis

oil, cetearyl olivate, sorbitan olivate, cetyl alcohol, ultrapure water, glycerin, benzyl alcohol, salicylic acid, and sorbic acid. The O/W formulation was prepared by the addition of the aqueous phase into the oily phase with continuous agitation. The proportion of the oily phase to aqueous phase was 27: 73. The bilberry glycero-alcoholic extract, in a concentration of 10% was added at the end. The antioxidant activity of the cream was determined using the DPPH radical scavenging assay imediately after preparation and after 7 months of storage at room temperature in air-tight containers.

Data analysis

Results were expressed as Mean ± Standard Deviation (SD) of three independent experiments for each determination. Data analysis was performed using GraphPad Prism® version 9. Student *t* test was used to compare the differences between two means. One-way ANOVA followed by posthoc Tukey test was used to compare the differences between samples. A value of *p* less than 0.05 was considered significant.

3. Results and discussion

The average anthocyanin content determined for the two extracts was higher in the glycero-alcoholic extract (**Fig. 2.**) with an average of 218.3 mg CGE/100 g herbal drug, compared with the macerate obtained with glycerol-water with an average of 187.6 mg CGE / 100 g. Considering these results, the glycero-alcoholic extract was further used in the cream formulation.

Vaccinium myrtillus is one of the best sources of anthocyanins, and until now there have been identified 15 different anthocyanins in the fruits. The identified anthocyanins are mainly glycosides of cyanidin, malvidin, peonidin, petunidin, or delphinidin (Lätti et al., 2008). Anthocyanins are a group of water-soluble pigments widely distributed in plants, with important therapeutic effects, such as antioxidant, anti-inflammatory, and anti-carcinogenic activities (Luca et al., 2020; Tena et al., 2020). The molecular structure of anthocyanins consists of a flavylium cation and one or more glycosyl groups attached to the phenolic hydroxyl groups (Hăncianu & Gîrd, 2020). The stability, bioavailability, and physiological activity of anthocyanins depend on various factors, such as pH, temperature, light, and enzymatic and chemical reactions (Dossett et al., 2011; He & Giusti, 2010). There is a significant variance in anthocyanin level and composition among bilberry populations, and is usually influenced by numerous factors, such as altitude, soil, precipitations and sun exposure (Lätti et al., 2008). It is thus very important that qualitative and quantitative determinations to be performed prior any preclinical and clinical studies.

Although there are many types of *Vaccinium* berries on the market, such as *Vaccinium corymbosum*, or *Vaccinium angustifolium*, the highest content of anthocyanins is found in *Vaccinium myrtillus* fruits, with 60-70% more than in the species mentioned above (Ștefănescu et al., 2017).

In the present study, a comparison with the anthocyanin standards mixture shown in **Figure 3A** revealed the presence of cyanidin 3-*O*-glucoside (Rt = 5.23 min) and delphinidin (Rt = 6.24) in the bilberry extract (**Fig. 3B**).

As it was expected delphinidin and cyanidin rutinoside were not present in the extract, because the anthocyanidins found in bilberries can be combined with three sugar moieties: glucose, galactose and arabinose. The presence of anthocyanins with rutinoside as a glycoside, is an indicator of adulteration of the sample with other berries (Govindaraghavan, 2014; Lätti et al., 2008).

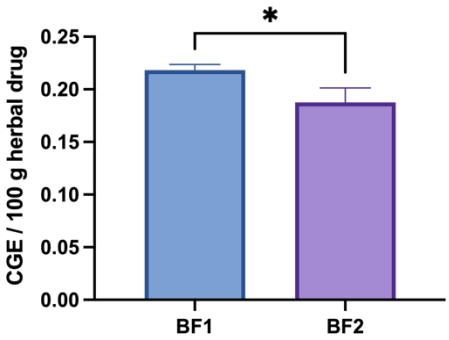

Fig. 2. Anthocyanin concentration in the extracts (*statistically significant difference at $p < 0.05$)

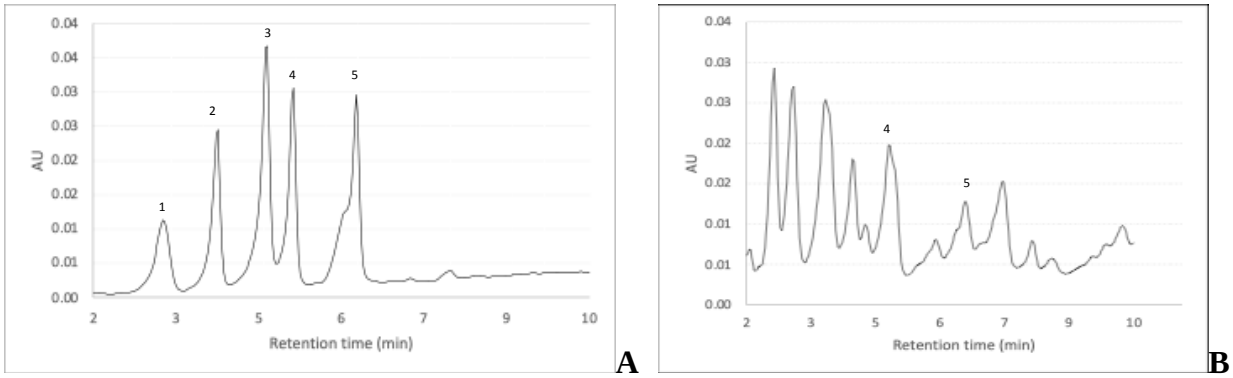

Fig. 3. Representative chromatograms of the standard mixture (**A**) and bilberry extract (**B**) 1 - cyanidin, 2 - delphinidin 3-O-rutinoside, 3 - cyanidin 3-O-rutinoside, 4 - cyanidin 3-O-glucoside, 5 - delphinidin

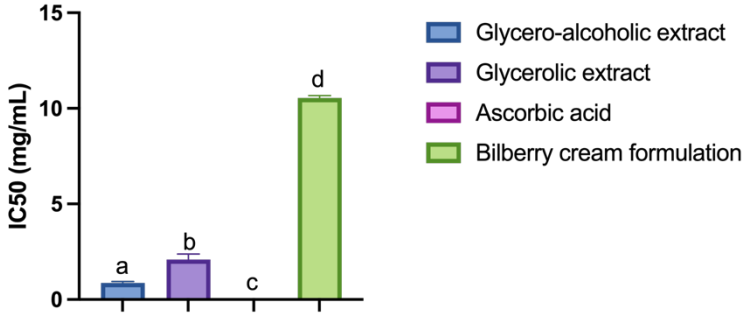

Fig. 4. Antioxidant activity with DPPH (* different letters above column, mean statistically significant differences at $p < 0.05$)

Regarding the antioxidant activity, our results have shown that all the tested samples had a good antioxidant potential. The highest antioxidant activity between the tested samples was observed for the glycero-alcoholic extract, with an IC_{50} of 0.87 ± 0.07 (**Fig. 4.**), while ascorbic acid solution had a more than 15 times lower IC_{50}.

The solvent used for maceration proved to be a significant factor for the antioxidant activity, probably correlated with the phytochemical profile.

The cream formulation, with 10% bilberry extract had a good antioxidant activity. However, statistically significant decline in antioxidant capacity is seen from 67.7% to 48.8% when comparing the percentage inhibition values of newly made cream versus the same cream after seven months. Data from other studies also suggest that formulations

with anthocyanins have stability issues, due to their chemical structure (Lee & Na, 2020).

Moreover, after seven months of storage under normal conditions, at room temperature, in a tightly closed container, protected from light, the preparation also underwent color changes from bright pink to a more faded pink. Because the color of anthocyanins is influenced by the pH, we have presumed that changes in pH occured during storage, but after the measurement of pH, we concluded that no changes appeard (pH = 5.6). No other changes of the formulation could be noticed, therefore the results confirm the instability of anthocyanins (Cai et al., 2022).

Abdellatif et al have also evaluated the stability of anthocyanins in a cream formulation and their results indicated that after 60 days, the formulation had a good stability and no changes appeared in the cream's colour (Abdellatif et al., 2021). The different results could be explain the by the longer storage time in our case. Different studies suggest an increased stability of anthocyanins in lyophilized form or formulated by microencapsulation (Gradinaru et al., 2003; Wang et al., 2017). Because more research is needed, until more stable extracts will be available, there is the possibility of offering consumers dual preparations, that can be extemporaneously prepared, in order to benefit from the effects of anthocyanin-rich extracts.

The limitations of the current study center around the methods for evaluating the antioxidant potential. Usually, different methods are used, due to different antioxidant mechanisms, but in this study only the DPPH method was used. This is due to the fact that the ABTS radical scavenging activity test produced clouding and opacification of the sample, rendering the method unsuitable for assessing the antioxidant potential of the cosmetic products.

Conclusions

The objective of the current study was to evaluate the antioxidant potential of a skincare product made with a bilberry fruit extract with a high anthocyanin concentration. In order to emphasize solely the anthocyanin-related qualities of the billberry fruit's glycerol-alcoholic extract, a simple cream base, while yet having moisturizing effects, was chosen. Although anthocyanin stability in cosmetics is limited, and more research is needed, anthocyanins have a longer shelf life in lyophilized form. This highlights the possiblity of producing dual cosmetic products that can be prepared extemporaneously, at the moment of use by the consumer.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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