South African Journal of Botany 108 (2017) 126–131

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Antimicrobial, anti-inflammatory activity and cytotoxicity of *Funtumia africana* leaf extracts, fractions and the isolated methyl ursolate

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ARTICLE INFO

Article history:
Received 1 June 2016
Received in revised form 18 August 2016
Accepted 13 October 2016
Available online xxxx

Edited by J Van Staden

Keywords:
Minimum inhibitory concentration
Bioautography
Cytotoxicity
Traditional knowledge
Fungal phytopathogens

ABSTRACT

Funtumia africana is used to treat and manage diverse ailments including fever, inflammation, malaria, cancer and urinary incontinence in South Africa. In this study, the antibacterial, antifungal, anti-inflammatory activities and cytotoxicity of the crude extracts, fractions and an isolated compound were determined. Serial microplate dilution and bioautography methods were used to determine the antimicrobial activities. The bacteria tested were ATCC reference strains of *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus*. The fungal test organisms used were three clinical isolates (*Aspergillus fumigatus, Cryptococcus neoformans* and *Candida albicans*), *C. albicans* ATCC 10231 and three phytopathogenic fungi (*Fusarium oxysporum, Penicillium janthinellum* and *Rhizoctonia solani*). The anti-inflammatory activity was determined using cyclooxygenase (COX) enzymes and the MTT assay was used to determine cellular toxicity against Vero and human liver (C3A) cells. The crude extract had MICs as low as 80 μg/ml against both bacteria and fungi. The chloroform fraction had the lowest MIC of 20 μg/ml against *P. aeruginosa*. The hexane and chloroform fractions had MIC of 40 μg/ml against *C. albicans* ATCC 10231. The crude extract, hexane and chloroform fractions had moderate activity against both COX-1 and COX-2. The chloroform fraction was more active than the crude extract (59.7%) with an inhibition of 68.2% against COX-1. Bioassay-guided fractionation using column chromatography led to the isolation of methyl ursolate (MU) with an MIC of 62.5 μg/ml against *F. oxysporum*. It was relatively toxic against Vero cells with an IC_{50} of 10.4 μg/ml. The antimicrobial and anti-inflammatory activities of the crude extract provide some support for the traditional use of the plant.

1. Introduction

As part of a search for plant based antimicrobial agents, a random screening of antimicrobial activities of acetone leaf extracts of more than 500 tree species was undertaken in the Phytomedicine Programme against six bacterial and two fungal pathogens (Pauw and Eloff, 2014). Nine tree species with promising activities were selected from this screening process for further investigation. *F. africana* had good antimicrobial activity against nosocomial bacterial and fungal pathogens with a minimal inhibitory concentration (MIC) of <100 μg/ml and contained a number of antimicrobial compounds on bioautograms (Ramadwa, 2010).

Funtumia africana (Benth.) Stapf belongs to the family Apocynaceae which generally contains alkaloids (Gurib-Fakim, 2006). The genus *Funtumia* consists of *F. africana* and *F. elastica*. The leaves of both species are very similar, glabrous, leathery, elongated, elliptic more or less acuminate, cuneate at the base with short stalks (Keay et al., 1964) the flowers and fruits of *F. africana* are longer than those of *F. elastica* (Burkill, 1960). Although *F. africana* is native and widely distributed in the east, central and west Africa, the species is also found in some southern Africa countries like Mozambique (Wagner et al., 1987; Beentje, 1994; Orwa et al., 2009). In West Africa, *F. africana* is used for the treatment of fever, inflammation, malaria, cancer, amoebic dysentery, urinary incontinence and burns (Adjanohoun and Aké, 1979; Adjanohoun et al., 1986; Wagner et al., 1987; Odugbemi et al., 2007; Ashidi et al., 2010).

Many steroidal alkaloids from the genus *Funtumia* have been reported (Janot et al., 1963; Truong-Ho et al., 1963; Mukam et al., 1973; Zirihi et al., 2005). Previous phytochemical investigation of the stem bark of *Funtumia africana* led to the isolation of steroidal alkaloids of the conanine group, named 12α-hydroxy norcona-*N* (18).1.4-trienin-3-one, 11α, 12α-dihydroxy norcona-*N* (18).1.4-trienin-3-one and 11α-hydroxy norcona-*N*(18).1.4-trienin-3-one (Wagner et al., 1987).

The aim of this study was to determine the antibacterial, antifungal, anti-inflammatory activities and the cytotoxicity of the crude extract, fractions and bioactive compounds of *F. africana* to evaluate its potential use as an antimicrobial agent.

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http://dx.doi.org/10.1016/j.sajb.2016.10.019
0254-6299/

2. Materials and methods

2.1. Plant collection and extraction

The leaves of *F. africana* were collected from the Lowveld National Botanical Gardens, Nelspruit, Mpumalanga, in November. The leaves were collected in open weave orange bags, dried at room temperature in the shade and powdered using a Macsalab mill (Model 200 Lab). The powdered materials were then stored in closed honey jars at room temperature in the dark until needed. Voucher specimens (PRU 114782) was prepared and kept at the H.G.W.J. Schweickerdt Herbarium of the University of Pretoria.

Acetone was used as extractant because it is the best for investigating antimicrobial activity of plant leaf extracts (Eloff, 1998b). The powdered plant leaf material (400 g) of *F. africana* were extracted with 4 L of acetone and shaken vigorously for eight hours on a Labotec shaking machine. The supernatant was filtered through Whatman No. 1 filter paper using a Buchner funnel and evaporated under vacuum using a Büchi rotavaporator R-114 (Labotec). The concentrated extract was poured into a pre-weighed beaker. The same procedure was repeated twice on the marc (remainder of plant material). The extract was then left to dry under a stream of cold air. The quantity extracted was 31.34 g. Solvent–solvent fractionation was used to fractionate the acetone extract based on polarity of the compounds (Suffness and Douros, 1979).

The acetone extract was reconstituted in 300 ml of chloroform and mixed with an equal volume of distilled water in a separatory funnel to give a chloroform fraction. The water fraction was then mixed with an equal volume of *n*-butanol to yield the water and butanol fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water in methanol mixture, which yielded the hexane fraction. The 10% water:methanol fraction was then further diluted to 30% water in methanol and mixed with chloroform to yield 30% water in methanol fraction and chloroform fraction. A total of five fractions were collected, namely water, butanol, 30% water in methanol, chloroform and hexane.

2.2. Antimicrobial activity

2.2.1. Bacterial and fungal species

Four most important nosocomial pathogenic bacterial species were selected for the study, namely the Gramme-positive *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), and the Gramme-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) (Sacho and Schoub, 1993). The selection of the specific bacterial strains was based on the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS, 1990), now known as the Clinical Laboratory Standards Institute (CLSI). All the cultures were maintained on Mueller-Hilton (MH) agar (Fluka, Switzerland) at 4 °C. The cells were inoculated and incubated at 37 °C in MH broth (Fluka, Switzerland) for 12 h prior to determining the activities. The densities of bacterial cultures for use in the screening procedures were as follows; *S. aureus* (2.6×10^{12} cfu/ml), *E. faecalis* (1.5×10^{10} cfu/ml), *P. aeruginosa,* (5.2 × 10 cfu/ml9), *E. coli* (3.0×10^{11} cfu/ml) (Shai et al., 2008).

The fungal pathogens that were used included a *Candida albicans* reference strain (ATCC 10231) and three clinical isolates from the National Health Laboratory Service (NHLS), Department of Microbiology, Pretoria. *Candida albicans,* isolated from a Gouldian finch, *Cryptococcus neoformans,* isolated from a cheetah, and *A. fumigatus*, isolated from a chicken which suffered from systematic mycosis, were obtained from the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. The plant pathogenic fungi, namely *Fusarium oxysporum, Penicillium janthinellum* and *Rhizoctonia solani,* which are among the most important fungi of economic significance to plants, were obtained from the Department of Microbiology and Plant Pathology at the University of Pretoria. All the fungal organisms were maintained on Sabouraud (SD) agar (Oxoid Basingstoke, UK) at 4 °C until use. For the antifungal assay, the fungal species were subcultured in SD broth at 35 °C before screening (Mahlo et al., 2010; Suleiman et al., 2010).

2.2.2. Bioautographic antibacterial method

Qualitative analyses of the number of antibacterial compounds were determined by the bioautography method (Begue and Kline, 1972). Exactly 100 μg of the crude extract was loaded in a line of 1 cm wide on the thin layer chromatography (TLC) plates and developed in the benzene:ethanol:ammonium hydroxide (90:10:1) (BEA), non-polar/basic eluent system (Kotze and Eloff, 2002). The TLC plates were dried under a stream of air to evaporate the solvents. Overnight bacterial cultures which were grown in MHin an incubator at 37 °C were centrifuged at 3000 × *g* for 10 min. The pellets were resuspended in 10 ml of fresh MH broth. The developed plates were sprayed with the fresh bacterial culture of *S. aureus* until completely moist using a spraying gun. The moist plates were incubated at 37 °C in a humidified atmosphere for about 18 h. The plates were sprayed with a 2 mg/ml aqueous solution of *p*-iodonitrotetrazolium violet (INT) (Sigma) and incubated for a further 2–6 h. Bacterial growth led to the emergence of a purple–red colour resulting from the reduction of INT into the corresponding formazan salt. Clear zones indicated the inhibition of the bacteria by the compound present at that R_f value on the chromatogram. For fungi a variation was used by collecting conidia and growing them overnight before spraying on a chromatogram (Masoko and Eloff, 2005).

2.2.3. Minimal inhibitory concentration (MIC)

A serial microdilution assay (Eloff, 1998a) with slight modification (Masoko et al., 2005) was used to determine the minimum inhibitory concentration value of the crude extracts, fractions and isolated compound using INT reduction as an indicator. This was determined against all the plant and animal fungi chosen for the study. The samples were tested in triplicate in each assay, and the assays were repeated in their entirety to confirm results.

Aliquots of the crude acetone extract and fractions were dissolved in acetone to final concentrations of 10 mg/ml. The isolated compound was dissolved in acetone to a final concentration of 1 mg/ml. Exactly 100 μl of the extracts, fractions and compound were serially diluted with 50% water in 96-well microtitre plates and 100 ml of microbial culture was added to each well. Amphotericin B and gentamicin were used as the positive controls, while serially diluted acetone was used as the negative control. As an indicator of growth, 40 μl of 0.2 mg/ml INT dissolved in hot water was added to the microplate wells (Eloff, 1998a). The covered microplates were incubated and examined after 24 and 48 h at 35 °C at 100% relative humidity after being sealed in a plastic bag to minimize fungal contamination in the laboratory. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (Eloff, 1998a). Where fungal growth is inhibited, the solution in the well shows a marked reduction in intensity of colour after incubation with INT or remains clear.

2.3. Anti-inflammatory activity of the crude extract of F. africana and fractions

Anti-inflammatory activity was determined using both the COX-1 and COX-2 assays. The COX-1 bioassay was performed as described by White and Glassman (1974) with slight modifications (Jager et al., 1996). All the fractions and the crude extract were reconstituted in ethanol. Cyclooxygenase enzyme (3 units protein prepared from ram seminal vesicles [Sigma Aldrich] was mixed with co-factor solution (0.3 mg/ml adrenalin and 0.3 mg/ml reduced glutathione in 0.1 M Tris