Natural Preservative Solutions for Personal Care Products

Antimicrobial Potential of Natural Bioactive Compounds in Synergy with Phenoxyethanol

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INNOVATIVE HEALTHCARE SOLUTIONS

Skincare Products Preservatives: Anti-microbial Potential of Natural Bioactive Compounds in Synergy with Phenoxyethanol

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Summary

Recent steady increase in modern personal care formulations require the inclusion of potent antimicrobial preservatives to ensure a long shelf-life of the product. Such formulations must include preservatives that are efficient, chemically stable and safe; application of novel natural ingredients in the cosmetics industry represents a promising investment, according to the current trends. These ingredients could be used alongside the established synthetic preservatives to boost their anti-microbial capacities. Alternatively, they could also enable formulation of the next-generation cosmetics where the choice of specific preservative would enhance the desired effect of the personalized product based on the specific molecular targets in the skin through to poly-pharmacology effect.

Using deep learning algorithms and in-silico screening of molecules from natural sources, we identified a panel of bioactive compounds with predicted microbiostatic capacities against a number of microorganisms. Several single compounds and their synergies have been initially tested in vitro alongside phenoxyethanol (PE). The result confirmed antimicrobial activity in Minimum Inhibitory Concentration (MIC) assay that allowed the selection of candidates with the antimicrobial strength comparable to that of PE. We analyzed the antimicrobial activity of three selected candidates and PE further in time-kill kinetics assay. The compounds demonstrate two major characteristics as a platform for proposed future applications. First, they increase the efficacy of PE thus enabling the potential reduction in its concentration. Second, the antimicrobial efficacy of the compounds in synergy is also comparable to the efficacy of PE. Complementary analysis of the compounds in the formulation-stability test confirms that the preservatives are stable, and their additional microbiological safety in the potential cosmetic products.

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The AI-guided approach to the discovery of antimicrobial agents offers a fast and cost-effective route to isolation, characterization and formulation of novel preservatives for personal care products. Our initial objective is to find the compounds that work synergistically with PE to improve preservation.

Pro.X® identification of novel antimicrobial compounds for use in the cosmetic preservatives industry

In recent years, the increasing demand for preservatives has been accelerated by growing trend in the design of new cosmetic products. Many of the cosmetic product include preservatives that are either synthetic or natural, such as Ethylhexylglycerin (synthetic or derived from glycerin), Hexylene glycol (synthetic), Capryl glycol (derived from caprylic acid, natural fatty acid), or Phenoxyethanol (synthetic or derived from green tea) [1]. The compounds are usually chemically stable over a wide 3-12 pH range and compatible with the majority of molecules in most types of aqueous formulations, based on anionic, nonionic and amphoteric surfactants.

Phenoxyethanol (PE) is a globally-approved, non-formaldehyde- releasing antimicrobial preservative used in many types of personal care formulations [1]. PE has been considered a non-toxic synthetic preservative and recommended safe to use as a cosmetic ingredient by the Cosmetic Ingredient Review expert panel. It has a strong microbiostatic activity against a broad spectrum of microorganisms including E.coli (Gram negative bacteria); P.aeruginosa (Gram negative bacteria); S.aureus (Gram positive bacteria); C.albicans (yeast) and A.brasiliensis (mold). PE based products have been marketed under several names such as Phenoxetol, Optiphen PO, Purolan PE, Euxyl K ® 400, 2-Phenoxyethanol, Phenoxyethanol. [2,3]. In the EU, PE is permitted at low dose use; the EEC Cosmetic Derivative and the Cosmetics regulation of the EU approved PE in concentrations up to 1% in cosmetics and other common products. Recent review of 43 cosmetic products demonstrated the mean concentration of PE at 0.46%; with the concentrations >0.6% in only 25% of the products [3].

Growing range of the new cosmetic products requires an approach to discovery that would enable rapid and cost-effective design, and validation of antimicrobials for applications as preservatives that can be used in relatively low concentrations. Such approach includes the review of alternate functionalities of known compounds, re-purposing them for new applications. Hexis Lab ProX® discovery platform is built on proprietary deep learning algorithms and a big data cloud computing approach that allows conducting computer model simulations and in-silico screening of natural compounds. The AI system is also integrated with advanced computing power that resolves the high complexity of biological systems. The proprietary database is constantly expanding and contains thousands of natural products, with the properties and the benefits of each compound identified. This is complemented

further by the application of a methodology adopted by the pharmaceutical industry when identifying and combining the ingredients.

Using the in-silico platform, the database of natural compounds was screened for the predicted antimicrobial capacities against a range of singular classified microorganisms. For the initial in-vitro verification, up to 10 candidates were randomly selected, based on the signal quality in the in-silico screening. Additional criteria for the selection were also based on known biological effects of the compounds on the skin, with the aim to place the future applications of specific preservative in a context of enhanced desired effect on the skin.

In vitro verification of the antimicrobial potencies of selected compounds in comparison with PE

The compounds were initially tested in the standard Minimum Inhibitory Concentration (MIC) assay. The microbiostatic capacities were calculated from growth curves at: 1) OD_{600nm} =0.05 (total growth inhibition, MIC); 2) OD_{600nm} =0.5 (50% growth inhibition); 3) OD_{600nm} =1 (0% growth inhibition). Compounds with the MIC values consistently close to the MIC for PE across all five microbial strains (P.aeruginosa, E.coli, S.aureus. C.albicans and A.brasilensis) were selected for further analysis. The MIC values of the compounds were between 0.7%-0.2%, the antimicrobial capacities could be subsequently enhanced by the synergy (1:1 v/v).

Three compounds, HX1, HX2 and HX3 were selected for further analysis in time-kill and formulation-stability assays alongside the PE. The compounds were tested as single, in combination with each other, as well as in the synergy with the decreasing concentrations of PE (**Table 1**). The concentrations of compounds were dictated by both their previously identified MIC values and solubility properties.

A Synergy: time-kill kinetics in the presence of single and combined compounds

For the time-kill assay, the compounds in the above combinations were tested in five microbial strains (P.aeruginosa, E.coli, S.aureus. C.albicans and A.brasilensis), exposed to the compounds for 0, 3, 6 and 24 hours or cultured without the preservatives as control. In the synergistic response, the compounds work together to produce a growth inhibition more potent than each compound applied separately; which results in a marked increase in microbiostatic rate within the first 24 hrs of exposure in vitro [4].

Table 1. The mixtures of the preservatives tested in time-kill assay.

0.9% PE	0.9% PE	0.9% PE	0.7% HX1+0.9% HX2
0.7% HX1	0.9% HX2	0.2% HX3	0.7% HX1+0.2% HX3
0.9% PE+0.7% HX1	0.9% PE+0.9% HX2	0.9% PE+0.2% HX3	0.9% HX2 +0.2% HX3
0.45% PE+0.7% HX1	0.45% PE+0.9% HX2	0.45% PE+0.2% HX3	0.2% PE+0.7% HX1+0.9% HX2
0.2% PE+0.7% HX1	0.2% PE+0.9% HX2	0.2% PE+0.2% HX3	0.2% PE+0.7% HX1+0.2% HX3
			0.2% PE+0.9% HX2+0.2% HX3

i CFU evaluation

1 mL of the growth media containing test compounds were inoculated with 0.01 mL microorganism suspension (initial count approx. 10^6 CFU/mL). The samples were incubated for 0, 3, 6 and 24 hrs and then plated onto agar plates in serial dilutions. The plates were incubated for 24-48 hrs and the evaluation made on the basis of quantitative assessment of the colonies (CFU/mL=no. of colonies x dilution factor/volume of culture plated).

ii Determination of survivors at time intervals and decimal reduction time (D-value)

For the antimicrobial activity comparison of the compounds, the number of the survivors/10^6 CFU control was calculated for each sample and the resulting CFU/mL converted to Log. The decimal reduction time (D-value, the time required to kill 1 Log of microorganisms) was calculated from the (Log CFU/mL vs. time) linear regression plots. D-value= -1/(y), where y=slope value.

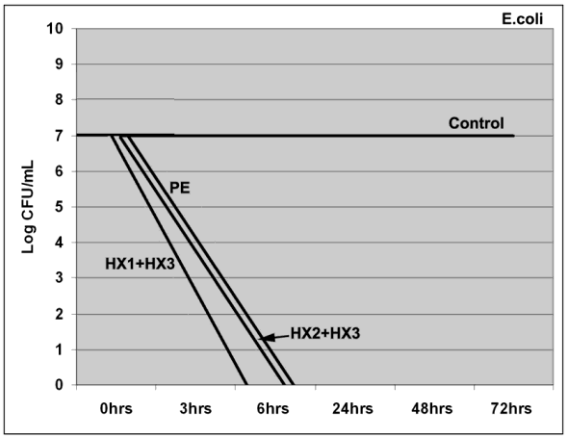
The D-values for the select samples are presented in **Table 2**. This analysis demonstrated that the time required to kill the microorganisms is reduced for HX1, HX2 and HX3 compared to PE in most, however not all samples, accross the five microbial strains. Significantly, the D-values are reduced further in the samples containing both the natural compound and PE, showing a consistent decrease across all samples comparing to single compounds and PE only (HX1+PE, HX2+PE, HX3+PE). In addition, a mixture of the natural compounds shows also a reduction in D-value compared to PE accross all five microbial strains (HX2+HX3).

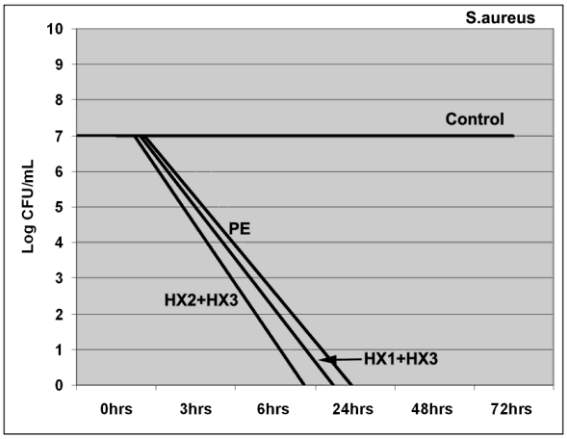
Table. 2. D-values (in hours) calculated in the samples. PE concentration in HX1+PE, HX2+PE, HX3+PE is aditionally reduced up to 2 times of the concentration used in PE samples only. The reduced D-values between single and mixed compounds indicate synergistic capacity for faster killing of the microorganism therefore higher anti-microbial strength.

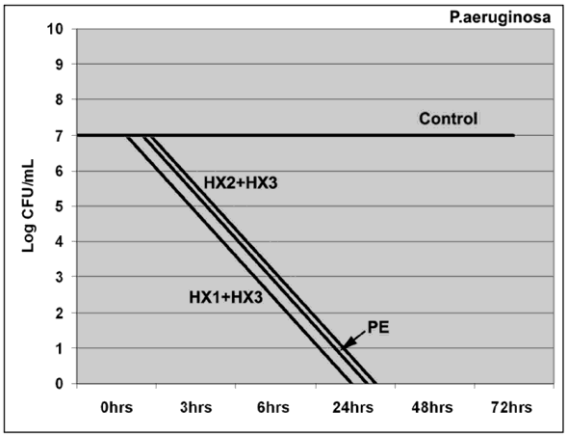
Compound	P.aeruginosa	E.coli	S.aureus	C.albicans	A.brasiliensis
PE	3.53	4.12	3.35	4.46	5.21
HX1	4.26	3.42	3.26	3.01	4.51
HX2	4.25	3.13	3.22	3.31	6.11
HX3	3.12	3.17	2.99	3.31	5.33
HX1+PE	3.46	2.11	2.09	2.59	4.52
HX2+PE	2.19	2.31	2.14	2.21	4.36
HX3+PE	2.38	3.13	2.28	2.26	5.03
HX1+HX3	4.21	6.08	3.06	2.58	5.23
HX2+HX3	3.16	4.01	2.43	2.46	4.56

iii Time-kill linear regression profiles

The anti-microbial capacities of the compounds and the possible synergistic potential with PE were analysed further in the lethality kinetic profiles based on Log CFU/mL x time (**Fig.1**).


E.coli
Log CFU/mL
10
9
8
7
6
5
4
3
2
1
0
Control
PE
HX1+HX3
HX2+HX3
0hrs
3hrs
6hrs
24hrs
48hrs
72hrs


S.aureus
Log CFU/mL
10
9
8
7
6
5
4
3
2
1
0
Control
PE
HX2+HX3
HX1+HX3
0hrs
3hrs
6hrs
24hrs
48hrs
72hrs


P.aeruginosa
Log CFU/mL
10
9
8
7
6
5
4
3
2
1
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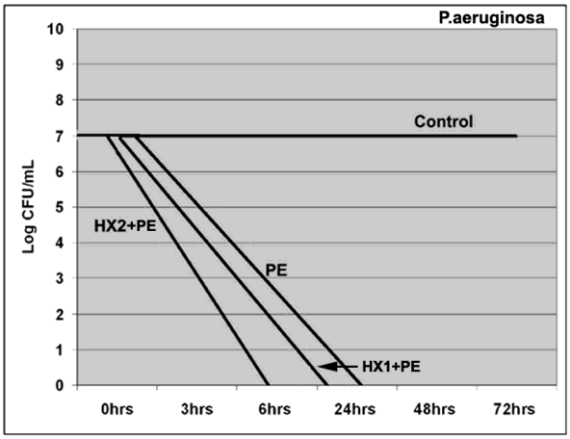
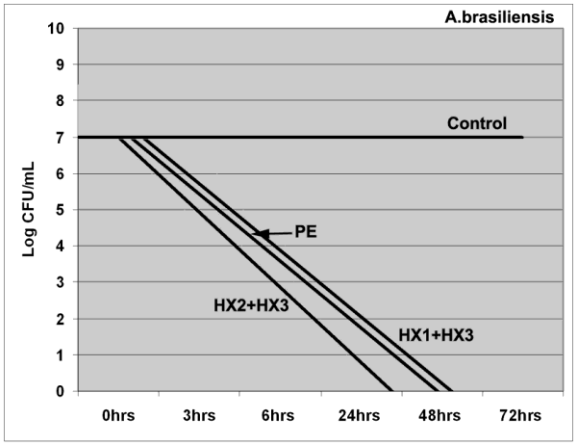
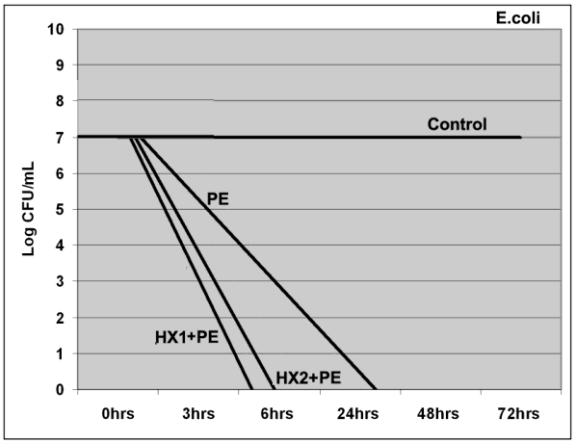
Control
HX2+HX3
HX1+HX3
PE
0hrs
3hrs
6hrs
24hrs
48hrs
72hrs


P.aeruginosa
Log CFU/mL
10
9
8
7
6
5
4
3
2
1
0
Control
HX2+PE
PE
HX1+PE
0hrs
3hrs
6hrs
24hrs
48hrs
72hrs


A.brasiliensis
Log CFU/mL
10
9
8
7
6
5
4
3
2
1
0
Control
PE
HX2+HX3
HX1+HX3
0hrs
3hrs
6hrs
24hrs
48hrs
72hrs


E.coli
Log CFU/mL
10
9
8
7
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5
4
3
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Control
PE
HX1+PE
HX2+PE
0hrs
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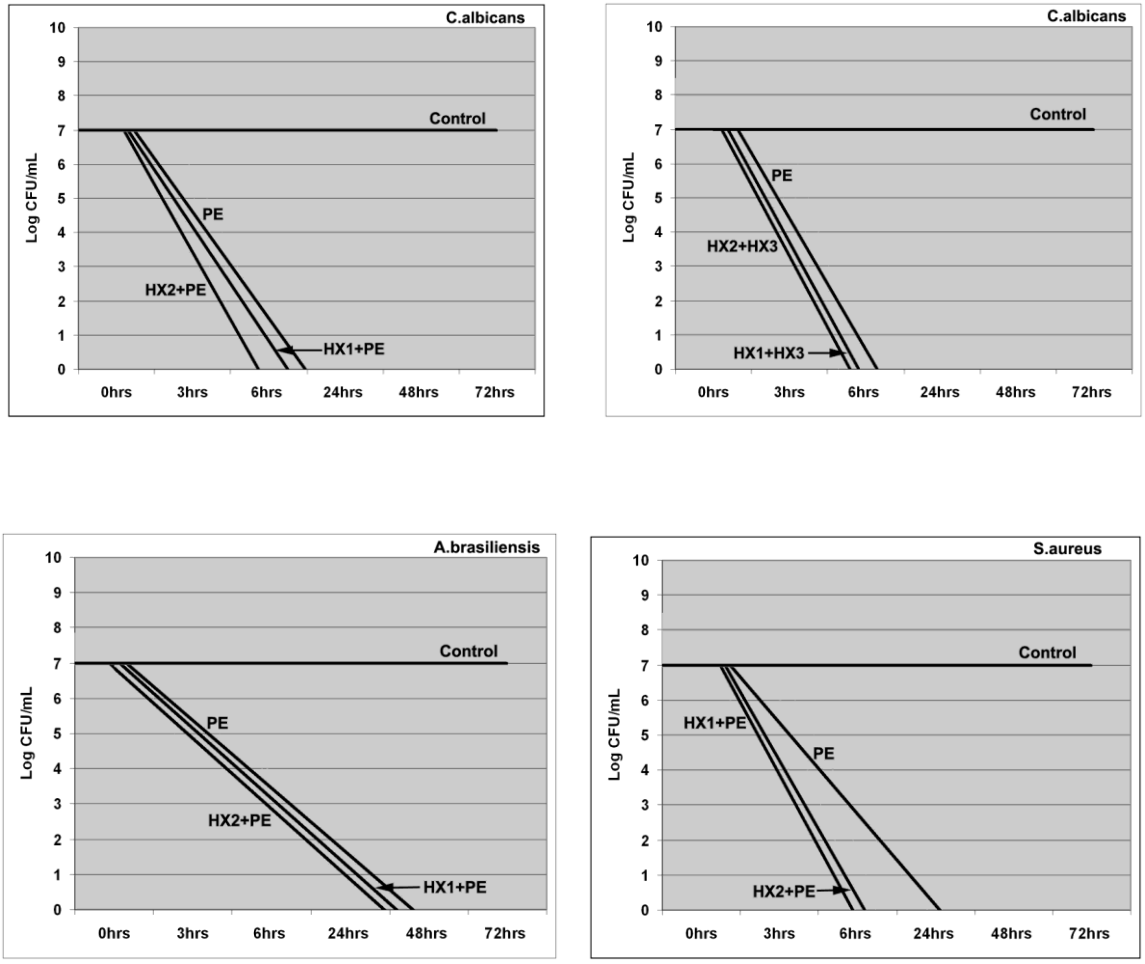

Fig.1. Time-kill profiles (Log CFU/mL x time) of P.aeruginosa, E.coli, S.aureus, C.albicans and A.brasiliensis for PE, HX1+PE, HX2+PE, HX1+HX3, HX2+HX3 and Control. PE concentration in the samples containing HX1 and HX2 has been reduced by 2 –fold.

The time kill profiles generated for PE and the mixtures of HX1 or HX2 with reduced concentration of PE demonstrate the increased efficacy of PE in the presence of HX compounds across all five microbial strains On average, the HX2 is slightly more efficient in synergy with PE than HX1 (**Fig.1**, left panels). The natural compounds in the combinations of HX1-HX3 or HX2-HX3 have also similar or enhanced time-kill profiles compared to PE. These data indicate that the HX compounds could enhance the antimicrobial activity of PE, leading to the potential reduction of the required MIC concentrations, alternatively the HX compounds could also fulfill the criteria of anti-microbial agents on their own.

B Testing synergy and preservation of Phenoxyethanol with HX antimicrobial compounds in formulation

The natural compounds tested here show reduction in the microbial colonies cultured on agar similarly to that obverved for PE. The killing of the microorganisms is evident at the

relatively short time points (up tp 72hrs, **Fig.2**), however the question remains on the long-term stability of the ingredients and their activity in the formulation compatible with cosmetic products.

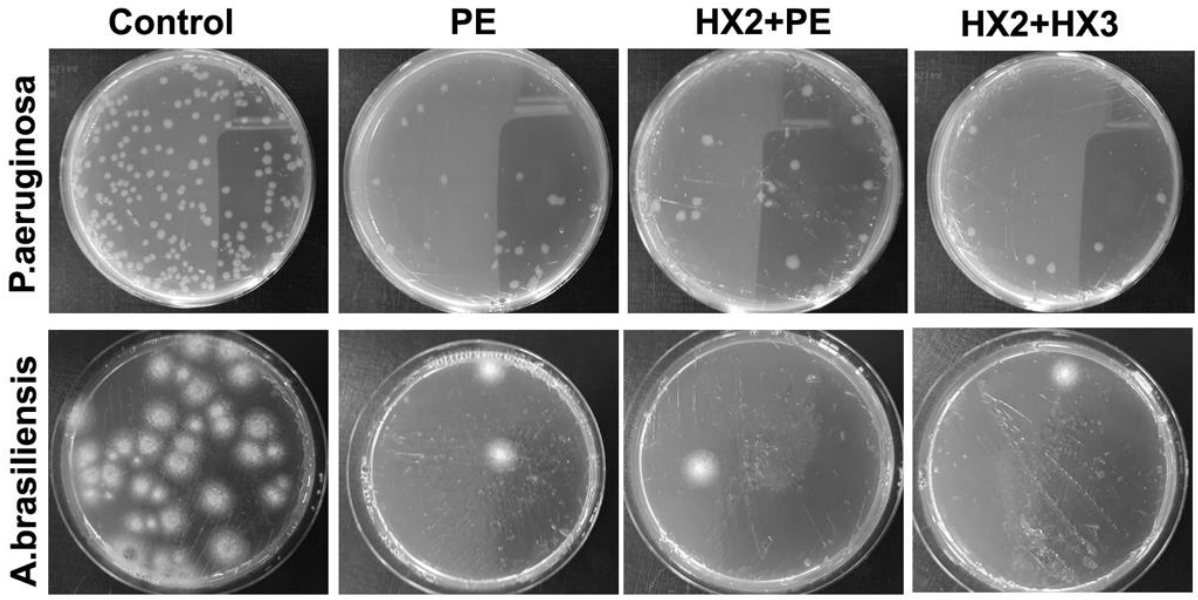

Fig.2. Examples of the microorganisms (P.aeruginosa and A.brasiliensis) cultured in the presence of PE, HX2+PE and HX2+HX3 for 24 hours and incubated at the same dilutions on agar plates for further 24-48 hours. All compounds show reduction of colonies compared to control.

For longer term stability and formulation compatibility, the single compounds at MIC concentrations and their synergies listed in **Table 1** are currently subject to the repeated challenge test as recommended by Schülke & Mayr (4). The preservatives have been mixed with the formulation containing 0.4% Carbopol ETD 2020 (Carbomer); 15% Glycerine; 0.9% sodium hydroxide; control includes formulation without the compounds. The formulation samples are inoculated with the mixed suspension of all five cultivated microorganisms (10^8 CFU/mL new suspension prepared for each inoculation cycle), once a week over a six week period. Before each inoculation the sample is streaked out onto CS-agar and SA-agar plates, incubated for 3 days at 25°C and evaluated semi-quantitatively. The samples are presently in the fourth inoculation cycles, with all preservatives tested assessed as free of growth at this point.

Potential further applications of the HX compounds in personalized skincare formulations

Human skin is continuously exposed to detrimental factors in the environment such as UV radiation, air pollution or changes in air humidity. This can lead to excessive damage and

premature ageing of the skin. These changes are characterized by the loss of the structural integrity of the skin, hype-rpigmentation and inflammation [5,6]. Targeting these activities is an attractive way towards skin protection strategies for applications in the cosmetic industry. There is presently a great demand for supply of raw materials for cosmetic industries, especially with increasing preference for organic and natural products [7].

Formulations containing HX compounds are differentially tinted in appearance therefore would be compatible with the natural hues and different tones of human skin (**Fig.3**).

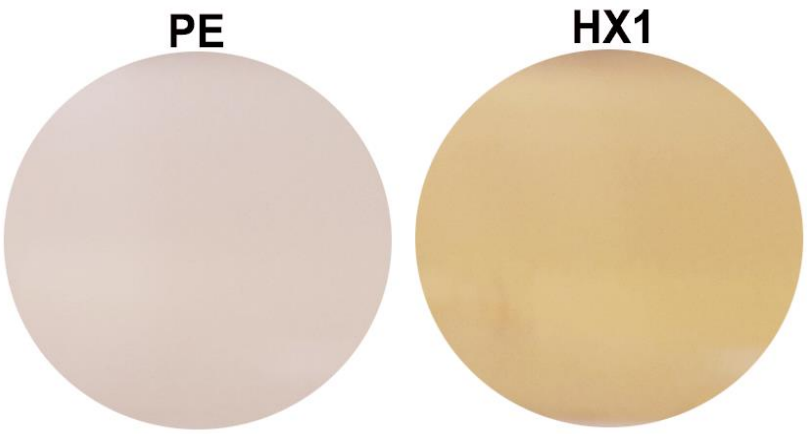

Fig. 3. Formulations containing PE or HX1 natural compound used in current Schulke KoKo test

In addition to the potential preservative capacities, the HX natural compounds also offer a broad range of applications in skincare due to their poly-pharmacology activity towards multiple and selective molecular targets: 1) Antioxidants. Due to the chemical structure the compounds are able to augment the depleted antioxidant defense system and neutralise and reduce free radicals. 2) Preservation of the ECM for anti-wrinkle application. The compounds can stimulate the collagen production or act as inhibitors of the enzymes involved in the degradation of extracellular matrix. 3) UV filters and protection against DNA damage. The compounds can act in photoprotection due to the ability to filter or absorb UV in broad spectra ranging from 200-400nm and protective effects against UV-induced DNA damage of mitochondrial and genomic origin. 4) Hypo-pigmentation agents. The compounds can act as inhibitors of the enzymes responsible for hyper-pigmentation and age spots due to the excessive synthesis of melanin.

Based on this property, the compounds could be included in the personalized cosmetic products for enhanced performance, when the choice of the preservative is also dictated by the desired final effect of the product. For example, HX1 and HX3 could be a first choice as the preservatives in the products targeting hyper-pigmentation or enhancing skin elasticity, whereas HX1 and HX2 could be an efficient complement in sun-protection products (**Fig.4**).

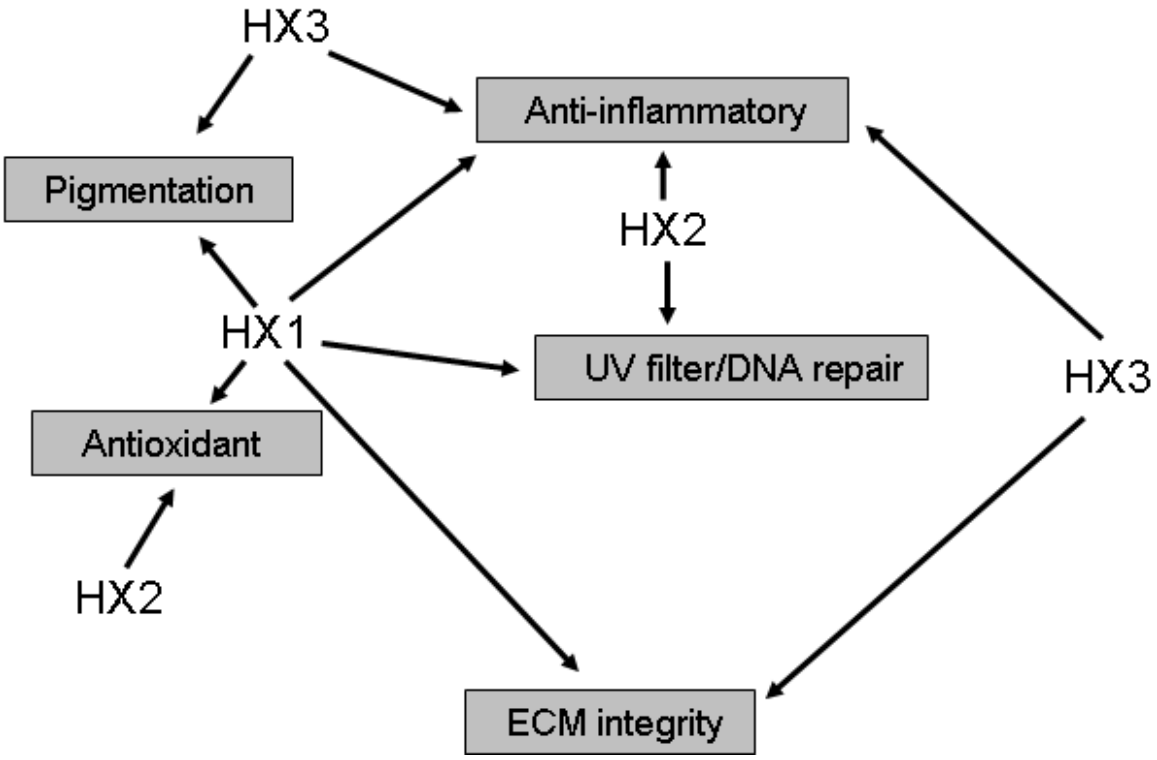

Fig 4. Biological activities of the natural anti-microbial compounds for enhanced performance of skincare products

Conclusions

Here, we provided an assessment of the AI-selected and in-vitro verified anti-microbial capacities of three selected natural compounds and proposed their application as a promising way towards discovery of alternative novel preservatives in personal care products.

The antimicrobial capacities of the individual HX compounds were assessed in a range of formulas that also included their mixtures with decreasing concentrations of PE. Time-kill assays yielding CFU/mL and corresponding graphs from each microbial strain revealed that the mixtures containing HX compounds and PE have significantly enhanced killing rate compared to the individual compounds, indicating their synergy. Similarly, enhanced time-kill profiles could also be recorded for the mixtures of HX compounds compared to PE only. The potential applications of HX compounds could be explored in the context of boosting the efficacy, reduction or replacing the PE with alternative active ingredients. The combination with AI-based screening and selection offers a credible solution within increasing demand towards formulating with alternative preservatives in personal care products.

One important additional aspect of this approach is the inclusion of the functional relations between antimicrobial capacities of the compound and its biological targets in skin cell affecting function/appearance of the skin. Such relations will likely dictate the choice of the

preservative to be included in the formulation in order to enhance to desired effect and performance of the product.

All compounds are included in the list of ingredients approved for cosmetics use in the EU. The natural compounds can be used within safe limits specified by regulators and suited to the required certification and product type. The natural antibiotics offer a wide range of complex and flexible synergistic capacities that could complement many formulations and product types as preservatives in cosmetics. The ingredients based on natural and sustainable origin could be also beneficial to the emerging brands driving innovation in the competitive markets. Further work would be required to not only determine the best possible candidates but also to ensure the highest qualities compatible with broad range pH, solubility and stability of the compounds in the formulations.

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