ANTIBACTERIAL AND ANTIOXIDANT ACTIVITY OF COSMETIC CREAMS WITH EXTRACTS OF DRY FLOWERS AND FRUITS OF *SAMBUCUS NIGRA* L. - USE OF *SAMBUCUS NIGRA* L. AS A NON-TRADITIONAL CHEMICAL PRESERVATIVE

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ABSTRACT

Natural ingredients rich in antioxidants and polyphenols in the forme of extracts, essential oils, organic acids have been used in the production of different cosmetic products. In the development of new cosmetic compositions, or the improvement of products already implemented in production, the evaluation of the user is of great importance, as it directly affects the demand for a given product. The present study aimed to produce phytocosmetic creams based on stearic acid with extracts of dry flower and fruit juice of Sambucus nigra L. (S. nigra) and to investigate in parallel their potential as substitutes for conventional preservatives. The obtained creams were determined for minimum inhibitory, and minimum bactericidal activity using three bacterial strains (Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC25923, Bacillus subtilis ATCC 6633). In addition, the antibacterial effect of creams against Escherichia coli (BW 28357) was determined. In our experiments we found that addition of dried flower extracts or elderberry fruit extracts from S. nigra in the emulsion does not disturb the compositional system. In conclusion, according to the results, the Sambucus nigra L. extracts from dry flowers and elderberry fruits are appropriate for use in cosmetic emulsions that demonstated antioxidant stability and antibacterial properties.

Keywords: plant antioxidants, S. nigra, natural cosmetics, natural preservatives.

INTRODUCTION

Cosmetic and personal care products are applied to the body to beautify, correct odors, change the appearance of the skin, and increase aesthetic appeal [1, 2]. Cosmetic formulas include complex systems of emulsions (including oil, water, surfactants, preservatives, and vitamins), which are part of soaps, shampoos, creams, decorative cosmetics, and protection against sunburn [3, 4]. In recent years, natural bioactive components with pronounced antioxidant activity, ultraviolet (UV) protection, reduced radical damage, and reduction of aging processes and oxidative damage to the skin have been increasingly demanded [5]. Natural and organic cosmetics differ in several specifics: 1) natural cosmetics - contain one or more natural components extracted directly from plants or minerals; 2) organic cosmetics - contain a minimum of 95 % certified organic ingredients, and the natural product does not necessarily have to be organic [6]. Natural extracts and essential oils rich in polyphenols, flavonoids, and organic acids are gaining popularity

in the production of cosmetic products as a defense strategy against reactive oxygen/nitrogen species (ROS/RNS) and oxidative damage. On the other hand, natural components successfully replace synthetic preservatives in obtaining effective, safe, and natural products [7].

The flowers and fruits of black elderberry (*Sambucus nigra* L., *S. nigra*) belong to *Adoxaceae*. They are rich in bioactive components and have powerful antioxidant properties. According to the studies, a high content of flavonoids (rutin, kaempferol, iso/quercetin), phenolic acids (caffeic, p-coumaric, ferulic, chlorogenic), tannins, vitamins (B, C), lipophilic substances (triterpenes (β -/α - amyrin, lupeol, cycloartenol), phytosterols, amino acids (valine, threonine, methionine, isoleucine, leucine, lysine) was found [8]. Rutin extracted from *S. nigra* is commonly used as an antimicrobial, antifungal, and antiallergic agent [9]. Studies of fresh juice and extracts of *S. nigra* showed no mutagenic effect and high *in vitro* activity [10].

The present research aims to prepare phytocosmetic creams based on stearic acid with dry elderflower and elderberry extracts from *S. nigra*, used as substitutes for conventional preservatives. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), using three bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 6633), and antibacterial effect of creams against *E. coli* (BW 28357) were determined. In addition, lipoxygenase activity (LOX), antioxidant activity, and lipid peroxidation *in vitro* were evaluated.

EXPERIMENTAL

Materials and chemicals

Stearic Acid and Tween 80 were were obtained from Sigma - Aldrich, Germany. Glycerin and Bronopol were from Alfa Aesar, Germany. Strains *Escherichia coli* BW 28357 [rrnB3 Δ lac Z 4787 hsd R514 Δ ara BA-D567 Δrha BAD 568 rph+]., *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 6633 were purchased form the National Bank for Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria). The rich Luria-Bertani (LB) medium contained 1 % protein hydrolysate, 0.5 % yeast extract and 0.5 % NaCl was from Scharlau chemicals.

Preparation of creams containing natural extracts of *S. nigra*

Cosmetic emulsions of oil/water type (O/W, oil - in - water), with the oil phase including stearic acid (Stearic Acid, $C_{17}H_{35}COOH$) and glycerin, added emulsifier non-ionic surfactant Tween 80 (Sigma - Aldrich, Germany) and used Bronopol (Bronopol - 2 - bromo - 2 - nitroropane - 1,3 - diol, Alfa Aesar, Germany) as a preservative. Stearic acid and glycerin are melted in an oil bath (silicone oil, 70°C) while adding the components of the aqueous phase - d. H_2O, extract, and / or preservative, with continuous stirring with a Teflon stirrer (120 - 250 rpm). The resulting homogeneous emulsions are cooled to 22°C.

Plants material and extract preparation

Dry elderflower from *S. nigra* (without impurities) was purchased from the manufacturer "Alin" Bulgaria. Each sample from dry elderflower was weighed with a balance (Sartorius analytic, A 200 S, Germany, accuracy 0.1 mg) and placed in a laboratory flask (250 mL).

Elderberry fruits for juice preparation were collected from the Southern Bulgaria region. After pressing, the obtained mass was filtered through gauze and a membrane filter - PTFE (pore size 0.45 μm). Deionized water (d. H_2O) was used as the extracting solvent, and the solid: liquid phase ratio was 1/30. The extraction process for both samples was performed in a water bath shaking machine (Gyrotory, model 676, New Brunswick Scientific, Edison N.J., USA). The flasks were placed in a shaker at 50 ºC and the solvent d. H_2O was contacted with the solid. The extracts were divided into E_1/b - *S. nigra* dry flower extract + bronopol; E_1 - *S. nigra* dry flower extract; E_2/b - *S. nigra* juice + bronopol; E_2 - *S. nigra* fresh juice.

Stability Tests and Heat-Shock Cycles

A part of the emulsions (B, E_1 and E_2) is placed on the top of a test tube and after 24 hours their stability is recorded (the absence of a drop at the bottom of the test tube proves a stable cosmetic composition). Through cycles of heating (50°C - 24 h), cooling (4°C - 24 h), the thermal resistance of the emulsions was checked, as the procedure was repeated three times [7, 11].

pH measurement

Horiba Laquatwin PH - 33, Japan is used to measure the pH of cosmetic creams at 25°C.

Antimicrobial Screening

Agar diffusion test

Bacterial cultures were cultivated under aerobic conditions on agar (Mueller-Hinton (Himedia, Mumbai, India)) until stationary phase. After that 20 mL of molten Mueller Hinton agar was added to each Petri dishes. The surface of each agar plate was inoculated with 1.5 × 10^8 CFU mL^{-1} bacteria. Cosmetic creams - containing paper disks are then applied to the agar and the plate is incubated on 37°C for 24 hours. As positive control it was used gentamicin (10 mg mL^{-1} concentration) containing paper disks. If tested substances stopped the bacteria growth killed the bacteria, there would be an area around the disk where the bacteria had not grown enough to be visible. This is called a zone of inhibition. The susceptibility of the bacterial isolate to each cosmetic creams could then be semi - quantified by comparing the size of these zones of inhibition. The zones of inhibition of growth of gentamicin against the reference strains used in the present study were fully consistent with the interpretive criteria of Clinical and Laboratory Standards Institute (CLSI). The zones of inhibition were measured with a transparent ruler on the outside of the bottom of the plates and the diameters of the zones of inhibition in millimeters to the nearest whole millimeter, were determined. The measured zones of inhibition are interpreted according to the three - level system *low antibacterial activity (IZ = 8 - 10 mm); **medium antibacterial activity (IZ = 11 - 14 mm); *** high antibacterial activity (IZ ≥ 15 mm) [12].

Determination of minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations

Weight 0.2 g of the respective cream is dissolved in 1 ml of chloroform. Bacteria of strains *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 6633) with concentration of 1 x 10^5 mL were cultured in 1 mL of LB (Luria-Bertrani) medium containing the respective cream at different concentrations of 1000 - 500 - 250 - 125 - 60 μg mL^{-1}, and 30 μg mL^{-1}. After that bacteria were cultivated for 12 hours at 37°C, with continuous agitation.

Minimum bactericidal (MBC) and minimum inhibitory concentrations (MIC) of tested creams were determined by taking aliquots of 100 μl from all tested tubes and seeded in LB agar plates. After 24 h incubation at 37ºC. bacterial colonies were counted [11].

Determining antibacterial effect of creams against bacteria E. coli (BW 28357)

The antimicrobial efficacy of phytoextracts was determined using the standard dilution method. Bacterial culture was cultivated until stationary phase (2 O.D). After that we took aliquots of 10 μL bacterial culture and diluted to 1000 μL LB medium. This procedure was twice repeat. It was taken 100 μL from last dilution and seeded in LB agar plates which were treated with different phytoextracts. As positive control we used bacteria cultivated in LB agar without phytoextracts. After 24 h incubation at 37 ºC bacterial colonies were counted.

$$\text{Percent reduction (Antibacterial efficacy)} = \frac{B - A}{B}.100,$$

where B - number of viable test microorganisms on the control sample; A - number of viable test microorganisms on the test sample.

Determination of lipoxygenase, antioxidant activity in vitro, and lipid peroxidation of the prepared emulsions

Enzyme inhibition, antioxidant activity, and lipid peroxidation of emulsions for 100 μL (125 μg mL^{-1}) sample of E_1/b, E_1, E_2/b, and E_2 were determined by: a) inhibition of lipoxygenase (LOX; 7126.1 U mL^{-1}) [13, 14] in positive control - indomethacin and absorption 234 nm; b) the DPPH/ R analysis (0.02 mM) after 30 min incubation, absorbance - 518 nm with positive control - ascorbic acid [14, 15]; c) Scavenging of superoxide ($\bullet O_2^-$) anion radicals in xanthine-xanthine oxidase (xanthine 0.5 mM/ PBS; 1:1v/v; NBT 0.16 mM/ PBS; xanthine oxidase 3.92 mU/ PBS) system [14, 16] at absorption 560 nm, with positive control - ascorbic acid; d) H_2O_2 inhibition (50 μL peroxidase / PBS; 50 μL H_2O_2 (0.007 %) and 50 μL / PBS luminol (0.005 μg mL^{-1}) [14, 16] was run on positive control - ascorbic acid; f) NO (nitric oxide) activity [17] with a positive control - ascorbic acid, performed at absorption 546 nm; f) EPR - Bruker ER - 116 - DS measurement of lipid peroxidation [18] against phenyl N - tert - butylnitrone (PBN/ DMSO; 5:0.04 mM) against the PBN/ DMSO standard.

Statistical methods for data processing

Statistical analyzes were performed using the STATISTICA 10 statistical software package, Stat Soft, Inc USA. The difference between the means of quantitative variables of two independent groups was assessed using Tuckey's post hoc test and the Students' t - test for independent groups. EPR spectral processing was performed using Bruker Win - EPR and Simfonia software. Three independent experiments were performed in triplicate for each method. Differences with a significance level of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

pH measurement and phytocosmetic creams composition

Table 1 presents the pH values and the composition of the different creams at 25°C. The pH values at E_1/E_2 relative to the base are almost identical. Due to the high bioactivity (anthocyanins) in *S. nigra* species, different coloration, high stability, resistance to temperature changes, moisture, and light, due to the stable pH of the target *S. nigra* matrix. In addition, the processing conditions also did not change the pH values and could be a beneficial technological solution for *S. nigra* use in skin treatment [19].

Extracts from various plants are widely used to inhibit the microbial growth of microorganisms that are responsible for primary and secondary microbial contamination [20]. The resulting cosmetic creams have excellent organoleptic characteristics and include unchanged pearly luster, pleasant color, low aroma intensity, and creamy texture, which are preserved for up to 6 months (Fig. 1), i.e. E_1/E_2 compositions show temporal stability.

Agar diffusion test

Antibacterial activity of cosmetic creams from *S. nigra* was evaluated against four bacterial strains (*Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 6633 and *E. coli* BW 28357) using the agar diffusion method. Gentamicin was chosen as a positive control for the antimicrobial studies because it showed satisfactory antimicrobial efficacy against the four tested strains. The method allows us to determine how the bacteria was sensitive, intermediate, or resistant to the creams we tested.

Positive control (gentamicin) 18.2 ± 0.5 1.0 ± 0.5 15.6 ± 1.0 10.7 ± 0.6

IZ- inhibition zone, mm; *low antibacterial activity (IZ= 8 -10 mm); **medium antibacterial activity (IZ = 11 - 14 mm); *** high antibacterial activity (IZ ≥ 15 mm).

P. aeruginosa (ATCC 27853), *S. aureus* (ATCC

Fig. 1. Cosmetic emulsions: Base /1/; emulsion with *S. nigra* flower extract without bronopol /2/; Emulsion with added *S. nigra* juice without bronopol /3/.

Table 1. Composition of the different creams: Base (B); emulsion with dry flower extract of *S. nigra* (E_1); emulsion with added *S. nigra* juice (E_2); - emulsion with added *S. nigra* dry flower + bronopol (E_1/b); *S. nigra* juice + bronopol (E_2/b).

Composition, mass % part	Base	E_1	E_2	E_1/b	E_2/b
Stearic acid	15.0	15.0	15.0	15.0	15.0
Extract (1 or 2)	none	0.3	0.2	0.3	0.2
Glycerin	10.0	10.0	10.0	10.0	10.0
Emulsifier	0.5	0.5	0.5	0.5	0.5
Preservative (bronopol (b))	0.5	none	none	0.5	0.5
Distilled water	74	74.2	74.3	73.7	73.8
pH with bronopol	5.5	5.3	5.0	5.4	5.1
Total mass % part	100	100	100	100	100

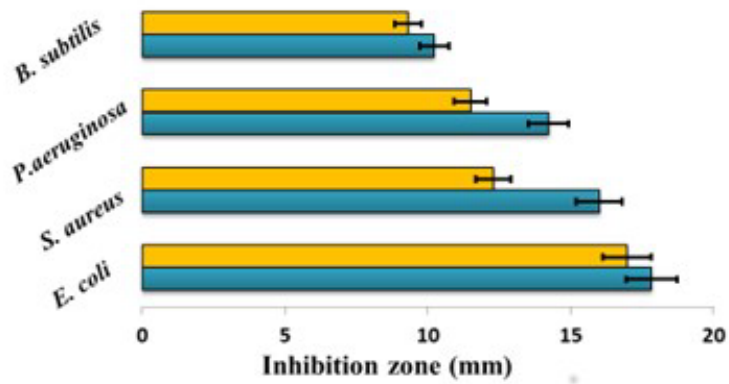

Fig. 2. Antimicrobial s test results (IZ ± SD) of cosmetic emulsions: E_1 (*S. nigra* dry flower extract) and E_2 (S. *nigra* fresh juice) against four bacterial strains: *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* ATCC 6633 and *E. coli* BW 28357.

25923) and *E. coli* (BW 28357) are considered the main potential pathogens in cosmetic products [21, 22]. The obtained results demonstrated those two tested cosmetic creams inhibited the growth of various microorganisms (Fig. 2). The greatest inhibition of bacterial growth was observed at *E. coli* (BW 28357*)*, followed by *S. aureus* (ATCC 25923) and *P. aeruginosa (*ATCC 27853*)* In the case of *B. subtilis* (ATCC 6633) we observed the weakest inhibition of bacterial growth, compared to others tested bacteria.

Minimum bactericidal (MBC) and minimum inhibitory concentrations (MIC)

The MIC and MBC of both creams against gram-positive and gram - negative bacteria were determined (Table 2, Table 3).

The obtained results confirmed the antibacterial properties of cosmetic creams E_1 and E_2 (Table 2 and Table 3). In case of cosmetic cream E_2 (emulsion with added *S. nigra* juice without bronopol) we observed lowest MIC and MBC values for all tested bacteria strains (Table 3). This higher antibacterial activity of cream E_2 may be due to the type and concentration of polyphenols extracted from the juice of *S. Nigra* [23].

Determining antibacterial effect of creams against bacteria E. coli (Strains BW 28357)

In all types of cosmetics, the presence of *E. coli* (BW 28357) *is* absolutely prohibited (Scientific Committee on Consumer Safety (SCCP)), [24]. For this reason, we studied antibacterial properties of creams E_1 and E_2 against *E. coli* (BW 28357) (Table 4.).

From the results shown it Table 4 it became clear that the cream (E_2) demonstrated 100 % antibacterial

Table 2. MIC and MBC data for *S. nigra* flower extract emulsions (E_1) without bronopol.

	Pseudomonas aeruginosa ATCC 27853	*Staphylococcus aureus* ATCC25923	*Bacillus subtilis* ATCC 6633
MIC (μg mL^{-1})	500	500	500
MBC (μg mL^{-1})	1000	1000	1000

Table 3. MIC and MBC data for emulsion with added *S. nigra* juice (E_2) without bronopol.

	Pseudomonas aeruginosa ATCC 27853	*Staphylococcus aureus* ATCC25923	*Bacillus subtilis* ATCC 6633
MIC (μg mL^{-1})	500	500	500
MBC (μg mL^{-1})	700	700	700

Table 4. Antibacterial effect of creams against bacteria *E. coli* (Strains BW 28357).

Samples	colony forming unit of *E. coli* (BW 28357)	Reduction %
control	76	
Cream E_2	0	100
Cream E_1	34	55

efficacy against *E. coli* (BW 28357) (100 % reduction of bacterial growth) while with cream E_1 we observed only 55 % inhibition of bacterial growth. The experiment confirmed again the great antibacterial potential of cream E_2 compared to cream E_1.

Increased lipoxygenase and in vitro antioxidant activity of the emulsions

The creams E_1 and E_2 were found to inhibit LOX activity in vitro statistically significantly 72.4 ± 6.24 % and 63.5 ± 8.33 %, respectively ($p < 0.05$), compared to the positive indomethacin control - 57.2 ± 9.7 % (Fig. 3). E_2 registered statistically significant ($p < 0.05$), the highest DPPH radical scavenging activity (93.5 ± 11.09 %) at the tested concentration of 125 μg mL^{-1}. Our results are in agreement with previous studies on *S. nigra* biological activity showing that aqueous and ethanolic extracts of *S. nigra* leaves and fruits strongly scavenge ROS and inhibit LOX [13, 14]. The increased antioxidant activity of E_2 and E_1 creams is directly related to the stable DPPH / R inhibition of the applied *S. nigra* extracts, especially the use of *S. nigra* fresh juice, more potent than ascorbic acid [13, 14, 22, 25].

Increased radical-trapping capacity and reduced lipid peroxidation in vitro of emulsions

Deterioration of the quality of cosmetic products is due to increased lipid peroxidation, mediated by the inability of natural or synthetic components to neutralize $\bullet O_2^-$, H_2O_2, and NO [13, 14].

Recent research indicated that dry *S. nigra* flower extracts obtained from cultivated European elderberry specimens demonstrated increased antioxidant activity and actively inhibited lipid peroxidation (LO•) [25]. The independent participation of *S. nigra* in E_2 and E_1 emulsions not only inhibits $\bullet O_2^-$ (~85.6 ± 7.05 %, $p < 0.003$), H_2O_2 (~ 97.09 ± 13.11 %, $p < 0.002$), NO• (~ 92.7 ± 16.01 %, $p < 0.05$), and LO• (~43.6 ± 1.37 %, $p< 0.002$) radicals and their derivatives (Fig 4). Moreover, *S. nigra* also prevent the subsequent initiation of additional re - oxidation in the investigated cosmetic products. The E_2 - emulsion containing fresh *S. nigra* fruit juice has maximal ROS / RNS inhibitory activity and minimal lipid peroxidation, which is also confirmed by other studies [26]. The maintenance of the antioxidant-prooxidant balance in E_2 and E_1 cosmetic emulsions was established after the use of *S. nigra* extracts, at a concentration of 125 μg mL^{-1}.

The high antioxidant capacity and amount of total polyphenolic compounds of the ethanolic *S. nigra* extracts used by us have been established and commented on in our other publications [27] and confirmed by other authors [8, 13, 14, 19]. Phenolic extracts contain various bioactive compounds (n-rutin; quercetin 3-O-rutinoside)

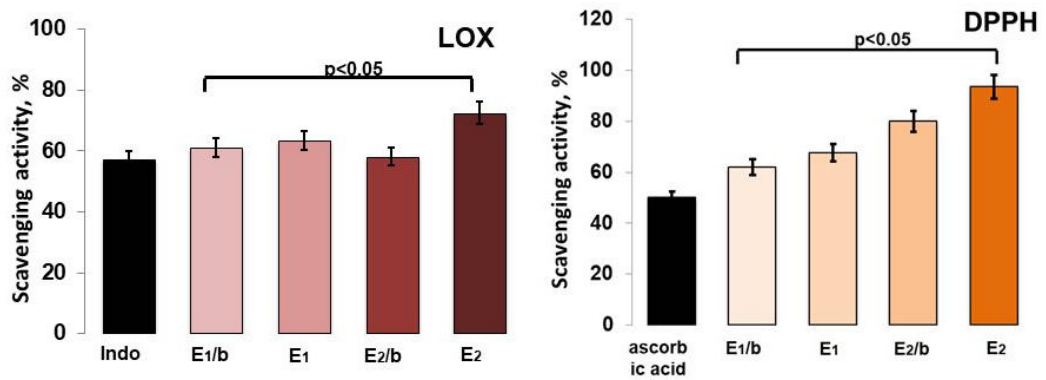

Fig. 3. Lipoxygenase activity and DPPH - trapping ability of emulsions - E_1/b - *S. nigra* dry flower extract + bronopol; E_1 - *S. nigra* dry flower extract; E_2/b - *S. nigra* juice + bronopol; E_2 - *S. nigra* fresh juice.

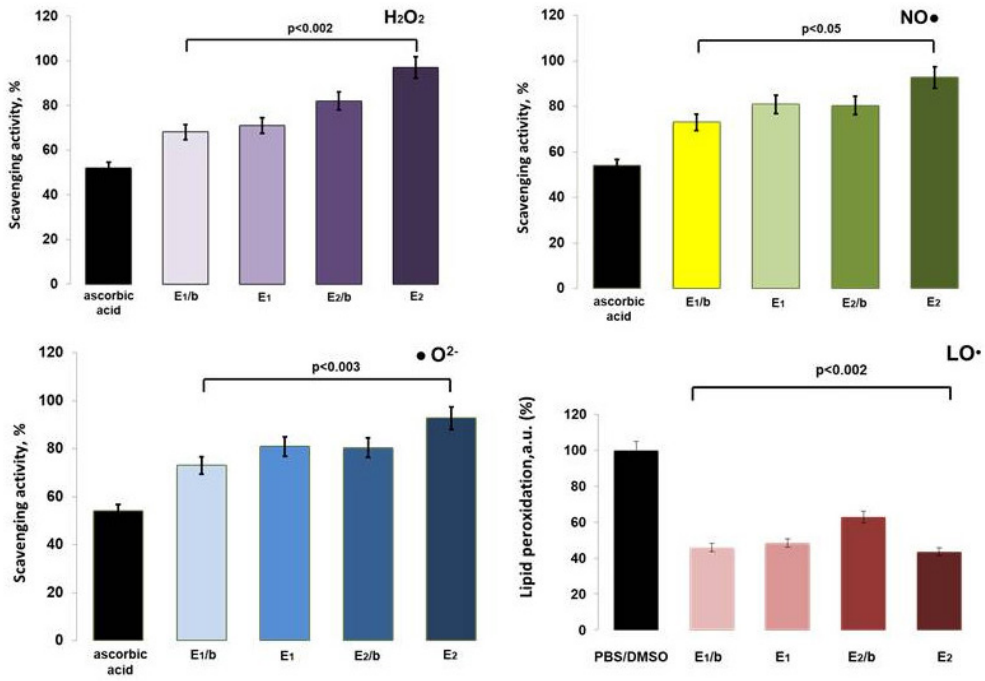

Fig. 4. The radical scavenging ability against superoxide ($•O_2^-$) anion radicals, hydrogen peroxide (H_2O_2), nitric oxide (NO), and lipid peroxidation degree (LO•) of emulsions - E_1/b - *S. nigra* dry flower extract + bronopol; E_1 - *S. nigra* dry flower extract; E_2/b - *S. nigra* juice + bronopol; E_2 - *S. nigra* fresh juice.

that can potentiate antimicrobial activity as well as add different biological activities to cosmetic products, increasing their potential as natural preservatives over synthetic ones in cosmetics [28, 29]. The concentration of the used extracts should be optimized in further studies, and the possible synergistic and antagonistic effects of preservatives such as sodium benzoate should be investigated.

CONCLUSIONS

The antioxidant, antibacterial activity, and inhibition of lipid peroxidation of traditionally used extracts of dry flower and fresh fruit of *Sambucus nigra* L. in cosmetic emulsions were investigated. The present study highlights the benefits of the application of fresh *S. nigra* fruits in cosmetics, presenting the increased anti-inflammatory, radical - scavenging, and antibacterial activity, *in vitro*, and provides a basis for conducting a wide range of future studies on the application and effectiveness of plant extracts as alternative substitutes of synthetic preservatives.

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