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(54) Title: ANTI-FUNGAL COMPOSITIONS

(57) Abstract: The present invention relates to anti-fungal uses of plant-derived saponins. In particular, the invention deals with the anti-fungal uses of momordin Icand its glucopyranosyl derivatives for the treatment of fungal infections such as for example caused by the genus *Candida*, more particularly the species *Candida albicans* and *Candida glabrata*.



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ANTI-FUNGAL COMPOSITIONS

Field of the invention

The present invention relates to anti-fungal uses of plant-derived saponins. In particular, the invention deals with the anti-fungal uses of momordin Ic and its glucopyranosyl derivatives for the treatment of fungal infections such as for example caused by the genus *Candida*, more particularly the species *Candida albicans* and *Candida glabrata*.

Introduction to the invention

Fungal infections are becoming a major health concern for a number of reasons, including the limited number of anti-fungal agents available, the increasing incidence of species resistant to older anti-fungal agents, and the growing population of immunocompromised patients at risk for opportunistic fungal infections. The most common clinical isolate is *Candida albicans* (comprising about 19% of all isolates). Neutropenic patients (neutropenia due to, e.g. chemotherapy, immunosuppressive therapy, infection, including AIDS, or an otherwise dysfunctional immune system) are predisposed to the development of invasive fungal infections, most commonly by *Candida* or *Aspergillus* species, or, on occasion, *Fusarium*, *Trichosporon*, *Cryptococcus* and *Dreschlera*. Fungal biofilms also represent a persistent source of disseminated infections in high-risk patients and are recalcitrant to antifungal therapy. There are a large number of anti-fungal compounds or agents currently on the market that have limited clinical applications due to either the emergence of fungal resistance or unwanted adverse effects. One major group includes polyene derivatives, including amphotericin B and the structurally related compounds nystatin and pimaricin, which are only administered intravenously. These are broad-spectrum anti-fungals that bind to ergosterol, a component of fungal cell membranes, and thereby disrupt the membranes, leading to cell death. The second major group of anti-fungal agents includes azole derivatives which impair synthesis of ergosterol and lead to accumulation of metabolites that disrupt the function of fungal membrane-bound enzyme systems (e.g. cytochrome P450) and inhibit fungal growth. Significant inhibition of mammalian P450 enzymes results in important drug interactions. This group of agents includes ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, fluconazole and itraconazole. These agents may be administered to treat systemic mycoses. The third major group of anti-fungal agents includes allylamines-thiocarbamates, which are generally used to treat skin infections. This group

includes tolnaftate and naftifine. Another anti-fungal agent is griseofulvin, a fungistatic agent which is administered orally for fungal infections of skin, hair or nails that do not respond to topical treatment. Echinocandins are a further well-known group of anti-fungal compounds. Echinocandins (e.g. caspofungin) are lipopeptides that inhibit the synthesis of glucan in the cell wall, probably via non-competitive inhibition of the enzyme 1,3-beta glucan synthase, and structure-function studies have indicated that the fatty acid side chain of the echinocandin B ring is required for anti-fungal activity. Echinocandins are limited to intravenous administration and cannot be applied orally. Furthermore, several clinically relevant *Candida* species such as *C. glabrata* cannot be eradicated with echinocandins.

The present invention identified oleanolic acid-based saponins like momordin 1c and its glucopyranosyl derivatives as promising anti-fungal compounds. Momordin 1c is one of several saponins derived from oleanolic acid, a triterpenoid. These chemical compounds are found in some plants of the *Momordica* genus as well as in other Asian herbal medicine plants such as *Kochia scoparia* and *Ampelopsis radix*. Momordin 1c has been found to be safe, orally bioavailable, well tolerated and has amongst many potential uses anti-pruritic, anti-inflammatory and anti-allergic activities (Grover JK *et al* (2004) *J. of Ethnopharmacology* 93, 123-132). In the present invention we demonstrate that a particular important use of momordin 1c and its glucopyranosyl derivatives is in the treatment of *Candida* infections, in particular *Candida glabrata* biofilms. Surprisingly, similar concentrations of momordin 1c are equally effective against planktonic and biofilm fungal populations.

Figures

Figure 1: Activity of four extracts of *Kochia scoparia* on preformed biofilms of *Candida albicans* (A) and *C. glabrata* (B). Extracts were added at a final concentration of 1% to 24h-grown biofilms. Metabolic activity was measured by XTT assay in triplicate.

Figure 2: Growth inhibitory activity of the ethanol extract of *Kochia scoparia* in liquid assays. Cells of *C. albicans* wild type strain were treated for up to 27h with three concentrations of the extract (1%, full circles; 0.5%, full squares; 0.25%, full triangles) or with DMSO only (open circles) as a control condition.

Figure 3: Identification of the active fractions of the ethanol extract of *K. scoparia*. Three solvents were used in the separation procedure of the original dried ethanol extract: 40% ACN (A), 60% ACN (B), and 80% ACN (C). Fractions were collected every minute during 60 to 70 minutes. In each panel, the upper graph represents the HPLC chromatogram of the fractions,

while the lower graphs illustrate *C. albicans* biofilm assay treated with each fraction at a final concentration of 1% (as measured by XTT reduction assay in triplicate, and calculated as percentage of metabolic activity compared to DMSO control (labelled 1 in the fractions axis)).

Figure 4: Identification of two active peaks from three active fractions of the ACN eluates. (A)

5 Biofilm assays were conducted in presence of 1% final concentration of each of the samples, or DMSO as a control sample. *C. albicans* biofilms (left panel) and *C. glabrata* biofilms (right panel) were tested for their susceptibility to the five purified peaks. (B) Fractions 43, 36 and 31 of the 40%, 60%, and 80% ACN eluates respectively were pooled together and analysed as one sample, with a gradient from 40% to 100% solvent B in 60 minutes.

10 Figure 5: Anti-biofilm activity of momordin Ic and its aglycon, oleanolic acid. Momordin Ic (A) and oleanolic acid (B) were dissolved in DMSO and tested at the indicated concentrations (final DMSO concentration of 1% in each treatment). The graphs on the left represent *C. albicans* mature biofilm assays. The graphs on the right illustrate *C. glabrata* mature biofilm assays.

15 Figure 6: Growth inhibitory activity of momordin Ic and oleanolic acid in liquid assays. Cells of *C. albicans* (left) and *C. glabrata* (right) were grown for 27h in presence of momordin Ic at 100 µg/ml (full circles), 50 µg/ml (full squares), 25 µg/ml (full triangles), of oleanolic acid at 100 µg/ml (open diamonds), and of DMSO only (open circles) as a control condition.

20 Figure 7: Anti-biofilm activity of the glucopyranosyl derivative of momordin Ic (peak1) and its potential synergism with momordin Ic. Mature biofilms of *C. albicans* (A) and *C. glabrata* (B) were treated for 24h with concentrations ranging from 100 to 12.5 µg/ml of the glucopyranosyl derivative of momordin Ic (peak1) alone, or with momordin Ic alone at 12.5 µg/ml, or with both compounds at the stated concentrations (grey bars). The final DMSO concentration in each treatment was 1%.

25 Figure 8: Anti-biofilm activity of momordin Ic *in vivo*. Momordin Ic was injected daily by intraperitoneal injection into rats challenged with catheters infected with *C. albicans* biofilms. Biofilms, 9 per animal, were analysed by colony-forming units (CFU) after seven days of treatment, with DMSO alone in saline solution (control group, left line)), with 1mg/kg/day momordin Ic (middle line), or with 2 mg/kg/day momordin Ic (right line). Each data point (dot)
30 represents one biofilm developed in one catheter piece. For each treatment, 27 biofilms were analysed and the mean value of CFU recovered in each biofilm is represented by a red diamond. Statistical significance is highlighted (*) with a p-value below 0.001.

Detailed description of the invention

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., *current Protocols in Molecular Biology* (Supplement 100), John Wiley & Sons, New York (2012), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

Candida species are increasingly resistant to common therapeutic treatments, and in particular to azoles. While resistance to echinocandins is less prevalent, some *Candida* species, such as *C. glabrata* and *C. parapsilosis* can present some intrinsic resistance. Extracts from 35 different Chinese plants were tested for their antifungal activity against *C. albicans* and *C. glabrata* planktonic and biofilm growths (Liu Q *et al* (2012) *J. Ethnopharmacol.* 143(3):772-8). Ethanol and/or acetone extracts of *Kochia scoparia* displayed a high inhibitory activity against mature *Candida* biofilms. Fractionation of the ethanol extract of *Kochia scoparia* followed by NMR analysis revealed two compounds with antifungal activity belonging to the triterpenoid saponin family.

Accordingly the invention provides in a first embodiment momordin Ic and its glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid (CAS Registry number: 152203-45-7) for use as a medicament.

The compound momordin Ic is one of several saponins derived from oleanolic acid, a triterpenoid. More specifically Momordin Ic is known as (3 β)-17-carboxy-28-norolean-12-en-3-yl 3'-O-(β -D-xylopyranosyl)- β -D-glucuronide and the CAS Registry number of momordin Ic is 96990-18-0. Momordin Ic is obtained as colorless fine crystals that decompose at 240 °C. Momordin Ic has been described as a compound inhibiting gastric emptying and having anti-pruritic and anti-inflammatory effects. Interestingly, momordin Ic is orally bioavailable and is non-toxic.

In a particular embodiment said momordin Ic and its glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid are administered sequentially as a medicament.

In another particular embodiment the administration of momordin Ic precedes the administration of its glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid.

In another embodiment the administration of the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid precedes the administration of momordin Ic.

In yet another embodiment the invention provides momordin Ic thereof for use as an anti-fungal agent.

In yet another embodiment the invention provides glucopyranosyl derivatives of momordin Ic for use as an anti-fungal agent.

In yet another embodiment the invention provides the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid for use as an anti-fungal agent.

In yet another embodiment the invention provides momordin Ic and/or glucopyranosyl derivatives thereof for use as an anti-fungal agent.

In yet another embodiment the invention provides momordin Ic and glucopyranosyl derivatives thereof for use as an anti-fungal agent.

In yet another embodiment the invention provides momordin Ic and the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid for use as an anti-fungal agent.

In yet another embodiment the invention provides momordin Ic and/or the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid for use as an anti-fungal agent.

It should be clear to the skilled practitioner that the dose and dosage regimen will depend mainly on whether momordin Ic or a combination between momordin Ic and a glucopyranosyl derivative thereof, in particular the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid, is (are) being administered separately or as a combination (a mixture), the type of fungal infection to treat, the specific application, the patient and the patient's history. The amount must be effective to achieve a fungicidal or fungistatic effect or an amount, which is synergistic particularly when used in a combination (i.e. momordin Ic and a glucopyranosyl derivative of momordin Ic). The practitioner will be able to ascertain upon routine experimentation which route of administration and frequency of administration are most effective in any particular case.

It is understood that the anti-fungal activity of momordin Ic and/or a glucopyranosyl derivative of momordin Ic such as for example the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid can be tested using standard techniques known in the art to determine whether the separate compound or the combination is suitable for the use as a specific anti-fungal compound. As used herein a compound of the invention (or compounds of the invention) refers to momordin Ic alone or the combination of momordin Ic with a glucopyranosyl derivative of momordin Ic or more particularly the combination between momordin Ic and the specific glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid. As is known in the art, anti-fungal activity of a compound (or a combination of compounds) may result in the killing or eradication of fungal cells (i.e. fungicidal activity), in the slowing or arrest of the growth or proliferation of fungal cells (i.e. fungistatic activity), and/or in the prevention of fungal cell growth, and/or the prevention of the formation of a fungal biofilm and/or the destruction of an established fungal biofilm. Thus, the compounds of the invention may be fungicidal, fungistatic, and/or may prevent the growth or the biofilm formation of fungal cells. It is understood that compounds of the invention (i.e. momordin Ic and/or a glucopyranosyl derivative thereof) that slow or arrest fungal cell growth may also be useful in combination treatments with other known anti-fungal agents. Exemplary methods of testing the compounds of the invention are

provided below and in the examples included herein. These methods can be used to test the anti-fungal activity of momordin Ic, or in combination with a glucopyranosyl derivative, or in still a further combination with other anti-fungal agents. One skilled in the art will understand that other methods of testing the anti-fungal activity of compounds are known in the art and are also suitable for testing compounds.

In vitro methods of determining the ability of the compounds of the invention to inhibit the growth of fungal cells are well-known in the art. In general, these methods involve contacting a culture of the cells of interest with various concentrations of the compounds and monitoring the growth of the cell culture relative to an untreated control culture. A second control culture comprising cells contacted with a known anti-fungal agent may also be included in such tests, if desired. For example, the ability of a compound of the invention to inhibit the growth of specific fungal cells can readily be determined by measurement of the minimum inhibitory concentration (MIC) for the compound. The MIC is defined as the lowest concentration that inhibits growth of the specific fungus to a pre-determined extent. For example, a MIC₁₀₀ value is defined as the lowest concentration that completely inhibits growth of the organism, whereas a MIC₉₀ value is defined as the lowest concentration that inhibits growth by 90% and a MIC₅₀ value is defined as the lowest concentration that inhibits growth by 50%. MIC values are sometimes expressed as ranges, for example, the MIC₁₀₀ for a compound may be expressed as the concentration at which no growth is observed or as a range between the concentration at which no growth is observed and the concentration of the dilution which immediately follows. Techniques for determining anti-fungal MIC values for candidate compounds include both macrodilution and microdilution methods (see for example Pfaller, M. A. *et al* (1997), *Clin. Infect. Dis.* 24:776-84). As is known in the art, different types of fungi may require different testing methods. For example, suitable reference methods for testing MIC values for candidate compounds in yeasts, include the NCCLS reference method for broth dilution anti-fungal susceptibility testing of yeasts (M27-A2, Vol. 22 No. 15). This method can be used to test MIC values in yeasts such as *Candida* species, and *Cryptococcus neoformans*, for example. Alternatively, suitable reference methods for determining MIC values for candidate compounds in filamentous fungi include the NCCLS reference method for broth dilution anti-fungal susceptibility testing of filamentous fungi. In the classical broth microdilution method, the anti-fungal compound is diluted in culture medium in a sterile, covered 96-well microtiter plate. An overnight culture of a single fungal colony is diluted in sterile medium such that, after inoculation, each well in the microtiter plate contains an appropriate number of CFU/mL (typically, approximately $5 \cdot 10^3$ CFU/mL). Culture medium only (containing no fungal cells) is also included as a negative control for each plate, and known anti-fungal compounds are often included as positive controls. The inoculated microtiter plate is subsequently incubated at an

appropriate temperature (for example, 35°C for 16-48 hours). The turbidity of each well is then determined by visual inspection and/or by measuring the absorbance, or optical density (OD), at 595 nm or 600 nm using a microplate reader and is used as an indication of the extent of fungal growth.

- 5 In accordance with one embodiment of the invention, a compound of the invention is considered to have an anti-fungal effect against a given fungus when the MIC of the compound (when used alone) for 80% inhibition of growth of the fungus is about 75 µg/mL or less. In one embodiment, a compound of the invention is considered to have an anti-fungal effect against a given fungus when the compound has a MIC for 80% inhibition of growth of about 64 µg/mL or
10 less. In another embodiment, a compound of the invention is considered to have an anti-fungal effect against a given fungus when the compound has a MIC for 80% inhibition of growth of about 50 µg/mL or less. In another embodiment, a compound of the invention is considered to have an anti-fungal effect against a given fungus when the compound has a MIC for 80% inhibition of growth of about 35 µg/mL or less. In other embodiments, a compound of the
15 invention is considered to have an anti-fungal effect against a given fungus when the compound has a MIC for 80% inhibition of growth of about 25 µg/mL or less, about 16 µg/mL or less and about 12.5 µg/mL or less.

- Anti-fungal effects may also be expressed as the percentage (%) inhibition of planktonic growth or of biofilm-associated growth of a given fungus over a pre-determined period of time
20 by treatment with a single concentration of a candidate compound. This method provides a rapid method of assessing the ability of a compound to inhibit fungal growth, for example, prior to conducting more in-depth tests, such as MIC determinations or *in vivo* testing. The wording 'fungal cell growth' refers to either biofilm-associated growth or planktonic growth or either biofilm-associated growth and planktonic growth. The predetermined period of time depends
25 on the given fungus being tested. Thus in one embodiment, the ability of a candidate compound to inhibit fungal cell growth is tested over a predetermined amount of time of between about 18 to about 24 hours. In another embodiment, the ability of a candidate compound to inhibit fungal cell growth is tested over a predetermined amount of time of about 48 hours. In yet another embodiment, the ability of a candidate compound to inhibit fungal cell
30 growth is tested over a predetermined amount of time of between about 48 to about 72 hours.

In another embodiment of the invention, a compound of the invention is considered to be an anti-fungal agent when it is capable of inhibiting the growth of a given fungus by about 25% when used at a concentration of about 25 µg/mL, with growth of the fungus being assessed over the appropriate predetermined amount of time. In another embodiment, a candidate

compound is considered to be a potential anti-fungal agent when it is capable of inhibiting the growth of a given fungus by about 50% when used at a concentration of about 25 µg/mL, with growth of the fungus being assessed over the appropriate predetermined amount of time. In another embodiment, a candidate compound is considered to be a potential anti-fungal agent when it is capable of inhibiting the growth of a given fungus by about 75% when used at a concentration of about 25 µg/mL, with growth of the fungus being assessed over the appropriate predetermined amount of time. In another embodiment, a candidate compound is considered to be a potential anti-fungal agent when it is capable of inhibiting the growth of a given fungus by about 80% when used at a concentration of about 25 µg/mL, with growth of the fungus being assessed over the appropriate predetermined amount of time.

One skilled in the art will appreciate that compounds that exhibit poor anti-fungal activity for a certain fungus when used alone may still be capable of good anti-fungal activity when used in combination with one or more known anti-fungal agents. For example, the compound may sensitize the fungal cells to the action of the other agent(s), it may act in synergy with agent(s), or it may otherwise potentiate the activity of the agent(s). Compounds of the invention thus include compounds that exhibit poor activity as sole agents but good activity in combination with other anti-fungal agents. The ability of a compound to exert an effect in combination with a known anti-fungal agent can be tested using standard methods, such as those described above. In addition, the ability of a compound of the invention to exhibit a synergistic effect in combination with another anti-fungal agent can be tested by standard methods, such as the measurement of the fractional inhibitory concentration (FIC) index.

The ability of a compound of the invention to act as an anti-fungal agent can also be tested *in vivo* using standard techniques. A number of animal models are known in the art that are suitable for testing the activity of anti-fungal compounds and are readily available. Representative examples of animal models suitable for testing the anti-fungal activity of a compound of the invention *in vivo* include, but are not limited to, the severe combined immunodeficiency (SCID) mouse model and a colostrum-deprived SPF piglet model for *Cryptosporidium parvum* infection, a granulocytopenic rabbit model of disseminated Candidiasis, a mouse model of disseminated Aspergillosis, a neutropenic rat model of disseminated Candidiasis and the *in vivo* biofilm animal models used in the present examples.

Methods for conducting *in vivo* tests to determine the activity of anti-fungal compounds are well-known in the art. Typically, *in vivo* testing comprises introducing a selected fungus into the appropriate animal model in a sufficient amount to cause infection, followed by administration of one or more doses of the compounds of the invention. Methods of administration will vary

depending on the compound being employed, but can be, for example, by way of bolus infusion into a suitable vein (such as the tail vein of mice or rats), or preferably by oral administration. In a specific embodiment the compounds of the invention are applied in a topical administration. Topical administration of the compounds of the invention is particularly useful for the treatment of mycotic (fungal) vaginitis. It is for example known in the prior art that antipruritic and anti-inflammatory effects are attributed to the use of momordin Ic which is a particular advantage over other antifungal compounds. Indeed patients suffering from fungal infections often complain of itching. Animals treated with a known anti-fungal agent and/or with a saline or buffer control solution serve as controls. Repeat doses of the compounds of the invention may be administered to the animal, if necessary, at appropriate time intervals. The animals are subsequently monitored daily for mortality. When tested by such methods, a compound of the invention is considered to exert an *in vivo* anti-fungal effect if it results in a decrease in mortality of at least about 15% in treated animals compared to test animals. In one embodiment of the invention, a compound of the invention is considered to exert an *in vivo* anti-fungal effect if it results in a decrease in mortality of at least about 25% in the treated animals. In another embodiment, a compound of the invention is considered to exert an *in vivo* anti-fungal effect if it results in a decrease in mortality of at least about 40% in the treated animals. In other embodiments, a compound of the invention is considered to exert an *in vivo* anti-fungal effect if it results in a decrease in mortality of at least about 50%, at least about 60%, at least about 70%, at least about 80% or at least about 90% in the treated animals.

In some contexts, for example when used *in vivo*, it is important that the anti-fungal compounds of the invention exhibit low toxicity. As such, the compounds of the invention may be submitted to toxicity tests, if desired, to determine their suitability for *in vivo* use. Toxicity tests for potential drugs are well-known in the art.

In yet another embodiment the invention provides for the use of a compound of the invention for the inhibition, prevention or eradication of the growth and/or proliferation of fungi, optionally in combination with one or more known anti-fungal agents. In one embodiment, the invention provides a method of inhibiting fungal growth by contacting a fungus with an effective amount of one or more compounds of the invention optionally in combination with one or more other anti-fungal agents. Representative examples of fungi that may be inhibited with compounds of the invention include, but are not limited to, *Histoplasma* (e.g. *H. capsulatum*), *Coccidioides*, *Blastomyces*, *Paracoccidioides*, *Cryptococcus* (e.g. *C. neoformans*), *Aspergillus* (e.g. *A. fumigatus*, *A. niger*, *A. nidulans*, *A. terreus*, *A. sydowi*, *A. flavatus*, and *A. glaucus*), *Zygomycetes* (e.g. *Basidiobolus*, *Conidiobolus*, *Rhizopus*, *Mucor*, *Absidia*, *Mortierella*, *Cunninghamella*, and *Saksenaaea*), *Candida* (e.g. *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C.*

stellatoidea, *C. krusei*, *C. parakrusei*, *C. lusitaniae*, *C. pseudotropicalis*, *C. guilliermondi* and *C. glabrata*), *Cryptosporidium parvum*, *Sporothrix schenckii*, *Piedraia hortae*, *Trichosporon beigeli*, *Malassezia furfur*, *Phialophora verrucosa*, *Fonsecae pedrosoi*, *Madurella mycetomatis* and *Pneumocystis carinii*. In a specific embodiment, the compounds according to the invention have anti-fungal activity against yeasts. Examples of yeasts that are susceptible to the anti-fungal effects of these compounds include, but are not limited to *Candida* sp. and *Cryptococcus* sp. In one embodiment, the compounds according to the invention exhibit anti-fungal activity including activity against *Cryptococcus neoformans*. In another embodiment, the compounds according to the invention exhibit anti-fungal activity including activity against *Candida albicans*. In still another embodiment, the compounds according to the invention exhibit anti-fungal activity including activity against *Candida glabrata*, *Candida krusei* or *Candida parapsilosis*.

In another embodiment, the compounds according to the invention have anti-fungal activity that includes activity against filamentous fungi. Examples of filamentous fungi include, but are not limited to *Aspergillus* sp., *Fusarium* sp., and *Rhizopus* sp. In one embodiment, the compounds according to the invention exhibit anti-fungal activity including activity against *Aspergillus* sp. In another embodiment, the compounds according to the invention exhibit anti-fungal activity including activity against *Aspergillus fumigatus*.

In another embodiment, the compounds of the invention exhibit anti-fungal activity against a broad spectrum of fungi and are suitable for use as broad-spectrum anti-fungal agents. One embodiment of the invention thus provides for the use of compounds of the invention as broad spectrum anti-fungal agents. Another embodiment of the invention provides for the use of compounds of the invention as broad spectrum anti-fungal agents.

In a specific embodiment, the compounds of the invention are capable of inhibiting the growth of or killing dermatophytes. Examples of dermatophytes include, but are not limited to *Epidermophyton* sp., *Microsporum* sp., and *Trichophyton* sp.

One embodiment of the invention provides for the use of the anti-fungal compounds of the invention in the treatment of subjects having an infection with a species of *Candida*, *Cryptococcus*, or *Aspergillus*, or having a disease or disorder related to infection with a species of *Candida*, *Cryptococcus*, or *Aspergillus*. Examples of diseases and disorders related to *Candida* infection include, but are not limited to, candidiasis, vaginitis, monilia, thrush, skin rash, diaper rash, nail bed infections and esophagitis. Examples of diseases and disorders related to *Cryptococcus* infection include, but are not limited to, cryptococcosis, meningitis, hepatitis, osteomyelitis, prostatitis, pyelonephritis and peritonitis. Examples of diseases and

disorders related to *Aspergillus* infection include, but are not limited to, aspergillosis, chronic lung irritation, hypersensitivity pneumonia, allergic bronchopulmonary aspergillosis, aspergilloma, tracheobronchitis, acute necrotising *Aspergillus* pneumonia, chronic necrotising *Aspergillus* pneumonia and granulomatous aspergillosis. In accordance with a further embodiment of the invention, one or more compounds of the invention may be used in combination with one or more known anti-fungal agents in combination or synergistic therapy for the treatment of fungal infection, or disorders or diseases associated therewith. The compounds of the invention can be administered before, during or after treatment with the known anti-fungal agent(s). Such combination therapy is known in the art and selection of the appropriate anti-fungal agent(s) to be administered with the compounds of the invention is readily discernible by one of skill in the art. For example, for the treatment of fungal infections and fungus-related diseases, known anti-fungal compounds include, but are not limited to, amphotericin B and the structurally related compounds nystatin and pimaricin; flucytosine; azole derivatives such as ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, fluconazole and itraconazole; allylamines-thiocarbamates, such as tolnaftate and naftifine, and griseofulvin. In yet another embodiment the invention also contemplates the use of compounds of the invention as the active ingredient in anti-fungal compositions for non-therapeutic uses including, for example, anti-fungal cleansers, polishes, paints, sprays, soaps, and detergents. The compounds of the invention can also be included as an anti-fungal agent in cosmetic, personal care, household and industrial products, for example, to improve shelf-life by inhibiting the growth of fungi within the products. The compounds may be formulated for application to surfaces to inhibit the growth of a fungal species thereon, for example, surfaces such as countertops, desks, chairs, laboratory benches, tables, floors, sinks, showers, toilets, bathtubs, bed stands, tools or equipment, doorknobs and windows. Alternatively, the compounds may be formulated for laundry applications, for example, for washing clothes, towels, sheets and other bed linen, washcloths or other cleaning articles. The anti-fungal cleansers, polishes, paints, sprays, soaps, and detergents comprising the compounds of the invention can optionally contain suitable solvent(s), carrier(s), thickeners, pigments, fragrances, deodorisers, emulsifiers, surfactants, wetting agents, waxes, or oils, as required for the formulation of such products as is known in the art. The cleansers, polishes, paints, sprays, soaps, and detergents comprising the compounds of the invention are useful in institutions, such as in hospital settings, for the prevention of nosocomial infections, as well as in home settings. In one embodiment, the invention provides a formulation containing one or more compounds of the invention for external use as a pharmaceutically acceptable skin cleanser. In yet another embodiment the

compounds of the invention can be used in agriculture. In the latter case they are formulated in the form of an agricultural formulation.

In addition, in one embodiment, the invention contemplates the use of compounds of the invention in formulations to inhibit the growth of fungal species in food preparations. In another embodiment, the invention contemplates the use of compounds of the invention in formulations to sterilise surgical and other medical equipment and implantable devices, including prosthetic joints. The compounds can also be formulated for use in the *in situ* sterilisation of indwelling invasive devices such as intravenous lines and catheters, which are often foci of infection. As such the compounds of the invention can be used for the coating of surfaces of devices or implants to prevent biofilm associated growth of fungi on the latter.

In another embodiment, the invention contemplates the use of the compounds of the invention as the active ingredient in personal care items, such as soaps, deodorants, shampoos, mouthwashes, toothpastes, and the like. Many compositions used in personal care applications are susceptible to fungal growth and it is thus desirable to incorporate into these compositions an effective anti-fungal agent. The anti-fungal agent may be incorporated into the personal care formulation using techniques known in the art. Thus, the anti-fungal agent may be added to the personal care formulation as a solution, emulsion or dispersion in a suitable liquid medium. Alternatively, the anti-fungal agent may be added, undiluted, to the personal care formulation or may be added with a suitable solid carrier or diluent. The anti-fungal agent may be added to the pre-formed personal care formulation or may be added during the formation of the personal care formulation, either separately or premixed with one of the other components of the formulation.

In yet another embodiment the invention provides a pharmaceutical anti-fungal composition comprising a compound of the invention and a pharmaceutical acceptable carrier. A pharmaceutical anti-fungal composition of the invention can be either: i) momordin Ic and a pharmaceutical acceptable carrier, ii) momordin Ic and a glucopyranosyl derivative thereof and a pharmaceutical acceptable carrier, or iii) momordin Ic and the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl) oleanolic acid and a pharmaceutical acceptable carrier. For use as therapeutic agents in the treatment of fungal infections, or disorders or diseases associated therewith in a subject, the anti-fungal compounds of the invention are typically formulated prior to administration. Therefore, the invention provides pharmaceutical formulations comprising a compound of the invention and a pharmaceutically-acceptable carrier, diluent, or excipient. The pharmaceutical formulations can be prepared by standard procedures using well-known and readily available ingredients. In

making the compositions of the invention, the active ingredient(s) may be mixed with a carrier, or diluted by a carrier, or enclosed within a carrier, and may be in the form of a capsule, sachet, paper, or other container. The carrier may also serve as a diluent and may be a solid, semi-solid, or liquid material. The pharmaceutical compositions comprising the anti-fungal compounds according to the invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g. by inhalation or insufflation of powders or aerosols, including by nebulizer, intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intra-arterial, subcutaneous, intraperitoneal or intramuscular injection or infusion, or intracranial, e.g. intrathecal or intraventricular administration.

The anti-fungal compounds of the invention may be delivered alone or in combination with other anti-fungal agents, and may be delivered along with a pharmaceutically acceptable vehicle. Ideally, such a vehicle would enhance the stability and/or delivery properties. The invention thus provides for administration of pharmaceutical compositions comprising one or more of the compounds of the invention using a suitable vehicle, such as an artificial membrane vesicle (including a liposome and the like), microparticle or microcapsule. The use of such vehicles may be beneficial, for example, in achieving sustained release of the anti-fungal compound(s). For administration to an individual for the treatment of an infection or disease, the invention also contemplates the formulation of the pharmaceutical compositions comprising the anti-fungal compounds into oral dosage forms such as tablets, capsules and the like. For this purpose, the compounds can be combined with conventional carriers, such as magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethyl-cellulose, low melting wax, cocoa butter and the like. Diluents, flavouring agents, solubilizers, lubricants, suspending agents, binders, tablet-disintegrating agents and the like can also be employed, if required. The anti-fungal compounds can be encapsulated with or without other carriers. In accordance with the invention, the proportion of anti-fungal compound(s) in any solid and liquid composition will be at least sufficient to impart the desired activity to the individual being treated upon oral administration. The invention further contemplates parenteral injection of the anti-fungal compounds, in which case the compounds are formulated as a sterile solution containing other solutes, for example, enough saline or glucose to make the solution isotonic.

For administration by inhalation or insufflation, the anti-fungal compounds can be formulated into an aqueous or partially aqueous solution, which can then be utilized in the form of an

aerosol. Aqueous formulations of the anti-fungal compounds of the invention may also be used in the form of ear or eye drops, or ophthalmic solutions. The invention further contemplates topical use of the anti-fungal compounds. For this purpose they can be formulated as dusting powders, creams or lotions in pharmaceutically acceptable vehicles, which are applied to affected portions of the skin.

Compositions intended for oral use may be prepared according to procedures known in the art for the manufacture of pharmaceutical compositions and such compositions may further contain one or more sweetening agents, flavouring agents, colouring agents, preserving agents, or a combination thereof, in order to provide pharmaceutically elegant and palatable preparations. Tablets typically contain the anti-fungal compound(s) in admixture with non-toxic pharmaceutically acceptable excipients suitable for the manufacture of tablets, such as inert diluents, for example, calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example, starch, gelatine or acacia, and lubricating agents, for example, magnesium stearate, stearic acid or talc. The tablets may be uncoated or they may be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monostearate or glyceryl distearate may be employed.

Formulations for oral use may also be presented as hard gelatin capsules wherein the anti-fungal compound(s) is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example, peanut oil, liquid paraffin or olive oil.

Aqueous suspensions typically contain the anti-fungal compound(s) in admixture with excipients suitable for the manufacture of aqueous suspensions, such as suspending agents (for example, sodium carboxymethylcellulose, methyl cellulose, hydropropylmethyl cellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia); dispersing or wetting agents such as a naturally-occurring phosphatide (for example, lecithin), or condensation products of an alkylene oxide with fatty acids (for example, polyoxyethylene stearate), or condensation products of ethylene oxide with long-chain aliphatic alcohols (for example, hepta-decaethyleneoxycetanol), or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol (for example, polyoxyethylene sorbitol monooleate), or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides (for example, polyethylene sorbitan monooleate). The aqueous suspensions may further contain one or more preservatives, for example, ethyl, or n-propyl-p-hydroxy benzoate;

one or more colouring agents; one or more flavouring agents, or one or more sweetening agents, such as sucrose or saccharin, or a combination thereof.

Oily suspensions may be formulated by suspending the anti-fungal compound(s) in a vegetable oil, for example, peanut oil, olive oil, sesame oil or coconut oil, or in a mineral oil
5 such as liquid paraffin. The oily suspensions may contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents such as those set forth above, and flavouring agents may be added to provide palatable oral preparations. These compositions may be preserved by the addition of an anti-oxidant such as ascorbic acid. Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of
10 water provide the anti-fungal compound in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents and suspending agents are exemplified by those mentioned above. Additional excipients, for example, sweetening, flavouring and colouring agents, may also be present. Pharmaceutical compositions of the invention may also be in the form of oil-in-water emulsions. The oil phase
15 may be a vegetable oil, for example, olive oil or peanut oil, or a mineral oil, for example, liquid paraffin, or mixtures thereof. Suitable emulsifying agents may be naturally-occurring gums (for example, gum acacia or gum tragacanth); naturally-occurring phosphatides (for example, soy bean lecithin), and esters or partial esters derived from fatty acids and hexitol anhydrides (for example, sorbitan monooleate), and condensation products of the partial esters with ethylene
20 oxide (for example, polyoxyethylene sorbitan monooleate). The emulsions may also contain sweetening and flavouring agents. Syrups and elixirs may be formulated with sweetening agents, for example, glycerol, propylene glycol, sorbitol or sucrose. Such formulations may also contain one or more demulcents, preservatives or flavouring and colouring agents, or combinations thereof. The pharmaceutical compositions may be in the form of a sterile
25 injectable aqueous or oleaginous suspension. This suspension may be formulated according to known art using suitable dispersing or wetting agents and suspending agents as described above. The sterile injectable preparation may also be a solution or a suspension in a non-toxic, parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution and
30 isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. Typically, a bland fixed oil is employed for this purpose such as a synthetic mono- or diglyceride. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Adjuvants, local anaesthetics, preservatives and/or buffering agents, may also be included in the injectable formulation. The one or more compounds of the
35 invention may be administered, together or separately, in the form of suppositories for rectal or vaginal administration of the compound. These compositions can be prepared by mixing the

compound with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at the rectal/vaginal temperature and will therefore melt to release the compound. Examples of such materials include cocoa butter and polyethylene glycols. Another formulation of the invention employs transdermal delivery devices ("patches"). Such transdermal patches
5 may be used to provide continuous or discontinuous infusion of the anti-fungal compounds of the invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art (see for example, US5023252). Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents. It may be desirable or necessary to introduce the pharmaceutical composition to the
10 brain, either directly or indirectly. Direct techniques usually involve placement of a drug delivery catheter into the host's ventricular system to bypass the blood-brain barrier. An example of such an implantable delivery system, used for the transport of compounds to specific anatomical regions of the body, is described in US5011472. The dosage of the anti-fungal compound to be administered is not subject to defined limits, but will usually be an
15 effective amount. In general, the dosage will be the equivalent, on a molar basis, of the pharmacologically active free form produced from a dosage formulation upon the metabolic release of the active free drug to achieve its desired pharmacological and physiological effects. The pharmaceutical compositions are typically formulated in a unit dosage form, each dosage containing from, for example, about 0.01 to about 100 mg of the anti-fungal compound. The
20 term "unit dosage form" refers to physically discrete units suitable as unitary dosages for administration to human subjects and other animals, each unit containing a predetermined quantity of anti-fungal compound calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient. Typical daily dosages of the anti-fungal compounds fall within the range of about 0.01 to about 200 mg/kg of body weight in single or
25 divided dose. However, it will be understood that the amount of the compound actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the actual compound administered, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope
30 of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be adequate, while in other cases still larger doses may be employed without causing any harmful side effect, for example, by first dividing larger doses into several smaller doses for administration throughout the day.

In yet another embodiment the invention additionally provides for therapeutic kits containing
35 one or more compounds of the invention in pharmaceutical compositions or unit dosage forms, alone or in combination with one or more other anti-fungal agents, for use in the treatment of

5 fungal infections, or fungus-related diseases or disorders. Individual components of the kit can be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human or animal administration. The kit can optionally further contain one or more other anti-fungal agents for use in combination with the compound(s) of the invention. The kit may optionally contain instructions or directions outlining the method of use or dosing regimen for the compound(s) and/or additional anti-fungal agents. When the components of the kit are provided in one or more solutions, the solution can be an aqueous solution, for example a sterile aqueous solution. In this case the container means may itself be an inhalant, syringe, pipette, eye dropper, or other such like apparatus, from which the solution may be administered to a subject or applied to and mixed with the other components of the kit. The components of the kit may also be provided in dried or lyophilised forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the administration of the final composition or unit dosage form to a patient. Such an instrument may be an inhalant, syringe, pipette, forceps, measured spoon, eye dropper or similar medically approved delivery vehicle.

10 20 It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells, animals and humans and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

25

Examples

1.The ethanol extract of *Kochia scoparia* displays a strong inhibitory activity against *Candida* species planktonic growth and mature biofilms

30 The main objective of the screening approach with different types of extract was to identify the best one(s) for further analysis or for future use. Both the water extract and the ethanol extracts showed some anti-biofilm activity (Figure 1A). With a reduction of biofilm by almost 100%, as assayed by XTT, the ethanol extract was tested further. When added prior to biofilm

formation, it strongly reduced adhesion and biofilm development (data not shown). In addition, the ethanol extract of *K. scoparia* displayed a high potency against *C. glabrata* biofilms as well (Figure 1B).

These data prompted us to test the activity of the ethanol extract in liquid assays, and to assess the fungicidal or fungistatic effect of the plant extract on *Candida* species. Strong growth inhibitory activity was observed only with the highest final concentration of 1% (Figure 2) against *C. albicans* wild type strain, and appeared to have a fungistatic effect. Strains of *C. glabrata*, *C. krusei* and *C. parapsilosis* were also susceptible to the extract (data not shown).

2. Identification of the active fractions of the ethanol extract of *K. scoparia*

Three solvents were tested to generate fractions of the extract. Amongst dichloromethane (DCM), methanol (MeOH), and acetonitrile (ACN), 80% ACN revealed the best activity against biofilms and free-living cells (data not shown). Hence, the extract was separated on C18 columns from 100 g of plant material. The dried ethanol extract was dissolved in 40%, 60% and 80% ACN, and the successive elutions were analysed by HPLC and tested in biofilm assays to identify the active fractions (Figure 3). Activity against biofilm was observed in the fractions corresponding to the largest peak on the chromatograms, and that for each of the three solvents used. Sixty fractions for each of the 40% and 60% ACN, and 71 fractions from the 80% ACN elution were tested at a final concentration of 1%, and analysed in comparison to the DMSO control treatment.

Strong activity (similar to the original ethanol extract) against biofilms was obtained with successive fractions for each of the solvents. Fractions 43 to 47 of the 40% ACN elution showed the highest anti-biofilm activity with less than 10% of the biofilm's metabolic activity, as measured by the reduction assay, remaining in presence of these fractions (Figure 3A). Similarly, fractions 36 to 43 of the 60% ACN elution (Figure 3B) and fractions 31 to 34 of the 80% ACN elution (Figure 3C) abolished mature biofilm's activity by more than 90%. Interestingly, the same fractions were also potent at the same concentration against *C. glabrata* mature biofilms (data not shown). Three active fractions, fraction 36 of the 60% eluate, fraction 43 of the 40% eluate, and fraction 31 of the 80% ACN eluate were analysed then by chromatography. The analysis revealed the presence of two major peaks in the 40% and 60% ACN eluates, and of one peak in the 80% ACN sample. Each of these five peaks were purified and tested against *Candida* biofilms (Figure 4A).

The so-called peak 1 and peak 2 of each of the three fractions were named as such as they were suspected to be similar between the fractions due to their high resemblance on

chromatograms (data not shown). An inhibitory activity of at least 90% remained in the peak 2 samples from each fraction (Figure 4A), against both species of *Candida* tested. The peak 1 samples had an attenuated anti-biofilm activity of around 40 to 50% against *C. albicans* biofilms only. *C. glabrata* biofilms showed no susceptibility to these samples. The nomenclature of peak 1 and peak 2 was retained as indeed a chromatographic analysis of a mixed sample of the three fractions revealed the presence of two peaks only (Figure 4B), which illustrated the presence of two different compounds. Peak 2 was the most abundant, which may explain the higher anti-biofilm activity of the peak 2 samples. As the exact concentration of each of the two peaks was not established at this step of the procedure, further work and analysis were conducted for both peaks.

3.NMR analysis reveals the identity of one of the active compound as the saponin momordin Ic

The two potential active compounds present in the fractions were analysed by NMR for identification. One compound, corresponding to the most potent peak (peak 2), was identified as the glycoside momordin Ic, which belongs to the saponin family of secondary metabolites. Momordin Ic or (3 β)-17-carboxy-28-norolean-12-en-3-yl 3'-O-(β -D-xylopyranosyl)- β -D-glucuronide (C₄₁H₆₄O₁₃) derives from oleanolic acid, a triterpenoid (Wen *et al.*, 1995). The antifungal activity of the commercially available glycoside and its aglycon were tested in biofilm assays to determine their potential antifungal activity (Figure 5).

In biofilm assays, the saponin greatly inhibited mature biofilms of both *C. albicans* and *C. glabrata* species, even at the lowest concentration tested of 25 μ g/mL. More than 80% of the biofilm's activity was abolished in presence of the saponin. However, the aglycon component had a reduced activity against biofilms. Concentrations of 100 μ g/ml of oleanolic acid eliminated biofilm activity by less than 50%, and had little effect on *C. glabrata* biofilms. The momordin Ic activity was similar to the one of peak 2, at equal concentrations (data not shown), suggesting that most of the antifungal activity, if not all, is due to the presence of the saponin in these samples. Interestingly, the antifungal activity of momordin Ic in liquid assays required high doses of the compound, as 100 μ g/ml was the only active concentration against both species (Figure 6). MIC determinations showed that concentrations between 12.5 μ g/ml and 25 μ g/ml were required to inhibit the growth of both *C. albicans* and *C. glabrata* (data not shown). These data suggest that, the contrary to known antifungals, such as azoles and echinocandins for which 10 to 100 times higher concentrations are needed to establish an anti-biofilm activity, momordin Ic seems efficient against planktonic and biofilm populations at similar concentrations.

The second potential active compound (peak 1 in figure 4) was identified by HSQC-NMR as the glucopyranosyl derivative of momordin Ic, 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid (Wang *et al.*, 2003). The anti-biofilm activity of this compound was tested *in vitro*, and data are shown in figure 7. At equal concentrations, momordin Ic is more potent than its derivative. However, the combination of the two compounds was seemingly more potent than each of the compounds alone. At concentrations of the compounds alone that did not fully inhibit biofilms (20% and no inhibition by 12.5 μ g/mL of peak1, and 50% and no inhibition by 12.5 μ g/mL of momordin Ic against *C. albicans* and *C. glabrata* biofilms, respectively), the combinatorial effect of the two molecules led to an increased biofilm inhibition. More than 80% inhibition of *C. albicans* mature biofilms and more than 90% inhibition of *C. glabrata* mature biofilms were obtained in presence of both compounds at 12.5 μ g/mL.

4. The saponin momordin Ic significantly reduces biofilm burdens *in vivo*

In vivo efficacy of momordin Ic, previously described as non-toxic (Kim *et al.*, 2005), was assessed in a subcutaneous biofilm rat model (Riccova *et al.*, 2010). A seven-day daily treatment (intra-peritoneal delivery) of 1mg/kg proved to be effective in reducing significantly the biofilm biomass developed in animals (Figure 8). By comparison, we have previously shown that intraperitoneal injection of fluconazole at 125 mg/kg had no effect on *C. albicans* biofilm, whereas injection of anidulafungin at 10 mg/kg reduced biofilm burdens by one log₁₀ (Kucharikova *et al.*, 2010).

Materials and methods

1. Strains

Wild type *Candida albicans* SC5314 strain (Gillum *et al.*, 1984) and *C. glabrata* ATCC2001 strain (CBS138, American Type Culture Collection) were used in all assays. They were routinely propagated on rich medium. Strains were grown on Sabouraud dextrose agar plate for biofilm assays and MIC tests.

2. Chinese plants

Thirty-three medicinal plants were selected based on their documented use for treating fungal infections in China (Liu *et al.* 2012). Relevant information on these materials has been summarised in Table1.

Table 1: List of 15 traditional Chinese medicinal (TCM) plants and 18 folk medicinal plants.

	Chinese name	Taxonomic name	Medicinal part	Origin (Province)	Supplier
TCM plants	Baibu	<i>Stemona sessilifolia</i> (Miq.) Miq.	Radix <i>Stemonae</i>	Hubei	a
	Baixianpi	<i>Dictamnus dasycarpus</i> Turcz.	Cortex <i>Dictamni</i>	Neimenggu	
	Chuanjiao	<i>Zanthoxylum bungeanum</i> Maxim.	Pericarpium <i>Zanthoxyli</i>	Sichuan	
	Danpi	<i>Paeonia suffruticosa</i> Andr.	Cortex <i>Moutan</i>	Henan	
	Difuzi	<i>Kochia scoparia</i> (L.) Schrad.	Fructus <i>Kochiae</i>	Hebei	
	Dingxiang	<i>Eugenia caryophyllata</i> Thunb.	Flos <i>Caryophylli</i>	Guangdong	
	Gaoliangjiang	<i>Alpinia officinarum</i> Hance	Rhizoma <i>Alpiniae officinarum</i>	Guangdong	
	Huangbo	<i>Phellodendron chinense</i> Schneid.	Cortex <i>Phellodendri chinensis</i>	Sichuan	
	Guanghuoxiang	<i>Pogostemon cablin</i> (Blanco) Benth	Herba <i>Pogostemonis</i>	Guangdong	
	Kushen	<i>Sophora</i>	Radix <i>Sophorae</i>	Henan	

	Chinese name	Taxonomic name	Medicinal part	Origin (Province)	Supplier
	Longkui	<i>flavescens</i> Ait. <i>Solanum nigrum</i> L.	<i>flavescentis</i> Herba <i>Solani nigri</i>	Fujian	
	Qianliguang	<i>Senecio scandens</i> Buch, -Ham.	Herba <i>Senecionis</i> <i>scandentis</i>	Anhui	
	Shechuangzi	<i>Cnidium monnieri</i> (L.) Cuss.	Fructus <i>Cnidii</i>	Hebei	
	Tufuling	<i>Smilax glabra</i> Roxb.	Rhizoma <i>Smilacis</i> <i>glabrae</i>	Sichuan	
	Tujinpi	<i>Pseudolarix</i> <i>kaempferi</i> Gord.	Cortex <i>Pseudolaricis</i>	Zhejiang	
Folk plants	Baixiaoniang	<i>Mallotus apelta</i> (Lour.) Muell-Arg	Folium <i>Malloti</i> <i>apeltae</i>	Fujian	b
	Gangbangui	<i>Polygonum</i> <i>perfoliatum</i> L.	Herba <i>Polygoni</i> <i>perfoliati</i>	Jiangxi	
	Heimainshen	<i>Breynia fruticosa</i> (Linn.) Hook. f.	Folium et Cacumen <i>breyniae</i> <i>fruticosae</i>	Guangdong	
	Huercao	<i>Saxifraga</i> <i>stolonifera</i> Curt.	Herba <i>Saxifragae</i>	Hebei	
	Huotanmu	<i>Polygonum</i> <i>chinense</i> Linn.	Herba <i>Polygoni</i> <i>chinensis</i>	Fujian	
	Jiubiyang	<i>Ilex rotunda</i> Thunb.	Cortex <i>Ilicis</i> <i>rotundae</i>	Anhui	
	Jingangteng	<i>Smilax china</i> L.	Rhizoma <i>Smilacis</i>	Gansu	

	Chinese name	Taxonomic name	Medicinal part	Origin (Province)	Supplier
	Jishiteng	<i>Paederia scandens</i> (Lour.) Merr.	<i>chinensis</i> Herba <i>Paederiae</i>	Jiangsu	
	Kulianpi	<i>Melia toosendan</i> Sieb. et Zucc.	Cortex <i>Meliae</i>	Kulianpi	
	Laliao	<i>Polygonum hydropiper</i>	Herba <i>Polygoni hydropiperis</i>	Anhui	
	Mujinpi	<i>Hibiscus syriacus</i> L.	Cortex <i>hibisci</i> .	Guangxi	
	Niaotama	<i>Acalypha australis</i> L.	Herba <i>Acalyphae</i>	Guangdong	
	Qidagu	<i>Glochidion eriocarpum</i> Champ.ex Benth.	Folium <i>Glochidii eriocarpi</i>	Guangxi	
	Sanyaku	<i>Evodia lepta</i> (Spreng.) Merr.	Radix <i>Evodiae leptae</i>	Yunnan	
	Wujue	<i>Stenoloma chusanum</i> (L.) Ching.	Herba <i>Stenolomae</i>	Zhejiang	
	Xiaofei yang	<i>Euphorbia thymifolia</i> Linn.	herba <i>Euphorbiae thymifoliae</i>	Sichuan	
	Yangshupi	<i>Populus davidiana</i> Dode	Cortex <i>Populi davidianae</i>	Anhui	
	Yizhihuanghua	<i>Solidago decurrens</i>	Herba <i>Solidaginis</i>	Fujian	

Plant supplier: (a) Bozhou Qiancao Pharm. Co. Ltd. (Bozhou, Anhui Province, China). (b) Ningbo Dekang Biochem Co., Ltd. (Ningbo, Zhejiang Province, China).

3. Preparation of the plant extracts

5 The protocol for the preparation of the extracts has been described before (Liu, et al. 2012). Briefly, the dried raw botanical material was ground to a fine powder. Five g of powder was transferred into each of four 100 mL flasks, and 50 mL of sterile water, absolute ethanol (SZBA0290, Sigma-Aldrich), acetone (S76776-259, Sigma-Aldrich), and hexane (09953580, Acros), respectively, were added. The flasks were left standing for 24 h at ambient
10 temperature. The flasks were then placed in a bath sonicator for four times 15 min each. After each sonication, there was an interval of 15 min to let the suspension cool to ambient temperature. Each sample was transferred into a 100 mL polyvinyl chloride (PVC) bottle and centrifugated at 530 g (2,000 rpm) for 20 min. The supernatant was transferred to a fresh PVC bottle. One mL was transferred into a 2.5 mL PVC tube and the solvent was evaporated in a
15 SAVANT Speedvac Concentrator (SVC 200H, Stratech Scientific, London, UK). For the sample extracted with water, the residue was re-dissolved in 0.5 mL of water using a vortex shaker. For the samples extracted with organic solvents (ethanol, acetone, and hexane), the residue was dissolved in 0.5 mL of DMSO. The samples were stored at 4 °C till the antifungal test.

4. Chromatographic purification of the whole plant extracts

Active extracts were further extracted with different solvents to establish the solubility of the active compounds. Aliquots of the active plant extract were dried by evaporation in a SAVANT Speedvac Concentrator, and the residue of one aliquot was resuspended in DMSO (control sample). Parallel dried aliquots were resuspended in various solvents (dichloromethane
25 (DCM), ether, methanol (MeOH), 50% or pure acetonitrile (ACN)) by vigorous vortexing. The samples were briefly centrifugated and the liquid phase (containing the solvent and compounds that dissolved in it) was transferred to a fresh tube. The solvent was evaporated and the residue was dissolved in DMSO before testing.

The active extract was further separated by preparative HPLC on a delta-pak C18 column,
30 2×(40×100 mm). Mobile phases were phase A (0.1% TFA) and phase B (80% ACN containing 0.1% TFA). The flow rate was 40 mL/min, and the eluate was monitored at 214 nm for channel 1 and 254 nm for channel 2. One-minute fractions (40 mL each) were collected and stored at

4°C. One mL aliquots of these fractions were dried in a Speedvac and the residues were dissolved in 50 µL DMSO for antifungal testing.

5. Identification and purification of active compounds by ^1H and ^{13}C NMR

Approximately two mg of each purified fraction was dissolved in DMSO- d_6 and further analysed by proton and/or carbon NMR spectroscopy (^1H and ^{13}C NMR, Bruker Avance 600 II+ spectrometer operating at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR) as described previously.

6. Fungal planktonic growth

Liquid assays were performed in a Labsystems Bioscreen C apparatus (Life Sciences International), at 35°C with continuous shaking. Cells were grown overnight on Sabouraud plates at 37°C. Some biomass was resuspended in PBS and washed twice. Cell suspensions of 1.10^6 cells/ml were prepared in RPMI-MOPS, and inoculums of 100 µl were added in duplicate to 100-well plates. An additional 100 µl of RPMI was added containing DMSO, as a control, or extracts (final DMSO concentration of 1%). Percentages of growth inhibition were calculated relatively to the DMSO control, and from duplicate experiments. Optical measurements were determined every 30 min for 24 to 48 hours.

7. Antifungal susceptibility assays

Minimal inhibitory concentrations (MIC) were determined according to the American standard procedures for DMSO-soluble compounds (CLSI M27-A2).

8. *Candida* biofilm assay *in vitro*

Biofilm assays were performed in 96-well polystyrene plate as substrate (adapted from Pierce *et al.*, 2008). Cells were grown overnight on Sabouraud plates at 37°C. Biomass was collected and washed twice in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4). Cell suspensions were then prepared at a final density of 1.10^7 cells/ml in RPMI-MOPS. An inoculum of 100 µl was added to each flat-bottomed well. Plates were incubated for 90 min at 37°C (without shaking) for initial adhesion. Inoculated wells were washed twice with PBS, and fresh RPMI was added for another 24h at 37°C to allow for biofilm formation. Wells were washed to remove non-adherent cells, and 100 µl of RPMI with DMSO or with extracts (final concentration of 1% DMSO) were added. Treatment lasted 24h at 37°C. Measurements of metabolic activity of the remaining biofilms after treatment were determined using the water-soluble tetrazolium salt reduction assay, XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide). Each biofilm assay

for drug susceptibility testing was performed in triplicate, from which mean and standard deviation were calculated.

9. *Candida* biofilm assay *in vivo*

In vivo biofilm assays were performed using the subcutaneous model of infection as previously described (Riccova *et al.*, 2010). Animals were treated accordingly to the ethical and animal care regulations of the KU Leuven. Briefly, serum-treated catheter fragments of 1 cm long were challenged with $5 \cdot 10^4$ *C. albicans* cells/mL for 90 min at 37°C. After two rounds of washes with PBS, catheters (9 per animal) were implanted subcutaneously in female Sprague Dawley rats. Two days post implant, animals were treated daily with mormodin 1c intraperitoneally (at 1 mg/kg or at 2mg/kg) or with DMSO alone (in control animals). In total, 27 biofilms per treatment were examined by CFU counting after 7 days of treatment.

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- 20

Claims

1. Momordin Ic and the glucopyranosyl derivative 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid for use as a medicament.
2. Momordin Ic and/or glucopyranosyl derivatives thereof for use as an anti-fungal.
- 5 3. A glucopyranosyl derivative according to claim 2 which is 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid.
4. Momordin Ic and/or the 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid for use as an anti-fungal.
- 10 5. Anti-fungal use according to claims 2, 3 or 4 wherein the anti-fungal effect is a cytostatic or cytotoxic effect.
6. Anti-fungal use according to claims 2, 3 or 4 wherein the anti-fungal use is the prevention or disruption of fungal biofilms.
7. Anti-fungal use according to claims 2, 3 or 4 wherein the anti-fungal use is the prevention and/or treatment of mycotic vaginitis.
- 15 8. Anti-fungal use according to claim 5 wherein the fungi comprise *Candida* sp., *Aspergillus* sp. and *Cryptococcus* sp.
9. Anti-fungal use according to claim 6 wherein the fungal species are selected from *Candida glabrata*, *Candida albicans*, *Candida krusei* and *Candida parapsilosis*.
- 20 10. An anti-fungal composition comprising momordin Ic and/or glucopyranosyl derivatives thereof and a pharmaceutical acceptable carrier.
11. An anti-fungal composition according to claim 10 wherein the glucopyranosyl derivative is 3'-O-(β -D-glucopyranosyl- β -D-xylopyranosyl- β -D-glucuronopyranosyl oleanolic acid.

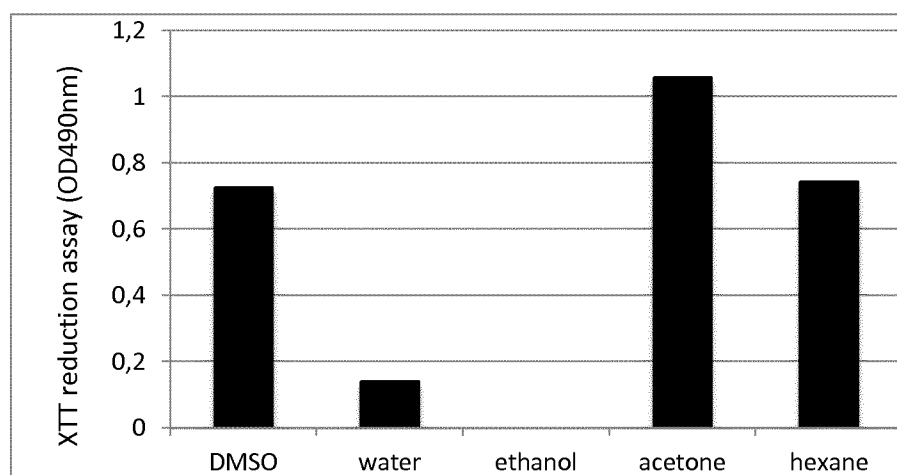
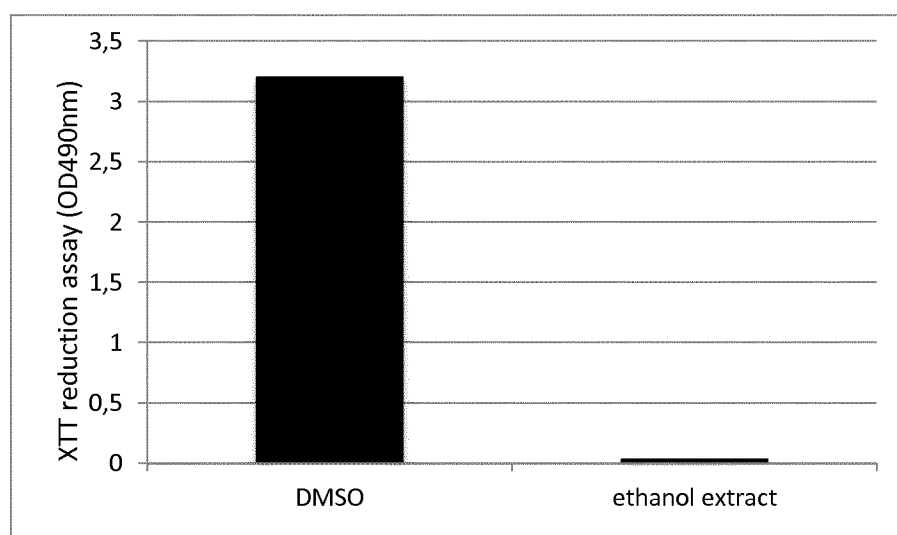
Figure 1**A****B**

Figure 2

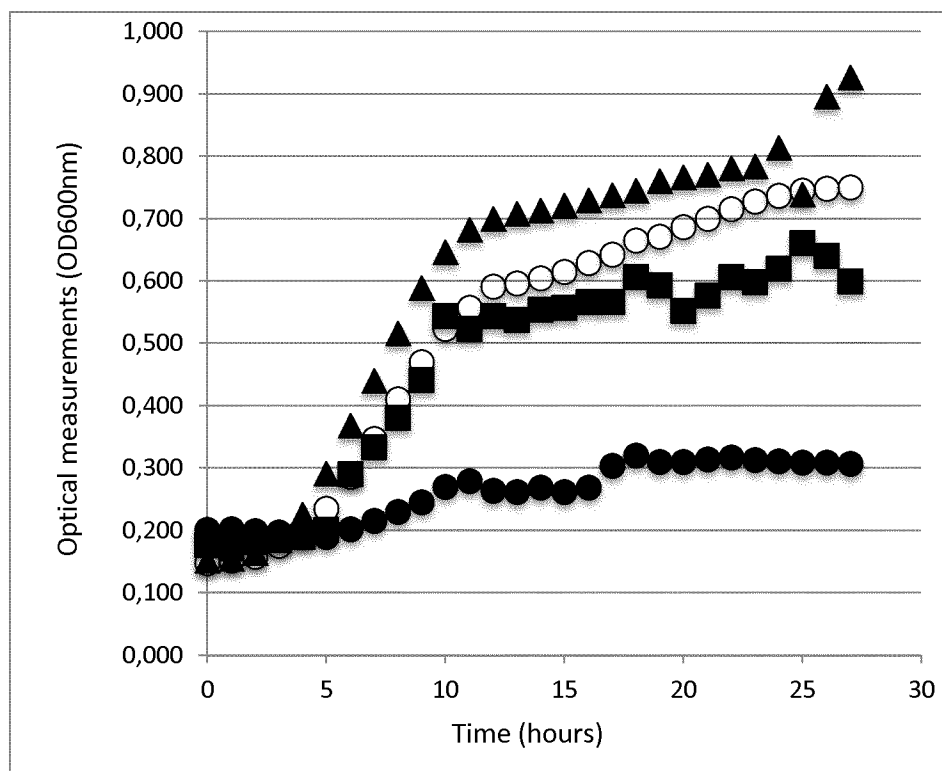
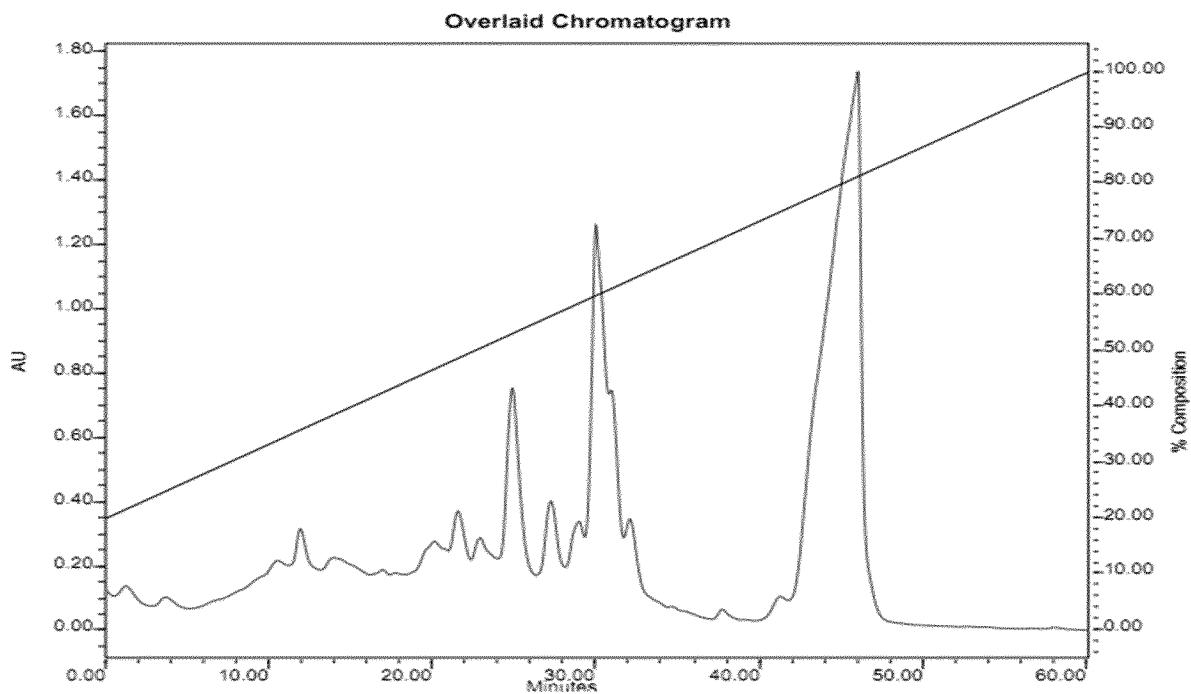
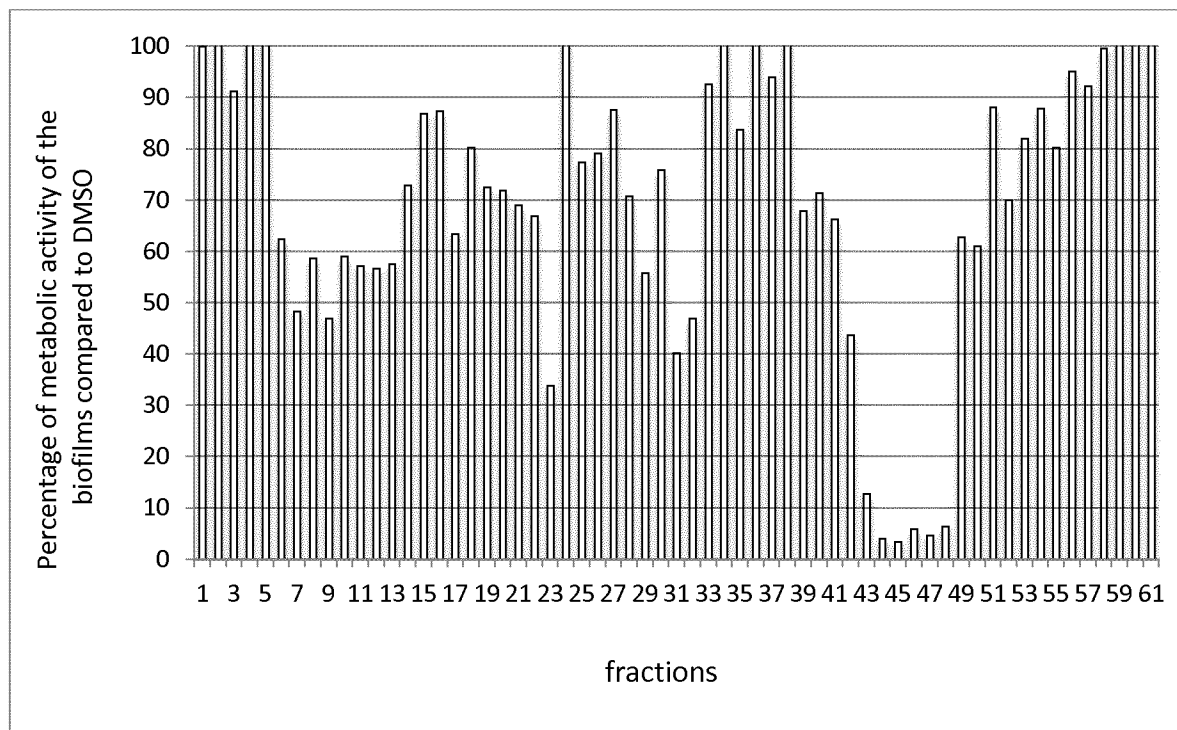


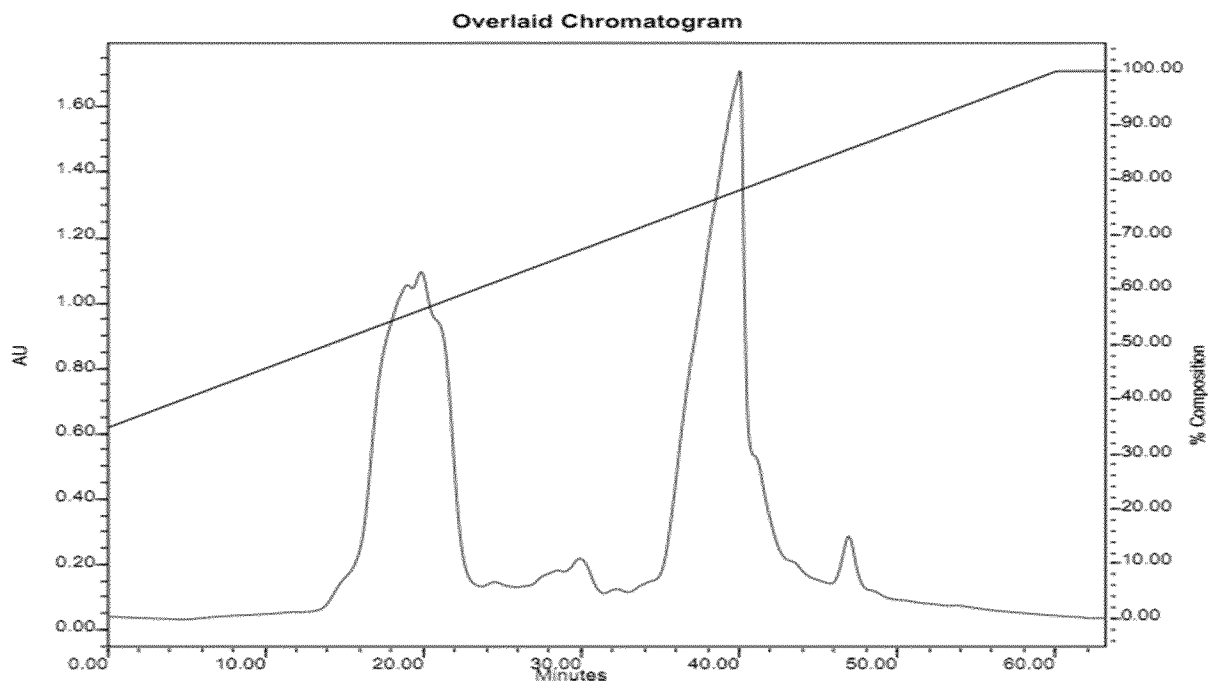
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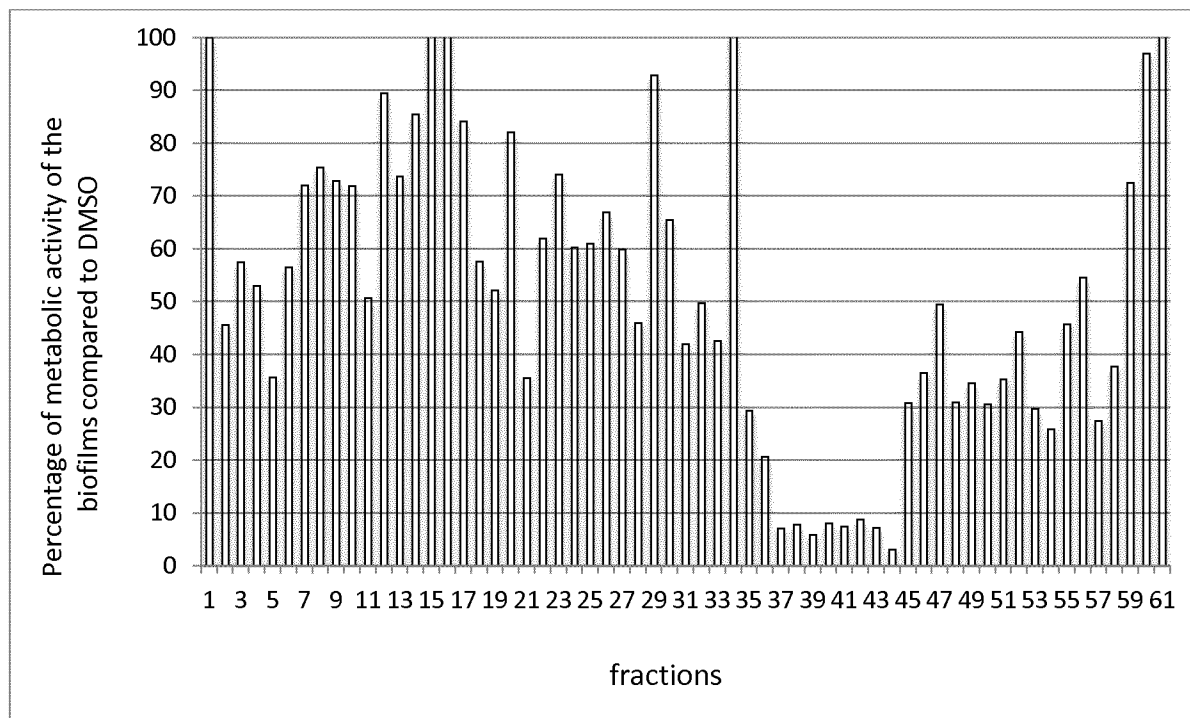
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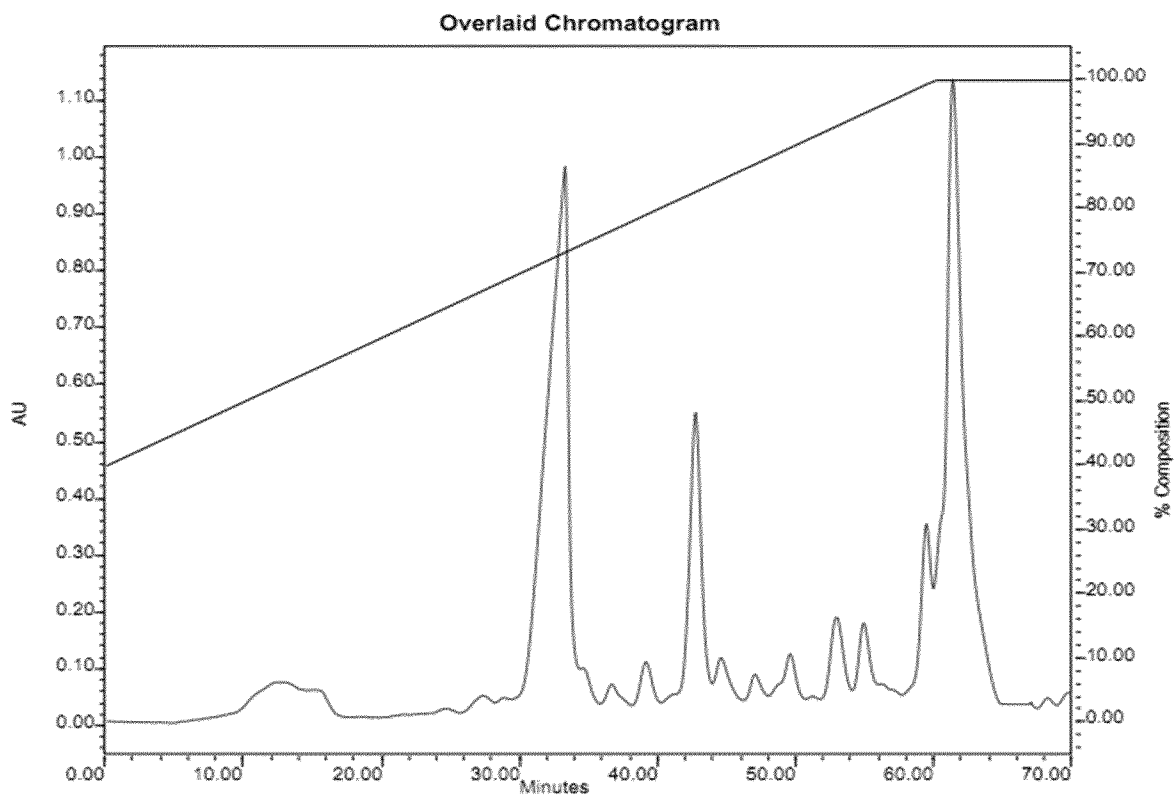


B





C



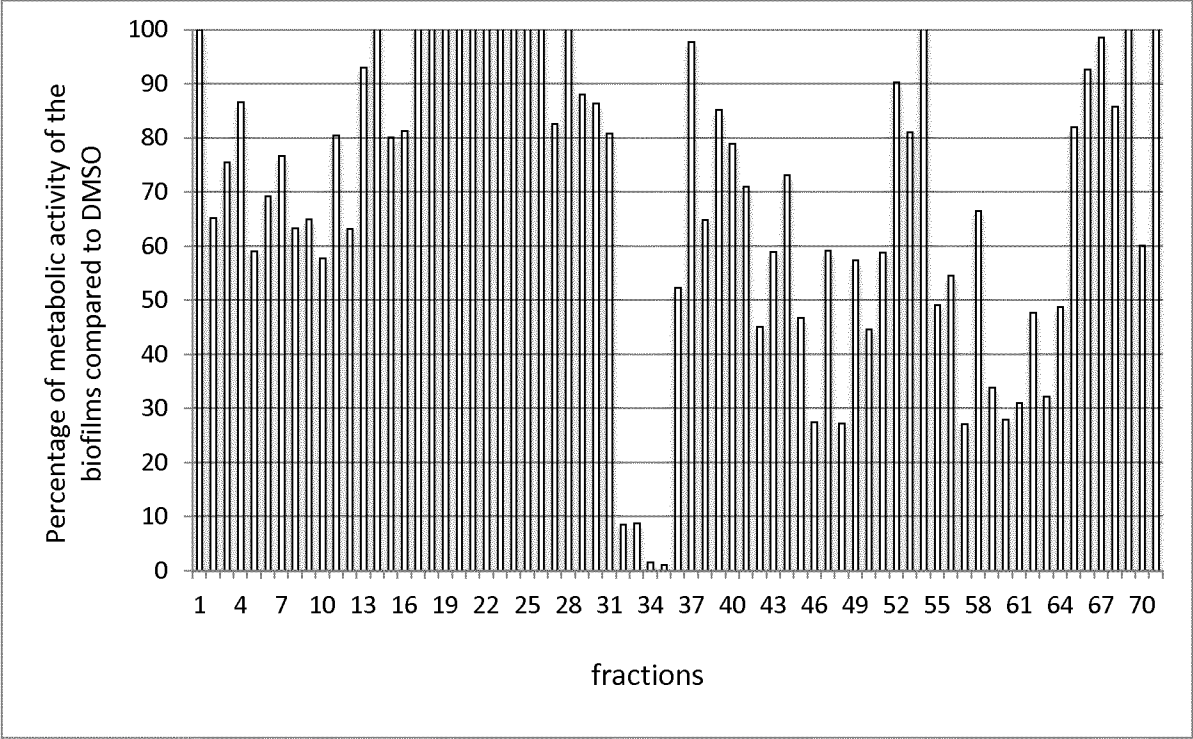
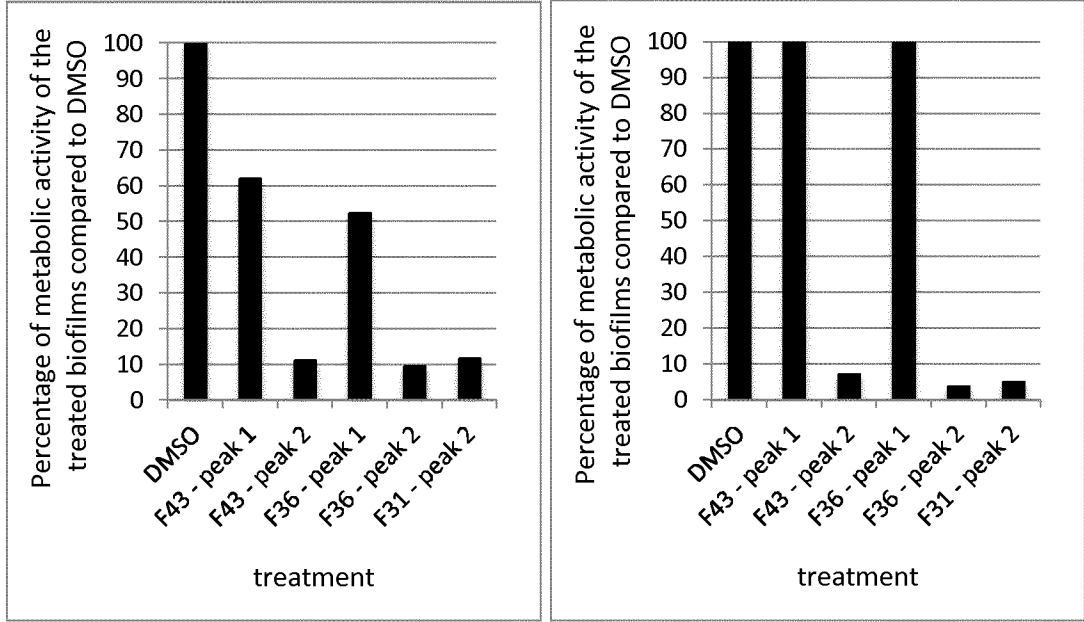


Figure 4

A



B

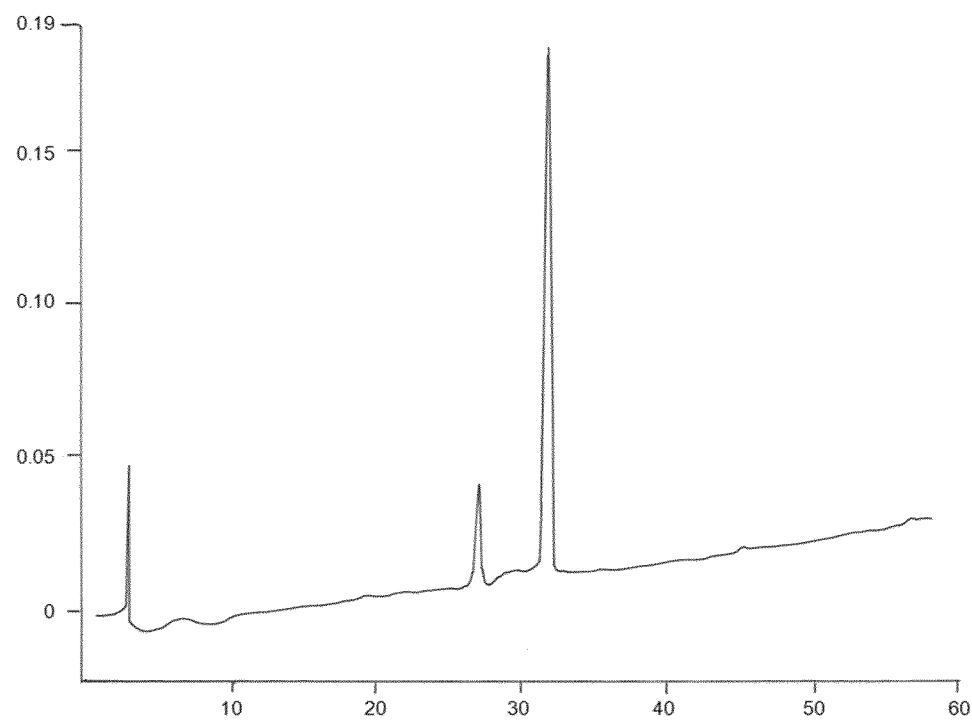
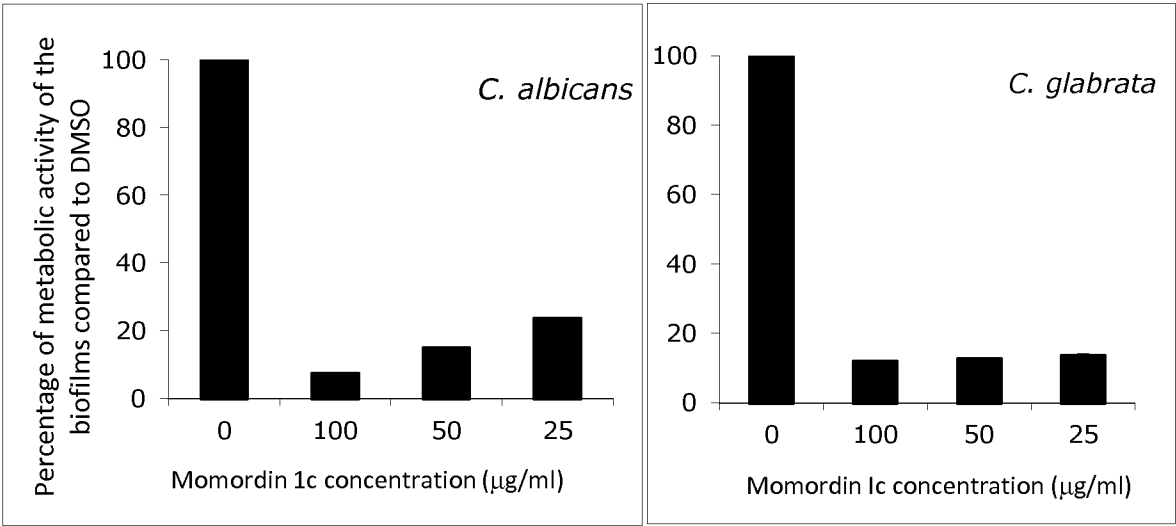


Figure 5

A



B

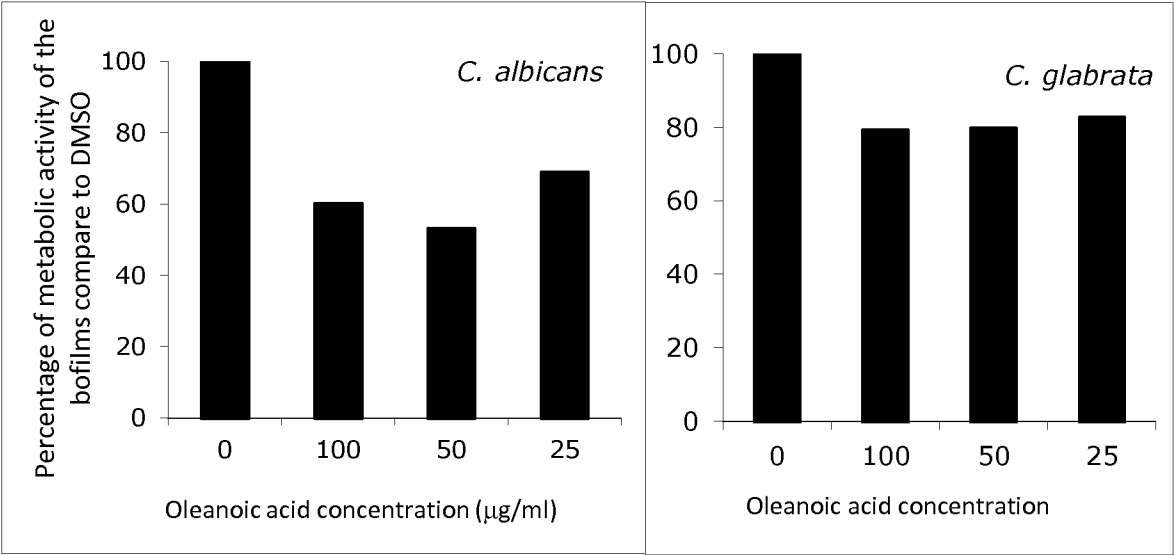


Figure 6

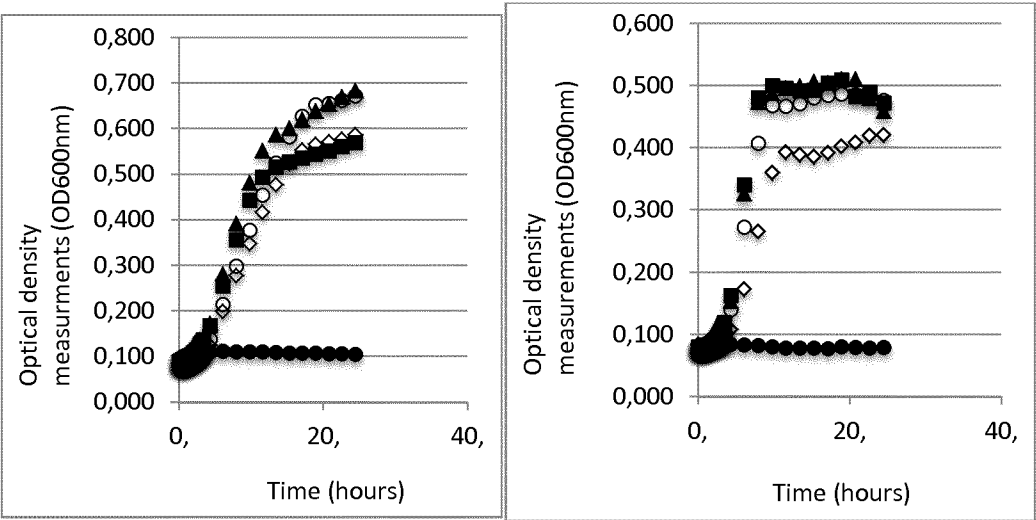
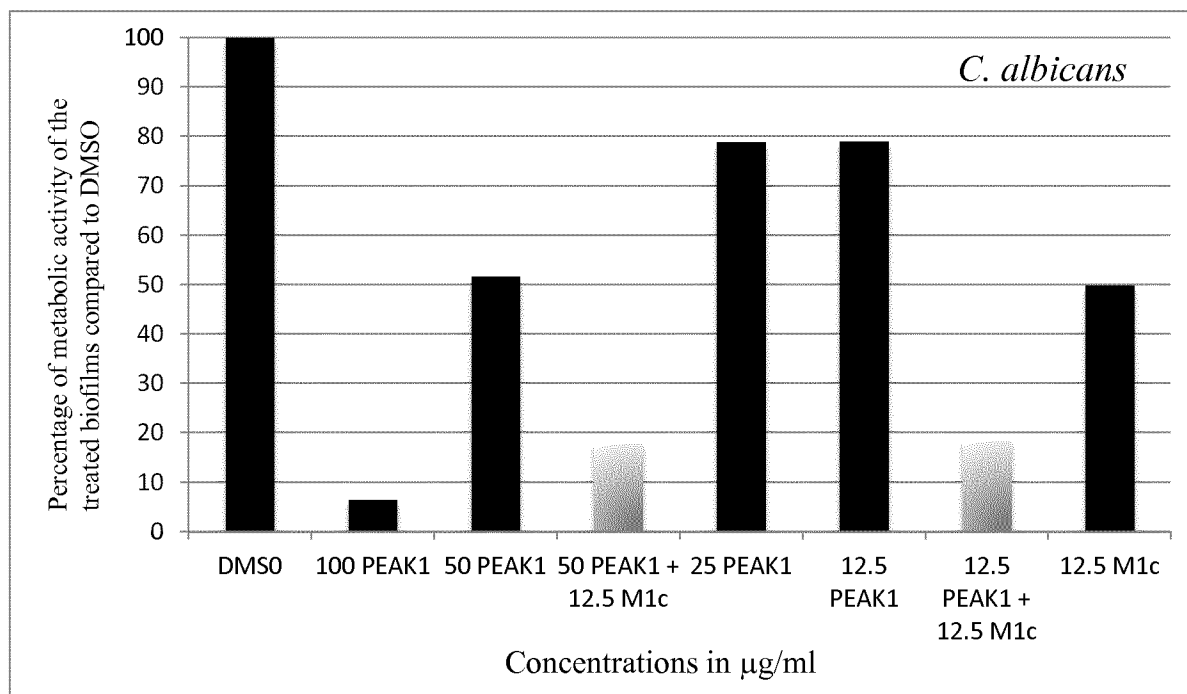


Figure 7

A



B

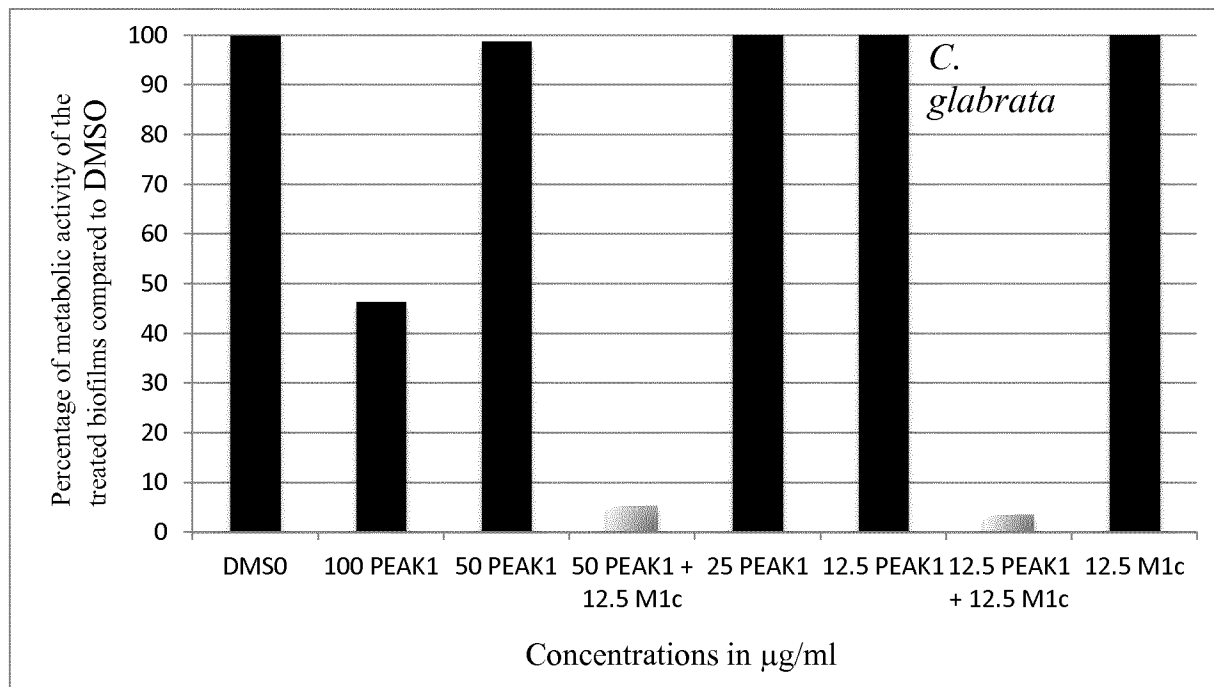
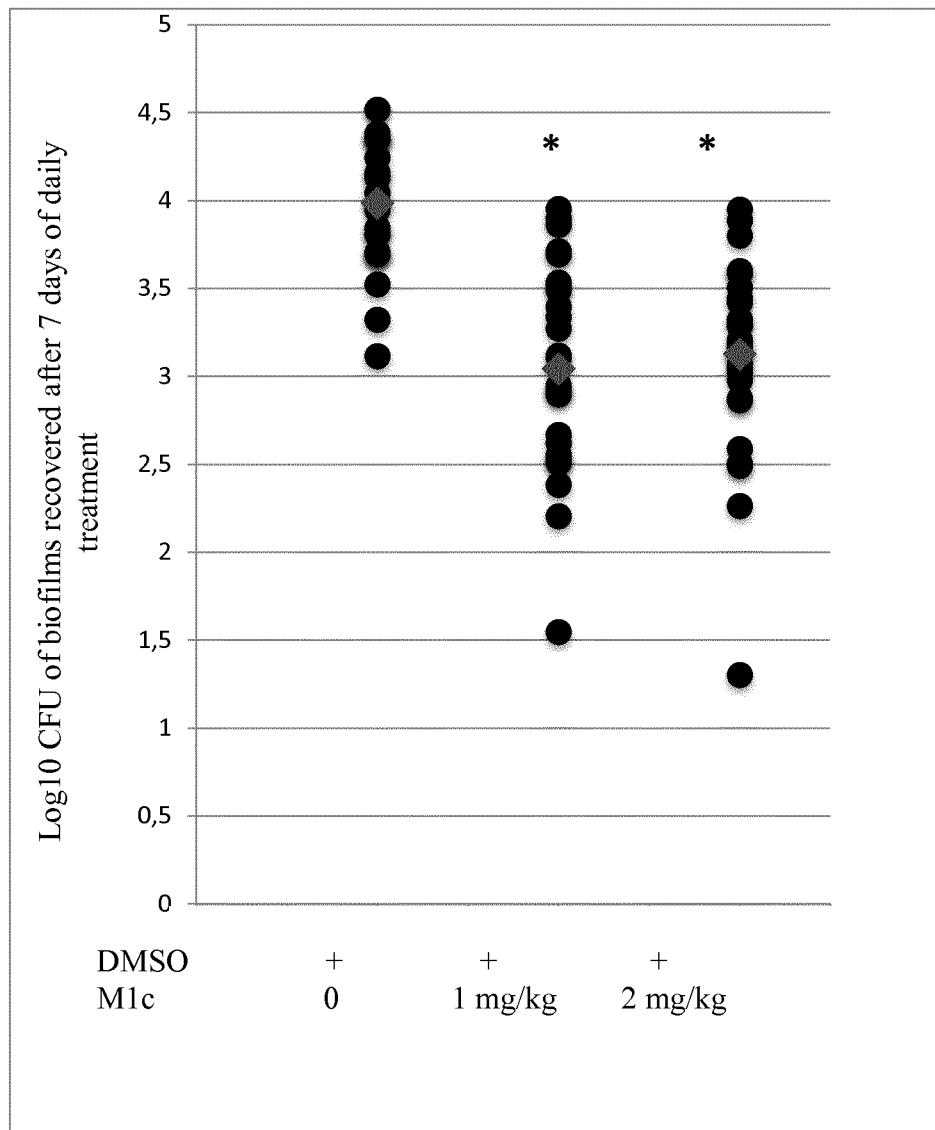


Figure 8



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/069159

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K31/704 A61P31/10
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

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, WPI Data, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	XU, YUN-HUI ET AL: "Chemical constituents of antifungal extract from Kochiae Fructus", ZHONGCHENGYAO , 34(9), 1726-1729 CODEN: ZHONBS; ISSN: 1001-1528, 2012, XP009180981, abstract -----	2-11
X	CN 103 083 340 A (SHANGHAI INST PHARM INDUSTRY; CHINA STATE INST PHARM IND) 8 May 2013 (2013-05-08) claims 1-3 ----- -/-	2-11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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"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

18 November 2014

Date of mailing of the international search report

24/11/2014

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Borst, Markus

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2014/069159

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIU QINGFEI ET AL: "Antifungal activity in plants from Chinese traditional and folk medicine", JOURNAL OF ETHNOPHARMACOLOGY, vol. 143, no. 3, 31 July 2012 (2012-07-31) , pages 772-778, XP028941608, ISSN: 0378-8741, DOI: 10.1016/J.JEP.2012.06.019 table 4 -----	10,11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2014/069159

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 103083340	A	08-05-2013	NONE
