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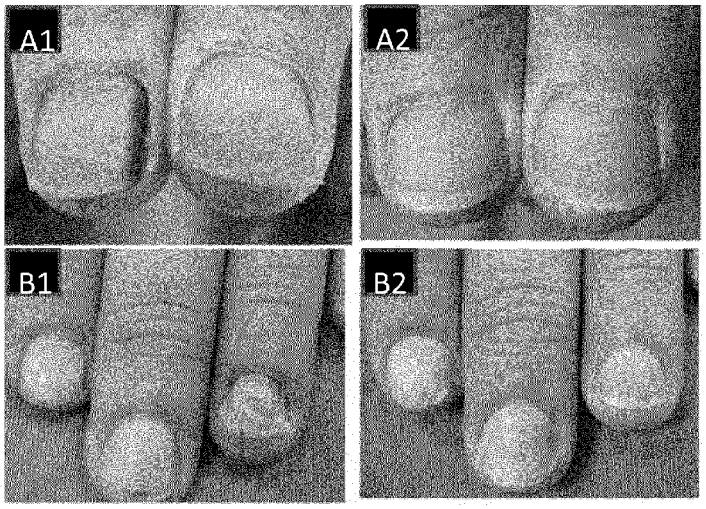
(54) Title: TREATMENT OF CANDIDIASIS WITH INDIGO NATURALIS OR INDIGO-PRODUCING PLANT EXTRACT

(57) Abstract: A pharmaceutical or cosmetic composition comprising Indigo Naturalis or Indigo-producing plant extract for treating candidiasis, and a method of treating candidiasis comprising administering therapeutically effective amount of Indigo Naturalis or Indigo-producing plant extract to a subject in need thereof are described.

FIGURE 1



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TREATMENT OF CANDIDIASIS WITH INDIGO NATURALIS OR INDIGO-PRODUCING PLANT EXTRACT

TECHNICAL FIELD

The present invention relates to a use of an Indigo Naturalis or Indigo-producing plant extract, particular in a use for treating candidiasis.

BACKGROUND

Candidiasis (or candidosis) refers to a diverse group of infections caused by Candida albicans or by other members of the genus Candida. These organisms typically infect the skin, nails, mucous membranes, and gastrointestinal tract, but they also may cause systemic disease.

These infections may be systemic or localized. C. albicans is a dimorphic yeast that is responsible for 70 percent to 80 percent of all candidal infections, which makes it the most common cause of superficial and systemic candidiasis.

C. albicans is often found as a saprophyte and colonizes the mucous membranes of warm-blooded animals. In up to 50 percent of normal individuals, the oropharynx is colonized. In addition, C. albicans exists as a commensal organism in the vaginal mucosa of 20 percent to 25 percent of asymptomatic, healthy women and up to 30 percent of pregnant women. The yeast is rarely isolated from normal human skin except occasionally from intertriginous areas. Likewise, the organism is infrequently found in soil, vegetation, and air samples.

The cutaneous and mucosal manifestations of candidiasis can be divided into several distinct clinical syndromes:

ORAL CANDIDIASIS

- Acute pseudomembranous candidiasis or thrush is the most common form of oral candidiasis. Oral candidiasis appears as discrete white patches that may become confluent on the buccal mucosa, tongue, palate, and gingivae.

- Candida cheilosis (angular cheilitis), so-called perlèche, is characterized by erythema, fissuring, maceration, and soreness at the angles of the mouth.

- Chronic atrophic candidiasis (denture stomatitis) is a common form of oral candidiasis seen in 24 percent to 60 percent of those wearing dentures.

- Acute atrophic candidiasis (erythematous candidiasis) commonly occurs after sloughing of the thrush pseudomembrane. This condition is associated with broad-spectrum antibiotic therapy, glucocorticoid use, and human immunodeficiency virus infection.

VAGINAL AND VULVOVAGINAL CANDIDIASIS

- Approximately three-fourths of all women will experience an episode of vulvovaginal candidiasis (VVC) in their lifetime. C. albicans causes 80 percent to 90 percent of cases of VVC, and C. glabrata is the next most common species involved. Patients generally present with a thick vaginal discharge associated with burning, itching, and occasional dysuria. Examination shows whitish plaques on the vaginal wall with underlying erythema and surrounding edema that can extend to the labia and perineum.

CANDIDA OF MALE GENITALIA: BALANITIS AND BALANOPOSTHITIS

- Candida sp. cause 30 percent to 35 percent of infectious balanitis. C. albicans balanitis presents as small papules or fragile papulopustules on the glans or in the coronal sulcus. These break to leave superficial erythematous erosions with a collarette of whitish scale. Infection may spread to the scrotum and inguinal areas.

CUTANEOUS CANDIDIASIS

- C. albicans has a predilection for colonizing moist, macerated folds of skin. Intertrigo is the most common clinical presentation on glabrous skin. Usual locations for intertrigo include the genitocrural, axillary, gluteal, interdigital, and inframammary areas and between folds of skin on the abdominal wall. Cutaneous candidiasis appears as pruritic, erythematous, macerated

skin in intertriginous areas with satellite vesicopustules. These pustules break open, leaving an erythematous base with a collarette of easily detachable necrotic epidermis.

- Candidal diaper dermatitis is caused by yeast colonization from patients' gastrointestinal tracts. Chronic occlusion by wet diapers furthers the infection. Lesions appear first in the perianal area and spread to the perineum and inguinal creases, which show pronounced erythema

- Candidal paronychia is common in individuals whose hands are habitually involved in wet work (e.g., housekeepers, bakers, fishermen, bartenders). Typically, there is redness, swelling, and tenderness of the paronychial area with prominent retraction of the cuticle toward the proximal nail fold. Secondary nail changes include onycholysis and transverse depressions of the nail plate (Beau's lines) with a brownish or green discoloration along the lateral borders.

DISSEMINATED CANDIDIASIS

CHRONIC MUCOCUTANEOUS CANDIDIASIS

It is desirable to develop medications for treating candidiasis.

SUMMARY

It has been discovered that Indigo Naturalis or Indigo-producing plant extract is particularly efficient for the treatment of candidiasis, more specifically for the topical treatment of candidiasis.

The present invention relates to an Indigo Naturalis or Indigo-producing plant extract that is effective for treating candidiasis.

In one aspect, the present invention provides a pharmaceutical or cosmetic composition comprising Indigo Naturalis or Indigo-producing plant extract for treating candidiasis.

In another aspect, the present invention provides use of Indigo Naturalis or Indigo-producing plant extract in the preparation of a medicament for treating candidiasis.

In further aspect, the present invention provides an Indigo Naturalis or Indigo-producing plant extract for a use in the treatment of candidiasis.

In another aspect, the present invention provides a method of treating candidiasis comprising administering therapeutically effective amount of Indigo Naturalis or Indigo-producing plant extract to a subject (e.g. human) in need thereof.

In the present invention, candidiasis in nail is suffering from nail psoriasis or not suffering from nail psoriasis.

In some embodiments, candidiasis is caused by Candida albicans.

In another aspect of the invention, the present invention provides use of Indigo Naturalis or Indigo-producing plant extract in the preparation of a medicament for treating candidiasis. In some embodiments, the candidiasis is caused by *Candida albicans* infection.

The Indigo Naturalis or Indigo-producing plant extract includes any extract obtained from an Indigo Naturalis or Indigo-producing or Indigo-bearing plant as starting material.

DESCRIPTION OF DRAWINGS

Figure 1:

A1 and B1: Two patients that had *Candida albicans* infection were diagnosed by a dermatologist.

A2 and B2: Improvements have been shown in the patients after treated by lindioil.

DETAILED DESCRIPTION

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art.

Indigo Naturalis, also known as Qingdai, is obtained from one or more plants including *Indigofera tinctoria L., Baphicacanthus cusia (Nees) Bremek (syn. Strobilanthes cusia (Nees), Persicaria tinctoria (Aiton) Spach. (syn. Polygonum tinctorium Aiton, P. tinctorium Lour.) and Isatis tinctoria L. (syn. Isatis indigotica Fort.)*, such as from the plant leaves or stems, and the

leaves and/or stems, after harvest and collection, may be processed by, for example, fermentation. Qingdai is the current name for Indigo Naturalis. Indigo Naturalis is usually a dark-blue powder. It is obtained from Indigo-bearing or Indigo-producing plants with a NaOH or KOH aqueous solution and corresponds to a mixture of around 5-15% organic compounds including alkaloids among which indigo and indirubin are present, and 85-95 % inorganic compounds such as calcium carbonate and calcium hydroxide.

An Indigo Naturalis or Indigo-producing plant extract, as used herein, refers to anextract from Indigo Naturalis or from the leaves and/or stems (or a part thereof) of one or more Indigo-bearing plant or Indigo-producing plant, where the extraction may be performed by using one or more organic solvents, one or more non-organic solvents, or a combination thereof. The extract may include at least one enriched ingredient (having a higher w/w percentage than that existing in Indigo Naturalis) such as tryptanthrin, isatin, indirubin, indigo, or qingdainone. The extract may be a solid, liquid, or any form in-between (*e.g.*, semi-solid).

In a particular embodiment, the Indigo Naturalis or Indigo-producing plant extract is enriched in indirubin, for example the extract may contain indirubin in an amount of at least 65% w/w of the extract, for example, 65%-90% w/w of the extract. The extract may further contain indigo in an amount of 0.1%-15% w/w of the extract. The extract may also contain tryptanthrin and/or qingdainone each in an amount of 0.1-5% w/w.

One example of the Indigo Naturalis or Indigo-producing plant extract is an ethyl acetate extract (EA-extract), which may be prepared as illustrated by Example 2 in this application. The content of each ingredient in the extract may vary. As an example, the extract may contain indirubin in an amount of at least 65% w/w of the extract, for example, 65%-90% w/w of the extract. The extract may further contain indigo in an amount of 0.1%-15% w/w of the extract. The extract may also contain tryptanthrin and/or qingdainone each in an amount of 0.1-5% w/w.

A further example of the Indigo Naturalis extract is an oil extract, particularly an olive oil extract. An oil extract can be prepared by the method disclosed in the patent US8784905. More specifically, the oil extract of is an oil-extracted product of Indigo Naturalis which is obtained by a process comprising extracting Indigo Naturalis powder with an oil under heating,

optionally followed by a refining treatment by filtration. More preferably, in said process, the oil-extracted product is obtained after the refining treatment has a decreased indigo content. In said process, extracting Indigo Naturalis powder is more particularly conducted at an elevated temperature not higher than 155° C, and preferably conducted at a temperature ranging from 100° C. to 155° C. The oil used in said process is preferably selected from the group consisting of vegetable oils, animal oils, mineral oils, and combinations thereof. More preferably, the oil is a vegetable oil and can be selected from the group consisting of olive oil, cottonseed oil, sesame oil, sunflower seed oil, peanut oil, wheat germ oil, soybean oil, jojoba oil, evening primrose oil, coconut oil, palm oil, sweet almond oil, aloe oil, apricot kernel oil, avocado oil, borage oil, hemp seed oil, macadamia nut oil, rose hip oil, pecan oil, hazelnut oil, sasanqua oil, rice bran oil, shea butter, corn oil, camellia oil, grape seed oil, canola oil, castor oil, and combinations thereof. The content of each ingredient in the oil extract may vary. As an example, the extract may contain indirubin in an amount of at least 65% w/w of the total amount of extracted alkaloids, for example, 65%-90% w/w of the total amount of extracted alkaloids. The extract may further contain indigo in an amount of 0.1%-15% w/w of the total amount of extracted alkaloids. The extract may also contain tryptanthrin and/or qingdainone each in an amount of 0.1-5% w/w of the total amount of extracted alkaloids.

Another example of Indigo Naturalis or Indigo-producing plant extract is an extract prepared by a process comprising the following steps:

a) an extraction step: extracting Indigo Naturalis or the leaves and/or stems of one or more Indigo-bearing plants or Indigo-producing plants, preferably selected from the group consisting of *Indigofera tinctoria L., Baphicacanthus cusia (Nees) Bremek (syn. Strobilanthes cusia (Nees)), Persicaria tinctoria (Aiton) Spach. (syn. Polygonum tinctorium Aiton, P. tinctorium Lour.) and Isatis tinctoria L. (syn. Isatis indigotica Fort.)* and/or *Strobilanthes Formosanus*, with a first polar solvent or moderately polar solvent to obtain a mixture of extraction;

b) a filtration step: filtering the mixture of extraction to obtain a filtrate;

c) a concentration step: concentrating the filtrate to obtain a crude extract;

d) a washing step: washing the crude extract with a non-polar solvent, and optionally a second polar solvent, to obtain a washing mixture; and

e) a filtration step: filtering the washing mixture to obtain a refined extract optionally after a drying step, for example, according to a conventional method for drying.

In a particular embodiment, a crude extract obtained from the concentration step c) is subjected to the following procedure for at least one cycle till obtaining a refined extract: the crude extract is washed by a solvent (step d)), and filtered (step (e)) to yield a refined extract, optionally followed by a drying step. According to a specific embodiment, the washing step d) and filtration step e) are performed by only one cycle to obtain the refined extract. When more than one cycle is applied, the same or different solvents for washing can be used. Further, the crude extract can be washed with a solvent under reflux, the mixture can be cooled to room temperature and then filtered to yield a refined extract, optionally followed by a drying step.

In a preferred embodiment, two cycles are performed. Particularly, the crude extract obtained by the concentration step c) is washed in a non- polar solvent, preferably hexane (step d) and filtered (step e), optionally followed by a drying step. The hexane extract is then washed by an organic polar solvent, preferably ethanol (step d) and then filtered (step e) to obtain a refined extract, optionally followed by a drying step.

Optionally, a micronization step is performed after step e), providing thereby a refined extract having a particle size between 25 and 35 μm, preferably of about 30 μm.

In another preferred embodiment, when the refined extract is micronized in the last step, 99% of the obtained particles are less or equal to 30 μm.In a preferred embodiment, a refined extract may be prepared by a process comprising the following steps consisting of: a) (i) adding an extracting solvent, a polar or moderately polar solvent (such as an alcohol or ethyl acetate), to Indigo Naturalis powder to yield a mixture; (ii) heating and stirring the mixture for a period of time (*e.g.*, 30 min, 1 hour, 2 hours); b) (iii) filtering the heated mixture while hot to remove insoluble by-products to yield a filtrate; c) (iv) concentrating the filtrate to yield a crude extract; d) (v) adding a washing solvent (for example, water a non-polar and/or a polar solvent or a mixture thereof) to the crude extract to yield a washing mixture; (vi) heating and stirring the

washing mixture for a period of time (*e.g.*, 30 min, 1 hour, 2 hours); e) (vii) filtering the washing mixture, for example at room temperature (e.g. 18-35°C) to collect a refined extract; optionally (viii) repeating steps (v) to (vii) until the amount of indirubin (% w/w) in the refined extract is more than 55% (w/w), preferably more than 65% (w/w) as measured by HPLC method, and optionally (ix) drying the residue according to a conventional method (*e.g.*, air-drying, *lyophilization*) to obtain a dried extract. The washing solvent in steps (v) and (viii) can be the same or different.

In a more preferred embodiment, a refined extract is prepared by a process comprising the steps of:

a) extracting Indigo Naturalis with ethanol at reflux between 2 and 8 hours,

b) filtering the mixture at a temperature not less than 65°C to obtain a filtrate,

c) concentrating the filtrate, to obtain a crude extract, said crude extract is optionally filtered (with addition of water) in order to remove completely the solvent and the last components still present in the solvent and dried,

d) (i) washing the crude extract with hexane at a temperature not less than 50°C between 15 and 60 min,

(ii) filtering at room temperature the mixture obtained at step d) (i) to obtain a product, optionally rinsing it with ethanol and water

(iii) washing the product obtained at step d) (ii) with ethanol at reflux, and

e) filtering at room temperature the washing mixture obtained at step d) and drying the resulting product at a temperature less than 80°C to obtain an extract which is optionally micronized.

In another preferred embodiment, when the refined extract is micronized in the last step, the particle size is around 99% in the range 25 to 35 μm, preferably of about 30 μm.

As used herein, "about" or "around" will be understood by a person of ordinary skill in the art and will vary to some extent on the context in which it is used. If there are uses of the term which are not clear to persons of ordinary skill in the art given the context in which it is used, "about" or "around" will mean up to plus or minus 20%, preferably 10% of the particular term.

The term "refined extract", as used herein, refers to a solid, semi-solid or oily extract which contains less than 10% (w/w) of water and/or solvents used in the process for preparing the said refined extract. A refined extract is more preferably characterized by an increase amount of active ingredients, including alkaloids among which indigo, indirubin, tryptanthrin, and/or qingdainone are present, preferably enriched in indirubin, compared to Qingdai or Indigo Naturalis. More specifically, the refined extract according to the invention comprises at least 60%, or more preferably more than 65 %, (w/w) of active ingredients, including indigo, indirubin, tryptanthrin, and/or qingdainone.

The term "crude extract", as used herein, refers to a solid, semi-solid or oily extract which contains less than 15% (w/w) (e.g., 5-15%, 5-10%) of water and/or solvents used in the process for preparing the refined extract. The crude extract is less enriched in indirubin, than the refined extract as compared to Qingdai or Indigo Naturalis. The crude extract is obtained by the concentration step c) according to the invention. The concentration step is more particularly carried out by sending the filtrate to a concentrator (for instance at reduced pressure), as to remove water and/or solvents used in the process and concentrating thereby the active ingredients present in the extract, including indigo, indirubin, tryptanthrin, and/or qingdainone.

"one cycle", as used herein, refers to the two steps of the washing step d) and filtration step e) which are performed sequentially once. "two cycles", as used herein, refers to the two steps of the washing step d) and filtration step e) which are performed sequentially twice.

According to a specific embodiment, the Indigo Naturalis or Indigo-producing plant extract according to the invention is an oil extract as defined above or an extract of Indigo-producing plant obtained by the process as above detailed comprising steps (a)-(e), optionally including one of the above described specific embodiments.

Candidiasis, as used herein, should be understood as a disease or disorder of skin, mucosa or nail caused by *Candida albicans* wherein *Candida albicans* is a primary cause of the disease or a superinfecting agent.

A therapeutically effective amount refers to a dose level of an Indigo Naturalis or Indigo-producing plant extract that yields a therapeutic benefit (for example, amelioration, reduction or cure of the diseases, disorder or symptoms of candidiasis) to a patient on average.

The present invention provides a composition comprising an Indigo Naturalis or Indigo-producing plant extract for treating candidiasis, preferably to treat topically candidiasis. According to a specific embodiment, the composition comprises an Indigo-producing plant extract as the sole active ingredient.

The extract may be used directly without further formulation or included in a pharmaceutical or cosmetic composition that comprises the extract.

The extract may comprise indirubin in an amount of at least 65% w/w of the extract, for example, 65%-90% w/w of the extract. It may further comprise indigo in an amount of 0.1%-15% w/w of the extract, and in another further embodiment, the extract may also comprise indigo in an amount of 0.1%-15% w/w of the extract and tryptanthrin and/or qingdainone each in an amount of 0.1-5% w/w of the extract.

The compositions, methods or uses of the invention may be used alone (i.e., in replacement of current treatments) or in combination with current treatments to improve their efficacy.

In an embodiment, Indigo Naturalis or Indigo-producing plant extract is used as the sole active ingredient (e.g. as a single therapy). According to this embodiment, the composition preferably comprises an Indigo-producing plant extract as the sole active ingredient.

In another embodiment, the Indigo Naturalis or Indigo-producing plant extract can be used in combination with at least one other therapy.

A pharmaceutical composition may be formulated into a suitable dosage form for topical or oral administration using technology well known to those skilled in the art. The pharmaceutical composition can additionally comprise a pharmaceutically acceptable carrier such as those widely employed in the art of drug-manufacturing. For instance, the pharmaceutically acceptable carrier may include one or more of the following agents: solvents such as olive oil, olive oil refined, cottonseed oil, sesame oil, sunflower seed oil, peanut oil, wheat germ oil, soybean oil, jojoba oil, evening primrose oil, coconut oil, palm oil, sweet

almond oil, aloe oil, apricot kernel oil, avocado oil, borage oil, hemp seed oil, macadamia nut oil, rose hip oil, pecan oil, hazelnut oil, sasanqua oil, rice bran oil, shea butter, corn oil, camellia oil, grape seed oil, canola oil, castor oil, and combinations thereof, preferably olive oil refined, emulsifiers, suspending agents, decomposers, binding agents, excipients, stabilizing agents, chelating agents, diluents, gelling agents, thickening agent such as beeswax and/or petroleum jelly, preservatives, lubricants, absorption delaying agents, liposomes, such as butylhydroxytoluene or butylhydroxyanisole and the like. A topical formulation suitable for the pharmaceutical composition according to the present invention may be an emulsion, a gel, an ointment, a cream, a patch, an embrocation, an aerosol, a spray, a lotion, a serum, a paste, a foam, or a drop. In some embodiments, the pharmaceutical composition is formulated into an external preparation by admixing the extract according to the present invention with a base such as those that are well known and commonly used in the art.

According to a specific embodiment, the compositions, methods or uses of the invention are suitable for a topical treatment of candidiasis.

In some embodiments, the dosage and the frequency of administration of the pharmaceutical composition according to the present invention may vary depending on the following factors: the severity of the disease to be treated, the route of administration, and the weight, age, physical condition and response of the subject to be treated. In further or additional embodiments, the amount of the extract is in the range of about 0.001 to about 1000 mg/kg body weight/day, for example, about 0.01 to about 500, 300, or 100mg/kg body weight/day. In further or additional embodiments, administration can be performed daily or even several times per day, if necessary. By way of examples, the extract of the invention can be administered once, twice, three, four, five or six times a week or more, or once, twice, three or four times a day or more. Duration of the treatment may vary and depends on the severity of the subject. It may last for instance from one week to several months (such as from 2, 3, 4, 5, 6 or 7 weeks to 12, 18, 24, 30, or 36 weeks).

The present invention also provides a cosmetic composition comprising the extract. The composition may be present in a form adapted for topical application comprising a cosmetically

or dermatologically acceptable carrier or medium. "Cosmetically or dermatologically acceptable" means media which are suitable for a use in which they come into contact with the skin or human skin appendages without posing a risk of toxicity, intolerance, instability, allergic reaction, etc. In the cosmetic composition, the extract may be previously solubilized in one or more cosmetically or dermatologically acceptable solvents, such as water, glycerol, ethanol, propylene glycol, butylene glycol, dipropylene glycol, ethoxylated or propoxylated diglycols, cyclic polyols, petroleum jelly, a vegetable oil or any mixture of these solvents.

In some embodiments, a composition may contain 0.001-10 mg, for example 0.01-1 mg of one or more active ingredients per 1g composition.

The present invention provides a method of treating candidiasis, comprising administering a therapeutically effective amount of an Indigo Naturalis or Indigo-producing plant extract to a subject in need thereof. The extract and compositions above can be used in the treatment or alleviation of a disease or condition. By treatment it is meant at least an alleviation of the symptoms associated with the pathological condition afflicting the subject, where alleviation is used in a broad sense to refer to at least a reduction in the magnitude of a parameter, *e.g.* symptom, associated with the pathological condition being treated. As such, treatment also includes situations where the pathological condition, or at least symptoms associated therewith, are completely inhibited, *e.g.*, prevented from happening, or stopped, e.g., terminated, such that the host no longer suffers from the pathological condition, or at least the symptoms that characterize the pathological condition. As such, treatment includes both curing and managing a disease condition. Accordingly, the extract and compositions above can be used in the treatment or alleviation of candidiasis.

The efficacy of the extract and compositions can be evaluated by *in vivo* models with respect to their activities in treating diseases or disorders, for example, clinically trails on humans.

The subject in need of candidiasis treatment according to the invention is any mammal, including a human or a non-human mammal, preferably a human mammal.

Within the context of the invention, the term treatment denotes curative, symptomatic, and preventive treatment. The compositions of the invention will not necessarily cure the patient who has candidiasis but will diminish in a satisfactory manner delaying or slowing thereby the progression or preventing thereby complications of candidiasis. This will ameliorate consequently the patients' skin condition. The compositions of the invention can also be administered to those who do not have candidiasis yet but who would normally develop candidiasis or be at increased risk for said disease, they will not develop said disease. Treatment also includes delaying the development of candidiasis in an individual who will ultimately develop said disease or would be at risk for the disease due to age, familial history, or genetic abnormalities. By delaying the onset of candidiasis, compositions of the invention have prevented the individual from getting candidiasis during the period in which the individual would normally have gotten candidiasis or reduce the rate of development of candidiasis or some of its symptoms.

The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present application will be obtained by reference to the following detailed description that sets forth illustrative embodiments.

While embodiments of the present invention have been shown and described herein such embodiments are provided by way of example only. It should be understood that the above described embodiments may be combined if compatible and various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. Those ordinary skilled in the art will appreciate that numerous variations, changes, and substitutions are possible without departing from the invention. It is intended that the following claims define the scope of aspects of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art. All documents, or portions of documents, cited herein including, without

limitation, patents, patent applications, articles, books, manuals, and treatises are hereby expressly incorporated by reference in their entirety for any purpose.

The percentage herein is expressed by weight relative to the weight of the extract, unless otherwise specified.

Further aspects and advantages of the invention will be disclosed in the following illustrative experimental section.

EXAMPLES

1. Preparation of refined Indigo Naturalis or Indigo-producing plant extracts and analytical methods for analysis

Example 1: Preparation of a refined Indigo Naturalis extract

Qingdai as used in the following preparation is obtained from Delong Pharmaceutical (Indigo 2.62% and Indirubin 0.284% (HPLC method depicted in Example 7A) and tryptanthrin 0.0046%.

500g of Qingdai were suspended in 10L ethyl acetate. The mixture was stirred in reflux for two hours, and then filtered at 75°C. The filtrate was concentrated at reduced pressure to yield a dark solid. The crude extract was stirred in 250mL hexane and heated to reflux for one hour. After cooling to room temperature, the suspension was filtered to give a dark residue.

0.50g of the dark residue were refluxed in 25mL hexane again for one hour, and cooled to room temperature, followed by filtration to give a refined extract as a dark red solid 452mg. HPLC: 62.9% indirubin, 12.9% indigo, and 0.53% tryptanthrin.

Example 2: Preparation of a refined Indigo Naturalis extract

500g of Qingdai as used in Example 1 were suspended in 10L alcohol (ethanol). The mixture was stirred in reflux for two hours, and then filtered at 75°C. The filtrate was concentrated at reduced pressure to yield a dark solid, which was stirred in 260mL hexane and

heated to reflux for one hour. Upon cooling to room temperature, the suspension was filtered to give a dark residue.

0.80g of the dark residue were refluxed in 24mL alcohol (ethanol) for an additional hour, and then cooled to room temperature, followed by filtration to give a refined extract as a dark red solid (538mg). HPLC: 83.6% indirubin, 6.35% indigo, and 0.75% tryptanthrin.

Example 3: Preparation of a refined Indigo Naturalis extract

500g of Qingdai as used in Example 1 were suspended in 10L ethyl acetate. The mixture was stirred in reflux for two hours, and then filtered while hot. The filtrate was concentrated at reduced pressure to yield a dark solid. The crude extract was stirred in 250mL hexane and heated to reflux for one hour. After cooling to room temperature, the suspension was filtered to give a dark residue.

0.75g of the dark residue were refluxed in 22.5mL ethanol for one hour, and cooled to room temperature, followed by filtration to give a refined extract as a dark red solid (538mg). HPLC: 77.9% indirubin, 15.9% indigo, and 0.56% tryptanthrin.

Example 4: Preparation of a refined Indigo Naturalis extract

500g of Qingdai as used in Example 1 were suspended in 2.1L DMF. The mixture was stirred at 50°C for 40 minutes. Upon cooling to 20°C, the suspension was filtered. The filtrate was concentrated at reduced pressure to yield a dark solid, which was stirred in 130mL hexane and heated to reflux for one hour. Upon cooling to 20°C, the suspension was filtered to give a dark residue.

1.56g of the dark residue was washed with 46.8 ml ethanol, and heated to reflux for one hour, and then cooled to 20°C, followed by filtered to yield a refined extract (766mg). HPLC: 66.3%, indirubin, 9.76% indigo.

Example 5: Preparation of a refined Indigo Naturalis extract

500g of Qingdai as used in Example 1 were suspended in 3L DMF. The mixture was stirred

at 30°C for 1 hour, and then filtered. The filtrate was concentrated at reduced pressure to yield a dark solid, which was stirred in 230mL hexane and heated to reflux for one hour. Upon cooling to 20°C, the suspension was filtered to give a dark residue.

1.96g of the dark residue was washed with 59mL 85% ethanol (85% aq. alcohol), and heated to reflux for one hour followed by filtration while hot to yield a refined extract (1.02g). HPLC: 69.4% indirubin, 18.7% indigo, and 0.62% tryptanthrin.

Example 6: Preparation of a refined Indigo Naturalis extract

100g of Qingdai was extracted with 2L of ethanol 92% (92% aqueous ethanol) for 2 hours under reflux conditions. Upon completion, the mixture was filtered while hot on AF6 filter (Buchner) to obtain a dark blue-red solution as a filtrate. This filtrate was reduced under vacuum to dryness to give 2.4 g of dry residue. This residue was washed with 120 mL of hexane for 1h under reflux. Upon completion, the mixture was cooled to room temperature for 2h then filtered under vacuum to yield 312.9mg of a dark red refined extract.

280mg of this refined extract were washed with 15 mL of ethanol 92% (92% aqueous ethanol) for 1h under reflux. Upon completion the solution was cooled to room temperature, and then filtered to yield 159 mg of a dark red/burgundy refined extract after drying in oven (80°C) for 1h30. (0.18%); HPLC: 82.31% indirubin, 8.99% indigo, and 0.81% tryptanthrin.

Example 7: Micronization step

The micronization step of refined Indigo Naturalis extract obtained in the previous examples is performed with the following equipment:

- Micronizer: spiral jet Mill Diameter 200
- Feeder: this equipment is used for the dosage of powder to feed the micronizer. The dosage is made thanks to two screws. This system allows a regularity of the flow.

Micronization consists to project grains of powder with jet of air. The contact of grains permits their explosion.

Following parameters of micronization are recorded during the micronization:

- Ring pressure: 6 bar
- Injector pressure: 3 bar
- The flow of powder feed: 25 kg/h

The micronizer allows a cylindrical enclosure - holes around the enclosure for the injection of air.

Powder is introduced in the micronizer; grains are propelled thanks to jet of air. When grains have the good size, they are concentrated in the center of the micronizer and they are breathed. To avoid any contamination by foreign particles or broken pieces of the equipment, an additional sieving (sieve: 700 μm) is performed.

The step is done manually after the micronization and before the packaging.

A granulometric analysis of the homogeneous product obtained was carried out according to the particular size distribution (PSD) method [Analytical specifications: D99 ≤ 30 μm].

2. Clinical assay

Here we present two patients (A and B) who had psoriatic nails coexisting with candidiasis and paronychia who were dissatisfied with or did not respond to other treatment methods responding dramatically to topical treatment of Indigo Naturalis extract in oil (Lindioil, the refined olive oil extract prepared in example 2, US 8,784,905).

The patients' characteristics are shown in table 1. The age of these two patients is 72 (A) and 57 (B) years old.

Patients were instructed to drip and apply one to two drops of Lindioil (0.05ml) onto the nail plate fold and hyponychium of affected nails once or twice daily. The two patients had the paronychia and nail bed discoloration in their fingernails disappear, on average, after 6 months of treatment with Lindioil. The two patients' improvements were made with no adverse side effects.

Table 1. Demographics

Patient	Age,y	Gender	Duration of psoriasis (skin / nail), y	Date of treatment start	Date of the lesion disappear
A	72	Male	2 / 1	20090224	20090714
B	57	Male	3 / 2	20090801	20100210

3. **Microbiological assay**

3.1. The Preparation of Indigo Naturalis.

The species for Indigo Naturalis varies in different parts of the world. *Strobilanthes formosanus* Moore, which is the species available in Taiwan, was harvested in Sansia, New Taipei City, Taiwan. The harvested leaves of *Strobilanthes formosanus* Moore were immersed in water for several days until the leaves were decomposed by microbial activities. After that, lime was the only substance added to the suspension to precipitate Indigo Naturalis. The chemicals included in Indigo Naturalis are different from *Strobilanthes formosanus* Moore due to the fermentation process.

3.2. Preparation of Ethyl Acetate (EA) Extracts of Strobilanthes Formosanus Moore and Indigo Naturalis.

The leaves of *Strobilanthes formosanus* Moore were dried in an oven (40 °C) for three days. Twenty-five g of the dried *Strobilanthes formosanus* Moore leaves were extracted with ethyl acetate (EA, 300 mL) at 40 °C for one hour. Indigo Naturalis (25 g) was also extracted by the same procedure. The EA-extractable compounds of the two samples were then separated from the residue by centrifugation (12,000 × *g*, 20 min) at 15 °C. The supernatant was recovered and evaporated to dryness under reduced pressure and stored at −20 °C for bioassays.

The antifungal effects of Indigo Naturalis extract (extraction with ethyl acetate) were assayed in the following fungal species: *T. rubrum* ATCC 10,218, *M. gypseum* ATCC 144,683, *E. floccosum* ATCC 18,397, *C. albicans*, *A. fumigates* and *C. neoformans*. Results showed *A. fumigates* and *C. albicans* were mildly inhibited by Indigo Naturalis extract in a dose-dependent manner (Table 2). Furthermore, Indigo Naturalis extract also showed a weak inhibitory effect on *S. cerevisiae* and little, if any, effect on the other three tested dermatophytes (Table 2).

Table 2. The antifungal effects of Indigo Naturalis extract on tested Fungi.

Test Strains	Quantity of Indigo Naturalis EA-extract per disc (mg)				
	0 mg	1 mg	2 mg	3 mg	4 mg
Fungi					
A. fumigates	0 ± 0	39 ± 8	51 ± 9	68 ± 15	79 ± 13
C. albicans	0 ± 0	31 ± 5	43 ± 7	48 ± 11	53 ± 10
C. neoformans	0 ± 0	0 ± 0	0 ± 0	0 ± 0	18 ± 4
S. cerevisiae	0 ± 0	0 ± 0	0 ± 0	24 ± 9	28 ± 12
E. floccosum	0 ± 0	0 ± 0	0 ± 0	0 ± 0	9 ± 3
T. rubrum	0 ± 0	0 ± 0	0 ± 0	0 ± 0	14 ± 5

*The diameters of the inhibition zones (diameter of inhibition zone minus diameter of disc) were measured in mm after incubation for 24 hours at the optimal temperature for the individual strains tested. Data are the means of triplicates ± standard deviation of a representative experiment.

CLAIMS

1. A pharmaceutical or cosmetic composition comprising an Indigo Naturalis or Indigo-producing plant extract for a use in the treatment of candidiasis.

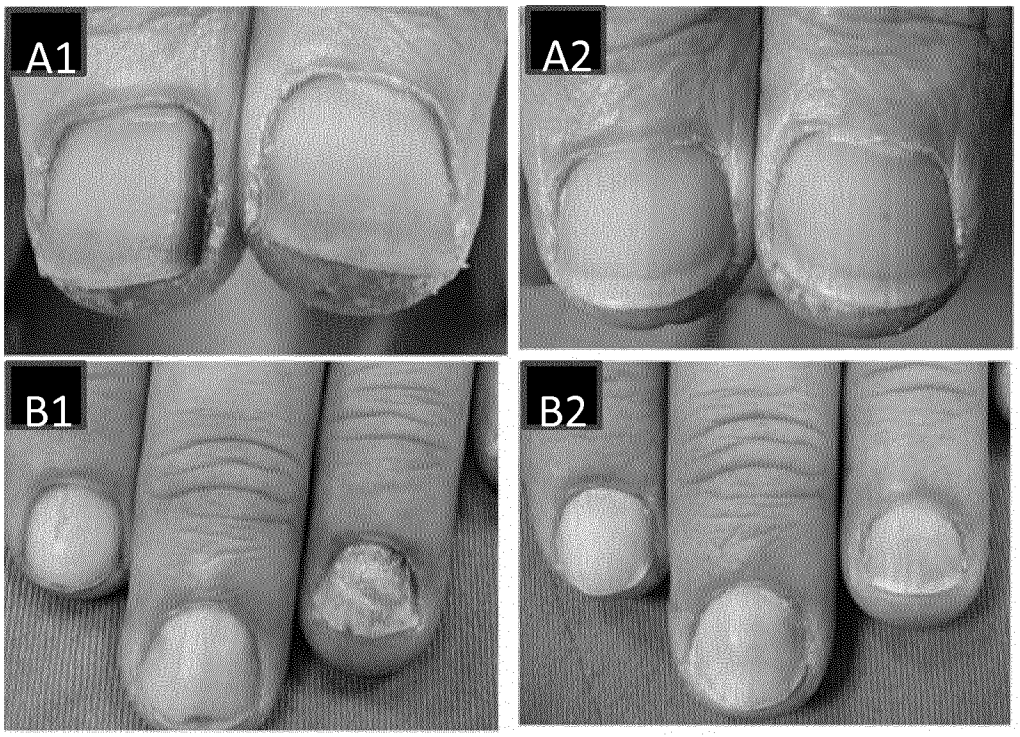
2. The pharmaceutical or cosmetic composition for a use according to claim 1, wherein the candidiasis is caused by *Candida albicans* infection.

3. An Indigo Naturalis or Indigo-producing plant extract for a use in the treatment of candidiasis.

4. The Indigo Naturalis or Indigo-producing plant extract for a use according to claim 3, comprising indirubin in an amount of at least 65% w/w of the extract, preferably 65%-90% w/w of the extract.

5. The Indigo Naturalis or Indigo-producing plant extract for a use according to any one of claims 3 or 4, wherein the candidiasis is caused by *Candida albicans* infection.

FIGURE 1



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/057766

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K36/19 A61K36/315 A61K36/48 A61P17/00
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 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, 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	DATABASE WPI Week 201450 Thomson Scientific, London, GB; AN 2014-M58458 XP002758541, & CN 103 766 415 A (TIANJIN ZHONGKEJIAN NEW MATERIAL TECHNOL) 7 May 2014 (2014-05-07) abstract ----- -/--	1-5

[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

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

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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
8 June 2016	21/06/2016

Name and mailing address of the ISA/	Authorized officer
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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.
X	CHIANG Y R ET AL: "An in vitro study of the antimicrobial effects of indigo naturalis prepared from Strobilanthes formosanus Moore", MOLECULES: A JOURNAL OF SYNTHETIC ORGANIC AND NATURAL PRODUCT CHEMISTRY, M D P I AG, CH, vol. 18, no. 11, 21 November 2013 (2013-11-21), pages 14381-14396, XP002720345, ISSN: 1420-3049, DOI: 10.3390/MOLECULES181114381 table 2	1-5
X	PONNUSAMY K ET AL: "In vitro antifungal activity of indirubin isolated from a South Indian ethnomedicinal plant Wrightia tinctoria R. Br", JOURNAL OF ETHNOPHARMACOLOGY, ELSEVIER IRELAND LTD, IE, vol. 132, no. 1, 28 October 2010 (2010-10-28), pages 349-354, XP027417971, ISSN: 0378-8741 [retrieved on 2010-10-13] abstract	1-5
X	EP 0 987 027 A1 (HAYASHIBARA BIOCHEM LAB [JP]) 22 March 2000 (2000-03-22) paragraph [0023]; claims; examples	1-5

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Information on patent family members

International application No
PCT/EP2016/057766

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