(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 June 2012 (21.06.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/082003 A1

(51) International Patent Classification:
A61K 36/06 (2006.01) *A61K 36/062* (2006.01)
A61P 27/02 (2006.01)

(21) International Application Number:
PCT/RO2011/000001

(22) International Filing Date:
14 January 2011 (14.01.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
a 2010 01351 15 December 2010 (15.12.2010) RO

(72) Inventor; and
(71) Applicant : **DAAS, Mohammed Marwan** [RO/—]; Str. Foişorului nr. bloc V51, apt. 129, sector 3, R-031171 Bucuresti (RO).

(72) Inventors: **MOSCOVICI, Mişu**; Str. Jean Steriadi, nr. 7, bloc I 22, scara b, etaj 2, apt. 16, sector 3, Bucureşti (RO). **CASARICA, Angela**; Str. Popa Stoica Farcaş nr. 19, sector 3, Bucureşti (RO). **COSA, Ortansa**; Str. Alexandro Moruzzi, nr. 9, bloc V54B, scara 3, etaj 3, apt. 39, sector 3, Bucureşti (RO). **NITA, Sultana**; Str. Barbat Voievod, nr. 21, sector 2, Bucureşti (RO). **RASIT, Iuksel**; Blv. Dinicu Golescu, nr. 37, bloc 4, scara B, apt. 40, sector 1 (RO). **DAAS, Ahmad**; Str. Foişorului nr. 1, bloc V51, apt. 129, sector 3, Bucureşti (RO). **PANTELI, Irina Minerva**; Str. Spatar Nicolae Milescu, nr. 46-48, sector 2, Bucureşti (RO). **CREŢU, Alexandra**; Str. David Constantin, nr. 6, Oraş Voluntari, Jud. Ilfov (RO). **DAAS, Marwa**; Str. Foişorului nr. bloc V51, apt. 129, sector 3, Bucureşti (RO). **DAAS, Arij**; Str. Foişorului nr. bloc V51, apt. 129, sector 3, Bucureşti (RO). **DAAS,Morouj**; Str. Foişorului nr. bloc V51, apt. 129, sector 3, Bucureşti (RO). **GHERA, Daniela**; Str. Ion Brezoianu nr. 18, et. 2, apt. 5, secor 1, Bucureşti (RO). **DAAS, Nour**; Str. Foişorului nr. bloc V51, apt. 129, sector 3, Bucureşti (RO). **BOGHIU, Anastasia**; Str. Baltita nr. 3, bloc B19, scara 3, apt. 69, sector 4 (RO).

(74) Agent: **ANDRONACHE, Paul**; Cabinet Individual de Proprietate Industrială, Aleea Compozitorilor nr. 1, bloc E21, apt. 35, sector 6, R-061601 Bucuresti (RO).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: PRODUCT OF NATURAL ORIGIN FOR OPHTHALMIC TREATMENT AND OBTAINING PROCEDURE

(57) Abstract: The invention refers to a natural product consisting in an aqueus extract of brown desert truffles of the *Terfezia*-genus fungi, which contains proteins, aminoacids, carbohydrates, utilizable as eye drops in the treatment of ocular affections of cornea and in glaucoma, as well as to its obtaining procedure. The truffles are extracted with demineralized water, in a ratio of 2-3: 1 (w/v) at 4°C; the extract is separated through filtration on filter aid layer or centrifugation, then it is purified through ultrafiltration on membranes with cut-off limit of 10 kDa; the permeate solution obtained is concentrated under vacuum to 1/6-1/9 (v/v), then benzalkonium chloride 0.01 % (w/v) and sodium chloride 0.6% (w/v) are added, thus obtaining an eye drops solution which fills in sterile bottles of 5-10 ml.

WO 2012/082003 A1

PRODUCT OF NATURAL ORIGIN FOR OPHTHALMIC TREATMENT AND OBTAINING PROCEDURE

The invention refers to a product of plant extraction having therapeutic action in ophthalmic disorders and its obtaining procedure.

There are many ophthalmic affections that harm the cornea: corneal ulcer, keratitis (infections of different origins, namely viral or microbial). Other lesions are due to the abrasion brought on by foreign bodies or mechanical shocks.. On a global scale, corneal affections rank second, right after the cataract, among the decisive factors for blindness [1-6].

For the treatment of the affections mentioned above, antibiotics or antivirals are used, both local and systemic. Many of these preparations can cause adverse or allergic reactions, having unpleasant or even serious effects. Moreover, the healing can be accompanied by proliferative processes, with upsetting scars, affecting sight.

Glaucomas are a group of eye diseases characterized by progressive lesions of the optic nerve and of the visual field, due to the increase of the intraocular pressure, being the third cause of blindness on worldwide ranking.

Anti-glaucoma medication comprises topical agents (analogues of prostaglandin, ß- blocking agents), as well as systemic medication. The administration of these can be limited by allergic reactions or adverse effects, some important, such as cardiac or central nervous system disorders [1,3,4,5,6,7].

The patent applications CN 101579488(A) and CN 101647913(A) present Chinese medicines of plant origin on traditional basis for the treatment of ophtalmic disorders.

The patent application CN 101579488(A) concerns to a medicine for cataracts and glaucoma treatment prepared with 8 natural ingredients, 5 of them being of natural origin [8].

In the patent application CN 101647913(A) a preparation procedure of a medicine for the treatment of senile cataracts and viral keratitis from 12 plant secies is claimed, promising statistical data regarding the improvement of patients'eyesight are shown [9].

From Islamic religious texts [10] and from popular medicine in the Middle East, one knows the use of the juice extracted from desert truffles in treating some eye diseases. [11].

Generally, the truffles are widely used in alimentation, having a high content of proteins and lipids, other nutritional substances being carbohydrates, amino acids and vitamins (especially vitamin C) [12].

Nevertheless, there are few scientific studies that have identified antimicrobial peptides in the extract of these desert truffles, without establishing any therapeutic action [13,14].

The technical problem solved by this invention is the achievement of a natural origin product of therapeutic use which is very active in ocular affections of the cornea and moderately active in the treatment of glaucoma, by a procedure involving pre-established steps and parameters. The plant source consists of brown desert truffles of the *Terfezia* type fungi.

The product according to the invention consists in an aqueous extract of brown desert truffles of *Terfezia* genus fungi, having a concentration of 13...17%(w/v) as dry matter, containing %(w/v):

-proteins 3.0...5.5
-aminoacids 0.1...0.2
-carbohydrates (as glucose) 0.4...0.6, to which are added
-benzalkonium chloride 0.01 as preservation agent
-sodium chloride 0.6 as isotonization agent and
-demineralized water to 100.

The product is sterile, for ophthalmic treatment as eye drops.

The product obtaining procedure according to the invention consists in that:

Fresh or frozen truffles are extracted in demineralized water at 4 °C by a ratio of 2-3:1 (v/w), the extract is separed by filtration on a filter aid layer (celite) or centrifugation and it is submitted to purification by ultrafiltration on membranes with a cut-off limit of 10 kDa. The obtained permeate solution is then concentrated under vacuum to 1/6-1/9 (v/v) and benzalkonium chloride 0.01% (w/v), as well as sodium chloride 0.6 % (w/v) are added, after which it is sterilely filtered and thus an eye drops solution is obtained and filled in sterile bottles of 5-10 ml.

The advantages of the invention are mainly that: a product of natural origin from a single plant source is obtained, which is very active in the affections of cornea and moderately active in glaucoma, having no proliferative effect, the regeneration taking place without leaving any scars; the product is not toxic and non-irritative.

The present invention is now illustrated by two following examples.

Example 1

A 2 kg quantity of brown desert truffles (of the *Terfezia* genus), frozen for 4 months at -17...-19 °C is submitted to extraction by shaking with 4 l demineralized water for 24 h at 4 °C. Following extraction, the solid phase is separated through filtration under vacuum, on a Buchner funnel, through a filtration aid layer (celite). Thus 3370 ml filtered product (aqueous extract) having a protein concentration of 6.7 mg/ml (the Lowry method) is obtained.

The extract is purified of the ballast proteins by ultrafiltration on a module with a cellulose membrane cassette with a cut-off limit of 10 kDa and a filtration area of 0.5 m^2 (PLCGC – Pellicon, Millipore), with the recirculation of the retentate and adding of demineralized water at the end in order to recover the active product. 3250 ml of permeate solution (filtrate) having a protein concentration of 5.5 mg/ml is obtained, this being the active fraction and 150 ml of retentate with 17.4 mg/ml (protein ballast). The filtrate is concentrated on a rotary evaporator, under vacuum (maximum temperature of 37°C) up to a volume of 450 ml.

The active solution so obtained has 35.8 mg protein/ml. This is formulated as eye-drops by adding at 100 ml solution of 0.5 ml. benzalkonium chloride 2% (w/v) – 0.01% (w/v) as refered to the active solution and 0.6 g NaCl – 0.6% (w/v), followed by aseptic filtration on sterile filter with a membrane of 0.22 μm and fills in sterile dropper bottles of 5 and 10 ml.

The chemical and physical characterization of the active solution and of the eye drops was carried out by of high performance liquid chromatography HPLC-DAD, coupled with mass spectrometry (HPLC-MS-MS), atomic emission (ICP-MS) and atomic absorption spectrometry.

HPLC determination (Kromasil column 100-5CB, two mobile phases, Merck-Hitachi LaChrom chromatograph) showed a protein complex (polypeptides).

HPLC-MS-MS chromatograms (Zorbax-SB-C18 column, two mobile phases, Agilent Triple Quad LC/MS chromatograph) showed a molecular weight of the components of up to 420 Da.

The spectrometric analysis of inductively coupled plasma atomic emission (ICP-MS Elan DRC-e Perkin Elmer) and atomic absorption (Analyst 800, Perkin Elmer) showed the presence as macro- and microelements of the metals Na, K, Mg, Fe, Zn, Cu, Cr, Cd, As, Se, Pb. Zinc, chromium and selenium can be useful in the therapeutic action, and the toxic metals (Cd, As, Pb) are within the limits approved by the European Pharmacopoeia, the 6^{th} edition.

In the following table the chemical composition and the metal content of the product are shown.

Table 1. Chemical composition of the product.

Composition	Concentrations, %(w/v)
Dry matter content	14.996
Total proteins, represented by polypeptides having a mol. wt. between 300 and 420 Da	3,4673
Aminoacds,	0,17
From which	
- Alanine	0,067
- Serine	0,033
- Proline	0,045
- Methionine	0,005
- Tyrosine	0,021
Carbohydrates, as glucose	0,499
Minerals (ash)	1,61

Tabel 2. Metal content of the product.

Element	Content	
	ppm (µg/g)	mg/100g
Sodium	1435,5	143,50
Potassium	4497,4	449,74
Magnesium	179,5800	17,9580
Cromium	0,3379	0,0338
Iron	11,0600	1,1000
Copper	0,4756	0,0476
Zinc	7,5723	0,7572
Manganese	11,53	1,1530
Cobalt	7,0371	0,7037
Arsenum	0,0321	0,0032
Selenium	< 0,0010	< 0,0001

Cadmium	0,0323	0,00323
Lead	< 0,0010	< 0,0001

Example 2

One proceed as in example 1, with the difference that the raw extraction material is represented by fresh truffles, extracted with demineralized water in a ratio of 3:1 (w/v), and the extract is separated by centrifuging at 7000-8000 rpm. From 2 kg of fresh truffles 4580 ml of extract with 5.1 mg protein/ml are obtained; one proceed further as in example 1.

The product showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923 şi *Escherichia coli* ATCC 25922.

The following tables give such examples.

Table 3. Antimicrobial activity assay at different dilution rates of the product (simple broth medium for bacteria, pH 7.4).

Dilution %(v/v)	Microorganism	
	Staphylococcus aureus ATCC 2592	*Escherichia coli* ATCC 25922
50	active	active
25	active	active
12,5	active	active
6,2	active	active
3,1	non-active	non-active

Table 4. Compared assay of antimicrobial activity against *Staphylococcus aureus* ATCC 2592 (TSA Agar medium, buffer pH 6.0, final pH 7.4).

Sample	Inhibition zone(average) mm
Product according to the invention	25,5
Cephalexin solution 10 µg/mL 20 µg/mL	 13,8 17,1

The single dose toxicity test was performed in NMRI mice, after a single oral dose by intragastric gavage. The maximum sample volume was 25 ml/kg bwt. The animals were supervised and observed until the end of a 14 days period.

After oral administration in mice, the product did not induce any toxic phenomena at this maximum possible dose.

The testing of the ocular tolerance of the eye drops was carried out in New Zealand rabbits, in two steps, after single and repeated administration.

The single dose tolerance was performed by unique instillation of a drop (0,02 ml) on the eye conjunctiva with an observation period of 72 hrs. In the repeated administration test, the sample was instilled daily, for 28 days, with a 2 weeks watching period after ceasing the administration.

According to the Draize ocular assessment scale, the product was classified as "non-irritative" by the local tolerance test after single administration and "practically non-irritative" after repeated administration for 28 days. .

There was a clinical trial carried out which comprised a number of 16 cases (7 cats and 9 dogs), that had been diagnosed with secondary glaucoma (secondary to distopias of the crystalline lens, tumors of the ciliary body) and keratitis of various etiologies (chronic superficial, eosinophilic, proliferative secondary to scarring, corneal sequestrum).

Secondary glaucoma cases associated to anterior lens luxation were diagnosed by clinical examination, ophthalmoscopy, tonometry, eye echography. The intraocular pressure IOP (the pressure inside the eye) was carried out with Tono Vet, 2 hours, 6 hours and 24 hours after eye-drops administration.

Diagnosis of keratitis cases was performed by slit lamp examination and the use of ophtalmic dyes (fluorescein).

The treatment consisted in administering of 1-2 drops three times/day, for 14 days, for all of the affections considered in this trial.

In the cases of glaucoma secondary to the distopias of the crystalline lens, the eye drops showed moderate action of decreasing the intraocular pressure (IOP).

In the cases of chronic superficial keratitis (of dog) and eosinophilic keratitis (of cat), an important action of decreasing proliferation action in the conjunctival and corneal neovascularization was noticed.

Bibliography

1. Leonard A. Levin, Daniel M.Albert, Ocular Disease: Mechanisms and Management, 2010, Elsevier Inc., (2010);
2. Douglas J Coster, Fundamentals of Clinical Ophthalmology – Cornea, BMJ Books, London, (2002);
3. David A. Palay, Jay H. Krachmer, Primary Care Ophthalmology, Elsevier Mosby Inc., (2005);
4. Jimmy D. Bartlett, Siret D Jaanus, Clinical Ocular Pharmacology, fifth edition, by Butterworth-Heinemann, Elsevier, (2008);
5. Jack J. Kanski, Clinical Ophthalmology - A Systematic Approach, sixth edition, by Butterworth-Heinemann, Elsevier, (2007);
6. Merck Manual Online, Corneal Disorders by Melvin I. Roat, Glaucoma by Douglas J. Rhee, (2008);
7. Peter A. Netland , Glaucoma Medical Therapy: Principles and Management, second edition, Oxford University Press, Inc., (2008);
8. Kai Tang, CN 101579488 (A), 2009;
9. Jian Ren, Lan Zhang, Junling Yang, CN 101647913 (A), 2010;
10. Zaghloul El-Nagar "Prophetic Medicine Part 2"-Truffles healing the eye, June 2009; http://www.elakiri.com/forum/showthread.php2t=4830238highlight=Truffles
11. Abu-Rabia A. (1983) "Folk medicine among the Bedouin tribes in the Negev", Social studies center of the Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Ierusalem, p. 17;
12. Murcia M. A., Martinez – Tome M., Vera A., Morte A., Gutierrez A., Honrubia M., Jimenez A. M. (2003) J.Sci. Food Agr. **83,** 535-541;
13. Janakat S., Al-Fakhiri S., Sallal A.-K. (2004) Phytother.Res. **18,** 810-813;
14. Janakat S.M., Al-Fakhiri S.M., Sallal A.-K.J. (2005) Saudi Med.J. **26** (6) 952-955.

PRODUCT OF NATURAL ORIGIN FOR OPHTHALMIC TREATMENT AND OBTAINING PROCEDURE

PATENT CLAIMS

1. Product of natural origin for ophthalmic treatment, active in affections of the cornea and moderately active in glaucoma, characterized by the fact that it consists in an aqueous extract of brown desert truffles of *Terfezia* genus fungi, having a concentration of 13...17%(w/v) as dry matter, containing %(w/v):

-proteins 3.0...5.5
-aminoacids 0.1...0.2
-carbohydrates (as glucose) 0.4...0.6, to which are added
-benzalkonium chloride 0.01 as preservative
-sodium chloride 0.6 as isotonizant and
-demineralized water to 100,
sterile, for ophthalmic treatment as eye drops.

2. Obtaining procedure of the product according to claim 1, characterized by the fact that brown desert truffles of the *Terfezia*-type fungi, fresh or frozen, are extracted with demineralized water, in a ratio of 2-3:1 (w/v) at 4°C; the extract is separated through filtration on a filter aid layer (celite) or centrifugation and it is purified through ultrafiltration on membranes with a cut-off limit of 10 kDa, the permeate solution so obtained is concentrated under vacuum to 1/6-1/9 (v/v), then benzalkonium chloride 0.01% (w/v) and sodium chloride 0.6% (w/v) are added, thus obtaining an eye drops solution which fills in sterile bottles of 5-10 ml.

3. The use of the product according to claim 1 for cornea affections and glaucoma in animals.

INTERNATIONAL SEARCH REPORT

International application No
PCT/RO2011/000001

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/06 A61P27/02 A61K36/062
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 practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MANDEEL ET AL: "Ethnomycological aspects of the desert truffle among native Bahraini and non-Bahraini peoples of the Kingdom of Bahrain", JOURNAL OF ETHNOPHARMACOLOGY, ELSEVIER, vol. 110, no. 1, 8 February 2007 (2007-02-08), pages 118-129, XP005879693, ISSN: 0378-8741, DOI: 10.1016/J.JEP.2006.09.014 the whole document	1-3

-/--

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

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"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

"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
30 January 2012	10/02/2012

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-De Meyere

2

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Zaghloul El-Naggar: "Truffles healing the eye", , 22 February 2010 (2010-02-22), XP002667246, Retrieved from the Internet: URL:www.elakiri.com/forum/showthread.php?t=483023 [retrieved on 2012-01-17] the whole document	1-3
Y	JANAKAT SANA M ET AL: "Evaluation of antibacterial activity of aqueous and methanolic extracts of the truffle Terfezia claveryi against Pseudomonas aeruginosa", SAUDI MEDICAL JOURNAL, vol. 26, no. 6, June 2005 (2005-06), pages 952-955, XP009155549, ISSN: 0379-5284 the whole document	1-3
Y	M. ANTONIA MURCIA ET AL.: "Effect of industrial processing on desert truffles Terfezia claveryi Chatin and Picoa juniperi Vittadini: proximate composition and fatty acids", J. SCI FOOD AGRIC., vol. 83, no. 6, 16 January 2003 (2003-01-16), page 535, XP009155559, JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE 2003 DEP. FOOD SCI., VET. FAC., CAMPUS DE ESPINARDO, UNIV. OF MURCIA, E-30008 MURCIA, SPAIN. E-MAIL MAMURCIA@UM.ES LNKD- DOI:10.1002/JSFA.1397 the whole document	1-3