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ORIGINAL RESEARCH ARTICLE

Unusual chemical composition of a Mexican propolis collected in Quintana Roo, Mexico

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Propolis is a resinous natural substance collected by honey bees from buds and exudates of various trees and plants. It is widely accepted that the composition of propolis depends on the phytogeographic characteristics of the site of collection. In this study, we have analyzed the chemical composition of a propolis collected in Quintana Roo, Mexico, and evaluated its antioxidant, antifungal, and antibacterial activities. Unexpectedly, the chemical analysis showed that the main components of the ethanolic extract of this Mexican propolis appeared to be pentacyclic triterpenoids, such as α and β -amyrin derivatives, and sterols. The crude extract did not show antioxidant activity when tested using the DPPH-reduction assay, and it also proved inactive when tested for antifungal and antibacterial activities using microdilution and agar diffusion assays, respectively. The fact that the presence of both α and β -amyrins and their derivatives have been reported from the resin of *Bursera simaruba*, one of the plants used by the bees for propolis production in Quintana Roo, Mexico, confirms the relationship that exists between the flora available to bees in a given region and the chemical composition of the propolis that they produce.

Composición química inusual de propóleos mexicano recogido en Quintana Roo, México

El propóleo es un producto natural resinoso colectado por abejas a partir de brotes y exudados de diferentes plantas y árboles; es ampliamente aceptado que la composición del propóleo depende de las características fitogeográficas del sitio de colecta. En este estudio analizamos la composición química de un propóleo colectado en Quintana Roo, México, y evaluamos sus actividades antioxidante, antifúngica y antibacteriana. Inesperadamente, el análisis químico mostró que los principales componentes del extracto etanólico del propóleo mexicano parecían ser triterpenoides pentacíclicos, tales como derivados de α y β -amyrina y esteroides. El extracto crudo no mostró actividad antioxidante al ser evaluado en el ensayo de reducción de DPPH, y también se mostró inactivo al ser evaluado en cuanto a su actividad antifúngica y antibacteriana utilizando los ensayos de microdilución y de difusión en agar, respectivamente. El hecho de que la presencia de α y β -amyrina y sus derivados ya se ha reportado en la resina de *Bursera simaruba*, una de las plantas utilizadas por las abejas para su producción de propóleo en Quintana Roo, México, confirma la relación que existe entre la flora disponible para las abejas en una región en particular y la composición química del propóleo que producen.

Keywords: Mexican propolis; pentacyclic triterpenes; amyrins; sterols; *Bursera simaruba*

Introduction

Propolis is a resinous natural substance collected by honey bees from buds and exudates of various trees and plants, mixed with beeswax and salivary enzymes. Bees generally use propolis to seal and smooth out the internal walls of the hive, as well as a protective barrier against fungal and bacterial infections. Propolis has been used in folk medicine since ancient times due to its pharmacological potential associated with antioxidant (Cottica et al., 2011; Gülçin, Bursal, Sehitoglu, Bilsel, & Gören, 2010; Miguel, Nunes, Dandlen, Cavaco, & Antunes, 2010), antifungal (Ota, Unterkircher, Fantinato, & Shimizu, 2001; Sawaya et al., 2002), antibacterial (Kujumgiev et al., 1999; Popova, Silici, Kaftanoglu, & Bankova, 2005; Raghukumar, Vali, Watson, Fearnley, &

Seidel, 2010), and anti-inflammatory (Castaldo & Capasso, 2002) properties.

Propolis is generally composed of 50% resin and balm (including polyphenolic compounds), 30% wax and fatty acids, 10% essential oils, 5% pollen, and 5% various organic and inorganic compounds. Presently, it is widely accepted that the composition of propolis depends on the phytogeographic characteristics of the site of collection (Marcucci, 1995; Sforcin & Bankova, 2011) and a number of attempts have been made to define propolis types taking into account geographic origin, chemical composition, and plant source (Park, Alencar, & Aguiar, 2002; Salatino, Teixeira, Negri, & Message, 2005; Sforcin & Bankova, 2011; Trusheva et al., 2006); recently, five propolis types have been defined according to their

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chemogeographic patterns (Salatino, Fernandes-Silva, Righi, & Salatino, 2011). It is also generally accepted that propolis from temperate climatic zones, like Europe, North America, and non-tropical regions of Asia, originate mainly from the bud exudates of *Populus* species and are rich in flavonoids, phenolic acids, and their esters (Bankova, de Castro, & Marcucci, 2000), while propolis from tropical regions, where no poplars and birches exist, are rich in prenylated derivatives of *p*-coumaric acid and benzophenones (Bankova, 2005a; Kumazawa, Hamasaka, & Nakayama, 2004; Sforcin & Bankova, 2011); other examples include lignan-containing propolis from Chile, Spain, and Kenya, and a propolis from Myanmar containing cycloartanes and prenylated flavanones (Salatino et al., 2011).

Mexico is considered as one of the three most important honey exporters in the world, with around 40% of its production located in and around the Yucatan Peninsula (Pino, Marbot, Delgado, Zumárraga, & Sauri, 2006). However, in spite of its potential importance, studies on the chemical composition and biological activity of Mexican propolis are limited. A chemical investigation of a red propolis sample collected in Campeche, in the southeast region of Mexico, reported the presence of flavanones, isoflavans, and pterocarpans, suggesting a possible relationship between the chemical composition of the Mexican red propolis and plants of the *Dalbergia* genus (Lotti et al., 2010). Other investigations report the antimicrobial activity against gram negative bacteria of several propolis samples collected in Campeche, Mexico (Tolosa & Canizares, 2002), the correlation between antimicrobial activity and composition of propolis and plants collected in central Mexico (Londono Orozco et al., 2010), and the identification by GC-MS of over 100 volatile constituents from two propolis samples collected in Yucatan, Mexico (Pino et al., 2006). Finally, a study on the chemical composition and biological activity of three propolis samples collected in different arid and semiarid regions of Sonora, Mexico showed cinnamic and phenylpropanoic acid derivatives and flavonoids as the main components, and antibacterial, free-radical scavenging, and cytotoxic activities (Li, Awale, Tezuka, Esumi, & Kadota, 2010; Velazquez et al., 2007).

We wish to report herein on the evaluation of the antioxidant, antifungal, and antibacterial activities of a tropical propolis sample from Quintana Roo, a state in the Yucatan Peninsula of Mexico, and the unexpected identification of pentacyclic triterpenoids and sterols as the only components present in its ethanolic extract.

Materials and methods

Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, formic acid, and gallic acid, all analytical grade, were purchased from Sigma-Aldrich (St Quentin Fallavier, France). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) and 5'-caffeoylquinic

acid (chlorogenic acid) were obtained from Acros Organics (Geel, Belgium).

Propolis sample

The propolis sample (224 g), collected from apiaries located in the area of Macario Gómez in Solidaridad, Quintana Roo, Mexico, was obtained from the Dzidzilche honey-distributing store of the "Productores y Realizadores de Miel Maya" cooperative.

Extractions

The propolis was first pulverized in the presence of liquid nitrogen into a homogenous powder. A 1 g portion of propolis powder was extracted three times with ethanol (20 ml) at room temperature for 2 h; the combined filtrates were maintained at -18°C overnight, filtered to remove waxes, and evaporated under reduced pressure to dryness to give the ethanolic extract (E1a). A second 1 g portion of propolis was successively extracted with cyclohexane (E2, 3×20 ml, 2 h), DCM (E3, 3×10 ml, 2 h), EtOAc (E4, 3×10 ml, 2 h), and MeOH (E5, 3×10 ml, 2 h) to produce the corresponding low, medium low, medium high, and high polarity extracts.

Determination of total polyphenol content

Total polyphenol content was determined according to the Folin-Ciocalteu colorimetric method (Rebiai, Lanez, & Belfar, 2011); 20 μl of extract solution (2.5 mg/ml) in MeOH was mixed with 280 μl of distilled water and 100 μl of Folin-Ciocalteu's phenol reagent. After 3 min, 1200 μl of distilled water and 400 μl of 20% aqueous sodium carbonate solution were added and 200 μl of each solution were put into a 96-well microtiter plate. A blank was prepared in the same way using MeOH instead of the extract solution. The absorbance was measured on a Tecan Infinite M200 microplate spectrophotometer, at 760 nm after 30 min in the dark, at room temperature. Gallic acid was used to calculate the calibration curve ($0.4\text{--}1.2$ mg/ml; $y = .5800x$; $r^2 = .9941$) and the total polyphenol content was expressed as milligram of gallic acid equivalent per gram of extract (mg GAE/g). The determination was performed in triplicate.

HPLC-DAD procedure

Ten milligram of extracts were dissolved in 1 ml of MeOH and centrifuged at 13,000 rpm for 10 min prior to injection (10 μl). HPLC analyses were run on a 2695 Waters® separation module equipped with a diode array detector 2996 Waters®. Separations were carried out on a Lichrospher® column 100 RP-18 (125 \times 4 mm i.d., 5 μm) protected with a Lichrocart® 4-4 guard

cartridge (4 × 4 mm i.d.), using a flow rate of 1 ml/min. The mobile phase consisted of .1% formic acid in water (solvent A) and methanol (solvent B) and the separation was performed by the following linear gradient: 25–100% B (0–40 min), 100% B (40–45 min). UV detection was achieved at three wavelengths: 254, 280, and 350 nm.

Analytical TLC

Analytical TLC was performed on TLC Alugram Xtra SIL G/UV₂₅₄, using a mixture of cyclohexane:AcOEt 85:15 as eluant. Spots in the chromatogram were visualized by spraying with vanillin–sulfuric acid reagent (2 ml of concentrated sulfuric acid in 98 ml of a 1:99 w/v vanillin: 95% ethanol solution) and heating the chromatograms at 110 °C for 5 min.

Fractionation by flash chromatography

Eighteen gram of propolis powder were macerated in ethanol (6 × 200 ml, 2 h) to give, after solvent evaporation, 4.2 g of crude extract E1b. E1b was totally dissolved in the minimum volume of DCM and the solution was mixed with 8.4 g of silica gel. The solvent was allowed to evaporate until a fine dry powder was obtained. The fractionation was performed using a CombiFlash Teledyne ISCO apparatus with a silica gel column (Redisep silica 80 g), with a gradient elution of 100% cyclohexane–100% EtOAc, and a flow rate of 35 ml/min. Two hundred tubes of 20 ml were collected and combined into 10 fractions on the basis of their TLC chromatographic profiles (cyclohexane: EtOAc 85: 15).

¹H and ¹³C NMR analyses

NMR spectra were recorded in CDCl₃ using a Bruker Avance spectrometer at 500 MHz for ¹H and 125 MHz for ¹³C.

GC–MS procedure

The GC–MS analysis was performed on non-derivatized samples using an Agilent Technologies Gas Chromatograph 6890 N connected to an Agilent Technologies 5975B mass detector system with an ionization voltage of 70 eV. Separations were carried out using a 30 m long, .32 mm i.d., .5 mm film ultra 1100% dimethylpolysiloxane column. The temperature was programmed as follows: 180 °C (3 min), 180–280 °C at the rate of 10 °C/min and 280 °C (30 min). Helium was used as a carrier gas at a flow rate 1.5 ml/min. Injector and detector temperatures were set at 250 and 280 °C, respectively. Metabolites were identified by comparing their retention times and/or their fragmentation patterns with those of authentic samples and/or those

contained in the fragmentation pattern library of the equipment (NIST Library in Chem Station G1701DA).

Evaluation of antioxidant activity using the DPPH assay

The assay was carried following a modified procedure on a reported methodology (Abdel-Lateff et al., 2002). Sample and standards were diluted in absolute ethanol at 0.02 mg/ml from stock solutions prepared at 1 mg/ml in DMSO; 100 µl aliquots of these solutions were placed in 96-well plates and the reaction was initiated by adding 25 µl of freshly prepared DPPH solution (1 mM) and 75 µl of absolute ethanol using the microplate reader's injector (Infinite® 200, Tecan, France) to obtain a final volume of 200 µl per well. After 30 min in the dark and at room temperature, the absorbance was determined at 517 nm. Ethanol was used as a blank, whereas 10, 25, 50, and 75 µM solutions of Trolox (hydrophilic α-tocopherol analog) were used as calibration solutions. A sample of 0.02 mg/ml chlorogenic acid was used as a quality control. The evaluation was carried out in triplicate. The DPPH-scavenging activity was compared with that of Trolox using the calibration curve. Result was expressed in terms of Trolox equivalents (micromoles of Trolox equivalents per gram of extract).

Evaluation of the antifungal activity

Antifungal activity was assayed on human pathogenic fungi, including a yeast (*Candida albicans* ATCC 66396) and an opportunistic mold (*Aspergillus fumigatus* CBS 11326). The strains were obtained from the parasitology and mycology laboratory, University Hospital Center, Angers, France. Both microorganisms were cultivated at 37 °C on yeast extract–peptone–dextrose agar (YPDA) containing .5 g/l chloramphenicol for two (*C. albicans*) or three (*A. fumigatus*) days. Tests were performed according to a procedure described by Alomar, Gaumet, Allain, Bouet, and Landreau (2012) following the guidelines of the approved reference method of the National Committee for Clinical Laboratory Standards (NCCLS) for yeasts (NCCLS, 1997) and filamentous fungi (NCCLS, 2002). Briefly, the yeast suspensions were prepared in RPMI-1640 culture medium and adjusted spectrophotometrically at 630 nm to reach a final concentration of ca. $.5 \times 10^3$ – 2.5×10^3 cells/ml. The tests were performed using sterile 96 flat-shaped well microtiter plates. Serial twofold sample dilutions were made in DMSO. Sample solutions (E1a and fractions A, B, and C) were dispensed at a volume of 5 µl in triplicate into the wells to obtain final concentrations from 250 to 1.95 µg/ml. After 48 h at 37 °C for *C. albicans*, and 72 h for *A. fumigatus*, the spectrophotometric MIC endpoint was calculated from the turbidimetric data as the lowest sample concentration causing a growth inhibition equal to or greater than 80% of the control (MIC₈₀). Amphotericin B was used as a positive control.

Evaluation of antibacterial activity

Antibacterial activity was evaluated on 21 bacterial strains obtained from the Laboratory of Bacteriology, University Hospital Center, Angers, France: seven strains of *Acinetobacter baumannii* (RCH, SAN008, 12, AYE, CIP7034, CIP107292, CIP5377), five of *Staphylococcus aureus* (ATCC25923, two methicillin-sensitive clinical isolates, two methicillin-resistant clinical isolates), two of *Escherichia coli* (ATCC25922 and a clinical isolate), three of *Pseudomonas aeruginosa* (ATCC27853 and two clinical isolates), and one clinical isolate each of *Enterobacter cloacae*, *Enterobacter aerogenes*, *Klebsiella oxytoca*, and *Salmonella enteritidis* (phage type 4). Tests were performed using the methodology described in the guidelines of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM, www.sfm.asso.fr). Briefly, a stock solution of each sample was prepared at 20 mg/ml in DMSO under sterile conditions. Serial dilutions were prepared (sample concentrations: 1, 10, 20, 30, 40, 50, 60, 80, and 100 µg/ml) and 100 µl of each dilution were added to 19.9 ml of Mueller–Hinton agar (Merck Germany) and transferred to Petri plates. Bacterial strains (2×10^4) were suspended in sterile NaCl aqueous solution (.15 M) and inoculated on the different Petri plates using the multipoint inoculator (AQS, England). After 24 h of incubation at 37 °C, the minimum inhibitory concentration (MIC₁₀₀ µg/ml) of each sample against each bacterial strain was determined.

Results

The propolis EtOH extract (E1a) did not show a significant polyphenol content (18.2 ± 2.9 mg GAE/g); this unexpected finding was confirmed by the lack of phenolic components in the HPLC profile of E1a and by its lack of antioxidant activity when tested in the DPPH radical reduction assay.

The sequential gradient extraction of the propolis sample using cyclohexane (E2), DCM (E3), EtOAc (E4), and MeOH (E5) showed that the majority of the components were extracted into the most abundant (>60%) low-polarity cyclohexane fraction (E2, Table I), which exhibited a TLC profile very similar to that of E1a. None of the remaining fractions was obtained in more than 5% yield and none showed the presence of significantly important components by TLC. These results implied that the studied propolis was predominantly composed of non-polar components in addition to

Table I. Yields of propolis extracts.

Extract	Solvent	Extraction yield (%)
E1a	EtOH	34.2
E2	Cyclohexane	61.6
E3	DCM	2.5
E4	EtOAc	1.0
E5	MeOH	4.6

beeswax. Since beeswax could be removed by cold filtration in EtOH, E1a was chosen to carry out the chemical characterization of the propolis.

Flash chromatography purification of E1b yielded three major fractions (A, B, and C; Figure 1), which accounted for more than 70% of the total crude extract (Table 2). ¹H NMR analyses of E1a and fractions A, B, and C showed predominantly signals in the range of .8–3.0 ppm, with a few between 4.0 and 5.5 ppm, while the majority of the signals in the corresponding ¹³C NMR spectra were located between 15 and 60 ppm, in addition to a few in the range of 110–150 ppm, that matches to triterpenes profiles. The GC/MS analysis of the most abundant fraction A, which showed a major low-polarity component by TLC ($R_f = .70$), allowed its identification as a mixture of α and β -amyrenone (I and II, respectively; structures of metabolites I–VIII are shown in Figure 2), containing a small amount of α -amyrin acetate (III). The TLC analysis of fraction B showed the presence of a single component ($R_f = .33$) that proved to be inseparable under TLC conditions using a number of solvent systems; GC/MS analysis of B allowed the identification of the main component as a mixture of α -amyrin (IV, major) and β -amyrin (V, minor). Finally, the GC/MS analysis of the least-abundant fraction C, which showed a major high-polarity component in its TLC profile ($R_f = .18$), allowed its identification as a mixture of sterols, with fucosterol (VI) being the major component, and a mixture of β/γ -sitosterol (VII/VIII) being the minor one.

Evaluation of the antimicrobial activities of E1b and fractions A, B, and C showed that they did not exhibit neither antifungal nor antibacterial activities when tested toward 2 fungal species (MIC₈₀ > 250 µg/ml) and 21 bacterial strains (MIC₁₀₀ > 100 µg/ml), respectively.

Discussion

The results obtained in this investigation were unexpected since, to date, there are only a limited number of reports about triterpenoids occurring as major

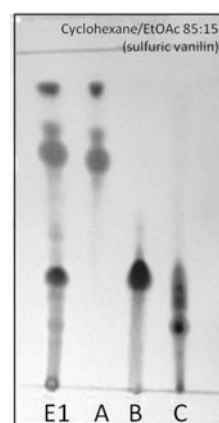


Figure 1. TLC profiles of E1b and fractions A, B, and C.

Table 2. Yields of fractions A, B, and C and R_f values of their major components on TLC.

Fraction	Yield (%)	R_f^*
A	47.7	.89, .77, .70
B	19.9	.33
C	4.1	.18
Total	71.7	

*Eluant system: cyclohexane: EtOAc 85:15.

components in propolis (de Castro Ishida, Negri, Salatino, & Bandeira, 2011; Furukawa et al., 2002; Ito et al., 2001; Kalogeropoulos, Konteles, Troullidou, Mourtzinou, & Karathanos, 2009; Melliou, Stratis, & Chinou, 2007; Velikova, Bankova, Marcucci, Tsvetkova, & Kujumgiev, 2000) and none of the propolis types that have been chemically described until now list triterpenes as their major components (Salatino et al., 2011; Sforcin & Bankova, 2011). Additionally, our results are not in agreement with reports stating that triterpenoids are rarely reported as propolis constituents because most chemical analyses of propolis samples are carried out with alcoholic extracts (Negri, Salatino, & Salatino, 2003), or that triterpenoids are only occasionally collected by bees along with other classes of secondary metabolites such as phenolics (Negri, Marcucci, Salatino, & Salatino, 2000).

The two plants used by the bees for their production of propolis in the region of Quintana Roo where the material was collected are commonly known with the Mayan names of "chakah" [*Bursera simaruba* (L.) Sarg.] and "tsalam" [*Lysiloma latisiliquum* (L.) Benth.]

(Luis Ignacio Hernández-Chávez, personal communication), and both species are found as part of the flora of what is known as a mature medium-statured semi-deciduous forest (*selva mediana*) (Schultz, 2009). While no phytochemical knowledge exists about *L. latisiliquum*, the bark and resin of *B. simaruba* have been reported to contain lignans and other phenolic metabolites (Maldini, Montoro, Piacente, & Pizza, 2009; Peraza-Sánchez & Peña-Rodríguez, 1992), with the resin also reported to contain pentacyclic triterpenes such as the ubiquitous α -amyrin (IV) and β -amyrin (V) (Peraza-Sánchez, Salazar-Aguilar, & Peña-Rodríguez, 1995). Although no lignans could be detected in the analyzed samples, these findings could confirm the reported relationship that exists between the flora available to bees in a given region and the chemical composition of the propolis that they produce (Adelmann et al., 2007; Bankova, 2005a; Dausch, Moraes, Fort, & Park, 2008; de Castro Ishida et al., 2011; Salatino et al., 2011; Sforcin & Bankova, 2011) and the fact that in tropical regions, the chemical composition of the propolis can be highly variable because of the richness and versatility of the local flora (Trusheva et al., 2004).

Finally, it is well known that propolis is mainly used by bees as a defense material against parasites (Salatino et al., 2011) and to prevent bacterial and fungal infections and that often, while the biological activity of different propolis samples is similar, the secondary metabolites responsible for the activity can be different (Bankova, 2005b; Trusheva et al., 2006). Although E1a

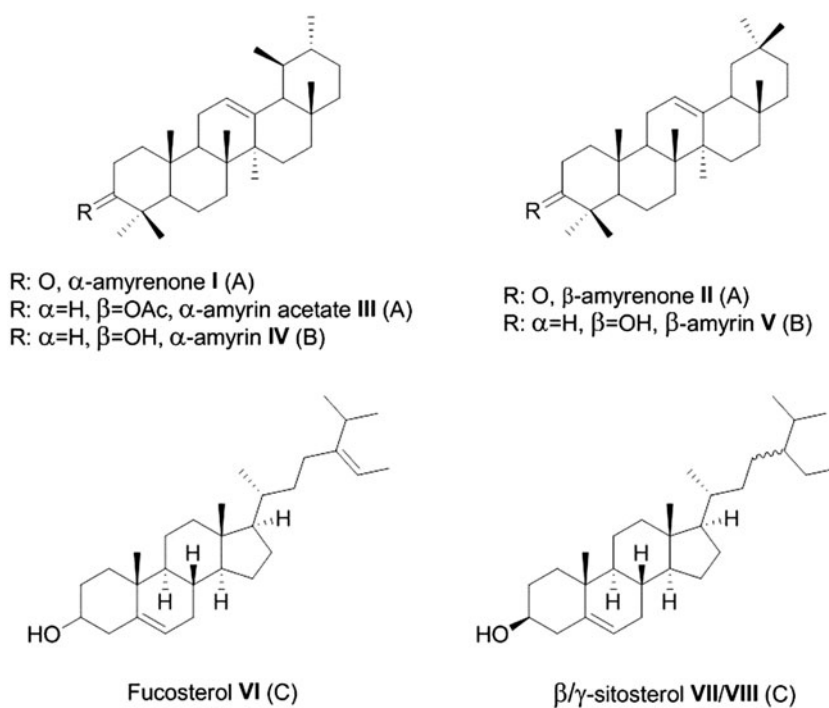


Figure 2. Structures of major constituents identified in E1b and fractions A, B, and C by GC-MS analyses.

did not show significant antioxidant or antimicrobial activity, pentacyclic triterpenoids which include α -amyrin (IV) and β -amyrin (V) and their various derivatives have been reported as antibacterials, antifungals, and with cytotoxic activity (Choi et al., 2012; Chung, Chung, & Navaratnam, 2013; Cota et al., 2011; Irshad et al., 2013; Sharma, Singh, & Vijayvergia, 2010); the lack of activity of both the propolis extract E1a and the purified fractions A–C in the assays for antioxidant and antimicrobial activity, suggests that the chemical composition of the propolis collected in Quintana Roo is of particular importance in the ecological interaction between the bees and the parasites and micro-organisms that occur specifically in that region. Therefore, additional research works will be needed to establish the mode of protective action of this Mexican propolis.

Supporting information

The HPLC profile of EtOH extract (E1a) and GC–MS data of E1b and fractions A–C are available in Supporting information.

Abbreviations

ATCC	American Type Culture Collection
CBS	Central Bureau voor Schimmelcultures
CDCl ₃	Deuterated chloroform
DCM	Dichloromethane
DMSO	Dimethylsulfoxide
DPPH	Diphenylpicrylhydrazyl
EtOAc	Ethyl acetate
GC/MS	Gas chromatography coupled with mass spectrometry
HPLC/DAD	High-performance liquid chromatography coupled with diode array detector
MIC	Minimum inhibitory concentration
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear magnetic resonance
RPMI	Roswell Park Memorial Institute
TLC	Thin-layer chromatography
UV	Ultraviolet
YPDA	Yeast–peptone–dextrose agar

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplemental data

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