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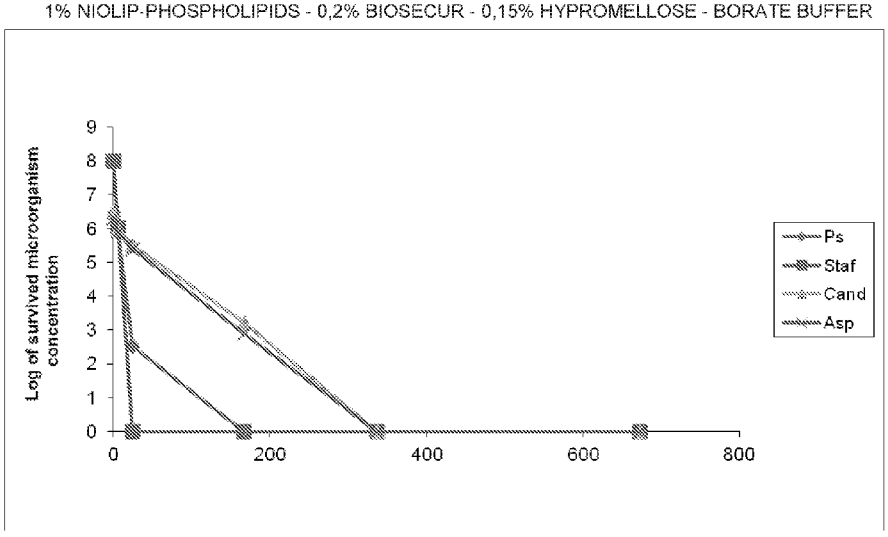

Fig. 7

(57) Abstract: An ophthalmic preparation consisting of an aqueous solution, or collyrium, containing a dispersion of BIOSECUR® liposomes of a mixture containing flavonoid- rich citrus extracts, in the form of a liposomal preparation, provides excellent antibacterial protection, in the parameters indicated by the A classification of the Pharmacopoeia XII ed. Preferably, such a collyrium, in borate isotonic buffer, is made viscous to improve its stability and mucoadhesiveness, with the addition of hydroxypropyl-methylcellulose (hypromellose). A method is also described for preparing such a solution for ophthalmic use comprising Biosecur® or a composition based on flavonoid-rich citrus extracts, in liposomal form, wherein the liposomes have a size smaller than 200 nm.

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COLLYRIUM CONTAINING CITRUS EXTRACT IN LIPOSOMAL FORM

DESCRIPTION

Summary of the invention

The present invention relates to the ophthalmic use of mixtures of natural origin, based on citrus extracts, which provide excellent antibacterial protection and are certified organic preservatives, such as the product marketed by the Canadian company BIOSECUR Lab. Inc. under the registered trademark BIOSECUR® and which is the subject of patent application US 2017/0079281, to which reference should be made for completeness. The antimicrobial compositions which are described generally comprise citrus extract as the main ingredient as well as a relatively low concentration of lauric arginate as an additive, which enhances the effect of citrus extracts.

The Biosecur product on the market is used as an agent to disinfect surfaces and for food storage.

The active ingredient of Biosecur® is represented by flavonoids extracted from the peel and the pulp of different types of citrus fruit which due to their chemical structure are poorly absorbed by cell membranes.

An object of the invention is an ophthalmic preparation consisting of an aqueous solution, or collyrium, consisting of a dispersion of liposomes containing BIOSECUR® or in any case a mixture of flavonoid-rich citrus extracts. In a preferred embodiment according to the present invention, such a collyrium, in borate isotonic buffer, is made viscous

to improve its stability and mucoadhesiveness, with the addition of hydroxypropyl-methylcellulose (hypromellose).

An object of the invention is also an industrial method for preparing a solution for ophthalmic use comprising a dispersion of liposomes containing Biosecur® or of liposomes containing a composition based on extracts of citrus fruits rich in flavonoid, which method does not damage, in its different operating steps, the liposomal structure and the active ingredients contained therein, and which allows obtaining liposomes of such a size as to make the same ophthalmic solution sterilizable by 0.2 micron filtration.

Background art

The BIOSECUR® product is characterized by the presence of a powerful citrus extract in glycerin of natural origin which, due to the strong concentration of flavonoids, provides an excellent antibacterial protection against bacteria, in particular against the Gram (-) and Gram (+) bacteria.

It is an organic preservative, certified, effective in the entire pH range, highly effective and specifically used as a disinfectant acting on surfaces, since flavonoids are not easily absorbed.

With the BIOSECUR® trademark, different formulations are marketed containing extracts of different types of citrus fruit in different concentrations, in glycerin, of natural origin, admixed with acids such as ascorbic acid, citric acid and lactic acid, but these formulations are always and only

usable as disinfectants which act on surfaces, and not really suitable for use in the ophthalmic field, due to the low capacity of the flavonoids to be absorbed by the ocular membranes.

In particular, the citrus extracts which bring bioflavonoids are: mandarin fruit extract, bitter orange fruit extract, and sweet orange peel extract.

For ease of description, in the following description reference will be made in particular to the BIOSECUR® product, but included within the scope of the invention are also similar mixtures of natural citrus extracts comprising mandarin fruit extract, bitter orange fruit extract, and sweet orange peel extract, where the possible presence of glycerin does not interfere with the antibacterial activity, but is used to make the preparation more viscous and therefore with greater permanence on the surfaces and to solubilize the flavonoids.

In literature there are several articles which highlight the characteristics of bio flavonoids:

1) as a surface cleaner;
2-3) in association with phospholipids to increase their absorption;
4) In association with propylene glycol (PG) and polyethylene glycol 400 (PEG 400) to increase their absorption and solubilization;
5) in association with Cyclodextrins to increase their absorption and their solubilization.

It is also known that collyrium is a liquid containing various medicinal substances which is applied in drops to the eye to treat eye diseases.

Collyrium may contain excipients:

to adjust the tonicity,

to adjust the viscosity of the preparation,

to adjust or stabilize the pH,

to increase the solubility of the active ingredient, and

to stabilize the preparation.

However, these substances must not affect the desired pharmacological action and give rise to local irritation.

From the technological point of view, the essential requirements of aqueous collyriums relate to:

sterility;

preservation from microbial pollution;

tonicity, and

pH.

In light of the excellent features of BIOSECUR as a disinfectant agent, the inventors have set themselves the task of developing a collyrium containing this agent, which meets all the above requirements, also to exploit the antioxidant and anti-inflammatory features of the flavonoids themselves even inside the membranes and not only at the superficial level, as is widely reported in the literature, also at the level of the ocular membranes.

The activity carried out for this purpose has led in a first stage to the development of an aqueous ophthalmic solution, with a buffer system to adjust the pH, slightly hypotonic or isotonic with an osmolality of between 260 and 310 mOsm/Kg, containing Biosecur® in a percentage concentration by weight, based on the

total weight of the composition, ranging from 0.05 to 0.2%, which was stable, provided with remarkable antibacterial activity, but which proved to be cytotoxic at in vitro toxicity tests and therefore unusable.

Following several successive experimental tests, the formulation of an aqueous ophthalmic solution was obtained, preferably in borate, isotonic buffer, in which Biosecur®, in a concentration of between 0.1% and 0.2% by weight, is in liposomal form, with phospholipids in a range of between 0.6 and 1.50% w/w, where the size of the liposomes, obtained through a high pressure extrusion as described below, result in a size smaller than 200nm. This formulation, while being less active than the previous one which included the simple presence of Biosecur®, was non-cytotoxic, allowing sterilization of the collyrium by filtration at 0.2 micron.

The partial and limited decrease in antibacterial activity is probably due to the peculiarity (polyphenols/flavonoids) of the Biosecur® product which interact with phospholipids.

This interaction does not cause precipitation but in a certain sense it modulates the antibacterial activity which remains good in the parameters indicated by the classification A Pharmacopoeia XII ed.

This modulating effect of the phospholipids is carried out also at the level of the cytotoxicity of the Biosecur® product, which loses any effect of cellular stress.

According to another aspect of the invention, in a

preferred embodiment thereof, to increase the permanence of the collyrium on the ocular surface, the liposomal formulation of Biosecur® was admixed with Hypromellose, in a percentage not higher than 0.3% w/w, as a viscosizing agent.

The choice of the Hypromellose does not derive from pure chance but from an articulated research which has involved a wide experimental activity, since the addition of other viscosizing agents of common use and widely used as the hyaluronic acid sodium salt or the cross-linked hyaluronic acid have proved completely unusable as they cause the precipitation of Biosecur®, with complete loss of its antibacterial activity.

Surprisingly however, the choice of Hypromellose, added in a percentage not higher than 0.15% w/w to the liposomal preparation containing Biosecur® at 0.2%, does not cause any precipitation, and the antibacterial activity is good, as coded by the Pharmacopoeia, always allowing the possibility of sterilizing the eye drops by 0.2 micron filtration.

The experimentation carried out allowed the development of a liposomal formulation of Biosecur® containing 0.15% w/w hypromellose, which has all the features of significantly improving the absorption of flavonoids at the level of the ocular membranes. This is due to the fact that the flavonoids, contained in Biosecur®, interacting with the phospholipids of the liposome (by complexing/aggregating) become liposoluble, and therefore spread in the eye membranes. In short, by performing their specific carrier (flavonoids are not particularly soluble in water) and

enhancer function, liposomes allow improving the absorption of flavonoids especially at the level of the ocular membrane.

It should be noted that the absorption of the bioflavonoids of which Biosecur® is made allows the latter not only to exercise the anti-bacterial activity locally in the membranes, but also to carry out other activities, more pharmacological, linked to their peculiar chemical structure, such as an antioxidant and anti-inflammatory effect, as reported widely in the literature.

Various documents are also known which refer to the use of liposomes for ophthalmic compositions comprising vegetable citrus orange extracts.

The patent document CN 103 860 625 A describes ophthalmic compositions comprising the extract of Pericarpium Citri tangerinae, which is rich in anthocyanidin, a strong antioxidant. The compositions can be in the form of liposomes. However, no specific activity or procedure is indicated to produce sterile citrus extract liposomes by 0.2 micron filtration.

WO 2017/161387 A1 describes a liposomal composition comprising an orange extract, prepared by mixing an aqueous and an organic phase containing phospholipids, evaporation of organic solvents, filtration (without any indication of the pore size of the filter used), and lyophilization of liposomes. No teaching is given to obtain liposomes so small that they are able to be filtered at 0.2 micron and then also lyophilized. The activity of obtaining nanosized liposomes does not occur spontaneously, but the action

of at least one high-energy source, such as ultrasounds or an ultra-high-pressure extruder (at least 900-1200 atm) is required. Furthermore, it is not clear how these spontaneously generated liposomes can be easily filtered and still freeze-dried, without taking into account the fact that normal liposomes when frozen to be freeze-dried break their well-organized structure, because ice crystals destroy the wall of liposomes themselves. The lack of knowledge on the lyophilization of liposomes by those who drafted the document is apparent from the fact that there is no mention of the need to add cryoprotectants to the liposomes before freezing to avoid breaking the liposomes and releasing the active ingredients contained. Finally, D2 does not reveal the presence of a buffer in combination with the liposomal formulation.

The patent document US 2010/143451 A1 describes eye gel compositions comprising an Osage orange extract which is encapsulated in a liposomal composition and then in an eye gel composition comprising polyacrylates and xanthan gum. A liposomal formulation (called EYE Gel Composition) is illustrated which, in addition to not being fully described, results in a composition which is not a collyrium: in fact, due to the presence of Carbopol, Xanthan gum and Dimethicone, as indicated, it is a gel/cream for topical use which due to the presence of phenoxyethanol and caprylyl glycol chlorphenesin (preservatives used in cosmetics) cannot be used on the eyes due to its toxicity.

Therefore, none of these documents provides a teaching for a collyrium consisting of an aqueous

solution in which there is a dispersion of liposomes based on a citrus extract, which is provided with a buffer system to adjust the pH and where the liposomes are smaller than 200 nm (normally 50-150 nm), which is the limit for filtration (sterilizing) at 0.2 microns, as prescribed by the pharmacopoeia.

Further features and advantages of the invention will become apparent from the following detailed description based on the experiments carried out which have allowed the identification of the optimal compositions for stable Biosecur® collyriums having good antibacterial activity.

In the figures:

fig. 1 is a graph illustrating the logarithmic reduction of the concentration of microorganisms which survived as a function of time for a sample of aqueous collyrium in isotonic borate buffer with Biosecur® at a concentration of 0.2% w/w;

fig. 2 is a graph like the previous one relating to a sample of collyrium with Biosecur® at 0.1% w/w;

fig. 3 is a graph like the previous one relating to a sample of collyrium with Biosecur® at 0.05% w/w.

fig. 4 is a graph like the previous one related to a sample of collyrium with Biosecur® at 0.2% w/w in liposomal form with phospholipids S80 at 1% w/w, where the acronym S80 indicates a mixture of phospholipids produced by the German company Lipoid Gmbh, of Ludwigshafen (EINECS No. 310-129-7, CAS No. 8030-76-0) and on the market under the name of LIPOID S 80, in which the concentration of phosphatidylcholine is between 73 and 79%;

fig. 5 is a graph like the previous one relating to a sample of collyrium with Biosecur® at 0.1% w/w in liposomal form with phospholipids S80 at 1% w/w;

fig. 6 is a graph like the previous one relating to a sample of collyrium with Biosecur® at 0.1% w/w in liposomal form with phospholipids S80 at 1% w/w and hypromellose at 0.15% w/w;

fig. 7 is a graph like the previous one related to a sample of collyrium with Biosecur® at 0.2% w/w in liposomal form with phospholipids S80 at 1% w/w and hypromellose at 0.15%.

Detailed description of the invention

As illustrated below, the inventors have succeeded in defining the composition and the features of an aqueous ophthalmic composition or collyrium containing Biosecur® (or similar mixtures of natural citrus extracts, including mandarin fruit extract, bitter orange fruit extract and sweet orange peel extract), in liposomal form following a long experimentation which allowed achieving the formulations as claimed in claim 1, namely:

an aqueous collyrium with buffer system to adjust the pH, preferably borate buffer, slightly hypotonic or isotonic comprised between 260 and 310 mOsm/Kg) sterile by filtration at 0.2 micron, where the Biosecur® is proposed in liposomal form, in a concentration not less than 1% by weight of non-hydrogenated phospholipids S80 and not higher than 0.2% by weight of Biosecur®, or equivalent extract of flavonoids from mandarin, bitter orange and sweet orange, with respect to the total weight of the aqueous collyrium, resorting or not to

the addition of Hypromellose to improve its stability and mucoadhesiveness, in a quantity not exceeding 0.15% by weight on the total weight, to always allow sterilization by (sterilizing) filtration of the liposomal solution without incurring the possible occlusion of the filters used.

Experimental tests

Feasibility study of a collyrium containing Biosecur®.

An aqueous collyrium sample was prepared in isotonic borate buffer, with Biosecur® at a concentration of 0.2%, 0.1% and 0.05% w/w.

The preparation of the collyrium did not involve particular difficulty. The only specific attention was to subject the aqueous collyrium with Biosecur® for ophthalmic use, to filtration at 0.2 micron to ensure sterility.

The product has shown a remarkable anti-bacterial capacity following the evaluation prescribed by the 12th edition of the Pharmacopoeia, and all the preparations were found to comply with criteria A recommended by the F.U.I. XII ed.

The microbiological results are reported for each sterile sample in Tables 1,2 and 3, shown below.

The "Challenge test" was carried out with the following strains:

Pseudomonas aeruginosa ATCC 9027,
Staphylococcus aureus ATCC 6538,
Candida albicans ATCC 10231, and
Aspergillus niger ATCC 16404.

Counts were performed after T_{6h}; T_{24h}; T_{7dd};

T_{14dd}; T_{28dd}.

In the following Table 1, the microbiological results for a sterile sample with Biosecur at a concentration of 0.2% by weight on the total weight of the solution are reported.

Sterile sample: of Niolip of Biosecur 0.2% Borate Buffer

TABLE 1

SAMPLE: Niolip 0.2% - Borate buffer

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$2x10^3$	UFC/ml	3.3
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^7$	UFC/ml	7.0
6h	$6x10^2$	UFC/ml	2.8
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$1x10^3$	UFC/ml	3.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$2x10^6$	UFC/ml	6.3
6h	$6x10^2$	UFC/ml	2.8
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

Since the liposomes used in the tests were produced by the company Niolip Srl headquartered in Pomezia, in the

tables the different samples are indicated as Niolip followed by the concentration of Biosecur by weight on the total weight of the solution.

The Logarithmic reduction of the number of microorganisms as a function of time has provided the diagram in fig. 1.

Sterile sample: Niolip Biosecur 0.1% Borate Buffer

A similar test was carried out relative to the sample at concentration 0.1, of which the microbiological results are listed in Table 2.

TABLE 2

SAMPLE: Niolip 0.1% - Borate buffer

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$1x10^4$	UFC/ml	4.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphilococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$3x10^4$	UFC/ml	4.5
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^8$	UFC/ml	6.0
6h	$3x10^3$	UFC/ml	3.5
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$2x10^6$	UFC/ml	6.3
6h	$9x10^3$	UFC/ml	3.9
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

The Logarithmic reduction of the number of microorganisms as a function of time has provided the diagram in fig. 2.

- **Sterile sample: Niolip Biosecur 0.05% Borate Buffer**

A similar test was carried out relative to the sample at concentration 0.05, of which the microbiological results are listed in Table 3.

TABLE 3

SAMPLE: Niolip 0.05% - Borate buffer

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$1x10^3$	UFC/ml	5.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^7$	UFC/ml	7.0
6h	$1x10^5$	UFC/ml	5.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$1x10^4$	UFC/ml	4.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$9x10^2$	UFC/ml	3.9
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

The Logarithmic reduction of the number of microorganisms as a function of time has provided the

diagram in fig. 3.

Improvement of the mucoadhesiveness of the collyrium.

Evaluation of the possibility of improving the mucoadhesiveness of the collyrium by making it viscous:

The possibility of viscosizing the collyrium was evaluated by preparing the following aqueous collyrium in which Biosecur is present in different percentages, using cross-linked Hyaluronic acid or Hyaluronic acid sodium salt as the viscosity agent.

The following results were obtained:

- Biosecur 0.2% Borate Buffer Cross-linked Hyaluronic Acid borate buffer: the product is not stable, it precipitates.
- Biosecur 0.2% Borate Buffer Cross Hyaluronic Acid sodium salt borate buffer: the product is not stable, it precipitates.
- Biosecur 0.1% Borate Buffer Cross-linked Hyaluronic Acid borate buffer: the product is not stable, it precipitates.
- Biosecur 0.1% Borate Buffer Cross Hyaluronic Acid sodium salt borate buffer: the product is not stable, it precipitates.

It may be concluded that:

all the products listed above cannot be used because they are unstable, and therefore they were not been evaluated from the antibacterial point of view.

We then proceeded to prepare samples of Biosecur at 0.2% w/w, adding Hypromellose as a viscosizing agent in a percentage not higher than 0.15%. Unlike the other viscosizing agents used in the previous procedure, such as Hyaluronic acid, the addition of Hypromellose does

not cause any precipitation, and the antibacterial activity is good, as codified by the Pharmacopoeia, always allowing the possibility of sterilizing the collyrium by filtration 0.2 microns.

Cytotoxicity tests

0.2% w/w samples of Biosecur admixed with 0.15% w/w Hypromellose were subjected to cell viability testing for cytotoxicity determination.

Test method:

ISO 10993-5:2009

ISO 10993-12:2012

Other references:

Cytotoxicity protocol

Method summary

The cells are grown in plates until an almost confluent monolayer is obtained. Three cell culture plates are prepared for each sample. Furthermore, three plates are prepared for the negative control, three for the positive control and three for the control of the culture medium (MEM control). In the plates to be treated with the sample, an aliquot of the test sample was placed in contact with the cellular monolayer.

Similarly, plates for negative controls and positive controls were prepared. Cell cultures are examined under a microscope after 24 and 48 hours of incubation and the possible presence of cytotoxic effects produced by the test sample is evaluated.

Cells: BSCL 56/L929 (connective tissue of mouse)

Culture medium: Minimum Essential Medium (MEM), with Earle's salts admixed with 5% fetal bovine serum, 1% L-glutamine, 0.6% penicillin/streptomycin and 0.3%

fungizone (complete MEM).

Sample preparation:

an aliquot of the sample is placed in direct contact with the cell culture.

Positive control: 70% ethanol aliquot.

Negative control: aliquot of sterile physiological solution.

Control medium: Complete MEM cell culture medium.

Incubation:

The plates treated with the sample under examination, those with the positive and negative controls and those with the culture control are incubated for 48 hours at 37 ± 1 °C.

Equipment:

CO2 thermostat (5%), maintained at 37 ± 1 °C;

Reverse phase optical microscope;

Sterile laminar flow hood;

Sterile single-use materials;

Cell culture plates.

Evaluation of the effects and interpretations of the results:

Cytotoxicity is assessed qualitatively by microscopic examination of the cells after 24 and 48 hours of incubation. The general morphology, the presence of vacuolization, detachments, cell lysis and integrity of the membranes are evaluated. The deviations from the normal morphology highlighted by the negative control are assigned a score from 0 to 4 (see scoring system). Furthermore, for the plates treated with the sample, the confluence of the monolayer is evaluated, and the color of the culture

medium is compared with that of the medium of the negative control plates.

Scoring system

The score varies from grade 0, which corresponds to no Reactivity i.e. a fair amount of intracellular granulations and no cell lysis, to grade 4 which indicates a severe Reactivity, i.e. the almost complete destruction of the cellular layer.

Results at 24 h of incubation	**Score**
Positive control	4
Positive control	4
Positive control	4
Negative control	0
Negative control	0
Negative control	0
Saline solution control	0
Saline solution control	0
Saline solution control	0
Sample	4
Sample	4
Sample	4
Confluence of the monolayer	Not confluent
Color of the culture medium	Not comparable to the medium of the negative control

Results at 48 h of incubation	**Score**
Positive control	4
Positive control	4
Positive control	4
Negative control	0
Negative control	0
Negative control	0
Saline solution control	0
Saline solution control	0
Saline solution control	0
Sample	4
Sample	4
Sample	4
Confluence of the monolayer	Not confluent
Color of the culture medium	Not comparable to the medium of the negative control

Conclusion:

The cells treated with the sample after 24 and 48 hours of incubation showed deviations from the normal morphology highlighted by the negative control. The sample under examination shows a severe reactivity.

Since hypromellose collyriums, known as 'artificial tears', are widely used in the eyes to alleviate dryness and irritation, the tests carried out show that the cytotoxicity of the samples is due to the presence of Biosecur®. Similar tests prove that all samples between 0.05% and 0.2% by weight are cytotoxic and therefore cannot be used.

IMPROVEMENT OF STABILITY AND BIO-COMPATIBILITY (ELIMINATION OF THE CYTOTOXICITY) OF THE COLLYRIUM

In order to improve Biocompatibility and eliminate especially the cytotoxicity of Biosecur collyriums given the negative results of the cytotoxicity test and in order to improve the stability of collyriums with Biosecur, given the negative results concerning Biosecur precipitation with both types of Hyaluronic acid, different formulations of Biosecur in liposomal form were prepared, respectively: 0.2% of Biosecur and 1% of Phospholipids S80 (LIPOID S80); - 0.1% of Biosecur and 1% of Phospholipids S80; - 0.05% of Biosecur and 1% of Phospholipids S80, also in the form of isotonic aqueous collyrium, made sterile by filtration at 0.2 micron.

These liposomal products have shown a good anti-bacterial capacity following the evaluation prescribed

by the 12th edition of the Pharmacopoeia, and all the preparations were found to comply with criteria A recommended by the FUI XII ed. The microbiological results are reported respectively in Tables 4 and 5, which follow.

Table 4 refers to an NIOLIP collyrium S80, consisting of liposomes with Phospholipids S80 at 1% containing Biosecur 0.2%

Sterile sample: Niolip S80 (liposomes prepared with Phospholipids S80) 1% Biosecur 0.1% Borate Buffer

TABLE 4

SAMPLE: Niolip S80 1% - Biosecur 0.2% - Borate buffer

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$6x10^8$	UFC/ml	8.8
6h	$3x10^6$	UFC/ml	6.5
24 h	$5x10^2$	UFC/ml	2.8
7d	$1,5x10^1$	UFC/ml	1.2
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$9x10^8$	UFC/ml	8.9
6h	$25x10^5$	UFC/ml	6.4
24 h	$9x10^5$	UFC/ml	5.9
7d	$2,5x10^1$	UFC/ml	1.4
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$3 x10^8$	UFC/ml	6.5
6h	$6 x10^1$	UFC/ml	1.8
24 h	$1 x10^1$	UFC/ml	1.0
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$1 x10^8$	UFC/ml	6.0
6h	$1 x10^2$	UFC/ml	2.0
24 h	1 x10	UFC/ml	1.0
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

Table 5 refers to an NIOLIP collyrium S80, with liposomes prepared with Phospholipids S80 at 1% containing Biosecur 0.1%

Sterile sample: Niolip S80 (liposomes prepared with Phospholipids S80) 1% Biosecur 0.1% Borate Buffer

TABLE 5

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$6x10^8$	UFC/ml	8.8
6h	$3x10^6$	UFC/ml	6.5
24 h	$5x10^2$	UFC/ml	2.8
7d	$1,5x10^1$	UFC/ml	1.2
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$9x10^8$	UFC/ml	8.9
6h	$25x10^5$	UFC/ml	6.4
24 h	$9x10^5$	UFC/ml	5.9
7d	$2,5x10^1$	UFC/ml	1.4
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$3 x10^8$	UFC/ml	6.5
6h	$6 x10^1$	UFC/ml	1.8
24 h	$1 x10^1$	UFC/ml	1.0
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$1 x10^8$	UFC/ml	6.0
6h	$1 x10^2$	UFC/ml	2.0
24 h	1 x10	UFC/ml	1.0
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

The partial and limited decrease of the antibacterial activity, compared to the use of the Biosecur not in liposomal form, can be ascribed perhaps to the peculiarity of the polyphenols/flavonoids of the Biosecur® product which probably interact with the phospholipids which form the liposome structure.

This interaction does not cause precipitation but in a certain sense it modulates the antibacterial activity which remains good in the parameters indicated by the classification A Pharmacopoeia XII ed.

This modulating effect of the phospholipids seems

to be carried out also at the level of the cytotoxicity of the Biosecur® product, which loses any cytotoxic type activity.

It should be pointed out that in the examples given, phospholipids S80 have always been used in which phosphatidylcholine has a concentration not higher than 80% by weight, in particular between 73 and 79

It was moreover noted that otherwise satisfactory results have been obtained with phospholipids in which the concentration of phosphatylcholine is in any case greater than 60% w/w.

Improved stability/mucoadhesiveness of Biosecur Liposomal collyrium

The possibility of improving the stability and mucoadhesiveness of collyrium containing Biosecur liposomes was evaluated by preparing the following liposomal collyrium:

- with cross-linked Hyaluronic acid, in sterile form by 0.2 micron filtration:
- 0.2% of Biosecur and 1% of Phospholipids S80, 0.1% cross-linked Hyaluronic acid
- 0.1% of Biosecur and 1% of Phospholipids S80, 0.1% cross-linked Hyaluronic acid
- 0.05% of Biosecur and 1% of Phospholipids S80, 0.1% cross-linked Hyaluronic acid
- the microbiological results are shown in tables 6 and 7 below.

Sterile sample: Niolip S80 (liposomes prepared with Phospholipids S80) 1% Biosecur 0.2%, cross-linked Hyaluronic acid 0.1%, Borate Buffer

TABLE 6

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$3x10^8$	UFC/ml	8.5
6h	$25x10^6$	UFC/ml	6.4
24 h	$1x10^6$	UFC/ml	6.0
7d	$3x10^3$	UFC/ml	1.5
14 d	$1x10^3$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$5x10^8$	UFC/ml	8.7
6h	$28x10^6$	UFC/ml	6.4
24 h	$14x10^6$	UFC/ml	6.1
7d	$2x10^3$	UFC/ml	1.3
14 d	$1x10^3$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$1x10^5$	UFC/ml	5.0
24 h	$3x10^4$	UFC/ml	4.5
7d	$3x10^2$	UFC/ml	2.5
14 d	$1x10^2$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$8x10^4$	UFC/ml	4.9
24 h	$3x10^4$	UFC/ml	4.5
7d	$1x10^3$	UFC/ml	3.0
14 d	$1x10^3$	UFC/ml	3.0
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** ; no increase

Sterile sample: Niolip S80 (liposomes prepared with Phospholipids S80) 1% Biosecur 0.1%, cross-linked Hyaluronic acid 0.1%, Borate Buffer

TABLE 7

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$1x10^8$	UFC/ml	6.0
24 h	$9x10^7$	UFC/ml	5.9
7d	$1x10^3$	UFC/ml	2.0
14 d	$1x10^1$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Staffococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^8$	UFC/ml	8.5
6h	$3x10^8$	UFC/ml	6.5
24 h	$25x10^7$	UFC/ml	6.4
7d	$3x10^1$	UFC/ml	1.5
14 d	$1x10^1$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$2x10^5$	UFC/ml	5.3
24 h	$1x10^5$	UFC/ml	5.0
7d	$1x10^3$	UFC/ml	3.0
14 d	$1x10^1$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$2x10^6$	UFC/ml	6.3
6h	$28x10^5$	UFC/ml	5.4
24 h	$12x10^5$	UFC/ml	5.1
7d	$15x10^2$	UFC/ml	3.2
14 d	$1,5x10^1$	UFC/ml	1.2
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

Sterile sample: Niolip S80 (liposomes prepared with Phospholipids S80) 1% Biosecur 0.05%, cross-linked Hyaluronic acid 0.1%, Borate Buffer

TABLE 8

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$2x10^7$	UFC/ml	6.3
24 h	$9x10^5$	UFC/ml	5.9
7d	$1x10^3$	UFC/ml	3.0
14 d	$1x10^2$	UFC/ml	2.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$3x10^6$	UFC/ml	6.5
24 h	$1x10^6$	UFC/ml	6.0
7d	$4x10^2$	UFC/ml	2.6
14 d	$1x10^1$	UFC/ml	1.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$9x10^5$	UFC/ml	5.9
24 h	$3x10^5$	UFC/ml	5.5
7d	$1x10^4$	UFC/ml	4.0
14 d	$1x10^3$	UFC/ml	3.0
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$1x10^6$	UFC/ml	6.0
6h	$9x10^5$	UFC/ml	5.9
24 h	$4x10^5$	UFC/ml	5.6
7d	$6x10^4$	UFC/ml	3.8
14 d	$6x10^4$	UFC/ml	2.8
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

- The three liposomal products viscosized with cross-linked Hyaluronic Acid tested above have shown a low anti-bacterial capacity; in fact, all the preparations were found to comply with the B criteria recommended by the F.U.I. XII ed.

COLLYRIUM with BIOSECUR feasibility study

Results

- Identification of a viscosizing mucoadhesive polymer compatible with the Biosecur Liposome collyrium

- The possibility of improving the stability and the

mucoadhesiveness of the Biosecur liposome collyrium was evaluated by adding hydroxypropyl-methylcellulose (Hypromellose) to the liposomal preparation in two different concentrations:

0.1% of Biosecur 1% of Phospholipids S80, Hypromellose 0.15%, Borate Buffer -

0.2% of Biosecur 1% of Phospholipids S80, Hypromellose 0.15%, Borate Buffer

The microbiological results are shown in the following table 9 and 10.

- **Sterile sample: Niolip S80 (with liposomes prepared with phospholipids S80) 1%, Biosecur 0.1%, Hypromellose 0.15%, Borate Buffer**

TABLE 9

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$9x10^7$	UFC/ml	7.9
6h	$2x10^6$	UFC/ml	6.3
24 h	$5x10^2$	UFC/ml	2.7
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staflococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$4x10^6$	UFC/ml	6.6
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$6x10^6$	UFC/ml	6.8
6h	$5x10^6$	UFC/ml	6.7
24 h	$4x10^6$	UFC/ml	6.6
7d	$1x10^4$	UFC/ml	4.0
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$5x10^6$	UFC/ml	6.7
6h	$3x10^6$	UFC/ml	6.5
24 h	$2x10^6$	UFC/ml	6.3
7d	$1x10^4$	UFC/ml	4.0
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

- The liposomal product containing 0.1% Biosecur® showed a weak/fair anti-bacterial capacity, the preparation was found to comply with the B criteria recommended by the F.U.I. XII. In the continuation of the experiment the product fell into the type A classification according to the Pharmacopoeia. (see fig. 6)

The liposomal product containing 0.2% Biosecur has instead shown a good anti-bacterial capacity following the evaluation prescribed by the Pharmacopoeia XII edition. All preparations comply with criteria A recommended by the F.U.I. XII ed.

TABLE 10

STRAIN: *Pseudomonas aeruginosa* ATCC 9027

			Log
Inoculum	$9x10^8$	UFC/ml	7.9
6h	$8x10^8$	UFC/ml	8.9
24 h	$3x10^2$	UFC/ml	2.5
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Staphylococcus aureus* ATCC 6538

			Log
Inoculum	$1x10^8$	UFC/ml	8.0
6h	$1x10^6$	UFC/ml	6.0
24 h	NR*	UFC/ml	NR*
7d	NR*	UFC/ml	NR*
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Candida albicans* ATCC 10231

			Log
Inoculum	$3x10^6$	UFC/ml	6.5
6h	$1x10^6$	UFC/ml	6.0
24 h	$35x10^5$	UFC/ml	5.5
7d	$15x10^2$	UFC/ml	3.2
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

STRAIN: *Aspergillus niger* ATCC 16404

			Log
Inoculum	$2x10^6$	UFC/ml	6.3
6h	$8x10^5$	UFC/ml	5.9
24 h	$25x10^4$	UFC/ml	5.4
7d	$8x10^2$	UFC/ml	2.9
14 d	NR*	UFC/ml	NR*
28 d	NR*	UFC/ml	NR*

NR* : no recovery
NI** : no increase

The challenge test was carried out on a sterile sample of Niolip with liposomes S80 1% - Biosecur ® 0.2% - Hypromellose 0.15% - Borate buffer.

Conclusions:

The liposome collyrium with the higher concentration of Biosecur® 0.2% above showed a good antibacterial capacity combined with excellent stability. There was no precipitation of the product containing the liposomes.

Therefore, the problem of the best mucoadhesiveness and stability of the product was solved by using collyrium with 1% Liposomes and 0.2% Biosecur, together with a viscosizing agent such as Hypromellose at a concentration not higher than 0.5% and preferably at 0.15% w/w.

The Logarithmic reduction of the number of microorganisms as a function of time has provided the diagram in fig. 7.

COLLYRIUM with BIOSECUR® feasibility study

Results

The experiments carried out up to now have therefore allowed:

- Identifying collyriums in liposomes containing Biosecur® at 0.2-0.1-0.05% with good antibacterial activity, free of cytotoxic activity; and
- Identifying Biosecur® collyriums in liposomes at 0.2-0.1%, viscosized with Hypromellose, without any precipitation of the preparation, unlike what happens in the presence of hyaluronic acid sodium salt or cross-linked, with good antibacterial activity and free of any cytotoxic activity.

According to another aspect, the present invention also provides a method for making an aqueous collyrium of Biosecur® or of a mixture of flavonoid-rich citrus

extracts, in the form of a liposomal preparation having antibacterial activity which provides for the steps as specified below:

Preparation of Biosecur® liposomes

1) Solubilization of Phospholipids and Biosecur® with an appropriate solvent, preferably ethyl alcohol: for the preparation of 100 liters of liposomal solution, 1 kg of SP80 phospholipids and 200 g of Biosecur® are used;

2) Ethanol drying and removal

The mixture of phospholipids and Biosecur® is dried under vacuum, using a rotavapor, at a temperature not higher than 45-50 °C.

A dry powder is obtained, where the Biosecur®, the flavonoids and the other lipophilic and hydrophilic components are in direct contact with phospholipids.

3) The dry powder is then suspended in the borate buffer (100 liters) and homogenized using a Silverson mixer: a mixture of micelles/Proliposomes is thus obtained with a very variable and large size (5-200 microns), certainly not sterilizable by 0.2 micron filtration

4) Preparation of the liposomal solution

The solution containing Proliposomes is extruded at high pressure (800 1000 atm) using a high-pressure mechanical extruder, to obtain liposomes with a size less than 200 nm; the high pressure extrusion procedure is repeated 4-6 times (100 liters x 4-6 times) as needed to obtain liposomes with a size smaller than 200 nm (limit for the 0.2 micron filterability required for

sterilization).

It should be emphasized that the forming process is not a spontaneous process but requires the application of great energy to obtain a real inclusion of the active ingredients and the desired size of the liposomes.

5) Preparation of the final collyrium

To the liposomal solution, already in buffer, sodium chloride is added, to regulate the isotonicity, and hypromellose to make the formulation viscous.

Controls: pH, isotonicity.

6) Preparation of sterile liposomal collyrium containing Biosecur®

Biosecur® is sterilized in line by filtration through a 0.2 micron filter.

Controls: pH, isotonicity, sterility, size, residual ethanol titer

Below is a summary table of the ingredients used and the role they play in a sterile collyrium containing Biosecur liposomes.

BIOSECUR LIPOSOMES WITH HYPROMELLOSE

Ingredients	%	
Phospholipids S80	1.00	Constituents liposomes, carrier- enhancer effect
Biosecur	0.20	Active ingredient
Boric acid	0.775	Buffer
Sodium tetraborate decahydrate	0.125	Buffer
Sodium chloride	0.350	Osmotic agent
Hypromellose	0.150	Viscosizing agent
pH	7-7.4	
Isotonicity mOsm/kg	275-310	
Sterile by 0.2 micron filtration	yes	
Size < 200 nm	Yes	Determination by light scattering

By way of non-limiting example, some preferred formulations of ophthalmic solutions containing Biosecur liposomes with and without Hypromellose additions are given below.

Formulation NLL-105E(ipr.):
Liposomes Biosecur 0.1%
Hypromellose 0.15%
(borate buffer)

Raw materials	%w/w	CAS number
Phospholipids S80	1,00	8030-76-0
Biosecur	***0,100***	-
Boric acid	0,775	10043-35-3
Sodium Tetraborate Decahydrate	0,060	1303-96-4
Sodium chloride	0,360	7647-14-5
Hypromellose	***0,150***	***9004-65-3***
Distilled water as needed	100	-

Sterile by filtration

Formulation NLL-105(ipr.)
Liposomes Biosecur 0.2%
Hypromellose 0.15%
(borate buffer)

Raw materials	%w/w	CAS number
Phospholipids S80	1,00	8030-76-0
Biosecur	***0,200***	-
Boric acid	0,775	10043-35-3
Sodium Tetraborate Decahydrate	0,060	1303-96-4
Sodium chloride	0,350	7647-14-5
Hypromellose	***0,150***	***9004-65-3***
Distilled water as needed	100	-

Sterile by filtration

NLL-102E: Liposomes Biosecur 0.1%
(borate buffer)

Raw materials	% w/w	CAS number
Phospholipids S80	1,00	8030-76-0
Biosecur	*0,100*	-
Boric acid	0,775	10043-35-3
Sodium Tetraborate Decahydrate	0,060	1303-96-4
Sodium chloride	0,430	7647-14-5
Distilled water as needed	100	-

Sterile by filtration

While the invention has been described on the basis of specific embodiments, it is clear that many alternatives, modifications and variations will become apparent to those skilled in the art. Accordingly, it is understood that these variations and modifications fall within the scope of the present invention as defined by the following claims.

CLAIMS

1) A solution for ophthalmic use in the form of an aqueous collyrium characterized in that it contains a dispersion of liposomes containing BiosecuR® or mixtures of natural extracts of citrus pulp and peel in the form of a liposomal preparation, with a buffer system to adjust the pH, wherein the liposome size is smaller than 200nm so that said solution is able to be sterilized by 0.2 micron filtration.

2) The solution for ophthalmic use in the form of collyrium according to the preceding claim wherein the concentration of Biosecur® in liposomes is between 0.1% and 0.2% by weight with respect to the total weight of the solution.

3) The solution for ophthalmic use in the form of collyrium according to any one of the preceding claims, characterized in that the liposomes containing Biosecur® or natural extracts of citrus pulp and peel are made of non-hydrogenated phospholipids from 0.5 to 1.5% by weight with respect to the total weight of the solution.

4) The solution for ophthalmic use in the form of collyrium according to any one of the preceding claims, wherein the phospholipids are phospholipids S80 in which the phosphatidylcholine has a concentration not higher than 80% by weight.

5) The solution for ophthalmic use in the form of collyrium according to any one of the preceding claims, wherein the phospholipids are phospholipids wherein the phosphatidylcholine has a concentration between 60 and 79% by weight.

6) The solution for ophthalmic use according to any one of the preceding claims, further comprising pH buffers based on monobasic and dibasic sodium and potassium phosphates, boric acid and sodium tetraborate, sodium or potassium bicarbonate, citric acid/citrates.

7) The solution for ophthalmic use according to any one of the preceding claims characterized in that the pH buffer is a buffering system based on boric acid and decahydrate sodium tetraborate.

8) The solution for ophthalmic use according to any one of the preceding claims characterized in that, in order to improve stability and mucoadhesiveness thereof, it is made viscous by addition of hydroxypropyl methylcellulose (hypromellose), in variable amounts not higher than 0.5% by total weight of the solution, in order to increase the time of its permanence on the ocular surface.

9) The ophthalmic solution according to any one of the preceding claims, characterized in that it is in the form of an aqueous collyrium comprising liposomes containing 0.2% Biosecur, viscosized with Hypromellose at a concentration ranging from 0.10 to 0.20% on the total weight of the solution.

10) The solution according to any one of the preceding claims characterized in that it is slightly hypotonic or isotonic, ranging from 260 to 310 mOsm/Kg.

11) The solution according to any one of the preceding claims characterized in that it has antibacterial, anti-inflammatory and antioxidant activity.

12) Liposomes containing Biosecur® or a mixture containing tangerine fruit extract, bitter orange fruit extract, sweet orange peel extract, ascorbic acid, ascorbic acid, citric acid, lactic acid and water, having a size smaller than 200 nm, for the preparation of an aqueous ophthalmic solution with an antibacterial, anti-inflammatory and antioxidant action which is able to be sterilized by 0.2 micron filtration.

13) Liposomes containing Biosecur® according to the preceding claim, in combination with Hypromellose as a viscosizing agent, for the preparation of an aqueous ophthalmic solution with an antibacterial, anti-inflammatory and antioxidant action which is able to be sterilized by 0.2 micron filtration.

14) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1, characterized in that it consists of:

phospholipids S80 0.6-1.5% w/w

Biosecur®	0.1-0.2% w/w
Boric acid	0.775%-0.8% w/w
Sodium Tetraborate Decahydrate	0.060-0.125% w/w
Sodium chloride	0.35-0.45% w/w
Distilled water	q.s.,

wherein the size of the liposomes is smaller than 200nm and said solution is able to be sterilized by 0.2 micron filtration.

15) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1,

characterized in that it consists of:

phospholipids S80 1% w/w	
Biosecur®	0.200% w/w
Boric acid	0.0775% w/w
Sodium Tetraborate Decahydrate	0.060% w/w
Sodium chloride	0.420% w/w
Distilled water	q.s. ,

wherein the size of the liposomes is smaller than 200nm and said solution is able to be sterilized by filtration.

16) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1, characterized in that it consists of:

phospholipids S80 1.00% w/w	
Biosecur®	0.100% w/w
Boric acid	0.775% w/w
Sodium Tetraborate Decahydrate	0.060% w/w
Sodium chloride	0.430% w/w
Distilled water	q.s. ,

wherein the size of the liposomes is smaller than 200nm and said solution is able to be sterilized by filtration.

17) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1, characterized in that it consists of:

phospholipids S80	1% w/w
Biosecur®	0.0050% w/w
Boric acid	0.775% w/w
Sodium Tetraborate Decahydrate	0.060% w/w
Sodium chloride	0.420% w/w
Distilled water	q.s.,

wherein the size of the liposomes is smaller than 200nm and said solution is able to be sterilized by filtration.

18) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1, characterized in that it consists of:

phospholipids S80	1% w/w
Biosecur®	0.200% w/w
Boric acid	0.775% w/w
Sodium Tetraborate Decahydrate	0.060% w/w
Sodium chloride	0.350% w/w
Hypromellose	0.150% w/w
Distilled water	q.s.,

wherein the size of the liposomes is smaller than 200nm and said solution is able to be sterilized by filtration.

19) The solution for ophthalmic use in the form of an aqueous collyrium according to claim 1, characterized in that it consists of:

phospholipids S80	1% w/w
Biosecur®	0.100% w/w
Boric acid	0.775% w/w
Sodium Tetraborate Decahydrate	0.060% w/w
Sodium chloride	0.360% w/w
Hypromellose	0.150% w/w
Distilled water	q.s.,

and is able to be sterilized by filtration.

20) A method for preparing a solution for ophthalmic use comprising a dispersion of liposomes containing Biosecur® or a composition based on citrus extracts in liposomal form, which method comprises the

following steps:

a) Solubilization of Phospholipids and Biosecur® with ethyl alcohol;

b) Drying and removal of the ethanol by vacuum drying by means of a rotavapor at a temperature not higher than 45-50 °C in order to obtain a dry powder wherein the Biosecur®, the flavonoids and the other lipophilic and hydrophilic components, are directly contacting the phospholipids;

c) Suspension of dry powder in the pH buffering system (100 liters) and homogenization by means of a Silverson mixer, to obtain a mixture of proliposomes with a very variable and large size (5-200 micron).

d) Preparation of the liposomal solution, wherein the solution containing proliposomes is extruded at high pressure (800-1000 atm) by means of a high pressure mechanical extruder, to obtain liposomes having a size smaller than 200 nm, in order to be able to sterilize said solution by filtration without damaging the liposomal structure and the active ingredients contained therein,

e) Preparation of the final collyrium, wherein to the liposomal solution sodium chloride is added, to regulate the isotonicity, and hypromellose to make the formulation viscous.

f) Preparation of the sterile liposomal collyrium containing Biosecur®, wherein the collyrium of Biosecur® liposomes is sterilized in-line by filtration through a 0.2 micron filter.

21) The method according to claim 20 characterized in that in step a) 1 kg of phospholipids are used for

the preparation of 100 liters of liposomal solution wherein the phosphodylcholine content is at least 60%, and 200 g of Biosecur®.

22) The method according to claim 20 characterized in that in step a) 1 kg of phospholipids S80 are used for the preparation of 100 liters of liposomal solution wherein the content of phosphodylcholine is at least 80%, and 200 gr of Biosecur®.

23) The method according to claim 20 characterized in that in step d) the high pressure extrusion procedure is repeated 4 to 6 times (100 liters x 4-6 times), until a real inclusion and the desired size of the liposomes are obtained.

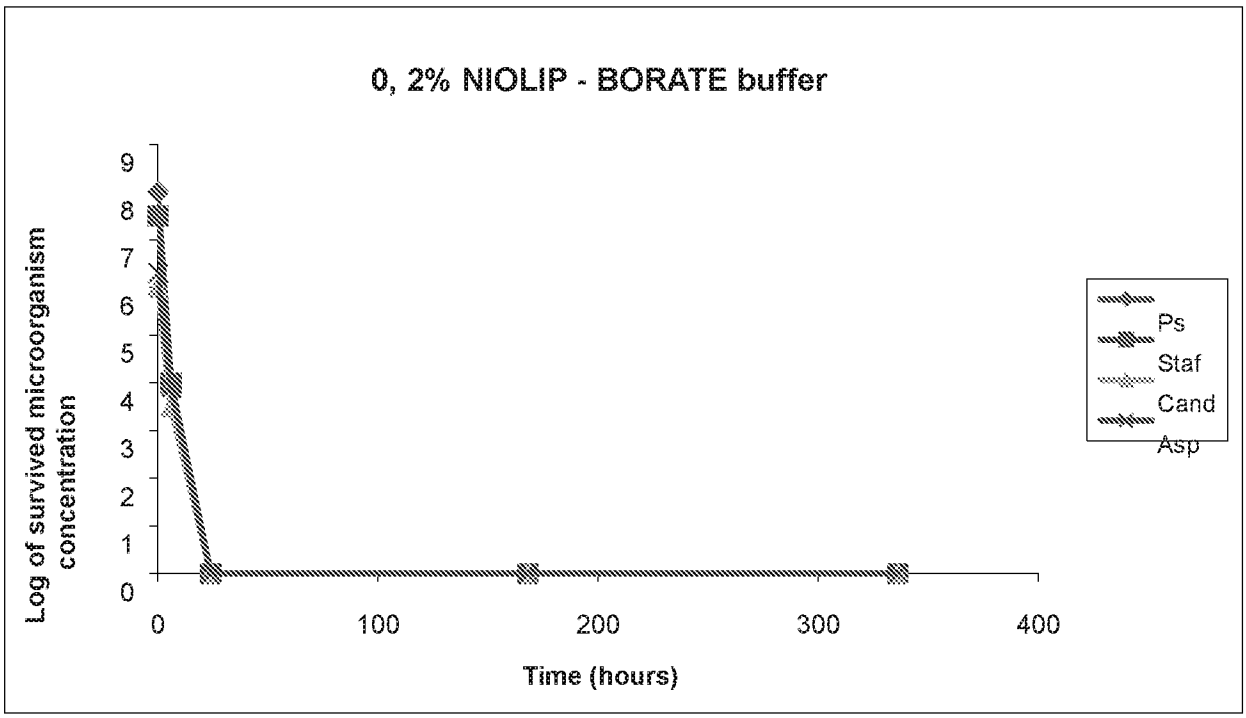
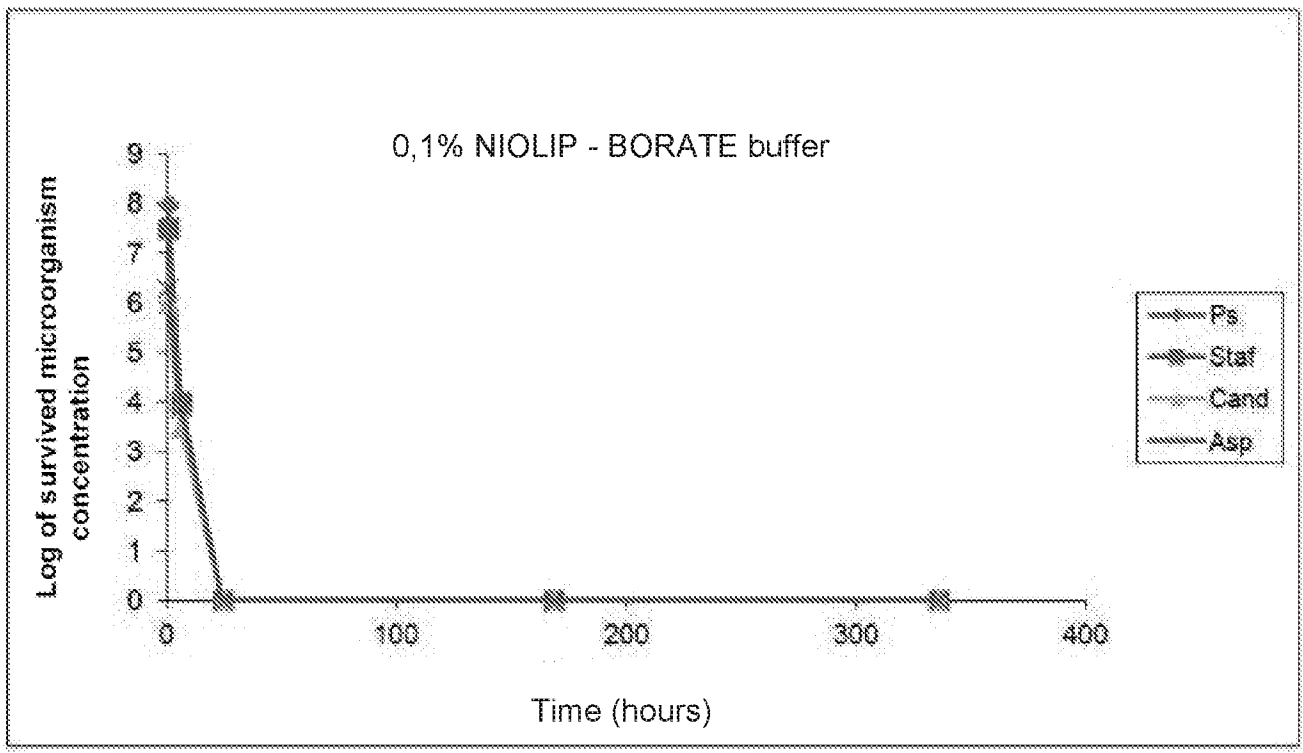

0, 2% NIOLIP - BORATE buffer
Log of survived microorganism concentration
9
8
7
6
5
4
3
2
1
0
0
100
200
300
400
Time (hours)
Ps
Staf
Cand
Asp

Fig. 1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Log of survived microorganism concentration
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8
7
6
5
4
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2
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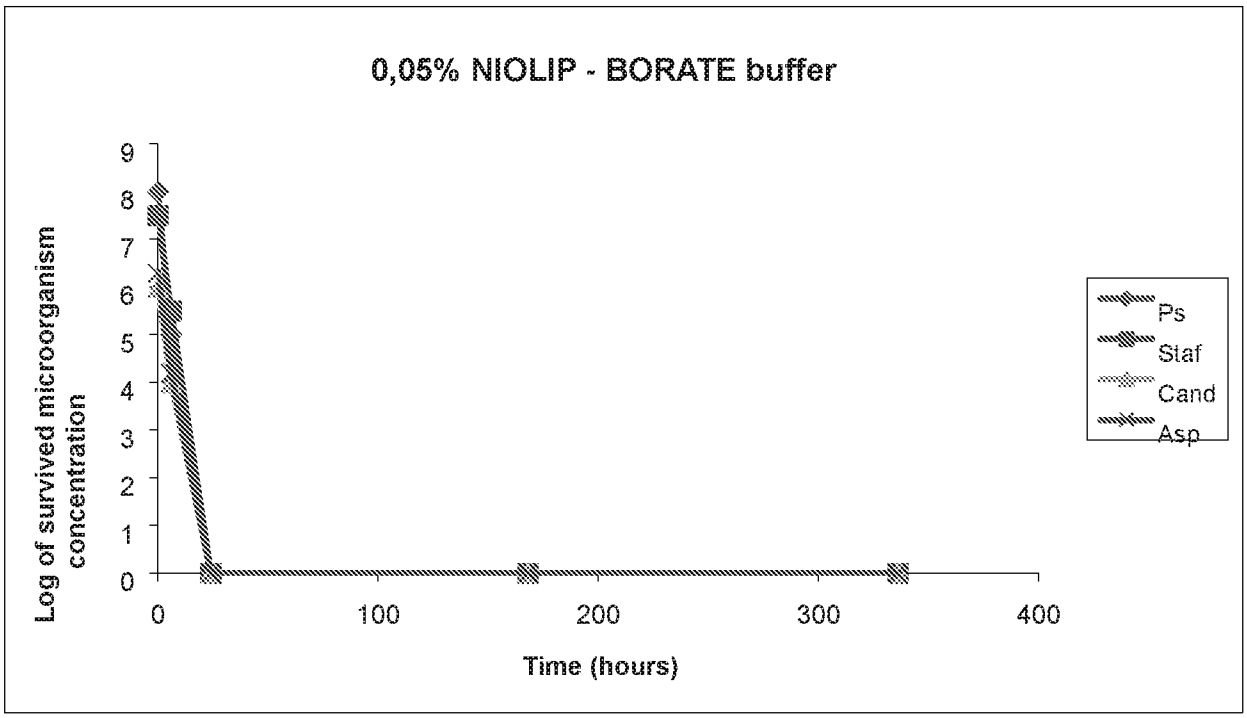
0
100
200
300
400
Time (hours)
Ps
Staf
Cand
Asp

Fig. 2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Log of survived microorganism concentration
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7
6
5
4
3
2
1
0
0
100
200
300
400
Time (hours)
Ps
Staf
Cand
Asp

Fig. 3

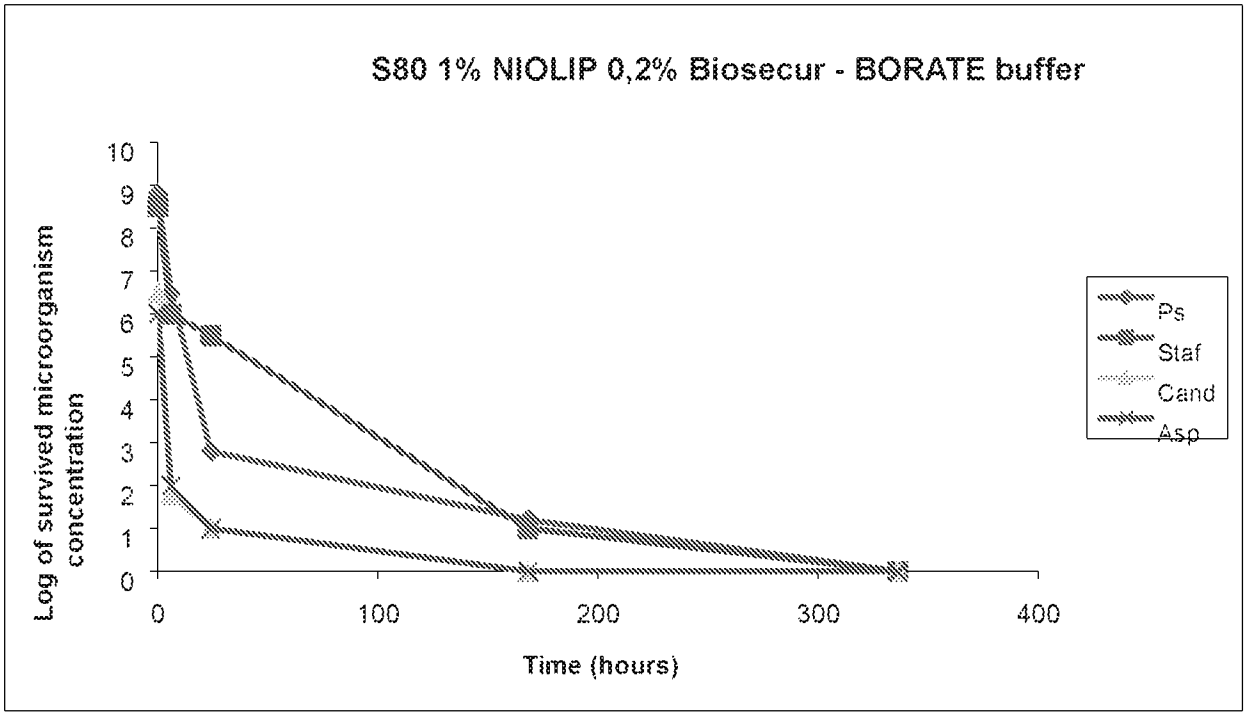
S80 1% NIOLIP 0,2% Biosecur - BORATE buffer
Log of survived microorganism concentration
10
9
8
7
6
5
4
3
2
1
0
0
100
200
300
400
Time (hours)
Ps
Staf
Cand
Asp

Fig. 4

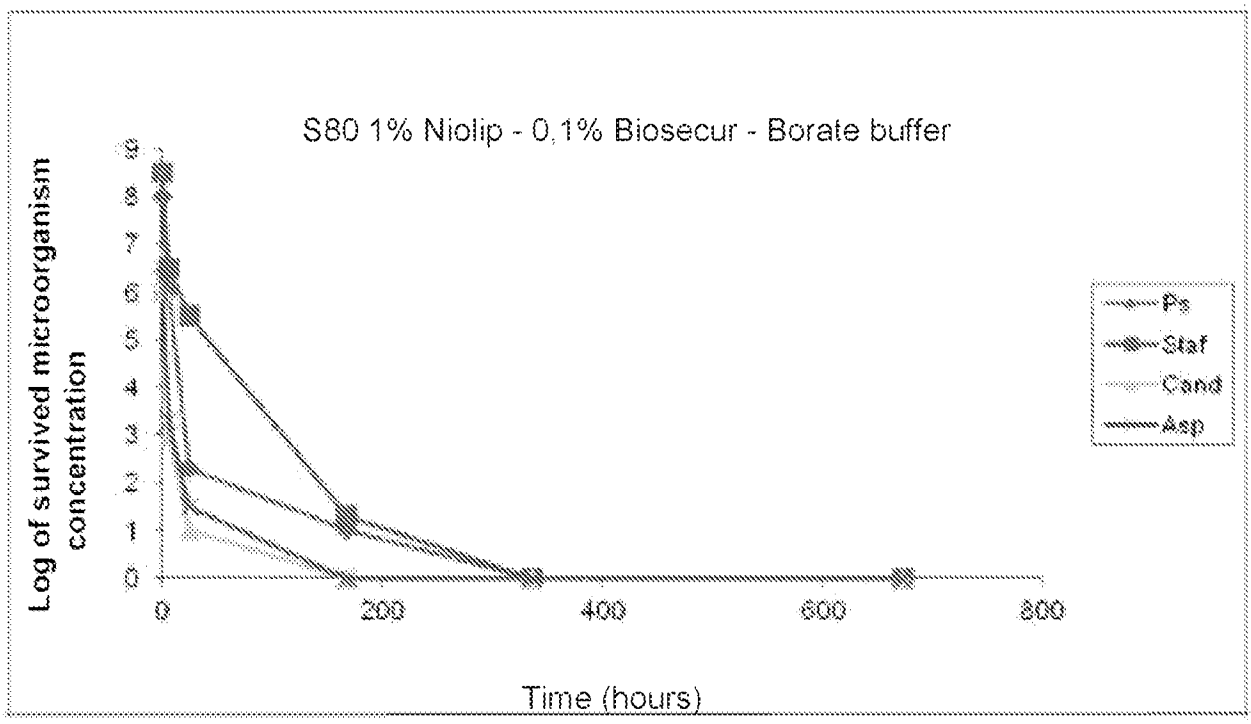
S80 1% Niolip - 0,1% Biosecur - Borate buffer
Log of survived microorganism concentration
0
200
400
600
800
Time (hours)
Ps
Staf
Cand
Asp

Fig. 5

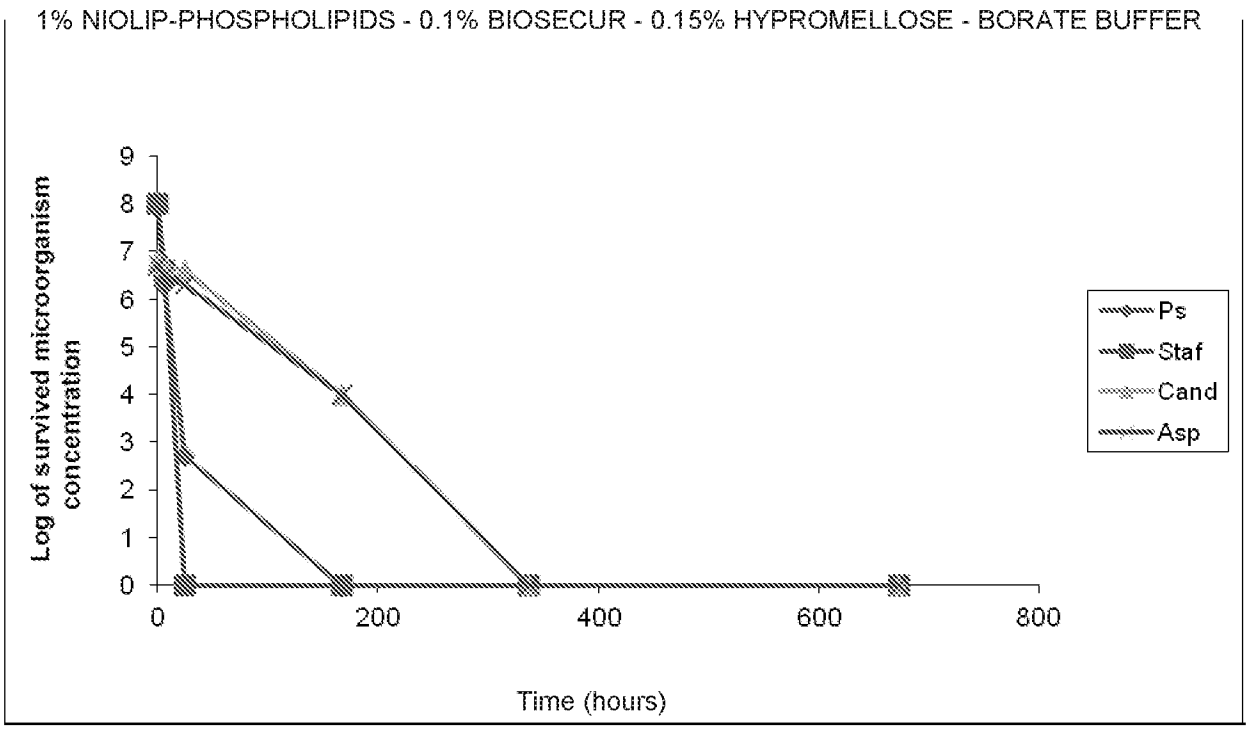
1% NIOLIP-PHOSPHOLIPIDS - 0.1% BIOSECUR - 0.15% HYPROMELLOSE - BORATE BUFFER
Log of survived microorganism concentration
0
1
2
3
4
5
6
7
8
9
0
200
400
600
800
Time (hours)
Ps
Staf
Cand
Asp

Fig.6

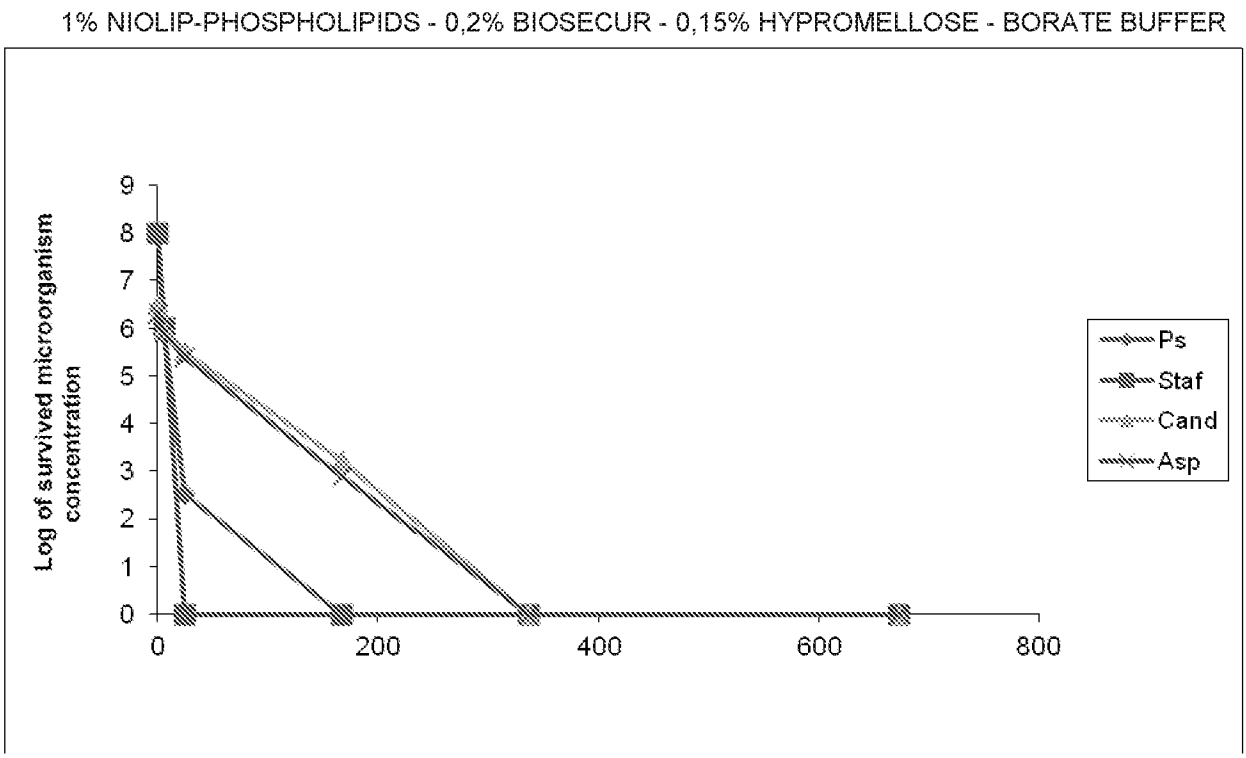
1% NIOLIP-PHOSPHOLIPIDS - 0,2% BIOSECUR - 0,15% HYPROMELLOSE - BORATE BUFFER
Log of survived microorganism concentration
0
1
2
3
4
5
6
7
8
9
0
200
400
600
800
Ps
Staf
Cand
Asp

Fig. 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/060123

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/127 A61K47/38 A61K36/752 A61K8/00
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, FSTA, 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 Y	CN 103 860 625 A (CHENGDU JIANKABIN SCIENCE & TECHNOLOGY CO LTD) 18 June 2014 (2014-06-18) paragraphs [0003], [0022], [0035]; claims 12, 15 -----	1-7, 10-12, 20-23 8,9, 13-19
Y	WO 2017/161387 A1 (REID CHRISTOPHER BRIAN [US]) 21 September 2017 (2017-09-21) examples 2, 15 -----	8,9, 13-19
A	US 2010/143451 A1 (GRUBER JAMES V [US]) 10 June 2010 (2010-06-10) page 3, paragraph 0035 page 4; example 1 page 9; example 7 -----	1-23
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2020	17/03/2020

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	van de Wetering, P

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

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2019/060123

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015/006863 A1 (VASUDEVAN HARISH [CA]; TAIT ANDREW [CA]) 22 January 2015 (2015-01-22) page 8, paragraph 0027 - page 9, paragraph 0033 page 21, paragraph 0087 - page 24, paragraph 0098	1-23
X	US 2016/303033 A1 (BEYER MONIKA [DE] ET AL) 20 October 2016 (2016-10-20) page 2, paragraph 0010-0015 page 6, paragraph 0054-0060 page 8, paragraph 0070-0076 examples 70, 77, 85	1,4-6,11
A	O GORTZI ET AL: "Enhanced bioactivity of Citrus limon (Lemon Greek cultivar) extracts, essential oil and isolated compounds before and after encapsulation in liposomes", PLANTA MEDICA, vol. 73, no. 09, 1 January 2007 (2007-01-01), page P_184, XP055674209, DE ISSN: 0032-0943, DOI: 10.1055/s-2007-986965 abstract	1-23

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2019/060123

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 103860625 A	18-06-2014	NONE	
WO 2017161387 A1	21-09-2017	BR 112018068986 A2	22-01-2019
		US 2019224193 A1	25-07-2019
		WO 2017161387 A1	21-09-2017
		ZA 201806128 B	26-02-2020
US 2010143451 A1	10-06-2010	EP 2303010 A1	06-04-2011
		JP 6223657 B2	01-11-2017
		JP 6257052 B2	10-01-2018
		JP 2011529908 A	15-12-2011
		JP 2016029090 A	03-03-2016
		US 2010143451 A1	10-06-2010
		WO 2010014861 A1	04-02-2010
WO 2015006863 A1	22-01-2015	CA 2955336 A1	22-01-2015
		US 2016158303 A1	09-06-2016
		WO 2015006863 A1	22-01-2015
US 2016303033 A1	20-10-2016	CA 2904105 A1	09-10-2014
		CN 105101938 A	25-11-2015
		EP 2981243 A2	10-02-2016
		EP 3395321 A2	31-10-2018
		JP 6298150 B2	20-03-2018
		JP 2016515622 A	30-05-2016
		KR 20150136537 A	07-12-2015
		US 2016303033 A1	20-10-2016
		WO 2014165490 A2	09-10-2014