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Two anti-staphylococcal triterpenoid acids isolated from *Psiloxylon mauritianum* (Bouton ex Hook.f.) Baillon, an endemic traditional medicinal plant of Mauritius

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ABSTRACT

Psiloxylon mauritianum (Bouton ex Hook.f.) Baillon (PM) is a traditional medicinal plant used for the treatment and management of dysentery and common infectious diseases in Mauritius. Locally, the plant is orally administered in the form of a broth made with the young leaves for its curative properties. Though the traditional use of this plant had been documented in early ethnomedicinal surveys, it had however never been studied. This study thus endeavors at validating the traditional use of PM and purifying the active bioactive secondary metabolites responsible for any observed biological activity. The crude extract was assessed *in vitro* for growth inhibiting activity against several microbial strains using both the broth microdilution assay and bioautography. Additionally, the antioxidant activity was assessed using standard assays and the most active extract was submitted to bio-guided fractionation for further evaluation. PM was found to be both strongly antibacterial and antioxidant. The crude acetone extract as well as its subsequent preliminary tannin-less fractions was found to be markedly anti-staphylococcal. Lowest Minimum Inhibitory Concentrations (MICs) of 51 μg/ml and 19 μg/ml were recorded for the crude extract and the most polar preliminary tannin-less (20% methanol in dichloromethane) fraction of PM, respectively. On the other hand, the plant was found to be only weakly antifungal, inhibiting the growth of *Candida albicans* at 3.25 mg/ml. Consequently, PM extract was further fractionated and the fractions were tested on *Staphylococcus aureus*. Bioassay guided fractionation led to the isolation and identification of (2α, 3β)-dihydroxyurs-12-en-28-oic acid and (2α, 3β) 23-trihydroxy-urs-12-en-28-oic acid, commonly known as, corosolic and asiatic acid respectively for the first time from this endemic species of Mauritius. The present study thus clearly indicates that PM possessed substantial antimicrobial, in particular anti-staphylococcal activity which corroborates with its use in the traditional Mauritian pharmacopoeia as a plant having potential anti-infective properties. The results so far obtained further substantiate, not only, the importance of screening medicinal plants as reliable sources of lead molecules, but also provide additional credence to the traditional uses of such plant.

1. Introduction

In developing countries, traditional medicine is often the only accessible and affordable treatment available to the population. Though the local people of Mauritius have access to conventional medicines and medications, medicinal plants have remained an important component in the treatment of a pantheon of diseases and hold favored positions amongst Mauritians (Gurib-Fakim, 2002). The local flora is particularly diverse and includes over 700 species of native flowering plants and ferns, out of which 311 species are endemic to the Mascarene Islands and 45% strictly endemic to Mauritius (Gurib-Fakim, 2002). About 150 species of this native endemic flora have been identified as critically endangered and are involved in the government's conservation program.

Psiloxylon mauritianum (Bouton ex Hook.f.) Baillon is a white-barked evergreen flowering plant. Formerly, it used to be considered as the unique species of the family Psiloxylaceae but is now classified as a basal member of the Myrtaceae (Myrtle) family and the sole representative of the genus *Psiloxylon* (Wilson et al., 2005). This endemic species is used in Mauritius for the treatment and management of common infectious diseases, dysentery and to alleviate symptoms of amenorrhea (Lavergne, 1990). Locally, the plant is orally administered in the form of a broth made with young leaves for its curative properties. Though the traditional use of this plant has been published in major ethnomedicinal surveys on the local flora (Lavergne, 1990; Gurib-Fakim et al., 1996), it

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has however not previously been subjected to any phytochemical screening or bioassay guided fractionation and purification. This endemic species is currently under the government's conservation program and considering its favored position among local traditional practitioners; its potential bioactivity was assessed *in vitro* using several bioassays. The antimicrobial, in particular, anti-staphylococcal activity, antioxidant properties and potential cytotoxicity on Chinese Hamster Ovary cells of the crude acetone extract of leaves of *P. mauritianum* (PM) and its subsequent tannin-free fractions was evaluated.

2. Materials and methods

2.1. Plant material

Fresh leaves were collected from trees grown in the natural reserve of Pétrin. Following their identification from the reserve botanist and the curator of Curepipe botanical garden, voucher specimen (PM0410) was prepared and deposited at the local herbarium, Réduit.

2.2. Extraction and isolation of pure compounds

Dried grounded leaves of PM (740 g) were exhaustively extracted at room temperature with acetone (1:10 *w/v*) for subsequent 24 h period and concentrated *in vacuo* to afford the crude acetone extract (79.6 g). The crude extract was assessed for possible antimicrobial, antioxidant and cytotoxic activities and then subjected to fractionation by column chromatography through polyamide SC 6 with a solvent gradient built with methanol in dichloromethane (DCM). This resulted in four preliminary fractions namely, 100% DCM fraction (PMF1, 27.8 g), 5% methanol in DCM fraction (PMF2, 7.66 g), 10% methanol in DCM fraction (PMF3, 18.4 g) and 20% methanol in DCM fraction (PMF4, 0.13 g). These four fractions, cleared of tannins by the polyamide were all tested against *Staphylococcus aureus* via both the broth microdilution assay and bioautography. Based on observed activity, PMF3 was further fractionated column chromatography over silica gel (mesh size 0.063–0.200 mm) under a mixture of DCM and methanol (9:1) and collected in 100 test tubes. These tubes were re-grouped according to their TLC profile into 5 sub-fractions (PMS1-PMS5). The most active subfraction (PMS3, 1.27 g) was fractionated via repeated column chromatography through silica gel, under 5% methanol in DCM. This yielded 21 pre-purified fractions among which PF3 to PF11 harbored anti-staphylococcal activity. PF4 and PF9 were collected as pure compound **1** (11.1 mg) and **2** (96.4 mg) respectively.

2.3. Characterization of the pure compounds

Structures of isolated compounds were elucidated by Mass and NMR spectroscopic methods. Mass spectra measurements were collected from a LTQ-orbitrap XL mass spectrometer equipped with a thermofinnigan PDA and a LTQ-orbitrap mass detector in both ESI and APCI positive and negative modes. Full MS scan from *m/z* 100 to 1000 were recorded. Samples were dissolved in deuterated solvents [methanol (CD_3OD), chloroform ($CDCl_3$), or mixture of $CD_3OD/CDCl_3$] and their 1D [^1H (500 MHz), and ^{13}C (125 MHz)] and 2D (^1H–^1H COSY, HMQC and HMBC) NMR spectra were recorded on a Bruker AVANCE 500 spectrometer.

2.4. Microorganisms

Antimicrobial testing was performed using a combination of Gram-positive, Gram-negative bacterial strains as well as yeast and mold fungi. Standard American Type Culture Collection (ATCC) strains of *Enterococcus faecalis* (ATCC 21212), *S. aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Candida albicans* (ATCC 26219) and *Aspergillus niger* (ATCC 16404) were use as recommended by the National Committee for Clinical Laboratory Standards (NCCLS, 1999).

2.5. Antimicrobial assays

2.5.1. Broth microdilution assay

Antimicrobial activity was sought according to the National Committee for Clinical Laboratory Standards procedures for aerobic testing (NCCLS, 1990) and with modification as per Eloff (1998) with the growth indicator INT [*p*-iodonitrotetrazolium violet (Sigma-Aldrich Chemical Co. Germany)]. Each of the bacteria was sub-cultured twice on Muller Hinton agar; colonies (5–7) were then transferred aseptically from the second transfer plate into individual tubes containing sterile nutrient broth (10 ml). The tubes were incubated for a period of 8–12 h at 37 °C to ensure that the bacteria were in the log phase. Subsequently, the bacterial suspensions were visually adjusted to 0.5 McFarland and then further diluted 1:100 with fresh sterile broth to yield starting inoculums of approximately 10^6 CFU/ml. Stock solutions of the different extracts at a known concentration were prepared as mentioned in Section 2.2. A known volume (100 μl) of each solution was placed in the first well of a 96-well microplate and 2-fold serially diluted with sterile distilled water. A known volume of the inoculum (100 μl) was then added to each well. The plates were then incubated at 37 °C for 24 h at 100% relative humidity (Eloff, 1998). After incubation, the growth indicator INT [*p*-iodonitrotetrazolium violet (Sigma-Aldrich Chemical Co. Germany)] (40 μl, 0.2 mg/ml) was added to each well and incubated for a further 20–30 min. Microbial growth is denoted by a red coloration of the wells. The well of lowest concentration in which no coloration is observed is taken as the MIC (Eloff, 1998). The *in vitro* antimicrobial tests were performed simultaneously with suitable solvent blanks and sterility tests. Standard antibiotic Streptomycin sulphate (Sigma-Aldrich Chemical Co. Germany), Gentamicin sulphate (Sigma-Aldrich Chemical Co. Germany), Nitrofurantoin (Sigma-Aldrich Chemical Co. Germany) and Amphotericin B (Sigma-Aldrich Chemical Co. Germany) were used as positive controls to compare the susceptibility of the different microorganisms. The procedure used was as described above, only instead of doing serial dilution of test samples; serial dilution of the standard antibiotics was done (Bonjar, 2004).

2.5.2. Bioautography using S. aureus

The fractions found most active were prepared at known concentration (10 mg/ml) and aliquots (10 μl) were spotted on a TLC plate, which was then developed in an appropriate solvent systems [DCM:MeOH (9:1)]. Once fully developed the plate was thoroughly dried using a hair drier to ensure that all solvents are evaporated. Mueller Hinton agar (homogenized with the bacterial strain in the log phase) was then poured over the plate allowed to set and incubated at 37 °C for 24 h. After this 24 h incubation activity was revealed with INT, white zones of inhibition on a pink background occur in the presence of *S. aureus* growth inhibiting constituents. All tests were done in duplicate and the retention factors of the zones of inhibition were recorded. For all samples, a triplicate chromatogram was eluted in parallel on the same plate, before the plate was cut into three parts. Two parts were used for the bioautography and the third plate was left intact for spot examination under UV and thence with the appropriate spray reagent (10% ethanolic sulphuric acid solution, NP/PEG, Dragendorff's reagent, Liebermann–Burchard's reagent, among others).

2.6. Cytotoxicity assay

Chinese hamster ovarian cell line (CHO) (ATCC, CCl-61) was used and cytotoxicity on these cells was assessed using the protocol by Block et al. (2002). For each experiment, cultures were seeded from frozen stocks. CHO cells were maintained in Ham's F12 medium supplemented with 10% fetal calf serum (FBS) and 1% antibiotic solution. All cell lines were incubated at 37 °C in a 5% CO_2 atmosphere and were in