TYROSINASE INHIBITION, ANTIOXIDATIVE AND ANTIMICROBIAL ACTIVITY OF ACTIVE SUBSTANCES AND CREAMS USED IN THE TREATMENT OF HYPERPIGMENTATION (KOJIC ACID, NIACINAMIDE AND GRAPEVINE EXTRACT)

ORIGINAL SCIENTIFIC ARTICLE

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ABSTRACT:
Hyperpigmentation is a skin disorder caused by increased melanin production. It appears in the form of dark spots and makes the complexion uneven. Pigmentation spots, such as age spots (also known as sun spots), manifest themselves most often on the palms, face and hands. The aim of this work was to investigate and evaluate which of the active substances used in hyperpigmentation treatments has the highest degree of tyrosinase inhibition, and the best antioxidant and antimicrobial activity. Kojic acid, niacinamide and grapevine extract were used as active substances in the formulations. When summarizing the results, kojic acid gave the best results, followed by niacinamide, and finally grapevine extract. However, it is necessary to perform a long-term *in vivo* study on volunteers that will show the effectiveness and efficiency of the mentioned substances. In addition to the treatment of hyperpigmentation, the mentioned substances can also be used in antiaging skin treatments due to the presence of antioxidant activity.

KEYWORDS: kojic acid, niacinamide, grapevine exctract, hyperpigmentation, tyrosinase inhibition

INTRODUCTION

Hyperpigmentation is a skin disorder caused by increased melanin production. It appears in the form of dark spots and makes the complexion uneven. Pigmentation spots, such as age spots (also known as sun spots), manifest themselves most often on the palms, face and hands.

Pigmentation disorders, dyschromia, acne scars and other disorders, along with a host of other dermatoses, are treatment challenges that dermatologists face every day. Through a detailed review of the literature, as well as recent research, we can conclude that the use of preparations for hyperpigmentation is very popular among all populations, but it is not always safe. The therapy that is most often prescribed to the patient, in addition to a positive effect on hyperpigmentation, also shows a cytotoxic effect. The goal of the therapy is that, in addition to the expected therapeutic effect, it is also safe, especially if the use of the drug requires a longer period of time.

The pigment melanin determines the color of the eyes, hair and skin of each person. Certain internal and external factors, e.g. sun exposure, genetics, hormonal

changes, inflammation and age can affect melanin production. For melasma, topical treatment options include retinoids, azelaic acid, hydroquinone, chemical peels and cosmetic medications.

Hydroquinone is considered the gold standard for the treatment of hyperpigmentation, but with long-term use, its cytotoxic effect is possible.

During the last decade, numerous cases and studies have been conducted that emphasize the use of products such as zinc, arbutin, kojic acid, basic compounds of vitamin C and green tea extracts, as newer therapies for the treatment of patients with melasma.

Kojic acid is a by-product of the Japanese rice wine, sake, and is a safer, natural ingredient, although its effectiveness in inhibiting melanin production is disputed. This acid (5-hydroxy-2 hydroxymethyl-4-pyrone) is a hydrophilic fungal product that occurs naturally in some species, such as *Aspergillus* and *Penicillium*. Kojic acid reduces hyperpigmentation by inhibiting the production of free tyrosinase, and is also a powerful antioxidant. Tyrosinase plays a pivotal role in the biosynthesis of melanin pigment in various organisms, including mammals [1]. It is predominantly localized within melanocytes, specialized cells responsible for synthesizing and secretion of pigment granules [2]. Kojic acid is used in a concentrations ranging from 1% to 4% [3].

Kojic acid presents a variety of applications for human use, especially as a depigmenting agent. Besides the depigmenting activity, kojic acid and derivatives can act as antioxidant, antimicrobial, anti-inflammatory, radioprotector, anticonvulsant and obesity management agents, and present potential as antitumor substances. Depigmenting activity is due to the molecules, after penetrating the cell, binding to tyrosinase active site, regulating melanogenesis factors, leucocytes modulation and free radical scavenging activity. Hence, polarity, size and ligands are also important factors for activity. Kojic acid and derivatives present cytotoxicity to some cancerous cell lines, including melanoma, hepatocellular carcinoma, ovarian cancer, breast cancer and colon cancer [4].

Niacinamide, an active form of vitamin B3, is recognised for its significant dermal benefits including skin brightening, anti-ageing properties and the protection of the skin barrier. Its widespread incorporation into cosmetic products, ranging from cleansers to serums, is attributed to its safety profile and proven efficacy. Topical niacinamide is used for some skin disorders, including dermatitis, acne vulgaris and actinic keratosis [4].

Niacinamide has been verified in treating almost every skin disorder, hyperpigmentation, acne, psoriasis, pruritus, dermatitis, fungal infections, epidermal melasma, non-melanoma skin cancer, etc. Long term use of niacinamide, regardless of the skin type, paves the way for new skin cells, making skin healthier, brighter, and hydrated [5,6,7]. It is used in creams in concentrations ranging 4% to 5%.

The inhibitory effect of leaf extracts from a few *Vitis vinifera* varieties concerning tyrosinase has been recorded in previous studies [8,9]. Therefore, investigating the potential of bioactive compounds of grapevine leaves becomes an attractive research direction due to the widespread occurrence of skin hyperpigmentation and the demand for natural and efficacious treatments [1].

MATERIALS AND METHODS

All chemical reagents were purchased from Semikem (Bosnia and Herzegovina) and Merck (Germany). Active substances used in this study were kojic acid (Terra Organica d.o.o., Zagreb, Croatia), niacinamide (Volimo prirodno d.o.o., Mostar, Bosnia and Herzegovina) and grapevine water-ethanol extract. Spectroscopic measurements were performed on a Perkin Elmer Lambda 25 spectrophotometer.

TYROSINASE INHIBITION ASSAY

The sample solution (120 μL) and 50 μL of tyrosinase solution in phosphate buffer (16 mM pH 6.8) were left at room temperature in the dark. After 10 minutes, 50 μL of L-DOPA solution (0.8 mg/mL in phosphate buffer) was added. After 10 min, the absorbance at 492 nm was measured.

Tyrosinase inhibitory activity (TyInh) was calculated as:

$$TyInh\ (\%) = (A0\text{-}As)/A0 \times 100\ [10]$$

where A0 is the absorbance of the negative control (where buffer was used instead of the extract) and As is the absorbance of the corresponding sample.

The concentration of the extract that inhibits 50% of tyrosinase activity (IC50) was calculated.

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical inhibition assay was performed according to the published method [11]. Substances were mixed with absolute methanol and then mixed with a DPPH radical solution. Absorbance measurements were performed at 517 nm, after which DPPH radical inhibition was calculated according to the equation:

$$I = Ac - As / Ac \times 100\ [\%]$$

where As is the absorbance of the solution containing the sample at 517 nm, and Ac is the absorbance of the DPPH solution.

Results are expressed as IC50 value. Vitamin C was used as a positive control.

FERRIC REDUCING ANTIOXIDANT POWER (FRAP) ASSAY

The ferric reducing antioxidant power of the substance, which reflects the antioxidant activity, was determined following the protocol [12]. 3 mL of prepared FRAP reagent was mixed with 100 μL of diluted substance. Absorbance at 593 nm was recorded after a 30 min incubation at 37°C. The FRAP value was calculated from the iron (II) sulfate heptahydrate calibration curve.

IN VITRO ANTIBACTERIAL ACTIVITY TESTING

Antibacterial activity was investigated by diffusion method [13] on reference microbial strains *E. coli*, *Listeria sp.*, *S. aureus*, *B. subtilis*, *P. aeruginosa and C.albicans*. From the microorganisms strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared. The strains were then placed on the surface of the nutrient substrate Mueller-Hinton agar, dispersed in sterile Petri dishes. Drill-shaped holes were made ("wells") in the agar, into which 100 μL of substances solutions in different concentration were added. After the plates were left at room temperature for 15 minutes, the substance was diffused into agar, and incubated at 37°C/24 h. After the incubation period, the inhibitory zones were measured.

FORMULATION AND CHARACTERIZATION OF O/W CREAMS

The formulations are given in Table 1. The cosmetic creams of the O/W type were made by using the dissolution method or the English method. The creams were made by dissolving the emulsifier (Phytocream) in the inner (fatty) phase and heating it in steam at a temperature of 40˚C with gentle mixing, and in a chemical beaker the ingredients of the outer (aqueous) phase were measured and heated to temperature of 40-50˚C with stirring. The active components (kojic acid, niacinamide or grapevine extract) were added to the heated water phase.

Table 1. Formulation of O/W creams

Components	Kojic acid cream	Niacinamide cream	Grapevine extract cream
Rose hydrolat	60.0	60.0	60.0
Almond oil	27.2	12.5	25.0
Geogard	0.8	0.8	0.8
Phytocream	11.0	11.0	10.0
Grapevine extract	-	-	5.0
Niacinamide	-	4.0	-
Kojic acid	2.0	-	-

After the creams were made, the color, feel on the skin, pH and electrical conductivity (by immersing the electrode directly into the creams) were recorded.

DETERMINATION OF THE ANTIOXIDANT CAPACITY OF ACTIVE COMPONENTS ISOLATED FROM O/W CREAMS

The antioxidant activity of the active components isolated from the creams involved testing and isolation after 72 hours after production.

The sample for determining the antioxidant capacity using the DPPH method was prepared by heating 5 g of the sample with 25 mL of purified water at a temperature of 60°C for 10 minutes. After cooling, the aqueous layer was separated by decantation or filtration. In this way, the fatty and aqueous phases of the cream were separated, and the aqueous layer was used for testing the antioxidant capacity of the cream [14].

RESULTS AND DISCUSSION

RESULTS OF TYROSINASE ACTIVITY

Niacinamide and grapevine extract did not show tyrosinase inhibition, which was confirmed by other authors. Hakozaki and others found that niacinamide had no effect on the catalytic activity of mushroom tyrosinase or on melanogenesis in cultured melanocytes. However, niacinamide gave 35-68% inhibition of melanosome transfer in the coculture model and reduced cutaneous pigmentation in the PREP model. In the clinical studies, niacinamide significantly decreased hyperpigmentation and increased skin lightness compared with vehicle alone after 4 weeks of use [10]. Kojic acid showed tyrosinase inhibition with IC_{50} 39.55 μg/mL.

Table 2. Tyrosinase inhibition of active substances

Sample	Tyrosinase inhibition IC_{50} value [μg/mL]
Kojic acid	39.55 μg/mL ± 0.86 μg/mL
Niacinamide	did not show tyrosinase inhibition
Grapevine extract	did not show tyrosinase inhibition

RESULTS OF ANTIOXIDATIVE ACTIVITY

Table 3 shows the results of the antioxidant activity of the tested samples. The antioxidant potential of kojic acid, niacinamide, and grapevine extract was determined *in vitro* using DPPH and FRAP colorimetric assays.

According to the obtained results, grapevine extract showed the best reducing properties in the FRAP assay. The obtained results can be explained by the presence of polyphenolic compounds in the grapevine extract [15]. Phenolic compounds act as electron donors, which reduce the yellow-colored ferric tripyridyltriazine complex (Fe(III)-TPTZ) to the blue-colored iron complex (Fe(II)-TPTZ). Thanks to their reducing properties, phenolic compounds are correlated with antioxidant activity. A lower IC_{50} value in the DPPH assay means a better antioxidant activity of the sample. An IC_{50} value of 1.31 mg/mL was recorded for grapevine extract, which confirms the antioxidant properties proven by the FRAP method.

In the DPPH and FRAP assay, niacinamide showed a weaker antioxidant activity compared to grapevine extract. Also, the IC_{50} value for niacinamide is higher than for kojic acid, which makes niacinamide a weaker antioxidant. Kojic acid is used as an antioxidant in cosmetic preparations, because it shows the ability to chelate iron ions. The lowest IC_{50} value was recorded for kojic acid, which means the best antioxidant activity of all tested samples. However, the ability to reduce ferric ions was unmeasurable, since, when mixed with the FRAP reagent, a dark yellow color appeared, with no subsequent visible changes during incubation. In the FRAP assay, a blue-colored Fe (II)-TPTZ complex appears, as a consequence of the reduction of ferric ions in the presence of antioxidants, which was not the case when testing kojic acid. It was assumed that a reaction occurs between some of the components of the FRAP reagent and the kojic acid itself. In addition, the results of antioxidant activity, measured by the FRAP assay, are affected by the concentration of kojic acid, as well as the pH value.

Table 3. Antioxidant activity of active substances

Sample	FRAP (μmol/g)	IC_{50} (mg/mL)
Kojic acid	-	0.38
Niacinamide	26.2	2.60
Grapevine extract	77.9	1.31

RESULTS OF ANTIMICROBIAL ACTIVITY

The most common causes of skin infections are *S. aureus, S. pyogenes* and *P. aerugionsa*. *S.aureus* and *S.pyogenes* often causes infections such as impetigo, folliculitis and cellulitis and necrotizing fasciitis. *P.aeruginosa* is known to cause infections in people with damaged skin, such as infections after injuries or surgery.

Kojic acid showed the largest inhibition zones, and grapevine extract did not show inhibition zones for any bacterial strain. A concentration of 1% kojic acid has shown excellent results on all strains except *B. subtilis* and *C. albicans*, and a concentration of 10% showed zones larger than 20 mm for all bacterial strains, including *C. albicans.*

Niacinamide is otherwise used in creams in a concentration of 5%, and has shown a zone of inhibition for *Listeria sp.* and *P. aeruginosa*. In a concentration of 10%, it showed zones of inhibition on *E.coli* and *S.aureus*. Grapevine extract did not show antimicrobial activity, which may depend on many factors such as chemical composition, geo-climatic location and growing condition of the plant, etc.

Table 4. Antimicrobial activity of the active substances

Name of the organism	*Kojic acid ZI (mm) 1% sol.*	*Kojic acid ZI (mm) 10% sol.*	*Niacinamide ZI (mm) 5% sol.*	*Niacinamide ZI (mm) 10% sol.*	*Grapevine extract ZI (mm) Concentrated*	*Grapevine extract ZI (mm) 20% sol.*
***E.coli* WDCM 00012**	**24**	**28**	**0**	**15**	**0**	**0**
Listeria sp. WDCM 00017	**10**	**30**	**10**	**18**	**0**	**0**
***S.aureus* WDCM 00034**	**18**	**30**	**0**	**14**	**0**	**0**
B. subtillis WDCM 00003	**0**	**23**	**0**	**0**	**0**	**0**
P.aeruginosa WDCM 00025	**21**	**33**	**15**	**25**	**0**	**0**
***C.albicans* WDCM 00054**	**0**	**20**	**0**	**0**	**0**	**0**

RESULTS OF THE CHARACTERISATION AND ANTIOXIDANT CAPACITY OF ACTIVE COMPONENTS ISOLATED FROM O/W CREAMS

The formulations differed in color, which depended on the active substance. pH ranged around 5, which corresponds to the requirements of the European pharmacopoeia (pH of semi-solid preparations for the skin is 3.5-8), and the electrical conductivity was above 50 $\mu S/cm$, which indicates O/W emulsions/creams.

Table 5. Characterisation of O/W creams

Sample	Color	Feel	pH	Eletrical conductivity (μS/cm)
Kojic acid cream	Light yellow	Smooth	5.57	51.4
Niacinamide cream	White	Smooth	5.80	58.9
Grapevine extract cream	Light brown	Smooth	4.84	59.3

Figure 1 graphically presents the antioxidant activity of creams made with kojic acid, niacinamide and grapevine extract.

The highest inhibition of DPPH radicals was recorded in the formulation with niacinamide, and the lowest in the formulation with kojic acid. Although kojic acid alone showed the lowest IC_{50} value in the DPPH assay, the formulation with kojic acid showed the weakest inhibition of DPPH radicals. Kojic acid is unstable at high temperature, so during technological and analytical processes, its decomposition and consequent reduction of antioxidant activity may occur [16]. Also, elevated temperature can lead to structural changes and degradation of polyphenols in plant extracts, which correlates with a weaker antioxidant activity of cream with grapevine extract [17, 18].

The 4% niacinamide cream showed the highest percentage of DPPH radical inhibition, although niacinamide individually showed a higher IC_{50} value compared to kojic acid and grapevine extract. Niacinamide is widely used as an antioxidant in topical antiaging formulations, usually in a concentration range between 4% and 5%. It has been shown to protect the integrity of cell membranes from oxidation, as it contributes to reducing the concentration of superoxide radicals in keratinocyte cultures [19].

Changes in the antioxidant activity of creams may be due to interactions with other ingredients of the creams and the amount of the starting substance in the creams. In creams, antioxidants can be dispersed in a matrix that can limit their freedom of movement and interaction with free radicals. The created O/W creams (aqueous phase) showed an antioxidant activity above 20% and have the potential to fight free radicals and prevent skin aging.

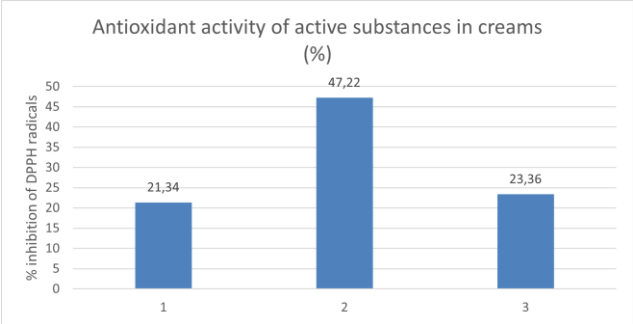

Figure 1. Antioxidant activity of active substances in creams

CONCLUSION

When summarizing the results, kojic acid gave the best results, followed by niacinamide, and finally grapevine extract. However, it is necessary to perform a long-term *in vivo* study on volunteers that will show the effectiveness and efficiency of the mentioned substances. In addition to the treatment of hyperpigmentation, the mentioned substances can also be used in antiaging skin treatments due to the presence of antioxidant activity.

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