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A Radish Root Ferment Filtrate for Cosmetic Preservation: A Study of Efficacy of Kopraphinol

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Abstract: Preserving cosmetic products from microbial contamination is crucial to ensuring their safety, efficacy, and shelf life. A number of synthetic antimicrobial preservatives are available, but, since the global market demand for natural ingredients is increasing, cosmetic manufacturers are considering replacing conventional preservatives with natural alternatives. In this context, the objective of this investigation was to characterize the antimicrobial activity of the natural preservative, intended for cosmetic purposes, Kopraphinol (INCI name: *Lactobacillus/Radish Root Ferment Extract Filtrate*). It was tested against a panel of selected bacteria and mycetes by using conventional microbiological techniques (determination of MIC, time killing assay), and a challenge test was used to verify its potential preservative in an O/W hydrophilic cream (Cetomacrogol cream base). Kopraphinol has shown an interesting antimicrobial effectiveness, with M.I.C.s ranging from 0.78% to 6.25% for bacteria and from 1.56% to 5% for mycetes. Moreover, it fulfilled challenge test criterion A and has proven effective against microbial contamination, leading to a 3 log reduction of inoculum after 7 days for bacteria and a 2 log reduction at 14 days for fungi. The results obtained show that Kopraphinol can be considered a promising and effective candidate for the antimicrobial preservation of cosmetics and could successfully complement or even replace conventional preservatives.

Keywords: cosmetics; preservative efficacy; natural preservatives; *Lactobacillus/Radish Root Ferment Extract Filtrate*; Kopraphinol©; methylparaben; challenge test; MIC

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1. Introduction

According to EU Regulation 1223/2009, which establishes rules to be complied with by any cosmetic circulating on European territory, "a cosmetic product made available on the market shall be safe for human health when used under normal or reasonably foreseeable conditions of use" [1]. With regard to safety, the microbial contamination of cosmetics is of special concern for industry because microbial contaminants can not only alter products, making them useless, but also pose a potential risk to the health of consumers [2]. Considering the conditions of use and the sites where they are applied, cosmetics are not expected to be sterile, but they are required to exhibit a controlled microbial content; however, they can undergo microbial contamination both in the various phases of industrial production and during the use by the consumer, from their opening until they are discarded. The Scientific Committee on Consumer Safety [3] provides guidelines on the microbiological quality of the finished cosmetic products and defines two separate categories of cosmetic products on the basis of their use and of their non-pathogenic microbial load:

Category 1: Products specifically intended for children under 3 years, to be used in the eye area and on mucous membranes (microbial limits 1×10^2 cfu per g or mL).

Category 2: Other products (microbial limits 1×10^3 cfu per g or mL).

To ensure that cosmetics and personal care products, especially those with a high content of water, are microbiologically safe and stable, microbial contamination must be

prevented by combining different strategies; they include the application of good manufacturing practices (GMPs), the use of suitable primary packaging, and, most importantly, the introduction into the formulation of an appropriate antimicrobial preservative system, one of the critical components in cosmetic formulations [4]. Antimicrobial preservatives have a double function: (i) to prevent the proliferation of microorganisms leading to deterioration of the product, and (ii) to prevent the growth of pathogenic microorganisms that may pose a potential health risk to the user during their repeated use, often by multiple persons, over extended periods of time; meeting these two requirements ensures that cosmetics are safe to use, stable, and effective during shelf-life. The availability of a wide and safe range of preservatives is one of the key challenges of the cosmetic industry. At present, Regulation 1223/2009 regulates the use of preservatives in cosmetic products marketed in the European Union, ruling that the only substances allowed as preservatives in cosmetics are those listed in Annex V of the Regulation. Preservatives allowed by the Regulation include different types of chemicals, such as parabens, alcohols, acids, quaternary ammonium compounds, and isothiazolinones. Parabens have been the most popular preservatives for quite a long time because they are effective and cheap, but European regulators put restrictions on them due to their endocrine-disrupting potential, and their safety status remains controversial [5]; on the other hand, isothiazolinones, which in recent times have largely replaced parabens due to their broad spectrum and high efficacy, present a high risk of sensitization and have been recently banned in leave-on cosmetics [6]. Due to evidence or suspicion of adverse effects of synthetic preservatives, consumer demand for natural ingredients in cosmetics and personal care products is constantly growing. Consequently, cosmetic manufacturers are increasingly oriented to replace conventional synthetic preservatives with suitable alternatives. The replacement of conventional preservatives is very demanding, and current strategies include the use of nature identical synthetic preservatives (e.g., benzoic acid, sorbic acid, benzyl alcohol); the use of natural plant-based preservatives (plant extracts, essential oils); the use of auto-preservation techniques; and the use of multifunctional ingredients [7]. Among all these, natural alternatives are especially gaining popularity not only because of consumers' preferences but also because of their sustainability and lower environmental impact [8]. The systematic search for new preservatives has led to the discovery of a wide variety of promising alternatives, and currently, the introduction into cosmetic formulations of a "preservative booster" to support or even completely replace legally recognized preservatives is one of the major trends in the field of cosmetic preservation [9].

The aim of the present study was to characterize the antimicrobial activity of a commercially available natural preservative obtained from radish by fermentation, Kopraphinol© (INCI name *Lactobacillus/Radish Root Ferment Extract Filtrate*), comparing it to that of methylparaben. It is obtained from white radish roots (*Raphanus sativus*) fermented in a solid state using *Lactobacillus* bacteria; the resulting ferment filtrate is stabilized. According to the information provided by the producer [10], Kopraphinol© is a water-based formulation declared suitable for body creams and lotions, facial products, shampoos, and conditioners, and its recommended dosage is 0.2–4.0%. Our interest in this preservative results from the fact that, although it is presented by the manufacturer as a natural and effective alternative to conventional preservatives and is present in some commercial formulations, there are, to our knowledge, no scientific works on its activity. Therefore, the present article study was undertaken with the aim of better defining the antimicrobial activity of Kopraphinol: conventional microbiological techniques were used to define the spectrum of action of the product, and a challenge test was used to verify its potential preservative in an O/W cream.

2. Materials and Methods

2.1. Materials

The test organisms used in this study were as follows: *Escherichia coli* (ATCC 8739), *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231), *Aspergillus brasiliensis* (*niger*) (ATCC 16404), and *Penicillium rubens*

(ATCC 9179) (all purchased from Oxoid-Thermofisher Scientific, Basingstoke, United Kingdom), as well as four *Candida* spp. clinical strains (respectively isolated from a vaginal, rectal, pharyngeal, and cheek swab, kindly supplied by Dr. M. Tidore, Laboratory of Clinical and Microbiological Analysis of Policlinic Hospital of Sassari, Italy). Mueller Hinton Agar (MHA), Mueller Hinton Broth (MHB), Sabouraud Liquid Medium (SLM), Sabouraud Dextrose Agar (SDA), Peptone Water (PW), Tryptone Soy Agar (TSA), and phosphate-buffered saline tablets (PBS, Dulbecco A, pH 7.3) were purchased from Oxoid-Thermofisher Scientific. Methylparaben (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO at 5% wt/vol. The culture media, PBS, and other solutions were prepared with MilliQ water.

Kopraphinol (INCI name: *Lactobacillus/Radish Root Ferment Extract Filtrate*) was kindly supplied by Kumar Organic Products Limited, Bangalore, India (batch number RFF/554/103). It is a clear, yellow, water-soluble liquid, with a pH from 4.0 to 6.0. The characteristics of Kopraphinol declared in the data sheet by the supplier are as follows: a specific gravity at 25 °C 1.000–1.100; solids (1 g at 105 °C for 1 h) 25.0–30.0%; phenolics (tested as salicylic acid) 18.0–22.0%; total aerobic microbial count < 10 cfu/mL; yeast and molds < 10 cfu/mL; and *E. coli* and *Salmonella* spp. absent in 25 mL.

2.2. Antibacterial Activity of Kopraphinol and Methylparaben

The antibacterial activity of Kopraphinol and methylparaben was determined as the Minimum Inhibitory Concentration (M.I.C.) by using a resazurin-based 96-well plate microdilution method [11]. This method is based on the use of resazurin dye as a redox indicator: viable bacteria reduce non-fluorescent blue resazurin to the fluorescent pink resorufin. Resazurin sodium salt (Sigma-Aldrich) was dissolved in water at 0.015% wt/vol, filter-sterilized (0.22 µm filter), and conserved at 4 °C for no longer than 2 weeks. To evaluate bacterial M.I.C.s, twofold dilutions of Kopraphinol (ranging from 50% to 0.39% vol/vol) and methylparaben (ranging from 0.2% to 0.025% wt/vol) were prepared in MHB in wells of microplates; the control wells contained only the liquid medium. It must be observed that, although the recommended maximum concentration for single parabens is 0.4% [6], in these assays, a lower concentration, but one that was still active, was tested, essentially for technical reasons related to the solubility of the compound. All assays were performed at least in triplicate. The microplates were inoculated with about 1×10^4 bacteria/well and aerobically incubated at 35 °C for 24 h. After the incubation of microplates for 24 h at 35 °C, 30 µL of resazurin solution was added to each well, and the microplates were further incubated at 35 °C for 2 h. After this time, the plates were visually inspected, and M.I.C. was defined as the lowest concentration of Kopraphinol with no color change (Figure 1).

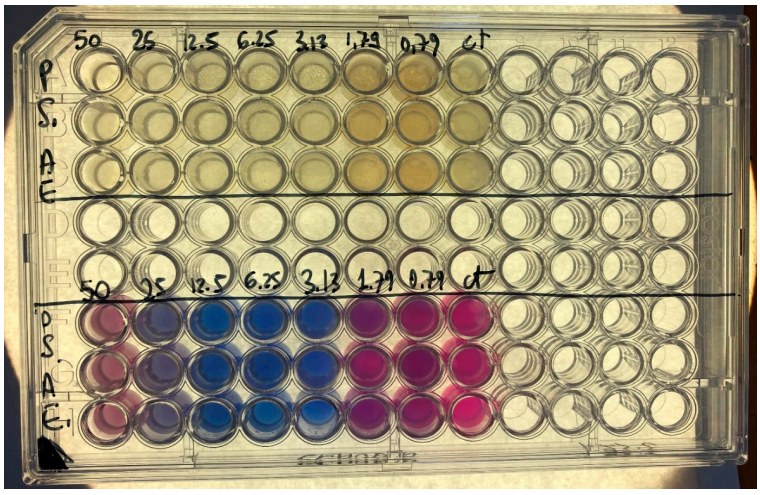

Figure 1. Evaluation of MIC of Kopraphinol against *P. aeruginosa* by using a resazurin-based 96-well plate microdilution method.

To determine the M.B.C. (Minimum Bactericidal Concentration), aliquots of 2 µL of the medium from each well with no visible growth were subcultured onto MHA plates, which were then incubated at 35 C for 24 h; M.B.C. was defined as the lowest concentration at which no growth was detectable. All tests were conducted at least in triplicate; at the concentrations tested, DMSO had no inhibitory effect on microorganisms' growth. The results are reported in Table 1.

Table 1. Evaluation of the antimicrobial activity of Kopraphinol and methylparaben. The values (±SD) represent the minimum inhibitory concentrations (MIC) (in brackets, the minimum bactericidal/fungicidal concentrations, MBC/MFC, are shown) (n.d. = not determined). MIC and MBC/MFC values are expressed as % vol/vol for Kopraphinol and as wt/vol for methylparaben.

Microorganisms	Kopraphinol (vol/vol)	Methylparaben (wt/vol)
Escherichia coli (ATCC 8739)	0.78 ± 0.00% (6.25 ± 0.00%)	0.2 ± 0.00% (0.2 ± 0.00%)
Staphylococcus aureus (ATCC 6538)	1.56 ± 0.00% (12.5 ± 0.00%)	0.2 ± 0.00% (0.2 ± 0.00%)
Pseudomonas aeruginosa (ATCC 9027)	6.25 ± 0.00% (6.25 ± 0.00%)	0.2 ± 0.00% (0.2 ± 0.00%)
Candida albicans (ATCC 10231)	1.56 ± 0.00% (3.13 ± 0.00%)	0.05 ± 0.00% (0.05 ± 0.00%)
Candida spp. (from rectal swab)	3.13 ± 0.00% (6.25 ± 0.00%)	n.d.
Candida spp. (from throat swab)	1.56 ± 0.00% (1.56 ± 0.00%)	n.d
Candida spp. (from vaginal swab)	1.56 ± 0.00% (6.25 ± 0.00%)	n.d.
Candida spp. (from mucosa of the cheek)	1.56 ± 0.00% (3.13 ± 0.00%)	n.d.
Aspergillus brasiliensis (ATCC 16404)	5.00 ± 0.00%	0.1 ± 0.00%
Penicillium rubens (ATCC 9179)	5.00 ± 0.00%	n.d.

2.3. Antifungal Activity of Kopraphinol and Methylparaben

The antifungal activity of Kopraphinol was assessed on *C. albicans*, *P. rubens*, and *A. brasiliensis* standard strains and on four *Candida* spp. clinical isolates. Fungi were grown on SDA plates at 35 °C (*Candida* strains) or 25 °C (other fungi).

The M.I.C.s of Kopraphinol for *Candida* strains were determined by using a 96-well plate microdilution method; twofold dilutions of the preservative, ranging from 50% to 0.39% vol/vol, were prepared in SLM. All assays were performed at least in triplicate. The microplates were inoculated with about 1×10^4 yeasts/well and aerobically incubated at 35 °C for 24 h. After the incubation, the plates were visually checked for fungal growth, and the M.I.C. was defined as the lowest concentration at which no growth was observed. To determine the M.F.C. (Minimum Fungicidal Concentration), aliquots of 2 µL of the medium from each well with no visible growth were subcultured onto SDA plates, which were then incubated at 35 C for 24 h; the M.F.C. was defined as the lowest concentration at which no growth was detectable. The results are reported in Table 1.

The M.I.C.s of Kopraphinol against *A. brasiliensis* and *P. ovale* were determined using an agar macrodilution method [12]. Twofold serial dilutions of the product in SDA were made in 5 mm Petri dishes (final volume 10 mL), obtaining final concentrations of 10%, 5%, 2.5%, and 1.25% vol/vol. The experiments were all performed in triplicate. Control plates containing only SDA were run simultaneously. The agar surface of the plates was then inoculated into the center with 1–3 µL of a conidial or yeast cell suspension prepared in sterile distilled water +0.05% Tween-80, containing 10^3–10^4 conidia or cells. The plates, wrapped with Parafilm to maintain the correct water activity in the medium, were inverted and incubated at room temperature (about 25 °C). The plates were visually checked for fungal growth; M.I.C.s were defined as the lowest concentrations of Kopraphinol preventing the growth of macroscopically visible colonies on Kopraphinol-containing plates when there was visible growth on the Kopraphinol-free control plates. The results are reported in Table 1.

2.4. Thermal Stability

Kopraphinol is declared resistant to heating up to 60 °C, does not need to be refrigerated, and retains its antimicrobial activity over time if kept in the dark at room temperature. To determine whether its antimicrobial activity is affected by exposure to temperatures far away from ambient temperature, Kopraphinol was exposed to extreme thermal conditions, and subsequently, its MICs were re-evaluated against *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*. More specifically, Kopraphinol was processed as follows:

- exposure to 50 °C in a thermostatic water bath for 1 h (K50);
- exposure to flowing steam at 100 °C in an autoclave for 1 h (K100);
- freezing at −80 °C for 24 h and subsequent thawing (K*).

The antimicrobial activity of the samples subjected to these treatments was reassessed with the method described above. The results are reported in Table 2.

Table 2. Antimicrobial activity of Kopraphinol subjected to thermal treatment (K = no heat treatment: K50 = heating to 50 °C for 1 h; K100 = heating to 100 °C for 1 h; K* = freezing at −80 °C for 24 h and subsequent thawing). The values (±SD) represent the minimum inhibitory concentrations (MIC) expressed as % vol/vol.

	***E. coli* (ATCC 8739)**	***P. aeruginosa* (ATCC 9027)**	***S. aureus* (ATCC 6538)**	***C. albicans* (ATCC 10231)**
MIC K	0.78 ± 0.00%	6.25 ± 0.00%	1.56 ± 0.00%	1.56 ± 0.00%
MIC K50	0.78 ± 0.00%	6.25 ± 0.00%	1.56 ± 0.00%	1.56 ± 0.00%
MIC K100	0.78 ± 0.00%	6.25 ± 0.00%	1.56 ± 0.00%	1.56 ± 0.00%
MIC K*	0.78 ± 0.00%	6.25 ± 0.00%	1.56 ± 0.00%	1.56 ± 0.00%

2.5. Contact Time (Killing Time)

The antimicrobial activity of Kopraphinol was also characterized by a "killing time" test, performed on *E. coli*, *P. aeruginosa*, *S. aureus*, and *C. albicans*; the purpose of this assay is to evaluate the reduction in the viable count when microorganisms are incubated with the product in a liquid medium that does not support cell growth. The tests, carried out in triplicate, were performed following the protocol developed by Juliano et al. [13]. Microorganisms in the logarithmic phase of growth were suspended at a density of 5×10^5–1×10^6 cfu/mL in a suitable volume of PBS (pH 7.3) containing 4% vol/vol of Kopraphinol (concentration suggested by the producer for practical use). A control tube (microorganisms suspended in PBS only) was included in each experiment. At time zero and after 1, 2, and 3 h of incubation at 35 °C, 0.5 mL of the suspensions was removed, subjected to serial tenfold dilutions in PBS, and seeded onto TSA (*E. coli*, *S. aureus*, and *P. aeruginosa*) or SDA (*Candida albicans*) plates. The number of surviving microorganisms at each time was evaluated by counting the colonies after incubation for 24 h at 35 °C. The results are reported in Table 3.

Table 3. Killing time test results. The values (±SD), the average of three experiments, represent the percentage of surviving bacteria after different times of contact with Kopraphinol 4% vol/vol.

STRAIN	**Time 0**	**1 h**	**2 h**	**3 h**
E. coli (ATCC 8739)	100%	30 ± 6.6%	0.46 ± 0.25%	0.17 ± 0.09%
P. aeruginosa (ATCC 9027)	100%	91.7 ± 7.6%	33.3 ± 5.6%	9.8 ± 4.5%
S. aureus (ATCC 6538)	100%	120.8 ± 7.7%	141.7 ± 9.9%	83.3 ± 7.5%
C. albicans (ATCC 10231)	100%	98.3 ± 8.7%	84.5 ± 5.0%	70.7 ± 10.5%

2.6. Challenge Test

The challenge test consists in artificially contaminating the cosmetic formulations with a standard inoculum of suitable microorganisms and evaluating the number of surviving microorganisms at predetermined intervals of time. Different challenge test protocols are commonly available in evaluating the microbiological stability of cosmetics [14]. In this investigation, to test the preservative efficacy of Kopraphinol compared to methylparaben, the method of the European Pharmacopoeia 7th Edition [15] was chosen, because it has the strictest acceptance criteria, which are difficult to achieve with many preservative systems [14].

Preliminary challenge tests were performed on several simple formulations to choose the most suitable one; more precisely, Carbopol 940- and hydroxyethylcellulose-based gels, emulsions formulated with Sepigel® as an emulsifier, and Cetomacrogol aqueous cream were prepared and tested. However, hydroxyethylcellulose-based gels have proven to be unsuitable because they already exhibit antimicrobial activity due to the presence of 10% glycerin, whereas Carbopol- and Sepigel-containing formulations show a sharp decrease in viscosity at the time of the addition of Kopraphinol. Therefore, challenge tests were performed on Cetomacrogol aqueous cream, which does not undergo alterations after the addition of the preservative; moreover, it is a formulation rich in free water, and for this reason, it represents a good substrate for microbial growth. Cetomacrogol cream was prepared as described by Juliano et al. [16]. Its composition is as follows: 15 g of lipophilic phase petrolatum, 6 g of mineral oil, 7.2 g of cetostearyl alcohol, 1.8 g of hydrophilic phase Cetomacrogol 1000, and 70 g of purified water. Kopraphinol was added to the creams at 4% vol/vol, as suggested by the manufacturer, while methylparaben was used at 0.2% wt/vol. The addition of Kopraphinol did not cause appreciable changes in the appearance and texture of the cream.

The following bacterial strains were employed in the challenge tests: *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC 9027), *S. aureus* (ATCC 6538), *C. albicans* (ATCC 10231), and *A. brasiliensis* (ATCC 16404). For each microorganism, fifty grams of cream were contaminated with a standard inoculum to obtain a concentration of about 10^6 colony-forming units (cfu)/g, and the preparations were stored at room temperature. At time 0 and after 1, 7, 14, and 28 days, one gram of each product was aseptically sampled and suspended in 10 mL of peptone water; the resulting suspension was subjected to decimal serial dilutions in sterile saline and analyzed for the enumeration of viable microorganisms onto TSA or SDA plates. The challenge tests were carried out in parallel with the corresponding controls (cream without preservative). The antimicrobial activity at each time was expressed in terms of the logarithmic reduction in the number of viable microorganisms compared to that at time 0. The reduction in the log number in each sample should comply with criterion A in the European Pharmacopoeia: 2 log reduction after 24 h and 3 log reduction after 7 days for bacteria and 2 log reduction at 14 days for fungi (with no increase at 28 days in both cases). The results are reported in Tables 4 and 5.

Table 4. Results of the challenge test performed on bacterial strains. The table shows the log reduction in the number of viable microorganisms during the incubation period with Kopraphinol and methylparaben. To facilitate the interpretation of the results, the values required by the European Pharmacopoeia are shown in the left column. (W = without preservative; K = with Kopraphinol; M = with methylparaben; NI = no increase.)

TIME	Eur. Ph. Criteria	*Escherichia coli*			*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
		W	**K**	**M**	**W**	**K**	**M**	**W**	**K**	**M**
Day 2	2	0.01	>5	>5	0.209	>5	0.017	−1.438	4.828	1.216
Day 7	3	−0.138	>5	>5	>5	>5	>5	−2.885	>5	−1.924

Table 4. *Cont.*

TIME	Eur. Ph. Criteria	*Escherichia coli*			*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
		W	**K**	**M**	**W**	**K**	**M**	**W**	**K**	**M**
Day 14	-	-	-	-	-	-	-	-	-	-
Day 28	NI	>5	>5	>5	>5	>5	>5	−2.793	>5	−0.711

Table 5. Results of the challenge test performed on fungal strains. The table shows the log reduction in the number of viable microorganisms during the incubation period with Kopraphinol and methylparaben. To facilitate the interpretation of the results, the values required by the European Pharmacopoeia are shown in the left column. (W = without preservative; K = with Kopraphinol; M = with methylparaben; NI = no increase.)

TIME	Eur. Ph. Criteria	*Candida albicans*			*Aspergillus brasiliensis*		
		W	**K**	**M**	**W**	**K**	**M**
Day 2	2	−3.117	>5	0	0	>5	0.405
Day 7	3	−3.569	>5	0.430	0	>5	0.310
Day 14	-	-	-	-	-	-	-
Day 28	NI	−3.944	>5	2.964	−0.109	>5	−0.182

3. Results

3.1. Antimicrobial Activity

The antimicrobial potential of Kopraphinol was determined by evaluating its MICs against a panel of selected microorganisms; the results are presented in Table 1. Overall, Kopraphinol exhibited significant antibacterial activity, with the lowest MIC value observed with *E. coli* (0.78%) and the higher with *P. aeruginosa* (6.25%); the effect was bacteriostatic, as can be seen from the higher values of MBC (except in the case of *P. aeruginosa*).

Against the various strains of *Candida*, Kopraphinol exhibited good inhibitory activity, with MIC values ranging from 1.56% and 3.13%. Higher concentrations (5.00%) of the preservative are required to totally inhibit the growth of *Aspergillus* and *Penicillium*.

As Figure 2 shows, the radial growth of *Aspergillus* was totally inhibited by Kopraphinol at a 5% concentration already from the fourth day; moreover, mycelial growth was considerably reduced at sub-MIC concentrations of the preservative, as shown by the reduction in the mycelial diameter compared to that of the control.

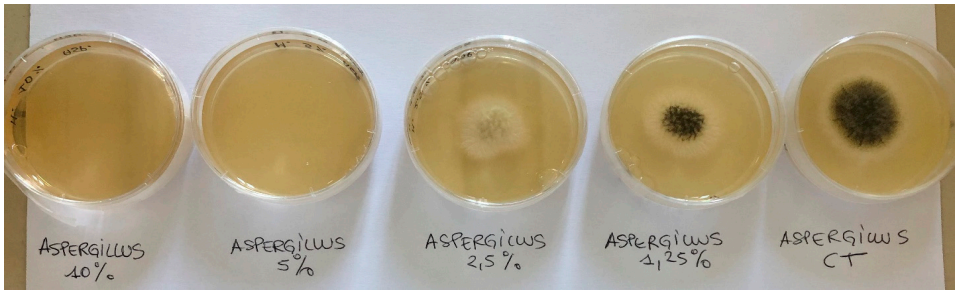

Figure 2. Evaluation of the inhibitory effect of Kopraphinol against *Aspergillus brasiliensis* (ATCC 16404) after 4 days. At 5%, the growth of fungus is totally inhibited, but a reduction in mycelial growth is already visible at lower concentrations.

3.2. Thermal Stability

The thermal stress imposed on Kopraphinol did not affect its antimicrobial activity; in fact, as can be seen in Table 2, the MICs of the preservative subjected to various treatments are perfectly superimposed to those of the untreated product.

3.3. Killing Time

The results of the killing time assays are quite different in relation to the strains tested, as can be observed in Table 3. After 3 h of contact with 4% of Kopraphinol, the viability of *E. coli* and *P. aeruginosa* was drastically reduced, with percentages of surviving bacteria of 0.17% and 9.8%, respectively. On the contrary, after 3 h of contact with the preservative, *S. aureus* and *C. albicans* were still largely viable, with values of surviving microorganisms of 83.3% and 70.7%, respectively. The much faster action of Kopraphinol against Gram-negative strains suggests that the peculiar structure of the outer envelope of these bacteria is more permeable to the preservative or is more easily damaged.

3.4. Challenge Test

According to criterion A of the European Pharmacopoeia, an antimicrobial preservation system is accepted if in the challenge test the bacteria number is reduced by 2 and 3 log units after, respectively, 2 and 14 days and no increase in plate counts is observed after day 14; with respect to molds and yeasts, their number must be reduced by 2 log units after 14 days, and no increase in plate counts can be observed after day 14. Table 4 shows that Kopraphinol, regarding the bacterial strains tested, largely meets this criterion, exhibiting a preservative efficacy quite comparable to that of methylparaben. It should be mentioned that in the control samples (without preservatives), the bacterial load of *E. coli* and *S. aureus* is reduced spontaneously to zero over time, while the *P. aeruginosa* count gradually increased, reaching a value about 1000 times higher than the initial one after 28 days. With regard to the mycetes tested, Kopraphinol exhibited effective and fast preservation activity against both *C. albicans* and *A. brasiliensis*. Indeed, as illustrated in Table 5, in formulations with Kopraphinol, the number of viable mycetes was already efficaciously reduced to satisfy criterion A from day 2 (fungal contamination reduced by more than 5 log units). It is noteworthy that Kopraphinol's performance was better than that of methylparaben; in fact, the latter reduced the *C. albicans* load but with values that do not meet criterion A, while at a 0.2% concentration, methylparaben failed to protect the cosmetic formulation from *A. brasiliensis* (at days 2 and 7, the reduction values did not fulfill criterion A, and after 28 days, the mold started to grow again)

4. Discussion

Recent trends in cosmetic antimicrobial preservation include the use of compounds of natural origin (e.g., essential oils, herbal extracts, and ferment filtrates) and the use of multifunctional ingredients (substances that offer preservation alongside other benefits, such as anti-aging or moisturizing effects). Kopraphinol fits perfectly into these trends for a number of reasons. First of all, our in vitro experiments confirmed the good broad spectrum antimicrobial activity of Kopraphinol, with a stronger inhibitory effect against the *E. coli* and *Candida* strains; moreover, challenge tests, performed on an O/W emulsion deliberately contaminated with high microbial loads, demonstrated the good conservative properties of Kopraphinol, complying with the requirements of current legislation and comparable with the efficacy of methylparaben, and sometimes better. It is also relevant that Kopraphinol is a natural ingredient obtained through a fermentation process. Fermentation is a process currently used to produce a wide range of cosmetic ingredients, such as emollients, surfactants, humectants, thickeners, and, indeed, preservatives [17]. Bioferments are innovative cosmetic ingredients, whose advantages include better biocompatibility, bioavailability, sustainability, and an increased efficacy compared to conventional ingredients [18]. Different cosmetic ingredients obtained by the *Lactobacillus* fermentation of different substrates are classified as multifunctional ingredients with moisturizing properties and a strong preservative efficacy due to bacteriocins and organic acids of bacterial origin [17]; Kopraphinol belongs to this category, and like all multifunctional ingredients, it would allow formulators to develop products that are less expensive, easier to manufacture, and better-performing [19]. Other arguments in favor of Kopraphinol are its ease of use and safety. As an aqueous liquid, Kopraphinol can be precisely dosed by volume, and due to its

temperature resistance, it can also be easily incorporated in hot aqueous formulations, in which it will be perfectly dispersed. However, an important limit to the use of Kopraphinol emerged during our investigation: the incompatibility of the preservative with polymeric emulsifiers (e.g., Sepigel©) and gels (e.g., Carbomer), which results in a drastic reduction in the viscosity of the formulations; therefore, any use of this preservative should be preceded by studies of physical compatibility. Regarding safety, the use of Kopraphinol does not seem to pose a significant problem. The Expert Panel for Cosmetic Ingredient Safety (CIR) [20] assessed the safety of seven radish root-derived ingredients, most of which are reported to function as hair- and skin-conditioning agents in cosmetic products. The Panel reviewed the available data and concluded that these ingredients are safe in cosmetics in the present practices of use and concentration described in that safety assessment when formulated to be non-sensitizing. However, as recently highlighted, the application of cosmetics on the skin can affect the resident microflora [21]; in this context, it should be verified that the antimicrobial activity of Kopraphinol does not constitute a problem for the skin microbiome, which could result from a lack of selectivity of the preservative.

In conclusion, the good antimicrobial activity, the natural origin, and the easy handling, together with the safety of use, make Kopraphinol a promising and effective candidate for the antimicrobial preservation of cosmetics, capable of successfully complementing or even replacing conventional preservatives.

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References

1. European Commission. *Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products*; Official Journal of the European Union L342/59; Publications Office of the European Union: Luxembourg, 2009.
2. Giorgio, A.; Miele, L.; De Bonis, S.; Conforti, I.; Palmiero, L.; Guida, M.; Libralato, G.; Aliberti, F. Microbiological stability of cosmetics by using Challenge Test procedure. *J. Pure App. Microbiol.* **2018**, *12*, 23–28. [CrossRef]
3. SCCS (Scientific Committee on Consumer Safety). Appendix 9: Guidelines on microbial quality of the finished cosmetic product. In *The SCCS Notes of Guidance for the Testing of Cosmetic Ingredients and Their Safety Evaluation*; 12th revision; SCCS: Brussels, Belgium, 2023; pp. 148–150.
4. Halla, N.; Fernandes, I.P.; Heleno, S.A.; Costa, P.; Boucherit-Otmani, Z.; Boucherit, K.; Rodrigues, A.E.; Ferreira, I.C.F.R.; Barreiro, M.F. Cosmetic preservation: A review on present strategies. *Molecules* **2018**, *23*, 1571. [CrossRef] [PubMed]
5. Nowak, K.; Jablónska, E.; Ratajczak-Wrona, W. Controversy around parabens: Alternatives strategies for preservative use in cosmetics and personal care products. *Environ. Res.* **2021**, *198*, 110488. [CrossRef] [PubMed]
6. European Commission. *Regulation (EU) N° 1004 (2014) of 18 September 2014 Amending Annex V to Regulation (EC) N° 1223/2009 of the European Parliament and of the Council on Cosmetic Products*; Official Journal of the European Union L282/5; Publications Office of the European Union: Luxembourg, 2009.
7. Martins, A.M.; Marto, J.M. A sustainable life cycle for cosmetics: From design and development to post-use phase. *Sustain. Chem. Pharm.* **2023**, *35*, 101178. [CrossRef]
8. Paramita, S.; Kusuma, I.W.; Sulistioadi, Y.B.; Kiswanto. Environmental and Safety Aspects of bio-based Cosmetics in Indonesia. In *Biomass-Based Cosmetics*; Arung, E.T., Fatriasari, W., Kusuma, I.W., Kuspradini, H., Shimizu, K., Kim, Y.-U., Azelee, N.I.W., Edis, Z., Eds.; Springer: Singapore, 2024; pp. 545–568. [CrossRef]
9. Poddebniak, P.; Kalinowska-Lis, U. A survey of preservatives used in cosmetic products. *Appl. Sci.* **2024**, *14*, 1581. [CrossRef]
10. Kopraphinol (Radish Root Ferment Filtrate). Available online: https://www.kumarorganic.net/kopraphinol/ (accessed on 8 July 2024).
11. Elshikh, M.; Ahmed, S.; Funston, S.; Dunlop, P.; McGaw, M.; Marchant, R.; Banat, I.M. Resazurin-based 96-well plate microdilution method for the determination of minimum inhibitory concentration of biosurfactants. *Biotechnol. Lett.* **2016**, *38*, 1015–1019. [CrossRef] [PubMed]

12. Therese, K.L.; Bagyalakshmi, R.; Madhavan, H.N.; Deepa, P. In-vitro susceptibility testing by agar dilution method to determine the minimum inhibitory concentrations of amphotericin B, fluconazole and ketoconazole against ocular fungal isolates. *Ind. J. Med. Microbiol.* **2006**, *24*, 273–279. [CrossRef]
13. Juliano, C.; Mattana, A.; Usai, M. Composition and in vitro Antimicrobial Activity of the Essential Oil of Thymus herba-barona Loisel growing wild in Sardinia. *J. Essent. Oil Res.* **2000**, *12*, 516–522. [CrossRef]
14. Siegert, W. Comparison of microbial challenge testing methods for cosmetics. *Househ. Pers. Care Today* **2013**, *8*, 32–39.
15. Council of Europe; European Directorate for the Quality of Medicines and Healthcare (Eds.). Chapter 5.1.3. Efficacy of antimicrobial preservation. In *European Pharmacopeia*, 7th ed.; Council of Europe: Strasbourg, France, 2011.
16. Juliano, C.; Gavini, E.; Giunchedi, P.; Magrini, G.A. Evaluation of Manuka honey as an adjuvant antimicrobial preservative in a O/A emulsion. *J. Appl. Cosmetol.* **2016**, *34*, 87–98.
17. Pérez-Rivero, C.; Lòpez-Gòmez, J.P. Unlocking the potential of fermentation in cosmetics: A review. *Fermentation* **2023**, *9*, 463. [CrossRef]
18. Majchrzak, W.; Motyl, I.; Smigielski, K. Biological and cosmetical importance of fermented raw material: An overview. *Molecules* **2022**, *27*, 4845. [CrossRef] [PubMed]
19. Romanowski, P.; Schueller, R. Definition and principles of multifunctional cosmetics. In *Multifunctional Cosmetics*, 1st ed.; Schueller, R., Romanowski, P., Eds.; Taylor & Francis: Boca Raton, FL, USA, 2001; pp. 1–11.
20. Cosmetic Ingredient Review. Safety Assessment of Radish Root-Derived Ingredients as Used in Cosmetics. 2022. Available online: https://cir-safety.org/sites/default/files/Radish.pdf (accessed on 2 July 2024).
21. Pinto, D.; Ciardiello, T.; Franzoni, M.; Pasini, F.; Giuliani, G.; Rinaldi, F. Effect of commonly used cosmetic preservatives on skin resident microflora dynamics. *Sci. Rep.* **2021**, *11*, 8695. [CrossRef] [PubMed]