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New alternatives to cosmetics preservation

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Synopsis

In recent years, there is a considerable interest in the development of preservative-free or self-preserving cosmetics. The aim of our work was to develop new cosmetic formulations by replacing chemical preservatives with ingredients with antimicrobial properties that are not legislated as preservatives according to Annex VI of Commission Directive 76/768/EEC. This paper describes the preservative efficacy of the well-known antimicrobial extracts of *Lonicera caprifoleum* and *Lonicera japonica* in combination with glyceryl caprylate and/or levulinic acid, *p*-anisic acid, and ethanol. We prepared a series of acidic (pH=5.5) aqueous and O/W formulations, i.e., tonic lotion, shampoo, shower gel, conditioning cream, anticellulite cream, cleansing milk and peeling cream, containing (0.2% w/w) *Lonicera* extracts, alone in the case of tonic lotion and in combination with (1% w/w) glyceryl caprylate in the other products, and we performed challenge tests according to the European Pharmacopoeia procedures and criteria. Formulations such as shampoo, shower gel, and conditioning cream fulfilled criterion A, while tonic lotion, anticellulite cream, cleansing milk, and peeling cream fulfilled criterion B, in regard to contamination from *A. niger*. Furthermore, we evaluated the efficacy of the antimicrobial systems in two states of use: the intact product and after three weeks of consumer use. The results showed that *A. niger* was also detected during use by consumers in the products that satisfied only criterion B in challenge tests. The addition of antimicrobial fragrance ingredients such (≤ 0.3% w/w) levulinic acid or (0.1% w/w) *p*-anisic acid and/or (5% w/w) ethanol afforded products that met criterion A in challenge tests and were also microbiologically safe during use. The small quantity (5% w/w) of ethanol gave an important assistance in order to boost the self-preserving system and to produce stable and safe products.

INTRODUCTION

Microbial spoilage of cosmetic formulations has always been of special concern for industry, since it can lead to product degradation or, in the case of pathogens, constitutes a threat

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to consumer safety. Chemical preservatives are added to cosmetics, pharmaceuticals, and foods in order to protect them against microbial contamination.

The growing skepticism of consumers regarding the safety of chemical preservatives in combination with the fact that long-lasting skin health is often associated with the use of natural ingredients has led the cosmetics industry to seek alternative approaches for cosmetics preservation (1,2). A recent trend in cosmetics preservation is the replacement of traditional chemical preservatives by antimicrobial agents that are not legislated as preservatives according to Annex VI of Commission Directive 1976/768/EEC (3-5) but that are safe and effective as preservatives.

An approach to acheive preservative-free cosmetics is the selection of natural compounds that have been characterized as safe and effective against microorganisms, in order to decrease or to eliminate the use of the traditional chemical preservatives and to formulate cosmetics with improved dermocosmetic properties, i.e., lower skin irritation and/or contact sensitization.

A number of well-known plant-derived essential oils and extracts have exhibited excellent antimicrobial properties; thus, they have been used for the effective preservation of cosmetic formulations. Among others, derivatives of *Rosmarinus officinalis* (7,8), *Lavandula officinalis* (9), *Pteronia incana* (8), *Artemisia afra* (8), *Thymus vulgaris* (10,11), *Eucalyptus globulus* (12), *Laurus nobilis* (12), *Salvia officinalis* (12), and *Melaleuca alternifolia* (13,14) have been reported to be effective natural preservatives.

Medium polar substances also belong to the class of alternative antimicrobial agents. Examples of such agents as caprylyl glycol and monoglycerides of capric acid and caprylic acid, i.e., glyceryl caprate and glyceryl caprylate, besides being moisturizing agents, exert antimicrobial activities (15–17). Due to their emulsifier-like structure, with a hydrophilic and lipophilic part, they interfere with the cellular structures of microorganisms and disintegrate cell membranes. Many studies have been reported concerning the use of glyceryl caprylate as an antimicrobial substance alone or in combination with other antimicrobial compounds for cosmetics preservation. (17–19).

We have to keep in mind that the chemical composition of fragrances plays a key role concerning the antimicrobial activity of essential oils and the extracts obtained from natural sources (20). Various aldehydes and alcohols, i.e., aromatic and aliphatic compounds, or terpenes and organic acids, are among the most active compounds. In the past, a fragrance mixture that mainly consisted of benzyl acetate, phenethyl alcohol, and linalool had been proposed as an alternative preservative in order to reduce the amount of parabens used in cosmetic formulations (21). Today, several antimicrobial fragrance ingredients are commercially available, such as, *p*-anisic acid (*p*-methoxy-benzoic acid) and levulinic acid (4-oxo-pentanoic acid), which were found to be the main compounds in *Pimpinella anisum* and other herbs and in *Dioscorea villosa* as a by-product in the production of diosgenin from wild yam, respectively (22).

Based on the above comments concerning the development of self-preserving cosmetics, we focused our research to evaluate the preservative efficacy of the antimicrobial extracts of *Lonicera caprifoleum* and *Lonicera japonica* (Table I) in combination with other antimicrobials such as glyceryl caprylate, *p*-anisic acid, levulinic acid (Table II), and ethanol in a series of aqueous and O/W emulsions. *Lonicera* extracts are described as being a mixture of esters of lonicerin and *p*-hydroxy benzoic acid, the structures of which are very similar

Table I
MIC Values of the Mixture Consisting of *Lonicera caprifoleum* and *Lonicera Japonica* Extracts (Plantservative WSr) (25)

Microorganism	MIC* (% w/v)
Staphylococcus aureus	0.125
Escherichia coli	0.125
Pseudomonas aeruginosa	0.125
Aspergilus niger	0.20
Candida albicans	0.10

*Minimum inhibitory concentration.

to those of parabens (23). Although *Lonicera caprifoleum* and *Lonicera japonica* extracts are well known for their antimicrobial properties (24–26), there are no studies in the literature regarding their incorporation as preservatives in cosmetic formulations. In order to evaluate the preservative efficacy of these multifunctional ingredients, we performed challenge tests (preservative efficacy tests, PETs) according to the standards proposed by the European Pharmacopoeia.

Furthermore, we examined the microbial purity of the formulations in two different states of use (the intact product and following use) because few published papers refer to the efficacy of preservative systems contained in cosmetic products during their use by consumers (27,28).

MATERIALS AND METHODS

COSMETIC FORMULATIONS

A series of aqueous formulations, i.e., tonic lotion, shampoo, and shower gel, and O/W cosmetic formulations such as conditioning cream, anticellulite cream, cleansing milk, and peeling cream was prepared.

Tonic lotion. Water, *Syringa vulgaris* (lilac) extract, lactic acid, cinnamyl alcohol, hydroxycitronellal, and preservative systems I, II, III, or IV (Table III) were used as the ingredients in the tonic lotion formulation.

Shampoo. Water, sodium cocoyl isethionate, lauryl glycoside, cocamidopropyl betaine, cocobetaine, glyceryl oleate, coco glycoside, hydrolyzed milk protein, sodium phytate, *Urtica dioica* leaf water, *Rosmarinus officinalis* (rosemary) leaf water, *Salix alba* (white willow)

Table II
Activity of the Alternative Preservatives Against Bacteria and Fungi (18,19)

Alternative preservative	Recommended dosage (% w/w)	Gram+	Gram-	Yeasts	Molds
Glyceryl caprylate	0.5–1.0	+++	+++	+++	+
p-Anisic acid	0.05–0.3	++	++	++	+++
Levulinic acid	0.2–0.3*	+++	+++	++	+++

+++Very good activity.
++Good activity.
+Activity depends on compatibility or dosage.
*As 2–3% w/w Dermosoft 1388®

Table III
Preservative Systems

Preservative systems	Concentration of preservative system in tested formulation (w/w)
I	*Lonicera* extracts 0.2 %
II	*Lonicera* extracts 0.2 % + *p*-anisic acid 0.1 %
III	*Lonicera* extracts 0.2 % + levulinic acid 0.3 %*
IV	*Lonicera* extracts 0.2 % + ethanol 5 %
V	*Lonicera* extracts 0.2 % + glyceryl caprylate 1 %
VI	*Lonicera* extracts 0.2 % + glyceryl caprylate 1 % + *p*-anisic Acid 0.1 %
VII	*Lonicera* extracts 0.2 % + glyceryl caprylate 1 % + levulinic acid 0.3 %
VIII	*Lonicera* extracts 0.2 % + glyceryl caprylate 1 % + levulinic acid 0.1 %**

*As 3 % w/w Dermosoft 1388.
**As 1 % w/w Dermosoft 1388.

leaf water, *Ginkgo biloba* leaf water, citric acid, fragrance, linalool, and preservative system V (Table III) were used as the shampoo ingredients.

Shower gel. The ingredients used in the shower gel formulation were: water, sodium cocoyl isethionate, lauryl glycoside, cocamidopropyl betaine, sodium lauryl glutamate, glyceryl oleate, cocoglucoside, cocobetaine, sodium phytate, *Aloe barbadensis* (*Aloe vera*) extract, *Avena sativa* (oat) leaf extract, *Calendula officinalis* leaf water, *Arnica montana* leaf water, *Lavandula angustifolia* (lavender) leaf water, hydrolyzed milk protein, parfum (fragrance), citric acid, D-limonene, and preservative system V (Table III).

Conditioning cream. The conditioning cream ingredients were: water, hydroxypropyl starch phosphate, cetyl alcohol, dioleyloylethyl hydroxyethylammonium methosulfate, sucrose laurate, cetearyl alcohol, *Macadamia ternifolia* seed oil, glycerin, polyglyceryl-10 laurate, meadowfoam (*Limnantes alba*) seed oil, fragrance, stearyl stearate, glycine soja, phospholipids, soy sterol, sodium phytate, ethanol, lauryl glycoside, tocopheryl acetate, *Urtica dioica* (nettle) leaf water, *Rosmarinus officinalis* (rosemary) leaf water, *Gingko biloba* leaf water, *Salix alba* (white willow) leaf water, hydrolyzed milk protein, tocopherol, and preservative system V (Table III).

Anticellulite cream. The ingredients used in the anticellulite cream formulation were: water, sodium stearoyl lactylate, caffeine, glycerin, tricaprylin, dicaprylyl carbonate, isopropyl myristate, polyglyceryl-3 stearate, dicaprylyl ether, cetyl alcohol, glyceryl stearate, panthenol, behenyl alcohol, glyceryl stearate, lecithin, glycine soja (soybean) sterols, *Lactobacillus*/*Trifolium pratense* (clover) flower ferment extract, *Lactobacillus*/*Theobroma cacao* (cocoa) ferment extract, *Lactobacillus*/*Camellia sinensis* leaf ferment extract, *Vitis vinifera* (grape) seed oil, *Prunus armeniaca* (apricot) kernel oil, sodium phytate, xanthan gum, escin, tocopherol, citric acid, *Olea europaea* (olive) fruit extract, parfum (fragrance), linalool, benzyl benzoate, benzyl salicylate, farnesol, geraniol, eugenol, and preservative system V, VI, or VII (Table III).

Cleansing milk. The cleansing milk ingredients were: water, isopropyl myristate, glyceryl stearate, polyglyceryl 3-stearate, myristyl myristate, glycerin, sodium stearoyl lactylate, tricaprylin, caprylic/capric triglyceride, *Calendula officinalis* oil, cetearyl alcohol, bisabolol, *Prunus armeniaca* (apricot) kernel oil, tocopherol, xanthan gum, sodium phytate, *Aloe barbadensis* extract, sodium hydroxide, citric acid, *Chamomilla recutita* (Matricaria) extract, and preservative system V, VI, VII, or VIII (Table III).

Peeling cream. The ingrediants used in the peeling cream formulation were: water, glycerin, isopropyl myristate, *Prunus dulcis amygdalus* (almond) shell granules, glyceryl stearate, myristyl myristate, polyglyceryl 3-stearate, tricaprylin, stearic acid, sodium stearoyl lactylate, cetyl alcohol, cetearyl alcohol, caprylic/capric triglyceride, *Prunus dulcis amygdalus* (almond) seed oil, tocopherol, xanthan gum, sodium stearate, *Simmondsia chinensis* (jojoba) seed oil, sodium phytate, sodium hydroxide, *Chamomilla recutita* (Matricaria) extract, citric acid, parfum (fragrance), limonene, and preservative system V, VI, or VII (Table III).

ESSENTIAL OILS AND MULTIFUNCTIONAL INGREDIENTS WITH ANTIMICROBIAL ACTIVITY

- Planteservative WSr® (Campo Cosmetics S Pte. Ltd., Singapore) = *Lonicera caprifoleum* flower extract and *Lonicera japonica* flower extract, water.
- Dermosoft GMCY® (Dr Straetmans Chemische Produkte GmbH, Hamburg, Germany) = glyceryl caprylate.
- Dermosoft 688® (Dr Straetmans Chemische Produkte GmbB, Hamburg, Germany) = *p*-anisic acid.
- Dermosoft 1388® (Dr Straetmans Chemische Produkte GmbH, Hamburg, Germany) = levulinic acid (10%), sodium hydroxide, glycerin, water.
- Ethanol (not denaturated).

ORGANISMS AND INOCULUM PREPARATION

Organisms. Staphylococcus aureus ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli, Aspergillus niger* ATCC 16404, and *Candida albicans* ATCC 10231 were used.

Inoculum preparation. For bacteria and *C. albicans* inoculum, the cells (10^8) were harvested into 0.1% peptone water by gentle agitation and adjusted to yield suspensions of approximately 10^6 cfu/ml. The count of *A. niger (*approx. 10^5*) was* achieved after the 1% (w/w) dilution of the initial suspension (10^7). The peptone water used for harvesting *A. niger* contained 0.05% v/v of Tween 80 (Sigma-Aldrich).

MICROBIAL CHALLENGE TESTS (PRESERVATIVE EFFICACY TESTS, PETS) ACCORDING TO THE EUROPEAN PHARMACOPOEIA (E. PH.)

Preliminary studies were performed in order to assure the ability of the unpreserved formulations to support the viability and/or microbial growth and also the effectiveness of the neutralizing medium for the inoculum recovery. The microbial challenge test was performed according to the standards proposed by the European Pharmacopoeia (E. Ph., 1996) concerning topical preparations.

The formulations (samples of 20 g) were placed in sterile containers and separately inoculated with bacterial and fungal suspensions to reach microbial levels of not less than 10^6 cfu/g for bacteria and 10^5 cfu/g for fungi. The test samples were mixed, diluted in Letheen broth, and assayed at 0, 2, 7, 14, 21, and 28 days. The assays were performed on 1 g or 1 ml of test sample and plated in triptic soy agar and Sabouraud dextroze agar for bacteria and fungi, respectively. Plates were incubated at 35°C for bacteria and at 25°C for fungi. After a five-day incubation, colonies of bacteria and fungi were counted and (cfu/g) calculated. The experiments were performed in triplicate. Products are judged adequately

preserved when bacteria are reduced by more than 99% (2 log) after two days and more than 99.9% (3 log) after seven days; yeasts and molds should be reduced by more than 99% (2 log for criterion A and 1 log for criterion B) after 14 days.

MICROBIOLOGICAL QUALITY IN TWO STATES OF USE: INTACT PRODUCT AND FOLLOWING THREE WEEKS OF USE

The collected samples of cosmetic products were analyzed for total aerobic plate count (*S. aureus, P. aeruginosa, E. coli, A. niger*, and *C. albicans*) in two different states of use, the intact product and after three weeks of use. One gram or 1 ml of test sample was serially diluted in Letheen broth and plated in triptic soy agar and Sabouraud dextroze agar for bacteria and fungi, respectively. Plates were incubated at 35°C for bacteria and at 25°C for fungi. After a five-day incubation, colonies of bacteria and fungi were counted and (cfu/g) calculated. The experiments were performed in triplicate. In some cases *A. niger* was identified as black colonies with the characteristic morphology of actinomycetes.

RESULTS

MICROBIOLOGICAL QUALITY OF THE TEST PRODUCT

The results regarding the microbiological safety of the formulations tested (challenge test according E. Ph., intact products, and following three weeks of use) are summarized in Tables IV, V, VIa–e, VII and VIII and in Figures 1a–e and 2.

Table IV
Challenge Test Criteria Regarding Aqueous and O/W Formulations Containing Preservative Systems I-VIII

Type formulation	Water content (%)	Water activity (a_w)	pH	Preservative system	Challenge Test criteria (E. Ph.)	Physicochemical stability
Tonic lotion	95	—	5.5	I	B regarding *A. niger* and *C. albicans*	OK
		—	5.5	II	A	No
		—	5.5	III	A	No
	90	0.9	5.5	IV	A	OK
Shampoo	74	0.932	5.5	V	A	OK
Shower gel	65	0.893	5.5	V	A	OK
Conditioning cream	67	0.903	5.5	V	A	OK
Anticellulite cream	65	—	5.5	V	B regarding *A. niger*	OK
		0.903	5.5	VI	A	OK
		—	5.5	VII	A	No
Cleansing milk	65	—	5.5	V	B regarding *A. niger*	OK
		—		VI	A	No
		0.919		VII	A	OK
		—		VIII	A	OK
Peeling cream	47	—	5.5	V	B regarding *A. niger*	OK
		0.865	5.5	VI	A	OK
		—	5.5	VII	A	No

—Not done.

Table V
In-Use Study (intact product and following three weeks of use) of Aqueous and O/W Formulations Containing Preservative Systems I-VIII

Type of formulation	Preservative system	Type of container	Total aerobic count (cfu/g) in intact product	Total aerobic count (cfu/g) following three weeks of use	Identified microorganisms
Tonic lotion	I	Glass bottle, pump	<10	10,000	*A. niger*
	II	Glass bottle, pump	<10	<10	Absence
	III	Glass bottle, pump	<10	<10	Absence
	IV	Glass bottle, pump	<10	<10	Absence
Shampoo	V	PE soft-touch bottle, pump	<10	<10	Absence
Shower gel	V	PE soft-touch bottle, pump	<10	<10	Absence
Conditioning cream	V	PE soft-touch bottle, pump	<10	<10	Absence
Anticellulite cream	V	Glass bottle, pump	<10	9000	*A niger*
	VI	Glass bottle, pump	<10	<10	Absence
	VII	Glass bottle, pump	<10	<10	Absence
Cleansing milk	V	Glass bottle, pump	<10	8800	*A. niger*
	VI	Glass bottle, pump	<10	<10	Absence
	VII	Glass bottle, pump	<10	<10	Absence
	VIII	Glass bottle, pump	<10	<10	Absence
Peeling cream	V	Glass jar, PP cap	<10	9500	*A. niger*
	VI	Glass jar, PP cap	<10	<10	Absence
	VII	Glass jar, PP cap	<10	<10	Absence

PE = polyethylene; PP = polypropylene.

Staphylococcus aureus. Systems I–IV preserved effectively the high-water-containing tonic lotion against this strain (Table IV). System V could not be used in the case of the tonic lotion due to solubility problems, but it was active in the cases of shampoo and shower gel and all the emulsified formulations tested (Figure 1a, Tables IV and VIa). Preservative systems VI and VII showed excellent activity in all the O/W formulations, i.e., the anticellulite cream, cleansing milk, and peeling cream. System VIII with the reduced percentage (0.1% w/w) of levulinic acid also proved to be effective. In all above cases, criterion A of the E. Ph. were fulfilled. Furthermore, no contamination from this strain was found, either in the intact products or following consumer use (Tables V, VII and VIII).

Pseudomonas aeruginosa. This Gram-negative microorganism was susceptible to preservative systems I–IV in the case of the tonic lotion (Table IV). System V effectively protected the shampoo, shower gel, and O/W preparations. (Figure 1b, Tables IV and VIb). System VI also preserved equally to system V all the O/W formulations tested, i.e., the anticellulite cream, cleansing milk, and peeling cream. As with the previous microorganism, levulinic acid (0.3% w/w, system VII, or 0.1% w/w, system VIII), was effective. In all of the above cases criterion A of the E. Ph. was met. Additionally, no contamination was found either in the intact products or following three weeks of use (Tables V, VII, and VIII).

Escherichia coli. The population of this test microorganism seemed to be effectively controlled by systems I–IV in the case of the tonic lotion, since criterion A of E. Ph. was satisfied (Table IV). System V was also active in the shampoo and shower gel and the

Table VIa
Challenge Test of Cosmetic Products Performed in Triplicate Regarding *Staphylococcus aureus* (system V)

S. aureus	Shampoo				Shower gel				Conditioning cream			
	Experiment				Experiment				Experiment			
	TAPC*				TAPC*				TAPC*			
	(log cfu/g)				(log cfu/g)				(log cfu/g)			
	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
Days 0	5.6	5.72	5.58	5.63	6	5.95	5.45	5.8	5.85	5.7	5.16	5.6
2	1.75	1.85	1.8	1.8	1.8	1.95	1.95	1.9	1.82	1.75	1.83	1.8
7	0.7	0.9	0.8	0.8	0.85	0.93	0.92	0.9	0.68	0.67	0.75	0.7
14	0.8	0.9	0.7	0.8	0.9	0.83	0.67	0.8	0.71	0.7	0.69	0.7
28	0.8	0.9	0.7	0.8	0.85	0.8	0.75	0.8	0.71	0.68	0.71	0.7

*TAPC = total aerobic plate count.
#MV = mean value.

Table VIb
Challenge Test of Cosmetic Products Performed in Triplicate Regarding *Pseudomonas aeruginosa* (system V)

P. aeruginosa	Shampoo				Shower gel				Conditioning cream			
	Experiment				Experiment				Experiment			
	TAPC*				TAPC*				TAPC*			
	(log cfu/g)				(log cfu/g)				(log cfu/g)			
	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
Days 0	5.57	5.8	5.73	5.7	5.65	5.55	5.6	5.6	5.85	5.7	5.16	5.6
2	1.85	1.92	1.93	1.9	1.35	1.41	1.44	1.4	1.82	1.75	1.83	1.8
7	0.91	1	0.94	0.95	0.85	0.93	0.92	0.9	0.82	0.91	0.82	0.9
14	0.85	0.92	0.93	0.9	0.71	0.7	0.69	0.7	0.82	0.91	0.82	0.9
28	0.85	0.92	0.93	0.9	0.71	0.7	0.69	0.7	0.82	0.91	0.82	0.9

*TAPC = total aerobic plate count.
#MV = mean value.

Table VIc
Challenge Test of Cosmetic Products Performed in Triplicate Regarding *Escherichia coli* (system V)

E. coli	Shampoo				Shower gel				Conditioning cream			
	Experiment				Experiment				Experiment			
	TAPC*				TAPC*				TAPC*			
	(log cfu/g)				(log cfu/g)				(log cfu/g)			
	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
Days 0	5.65	5.7	5.62	5.65	5.6	5.57	5.63	5.6	5.5	5.8	5.26	5.5
2	1.35	1.41	1.44	1.4	1.38	1.42	1.4	1.4	1.8	1.95	1.95	1.9
7	0.94	0.94	0.97	0.95	0.85	0.93	0.92	0.9	0.85	0.93	0.92	0.9
14	0.85	0.93	0.92	0.9	0.68	0.72	0.7	0.7	0.8	0.9	0.7	0.8
28	0.71	0.68	0.71	0.7	0.68	0.72	0.7	0.7	0.8	0.9	0.7	0.8

*TAPC = total aerobic plate count.
#MV = mean value.

Table VIa (cont'd)

Anticellulite cream				Cleansing milk				Peeling cream			
Experiment				Experiment				Experiment			
TAPC*				TAPC*				TAPC*			
(log cfu/g)				(log cfu/g)				(log cfu/g)			
No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
5.5	5.8	5.26	5.52	5.55	5.78	5.68	5.67	5.5	5.67	5.39	5.5
1.9	1.82	1.83	1.85	1.6	1.53	1.37	1.5	1.85	1.91	1.94	1.9
0.85	0.93	0.92	0.9	0.85	0.92	0.93	0.9	0.85	0.92	0.93	0.9
0.82	0.91	0.82	0.85	0.84	0.9	0.96	0.9	0.68	0.72	0.7	0.7
0.8	0.82	0.72	0.78	0.82	0.88	0.85	0.85	0.77	0.69	0.64	0.7

Table VIb (cont'd)

Anticellulite cream				Cleansing milk				Peeling cream			
Experiment				Experiment				Experiment			
TAPC*				TAPC*				TAPC*			
(log cfu/g)				(log cfu/g)				(log cfu/g)			
No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
5.6	5.67	5.68	5.65	5.7	5.69	5.77	5.72	5.55	5.78	5.68	5.7
0.85	0.92	0.93	0.9	1.8	1.95	1.95	1.9	1.65	1.72	1.43	1.6
0.82	0.91	0.82	0.85	0.94	0.94	0.97	0.95	0.68	0.67	0.75	0.7
0.71	0.7	0.69	0.7	0.85	0.93	0.92	0.9	0.85	0.93	0.92	0.9
0.71	0.7	0.69	0.7	0.71	0.7	0.69	0.7	0.85	0.93	0.92	0.9

Table VIc (cont'd)

Anticellulite cream				Cleansing milk				Peeling cream			
Experiment				Experiment				Experiment			
TAPC*				TAPC*				TAPC*			
(log cfu/g)				(log cfu/g)				(log cfu/g)			
No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
5.53	5.69	5.73	5.65	5.69	5.72	5.69	5.7	5.71	5.69	5.61	5.7
0.85	0.93	0.92	0.9	1.8	1.95	1.95	1.9	1.65	1.72	1.43	1.6
0.82	0.91	0.82	0.85	0.94	0.94	0.97	0.95	0.71	0.68	0.71	0.7
0.71	0.68	0.71	0.7	0.85	0.93	0.92	0.9	0.85	0.93	0.92	0.9
0.48	0.55	0.47	0.5	0.71	0.7	0.69	0.7	0.9	0.83	0.67	0.8

Table VId
Challenge Test of Cosmetic Products Performed in Triplicate Regarding *Aspergillus niger* (system V)

	Shampoo				Shower gel				Conditioning cream			
A. niger	Experiment				Experiment				Experiment			
	TAPC*				TAPC*				TAPC*			
	(log cfu/g)				(log cfu/g)				(log cfu/g)			
	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
Days 0	5.6	5.67	5.68	5.65	5.49	5.58	5.43	5.5	5.25	5.32	5.33	5.3
2	0.85	0.93	0.92	0.9	0.8	0.9	0.7	0.8	0.71	0.7	0.69	0.7
7	0.71	0.7	0.69	0.7	0.7	0.9	0.8	0.8	0.71	0.7	0.69	0.7
14	0.62	0.6	0.58	0.6	0.57	0.59	0.64	0.6	0.59	0.6	0.61	0.6
28	0.51	0.51	0.48	0.5	0.38	0.41	0.41	0.4	0.49	0.49	0.52	0.5

*TAPC = total aerobic plate count.
#MV = mean value.

Table VIe
Challenge Test of Cosmetic Products Performed in Triplicate Regarding *Candida albicans* (system V)

	Shampoo				Shower gel				Conditioning cream			
C. albicans	Experiment				Experiment				Experiment			
	TAPC*				TAPC*				TAPC*			
	(log cfu/g)				(log cfu/g)				(log cfu/g)			
	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
Days 0	5.28	5.31	5.31	5.3	5.71	5.76	5.78	5.8	5.29	5.31	5.42	5.3
2	1.75	1.85	1.8	1.8	1.94	1.95	2.05	2	1.87	1.97	2.1	2
7	0.49	0.49	0.52	0.5	0.84	0.9	0.96	0.9	0.93	0.94	0.98	1
14	0.49	0.49	0.52	0.5	0.8	0.9	0.7	0.8	0.9	0.83	0.67	0.8
28	0.31	0.3	0.29	0.3	0.37	0.41	0.42	0.4	0.9	0.83	0.67	0.8

*TAPC = total aerobic plate count.
#MV = mean value.

emulsified formulations (Figure 1c, Tables IV and VIc). Preservative systems VI and VII revealed excellent activity against this microorganism in the O/W formulations tested, i.e., the anticellulite cream, cleansing milk, and peeling cream. System VIII, with the reduced percentage of levulinic acid, was sufficient as well. The strain was not detected in the intact product (Tables V and VII). Furthermore, the strain was not recovered during the in-use consumer use test (Tables V and VIII).

Aspergillus niger. System I preserved marginally the tonic lotion against this mold in the challenge test, since only criterion B of E. Ph. was achieved (Figure 2 and Table IV). Although no contamination of the mold was detected in the intact product (Table VII), recovery was observed during the in-use study (Table VIII). Addition of (0.1% w/w) *p*-anisic acid (system II) or (0.3% w/w) levulinic acid (system III) or (5% w/w) ethanol (system IV) to *Lonicera* extracts (system I) enhanced the efficacy, and the resulting products were microbiologically safe either in the challenge test (Figure 2 and Table IV) or

Table VId (cont'd)

Anticellulite cream				Cleansing milk				Peeling cream			
Experiment				Experiment				Experiment			
TAPC*				TAPC*				TAPC*			
(log cfu/g)				(log cfu/g)				(log cfu/g)			
No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
5.4	5.45	5.65	5.5	5.49	5.49	5.52	5.5	5.81	5.82	5.77	5.8
3.9	4.1	4	4	3.4	3.55	3.55	3.5	3.85	3.95	3.91	3.9
3.12	3.25	3.53	3.3	3.38	3.39	3.43	3.4	3.35	3.42	3.45	3.4
3	3.25	3.05	3.1	2.8	3	3.2	3	3.1	3.35	3.15	3.2
2.95	3.1	2.95	3	2.5	2.8	3.1	2.8	2.95	3.05	3	3

Table VIe (cont'd)

Anticellulite cream				Cleansing milk				Peeling cream			
Experiment				Experiment				Experiment			
TAPC*				TAPC*				TAPC*			
(log cfu/g)				(log cfu/g)				(log cfu/g)			
No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#	No.1	No.2	No.3	MV#
5.33	5.32	5.37	5.34	5.52	5.59	5.63	5.58	5.37	5.3	5.29	5.3
1.92	1.96	1.97	1.95	1.92	1.96	1.97	1.95	1.85	1.91	1.94	1.9
0.93	0.94	0.98	0.95	0.71	0.7	0.69	0.7	0.85	0.92	0.93	0.9
0.85	0.93	0.92	0.9	0.48	0.51	0.51	0.5	0.85	0.92	0.93	0.9
0.85	0.93	0.92	0.9	0.37	0.41	0.42	0.4	0.82	0.91	0.82	0.9

during the consumer-use study (Tables VII and VIII). System V preserved efficiently the shampoo and shower gel (Figure 1d, Tables IV and VId). O/W formulations, besides the conditioning cream, were not completely protected against *A. niger* with system V, since in the cases of anticellulite cream, cleansing milk, and peeling cream only criterion B of E. Ph. was fulfilled (Figure 1d, Tables IV and VId). The relative activity exerted previously by system V against the three bacterial strains did not seem to affect this fungus in the emulsified formulations tested. A population of about 10^3 cfu/g was counted at the end of the challenge test (Figure 1d), whereas recovery was observed following three weeks of use (Table VIII). The addition of *p*-anisic acid (system VI) or levulinic acid (systems VII and VIII) inhibited the growth of the mold in the O/W formulations, i.e., the anticellulite cream, cleansing milk, and peeling cream, and criterion A was achieved in the challenge test. No contamination of the mold was detected either in the intact product or following use (Tables V, VII, and VIII), when systems VI, VII, or VIII were utilized.

Table VII
Microbiological Analysis of Intact Products in Triplicate

Preserv. system	Tonic lotion					Shampoo/shower gel					Conditioning cream				
	Microbiological analysis					Microbiological analysis					Microbiological analysis				
	TAPC*				Ident.	TAPC*				Ident.	TAPC*				Ident.
	(cfu/g)				Micr.	(cfu/g)				Micr.	(cfu/g)				Micr.
	No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#	
No. I	<10	<10	<10	<10	Abs.										
No. II	<10	<10	<10	<10	Abs.										
No. III	<10	<10	<10	<10	Abs.										
No. IV	<10	<10	<10	<10	Abs.										
No. V						<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
No. VI															
No. VII															
No. VIII															

*TAPC = total aerobic plate count.
#MV = mean value.

Table VIII
Microbiological Analysis in Triplicate Following Three Weeks of Use

Preserv. system	Tonic lotion					Shampoo/shower gel					Conditioning cream				
	Microbiological analysis					Microbiological analysis					Microbiological analysis				
	TAPC*				Ident.	TAPC*				Ident.	TAPC*				Ident.
	(cfu/g)				Micr.	(cfu/g)				Micr.	(cfu/g)				Micr.
	No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#	
No. I	9500	9500	11000	10000	A.Niger										
No. II	<10	<10	<10	<10	Abs.										
No. III	<10	<10	<10	<10	Abs.										
No. IV	<10	<10	<10	<10	Abs.										
No. V						<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
No. VI															
No. VII															
No. VIII															

*TAPC = total aerobic plate count.
#MV = mean value.

Candida albicans. System I fulfilled marginally criterion B in the case of the tonic lotion in the challenge test (Table IV). Systems II–IV were effective against yeast in the tonic lotion during the challenge test (Table IV) and in the in-use study (Tables V, VII, and VIII). Systems V (Figure 1e, Tables IV and VIe) and VI (Table IV) were active against

Table VII (cont'd)

Anticellulite cream					Cleansing milk					Peeling cream				
Microbiological analysis					Microbiological analysis					Microbiological analysis				
TAPC*				Ident.	TAPC*				Ident.	TAPC*				Ident.
(cfu/g)				Micr.	(cfu/g)				Micr.	(cfu/g)				Micr.
No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#	
<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
					<10	<10	<10	<10	Abs.					

Table VIII (cont'd)

Anticellulite cream					Cleansing milk					Peeling cream				
Microbiological analysis					Microbiological analysis					Microbiological analysis				
TAPC*				Ident.	TAPC*				Ident.	TAPC*				Ident.
(cfu/g)				Micr.	(cfu/g)				Micr.	(cfu/g)				Micr.
No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#		No.1	No.2	No.3	MV#	
7300	9500	10200	9000	*A. niger*	8500	8500	9400	8800	*A. niger*	9200	9600	9700	9500	*A. niger*
<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.	<10	<10	<10	<10	Abs.
					<10	<10	<10	<10	Abs.	NA	NA	NA	NA	NA

yeast in the O/W formulations tested. Systems VII and VIII with (0.3% w/w) levulinic acid and (0.1% w/w) *p*-anisic acid, respectively, preserved sufficiently the emulsified products and criterion A was fulfilled. No contamination from the mold was detected either in the intact product or during the in-use consumer study. (Tables V, VII, and VIII).

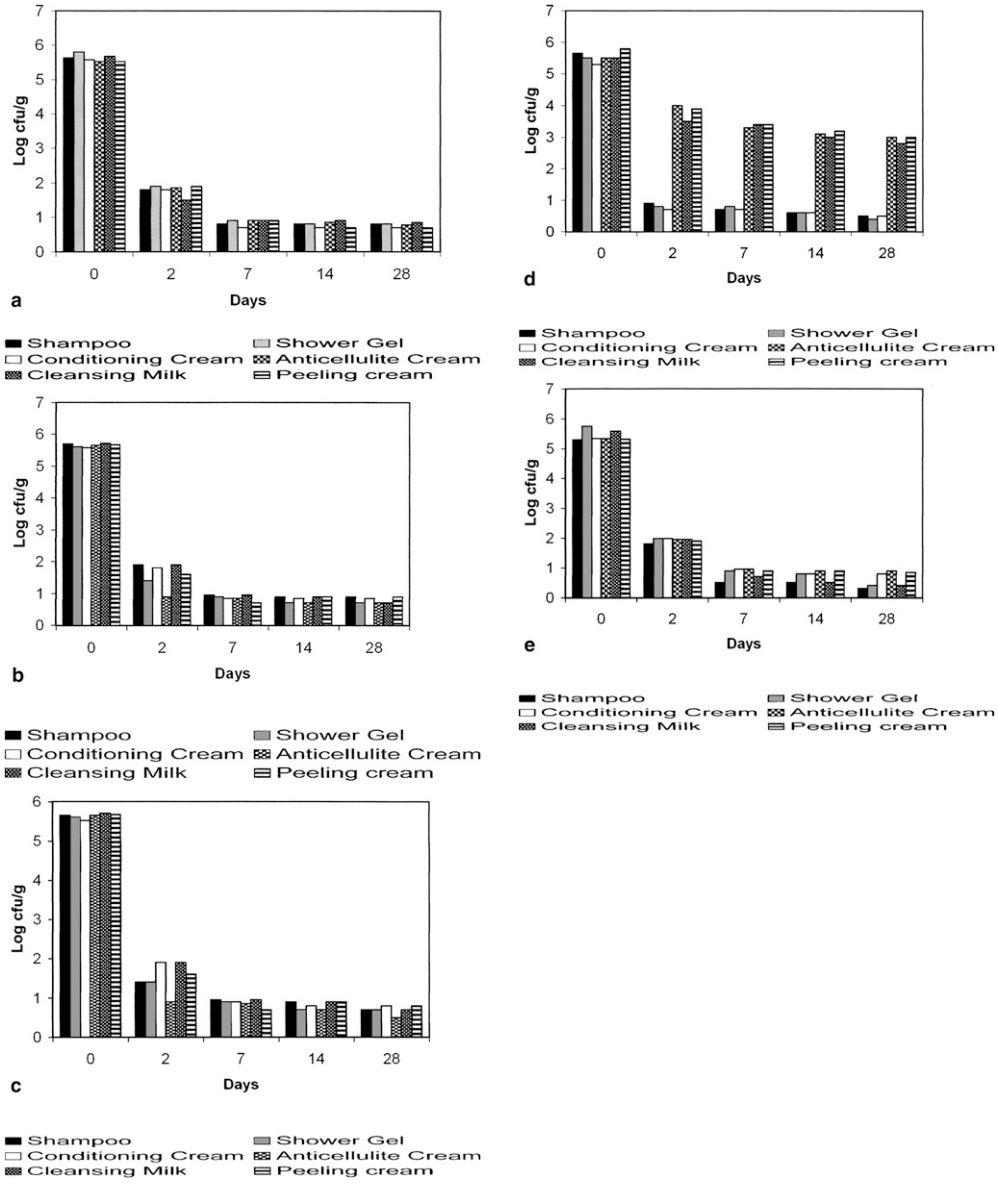

Figure 1. Challenge tests (E. Ph.) in various cosmetic forms containing prservative system V: (a) *S. aureus,* (b) *P. aeruginosa,* (c) *E. coli*, (d) *A. niger,* and (e) *C. albicans*.

PHYSICOCHEMICAL STABILITY

The results concerning physicochemical stability are summarized in Table IV. *Lonicera* extracts alone (system I) was used only in the case of the tonic lotion and did not cause stability problems. The addition of (0.1% w/w) *p*-anisic acid or (0.3% w/w) levulinic acid to system I (i.e, systems II and III), caused the precipitation of solids after a few days. System V afforded stable O/W emulsions, the shampoo and the shower gel. However, it could not be used in the case of the tonic lotion, where the water content was high, due

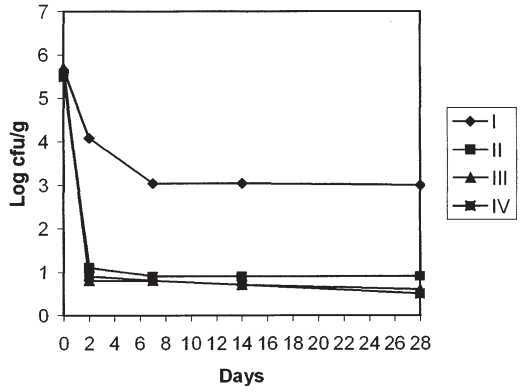

Figure 2. Results of the challenge test regarding the activity of preservative systems I–IV against *A. niger* in the case of tonic lotion.

to the solubility problems of glyceryl caprylate. Furthermore, the enrichment of system V with (0.1% w/w) anisic acid (system VI) or (0.3% w/w) levulinic acid (system VII) led to the separation of the phases in the cases of the cleansing milk and the anticellulite cream after 20 days. In contrast, addition of (0.1% w/w) levulinic acid (system VIII) did not influence the stability of the cleansing milk.

WATER ACTIVITY

Water activity (a_w) or equilibrium relative humidity quantifies the active part of the moisture content or "free water" as opposed to the total moisture content, which also includes "bound water." It indicates the amount of water in the total water content that is available to microorganisms. Each species of microorganism has its own minimum a_w value below which growth is no longer possible (6). The results of water activity measurements of the tested formulations are presented in Table IV.

DISCUSSION

All the tested antimicrobial systems (I–VIII) have exerted excellent activity against Gram-positive and Gram-negative bacteria in the acidic (pH=5.5) environment used. They protected efficiently the emulsified and aqueous formulations against Gram-positive and Gram-negative bacteria in challenge tests (criterion A of E. Ph.) and in in-use study (intact products and following three weeks of use). Antimicrobials I–VIII proved to be effective against Gram-positive bacteria, although 0.86 is the lowest a_w value permitting *S. aureus* growth. Acidic pH conditions may contribute to the increase in the minimum a_w value for this microorganism (6) and therefore improve the performance of alternative systems used. Of course, manipulation of a_w is only part of the preservative system.

The activity against Gram-negative bacteria could be partially attributed to the relatively low water activity values of the products (0.865–0.932) (Table VI), since water activity values lower than 0.95 prevent the growth of Gram-negative microorganisms (6). We note that these microorganisms are known to be very persistent and often are recovered in the in-use state, probably from the hands of the consumers (27), even in products containing effective traditional preservatives such as parabens and phenoxyethanol (28).

On the other hand, fungi were less susceptible to *Lonicera* extracts (system I) and *Lonicera* extracts/glyceryl caprylate (system V). Although, system I in the case of the tonic lotion showed moderate activity against *A. niger* and *C. albicans* in challenge tests (criterion B of E. Ph.), significant levels of mold were recovered following use. The moderate efficacy of system I in the preservation of the tonic lotion could be ascribed to the inability of *Lonicera* extracts (0.2% w/w) to inhibit mold in this formulation. System V protected the aqueous shampoo and shower gel against *A. niger*, where criterion A of E. Ph. was fulfilled. Probably the antimicrobial potencies of *Rosmarinus officinalis* leaf water in the shampoo and *Lavender angustifolia* leaf water in the shower gel enhanced the antifungal activity of system V. System V satisfied marginally criterion B of E. Ph against *A. niger* in all the emulsified formulations except the conditioning cream. The greater ability of system V in this cosmetic form could be ascribed to cationic agents, which might reinforce the antimicrobial activity (28,29). Furthermore, system V was unable to preserve the anticellulite cream, cleansing milk, and peeling cream, since *A. niger* was detected after three weeks of consumer use.

Another factor that might enhance contamination risk in the case of the peeling cream is the jar-container, which allows the entry of microorganisms into the product. The lack of efficacy in system V against *A. niger* in some emulsified formulations is in accordance with the findings reported previously that the preservative performance of glyceryl caprylate against molds depends on the formulation (18,19).

Regarding the fragrance ingredients, although the addition of (0.1% w/w) anisic acid (systems II and VI) or (0.3% w/w) levulinic acid (systems III and VII) significantly improved antifungal activity, in some cases it caused stability problems. The reduction of the concentration of levulinic acid to 0.1% w/w resulted in microbiologically and physicochemically stable products.

CONCLUSIONS

The results demonstrate that natural origin ingredients such as *Lonicera* extracts seem to be promising as antimicrobial substances for producing self-preserving cosmetic products. The addition of multifunctional ingredients such as glyceryl caprylate, levulinic acid or *p*-anisic acid and/or ethanol was beneficial in the majority of the products. Ethanol at low concentration (i.e., 5% w/w) may contribute to the performance of the antimicrobial. An interesting observation is that the products that fulfilled criterion B in the challenge tests proved to be inadequately preserved after of three weeks of consumer use. We note that this is not surprising since criterion B is more lenient than criterion A. We suggest that E. Ph. should be changed to recognize only criterion A for adequately preserved products in multiple-use containers. Furthermore, challenge tests should be performed not only during the preparation of cosmetic products, but should also be used to evaluate the protection efficacy of the preservative systems following periods of use.

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