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DEVELOPMENT OF A NATURAL CREAM ADDITIVE CONTAINING AN EXTRACT OF *ARONIA MELANOCARPA* BERRIES AND A PROBIOTIC TO INHIBIT MICROBIAL DEVELOPMENT AND INCREASE SOLAR PROTECTION

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Natural products have been used in cosmetics for centuries for skincare purposes. One of the most effective ways to protect skin flora and keep the skin healthy is to use plant extracts in cosmetic products. This study aimed to investigate the biological activities of an aronia berries ethanol extract (ABEE) obtained from *Aronia melanocarpa* (Aronia) as well as determine its potential use in the cosmetic and pharmaceutical industries. In the study, disc diffusion, minimum inhibitory concentration (MIC) and minimum fungicidal or bactericidal concentration (MFC or MBC) tests were applied to evaluate the antimicrobial potential of ABEE. Next, the antimicrobial activity of ABEE was accurately determined by counting live cells against *Staphylococcus aureus* ATCC 25923 using the macrodilution method. The sun protection factor (SPF) of ABEE and ABEE + commercial cream mixtures was obtained in vitro. Finally, cream formulations containing ABEE and a probiotic were developed for the treatment and prevention of clinical infections. In this context, the antimicrobial activities of the developed cream mixtures were determined using the well diffusion test. The inhibition zone diameter of ABEE against test microorganisms was measured to be between 8.32 and 14.49 mm. MIC and MBC or MFC values of the extract were measured to be within 12.5-50.0 µg/µl. ABEE exhibited the best antimicrobial activity against *S. aureus* ATCC 25923. At an ABEE concentration of 50 mg/ml, a significant decrease in the number of viable cells of *S. aureus* ATCC 25923 was observed compared to the control. An ABEE concentration of 100 mg/ml inhibited all bacterial growth. The SPF value of ABEE determined in vitro was high (25) and those of the cream mixtures also increased as the ABEE concentration increased. ABEE and probiotic-containing cream mixtures exhibited high antimicrobial activities on the tested microorganisms. With the synergistic effect of the extract and probiotic, the highest antimicrobial activity was determined against *Escherichia coli* O157:H7 with an inhibition zone diameter of 25.40 mm. ABEE may be used as a natural antimicrobial agent to prevent or treat clinical infections in the pharmaceutical industry and as a natural ingredient in sunscreen in the cosmetic industry.

Keywords: Aronia, extract, living cell, sun protection factor, antimicrobial cream

1. Introduction

Humans have used various plants for thousands of years to cure diseases [1]. Natural products, especially plants, exhibit important pharmacological effects and form the basis of modern medicines [2]. Herbal medicines use the fruit, seeds, leaves, roots, flowers or bark of plants for medicinal purposes [3]. Natural extracts obtained from plants are used in many areas such as the pharmaceutical [4], agricultural [5] and food industries [6] for their various biological activities.

Aronia melanocarpa (Aronia) is a shrub belonging to the Rosaceae family [7]. Aronia is widely consumed as a fresh fruit and used for various purposes, for example, as a herbal medicine and food colorant [8]-[9]. The presence of bioactive compounds such as polyphenolic compounds, minerals and vitamins in aronia berries has many beneficial effects on our health, e.g. anti-aging, antimicrobial, anticancer and anti-inflammatory [10]-[12]. Aronia extracts improve the health of the liver, intestines and brain [13]-[14].

Probiotics are defined as non-pathogenic live microorganisms that are administered to improve the microbial balance in the body, especially in the gastrointestinal tract and skin [15]. Probiotics also increase immune response, strengthen the epithelial barrier, degrade toxins and exhibit antimicrobial effects [16]. *Limosilactobacillus fermentum*, an important probiotic bacterium for human health, has antimicrobial, antioxidant and anti-photoaging activities by improving skin health and flora [17]-[18].

The skin, the largest organ of the human body, is exposed to environmental effects. The skin ages

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depending on chronological events and external factors. In addition, sunscreen products should be used to provide protection against the harmful effects of the sun [19]-[20]. Creams developed using chemical additives remain on the skin for a long time causing unwanted penetration into its epidermis, potentially negatively affecting skin biology [21]. Sunscreens containing herbal additives are known to exhibit more photoprotective activities with fewer or no side effects compared to sunscreens containing chemical additives [22].

Infections in the skin and soft tissues are common conditions that cause serious morbidity caused by yeasts and bacteria [23]. Topical creams with antimicrobial properties are used to treat superficial skin infections. Topical treatments are based on administering drugs in appropriate concentrations to the desired area [24]. For skin infections, new antimicrobial agents containing natural ingredients should be used instead of existing antibiotics [25]. The ability to select antimicrobial agents containing natural ingredients suitable for developing topical creams is highly advantageous [26]-[27]. Creams developed with plant extracts can be used to treat different skin disorders caused by microbial infections [28]. Protecting the skin from prolonged exposure to direct sunlight and applying sunscreen topically when exposed protects the skin from sunburn and photoaging as well as reduces the risk of developing skin cancer [29]. Sunscreen applied topically before or after sun exposure may help increase DNA repair, regulate DNA transcription, reduce inflammation and play an important role in skin cancer prevention [30].

This study aimed to determine the potential of aronia berries as a natural bioactive compound in the pharmaceutical and cosmetic industries. For this reason, the antimicrobial effect of an ethanol extract obtained from aronia berries (ABEE) on various test microorganisms was investigated. In addition, the antibacterial activity of this extract on *S. aureus* ATCC 25923 was examined in more detail by determining the viable cell count. The sun protection factor (SPF) values of ABEE and ABEE + commercial cream mixtures were measured to evaluate their potential as safer and cheaper alternatives to the harmful chemicals used in sunscreens. The antimicrobial activities of creams containing a mixture of ABEE and probiotic *L. fermentum* were investigated on test microorganisms for pharmaceutical purposes.

2. Experimental

2.1. Preparation of extracts

Aronia were purchased as fresh berries from Chokeberry Food and Health I.C (Yalova, Turkey) before being rinsed first with tap water and then distilled water. The berries were dried in an airy environment then ground using a blender manufactured by Waring Laboratory Science. The extraction was carried out with ethanol (99.9%) using a sonicator (Amplitude of 100%; Cycle of 1). After the solvent was evaporated, the extract was dissolved in dimethyl sulfoxide and then sterilized using a 0.45 µm syringe filter.

2.2. Test microorganisms

The antimicrobial activity of ABEE was tested against 5 test microorganisms. *Candida albicans* ATCC 10231 was cultured at 30 °C in yeast peptone dextrose medium for 24 hours. *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* O157:H7, *Staphylococcus epidermidis* ATCC 12228 and *Staphylococcus aureus* ATCC 25923 were grown at 37 °C for 24 hours.

2.3. Bioactivity of ABEE

2.3.1. Disc diffusion method

The antimicrobial activity of ABEE on test microorganisms was investigated using the disc diffusion method. The active cultures of the microorganisms were prepared as microbial suspensions at a density of a 0.5 McFarland standard ($1.5x10^8$ CFU/ml). The sterile discs, 6 mm in diameter, were placed on an agar medium and inoculated with a suspension equal to 0.5 McFarland standard before 20 µl of the extract (2 mg/disc) was dripped onto them. As a positive control, standard Ampicillin antibiotic (AM: 10 µg/disc), Kanamycin (K: 30 µg/disc) and Fluconazole (FCA: 25 µg/disc) discs were used. The discs were left to allow the test microorganisms to incubate at appropriate temperatures for 24 hours before the diameter of the inhibition zones were measured and recorded with a digital caliper. This method was repeated three times to obtain the means and standard deviations of the inhibition zone diameters of the ABEE.

2.3.2. Microdilution method

The minimum inhibitory (MIC) and minimum fungicidal or bactericidal (MFC or MBC) concentrations on the test microorganisms of ABEE were determined using the two-fold dilution method. At the first concentration (100 µg/µl), the broth medium and extract were added to the tube and homogenized before the other tubes containing the broth medium were subjected to two-fold dilutions. Finally, the suspension equal to 0.5 McFarland standard was added to the diluted tubes separately and incubated. The concentration of the extract at which no microbial growth occurred after incubation was determined to be the MIC. Next, the samples taken from the tubes were inoculated sequentially onto the solid medium and incubated for 24 hours. The ABEE concentration at which no microbial growth occurred in the solid medium after incubation was denoted as the MBC or MFC.

2.3.3. Macrodilution method

The biological activity of ABEE on clinically sourced *S. aureus* ATCC 25923 pathogenic bacteria was determined using the macrodilution method. A suspension of *S. aureus* ATCC 25923 equal to 0.5 McFarland was prepared and added to the mixture of

ABEE (50 and 100 mg/ml) and the growth medium. The control consisted of the broth without the extract. The test groups were incubated over 48 hours at the appropriate temperature for the bacteria. Samples were taken after 0, 24 and 48 hours before being diluted and then cultured on solid media. At the end of each incubation period, the number of viable cells was determined and this value recorded in CFU/ml.

2.4. Calculating the sun protection factor (SPF)

The sun protection factor of ABEE was determined by a spectrophotometric analysis [31]. The ABEE was homogenized in ethanol (96%) at a concentration of 2 µg/µl. 3 replicates of the mixture were measured by a UV-B spectrophotometer (Beckman Coulter Inc., USA) at 5 nm intervals between 290 and 320 nm before using the Mansur equation [32].

The SPF values of the mixtures developed with ABEE and a commercial cream were determined spectrophotometrically with some modifications [33]. The ABEE (5% w/v) was mixed with the commercial cream (10% w/v) and increased to its final volume by adding distilled water. Ethanol (10 ml, 40% v/v) was added to 0.1 g of the sample taken from the mixture. Next, the sample was sonicated (Amplitude of 100%, Cycle of 1) with an ultrasonicator (Hielscher UP100H) for 5 minutes. The mixtures of 2.5, 5.0 and 10.0 ml in volume were measured on the spectrophotometer as mentioned above. The Mansur equation was used to determine the SPFs of the ABEE and cream mixtures [32]:

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \qquad (1),$$

where

CF: correction factor (=10);
EE(λ): erythemal effect spectrum;
I(λ): intensity of sunlight at wavelength *λ*;
Abs(λ): OD value of ABEE at UV-B wavelength *λ*;
EE(λ) x I(λ): a constant specified by Sayre et al. [34].

2.5. Determining the antimicrobial activity of the cream developed with ABEE and L. fermentum MA-7

Groups of antimicrobial creams developed with ABEE and/or *L. fermentum* MA-7 in commercial creams were prepared by the methods used in the studies by Handali et al. [35] and Chen et al. [36]. The content of the groups of creams developed consisted of 10% commercial cream (w/v) and 20% ABEE (w/v). The volume of the groups with and without probiotic *L. fermentum* MA-7 were increased to their final volumes with a live culture (OD_{600nm} = 1.6) and distilled water, respectively. The negative control group of creams did not contain the extract nor *L. fermentum* MA-7 which were sterilized using a 0.45 µm syringe filter. The cream formulations were stored at 4 °C under dry conditions until used.

The antimicrobial activities of the developed groups of creams were tested on the tested microorganisms using the well diffusion method. The microbial suspensions at a concentration of 0.5 McFarland standard were dripped onto 100 µl of the solid media and spread. The cream formulations, 100 µl in volume, were inoculated into wells 6 mm in diameter. After incubation, the clean areas around the wells were measured and recorded with a digital caliper. This method was replicated three times to obtain the means and standard deviations of the inhibition zone diameter of the extract.

2.6. Statistical analysis

All data in the current study are reported as the mean and standard deviation (SD) of three different evaluation results. The data were analyzed using the GNU PSPP program and statistical significance was confirmed by the One-Way ANalysis Of VAriance (ANOVA) with Tukey's post-hoc test. The difference between the values was considered statistically significant when $p<0.05$.

3. Results and discussion

Excessive use and inappropriate selection of antibiotics is causing an increase in antimicrobial resistance in healthcare [37]. It is difficult to treat antibiotic-resistant microorganisms in bacterial infections [38]. Wounds and hospital-acquired infections often prolong healing and cause implant failure as well as lead to other serious health problems, even death [39]. Safe bioactive compounds that can control and inhibit the development of pathogenic microorganisms need to be developed. An alternative way to ensure a microbial balance in hospital services and cosmetic products is to use herbal extracts that are antimicrobially active [40]. An alternative to chemical preservatives in the cosmetic industry is the combination of herbal extracts and probiotics, increasing the number of properties exhibited by cosmetic products [41]-[42].

3.1. Biological activity of the extract

The biological activity of ABEE on test microorganisms is presented in *Table 1*. The results show that the largest inhibition zone diameter of ABEE was 14.49 mm on *S. aureus* ATCC 25923. The results show that the difference between the antimicrobial activity of the extract on *C. albicans* ATCC 10231 (8.32 mm in diameter) and *S. epidermidis* ATCC 12228 (8.66 mm in diameter) was not statistically significant ($p>0.05$). For other test microorganisms, the statistical difference between the means of *C. albicans* ATCC 10231 and *S. epidermidis* ATCC 12228 was significant ($p<0.05$).

Table 1: Antimicrobial activity of ABEE

Test microorganisms	Inhibition Zone Diameter (mm±SD)			
	ABEE (2 mg/µl)	Antibiotics		
		AM	K	FCA
C. albicans ATCC 10231	8.32±0.82[a]	-[a]	-[a]	21.85±0.14
P. aeruginosa ATCC 27853	13.09±0.59[b]	23.53±0.20[b]	20.37±0.32[b]	-
E. coli O157:H7	12.10±0.86[b,c]	17.76±0.03[c]	19.33±0.40[c]	-
S. epidermidis ATCC 12228	8.66±0.31[a]	20.52±0.09[d]	14.35±0.37[d]	-
S. aureus ATCC 25923	14.49±0.35[b]	21.04±0.11[e]	19.91±0.30[b,c]	-

*ABEE: Aronia berry ethanol extract, AM: Ampicillin, K: Kanamycin, FCA: Fluconazole, NA: No activity
*Different superscript values in the columns indicate significant differences ($p<0.05$) according to the one-way ANOVA followed by Tukey's post-hoc test. F(54.945) Sig.(0.000) for ABEE, F(21729.569) Sig.(0.000) for AM, F(2216.392) Sig.(0.000) for K

A study measured the antimicrobial activities of aronia berry extracts (96% ethanol, 50% ethanol and water) using the disc diffusion method. Although the extracts did not create an inhibition zone on *E. coli* JM109, the 50% ethanol extract did create an inhibition zone of 10.20 mm in diameter on *B. cereus* 407 according to Bräunlich et al. [43]. In another study, the ethanol extract prepared using aronia berries exhibited inhibition zones of 7.00 and 8.66 mm in diameter on *C. albicans* ATCC 10231 and *E. coli* ATCC 25922, respectively [44]. Compared with these studies, in the present study, ABEE exhibited good levels of antimicrobial activity on *E. coli* O157:H7 and *C. albicans* ATCC 10231 by forming inhibition zones of 12.10 and 8.32 mm in diameter, respectively.

The minimum inhibitory concentration of an antimicrobial that can inhibit the microorganism was determined using the microdilution method. The lowest concentration at which the extract inhibited the microorganism was denoted as the MIC of the extract and the lowest concentration at which the growth of a microorganism on solid media was completely inhibited was denoted as the MBC or MFC of the extract (*Figure 1*). The MIC and MFC of the extract on *C. albicans* ATCC 10231 was recorded to be 50 µg/µl. Among the bacteria, the lowest MIC and MBC of the extract were recorded as 12.5 µg/µl on *S. aureus* ATCC 25923. The most resistant microorganisms to the extract were *C. albicans* ATCC 10231 and *S. epidermidis* ATCC 12228.

In a study, Deng et al. [45] extracted anthocyanin (75% ethanol) from aronia berries. The MIC of anthocyanin on *E. coli* O157:H7 was determined to be 0.625 µg/µl and the MBC was 1.25 µg/µl. In another study, a hydrophilic ethanol extract was obtained from aronia branches using 96 and 50% ethanol. It was determined that the 96% ethanol extract yielded both MIC and MBC of 0.20 and 0.39 µg/µl on *E. coli* and *S. aureus*, respectively. The 50% ethanol extract showed 0.20 µg/µl MIC and 0.39 µg/µl MBC for *E. coli* and 0.20 µg/µl MIC and 0.20 µg/µl MBC for *S. aureus*. In addition, both 96% and 50% ethanol extracts resulted in MIC and MBC values of 0.39 µg/µl and 0.78 µg/µl, respectively, for *P. aeruginosa*. [46].

According to the disc diffusion test results, the ABEE did not exhibit the highest inhibitory activity among the test microorganisms on *S. aureus* ATCC 25923 by producing an inhibition zone diameter of

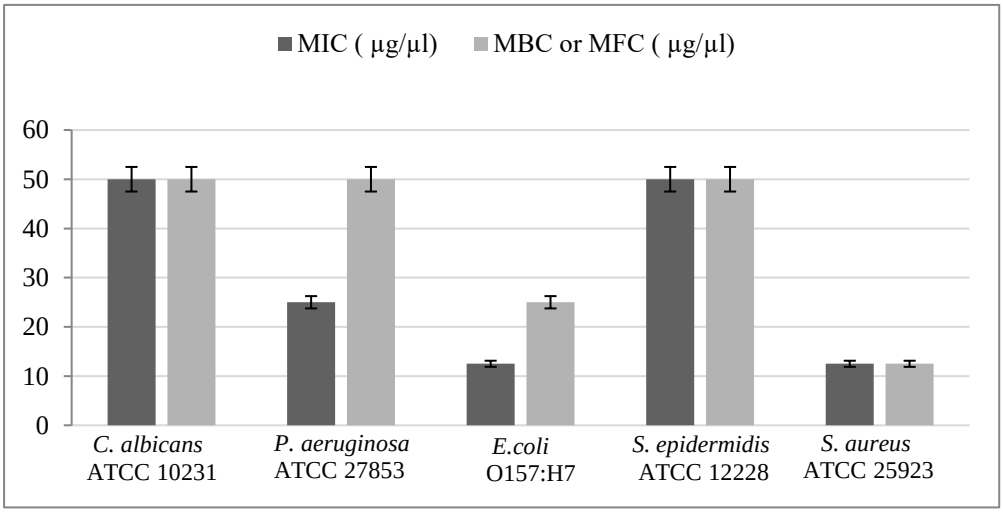

Figure 1: MIC and MBC or MFC of ABEE

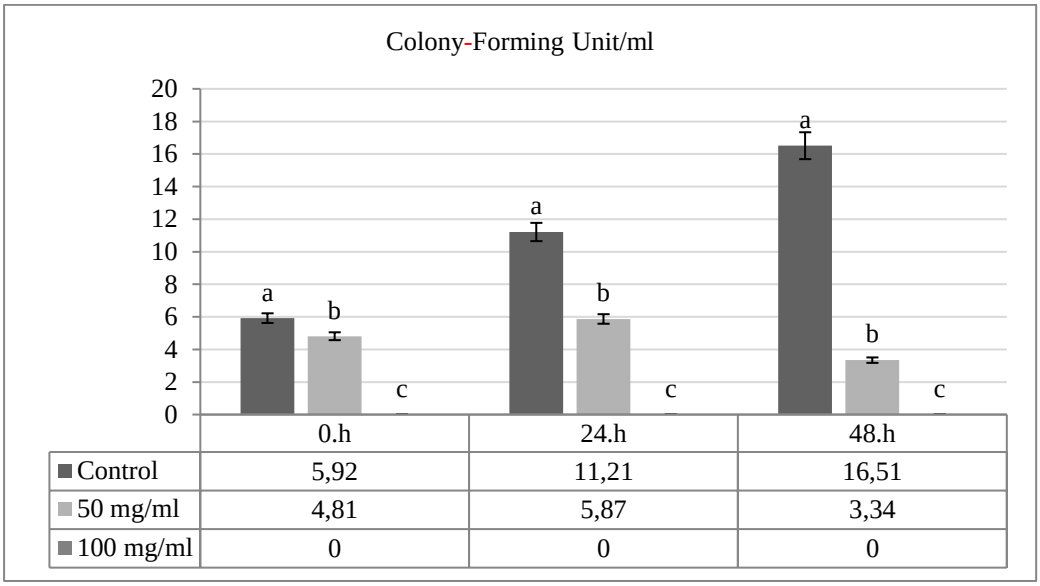

Figure 2: Macrodilution results of ABEE on *S. aureus* ATCC 25923
*F(668692.000) Sig.(0.000) after 0 h, F(2123403.250) Sig.(0.000) after 24 h, F(1583247.077) Sig.(0.000) after 48 h

14.49 mm, moreover, the lowest MIC and MBC of 12.5 µg/µl were determined. These results indicated that *S. aureus* ATCC 25923 was the most sensitive microorganism against ABEE since an ABEE concentration of 100 mg/ml inhibited the growth of this strain throughout the experiment with no live cells.

The concentration of 50 mg/ml ABEE (4.81 CFU/ml) exhibited a 1 log reduction compared to the control (5.92 CFU/ml) initially ($p<0.05$). After 24 and 48 hours, 50 mg/ml of ABEE inhibited the growth of bacteria as the number of viable cells significantly decreased compared to the control ($p<0.05$), as presented in *Figure 2*.

In a study conducted by Tamkutė et al. [47], the aronia pomace ethanol extract exhibited a higher antimicrobial activity than the water extract equivalent. The extracts were reported to be very effective on *L. monocytogenes* in pig slurry and burgers stored in a refrigerator for 16 days. They suggested that extracts from aronia pomace could be used as natural antimicrobial agents when added to meat.

Table 2: SPF of ABEE

λ (nm)	*Abs*	*CF* x *EE*(λ) x *I*(λ) x *Abs*(λ)
290	2.52	0.37
295	2.64	2.15
300	2.58	7.43
305	2.48	8.15
310	2.41	4.49
315	2.34	1.95
320	2.31	0.41
		SPF Value = 25.00±0.09

3.2. SPF of ABEE-cream mixtures

Prolonged exposure to UV rays increases the risk of various skin diseases such as cancer and photoallergic reactions [48]. In recent years, plants have become regarded as potential additives in cosmetics due to their ability to absorb UV rays and protect skin from the sun's harmful rays [49]-[50]. The ability of sunscreens to protect the skin against UV-B radiation is referred to as the SPF [51]. Sunscreen provides protection against the negative effects of the sun and UV rays [52]. Since the SPF of ABEE determined by spectrophotometry is high, that is, 25 (*Table 2*), ABEE may be a potential natural additive in sun cream.

In our study, the SPFs of the cream mixtures prepared with various amounts of ABEE, namely 2.5, 5.0 and 10.0 ml, and commercial creams are given in *Figure 3*. As the ABEE amount increased, the SPF of the cream mixtures increased proportionally ($p<0.05$). The

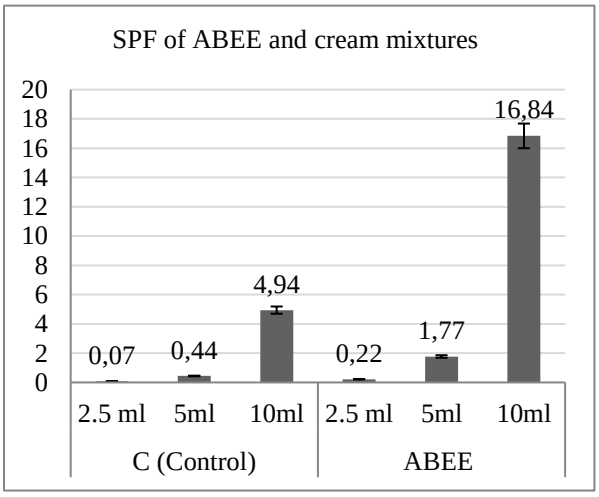

Figure 3: SPF of ABEE and cream mixtures
*F(397248.800) Sig.(0.000) for Control, F(37408.505) Sig.(0.000) for Cream+Extract

Table 3: Antimicrobial activity of the cream developed with ABEE and *L. fermentum* MA-7

	Inhibition Zone Diameter (mm±SD)				
	C	C-ABEE	C-P	C-ABEE-P	F(Sig)
C. albicans ATCC 10231	-[a]	2.76±0.39[a]	6.76±0.60[b]	12.84±2.06[c]	78.000(0.000)
P. aeruginosa ATCC 27853	2.44±0.85[a]	11.55±0.52[b]	3.28±0.30[a]	15.80±0.70[c]	353.231(0.000)
E. coli O157:H7	-[a]	11.75±0.41[b]	6.40±0.24[c]	25.40±0.73[d]	1809.603(0.000)
S. epidermidis ATCC 12228	-[a]	7.54±0.72[b]	10.43±0.38[c]	12.55±1.69[c]	102.156(0.000)
S. aureus ATCC 25923	-[a]	8.31±0.09[b]	10.80±0.38[b]	14.30±1.93[c]	114.180(0.000)

*C: Cream, C-ABEE: Cream+Extract, C-P: Cream+Probiotic, C-ABEE-P: Cream+Extract+Probiotic.
*Superscript values in the rows differed when the one-way ANOVA was applied followed by Tukey's post-hoc test ($p<0.05$).

highest SPF among the cream mixtures was determined to be 16.84 in 10 ml.

In one study, the protective effect of the water extract obtained from aronia berries against UV-B-induced photodamage was investigated on the dorsal skin of mice. The extract was applied topically once a day for 7 days to the skin of mice exposed to UV-B radiation. It was reported that the topical application of the extract protected against skin damage by reducing collagen degradation of the skin after UV-B irradiation [53]. In another study, oral supplementation of the aronia extract improved the antioxidant status of human skin [54] and the consumption of fresh aronia berries increased radical scavenging rates of the skin by 22-23% [55]. In addition, according to the literature, ABEE and cream mixtures can be potential sources for preventing skin damage with their high SPFs.

3.3. Antimicrobial activity of cream developed with ABEE and L. fermentum MA-7

Nowadays, the demand for chemical preservative-free cosmetic products is increasing. An alternative way to ensure a microbial balance in cosmetic products is to use herbal extracts that do not contain chemical preservatives but are also antimicrobially active [40]. Herbal extracts and probiotics are used as natural additives as an alternative to synthetic preservatives in the cosmetic industry, thereby providing extra properties to cosmetic products [41]. The biological activities of the developed groups of creams on clinical microorganisms using a well diffusion assay are presented in *Table 3*. The control, namely cream, exhibited no antimicrobial activity on test microorganisms except for *P. aeruginosa* ATCC 27853 with an inhibition zone diameter of 2.44 mm. The C-ABEE group exhibited the two highest antimicrobial activities on *E. coli* O157:H7 and *P. aeruginosa* ATCC 27853 with inhibition zone diameters of 11.75 and 11.55 mm, respectively. C-P created a large inhibition zone diameters on *S. epidermidis* ATCC 12228 and *S. aureus* ATCC 25923 of 10.43 and 10.80 mm, respectively. The C-ABEE-P group yielded the largest inhibition zone diameter among the test microorganisms of 25.40 mm on *E. coli* O157:H7. Among the groups of creams, the highest antimicrobial activity was detected in the C-ABEE-P group on all test bacteria, possibly due to the synergistic antimicrobial activity of ABEE and *L. fermentum* MA-7 on the microorganisms tested.

In another study, it was determined that the topical application of water extracts obtained using aronia berries reduced deterioration in the function of types I and III collagen in photodamaged mouse skin induced by UV-B radiation [53]. Aronia berry extract has been reported to reduce the activity of matrix metalloproteinases 1 and 3, which are associated with the delayed healing of chronic wounds [56], moreover, the main polyphenols found in aronia berry extracts can inhibit reactive oxygen species, including chlorogenic acid, rutin and hydroxyl radicals [57].

In addition, a study reported that an important interaction occurs between the skin and the fermentation of lactic acid bacteria (LAB), moreover, that the lactate and amino acids in the supernatant of LAB help moisturize the skin [58].

4. Conclusions

ABEE exhibited antimicrobial and antifungal activities against pathogen microorganisms and ABEE as well as cream mixtures yielded high SPFs. Therefore, it was determined that ABEE could add extra features to commercial creams as an additive. ABEE, which was combined with both probiotics and commercial creams, exhibited high levels of antimicrobial activity against pathogen microorganisms. Therefore, the ethanol extract obtained from aronia berries may be a promising in the cosmetic and pharmaceutical industries as a natural additive against infections and to protect the skin.

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