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Isolation of anti-*Candida albicans* compounds from *Markhamia obtusifolia* (Baker) Sprague (Bignoniaceae)

F. Nchu, M.A. Aderogba *, L.K. Mdee, J.N. Eloff *

Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria, PO Box X04, Onderstepoort 0110, South Africa

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Abstract

An increase in clinical cases of Candidiosis globally as well as fungal resistance to drugs prompted the search for novel anti-*Candida albicans* agents from plant sources. Leaf extracts of *Markhamia obtusifolia* were screened for activity against *C. albicans in vitro*. An acetone extract obtained following serial exhaustive extraction contained mainly the active components with at least four active zones on the bioautogram. Bioassay guided fractionation of this extract led to the isolation of three compounds which inhibited the growth of three *C. albicans* strains. Based on spectroscopy studies (NMR and MS), the compounds were identified as 3β-hydroxyurs-12-en-28-oic acid, ursolic acid (1) 3β, 19α-dihydroxyurs-12-en-28-oic acid, pomolic acid (2) and 2β, 3β, 19α -trihydroxy-urs-12-en-28-oic acid, 2-*epi*-tormentic acid (3). The most active compound was 3β, 19α-dihydroxy-12-ursen-28-oic acid (2) with a minimum inhibitory concentration (MIC) value of 12.5 μg/mL for *C. albicans* isolated from dog and 25.0 μg/mL for *C. albicans* from cat and ATCC 90028 at 24 h following incubation. However, at 48 h of incubation MICs were >400 μg/mL for all the three compounds isolated. This study indicated that *M. obtusifolia* could be a potential source of active principles against *C. albicans*.

Keywords: *Candida albicans*; *Markhamia obtusifolia*; Minimum inhibitory concentration

1. Introduction

In recent times, there has been a rapid increase in fungal infections globally and this could be attributed mainly to increased susceptibility of individuals to infection due to immunosuppression and invasive drug therapy (Schmidt-Westhausen et al., 2004; Sims et al., 2005). Schwab et al., 1997 reported the increased adherence of *Candida albicans* to buccal epithelial cells obtained from AIDS patients compared to non-sufferers. Multiple resistant phenotypes of *C. albicans* have been found to coexist during episodes of oropharyngeal candidiasis in AIDS patients (Lopez- Ribot et al., 1999). *C. albicans* cells are protected by cell walls which mediate interaction with the host for adhesion and modulation of antifungal immune response in their host (Poulain and Jouault, 2004). Hence, the cell membrane may serve as a potential target for antifungal drugs. The mechanism of action of some anti-fungal drugs is by binding to the cell membrane of pathogenic fungi in the presence of certain sterols, which subsequently disturb permeability and transport characteristics of the membrane, resulting in loss of intracellular cations (Katzung, 1984).

The current drugs available against *Candida* spp. include Amphotericin b and fluconazole (Sandven, 2000). However, there is an increasing rate of triazole resistance amongst *C. albicans* isolates worldwide, which may be attributed to the frequent use of triazoles as prophylaxis for fungal infections in AIDS patients (Wroblewska et al., 2002). The situation is even worse in Africa due to shortage of antifungal drugs, poverty and rise in the number of HIV patients. In order to alleviate the problem of reduced availability of drugs needed to treat candidiasis, traditional medicine derived from plants are still being used in parts of Africa (Motsei et al., 2003). This prompted the search for novel and active anti-*C. albicans* agents from plant sources.

In a systematic search for bioactive secondary metabolites from plant origin, *Markhamia obtusifolia* was selected for investigation as a result of our random screening of trees of southern Africa for antifungal activity. *M. obtusifolia* is a

* Corresponding authors. Tel.: +27 12 529 8244; fax: +27 12 5298525.
E-mail addresses: marogba@oauife.edu.ng (M.A. Aderogba), kobus.eloff@up.ac.za (J.N. Eloff).

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perennial shrub belonging to the family Bignoniaceae, which grows widely in southern and eastern Africa (Germishuizen and Meyer, 2003). In folk medicine, roots of *M. obtusifolia* are used for the treatment of hookworm in parts of Tanzania (Chhabra and Mahunnah, 1994) and there is no report of isolation of any secondary metabolites from the plant. In this study, we report the isolation and evaluation of anti-*C. albicans* compounds from the leaves of *M. obtusifolia*.

2. Materials and methods

2.1. Plant materials

Fresh leaves of *M. obtusifolia* were harvested from the Lowveld National Botanical Gardens Nelspruit, South Africa in April 2005. The origin of the tree is recorded in the database of the garden's herbarium with number 15/94. Leaves were dried under shade at room temperature 27 °C for a period of one month. The dried leaves were ground using a Jankel and Kunkel Model A 10 mill into fine powder.

2.2. Extraction of plant material

Powdered leaf material (700 g) was subjected to serial exhaustive extraction using 4 L each of hexane, dichloromethane, acetone and methanol in this order. Extraction was repeated three times per extractant by replacing solvent after each extraction. The third extraction for each solvent was left overnight while shaking. The extracts obtained were filtered under pressure and concentrated using a Büchi rotary evaporator. The following yields were obtained per extractant; Hexane (10.01 g, 1.43% w/w); dichloromethane (24.18 g, 3.45% w/w), acetone (21.03 g, 3% w/w) and methanol (19.2 g, 2.74% w/w).

2.3. Thin layer chromatography (TLC)

For each of the extracts obtained, 20 mg was dissolved in 2 mL of acetone and the mixture was homogenized using a sonicator to obtain a solution of 10 mg/mL. Five micro litre of each solution was spotted on TLC plates and components of the various extracts were separated on normal phase silica gel TLC plates (Merck®). Standard mobile phases (ethyl acetate:methanol:water (EMW), 10:1.35:1; benzene:ethyl acetate:ammonia (BEA), 18:2:0.2; chloroform:ethyl acetate:formic acid (CEF), 10:8:2) were used to separate components over a wide range of polarities. Some of the developed chromatograms were examined under ultra violet light at 254 and 354 nm (Camac Universal UV lamp TL 600) and sprayed with vanillin–sulphuric acid reagent, while others were kept for bioautography.

2.4. Bioautography

Three strains of *C. albicans* were used for this study. Two strains were isolated from clinical cases of dogs and cats and both provided to us by the Microbiology unit of the Department of Veterinary Tropical Diseases, University of Pretoria. The third strain was standard ATCC 90028. The fungi were sub-cultured on sabouraud dextrose agar (SDA) plates and kept at 3 °C in a cold room until use. A sterile swap was used to inoculate fungus into a liquid medium (sabouraud dextrose broth). A final fungal concentration (10^6 cells/ml) used for this study was determined by the aid of a Neubauer haemocytometer. The TLC plates were dried overnight next to a table fan, and then sprayed with fungal suspension (actively growing cells of *C. albicans*) using a spraying gun and incubated overnight in a chamber at 38 °C and 100% RH. Plates were sprayed with 2 mg/mL solution of p-iodonitrotetrazolium (INT) (Sigma). They were incubated for an hour and clear zones on the bioautogram indicated inhibition of growth and an indication of the bioactive components of the extracts. The retardation factor (R_f) values of active components in the bioautograms were noted and were targeted in bioassay guided fractionation.

2.5. Isolation of bioactive compounds

The acetone fraction (20 g) of *M. obtusifolia* leaves extract obtained following serial exhaustive extraction was fractionated on silica gel column and successively eluted with $CHCl_3$ (100%) followed by $CHCl_3$:MeOH (95:5 v/v). Column fractions collected in 50 mL test tubes were analysed on TLC plates. The fractions were pooled into five groups (1, 2, 3, 4 and 5). TLC analyses and bioautography results indicated that active components of the fractions were distributed in group 5 (4.78 g). This fraction (group 5) was chromatographed on a silica gel using $CHCl_3$ (100%) followed by an increasing gradient of EtOAc in $CHCl_3$ up to 50%. Fractions were collected in 50 mL test tubes and analysed on TLC plates. Based on TLC analysis, tubes 56–89 contained single spots and were pooled together. Evaporation of the solvent on a rotary evaporator yielded compound **1** (white powder, 100 mg). Test tubes 104–121 also contained a single spot on TLC, and evaporation of the solvent yielded compound **2** (white powder, 75 mg). Compound **3** (White powder, 50 mg = 1.05%) was obtained as a single spot from test tubes 155–168 from the eluted column fractions. The R_f values of the compounds in the CEF solvent system were: 0.826, 0.8 and 0.6 respectively.

2.6. Structure elucidation of compounds **1**–**3**

Structure elucidations of the isolated compounds were carried out using spectroscopic techniques: mass spectrometry, ^1H and ^{13}C NMR, DEPT together with 2D experiments (COSY, HMQC and HMBC). Spectroscopic data of the compounds were also compared with the appropriate literature. The spectra data agreed with the reported data [Ursolic acid, 3β-hydroxyurs-12-en-28-oic acid (**1**), (Seebacher et al., 2003; Thuong et al., 2005), Pomolic acid, 3β, 19α-dihydroxy-urs-12-en-28-oic acid (**2**), (Cheng and Cao, 1992), 2-*epi*-tormentic acid, 2β, 3β, 19α-trihydroxy-urs-12-en-28-oic acid (**3**), (Delgado et al., 1989), Fig. 1]. The compounds were identified as: 3β-hydroxyurs-12-en-28-oic acid (**1**), 3β, 19α-dihydroxy-urs-12-en-28-oic acid (**2**) and 2β, 3β, 19α-trihydroxy-urs-12-en-28-oic acid (**3**) from their spectroscopic data.

Table 1
Minimum inhibitory concentration (MIC) values of *Markhamia obtusifolia* crude extracts at 24 h and 48 h.

Crude extracts		MIC (mg/mL)	
		24 h	48 h
Hexane	D	>2.5	>2.5
	C	>2.5	>2.5
	Q	1.25	>2.5
Dichloromethane	D	0.16	>2.5
	C	0.32	>2.5
	Q	0.16	>2.5
Acetone	D	0.16	>2.5
	C	0.16	>2.5
	C	0.8	>2.5
Methanol	D	0.32	>2.5
	C	0.32	>2.5
	Q	1.25	>2.5

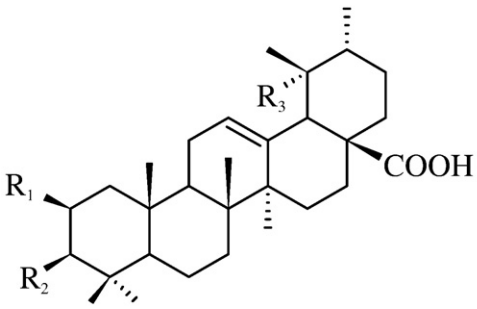
D indicates fungal isolate from dog, C indicates fungal isolate from cat and Q is the standard isolate of *C. albicans* 90028.

2.7. Microdilution assay (quantitative determination of anti-C. albicans activity)

The microdilution method described by Eloff (1998a) was employed with slight modifications in the determination of the minimum inhibitory concentration (MIC) for the extracts and compounds. Crude extracts (10 mg/mL), compounds **1**–**3** (1600 µg/mL) and Amphotericin b (160 µg/mL) were prepared as stock solutions in acetone. One hundred microlitre of solution containing crude extracts or pure compounds were serially diluted 50% with water in 96-well microplates (two-fold serial dilution). Fungal cultures were transferred into SDB, and 100 µL of fungal concentration (10^6 cells/mL) was added to each well. Amphotericin b was used as the reference antibiotic and positive control. Solvent blank (acetone) was included as negative control. Forty micro litre of 0.2 mg/mL of INT dissolved in water was added to each microplate well and incubated at 37 °C 100% RH (sealed in a plastic bag). The MIC values were recorded after 24 and 48 h. The colourless tetrazolium salt acted as electron acceptor and was reduced to a red coloured formazan product by biologically active organisms (Eloff, 1998a). Experiments for the MIC were carried out in replicate.

3. Results and discussion

Results obtained from this study indicated that the acetone extract of *M. obtusifolia* was the most active compared to extracts obtained with hexane, dichloromethane and methanol following serial exhaustive extraction (Table 1). Acetone is a very useful extractant in that it is less toxic, highly volatile and capable of extracting a wide range of compounds from lipophilic to hydrophilic compounds (Eloff, 1998b). The best resolution of bioactive compounds was obtained in the CEF solvent system, with at least four visible active spots in the bioautograms. The bioautography results of the three strains used were similar.



Compound	Trivial name	R_1	R_2	R_3
1	ursolic acid	H	OH	H
2	pomolic acid	H	OH	OH
3	epi-tormentic acid	OH	OH	OH

Fig. 1. Structures of isolated compounds from *Markhamia obtusifolia*.

Phytochemical investigation of the acetone fraction led to isolation of three compounds: Ursolic acid, 3β-hydroxyurs-12-en-28-oic acid (**1**), Pomolic acid, 3β, 19α-dihydroxy-urs-12-en-28-oic acid (**2**), 2-*epi*-tormentic acid, 2β, 3β, 19α-trihydroxy-urs-12-en-28-oic acid (**3**), which are active against *C. albicans*. Compound (**2**) was the most active with an MIC value of 12.5 µg/mL at 24 h (Table 2). Ursolic acid had been isolated from many plant species including *Rosemarinus officialis*, *Pyrus pyrifolia*, *Prunus domestica* and *Ocimum sanctum*. The compound exhibited potent anticancer potential in humans through inhibition of signal transducers and activators of transcription 3 (Pathak et al., 2007). Pomolic acid was previously reported from *Crysobalanus icaco* by Fernandes et al. (2003) and had been recently reported that it may be an effective agent for the treatment of chronic myeloid leukaemia (Vasconcelos et al., 2007). The compound, 2-*epi*-tormentic acid was previously reported from *Hoslundia opposite* Vahl (Ngadjui et al., 1995.) and had been reported to demonstrate mild growth inhibition of *M. tuberculosis* (Wachter et al., 1999).

Table 2
Minimum inhibitory concentration (MIC) values of compounds **1**–**3** from *Markhamia obtusifolia* at 24 h and 48 h.

	R_f values	MIC (µg/mL)	
		24 h	48 h
1	0.826	D 100	>400
		C 50	>400
		Q 50	>400
2	0.8	D 12.5	>400
		C 25	>400
		Q 25	>400
3	0.6	D 100	>400
		C 100	>400
		Q 50	>400
Positive control	–	0.3125	0.3125
Negative control	–	No effect	No effect

D indicates fungal isolate from dog, C indicates fungal isolate from cat and Q is the standard isolate of *C. albicans* 90028. (Amphotericin b) was used as the reference antibiotic and positive control.

Growth inhibition of *C. albicans* was observed during 24 h reading following the start of the experiment, however, the 48 h reading indicated that there was no growth inhibition of *C. albicans* by the extracts or pure compounds from *M. obtusifolia*. The standard *C. albicans* (ATCC 90028) was more susceptible when compared with the isolates from dog and cat in most instances during this study. Susceptibility to antifungal drugs may vary with isolates. To the best of our knowledge no previous report exists on the inhibitory effects of extracts of *M. obtusifolia* and its isolated compounds on *C. albicans*. This study demonstrated the importance of random screening of trees for antifungal activity and potential of *M. obtusifolia* extracts and isolated compounds in treatment of fungal infections.

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