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Obliquumol, a novel antifungal and a potential scaffold lead compound, isolated from the leaves of *Ptaeroxylon obliquum* (sneezewood) for treatment of *Candida albicans* infections

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In a random screening of the anti-Candida activity of more than 500 tree species, the acetone leaf extract of *Ptaeroxylon obliquum* (Thunb.) Radlk. (Sneezewood) had very good antifungal activity against *Candida albicans*. We isolated the compounds responsible for the antifungal activity by bio-assay guided fractionation. The acetone leaf extract was separated into five fractions by solvent-solvent fractionation. The chloroform fraction was subjected to repeated open column silica gel chromatography. We isolated two antifungal entities and determined the structures by nuclear magnetic resonance spectroscopy. The first entity was a mixture of β-amyrin and lupeol found frequently in other plants that we could not separate. The second entity was a novel compound not isolated before 8,11-Dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4H-pyrano[2,3-g][1]benzoxepin-4-one 12-O-acetate given the trivial name of obliquumol. Obliquumol had a higher activity against some *Candida albicans* isolates than amphotericin B, the positive control (MIC of 0.04-0.08 compared to 0.11mg/ml) and lower cellular cytotoxicity against mouse fibroblast cells than amphotericin B. The structure of obliquumol may represent a low toxicity new scaffold molecule for the development of antifungal compounds.

Keywords: antifungal, isolation, structure elucidation, minimum inhibitory concentration, cytoxicity

Obliquumol 'n nuwe antifungus en 'n potensiële raamwerkmolekuul uit die blare van *Ptaeroxylon obliquum* (NieshoutC) om *Candida albicans* infeksies te behandel: In 'n voorlopige ondersoek na die aktiwiteit van asetoon blaarekstrakte van meer as 500 boomspesies teen *Candida albicans* het die ekstrak van *Ptaeroxylon obliquum* (Thunb.) Radlk. (Nieshout) baie goeie aktiwiteit gehad. Ons het die aktiewe verbindings deur 'n bioaktiwiteit-geleide proses geïsoleer. Die asetoon blaarekstrak is in vyf fraksies verdeel deur oplosmiddel-oplosmiddel fraksionering. Die chloroform fraksie het die hoogste aktiwiteit gehad en is onderwerp aan herhaalde oop-kolom kolomchromatografie. Ons het twee antifungus entiteite geïsoleer en die strukture bepaal deur kernmagnetiese resonansspektroskopie. Die eerste entiteit was 'n mengsel van β-amirien en lupeol, verbindings wat dikwels in plante aangetref word. Die tweede entiteit was 'n molekuul wat nog nie tevore gevind is nie, naamlik 8,11-Dihidro-5-hidroksie-12-hidroksiemetiel-2 metiel-4H-pirano[2,3-g]bensoksiepin-4-oon-12-O-asetaat waaraan ons die algemene naam obliquumol toegeken het. Die verbinding het hoër aktiwiteit as die positiewe kontrole amfoterisien- B teenoor *Candida albicans* gehad (MIK 0.04-0.08 teenoor 0.11). Dit het ook 'n laer sitotoksisiteit op muis fibroblastselle as amfoterisien-B gehad. Obliquumol se buitengewone struktuur mag as 'n raamwerkmolekuul dien vir die ontwikkeling van nuwe antifungusverbindings.

Introduction

The growing resistance of microorganisms against antimicrobials is widely accepted. One author has even stated that we may be entering the antibiotic era (Berkowitz 1995). There is also a growing development of resistance against antifungal agents. The importance of finding new antifungal agents from African plants was stressed (Hostettman *et al.* 2000). Van Wyk *et al.* (2009) reviewed the current treatment and potential use of plant-based medicines on treating *Candida albicans* infections especially against oral candidiasis. HIV-positive patients may have an increased susceptability to *C. albicans* infections (Owotade *et al.* 2016).

C. albicans infection is a world-wide problem with an estimated 75 million cases per year for

mucosal vaginal infections and an estimated 9.5 million cases per year for digestive and urinary tract infections (Vandeputte *et al.* 2012). Africa and Abrantes (2016) provided a systematic review of drug resistance against *C. albicans* in sub-Saharan populations where there is a high HIV infection rate. There is also a growing resistance against other fungal pathogens and South Africa has the highest, close to 50%, non-Candida flucaconazole resistance of many countries investigated (Wiederhold 2017).

The Phytomedicine Programme has embarked on a project to determine the activity of acetone leaf extracts from trees growing in botanical gardens against eight important bacterial and fungal pathogens including *C. albicans*. The main object was to determine if there is a correlation between the taxonomy and antimicrobial activity. A degree of success was obtained at order level (Pauw & Eloff 2014).

In this project some plant extracts with excellent activity against one or more of the microbial pathogens were discovered. An acetone extract of Sneezewood (*Ptaeroxylon obliquum*) leaves collected from the Manie van der Schyff Botanical Garden at the University of Pretoria had minimum inhibitory activities of 0.07 mg/ml against an ATCC strain of *C. albicans*. An herbarium voucher specimen was housed in the JGW Schweikert Herbarium of the University of Pretoria. In this contribution we describe the isolation and characterisation of two known and a novel antifungal compound from the leaves of *Ptaeroxylon obliquum.*

Ptaeroxylon is a monotypic genus comprising only the southern African species, originally placed in the Ptaeroxylaceae family. The APG II system of classification (2003 and 2009), has now placed all species in this family within the Rutaceae. *Cedrelopsis* is a genus limited to Madagascar incorporating several species (Mulholland *et al.* 2000). *P. obliquum* bark contains a wide variety of simple and prenylated 6.7-dioxygenated coumarins and 5.7-dioxygenated prenylated chromones (Dean & Taylor 1967). An ethyl acetate extract of ground *P. obliquum* heartwood contains the chromone peucenin, found previously in the roots of *Peucedanum ostruthium* Koch (Umbelliferae); desoxykarenin and karenin; as well as isomeric coumarins 7-0-(3.3-dimethylallyl) scopoletin, nieshoutin and nieshoutol; and the ubiquitous β-sitosterol (McCabe *et al.* 1967). A re-investigation of the bark of *P. obliquum* yielded the unusual aromadendrane diterpenoid, cneorubin X (Mulholland *et al.* 2000). The wood is chemically highly complex and contains numerous unusual chromones and other phenolic compounds. It appears that no phytochemical work has been done on leaf extracts of sneezewood.

Ptaeroxylon obliquum (Thunb.) Radlk. (Sneezewood) is a medicinal plant used for several therapeutic purposes. It grows naturally along the eastern coastal parts of South Africa and northwards to the Northern Province (van Wyk *et al.* 2002)]. The powdered wood is used as a snuff to relieve headache and infusions of the powdered wood are taken for the treatment of rheumatism and heart disease. The bark is used for the treatment of rheumatism and arthritis (Watt & Breyer-Brandwijk, 1962). Sneezing is induced presumably by the chromones in the wood. Perforatin A, isolated from the leaves, has antihypertensive activity (van Wyk *et al.* 2002). The aim of this study was to isolate and characterise the compounds in the *P. obliquum* acetone leaf extract responsible with the antifungal activities against *C. albicans* standard strain (ATCC 10231).

Materials and methods

Healthy leaves were collected from a tree growing in the Manie van der Schijff Botanical Garden on the Hatfield campus of the University of Pretoria and also at a later stage from the Lowveld National Botanical Garden and the Pretoria National Botanical Garden. The leaves were dried in a stream of air at room temperature and then ground to a fine powder. The finely ground leaves were extracted with acetone (ratio 10 ml per g) because its efficacy in extracting antimicrobial compounds from plants has been established (Eloff 1998a). The extract was filtered using Whatman # 1 paper in a Büchner funnel and the acetone was removed in a Büchi rotavaporator under reduced pressure.

The solvent-solvent extraction/fractionation of plant extracts protocol developed by the National Cancer Institute was modified by elimination of the carbon tetrachloride step. Five solvent-solvent fractions containing compounds with different polarities from *P. obliquum* acetone leaf extract were obtained (Suffness & Douros 1979; Eloff 1998c).

To determine the MIC values of the different fractions and the isolated compounds, a serial microplate dilution method using *p*-iodonitrotetrazoliumviolet (INT) as growth indicator (Eloff 1998b) was used. Briefly a series of twofold serial dilutions of the 10 mg/ml acetone extracts were placed in wells of a 96-well microplate. The 100 µl volumes with different concentrations of the extract were inoculated with 100 µl of an actively growing *C. albicans* culture, sealed and incubated for 12 and 24 hours respectively. After incubating, 40 µl of a 2 mg/ml *p*-iodonitrotetrazoliumviolet (INT) in water was added to each well and incubated until the cultures turned red due to the reduction of INT to the formazan by live microorganism. The organisms would have been subjected to 2.5, 1.25, 0.63, etc. mg/ml of the extract. The lowest concentration of the extract that leads to a decrease in colour intensity represents the minimum inhibitory concentration. Acetone was used as negative control and 10 mg/ml serially diluted amphotericin B as positive control. All experiments were repeated three times and because it is based on a two-fold dilution, the standard deviation was usually 0. The different *C. albicans* isolates were obtained from the American Type Culture Collection (ATCC 10231) and from clinical isolates of patients at the dental hospital of the University of Pretoria. Isolates were cultured in potato dextrose broth (Oxoid, Basingstoke United Kingdom).

The cytotoxicity of the acetone leaf extract, the different solvent-solvent fractions of the acetone extract and the isolated compounds were determined by using the

TABLE 1: MIC in mg/ml (determined in triplicate), of three fractions from acetone leaf extracts and amphotericin B against one ATCC and six clinical isolates of *C. albicans*

	ATCC 10231	M0824	M0825	M0826	1051604	1051608	1051255	average
Hexane	0.169	0.683	0.336	0.169	0.169	0.169	0.266	0.28
Chloroform	0.179	0.179	0.045	0.022	0.022	0.022	0.070	0.08
Ethyl acetate	0.740	0.740	0.091	0.091	0.091	0.091	0.276	0.30
Amphotericin B	0.11	0.03	0.03	0.06	0.06	0.11	0.03	0.06

MTT assay procedure of Mossman (1983) against mouse fibroblast cells with berberine as the positive control.

Because the chloroform fraction had the highest antifungal activity and contained the most antifungal compounds determined by bioautography, it was selected for isolating the active compounds. Repeated open column chromatography of the chloroform fraction with solvents of increasing polarity was carried out. Further fractionation was based on antifungal activity and pooled TLC chromatograms of fractions based on similarity of composition.

Two entities were isolated that yielded only one separated compound by thin layer chromatography using different eluents. A Bruker Avance III-400 spectrometer was used for nuclear magnetic resonance spectroscopy. The structures were elucidated mainly by 1D NMR (^{1}H, ^{13}C and DEPT) and 2D NMR (HSQC, HMBC and COSY), mass spectroscopy and by comparison with the literature data.

Results and discussion

MIC of extracts and fractions

The *C. albicans* isolates differed substantially in sensitivity to the different solvent-solvent fractions with MICs varying from 22 to 740 µg/ml (Table 1). The chloroform fraction had a much higher average activity than the other fractions and had an activity better than amphotericin B with three of the isolates. This, with the fact that the chloroform fraction contained the most antimicrobial compounds by bioautography (results not shown) motivated the selection of the chloroform fraction for further investigation. The methanol-water fraction had practically no activity with an MIC of > 2 500 µg/ml.

TABLE 2: ^{13}C NMR data of obliquumol in $CDCl_3$

Atom	δC	δH	HMBC (H→C)
2	167.14 S		–
3	108.66 D	3 5.99 q (J 0.7)	C-2, C-4, C-4a, 2-CH3
4	182.68 S	–	–
4a	106.72 S	–	–
5	155.79 S	–	–
6	99.29 D	6.47 s	C-4, C-6a, C-5, C-11a, C-4a
6a	164.39 S	–	–
8	71.04 T	4 4.61 tt (*J* 1.6, 1.6)	C-6a, C-9, C-10, C-12, C-11
9	133.23 S	–	–
10	128.30 D	2 6.02 tt (*J* 5.5, 1.2)	C-11a, C-8, C-12, C-15
11	21.14 T	6 3.52 d (*J* 5.6, 1.2, 1.6)	C-6a, C-11b, C-9, C-10, C-11a
11a	115.82 S	–	–
11-b	158.06 S	–	–
12	66.45 T	5 4.41 br s	C-9, C-10, C-8, C-13
2-Me	20.78 Q	7 2.30 d (J 0.7)	C-2, C-3
5-OH	–	12.94 br s	–
13	170.61 S	–	–
14	20.42 Q	8 2.01	C-13

Isolation and characterisation of antifungal compounds

Two entities that could not be resolved into more than one compound by column chromatography or TLC with the solvent systems we frequently use (Kotze & Eloff 2002) and with good antifungal activity (Table 1) were isolated. Entity 1 turned out to be a mixture of two related compounds, β amyrin and lupeol, that we could not separate by column or thin layer chromatography. These compounds occur in many plants and their antifungal activity is known.

Compound 2 was obtained as a pure compound. 1D and 2D NMR spectra exhibited chemical shifts that could not be reconciled with a known compound (Table 2). The compound was identified as 8,11-dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*-pyrano [2,3-g][1]benzoxepin-4-one 12-*O*-acetate (Figure 1); the 12-O-acetate derivative of eranthin. We named the compound

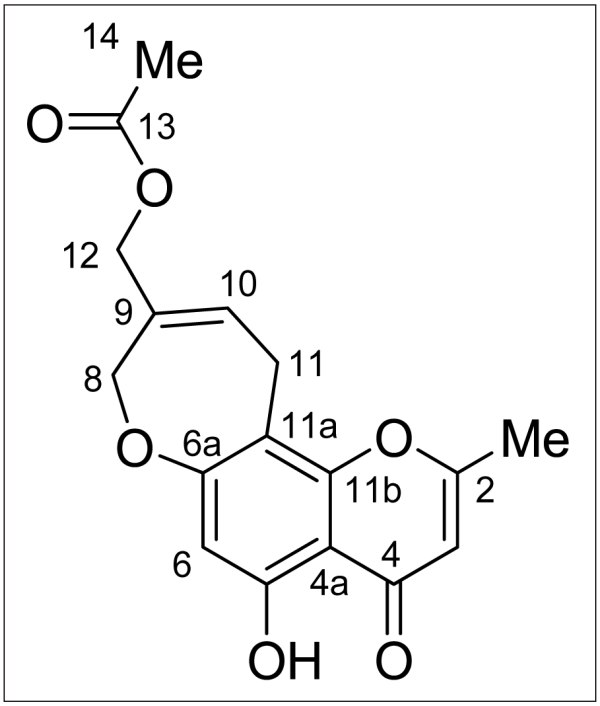

FIGURE 1: Structure of obliquumol isolated from *P. obliquum* leaves: 8,11-dihydro-5-hydroxy-12-hydroxymethyl-2-methyl-4*H*pyrano[2,3-g] [1] benzoxepin-4-one 12-*O*-acetate

TABLE 3: Minimal inhibitory concentrations in mg/ml of the isolated compounds against ATCC 10231 and clinical isolates of *C. albicans* and amphotericin B after 12 and 24 h incubation*

Compound	Incubation time h	ATCC 10231	M0824	M0825	M0826	1051604	1051608	1051255
lupeol/β-amyrin	12	0.004	> 0.25	> 0.25	> 0.25	> 0.25	0.004	> 0.25
	24	0.004	> 0.25	> 0.25	> 0.25	> 0.25	0.25	> 0.25
obliquumol	12	0.004	> 0.25	> 0.25	> 0.25	> 0.25	0.008	> 0.25
	24	0.004	> 0.25	> 0.25	> 0.25	> 0.25	0.25	> 0.25
amphotericin B	12	0.11	0.03	0.03	0.06	0.06	0.11	0.03
	24	0.11	0.11	0.06	0.11	0.06	0.11	1.23

** There was no growth inhibition with the highest concentration (25%) of the negative control acetone*

obliquumol after the species epitheton. The structure of the linear isomer of eranthin O-acetate has been reported, but is not the same compound as obliquumol (Bruder *et al.* 2010).

Comparison of obliquumol's ^{1}H NMR spectrum with the limited data reported by Epe and co-workers and eranthin-12-O- β-D-glycoside (Junior 1979) clearly shows the difference with obliquumol. The synthesis of the closely related isomeric oxepinochromone eranthin was also described (Bruder *et al.* 2010). The molecular formula of obliquumol was determined through accurate mass spectrometry as $C_{17}H_{16}O_6$. The [M+H]+m/z of 339.0840 for $C_{17}H_{16}O_6$ compared well with the calculated value of 339.0845. This compound has not yet been reported as far as we could ascertain from the Dictionary of Natural Products (1996).

The lupeol/β-amyrin mixture and obliquumol inhibited the growth of *C. albicans* standard strain (ATCC 10231) at a much lower concentration (0.004 mg/ml) than amphotericin B (0.11 mg/ml) (Table 3). Obliquumol had high activity against the ATCC strain, but was less active against most of the clinical isolates. Obliquumol had fungicidal activity against strain ATCC 10231 but probably fungistatic activity against isolate1051608 based on the MICs after 12 and 24 h.

TABLE 4: Cytotoxicity to mouse fibroblast cells in µg/ml (LC_{50}) and average antifungal activity to different *C. albicans* strains (MIC) of different extracts, fractions of acetone extracts and compounds

Tested entity	LC50	MIC in µg/ml
Sneezewood acetone extract	35.6	70
Hexane fraction	2012.0	280
Chloroform fraction	28.6	80
Ethyl acetate fraction	229.7	300
Water fraction	0.08	> 2 500
Mixture β amyrin and lupeol	0.001	*
Obliquumol	7.2	*
Amphotericin B	1.5	*
Berberine positive control	9.0	NA

** Values varied, see Table 3*

Cytotoxicity and selectivity index

The cytotoxicity of the compounds was determined against mouse fibroblast cells using the MTT assay. Berberine was used as a positive control and the concentration that killed 50% of the cells was calculated. Amphotericin B was more toxic with a LC_{50} of 1.46 µg/ml against mouse fibroblast cells (Table 4) than berberine. The cytotoxicity of obliquumol (LC_{50}=7.23 µg/ml) was substantially lower than that of amphotericin B (LC_{50}=1.46 µg/ml).

Selective activity of the compounds was calculated by dividing the LC_{50} against mouse fibroblast cells with the MIC in the same units.

The selectivity index indicates the relative safety of a test product to its cytotoxic concentration; the higher the number the safer the product. The selectivity index for the β **amyrin and lupeol** mixture was very low varying between 0.000004 and 0.00025. This mixture is therefore much more toxic to fibroblast cells than to the different *C. albicans* isolates. In some cases, the selectivity index of obliquumol was higher than that of amphotericin B indicating a higher safety level (Table 5). The average selectivity index for obliquumol (0.41) was 12.5 higher than that of amphotericin B (0.03). Although only in the standard ATCC strain, the selectivity index of obliquumol was higher than 1, it should be kept in mind that against many *Candida* infections topical applications, oral rinses or vaginal douches would be used. It is encouraging that obliquumol has a higher average selectivity index than amphotericin B, a drug currently used.

Conclusion

The last scaffold molecule for antifungal compounds, amphotericin B, was discovered in 1956. Obliquumol represents a totally new structure that had better antifungal activity against some *C. albicans* isolates and higher cellular safety than amphotericin B. Although it had lower

TABLE 5: Selectivity index (SI) of obliquumol and amphotericin B against *C. albicans* standard strain (ATCC 10231) and clinical isolates

Compounds	*Candida albicans*						
	Clinical isolates						
	ATCC 10231	M0824	M0825	M0826	1051604	1051608	1051255
Obliquumol	1.80	0.03	0.03	0.03	0.03	0.90	0.03
Amphotericin B	0.01	0.05	0.05	0.03	0.03	0.01	0.05

antifungal activity against some clinical isolates, there is a possibility that modifying the structure may increase its activity against some other resistant fungi. The animal safety should determine the feasibility of continuing work on this compound.

The cytotoxicity of obliquumol (LC_{50}=7.23 µg/ml) was four times lower than that of amphotericin B (LC_{50}=1.46 µg/ml). This is encouraging especially if an envisaged product is to be used as an oral rinse or vaginal douche against candidiasis. Agents inhibiting adhesion of *C. albicans* to the oral mucosa may be beneficial in managing oral candidiasis. The possibility therefore exists to develop this compound into a new oral or vaginal hygiene or antifungal product. Cytotoxicity is not necessarily a good indication of toxicity to animals or humans because several other factors could be involved. Isolation of sufficient obliquumol to determine *in vivo* toxicity in animal studies and activity towards other pathogens should be a priority.

Authors contributions

Dr C van Wyk isolated compound and determined biological activities.

Dr FS Botha compiled and wrote the first draft of the article. The late Prof R Vleggaar elucidated the structure of the isolated compounds.

Prof JN Eloff identified plant to be examined, supervised the isolation of compound and edited and submitted the manuscript.

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