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(54) Title: USE OF RHAMNOLIPIDS FOR THE COSMETIC TREATMENT OF REACTIVE SKIN

(57) Abstract: The present invention concerns a method for the cosmetic treatment of reactive skin, comprising administering to a subject in need thereof an effective amount of at least one rhamnolipid.

Use of rhamnolipids for the cosmetic treatment of reactive skin

The present invention concerns methods for the cosmetic treatment of reactive skin.

Generally, reactive skin is defined by a particular reactivity of the skin. However, as opposed to skin described as allergic, this reactivity is not due to an immunological process, that is to say it does not only occur in a skin already sensitized, in response to the presence of an allergen. Its mechanism is named non-specific.

This skin reactivity is generally reflected by the manifestation of signs of discomfort in response to a contact of the subject with a triggering element which may have various origins. It may be a cold or a hot application or the application of a product at the surface of the reactive skin (for example an acidic product), exposure to sudden temperature changes, etc. There are also associated factors such as age and skin type.

The appearance of these signs of discomfort, which appear in the minutes following the contact with the triggering element, is one of the essential features of reactive skin. These signs of discomfort are mainly dysaesthetic sensations, *i.e.* more or less unpleasant sensations experienced in a cutaneous area, such as stinging, tingling, itching, heating, discomfort, tightness, etc. These subjective signs can exist most in the absence of visible signs such as peeling. It is now known that these cutaneous reactions are related in particular to a release of neuropeptides from the nerve endings of the epidermis and dermis. Additionally, reactive skins are characterized by an abnormally high secretion of proinflammatory molecules which induce mastocytes degranulation.

However, fully satisfactory solutions to treat this type of skin described as reactive are still missing.

The present inventors surprisingly demonstrated that particular rhamnolipids could attenuate skin reactivity. Indeed, they showed that the application of particular rhamnolipids on a skin model stimulated by capsaicin, used as a model of reactive skin, significantly decreased dermal mastocytes degranulation further to capsaicin stimulation.

Rhamnolipids are biosurfactants secreted from *Pseudomonas aeruginosa* which provide great antibacterial and antifungal activity, which makes them an attractive alternative to chemical surfactants. They have been further proposed in the medical field to combat certain types of bacteria, viruses and fungi. However, to the inventor's knowledge, no effect of these particular rhamnolipids on dermal mastocytes degranulation, and thereby on skin reactivity, has been disclosed so far.

The present invention thus concerns a method for the cosmetic treatment of reactive skin, comprising administering to a subject in need thereof an effective amount of at least one rhamnolipid.

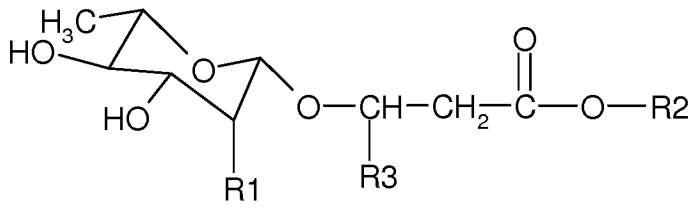
As used herein, the term “reactive skin” refers to an intolerant skin which reacts by sensations of heating, tightness and/or tingling, to various factors such as a cold or hot application or the application of cosmetic or dermatological products or soap. As indicated above, a reactive skin does not refer to an allergic skin since its reactivity only manifests by dysaesthetic sensations.

In a particular embodiment, the method for the cosmetic treatment of reactive skin according to the invention is for preventing and/or attenuating sensations of heating, tightness and/or tingling of the skin.

In the context of the invention, the term “skin” means any cutaneous surface of the body, preferentially the skin of the face.

As used herein, the term “treating” or “treatment” refers to any action that aims to improve the comfort or the well-being of an individual. This term therefore covers preventing, attenuating, relieving or suppressing the symptoms of reactive skin, *i.e.* dysaesthetic sensations, but is limited to a cosmetic treatment.

In a particular embodiment, the at least one rhamnolipid is at least one rhamnolipid of formula (I)



(I)

wherein R_1 is selected from the group consisting of H and α-L-rhamnopyranosyl,

R_2 is selected from the group consisting of H, a C_1-C_6 linear or branched alkyl, -CH(R_4)-CH_2-COOH and CH(R_4)-CH_2-COOR_5,

R_3 is –$(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6,

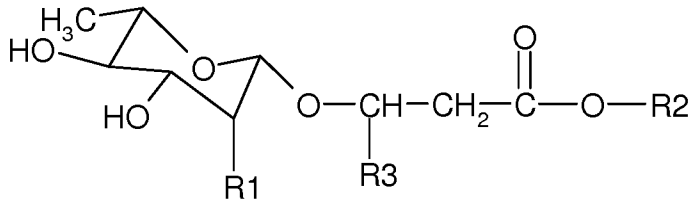
R_4 is –$(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8, and

R_5 is a C_1-C_6 linear or branched alkyl, preferably –CH_3.

In a preferred embodiment, the at least one rhamnolipid is a mixture of rhamnolipids, in particular a mixture of rhamnolipids of formula (I) as defined above. The

mixture of rhamnolipids may in particular comprise a mono-rhamnolipid, a di-rhamnolipid or a combination of both

In a particular embodiment the at least one rhamnolipid is a mono-rhamnolipid of formula (I):



(I)

wherein R_1 is H,

R_2 is selected from the group consisting of H, a C_1-C_6 linear or branched alkyl, -$CH(R_4)$-CH_2-COOH and $CH(R_4)$-CH_2-$COOR_5$,

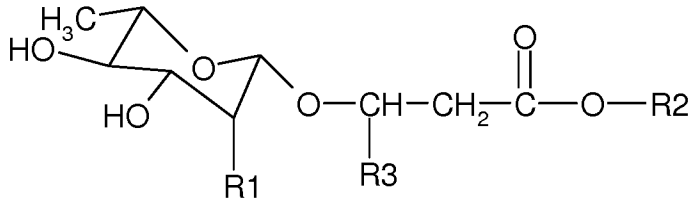
R_3 is $–(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6,

R_4 is $–(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8, and

R_5 is a C_1-C_6 linear or branched alkyl, preferably $–CH_3$,

or their salts, isomers or solvates.

In a particularly preferred embodiment, the at least one rhamnolipid is a mono-rhamnolipid of formula (I)



(I)

wherein R_1 is H,

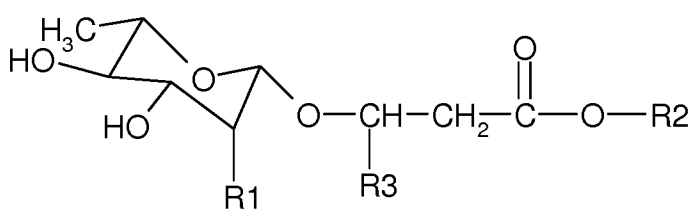
R_2 is -$CH(R_4)$-CH_2-COOH,

R_3 is $–(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6, and

R_4 is $–(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8,

or their salts, isomers or solvates.

In another particular embodiment the at least one rhamnolipid is a di-rhamnolipid of formula (I)



(I)

wherein R_1 is α-L-rhamnopyranosyl,

R_2 is selected from the group consisting of H, a C_1-C_6 linear or branched alkyl, -CH(R_4)-CH_2-COOH and CH(R_4)-CH_2-COOR_5,

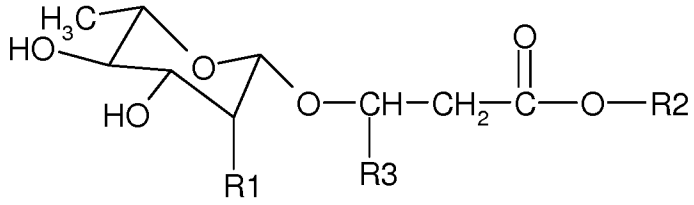
R_3 is –$(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6,

R_4 is –$(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8, and

R_5 is a C_1-C_6 linear or branched alkyl, preferably –CH_3,

or their salts, isomers or solvates.

In a particularly preferred embodiment, the at least one rhamnolipid is a di-rhamnolipid of formula (I)



(I)

wherein R_1 is α-L-rhamnopyranosyl,

R_2 is -CH(R_4)-CH_2-COOH,

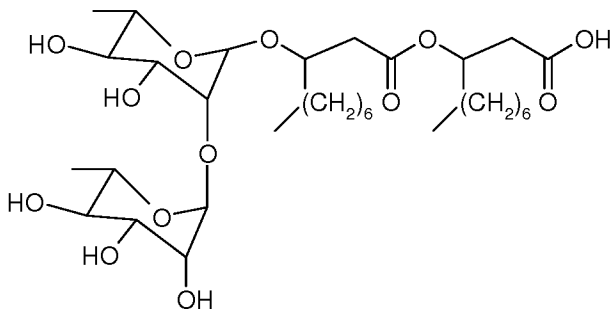
R_3 is –$(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6, and

R_4 is –$(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8,

or their salt, isomers or solvates.

In a particularly preferred embodiment, the at least one rhamnolipid is a mixture of at least one mono-rhamnolipid as defined above and at least one di-rhamnolipid as defined above.

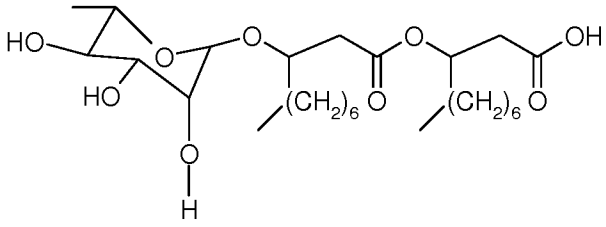
In another embodiment, said at least one rhamnolipid is a mixture comprising at least one di-rhamnolipid of formula (II)



(II), called DiC10C10,

or its salts, isomers or solvates,

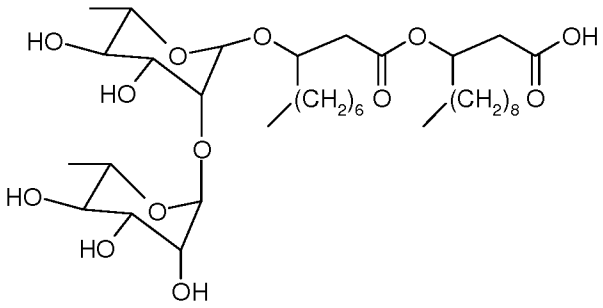
and at least one mono-rhamnolipid of formula (III)



(III), called MonoC10C10,

or its salts, isomers or solvates.

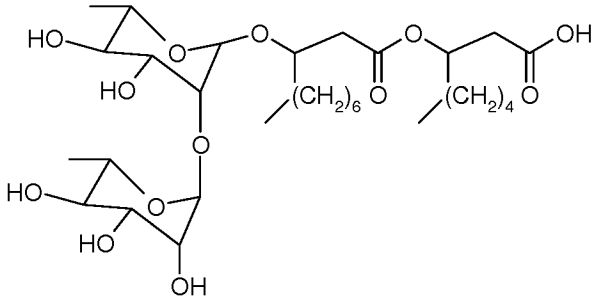
In another particular embodiment, said at least one rhamnolipid is a mixture comprising MonoC10C10 or its salts, isomers or solvates, DiC10C10 or its salts, isomers or solvates, the di-rhamnolipid of formula (IV)



(IV), called DiC10C12,

or its salts, isomers or solvates,

and the di-rhamnolipid of formula (V)



(V), called DiC10C8,

or its salts, isomers or solvates.

In a particularly preferred embodiment, said at least one rhamnolipid is a mixture exclusively comprising, as rhamnolipids, MonoC10C10, DiC10C10, DiC10C12 and DiC10C8, or their salts, isomers or solvates.

In a particular embodiment, said mixture comprises:

- from 30% to 40% by weight of MonoC10C10, or its salts, isomers or solvates, in particular from 31% to 39%, from 32% to 38%, from 33% to 37%, from 34% to 36%, or from 35% to 35.5% by weight of MonoC10C10;

- from 45% to 55% by weight of DiC10C10, or its salts, isomers or solvates, in particular from 46% to 54%, from 47% to 53%, from 48% to 52%, from 49% to 51% or from 50% to 51% by weight of DiC10C10;

- from 5% to 15% by weight of DiC10C12, or its salts, isomers or solvates, in particular from 6% to 14%, from 7% to 13%, from 8% to 12%, from 9% to 11% or from 9.5% to 10% by weight of DiC10C12; and

- from 0.5% to 10% by weight of DiC10C8, or its salts, isomers or solvates, in particular from 1% to 9%, from 2% to 8%, from 3% to 7%, from 3.5% to 6% or from 4% to 5% by weight of DiC10C8;

wherein the percentage by weight refers to the weight of said rhamnolipid calculated on the acidic form (COOH) compared to the total weight of rhamnolipids in the mixture calculated on the acidic form (COOH).

In a particularly preferred embodiment, said mixture comprises:

- from 34% to 36% by weight of MonoC10C10;
- from 48% to 52% by weight of DiC10C10;
- from 9% to 11% by weight of DiC10C12; and
- from 3% to 7% by weight of DiC10C8;

wherein the percentage by weight refers to the weight of said rhamnolipid calculated on the acidic form (COOH) compared to the total weight of rhamnolipids in the mixture calculated on the acidic form (COOH).

The carboxylic function of rhamnolipids described above can be present as carboxylate salt with an organic or inorganic cation.
The cation is preferably chosen among, sodium, potassium, calcium and ammonium.

As the cation provides electroneutrality to the molecule, it is understood that, when the cation comprises several cationic charges, then the same cation can provide electroneutrality for several anionic groups such as carboxylates in a same molecule or can provide electroneutrality for several molecules.

The rhamnolipids used in the context of the invention may be used as mixt or highly purified rhamnolipids. A mixt rhamnolipid is a rhamnolipid, having other components as Glycerid residues and/or a variety of various rhamnolipid mixtures. Highly purified rhamnolipid is a rhamnolipid whose external impurities have been removed and/or wherein the rhamnolipid have been purified from the other various rhamnolipids present in the crude form.

As well-known from the skilled person, rhamnolipids are secreted by bacteria. Bacteria capable of synthesizing the rhamnolipids used in the context of the present invention can be isolated from oil environnements, which have been found to contain

bacteria which produce rhamnolipids when the bacteria are grown on either a soluble carbon nutrient source (glucose) or an insoluble carbon nutrient source (glycerol, gas oil). In particular, the rhamnolipids used in the context of the invention may be produced by *Pseudomonas aeruginosa*.

Accordingly, in a particular embodiment, said at least one rhamnolipid is included in or in the form of a bacterial extract, *i.e.* is in crude form. Preferably, said at least one rhamnolipid is included in or in the form of a *Pseudomonas aeruginosa* bacterial extract.

In the context of the invention, the term "bacterial extract" refers both to a set of compounds produced and secreted by a bacterium, thus present in the culture medium of the bacterial culture (and therefore in the culture supernatant of the bacterial culture after centrifugation), and a set of compounds comprised in the bacterium, thus present in the bacterial pellet of the bacterial culture after centrifugation. Preferably, the bacterial extract refers to the culture medium of the bacterial culture, including compounds secreted by the bacterium.

The inventors demonstrated that the rhamnolipids produced by the *Pseudomonas aeruginosa* strain PA1, disclosed in Santa Anna *et al.* (2002) *Braz. J. Chem. Eng.* **19**:159-166, were particularly efficient in the treatment of reactive skin.

Accordingly, in a particular embodiment, said at least one rhamnolipid is included in or in the form of a bacterial extract from *Pseudomonas aeruginosa* strain PA1.

The rhamnolipids used in the context of the invention may be prepared by any conventional method well-known from the skilled person. The bacterial extract containing the rhamnolipids used in the context of the invention may in particular be obtained from a culture of at least one strain of *Pseudomonas aeruginosa*, preferably from the *Pseudomonas aeruginosa* strain PA1.

Conventional methods to prepare rhamnolipids from producing bacteria, in particular to prepare bacterial extracts comprising rhamnolipids, are well-known from the skilled person and typically comprise fermentation, isolation and purification as described in U.S patent 5,455,232 and US 5,466,675, in Santa Anna *et al.* (2002) *Braz. J. Chem. Eng.* **19**:159-166 or in Dos Santos *et al.* (2016) *PeerJ* **4**:e2078.

Typically, the bacterium *Pseudomonas aeruginosa* is cultured in a suitable medium and grown to a desired density. Preferably, the bacterial themselves are removed from the culture media by any method known in the art, such as centrifugation. The supernatant may then be used directly as the crude preparation, or further processing steps well-known from the skilled person may be carried out such as concentration, dehydration, filtration, purification, column chromatography, and the like. Preferably, the final rhamnolipid preparation is not highly purified and corresponds to a crude preparation,

preferably comprising a mixture of mono-rhamnolipids and di-rhamnolipids as defined above.

In a particular embodiment, the bacterial extract containing the rhamnolipids used in the context of the invention is obtained by fermenting at least one strain of *Pseudomonas aeruginosa*, preferably the strain *Pseudomonas aeruginosa* PA1, in a culture medium, separating the supernatant comprising the at least one rhamnolipid from the bacterial cells, and optionally at least partially dehydrating, sterilizing, purifying, grinding and/or acylating the bacterial extract thus obtained.

Preferably, the process of preparation of the rhamnolipids used in the context of the invention is carried out so that the rhamnolipids represent 40% to 65% by weight of the total dry matter weight of the bacterial extract.

Accordingly, in a particular embodiment, the at least one rhamnolipid represents 40% to 65% by weight of the total dry matter weight of the bacterial extract, preferably 45% to 60% by weight of the total dry matter weight of the bacterial extract.

As used herein, the term “effective amount” refers to the amount of the rhamnolipid, that, as a whole, enables treating reactive skin.

According to the present invention, the process is a non-therapeutic process.

Preferably, the at least one rhamnolipid used in the context of the invention is contained in a cosmetic composition further comprising a cosmetically acceptable carrier.

By “cosmetically acceptable carrier” is meant herein a non-toxic carrier which can be applied to the skin.

Preferably, the at least one rhamnolipid is comprised in the cosmetic composition at a concentration of 0.0001% to 30% by weight compared to the total weight of the composition, in particular at a concentration of 0.01% to 15% by weight, more particularly of 0.1% to 10% by weight compared to the total weight of the composition.

The present invention also concerns a composition, in particular a cosmetic composition, comprising at least one rhamnolipid as defined above.

In a particular embodiment, said composition comprises a mixture comprising or exclusively comprising, as rhamnolipids, MonoC10C10, DiC10C10, DiC10C12 and DiC10C8, as defined above, or their salts, isomers or solvates.

In a particular embodiment, said mixture comprises:

- from 30% to 40% by weight of MonoC10C10, or its salts, isomers or solvates, in particular from 31% to 39%, from 32% to 38%, from 33% to 37%, from 34% to 36%, or from 35% to 35.5% by weight of MonoC10C10;

- from 45% to 55% by weight of DiC10C10, or its salts, isomers or solvates, in particular from 46% to 54%, from 47% to 53%, from 48% to 52%, from 49% to 51% or from 50% to 51% by weight of DiC10C10;

- from 5% to 15% by weight of DiC10C12, or its salts, isomers or solvates, in particular from 6% to 14%, from 7% to 13%, from 8% to 12%, from 9% to 11% or from 9.5% to 10% by weight of DiC10C12; and

- from 0.5% to 10% by weight of DiC10C8, or its salts, isomers or solvates, in particular from 1% to 9%, from 2% to 8%, from 3% to 7%, from 3.5% to 6% or from 4% to 5% by weight of DiC10C8;

wherein the percentage by weight refers to the weight of said rhamnolipid calculated on the acidic form (COOH) compared to the total weight of rhamnolipids in the mixture calculated on the acidic form (COOH).

In a particularly preferred embodiment, said mixture comprises:

- from 34% to 36% by weight of MonoC10C10;
- from 48% to 52% by weight of DiC10C10;
- from 9% to 11% by weight of DiC10C12; and
- from 3% to 7% by weight of DiC10C8;

wherein the percentage by weight refers to the weight of said rhamnolipid calculated on the acidic form (COOH) compared to the total weight of rhamnolipids in the mixture calculated on the acidic form (COOH).

In another particular embodiment, said composition comprises at least one rhamnolipid, in particular a mixture of rhamnolipids as defined above, included in or in the form of a *Pseudomonas aeruginosa* bacterial extract, as defined above.

The composition of the invention may be in any galenic form normally used in the cosmetic field. In particular, the composition may be in the form of a powder, *i.e.* in dry form, or in the form of a suspension, an emulsion or a solution.

The cosmetically acceptable carrier may be of diverse nature depending on the type of composition considered.

In particular, in the case of compositions intended for topical application, the composition may be in the form of an aqueous solution, an aqueous-alcoholic or oily solution, a lotion- or serum-type dispersion, emulsions of liquid or semi-liquid consistency of the milk type, a cream-type suspension or emulsion, an anhydrous or aqueous gel, microemulsions, microcapsules, microparticles, or vesicular dispersions of ionic and/or nonionic type.

These compositions can be formulated according to the usual techniques.

These compositions in particular constitute a cleansing, protective or care cream for the face, for the hands, for the feet, for the major anatomical folds or for the body (for example day creams, night creams, makeup-removing creams, foundation creams), makeup products such as fluid foundations, makeup-removing milks, protective or care body milks, aftersun milks, skincare lotions, gels or mousses, as cleansing lotions, bath compositions, deodorizing compositions containing a bactericidal agent, aftershave lotions or gels or hair-removing creams.

These compositions can also be formulated as a solid preparation constituting a cleansing bar or a soap.

When the composition is an emulsion, the proportion of the fatty phase may range from 5% to 80% by weight and preferably from 8% to 50% by weight relative to the total weight of the composition. The oils, emulsifiers and coemulsifiers may be present in a proportion ranging from 0.3% to 30% by weight and preferably from 0.5% to 20% by weight relative to the total weight of the composition.

When the composition is an oily solution or gel, the fatty phase may represent more than 90% of the total weight of the composition.

As well-known from the skilled person, the formulations for topical application can also contain adjuvants that are common in the cosmetic field, such as hydrophilic or lipophilic gelling agents, hydrophilic or lipophilic active agents, preservatives, antioxidants, solvents, fragrances, bactericides, odor adsorbing agents, dyestuffs and colorants. The amounts of these various adjuvants are those conventionally used in the particular field, and, for example, range from 0.01% to 20% of the total weight of the composition. Depending on their nature, these adjuvants can be introduced into the fatty phase and/or into the aqueous phase.

As an example, fatty materials that can be used according to the invention include mineral oils such as hydrogenated polyisobutene and vaseline oil, plant oils such as a liquid fraction of karite butter, sunflower oil and apricot seed oil, animal oils such as perhydrosqualene, synthetic oils such as purcellin oil, isopropyl myristate and hexyl ethyl palmitate, unsaturated fatty acids, and fluoro oils such as perfluoropolyethers. Fatty alcohols and fatty acids such as stearic acid can also be used. Waxes such as paraffin, carnauba and beeswax can further be used. It is also possible to use silicone oils such as cyclomethicone and dimethicone, and siliconed waxes, resins and gums.

As an example, emulsifiers usable in the compositions of the invention include glyceryl stearate, polysorbate 60, the mixture cetylstearyl alcohol/cetylstearyl alcohol oxyethylenated with 33 moles of ethylene oxide marketed under the trademark Sinnowax AO® by Henkel, the mixture PEG-6/PEG-32/glycol stearate marketed under the trademark

Tefose®63 by Gattefosse, PPG-3 myristyl ether, siliconed emulsifiers such as cetyldimethicone copolyol and sorbitan mono- or tristearate, PEG-40 stearate and oxyethylenated sorbitan monostearate (20OE).

Representative solvents which can be used include the lower alcohols, in particular ethanol and isopropanol, propylene glycol.

As an example, hydrophilic gelling agents include carboxylic polymers such as carbomer, acrylic copolymers such as acrylate/alkylacrylate copolymers, polyacrylamides in particular the mixture of polyacrylamide, C13-14-isoparaffin and Laureth-7 marketed under the trademark Sepigel 305® by Seppic, polysaccharides such a cellulose derivatives including hydroxyalkylcelluloses, in particular hydroxypropylcellulose and hydroxyethylcellulose, natural gums such as guar, carob and xanthan, and clays.

As an example, lipophilic gelling agents include modified clays such as bentones, metal salts of fatty acids such as aluminum stearates, and hydrophobic silica, or still ethylcellulose and polyethylene.

Of course, the compositions of the invention can additionally contain several other compounds.

In a preferred embodiment, the compositions used in the context of the invention are for topical administration.

Preferably, in the methods of the invention, the at least one rhamnolipid is administered topically. Preferably, the at least one rhamnolipid is applied on the face, the hands, the feet, the major anatomical folds or the body, more preferably on the face.

The method of the invention may comprise a single administration. In another embodiment, the administration is repeated for example 2 to 3 times daily for a day or more and generally for an extended period of at least 4 weeks or 4 to 15 weeks, or as much as necessary with possible break if needed.

In the whole description, the expression « comprising a » or « containing a » means « comprising at least one » or « containing at least one », unless otherwise specified.

In the description and in the following examples, unless stated otherwise, the percentages are percentages by weight and the ranges of values written as "between ... and ..." include the upper and lower limits specified. The ingredients are mixed, before their shaping, in order and under conditions easily determined by the skilled person.

The present invention will be further illustrated by the examples below.

Examples

Example 1: Production of rhamnolipids from *Pseudomonas aeruginosa* strain PA1.

This example describes the production of the rhamnolipids used in Example 2.

1. Microorganism maintenance and preinoculum preparation

The PA1 strain of *Pseudomonas aeruginosa*, disclosed in Santa Anna *et al.* (2002) *Braz. J. Chem. Eng.* **19**:159-166, was preserved in 10 % glycerol in an ultrafreezer at -80°C. The preinoculum was grown on a plate with YPDA (yeast extract 0.3%, peptone 1.5%, dextrose 0.1%, agar 1.2%) at 30°C for 48 hours and was transferred to 1000 ml flasks with 300 ml of medium of the following composition (g/l): $NaNO_3$ 1.0, KH_2PO_4 3.0, K_2HPO_4 7.0, $MgSO_4.7H_2O$ 0.2, yeast extract 5.0, peptone 5.0 and glycerol P.A. 30.0. After 24 hours of cultivation, the fermentation medium containing the cells was stored in cryotubes with a glycerol/fermentation medium ratio of 1:3 to act as standard preinoculum in all fermentations.

2. Preinoculum preparation

1.0 ml of the contents of a cryotube was inoculated into 300 ml of fermentation medium of the following composition (g/l): glycerol 30.0, $NaNO_3$ 1.0, K_2HPO_4 7.0, KH_2PO_4 3.0, $MgSO_4.7H_2O$ 0.2, yeast extract 5.0 and peptone 5.0. The flasks were then incubated on rotary agitators at 30°C and 170 rpm for 40 hours. At the end of this period the cells from each flask were recovered by centrifuging (5000 g for 20 minutes) and utilised as inoculum in the bioreactors.

3. Sterilisation

The fermenter, containing the culture medium to be utilised, was autoclave-sterilized at 121°C for 15 minutes prior to each production run. The oxygenation system was sterilised by circulating a 1.0 % solution of sodium hypochlorite for 1 hour. Following this procedure, sterile distilled water was circulated through the system to eliminate traces of chlorine. Only then was inoculation of the microorganisms carried out.

4. Production of rhamnolipids by Pseudomonas aeruginosa PA1

The culture medium utilised in the fermentations had the following composition (g/l): glycerol 30.0, $NaNO_3$ 1. in 4, K_2HPO_4 7.0, KH_2PO_4 3.0 and $MgSO_4.7H_2O$ 0.2, there resulting a carbon/nitrogen ratio of 60.

The principal fermentations were carried out in a BioFlo IIc bioreactor (Batch/Continuous Fermenter, New Brunswick Scientific, USA) of 5.0 litres nominal capacity located in a fume cabinet with extraction. The average working volume utilised in the fermentations was 3.0 litres. The temperature was maintained at 30°C with agitation at 100 rpm. Oxygenation was carried out non-dispersively by means of a gas/liquid contactor.
Oxygenation was carried out with compressed air or employing a cylinder of pure oxygen. Oxygenation conditions were initially defined in accordance with the results of the oxygenation tests with the module utilised.

5. Sterilization and cell removal

After the fermentation (7-9 days, in simple batches), the suspension was sterilized in an autoclave at 121°C for 15 min. The bacterial cells were removed by centrifugation, at 10,000 rpm for 20 minutes.

6. Lyophilization

The solution containing the biosurfactants was then concentrated. Water was removed by lyophilization.

7. Rhamnolipid quantification and analytical data of the samples

The rhamnolipid quantification was carried out in an indirect way, using rhamnose as a reference – rhamnose is a product of the acid hydrolysis of the rhamnolipids. A method adapted from the one described by Pham *et al.* (2004) *Microbiology* **150**:3405-3413, was used - the extraction step was suppressed.
The biosurfactant solution produced by this strain of *Pseudomonas aeruginosa* was also characterized by HPLC. A solution of H_2SO_4 was added to the biosurfactant solution until pH 2.0 was achieved, necessary for the precipitation of the rhamnolipids. The precipitated material was then dissolved in a chloroform:ethanol (2:1) solution and filtered. After the evaporation of the solvent, the purified rhamnolipids were derivatized and analyzed in the HPLC, using the patterns reported by Mata-Sandoval *et al.* (1999) *Journal of Chromatography* **864**:211-220 as a reference.

The average composition of a rhamnolipid type biosurfactant solution produced as shown above is shown in Table 1.

Table 1: Composition of a rhamnolipid solution

Rhamnolipid type	Molar mass (g/mol)	Mass concentration (%)
MonoC10C10	492	35.3
DiC10C10	638	51.0
DiC10C12	666	9.8
DiC10C8	610	3.9

The lyophilized extract obtained as shown above displayed the following specification:
Rhamnolipid content: 50-65%
Glycerol residue: 4-10%
Protein residue: 3-10%
Ashes: 15%

Example 2: Effect of rhamnolipids on dermal mastocytes degranulation

This example demonstrates the effect of rhamnolipids on dermal mastocytes degranulation induced in a model of reactive skin.

Degranulation of the mastocyte is a manifestation which is observed in reactive skin after contact with a particular stimulus which will leads to this discomfort.

Capsaicin can be used as discomfort inducer. Indeed, in reactive skins, it is observed, after capsaicin application, burning sensations, pulling sensations, stinging sensations, to different stimuli such as application of cosmetic or dermatologic products or soap, cold or hot application.

This example demonstrates the modulation of dermal mastocytes degranulation, induced by rhamnolipids in a human skin model maintained in survival after capsaicin stimulation.

Material and methods

1. Skin preparation

8 human skins from different donors were obtained from women (aged 30 to 55 years) after plastic surgery of the scalp and maintained in survival *ex vivo*.

Skin fragments were put in inserts which were in suspension above a culture well. Culture medium (DMEM including antibiotics and FCS) was added in the well, a passage occurring by slow diffusion between the two compartments through a porous membrane (3 µm). 5 hour-equilibration was carried out before the beginning of the experiment.

2. Experimental model of mastocyte degranulation using capsaicin and application of the 4 products

After the 5-hour equilibration, the experimental mastocyte degranulation model was performed by adding 10 µM of capsaicin in the culture medium. Simultaneously, a product comprising the rhamnolipid mixture obtained in Example 1 was tested at 2 concentrations. Skin fragments were maintained in organ culture for 24 hours in a humid atmosphere chamber at 37°C and 5% CO_2.

A comparative study was then carried out:

- Control skin (basal condition: non-stimulated, non-treated skin)
- Skin stimulated with capsaicin at 10 µM (inflammatory condition)
- Skin stimulated with capsaicin and treated with the rhamnolipid mixture of Example 1 at 0.5% and 0.1%.

3. Analysis

- Histologic evaluation of mastocytes degranulation

Mastocytes which are present in the dermis are revealed in blue-violet by staining with toluidine blue. Histologically, it is observed a granular and more or less intense blue-violet aspect of mastocytes, linked to the more or less important presence of basophile and metachromatic granulations in their cytoplasm, said granulation notably comprising histamine.

Mastocytes were then counted using an optical microscope (15 fields at magnification x40) and classified according to the three following scores:

- score 2: strongly basophile cells with the presence of an intense blue-violet coloration covering the whole cytoplasm or preferentially located at one pole of the cell (mastocytes including a high number of basophile granulations),
- score 1: degranulated mastocytes with disappearance of the blue-violet coloration of the cytoplasm or persistence of a few rare grains sparsely located in the cytoplasm (mastocytes including a low number of basophile granulations).

The degranulation obtained after capsaicin application is responsible of a decrease in or a negativity of the toluidine blue coloration of mastocytes. This decrease in coloration is associated with a decrease or an almost complete disappearance of the number of granulations initially present in the mastocytes cytoplasm.

The results are expressed as followed: for each subject, the percentage of mastocytes from each score to the total number of mastocytes is calculated.

- Statistical analysis

A mean was calculated from the results obtained on the 8 skins. The statistical analysis was performed with the Student test with a risk α of 5%.

Results

Histological evaluation of mastocytes degranulation

The results concerning the evaluation of mastocytes degranulation are shown in Tables 2 and 3 (mean±standard error).

Table 2: Histologic evaluation of mastocytes of score 1 after toluidine blue staining (degranulated mastocytes)

	Percentage of Score 1
Control skin	**8.5** ± 6.6
Skin + Capsaicin	**38.3** ± 21.2 * p = 0.002
Skin + Capsaicin + Rhamnolipids at 0.5%	**16.7** ± 10.5
Skin + Capsaicin + Rhamnolipids at 0.1%	**17.6** ± 10.3

* : statistically significantly different from the control skin (Student's test, $p < 0.05$)

: statistically significantly different from the Capsaicin condition (Student's test, $p < 0.05$)

Rhamnolipids at 0,5% means 0,5% of rhamnolipids from *Pseudomonas aeruginosa* strain PA1 described in example 1.

Rhamnolipids at 0,1% means 0,1% of rhamnolipids from *Pseudomonas aeruginosa* strain PA1 described in example 1.

Table 3: Histologic evaluation of mastocytes of score 2 after toluidine blue staining

	Percentage of Score 3
Control skin	**25** ± 14.1
Skin + Capsaicin	**12.9** ± 9.6 * p = 0.0004
Skin + Capsaicin + Rhamnolipids at 0.5%	**15.4** ± 8.1
Skin + Capsaicin + Rhamnolipids at 0.1%	**15.6** ± 10.2

* : statistically significantly different from the control skin (Student's test, $p < 0.05$)

: statistically significantly different from the Capsaicin condition (Student's test, $p < 0.05$)

In the model of skin maintained in survival and stimulated with capsaicin (n = 8), a statistically significant decrease of the percentage of strongly granulated mastocytes was observed compared to the control skin: 12.9% *vs.* 25% (p = 0.0004). Furthermore, an increase in the percentage of cells of score 1 (degranulated mastocytes) was also

observed: 38.3% *v.s.* 8.5% (p = 0.002). Capsaicin thus indeed induces mastocytes degranulation.

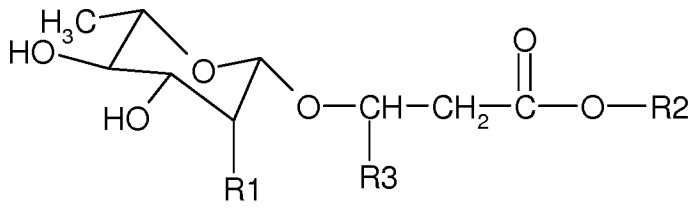
The application of rhamnolipids at 0.5% enables significantly decreasing degranulation of mastocytes compared to the skin stimulated with capsaicin, with an increase of the percentage of partially degranulated mastocytes (score 2), with respectively 67.8% for the skin treated with rhamnolipids at 0.5% *v.s.* 52.2% for the skin stimulated with capsaicin (p = 0.03, n = 6).

These results thus demonstrate that, in a model of human skin maintained in survival, an effect of rhamnolipids, used at 0.5% and 0.1%, was observed on mastocyte degranulation, a characteristic of reactive skin.

CLAIMS

1. Method for the cosmetic treatment of reactive skin, comprising administering to a subject in need thereof an effective amount of at least one rhamnolipid.

2. The method according to claim 1, wherein said at least one rhamnolipid is at least one rhamnolipid of formula (I)



(I)

wherein R_1 is selected from the group consisting of H and α-L-rhamnopyranosyl,

R_2 is selected from the group consisting of H, a C_1-C_6 linear or branched alkyl, -CH(R_4)-CH_2-COOH and CH(R_4)-CH_2-COOR_5,

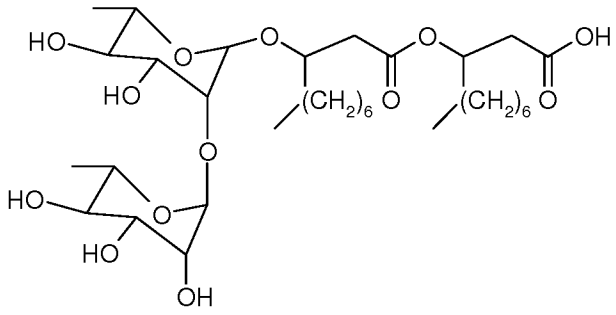
R_3 is –$(CH_2)_xCH_3$, where x is an integer from 4 to 12, preferably x is 6,

R_4 is –$(CH_2)_yCH_3$, where y is an integer from 1 to 10, preferably y is 4, 6 or 8, and

R_5 is a C_1-C_6 linear or branched alkyl, preferably –CH_3.

3. The method according to claim 1 or 2, wherein said at least one rhamnolipid is a mixture of rhamnolipids.

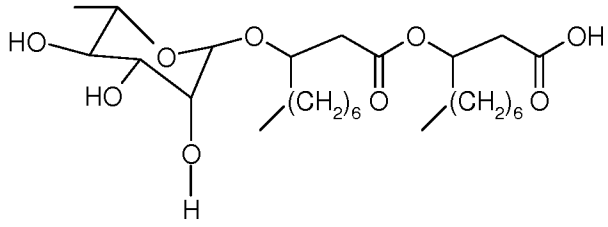
4. The method according to claim 3, wherein said at least one rhamnolipid is a mixture comprising at least one di-rhamnolipid of formula (II)



(II), called DiC10C10,

or its salts, isomers or solvates

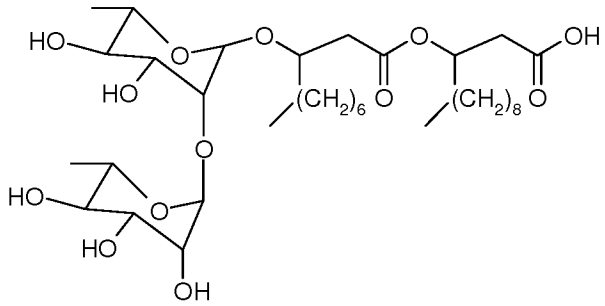
and at least one mono-rhamnolipid of formula (III)



(III), called MonoC10C10,

or its salts, isomers or solvates.

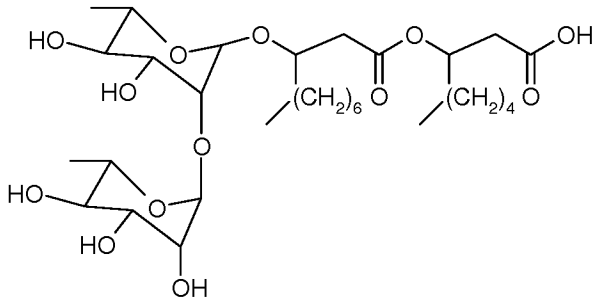
5. The method according to claim 3 or 4, wherein said at least one rhamnolipid is a mixture comprising MonoC10C10 or its salts, isomers or solvates, DiC10C10 or its salts, isomers or solvates, the di-rhamnolipid of formula (IV)



(IV), called DiC10C12,

or its salts, isomers or solvates,

and the di-rhamnolipid of formula (V)



(V), called DiC10C8,

or its salts, isomers or solvates.

6. The method according to any one of claims 2 to 5, wherein the mixture comprises:

- from 30% to 40% by weight of MonoC10C10 or its salts, isomers or solvates,
- from 45% to 55% by weight of DiC10C10 or its salts, isomers or solvates,
- from 5% to 15% by weight of DiC10C12 or its salts, isomers or solvates, and
- from 0.5% to 10% by weight of DiC10C8 or its salts, isomers or solvates,

wherein the percentage by weight refers to the weight of said rhamnolipid calculated on the acidic form (COOH) compared to the total weight of rhamnolipids in the mixture calculated on the acidic form (COOH).

7. The method according to any one of claims 1 to 6, wherein the at least one rhamnolipid is included in a bacterial extract.

8. The method according to claim 7, wherein the at least one rhamnolipid represents 40% to 65% by weight of the total dry matter weight of the bacterial extract.

9. The method according to claim 7 or 8, wherein the bacterial extract is obtained from a culture of at least one strain of *Pseudomonas aeruginosa*.

10. The method according to any one of claims 7 to 9, wherein the bacterial extract is obtained by fermenting at least one strain of *Pseudomonas aeruginosa* in a culture medium, separating the bacterial extract comprising the at least one rhamnolipid from the bacterial cells, and optionally at least partially dehydrating, sterilizing, purifying, grinding and/or acylating the bacterial extract.

11. The method according to any one of claims 1 to 10, wherein the at least one rhamnolipid is contained in a cosmetic composition further comprising a cosmetically acceptable carrier.

12. The method according to claim 11, wherein the at least one rhamnolipid is comprised in the cosmetic composition at a concentration of 0.0001% to 30% by weight compared to the total weight of the composition.

13. The method according to any one of claims 1 to 12, wherein said at least one rhamnolipid is administered topically.

14. The method according to any one of claims 1 to 13, for preventing and/or attenuating sensations of heating, tightness and/or tingling of the skin.

15. Composition comprising at least one rhamnolipid as defined in any one of claims 1 to 10.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/084115

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K8/60 A61Q19/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/056871 A2 (DESANTO KEITH [US]) 12 May 2011 (2011-05-12) page 6, lines 10-19 page 7, lines 21-28 page 18, lines 1-20 claim 5 ----- -/--	1-3,13, 15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 January 2018	15/02/2018

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Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/084115

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STIPCEVIC T ET AL: "Di-rhamnolipid from Pseudomonas aeruginosa displays differential effects on human keratinocyte and fibroblast cultures", JOURNAL OF DERMATOLOGICAL SCIENCE, ELSEVIER, AMSTERDAM, NL, vol. 40, no. 2, 1 November 2005 (2005-11-01), pages 141-143, XP027793006, ISSN: 0923-1811 [retrieved on 2005-11-01] page 141, left-hand column, lines 13-17	1,9
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Y	EP 0 153 634 A2 (WINTERSHALL AG [DE]) 4 September 1985 (1985-09-04) page 11; table 1 example 5	1-15
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1

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Information on patent family members

International application No
PCT/EP2017/084115

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