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(72) Inventors; and
(71) Applicants : **PESZKE, Jerzy Przemysław** [PL/PL]; 19 Kmicica St., 42-282 Kruszyna (PL). **SZYMANSKI, Zygmunt Jerzy** [PL/PL]; 31 Lubelska St., 08-500 Ryki (PL).

(74) Agent: **BELZ, Anna**; 6/24 Polnocna St., 20-064 Lublin (PL).

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(54) Title: PROPOLIS-SILVER PREPARATION ACCELERATING THE REGENERATION OF SOFT TISSUES AND METHOD OF OBTAINING THEREOF

(57) Abstract: A propolis-silver formulation is characterized in that comprises an extract of propolis in ethanol at a weight ratio of 1: 1- 4: 1 devoiding of any waxy substances, and silver in a form of complex with polyethylene glycol (PEG) in an amount of 0.001-0.1% by weight, wherein silver is in a complex in amount of 10 ppm/lml (or 1 g of fluid) and polyethylene glycol (PEG) is present in a total amount of 20-50% by weight, lanolin in amount of 10-50% by weight, petrolatum and optionally oils preferably in amount of 1-20% by weight, relative to the total mass of formulation. A process for preparing a propolis- silver formulation is characterized in that the extract of propolis obtained by known method by washing propolis with ethanol or ethanol and water mixture or other polar compound, after separating any waxy substances, preferably by vacuum filtration, is cooled to temperature of crystallization of the wax, after separation of which preferably by filtration under pressure, the filtrate after evaporation is poured with water/ethanol/isopropanol mixture made preferably in a volume ratio of 65:20:15 at a weight amount of 3:1 - 5:1 relative to the extract, and then it is shaken for 6 -24 hours, filtered, cooled to temperature about 3 °C, and then the solution after separation of waxy substances, is re-evaporated to dryness and the residue is treated with ethanol at a weight ratio of 1: 1 - 4: 1 on a dry mass, and further the obtained solution in an amount of 1 - 60% relative to the mass of the final product, is mixed with hydrophobic medium preheated to temperature of 25 - 45 °C, comprising 10 to 50% by weight lanolin, petrolatum or optionally oils, and finally after its homogenization it is added of 0.001 - 0.1% silver in complex form with polyethylene glycol (PEG).

TITLE: A propolis-silver formulation accelerating a regeneration of soft tissues and process for its preparation

DESCRIPTION

The present invention relates to a propolis-silver formulation which accelerates a regeneration of soft tissue and a process for preparation the same.

Propolis ointments are widely used in medical treatments. Propolis has a bactericidal effect and promotes natural regeneration processes. Organic compounds contained in propolis have a wide range of biological effects. The high content of plant flavonoids - antioxidants and both systems regulating cellular metabolism by binding free radicals - effect very positive on tissue regeneration processes. Besides flavonoids, propolis contains a group of anti-inflammatory substances and ones stimulating development of new blood vessels - angiogenesis. Antiseptic activity is associated with a presence of series of active substances combating microorganisms.

As a result, tissue regeneration and thereby scarring is smooth, and time necessary to complete coalescence itself is faster by up to 30%. Propolis extracts presently produced are mainly based on a method relying on their dissolution in ethanol or mixtures of ethanol and water. They are offered on the market as raw material for cosmetics and medicinal preparations in concentrated form - namely as Propolis plus

ethanol. In order to obtain the formulation they are mixed among others, with lanolin or other emulsifiers.

Silver is also one of the oldest active substance used in medicine. Presently, due to the increasing antibiotic resistance of micro-organisms, a reversal is observed towards preparations based on silver. Silver formulations exhibit a high biological activity against microorganisms as opposed to organic compounds. A permanent effect on a number of pathogens has been proven what qualifies this material as one of the strongest aids which support tissue regeneration processes. Silver nitrate is the most often used source of silver applied in pharmaceutical formulations (lapis, Mikulicz's ointment). The silver ions fight off pathogens. By this way it protects the wound from infection and development of pathogenic micro-organisms.

Another well-known preparation is Dermazin used in burns treatment of first and second degree. It works synergistically to pathogens - by silver ions plus sulfonamide. In the case of cosmetics - there are generally used colloidal silver, produced by physical processes: by arc discharge method or plasma method. To the known preparations it is added in the form of a powder. When using silver ions a detrimental effect is occurred - a precipitation of the pure metal by reducing ions with light. The present invention solves the problem of preparing ointments propolis-silver of high biological activity from the substrates, and therefore preparation thereof and

combining the same provides the subject of a present invention.

One of the main substrates of an ointment is propolis extract separated from the waxes ballast material. Its biological activity and efficacy determines the degree of purity, in particular ridding it of waxy substances so that their content in the final product will be reduced to trace levels. Furthermore, in the separation process we isolate biologically active ingredients and the others do not having any biological activity.

Various methods are known for isolating active ingredients of propolis. These methods generally rely on the elution of flavonoid fraction using ethanol or a mixture of ethanol and water and optionally other polar compounds.
US Patent no. 8257747 teaches a method of selecting biologically active compounds from the material constituting the previously waste from the extraction - i.e. a wax fraction. This material contains a lot of hydrophobic compounds with proven biological activity. The extraction process involves the selective elution of the oil.
The described methods of extracting propolis not provide obtaining an extract completely devoid of waxy substances what, as already indicated, significantly reduces its biological activity. Propolis-silver ointment containing silver in a powder form, is known from patent specification no. CN 1669434, where the silver with the propolis extract is connected in various proportions. To prepare emulsions alcohols and emulsifiers are used.

From another patent specification no. CN101947257 there are known aerosols containing propolis extract and silver and selenium. This product is used in the treatment of influenza infections.

According to the invention an extraction process of a biologically active system from propolis comprising active components relies on that propolis extract obtained by a known method, after vacuum filtration is cooled to a temperature at which the crystallization of waxes is occurred, and after separation of the waxy substances by filtrating on the pressure, the filtrate is evaporated to dryness , preferably under vacuum, and the residue is poured with water / ethanol / isopropanol mixture made preferably in a volume ratio of 65:20:15 in a weight ratio of 3:1 - 5:1 relative to the extract mass, and further it is shaken for 6-24 hours, then filtered, and cooled to a temperature of about 3 ^{0}C. After separation of the wax material, the solution is then re-evaporated to dryness and the residue is treated with ethanol in a weight ratio of 1:1 - 4:1 relative to a dry mass. A biological activity of the extract so obtained is dependent on the content of active substances contained in starting material, and is about 20 - 200% higher than the biological activity of the extract obtained by the prior known method.

Silver nano-material according to the invention is obtained by reduction of silver ions on a matrix of polyethylene glycol (PEG), for this purpose into PEG solution containing 50-80% by weight of PEG in water, silver nitrate is added in

an amount from 0.1 to 10% by weight relative to PEG mass. After dissolution of the mixture it is filtered preferably onto polyethylene filter, and is placed at the microwave pool of the power of 500 - 1200 W for approximately 1 -20 minutes, and then it is dissolved in ethanol and / or isopropanol to obtain a silver concentration of 0, 01-2% by weight in the mixture.

According to the invention, being a subject thereof, a propolis-silver formulation is a homogenous mixture comprising: propolis extract in ethanol at a weight ratio of 1:1 - 4:1 (in an amount of 1 - 60% by weight) devoiding of any waxy substances, silver in complex form with polyethylene glycol (PEG) in a quantity 0.001-0.1% by weight, wherein the silver is present in the complex in an amount of 10 ppm / 1 ml (or 1 gram of fluid) and polyethylene glycol (PEG) is present in a total amount of 20-50% by weight, and lanolin in an amount of 10 - 50% by weight, petrolatum and optionally oils, preferably linseed, hemp or jojoba oils in an amount of 1-20% by weight, relative to the total mass of formulation. The formulation contains ethyl alcohol being added to the final form of a formulation in an amount needed to stabilize oil emulsion in petroleum.

The present invention further provides a process for preparing a propolis-silver ointment, which is relying on that the propolis extract obtained as described in above method in an amount of 1-60% by weight is mixed with hydrophobic medium preheated to temperature of 25-45 ^{0}C, where the above medium comprises 10-50% by weight of

lanolin, and petrolatum, and further after mixing to a homogeneous mass into this mixture it is added a silver formulation obtained according to the invention in an amount of 0.001 to 0.1% by weight of silver, and polyethylene glycol, wherein the amount of glycol is 20-50% by weight relative to the mixture mass, and optionally polyunsaturated fatty acids in an amount of 1-20% by weight. As fatty acids, linseed, hempseed, jojoba oils or mixtures thereof are preferably used. Preferably, when added to the final form of the formulation, ethyl alcohol and polyethylene glycol is used in an amount necessary to stabilize oil emulsion in petroleum. Preferably, the sequence of addition of the active constituents into the medium is facultative.

The product according to the invention which is a propolis extract, devoid of wax and other ballasts, combined with the silver complexes, exhibits a high activity in the treatment of wounds. Propolis extract by its high content of flavonoids in combination with complexes of silver, leads to their higher activity (synergism) against bacterial pathogens rather than each of these substances individually. Plant flavonoids content accelerates the process of angiogenesis and thus the blood supply to the place of local applying of a formulation. The hydrophobicity of the formulation results in an increased affinity for cell membranes.

In addition, an enrichment of the formulation by polyunsaturated fatty acids accelerates regeneration processes. A presence in the composition of plant sterols and steroids causes analgesic and anti-inflammatory action

thereof. An addition of polyethylene glycol during a final stage of the preparation of a formulation has a good influence on homogeneity of an emulsion of ethanol / petrolatum / lanolin / propolis extract.

Description of testing a formulation

The study was performed by diffusion method in accordance with the test procedure of Specialist Research Laboratory - ITA-TEST PB 33 / ChM ed. 3 dated 18.07.2011 "Evaluation of the antimicrobial action of cosmetics by diffusion method".

Test strains:

a) *Staphylococcus aureus* (ATCC 6538) (purchased from Biomedica)

b) *Pseudomonas aeruginosa* (ATCC 9027) (purchased from Biomedica)

Materials and reagents

Culture media:

Baird-Parker Agar (for *Staphylococcus aureus*)

Acetrimid Agar (for *Pseudomonas aeruginosa*)

Saline solution

Nutrient broth TSB

A study course: An assestment of the antimicrobial activity of the tested formulation with respect to pathogenic strains *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027).

1) Preparation of individual strains culture from a reference stock? culture collection (test strains)

For the study there were used two known and well-defined pathogens *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027) : *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027), in order to demonstrate antimicrobial properties of the product relative to the strains with increased resistance to antibacterial agents. Selected for study test strains were revived according to Technical Manuals IT 25 / ChM "Dealing with the strains for microbiological examination." Strains were obtained from the fifth passage. Using densitometer the standardized suspension of strains was prepared. Both in the case of *Staphylococcus aureus* (ATCC 6538) and *Pseudomonas aeruginosa* (ATCC 9027) as well, a density of 2 in MacFarland scale was applied. In order to obtain an inoculum with an appropriate dilution, further decimal dilutions in saline of 10-2 microorganisms were made.

2) Performing of a study

Onto test plates with solidified medium specific to the relevant microorganism it was sterile loaded in 0.1 ml of the prepared inoculum of both microorganisms which were then gently stroked and let stand them for 15 minutes at room temperature. On thus prepared lawn of microorganisms there were centrally placed discs bearing with 0.3 ml of formulation. The plates were incubated for 24 hours at temperature of 36 ± 2 °C.

After completing incubation time the zones of inhibition of microbial growth were determinated. Evaluation was based on the determination of bacterial growth or lack thereof in the zone between agar and disc and on possible determination of inhibition zone around the product. Attention was also drawn to the intensity of microbial growth under formulation, to changes in the appearance of colonies grown, their shape, color and size. The results obtained are shown in Table 1.

Table 1. The size of the zones of growth inhibition of *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538).

Test strain	Test product	
	The diameter of growth inhibition zone (mm) of isolated microorganisms on TSA medium*	The growth intensity under preparation**
Pseudomonas aeruginosa (ATCC 9027)	0	-
Staphylococcus aureus (ATCC 6538)	0	-

** *The scale of growth intensity under preparation:*

- *a lack of growth*

\+ *weak growth*

++ *strong growth*

+++ *confluent growth*

Propolis ointment with active particles of silver exhibits antibacterial effect against strains of the test strains: *Pseudomonas aeruginosa* (ATCC 9027) and *Staphylococcus aureus* (ATCC 6538). Under imposed product it was observed a complete inhibition of growth of the test strains with simultaneous absence of growth inhibition zone. (Table 1).

Other features and advantages of the invention appear on reading the description given hereafter of a particular embodiment of the invention given by way of non-limiting example.

EXAMPLES

Preparation of biologically active systems from propolis raw material

A propolis raw material was quenched with pure ethanol in a weight ratio of 3: 1. The mixture was placed on a shaker and extracted for 35 hours. The mixture was then placed on homogenizing mixer and stirred for 12 hours. The crude extract was filtered through a polyethylene filter under vacuum of 10^{-2} Tor. The filtrate was cooled to -5 °C and then again filtered and evaporated to dryness under vacuum.

A propolis crude material was quenched with mixture of water / ethanol / isopropanol prepared in a volume ratio of 65:20:15 at a weight of 4: 1 relative to extract and placed on a shaker for 20 hours and then filtered through a polyethylene filter and finally cooled to 3 °C. The resulting product was passed through a cellulose filter to separate wax residues, and then evaporated to dryness. Into the dry

extract of propolis an ethanol in an amount by weight of 1: 2 was added.

Preparation of the silver nano-material

Into 100 g of 65%_by weight of polyethylene glycol (PEG) solution in distilled water 3 g of silver nitrate was added. The mixture was placed on a magnetic stirrer in order to dissolve, then filtered through a polyethylene filter and finally placed at microwave field with power of 700W for 15 minutes. The resulting product was dissolved in ethanol to obtain a concentration of 1.5% by weight.

Example 1

Preparation of anti-acne formulation in an ointment form

The product formulation was obtained as follows: 10 g of silver-containing material having in its composition of 10 ppm of nanosilver and 1 g of PEG 400, as well as 6 g of ethanol and 3 g of water, was added to 50 g of cosmetic vaseline. The whole was heated to 35 °C and slowly stirred with a mechanical mixer at 20 rev/min. After exact mixing, 8 g of 50% by weight of propolis extract in ethanol, and then 25 g of lanolin and 4 grams of PEG 400 were added. When homogeneous consistency is obtained, the remaining amount of petroleum jelly was added into this mixture to obtain a formulation in an amount of 100 g. The product contained in its composition 100 ppm silver / 1 kg.

Acne is a pathological lesion caused by bacteria Propionibacterium acnes difficult to treat. The ointment

obtained according to the invention was applied to the affected skin three times a day. After three days it was found a clear improvement and reducing of redness. As a result of applying the ointment regularly for 6 days acne completely regressed.

The formulation according to the invention provides significant effects in the treatment of acne in a shorter time of its application in relation to prior known medicinal preparations based on benzoyl peroxide, eg. Benzacne, Clerasil or Acnidazil. The use of these formulations involves, however, a change in epidermis structure of the skin caused by an influence of oxygen on normal cells. In our case, an active agent consists in organic antioxidants and silver. Their synergistic interaction increases blood flow in the skin and acts destructive to microorganisms colonizing the sebaceous glands of the skin.

Example 2

Preparation of the formulation as a product for foot care

Foot care product formulation was prepared as follows: into 30 g of cosmetic vaseline it was added 30 g of lanolin and the whole was heated to temperature of 40°C and then stirred with the low-speed of 150 rev / min. When a homogenous consistency is obtained, 20 g of linseed oil and 10 grams of the hemp oil were added into this mixture and stirring was continued for at least 5 minutes. During this period of time, the mixture became consistency of a cream. Further, with

continuation of stirring, it was added 4 grams of the silver formulation comprising in its composition of 2 ppm of silver, 2 g of PEG 400 and 2 g of ethanol.

After adding the whole amount of silver formulation, it was then added 6 g of 50% by weight of propolis extract in ethanol and the resulting mixture was stirred for another 10 minutes until a homogenous consistency is obtained. In the next step, 4 g of PEG 400 and petrolatum were added into this mixture to give 100 g of product. After a complete mixing, the formulation contained in its composition of 20 ppm of silver and 3% by weight of mass of pure extract of propolis.

?The final? formulation is obtained being a product for foot care. It is used to protect feet against hyperkeratosis and maintaining their right environment.

CLAIMS

1. A propolis-silver formulation comprising lanolin, petrolatum and optionally oil constituting a hydrophobic medium and propolis and silver being active agents of a formulation, **characterized in that** comprises an extract of propolis in ethanol at a weight ratio of 1: 1- 4: 1 devoiding of any waxy substances, and silver in a form of complex with polyethylene glycol (PEG) in an amount of 0.001-0.1% by weight, wherein silver is in a complex in amount of 10 ppm/1ml (or 1 g of fluid) and polyethylene glycol (PEG) is present in a total amount of 20-50% by weight, lanolin in amount of 10-50% by weight, petrolatum and optionally oils preferably in amount of 1-20% by weight, relative to the total mass of formulation.

2. A formulation according to claim 1, **characterized in that** comprises additionally ethyl alcohol added to final form of a formulation in an amount necessary to stabilize oil emulsion in petrolatum.

3. A formulation according to claim 1, **characterized in that** as an emulgator and additional source of biologically active compounds comprises linseed, hemp or jojoba oils.

4. A formulation according to claim 1, **characterized in that** comprises petrolatum in an amount necessary to adjust the concentration of a propolis extract with respect to the other ingredients.

5. A process for preparation of a propolis-silver formulation by mixing the active ingredients with a hydrophobic carrier such as lanolin, petrolatum and/or oil, **characterized in that** propolis extract obtained in known manner by washing propolis with ethanol or ethanol and water mixture or other polar compound, after separation of the waxy substances, preferably by filtrating off under vacuum, is cooled to the wax crystallization temperature, and after separation thereof preferably by filtration under pressure, the filtrate after evaporation is poured with water / ethanol / isopropanol mixture made preferably in a volume ratio of 65:20:15 at a weight amount of 3 : 1 - 5 : 1 relative to the extract mass, and further it is shaken for 6 - 24 hours, filtered, cooled to about 3 °C, and then the solution after the separation of waxy substances is re-evaporated to dryness and the residue is treated with ethanol at a weight ratio of 1:1 - 4:1 relative to a dry mass , and the obtained solution in an amount of 1 - 60% by weight of the final product is mixed with hydrophobic medium preheated to temperature between 25-45°C and comprising 10-50% by weight of lanolin, petrolatum or oil, to which after the homogenization 0.001 - 0.1% by weight of silver in a complex form with polyethylene glycol (PEG) is added, wherein the amount of glycol is 20-50% by weight, relative to the total mass of formulation.

6. A process according to claim 4, **characterized in that** additionally comprises ethyl alcohol added to a final form of

a formulation in an amount necessary to stabilize oil emulsion in petrolatum.

7. A process according to claim 4, **characterized in that** linseed, hemp or jojoba oil is added into a hydrophobic medium in an amount of 1-20% by weight relative to the product mass.

8. A process according to claim 4, **characterized in that** addition order of the active ingredients into the medium components is facultative.

INTERNATIONAL SEARCH REPORT

International application No
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A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P17/02 A61K35/64 A61P17/10 A61K33/38
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 A61P

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, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE WPI Week 200381 Thomson Scientific, London, GB; AN 2003-874047 XP002754592, & RU 2 209 068 C1 (ORLOVSKII E V) 27 July 2003 (2003-07-27) abstract -----	1-8
Y	DATABASE WPI Week 200974 Thomson Scientific, London, GB; AN 2009-P35156 XP002754593, & KR 2009 0099110 A (SUN-TEK J) 22 September 2009 (2009-09-22) abstract ----- -/--	1-8

[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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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	Thalmair, Michaela

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2015/059363

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 2004/022034 A1 (DERMAPHYT LTD [PL]; MASLANKY JERZY [PL]) 18 March 2004 (2004-03-18) claims 1,11,12,15	1-8
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2015/059363

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