Effect of Bacterial Nanocellulose and Plant-Containing Facial Serum on Hyperpigmentation in *in-vitro* Conditions

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This study investigated the effect of some herbal extracts, such as licorice root, white mulberry leaf, green tea leaf, and grape seed, with a combination of bacterial nanocellulose and some bioactive materials, such as ascorbic acid, niacinamide, hexylresorcinol, and alpha-arbutin, on treatment of hyperpigmentation. The effect of the prepared emulsions on hyperpigmentation was revealed by analyzing their tyrosinase inhibition properties, their ability to stop melanin production, or their properties of whitening the brown spot on the skin. In addition to the physicochemical properties of the 5 different emulsions obtained, tyrosinase, collagenase, and elastase enzyme activities, antioxidant properties, cytotoxicity, and microbiological analyzes were performed by cell-culture modelling. Finally, a dermocosmetic facial serum was designed that is compatible with skin pH, is homogeneously mixed, has good spreading properties, does not cause any microbiological growth, does not inhibit elastase activity while stimulating collagenase activity, reduces melanin production by inhibiting the tyrosinase enzyme, and does not have any toxic effects.

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INTRODUCTION

Hyperpigmentation is a skin breakdown due to the accumulation of melanin pigment in the epidermis and/or dermis that manifests itself as spotted, irregularly patterned brown or sometimes gray-brown hyper-melanosis that occurs in sun-exposed areas of the face, sometimes on the neck, cheeks, forehead, upper lip, nose, and chin (Sehgal *et al.* 2011). Melanin is a skin pigment that gives colour to the skin, hair, and eyes. Its production can be affected by various internal factors, such as hormone regulation and inflammation, as well as external factors such as UV exposure and medications (Resende *et al.* 2022). Against electromagnetic radiation hitting human skin, melanin acts as an optical filter to reduce radiation and a chemical filter with a stable free radical function to absorb compounds produced by photochemical action that may be toxic or carcinogenic (Kollias *et al.* 1991). Biosynthesis of melanin plays a crucial role in protecting the skin by protecting it from sunlight damage (UV radiation absorption), ion accumulation, as well as sequestration of reactive oxygen species (ROS) (Betteridge 2000; Trouba *et al.* 2002;

Yamakoshi *et al.* 2004; Kaur *et al*. 2006).

Melanogenesis is the physiological process of melanin production. It takes place in melanosomes, which are membrane-bound organelles located within melanocytes in the stratum basale layer (Maranduca *et al.* 2019). The biggest factor that induces melanogenesis is UV rays. UV rays directly or indirectly induce melanogenesis by activating intracellular signaling mechanisms such as protein kinase C (PKC), nitric oxide (NO), cyclic AMP (cAMP), proopiomelanocortin (POMC)-derived peptides such as melanocyte-stimulating hormone (MSH), adrenocorticotropic hormone (ACTH) released from keratinocytes or fibroblasts, melanogenic factors such as endothelin-1 (ET-1), stem cell factor (SCF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), or histamine synthesized from mast cells.

Melanogenesis is a complex process regulated by several enzymes, including tyrosinase, phenylalanine hydroxylase (PHA), and tyrosinase-related proteins (TRP-1 and TRP-2) (Pillaiyar *et al.* 2016). Tyrosinase is the key enzyme mainly involved in the initial rate-limiting reactions in melanogenesis, such as the hydroxylation of L-tyrosine (monophenolase activity) to L-3,4-dihydroxy-phenylalanine (L-DOPA) and its subsequent oxidation to yield L-dopaquinone (diphenolase activity) (Ito and Wakamatsu 2003). Inhibiting the tyrosinase enzyme will stop melanin production, as this enzyme plays a central role in this process and catalyzes the first step of melanin formation. Tyrosinase inhibitors have begun to be used as the main whitening agents in commercial skin whitening products (Wang *et al.* 2015). Substances such as hydroquinone, corticosteroids, mercury, kojic acid, *etc*., are very effective, but long-term exposure causes serious adverse effects on the skin, such as ochronosis, atrophy, carcinogenesis, and other local or systemic side effects (Rendon and Horwitz 2012). As a result of increasing studies on the toxicity of hydroquinone and its metabolites, the European Union Scientific Committee on Consumer Safety (SCCS) banned the use of hydroquinone in cosmetic products as of January 1, 2001 (Draelos 2007). The ban on hydroquinone has led cosmetic researchers to study different non-cytotoxic and non-mutagenic active ingredients that suppress melanin synthesis by interfering with the melanogenesis process (Baurin *et al*. 2002; Batubara *et al.* 2010; Kamakshi 2012; Almeda *et al.* 2015; Mann *et al.* 2018).

The general term arbutin refers to α-arbutin (4-hydroxyphenyl-α-D-glucopyranoside) or β-arbutin (4-hydroxyphenyl-β-D-glucopyranoside), both of which are glycoside derivatives of hydroquinone. Both compounds have whitening effects comparable to hydroquinone by functioning as competitive tyrosinase inhibitors, with α-arbutin being much more effective than native arbutin in inhibiting tyrosinase activity (Jimenez *et al.* 2017). Although the molecular structure of α-arbutin is similar to hydroquinone, it does not cause exogenous ochronosis and is less likely to cause irritation or sensitization, making it a more tolerable alternative to hydroquinone. It brightens skin tone by reducing discoloration caused by inflammation and environmental stress, and also improves sugar-related skin sallowness and loss of elasticity (Notaroberto 2016; Chandorkar *et al.* 2021).

Other tyrosinase inhibitors include kojic acid produced by various species of fungi, particularly *Aspergillus oryzae*, and resorcinols found in breadfruit (*Artocarpus incisus*) fruit and argan tree (*Argania spinosa*) (Rendon and Gaviria 2005; Stratford *et al.* 2013). Especially, 4-hexylresorcinol has been shown through *in vitro* and clinical studies to decrease hyperpigmentation with its tyrosinase inhibition activity and anti-inflammatory potential (Kim *et al*. 2011; Fidalgo *et al.* 2018).

In addition, although some whitening agents do not cause tyrosinase inhibition,

they can interfere with melanogenesis through some mechanisms. Antioxidant compounds (*e.g.*, ascorbic acid) can prevent skin pigmentation by multiple mechanisms (Fujiwara *et al.* 2004). There are studies showing that ascorbic acid significantly inhibits melanin formation on purified tyrosinase or cultured cells and inhibits melanin formation in cultured human melanoma cells (Kameyama *et al.* 1996). In addition, nicotinamide, also known as niacinamide, can affect skin tone by inhibiting melanosome transfer. This mechanism has been found to reduce UVB-induced pigmentation in animal models but does not affect tyrosinase activity or melanin synthesis (Fujiwara *et al.* 2004; Smit *et al.* 2009). The chemical structures of actively used skin whitening products are shown in Fig. 1 (Wang *et al.* 2015).

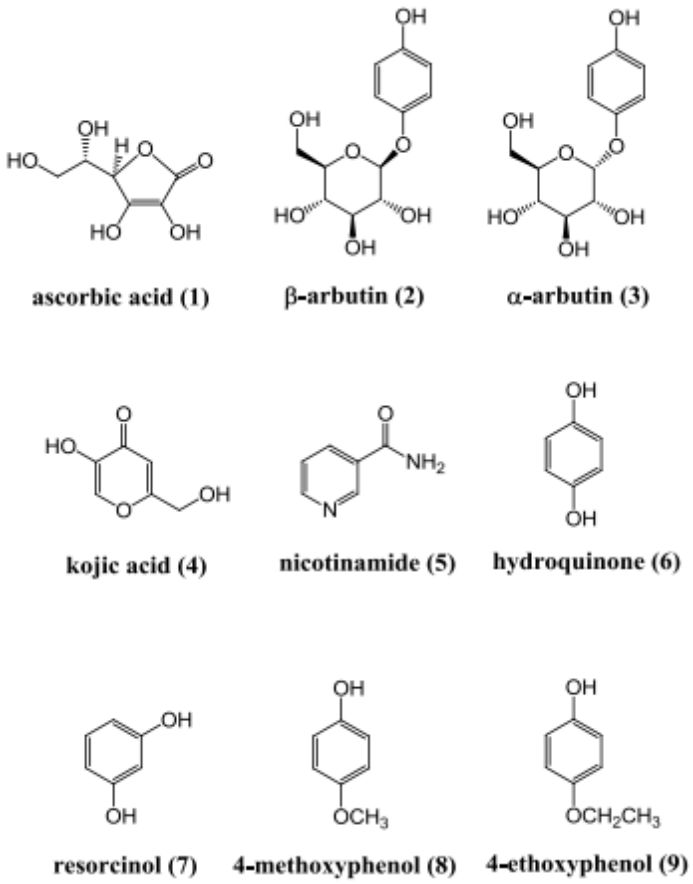


Fig. 1. Chemical structures of actives used as skin whitening products

In addition to these products, natural melanogenesis inhibitors have recently been investigated. It was demonstrated that non-cytotoxic and non-mutagenic plant extracts may be as potent as synthetic whitening agents (Baurin *et al.* 2002; Batubara *et al.* 2010; Kamakshi 2012; Almeda *et al.* 2015). The most important of these is licorice root (*Glycyrrhiza glabra*), which contains isoflavonoids and chalcone as active ingredients (Nerya *et al*. 2003; Kao *et al*. 2014). Extracts of oregano (*Origanum vulgare*, whitening agent: origanoside, a glycosylated phenolic compound) and Chinese skullcap (*Scutellaria baicalensis*, whitening agent: baicalein, a flavone), as well as white mulberry (*Morus alba*) containing chalcones and stilbenoids, and citrus (*Citrus*) fruits containing flavones and flavonelles species also show interesting whitening properties (Lee *et al*. 2002; Jeong *et al.*

2009; Kang *et al.* 2013). Additionally, one-year oral intake of pro-anthocyanidin-rich extract from grape seed (*Vitis vinifera*) has been shown to reduce hyperpigmentation in women suffering from chloasma (Jimbow *et al*. 1976). Green tea extracts, which contain four catechin derivatives: epicatechin, epigallo-catechin, epicaticingalato, and epigallocatechin-3-gallate (EGCG), can also inhibit the acute effects caused by exposure to UVB radiation on the skin, due to their high concentration of polyphenol compounds (Alexis *et al.* 1999; Dal'Belo *et al.* 2009).

Bacterial nanocellulose (BNC), a nanoscale form of cellulose that combines the properties of cellulose with those of nanomaterials is produced biotechnologically as an exopolysaccharide by some aerobic non-pathogenic bacteria (*e.g.*, *Komagataeibacter* (only for *Acetobacter*), *Agrobacterium*, *Aerobacter*, *Achromobacter*, *Azotobacter*, *Rhizobium*, *Sarcina*, *Salmonella*, and *Escherichia*). In addition to outstanding physicochemical and mechanical properties, BNC also offers high *in vivo* skin biocompatibility, as demonstrated in studies with human volunteers (Almeida *et al*. 2014; Silva *et al.* 2020). The use of BNC in cosmetics, especially as a carrier of skin active ingredients, enzyme immobilizer, emulsion stabilizer, and/or alternative to microplastics, demonstrates the tremendous potential of this remarkable biopolymer as a sustainable alternative for green cosmetic design. BNC has received great attention as a delivery system due to its unique porous nanostructure that supports the incorporation and release of active substances of interest in fields such as biomedical or cosmetics (Perugini *et al.* 2018). The high surface area of BNC fibrils contributes to the establishment of interactions between the hydroxyl groups abundant on the BNC surface and the functional groups of other components such as active low molecular weight compounds, polymers, metal oxides or metal nanoparticles. Its combination with other materials endows BNC with new or improved properties such as bioactivity (e.g. antioxidant, anti-inflammatory and antimicrobial activities), conductivity, optical or magnetic properties and even improved mechanical properties, greatly increasing its potential (Hu *et al.* 2014). Considering the studies in which BNC is combined with different bioactive macromolecules in cosmetics and used as a carrier, it is seen that it increases the penetration of the active ingredients in the formulation (Fonseca *et al.* 2021). Bacterial nanocellulose, which has emerged as an innovative material of recent times, was used in this study to increase the biological effectiveness of the formulation by increasing skin penetration without creating toxic effects. In this study it is aimed to develop a skin serum that could be used in the treatment of hyperpigmentation. While obtaining this serum, the synergistic effect of natural products effective on skin blemishes and the combination of these products with plant extracts and bacterial nanocellulose on tyrosinase enzyme, melanin production, and pigmentation will be revealed.

EXPERIMENTAL

Materials

In the formulations prepared within the scope of the study, 4 different natural products and 4 different herbal extracts were used as active ingredients. Among the raw materials used, niacinamide was supplied from Doğa İlaç (Istanbul, Turkey), α-arbutin from HD International (Kocaeli, Turkey), ascorbic acid from Arkem Kimya (Istanbul, Turkey), and hexylresorcinol from ATS Kimya (Istanbul, Turkey). Among the plant extracts, grape seed, green tea leaf, and licorice root extracts were purchased from Naturalya company with all analysis reports and documents. All extracts were obtained by

water extraction and are 100% herbal. According to the chromatographic analysis results of the extracts, the grape seed extract contained 96.2% oligomeric proanthocyanidin, green tea leaf extract contained 52.1% EGCG and 98.7% polyphenol, and licorice root extract contained 20.4% glycyrrhizin. White mulberry leaf extract had been extracted using methanol, and it had been purchased from Immunflex company with all analysis reports. Hyaluronic acid, used as a moisturizing and viscosity adjusting auxiliary in the formulations, was purchased from HD International, and BNC, used as a carrier, was purchased from Nanografi company (Ankara, Turkey).

Preparation of Formulations

In the formulations, the combination of 4 different active ingredients (α-arbutin, niacinamide, ascorbic acid, and hexylresorcinol) with 4 different plant extracts (licorice root, white mulberry leaf, green tea leaf, and grape seed) were studied. The aim was to achieve an optimum synergistic effect. The prepared formulations were named according to the active ingredient from which they were produced, such as Arb5, Nia5, C5, and Rez5, respectively. In each formulation, the active ingredient was used at a level of 5%, grape seed and green tea extract was used at 1%, and licorice and white mulberry leaf extract was used at 2%. In the other, 5th formulation, no active ingredient was added, aiming only to examine the effect of the combination of plant extracts, and the formulation was named Ext5. The extracts used in the Ext5 formula were used in the same proportions as those in the other formulas.

The active ingredients were mixed in ultrapure water using a heated magnetic stirrer at 300 rpm for 10 min at 75 °C, and after the active ingredients were dissolved, the plant extracts were added to the emulsion as licorice root, white mulberry leaf, grape seed, and green tea liquid extracts, respectively. Subsequently, BNC was added to the emulsion and mixed in a heated magnetic stirrer at a speed of 300 rpm and a temperature of 75 °C for 15 min. After the other raw materials were added to the emulsion and dissolved by mixing, BNC was added and mixed in a heated magnetic stirrer at 300 rpm and 75 °C for 15 min. Then the emulsion was transferred to a mechanical mixer and mixed for another 15 min at 700 rpm. Finally, the emulsion to which hyaluronic acid (as sodium hyaluronate) was added to adjust its viscosity was mixed in a mechanical mixer at 500 rpm for another 30 min.

Physicochemical Properties

The physicochemical properties of the 5 formulations obtained in the previous stage were evaluated by checking their physical appearance, pH, density, viscosity values, as well as response to centrifugation and stability characteristics. While their physical appearances were checked visually, pH values were measured with a pH meter, density values with a density kit, and viscosity values with a viscometer at room temperature. A total of 2 mL samples taken from the formulations into Eppendorf tubes were centrifuged in a centrifuge device at 13,000 rpm for 30 min and it was examined whether there was any phase separation at the end of the centrifugation. Additionally, the prepared formulations were stored at 4 ± 2 °C and 23 ± 2 °C for six months for stability control. Physical appearance, pH, turbidity, phase separation, viscosity and microbiological analyzes of the formulations were examined at the beginning (at time t = 0), in the third and sixth months.

In-vitro Model

Antioxidant activity assay

An antioxidant assay kit (ref MAK334-1KT; Merck, Germany) was used according to manufacturer instructions. Various concentrations of compounds (% v/v) were tested ranging from pure down to zero.

Microbiological analysis

Pseudomonas aeruginosa, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* are the main potential pathogens in cosmetic products. *In vitro* microbiological analyzes of formulations were evaluated against three human pathogenic bacteria, namely *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Staphylococcus aureus* ATCC 6538, and the yeast isolate *Candida albicans* ATCC 10231.

Microbiological analysis was performed according to a standard (Turkish Standard Institute 2019), and the medium of the tested microorganisms and the method used for microbiological analysis are given in Table 1.

Microorganisms were first grown in Brain Heart Infusion Broth for 18 to 24 h. Subsequently, the bacterial suspension was adjusted to turbidity equivalent to 0.5 McFarland solution (1×10^8 CFU mL^{-1}) in sterile physiological water tubes. Finally, antimicrobial activity analysis was performed by diluting 0.5 McFarland tubes to 5×10^5 CFU mL^{-1}.

Table 1. Microbiological Analysis Method

Analysis	Method	Medium
Staphylococcus aureus ATCC 6538	Enrichment and Inoculation of Culture Media	Baird Parker Agar Medium
Pseudomonas aeruginosa ATCC 9027	Enrichment and Inoculation of Culture Media	Cetrimide Agar Medium
Escherichia coli ATCC 8739	Enrichment and Inoculation of Culture Media	MacConkey Agar Medium + Levine Eosin - Methylene Blue Agar Medium
Candida albicans ATCC 10231	Enrichment and Inoculation of Culture Media	Sabouraud 4% Dextrose Agar + Supplement
Moulds - Yeast	Pour Plate or the Spread Plate Technique	Sabouraud 4% Dextrose Agar + Supplement
Total Aerobic Mesophilic Microorganisms	Pour Plate or the Spread Plate Technique	Tryptic soy Agar with Polysorbate 80 and Lecithin

MNT-1 Cell culture

The MNT-1 cells (from human melanoma) were purchased from the cell provider ATCC (CRL-3450; American Type Culture Collection, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, penicillin 100 U/mL, and streptomycin 100 µg/mL. Cells were maintained at 37 °C in a humidified incubator in a 95% air/5% CO_2 atmosphere and passaged by detaching cells with 0.05% trypsin-EDTA (w/v) solution.

Screening against Human Tyrosinase from MNT-1 Cell Lysate

A total of 10×10^6 MNT-1 cells were collected by detaching them with 0.05% trypsin-EDTA (w/v). The cells suspension was washed two times by centrifugation (300 g, 5 min) with phosphate buffer saline solution (PBS 1X). After a final centrifugation at

300 g during 5 min, the supernatant was discarded and the cell pellet was dispersed in 2 mL lysis buffer (PBS with 1% (v/v) Triton X-100). After the lysis (homogenization by pipetting, 5 min, 4 °C), the volume was extended to 10 mL with PBS 1X prior a final centrifugation at 3000 g for 5 min, at 4 °C. Then, 90 µL of the collected supernatant were dispensed per well of a 96-well plate. Increasing concentrations of test compounds dissolved in DMSO were added to wells of the 96-well plate (10 µL/well), all wells (including negative controls) receiving the same amount of vehicle (5% final concentration of DMSO (v/v)). The plate was then incubated for 10 min prior the addition of 100 µL of L-DOPA at 4 mM in PBS 1X. After 4 h of incubation at 37 °C, the formation concentrations of black/brown products were determined by measuring the absorbance at 600 nm using a Multiskan SkyHigh microplate reader (Thermo Scientific, Waltham, MA, USA). Each experiment was performed three times, and the obtained data were normalized and presented as curves defined between 0 to 100% activity, related to the appropriate blank and control experiments (Roulier *et al.* 2023).

Enzyme Activity Analysis

Both collagenase and elastase inhibitory activities were performed with an EnzCheckTM Gelatinase/collagenase assay kit (Invitrogen, ref E12055) and an EnCheckTM Elastase assay kit (Invitrogen, ref E12056) according to manufacturer's instructions. Various concentrations of compounds (% v/v) were tested, ranging from pure down to zero. Inhibitory activity was expressed in %, considering the 0 of concentration as the reference for 100% of activity.

Cytotoxicity

MNT-1 cells were seeded in a 96-well plate at 5000 cells/well in a final volume of 200 µL of complete medium. After 24 h, cell culture medium was removed and compounds to be tested were added at various concentrations, ranging from 0 to 10% (v/v). The toxicity of these compounds was evaluated after 24 h of incubation by the AlamarBlue Cell Viability Reagent (Invitrogen). Briefly, compounds were removed, and cells were washed once with PBS (with Ca^{2+}/Mg^{2+}) before the incubation at 37 °C with the AlamarBlue reagent diluted to 1:10 in PBS, with the final volume of 100 µL/well. After 2 to 3 h at 37 °C, the fluorescence of the 96-well plates were read with a TECAN INFINITE M PLEX (Tecan, Männedorf, Switzerland) 200 pro plate reader (excitation wavelengths 560/9 nm, emission wavelengths 590/20 nm). Cell viability was expressed in %, considering the untreated cells as the reference for 100% of viability.

RESULTS AND DISCUSSION

Preparation of Formulations

According to preliminary trials hyaluronic acid was used to adjust the viscosity of the formulations, and it was tried as 0.2%, 0.4%, 0.6%, 0.8%, and 1.0%, respectively, and the optimum rate for the serum form was determined as 0.7%.

Preliminary trials were also conducted for BNC used as a carrier, and its homogenous dispersion was checked at 0.1%, 0.2% and 0.3%, respectively. It has been determined that BNC forms clumps in water when added at levels of 0.2% and above due to its nano size and large surface area. Thus, the optimum level for BNC was accepted as 0.1%.

Physicochemical Properties

Physical appearance

The prepared formulations are in serum gel form and are a transparent light brown color with a droplet structure due to the presence of nanocellulose and hyaluronic acid. The appearance of each formulation is shown in Table 2. As can be seen in Table 2, the formulas with the largest droplet numbers and sizes were Nia5 and Ext5. In the Rez5 formula, the droplets were small but few in number. The only formulations that had both small droplet size and large number were Arb5 and C5.

Table 2. Physical Appearance of Formulations

Formulation	Physical Appearance
Arb5	
Nia5	
C5	
Rez5	
Ext5	

pH Values

Recently, cleansers and skin care products with a pH of 5.5 have attracted special attention because they do not change the skin pH (Schmid and Korting 1995). For this reason, pH measurements of formulations were controlled after preliminary trials, and sodium hydroxide (NaOH) and citric acid ($C_6H_8O_7$) solutions were used to set pH values to the range of 5.40 to 5.60, which is the ideal pH rate of the skin. The pH values were measured after preliminary trials, the solution used for pH adjustment and the pH values of final products are shown in Table 3.

Table 3. pH Values of Formulations

Formulation	pH Values Preliminary Trials	pH Adjustment	pH Values Final Products
Arb5	5.461	-	5.461
Nia5	5.810	2 mL %16 (w/v) $C_6H_8O_7$ solution	5.418
C5	3.100	16 mL %10 (w/v) NaOH solution	5.591
Rez5	5.207	2 mL %10 (w/v) NaOH solution	5.534
Ext5	5.510	-	5.510

The pH values of the final product formulations are within the skin's ideal pH value range as shown in Fig. 2.

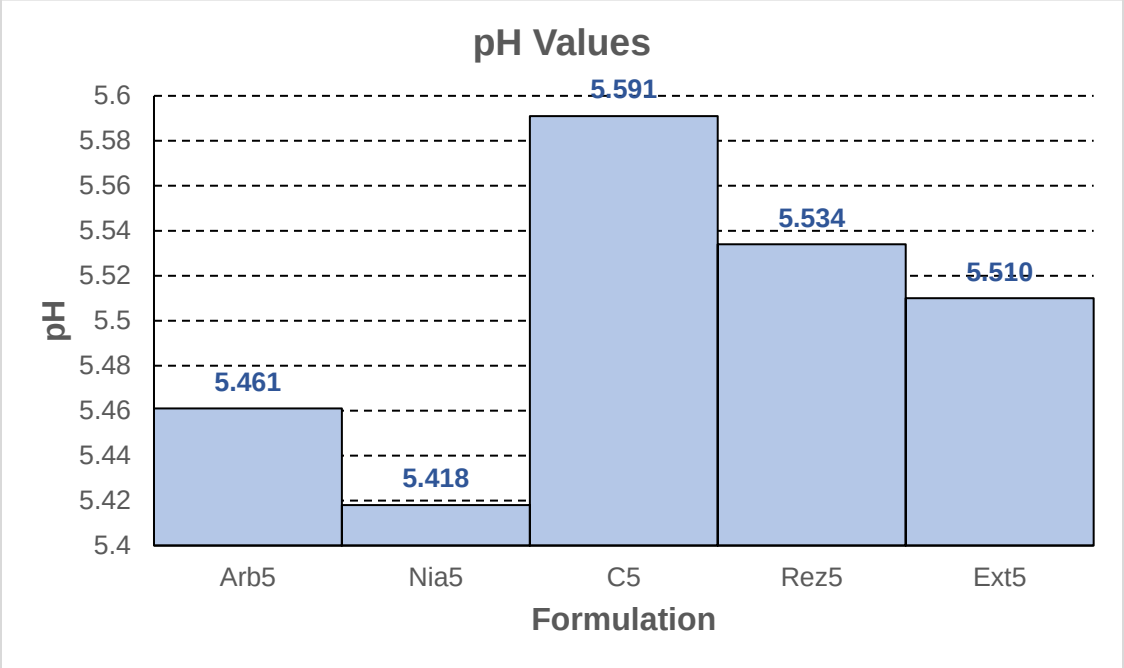


Fig. 2. pH values of formulations

Density values

The densities of the prepared formulations were checked and are given in Table 4.

Table 4. Density Values of Formulations

Formulation	Density (g/mL)
Arb5	2.004
Nia5	2.084
C5	2.376
Rez5	2.059
Ext5	1.928

As can be seen in Table 4, there were small differences between the densities of the formulations. The Ext5 formulation had the lowest density. This is attributed to the fact that only plant extracts were added and no active ingredients were added. A total of 5% of different active ingredients added to other formulas increased the density of the formulations. The reason for the higher density values of Nia5, C5, and Rez5 formulas than that of Arb5 is that NaOH or $C_6H_8O_7$ solution was added to these formulas to adjust the pH. The added solutions increased the density of the formulas in direct proportion to the rate at which they were added.

Viscosity values

Viscosity is defined as the resistance of fluids to flow. In the cosmetics industry, viscosity measurements are an important parameter of quality control both in the production process and in finished products (Deepika *et al.* 2020). Statistical analysis of physicochemical and spreading value data revealed that viscosity has a significant impact on the spreading behavior of cosmetic products, regardless of chemical type (Gorcea and Laura 2010). Many studies show that while physicochemical values, such as surface tension and density do not have a significant effect, a clear correlation can be established between viscosity and spreadability. Especially if log viscosity values are higher than 1, the spreadability of cosmetic products will be high (Hughes *et al.* 2006; Savary *et al.* 2013). In another study, it was shown that the spreading properties will decrease significantly as the log viscosity increases (Douguet *et al.* 2017). The viscosity and log viscosity values of the formulations are shown in Table 5 and Fig. 4.

Table 5. Viscosity and log Viscosity Values of Formulations

Formulation	Viscosity (mPas)	log Viscosity	Temperature (ºC)
Arb5	459.6	2.662	22.3
Nia5	348.6	2.542	21.8
C5	282.9	2.452	22.1
Rez5	256.6	2.409	21.9
Ext5	441.0	2.644	22.4

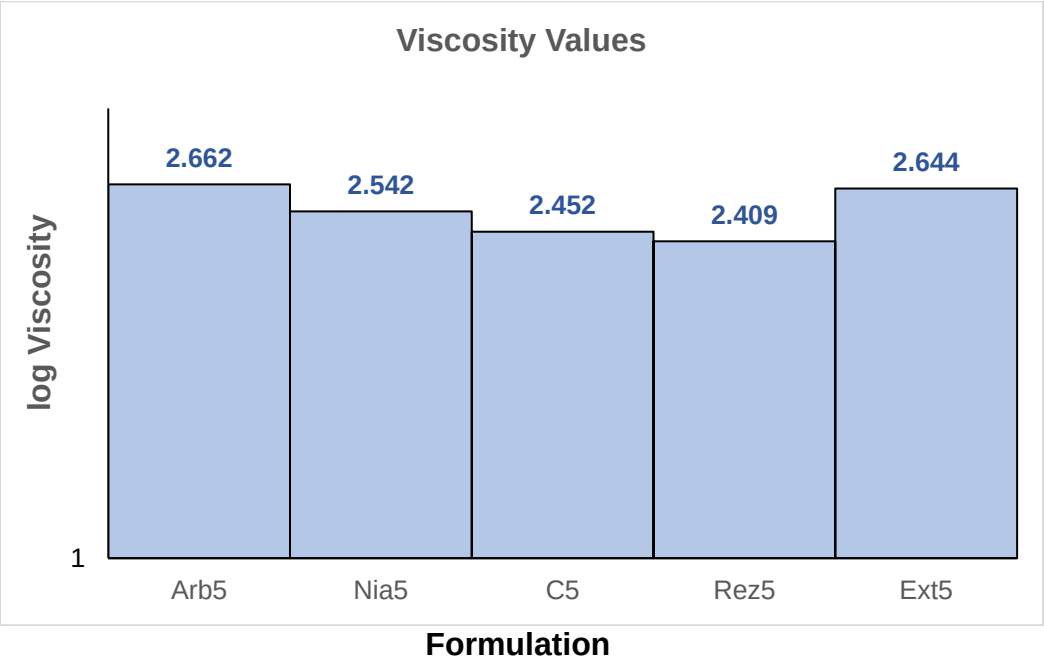


Fig. 3. Log viscosity values of formulations

As shown in Fig. 3, the log viscosity values of all formulations were greater than 1. This shows that all formulations will have good spreading properties and will not create a problem in practice. The log viscosity values of all formulations were close to each other, and C5 and Rez5 formulations had higher spreading values than the others. Differences in viscosity and log viscosity values between formulations arise from the difference in the diffusion/absorption properties of the active substance added.

Centrifuge Properties

Centrifuge analysis evaluated the stability of the emulsions by creating a certain stress in the prepared formulations. Centrifugation is a useful method to predict the shelf life of emulsions.

The formulations were centrifuged as soon as they were prepared (t = 0), after being kept at 23 ± 2 °C for 3 months (t = 3 months), and at 23 ± 2 °C for 6 months (t = 6 months). Their appearance after centrifugation is shown in Table 6.

As shown in Table 6, no phase separation or blurring was observed in the formulations prepared as emulsions, either initially or after waiting for at least 6 months. Thus, the stability and shelf life of the prepared emulsions are sufficient.

Stability Analysis

The purpose of stability analysis is to provide evidence of how the quality of a product changes over time under the influence of a range of environmental factors such as temperature, humidity, and light; to determine both the retest period and shelf life for the product as well as recommended storage conditions. In this study, after the formulations were prepared, they were stored at 4 ± 2 °C and 23 ± 2 °C for six months.

Table 6. Appearance of Formulations After Centrifugation

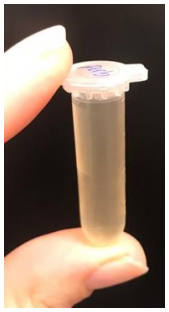
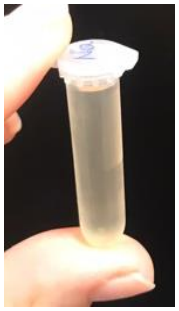
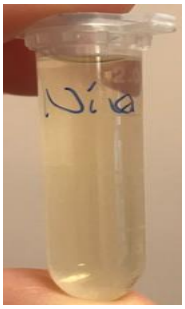
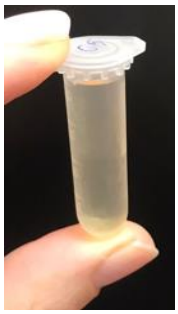
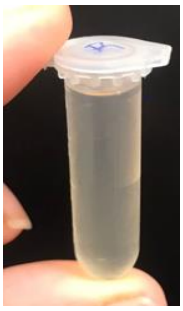
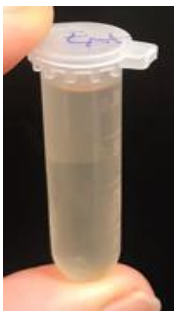
Formulation	Appearance After Centrifugation *t* = 0	Appearance After Centrifugation *t* = 3 months	Appearance After Centrifugation *t* = 6 months
Arb5			
Nia5			
C5			
Rez5			
Ext5			

Table 7. Stability Studies of Formulations

Analysis	*t* = 0 (23 °C)	*t* = 3 months (4 °C/23 °C)	*t* = 6 months (4 °C/23 °C)
Physical Appearance			
Arb5	Light brown Transparent gel	Light brown Transparent gel	Light brown Transparent gel
Nia5	Light brown Transparent gel	Light brown Transparent gel	Light brown Transparent gel
C5	Light brown Transparent gel	Light brown Transparent gel	Light brown Transparent gel
Rez5	Light brown Transparent gel	Light brown Transparent gel	Light brown Transparent gel
Ext5	Light brown Transparent gel	Light brown Transparent gel	Light brown Transparent gel
pH			
Arb5	5.461	5.459 / 5.451	5.435 / 5.447
Nia5	5.418	5.423 / 5.429	5.409 / 5.423
C5	5.591	5.595 / 5.589	5.587 / 5.581
Rez5	5.534	5.521 / 5.532	5.548 / 5.380
Ext5	5.510	5.517 / 5.511	5.532 / 5.527
Viscosity			
Arb5	459.6	458.9 / 459.2	459.1 / 458.6
Nia5	348.6	347.5 / 348.2	346.2 / 347.1
C5	282.9	280.9 / 281.5	281.3 / 280.8
Rez5	256.6	251.2 / 253.4	254.3 / 253.9
Ext5	441.0	438.7 / 438.1	439.5 / 438.9
Centrifugate			
Arb5	No phase separation/blurring	No phase separation/blurring	No phase separation/blurring
Nia5	No phase separation/blurring	No phase separation/blurring	No phase separation/blurring
C5	No phase separation/blurring	No phase separation/blurring	No phase separation/blurring
Rez5	No phase separation/blurring	No phase separation/blurring	No phase separation/blurring
Ext5	No phase separation/blurring	No phase separation/blurring	No phase separation/blurring
Microbiological Property			
Arb5	No microbiological growth	No microbiological growth	No microbiological growth
Nia5	No microbiological growth	No microbiological growth	No microbiological growth
C5	No microbiological growth	No microbiological growth	No microbiological growth
Rez5	No microbiological growth	No microbiological growth	No microbiological growth
Ext5	No microbiological growth	No microbiological growth	No microbiological growth

Physical appearance, pH, viscosity, turbidity, phase separation, and microbiological analyses of the formulations were examined at the beginning (at time t = 0), in the third (t = 3 months), and in the sixth (t = 6 months) months. The results are given in Table 7. According to the stability analysis, there was no change in color, odor, and appearance in the samples that were kept, and no turbidity or phase separation was observed in the samples after centrifugation. Changes in pH and viscosity values in the samples stored for six months at 4 ± 2 °C and 23 ± 2 °C were negligible, and it was determined that there was no microbiological growth. This gives us the chance to comment positively on the shelf life of the products.

In vitro Model

Antioxidant activities

The antioxidant properties of all five formulations were evaluated. However, no quantitative values were obtained due to a lack of linearity along dilutions. This could be explained by the complexity of the prepared formulations and the number of molecules mixed within each one. Nevertheless, qualitative data revealed that higher antioxidant properties were observed for C5, followed by Arb5, and then by Rez5. Very low antioxidant properties were observed for Nia5 and Ext5 as shown in Table 8.

Table 8. Antioxidant Effect of Formulations *

Final concentration (% vol:vol)	Antioxidant effect of Ext5	Antioxidant effect of Arb5	Antioxidant effect of C5	Antioxidant effect of Nia5	Antioxidant effect of Rez5
16%	+	Out of Range	Out of Range	+	+++
3.2%	-	Out of Range	Out of Range	-	+++
0.64%	-	+++	Out of Range	-	++
0.13%	-	++	+	-	+
0.026%	-	+	-	-	-
0.005%	-	-	-	-	-
0.001%	-	-	-	-	-
0.0002%	-	-	-	-	-

* Notes: - indicates no antioxidant activity, + indicates antioxidant activity, ++ indicates moderate antioxidant activity, +++ indicates high level antioxidant activity, "out of range indicates value above the upper point of the reference molecule.

Microbiological analysis

Cosmetic products were grouped into two categories based on the guide (Turkish Medicines and Medical Devices Agency 2005). Products specifically intended for children under 3 years old, for use in the eye area, for use on mucous membranes, and leave-in products are evaluated in scope of Category 1, while rinsing products are in Category 2. For cosmetics classified in category 2, the total viable count for aerobic mesophilic microorganisms should not exceed 10^3 CFU/g or 10^3 CFU/mL when tested in 1 g or 1 mL of the product. The main potential pathogens in cosmetic products, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, must not be present in 1 g or 1 mL of any Category 1 or Category 2 cosmetic product. As seen in Table 9, no microbiological growth occurred in the formulations.

Table 9. Microbiological Analysis of Formulations

Parameter	Unit	Microbiological Analysis Results	Tolerances
Total Aerobic Mesophilic Microorganisms	CFU/g	< 10	< 100
Staphylococcus aureus	/1g-mL	Absence/1g-mL	Absence/1g-mL
Pseudomonas aeruginosa	/1g-mL	Absence/1g-mL	Absence/1g-mL
Escherichia coli	/1g-mL	Absence/1g-mL	Absence/1g-mL
Candida albicans	/1g-mL	Absence/1g-mL	Absence/1g-mL
Total Yeast and Moulds	CFU/g	< 10	< 100

Screening Against Human Tyrosinase from MNT-1 Cell Lysate

Despite years of evaluation achieved on mushroom tyrosinase isolated from *Agaricus bisporus* (abTyr) and considering the low percentage of homology with the human form (hsTyr) (Roulier *et al.* 2020), all experiments were conducted on human tyrosinase extracted from the human MNT-1 melanocyte cell line. No inhibitory activities were obtained with formulations Ext5 and Nia5 as seen in Figs. 4 and 5.

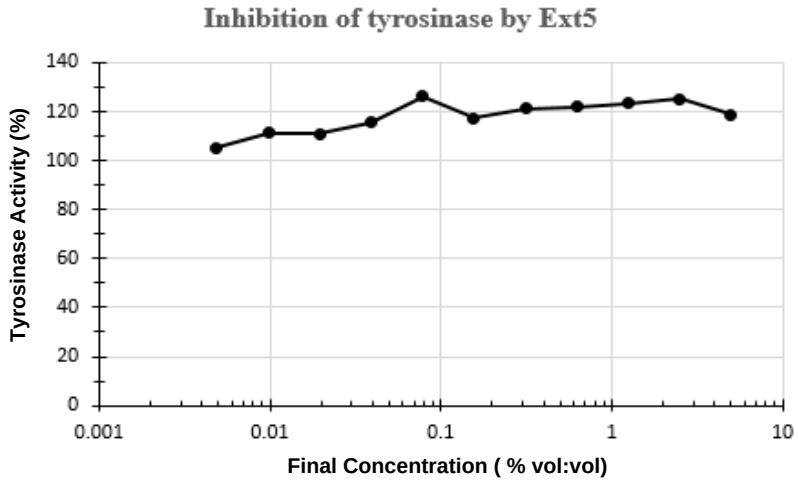


Fig. 4. Tyrosinase Inhibition of Ext5

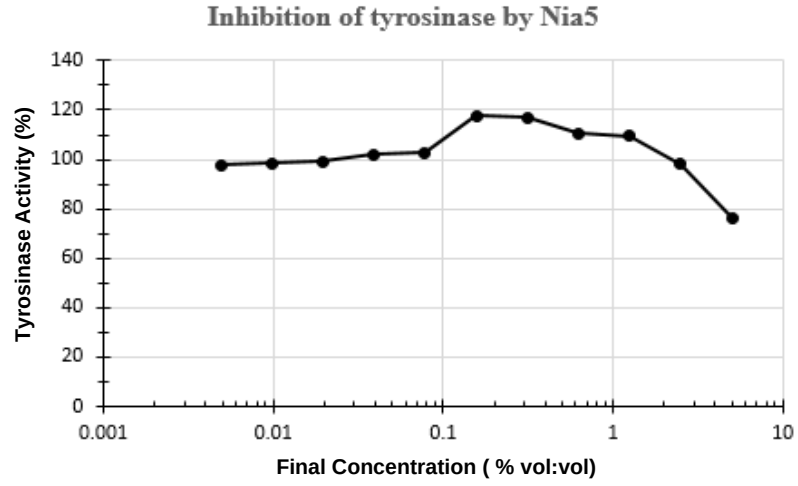


Fig. 5. Tyrosinase Inhibition of Nia5

The three other formulations show an inhibition of tyrosinase activity and a decrease of produced melanin (Figs. 6 to 8).

These inhibitions were characterized by the IC50, which is the concentration of compounds inhibiting 50% of the tyrosinase activity. The best inhibition was observed with the Rez5 formulation displaying an IC50 at 0.025% (v/v). Arb5 and C5 show an IC50 at 0.26% and 1.6% (v/v), respectively.

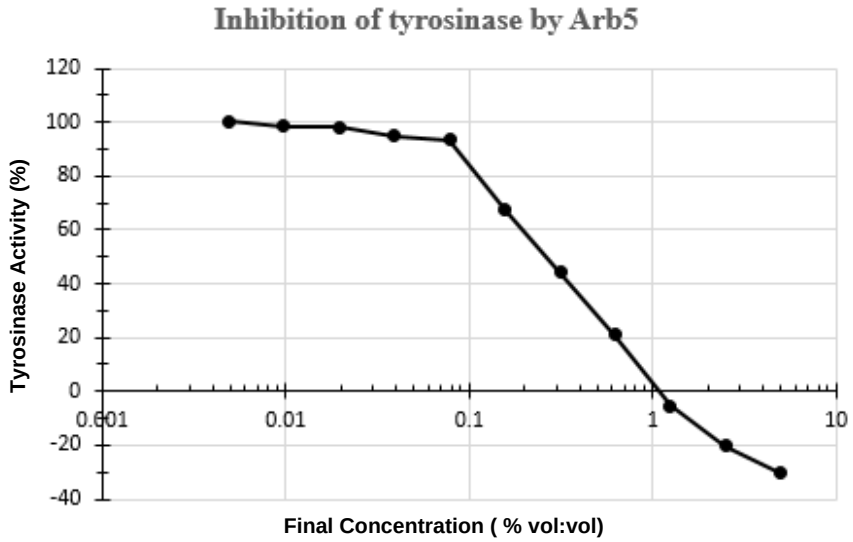


Fig. 6. Tyrosinase Inhibition of Arb5

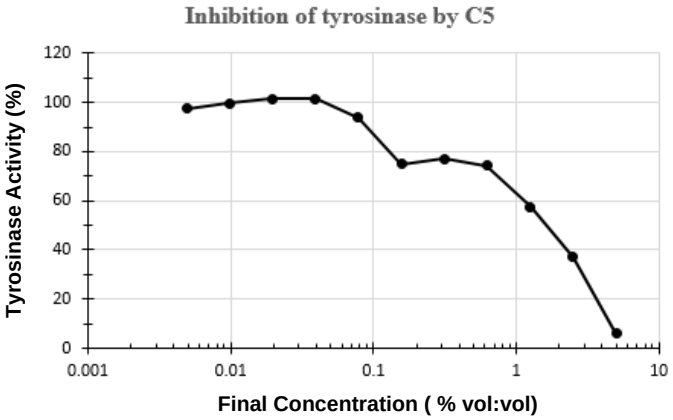


Fig. 7. Tyrosinase Inhibition of C5

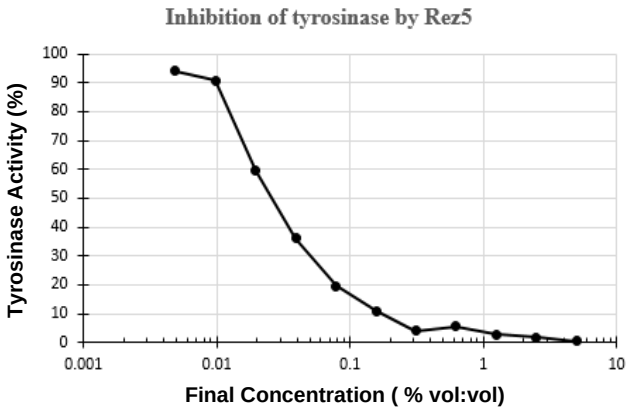


Fig. 8. Tyrosinase Inhibition of Rez5

Enzyme Activity Analysis

Inhibition activities on key enzymes for skin remodeling, such as collagenase and elastase, were measured. Considering collagenase activities, only C5 formulation showed inhibition even at low concentrations. Ext5 and Nia5 did not reveal any inhibitory activities, whereas surprisingly Rez5 and Arb5 seemed to stimulate the enzyme activities (Figs. 9 through 13).

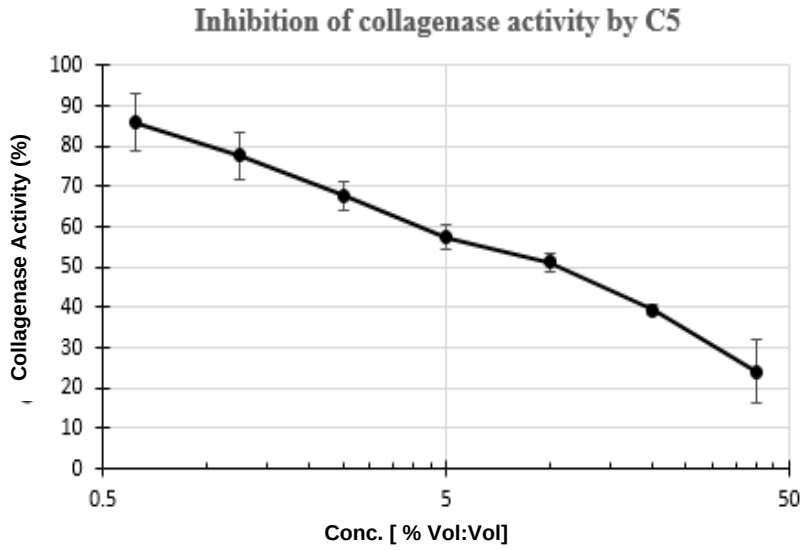


Fig. 9. Collagenase activity of C5

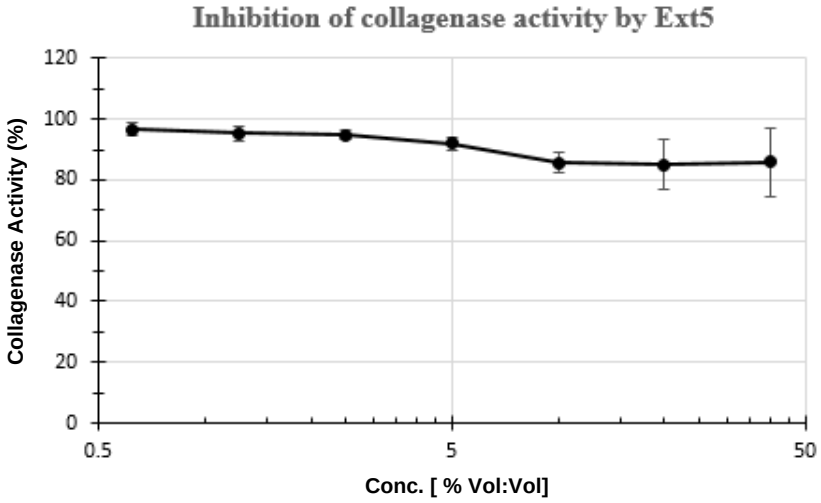


Fig. 10. Collagenase activity of Ext5

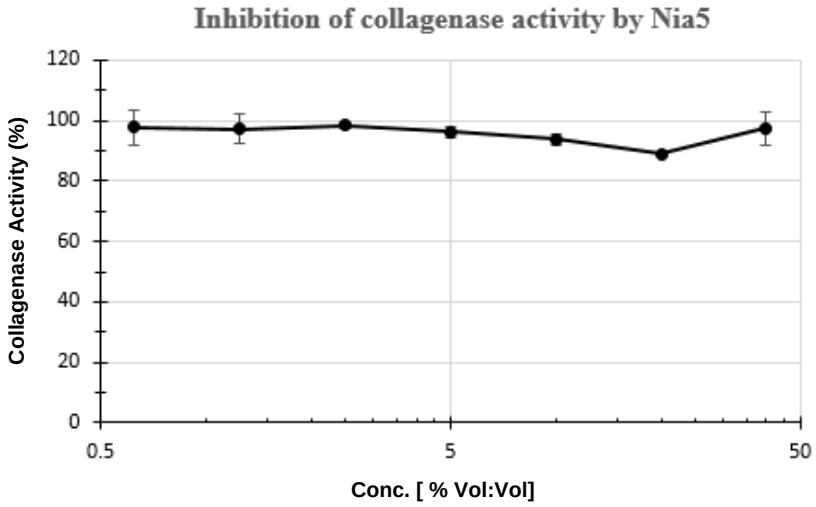


Fig. 11. Collagenase activity of Nia5

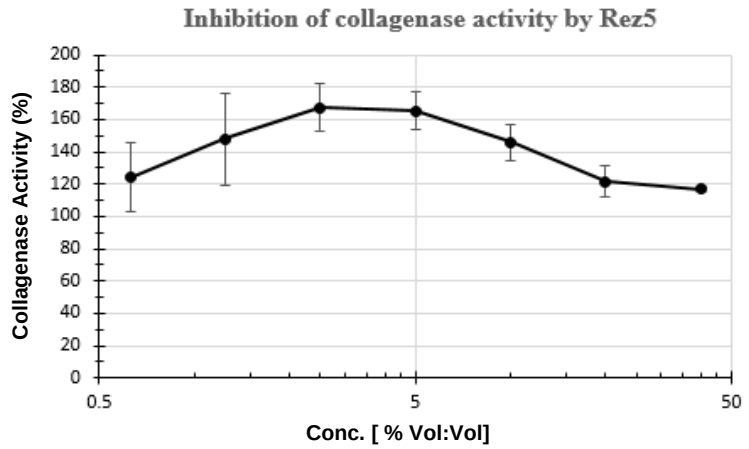


Fig. 12. Collagenase activity of Rez5

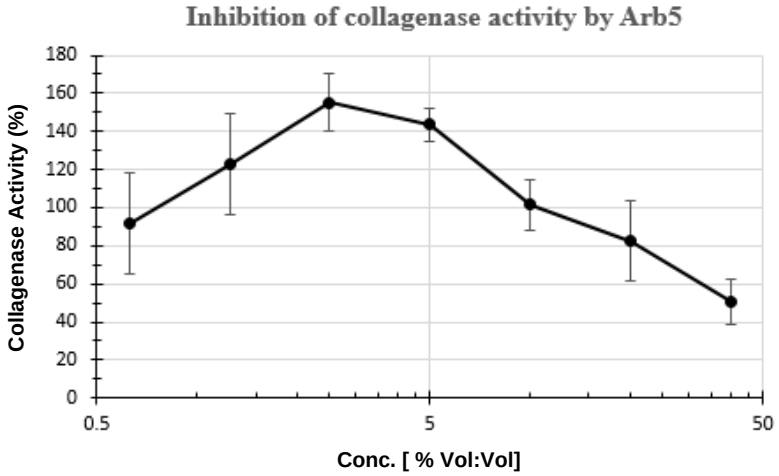


Fig. 13. Collagenase activity of Arb5

Considering elastase activities, only moderate inhibition activities were observed at concentrations higher than 5% (v/v). The best effect was obtained with the C5 formulation (Figs. 14 through 18).

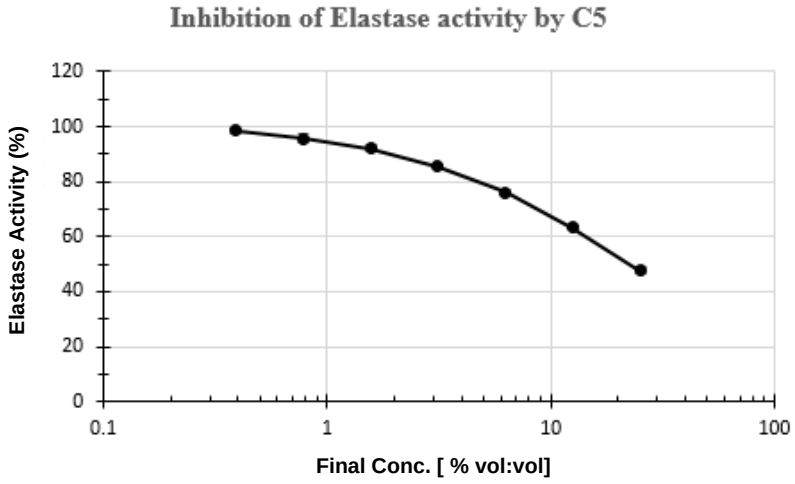


Fig. 14. Elastase activity of C5

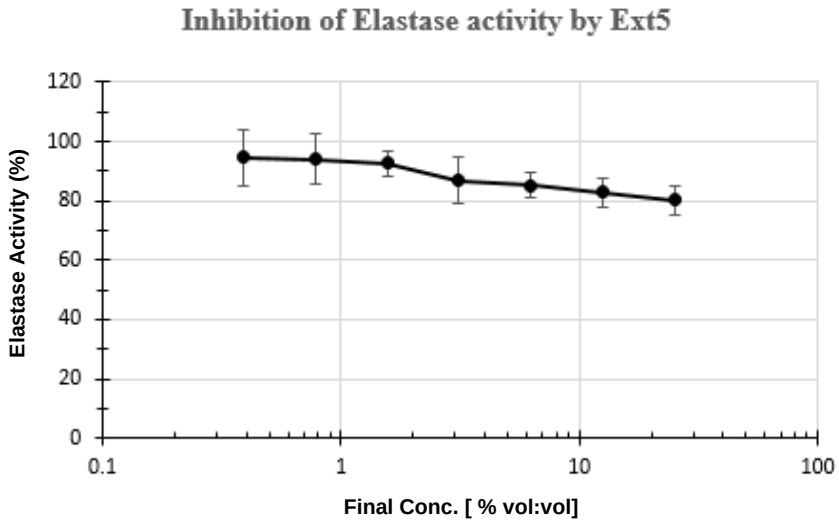


Fig. 15. Elastase activity of Ext5

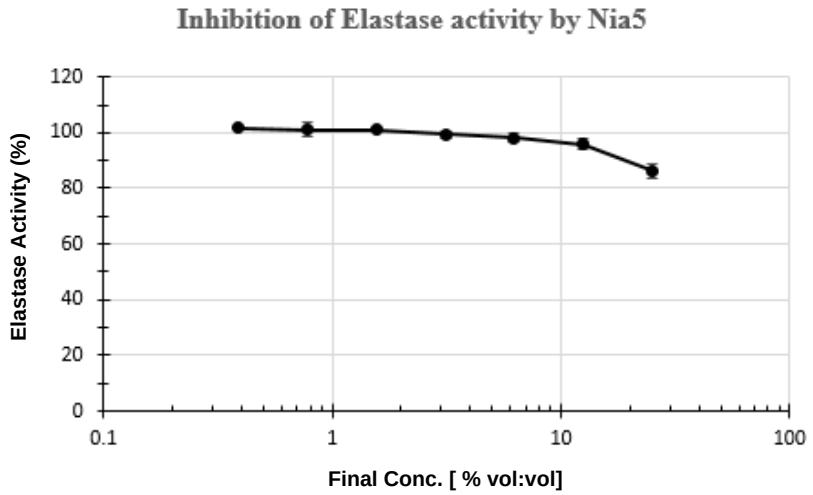


Fig. 16. Elastase activity of Nia5

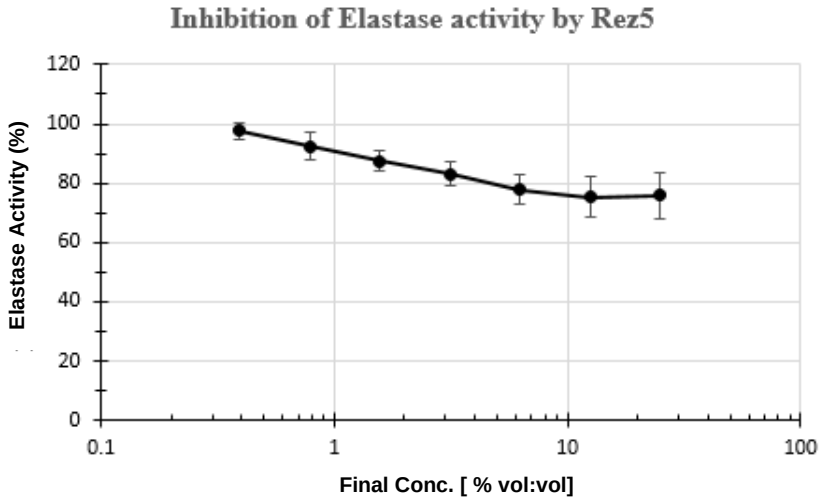


Fig. 17. Elastase activity of Rez5

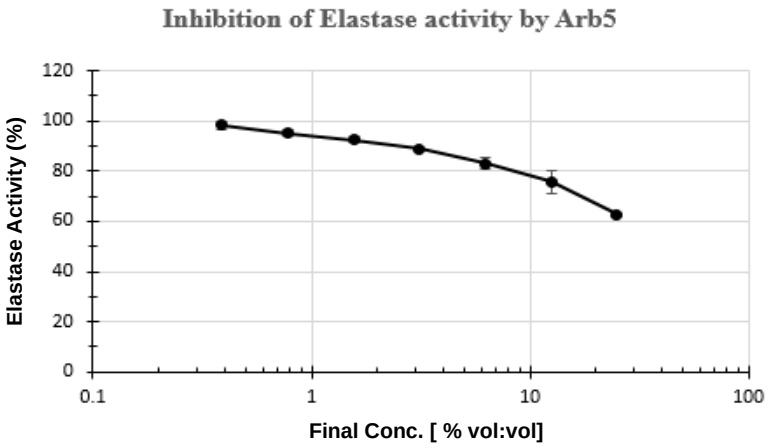


Fig. 18. Elastase activity of Arb5

Melanocyte Cytotoxicity

Because an inhibitory activity has been observed on human tyrosinase activity for some formulations, the toxicity toward the cells expressing this enzyme, namely the melanocytes, is very interesting. Consequently, an experiment of cytotoxicity was assessed on these highly specialized cell type present in the skin after 24 h of incubation with the different formulations. The obtained data revealed an absence of toxicity for Ext5 and Arb5 (Figs. 19 and 20, respectively).

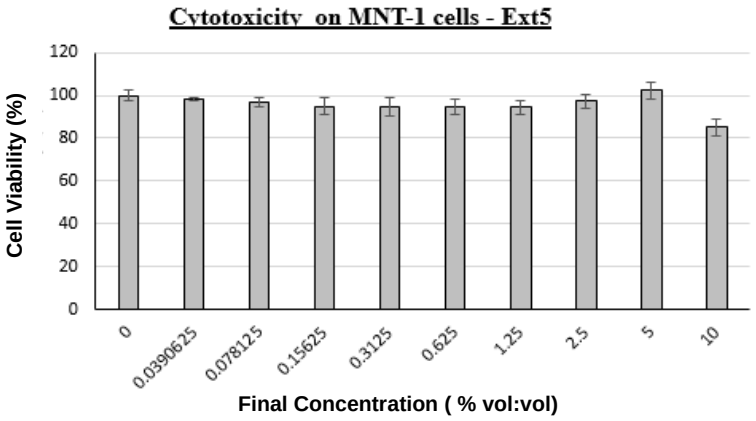


Fig. 19. Cytotoxicity of Ext5

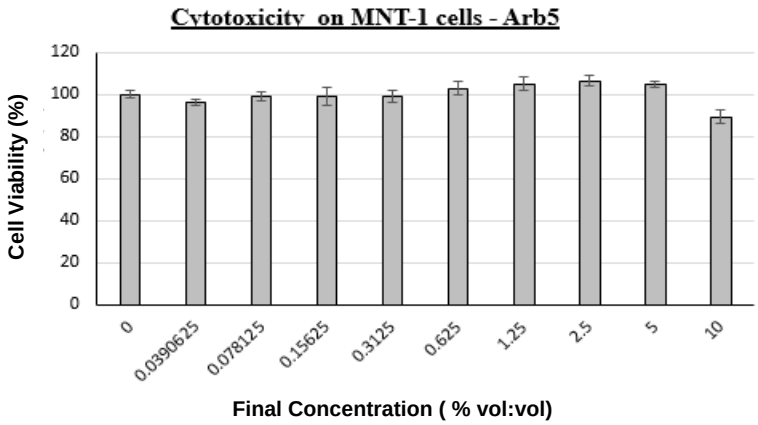


Fig. 20. Cytotoxicity of Arb5

All other 3 formulations showed some toxicity for the higher concentrations tested. Nia5 exhibited a moderate toxicity compared to Rez5 and C5 formulation toxic even at low concentration (around 0.1 % v/v) (Figs. 21, 22, and 23, respectively).

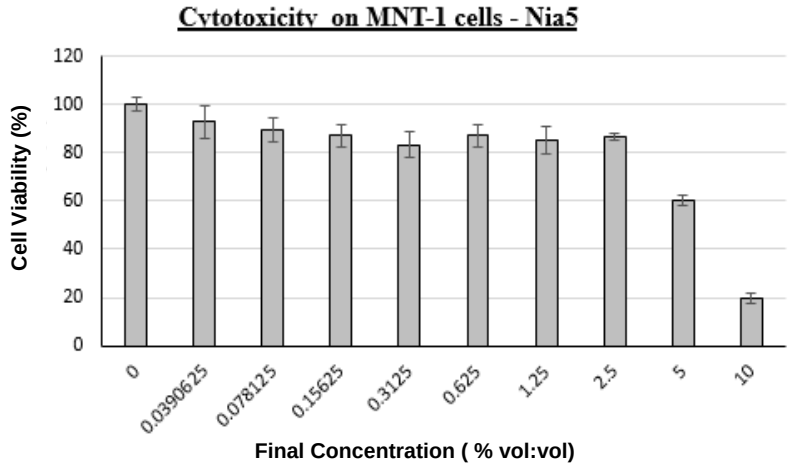


Fig. 21. Cytotoxicity of Nia5

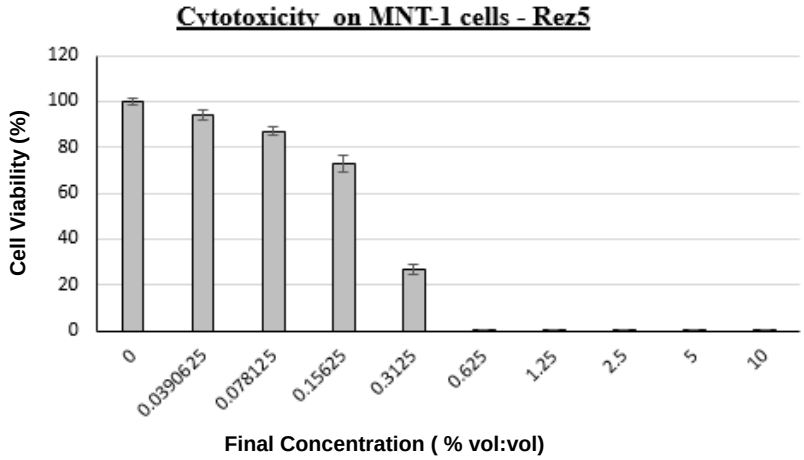


Fig. 22. Cytotoxicity of Rez5

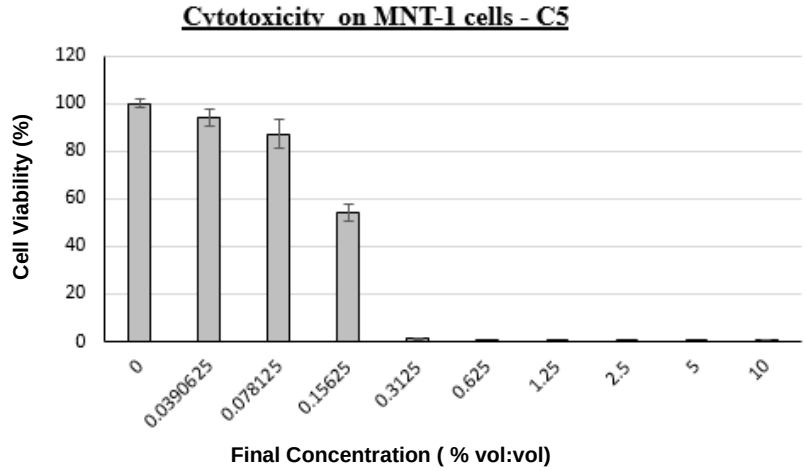


Fig. 23. Cytotoxicity of C5

CONCLUSIONS

1. The prepared samples by the combination of bioactive materials, such as ascorbic acid, niacinamide, hexylresorcinol, alpha-arbutin, and herbal extracts, with bacterial nanocellulose were in serum gel form and transparent in light brown color.
2. According to the physicochemical analysis, all prepared emulsions were suitable for skin, having an appropriate pH and being homogeneously mixed with good spreading properties.
3. Based on the results of the stability analyses performed for shelf-life determination, no microbiological growth or phase separation was observed at room temperature and 4 ℃ for 6 months.
4. The higher antioxidant property was observed for C5, followed by Arb5, and then by Rez5. The antioxidant properties of Nia5 and Ext5 were very low.
5. While Ext5 and Nia5 did not show any tyrosinase inhibition, Arb5, C5 and Rez5 inhibited tyrosinase and decreased the production of melanin. Rez5 showed the highest performance.
6. When collagenase and elastase enzyme activities were investigated, it was determined that Rez5 and Arb5 increased collagenase activity and C5 inhibits elastase.
7. The cytotoxicity analysis showed that Ext5 and Arb5 had no toxicity, whereas all 3 other formulations showed some toxicity.
8. Five serum samples were prepared by combining 5 different bioactive materials with 4 different herbal extracts and bacterial nanocellulose. Of these Arb5 formulation was analyzed to be best for hyperpigmentation without any toxic effects.

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