Biofilm inhibiting properties of compounds from the leaves of *Warburgia ugandensis* Sprague subsp *ugandensis* against *Candida* and staphylococcal biofilms

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Abstract

Ethnopharmacological relevance

Warburgia ugandensis Sprague subspecies *ugandensis* is a plant widely distributed in Eastern, Central and Southern Africa. In humans, it is used to treat respiratory infections, tooth aches, malaria, skin infections, venereal diseases, diarrhea, fevers and aches.

Aim of the study

This study aims to identify the bioactive compounds against clinically important biofilm-forming strains of *Candida* and staphylococci that are responsible for tissue and implanted device-related infections.

Methods

Using a bioassay-guided fractionation approach, hexane -, ethanol -, acetone - and water extracts from the leaves of *W. ugandensis*, their subsequent fractions and isolated compounds were tested against both developing and preformed 24h-biofilms of *Candida albicans* SC5314, *Candida glabrata* BG2 *Candida glabrata* ATCC 2001, *Staphylococcus epidermidis* 1457 and *Staphylococcus aureus* USA 300 using microtiter susceptibility tests. Planktonic cells were also tested in parallel for comparison purposes. Confocal scanning laser microscopy was also used to visualize effects of isolated compounds on biofilm formation.

Results

Warburganal, polygodial and alpha-linolenic acid (ALA) were the major bioactive compounds isolated from the acetone extract of *W. ugandensis*. For both warburganal and polygodial, the biofilm inhibitory concentration that inhibits 50% of *C. albicans* developing biofilms (BIC_{50}) was 4.5±1 and 10.8±5 µg/mL respectively. Against *S. aureus* developing biofilms, this value was 37.9±8 µg/mL and 25 µg/mL with warburganal and ALA respectively. Eradication of preformed

24 h biofilms was also observed. Interestingly, synergy between the sesquiterpenoids and azoles against developing *C. albicans* biofilms resulted in an approximately ten-fold decrease of the effective concentration required to completely inhibit growth of the biofilms by individual compounds. The hydroxyl group in position C-9 in warburganal was identified as essential for activity against staphylococcal biofilms. We also identified additional promising bioactive sesquiterpenoids; drimenol and drimendiol from the structure-activity relationship (SAR) studies.

Conclusions

ALA and four sesquiterpenoids: polygodial, warburganal, drimenol and drimendiol, have shown biofilm-inhibitory activity that has not been reported before and is worth following up. These compounds are potential drug candidates to manage biofilm-based infections, possibly in combination with azoles.

Keywords

Chromatography, multi-drug resistance, sesquiterpenoids, *W. ugandensis*, synergy, azoles

1 Introduction

Biofilms are the most prevalent forms observed in microbial growth and development (Dowd et al., 2008). They comprise microbial populations that are attached to surfaces and embedded in a self-produced extracellular matrix (Frade and Arthington-Skaggs, 2011). Biofilm formation occurs in several phases, namely early, intermediate and maturation phases. Pathogenic microbes such as *Candida* and staphylococcal strains in particular, use this as a virulence strategy to ensure successful colonization and survival in tissues and on implanted medical devices (Silva et al., 2011). Notably, the most offensive clinical consequence of these biofilm infections has been

increased resistance to current treatments (Van Acker et al., 2014), consequently increasing morbidity and mortality rates, especially among immunocompromised patients (Fidel et al., 1999; Otto, 2013). The paucity of effective medicines to combat these biofilm infections caused by common pathogens is alarming and justifies increased drug discovery efforts to surmount this challenge.

Plants offer a rich source of chemical diversity from which novel compounds can be harnessed to boost the current antimicrobial drug pipeline (Karygianni et al., 2019; Rishton, 2008; Sardi et al., 2013). *Warburgia ugandensis* Sprague subsp. *ugandensis* is a highly valuable medicinal plant in the Canellaceae family. It is also used as a spice in East Africa suggesting considerable safety upon oral use (Kokwaro, 1976).

It has been used traditionally in the treatment of a broad range of diseases in Kenya, Ethiopia, Tanzania, Uganda, Congo and Malawi (Leonard and Viljoen, 2015; Maroyi, 2014). In Kenya, decoctions from either the bark or leaves are used to treat infections of lungs, throat, gastric ulcers and venereal diseases (Kiringe, 2006). The bark is used for treating skin diseases (Wamalwa et al., 2006), toothaches, malaria, diarrhea, fevers, joint-, chest- and headaches (Kokwaro, 2009) and as an antiviral against measles (Parker et al., 2007). Kubo *et al*. showed that both polygodial and warburganal have potent antifungal activities against planktonic (non-adherent) *C. albicans* (Kubo et al., 1977; Lunde and Kubo, 2000). Mbwambo and colleagues (2009) later reported on the antibacterial and antifungal activity of ethanolic extracts of *W. ugandensis* on planktonic forms of *S. aureus* and *C. albicans*. However, biofilms (sessile cells) are generally more difficult to eradicate by antimicrobials compared to planktonic cells, in part due to the extracellular matrix that restricts penetration of antimicrobials and also due to decreased/heterogenous growth rates as well as drug-efflux systems, amongst other factors (Kuhn et al., 2002; Lewis, 2001; Van Acker et

al., 2014). Biofilm-associated infections account for 60-80% of all infections in humans (Omar et al., 2017), and are often considered the underlying cause of failed treatments in clinical settings, posing a serious challenge to human health. Moreover, drugs/compounds that act on planktonic cells are not necessarily active against biofilms; fluconazole e.g. shows potent activity against planktonic *C. albicans* at 4 µg/mL but its activity against biofilms was >1024 µg/mL (Ramage et al., 2001). Thus, in our search for novel compounds that act on biofilms, we probed on the potential of *W. ugandensis* to inhibit developing or preformed biofilms, and tried to identify the active compounds by bioassay-guided fractionation. Consequently, extracts from this plant and their subsequent fractions and bioactive compounds were tested for activity against biofilms of *S. aureus*, *S. epidermidis*, *C. albicans* and two strains of *C. glabrata*. To the best of our knowledge, this is the first report documenting the biofilm-inhibitory activity of *W. ugandensis*.

2 Materials and methods

2.1 Chemicals and reagents

Resazurin salt was purchased from Acros Organics (Geel, Belgium). All the other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Flat-bottomed Costar™ plates were used for the antimicrobial tests (Kipanga and Luyten, 2017). Stock solutions of fluconazole (FLZ) 1 mg/mL, miconazole (MCZ) 2.5 mg/mL and Amphotericin B (AmB) 10 mg/mL were prepared in dimethyl sulfoxide (DMSO), while vancomycin (VAN) stock, 10mg/mL was prepared in sterile MilliQ water. These stock solutions were aliquoted and stored at -20 °C. A stock solution of Resazurin powder was prepared by mixing 1 g with 100 mL 1x phosphate-buffered saline (PBS) (1 % w/v) in a tube, and covering it with aluminium foil. From this, a working solution was prepared by mixing 200 µL of the stock solution with 50 mL 1x PBS (0.4 % v/v).

115 **2.2 Plant collection**

116 Fresh leaves of *W. ugandensis* were collected from the Kakamega forest in Western Kenya, air-
117 dried at ambient temperature, and milled to a fine powder. A voucher specimen of the plant (NWB
118 237) was deposited at the Egerton University's herbarium.

119 **2.3 Bioassay-guided fractionation**

120 **2.3.1 Small-scale plant extraction**

121 One g *W. ugandensis* powder was added to each of 4 Falcon tubes and extracted separately with
122 10 mL water, acetone, ethanol, and hexane. After extractions, 1 mL aliquots of the crude extracts
123 were transferred into pre-weighed 2 mL Eppendorf tubes, then dried in a Savant™ SpeedVac™
124 apparatus, weighed and stored in a cold room at 4 °C. Just before a biological assay, the dried
125 residue was dissolved in DMSO (for organic extracts) or water for the aqueous extracts; the final
126 concentration in the assay was 1 mg/mL.

127 **2.3.2 Large-scale extraction**

128 One hundred gram of *W. ugandensis* powder was mixed with 1 L of acetone in a screw-capped
129 glass bottle, shaken and sonicated in a sonication water bath for 4 times 30 min over a 24h-period
130 to maximize extraction. The supernatant was decanted through filter paper (MN 615 ¼; Ø 18.5
131 cm, Macherey-Nagel) and the solvent of the filtrate was evaporated in a Buchi® rotary evaporator.
132 The dry residue of the combined filtered supernatants (8 g) was dissolved in a small amount of
133 acetone and adsorbed to about 14 g of silica gel (63-200 µm). The slurry mixture was then
134 evaporated to dryness and stored at 4 °C until dry-loading on a silica gel column.

135 **2.3.3 Preparative silica gel column chromatography**

136 A glass column (20 x 8 cm) was packed with 200 g silica gel (63-200 µm), suspended in 500 mL
137 hexane to a slurry consistency. Once the silica gel had settled in the column, the dried acetone

extract adsorbed to silica gel was evenly layered over the bed surface. The column was eluted using a step gradient starting with 400 mL of 100% hexane, labelled as fraction 1 (F1). This was then followed by the same volume of hexane:ethyl acetate (EtOAc) mixtures, yielding F2-F5 (75:25, 50:50, 25:75, 0:100), then EtOAc:methanol (MeOH) mixtures, F6-F12 (95:5, 90:10, 85:15, 80:20, 60:40, 40:60, 0:100) and finally MeOH:acetic acid mixtures, F13-F15 (95:5, 90:10, 85:15). All 15 fractions (400 mL each) were stored in dark glass bottles at 4 °C. One mL from each fraction was aliquoted into pre-weighed 2 mL Eppendorf tubes and dried. One hundred µL of DMSO was then added to each tube to dissolve the dried residue. Ten and four µL of this solution was tested against *Staphylococcus* and *Candida*, respectively (see section 2.4.2).

2.3.4 High Performance Liquid Chromatography (HPLC)

Active fractions from the silica gel column were fractionated further on a Shimadzu LC-20 AT HPLC system with a DAD detector using a C18 column (Sunfire®, 10 x 250 mm, 5 µm) and acetonitrile (ACN)/water gradients at a flow rate of 4 mL/min, with all mobile phases containing 0.1% trifluoro acetic acid (TFA). Fractions were collected every minute (Gilson model 201 fraction collector) and dried, dissolved in 20 µL DMSO and tested for antimicrobial activity in duplicate. Active peaks (inhibition >75%) were determined by superimposing the chromatogram with the activity profiles of the fractions. Active peaks were collected manually for structure elucidation by nuclear magnetic resonance (NMR) (Liu et al., 2018). Comparison with spectra from published literature enabled confirmation of the identity of the compounds.

2.4 Bioassays

2.4.1 Microbial strains and cultures

Cultures of *S. aureus* USA 300 (Tenover and Goering, 2009), *S. epidermidis* 1457 (Mack et al., 1992), *C. albicans* SC5314 (Gillum et al., 1984), *C. glabrata* ATCC 2001, and *C. glabrata* BG2

(Kaur et al., 2005) were propagated at 37 ˚C for 24 h on either tryptic soy agar (TSA) plates (for bacteria) or YPD agar plates (1% yeast extract, 2% peptone, 2% D-glucose, supplemented with 2% agar) (for *Candida*). These strains were selected as they are the typical standard strains used in many research laboratories.

2.4.2 Antimicrobial test against planktonic cells

We followed the antimicrobial activity using a broth microdilution method throughout the bioassay-guided purification as described earlier (Kerkoub et al., 2018; Panda et al., 2017). Briefly, Roswell Park Memorial Institute (RPMI 1640) medium buffered with MOPS (3-(*N*-morpholino) propanesulfonic acid) to pH 7.0 was used to prepare a *C. albicans* cell suspension at an optical density (OD) 0.1 at 600 nm. Tryptic soy broth (TSB) was used for bacterial suspensions at OD_{600nm} 0.1. In a final assay volume of 200 µL, 5% DMSO was used for *S. aureus* and 2% DMSO for *C. albicans* experiments (Panda et al., 2017). These DMSO concentrations did not affect microbial growth, as confirmed by growth experiments in presence and absence of DMSO. After 24 h in a shaking incubator at 37 °C, absorbance was measured at 490 nm (yeast) or 620 nm (bacteria), and the percentage growth inhibition calculated relative to solvent controls.

$$100 - \left(\frac{A}{B} \times 100\right)$$

Assuming A is the OD value of a well with treated cells and B the OD value of the vehicle control(s) with cells. Antibiotics VAN and AmB (250 µg/mL) were used as positive controls for bacteria and fungi, respectively.

2.4.3 Antibiofilm test on developing and preformed biofilms

Against developing biofilms, a broth microdilution test was performed according to Kerkoub *et al* (2018). Briefly, 100 µL cell suspensions at OD_{600nm} 0.1 in either TSB or RPMI 1640-MOPS were aliquoted into flat-bottomed 96-well (Costar™) plates and incubated for 90 minutes at 37 ˚C in a

184 stationary incubator to permit adhesion of the cells to the bottom of the polystyrene plates. The
185 medium was carefully aspirated and the wells carefully rinsed with 1x PBS (Kipanga and Luyten,
186 2017). Fresh TSB (190 µL) was added to the wells and gently mixed with 10 µL of a plant
187 extract/fraction/test compound. For yeast, 196 µL fresh RPMI 1640-MOPS was gently mixed with
188 4 µL of a test compound. The plates were then incubated at 37 ˚C for 24 h in a stationary incubator.
189 Afterwards, the medium was carefully aspirated and the cells washed twice with PBS, and stained
190 with 100 µL resazurin dye (see section 2.1). After 1 h incubation at 37 ˚C, fluorescence was
191 measured; λ_{ex} at 535 nm and λ_{em} at 590 nm. For *C. glabrata* strains, an XTT, tetrazolium salt 2,3-
192 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide
193 reduction assay at 490 nm was used to quantify the surviving biofilm cells, as *C. glabrata* is not
194 able to metabolize resazurin dye within hours, as also observed with cell titer blue stain (Delattin
195 et al., 2014). The percentage of surviving biofilm cells was calculated relative to the growth
196 controls. Assuming A is the OD/fluorescence value of a well with biofilm treated with compound,
197 B the OD value of the dye in a well (s) without cells and C the OD value of the vehicle control (s)
198 with cells. Antibiotics VAN and AmB (250 µg/mL) were included as positive controls.

199 $$\left(\frac{A-B}{C}\times 100\right)$$

200 For 24 h-preformed biofilms in 96-well plates, treatment with compounds was started 24 h after
201 biofilm formation. Additionally, treated developing biofilms on highly adhesive, round tissue-
202 culture coverslips (diameter 13 mm; Sarstedt) were separately prepared and visualized using
203 confocal scanning laser microscopy (CSLM), essentially as described before (Kucharíková et al.,
204 2011). Briefly, the medium was removed from the wells, the coverslips were gently washed with
205 1 mL PBS, placed into a clean 24-well tissue-culture plate, and 400 µL PBS was added together

with 5 µL (50 µg/mL) concanavalin A–Alexa Fluor 488 conjugate (C-11252; Molecular Probes). After incubation for 10 min at 37 °C in the dark, the stained biofilms were then visualized.

2.4.4 Determination of biofilm inhibitory concentration-50 (BIC_{50}), biofilm eradication concentration-50 (BEC_{50}), inhibitory concentration-50 (IC_{50}), and minimum fungicidal concentration (MFC)

Two-fold serial dilutions of test compounds in the range of 200 µg/mL-0.78 µg/mL (final concentration) were prepared in DMSO and tested against biofilms and planktonic cells. The BIC_{50} (minimum concentration required to inhibit 50% of biofilm growth), BEC_{50} (minimum concentration required to eradicate 50% of formed biofilms) and IC_{50} (minimum concentration required to inhibit 50% of planktonic cell growth) were then calculated by non-linear regression (see section 2.6). The MFC was then determined for the treated cells (from wells where no cells grew) by plating ten-fold dilutions (100 µL) of the cultures on YPD plates and incubating overnight at 37 °C, then counting colonies.

2.4.5 Synergy studies using the checkerboard assay

A combination of an azole (MCZ/FLZ) and a test compound, two-fold serially diluted in DMSO across rows and columns, respectively, of a microplate, was tested on *C. albicans* SC5314 developing biofilms. Fractional inhibitory concentration index (FICI) was calculated as $FICI = (C_A/IC_A) + (C_B/IC_B)$, where IC_A and IC_B are the inhibitory concentrations of drugs A and B alone, respectively, and C_A and C_B are the concentrations of the drugs in combination. The interaction was considered synergistic if the value was ≤ 0.5, indifferent for $0.5 < FICI < 4$ and antagonistic for a FICI value ≥ 4 (Odds, 2003).

2.5 Structure-activity relationship (SAR) studies

Analogues of isolated compounds were purchased from the Sigma-Aldrich (St. Louis, MO, USA) and Specs® companies, and tested for antibiofilm activity.

2.6 Statistical analyses

Non-linear regression was used to determine the BIC_{50}, BEC_{50} and IC_{50} of the compounds used against the pathogens using Graphpad Prism 5 software (San Diego, CA). All bioassays were independently repeated at least twice with duplicate technical repeats. Error bars represent standard deviations.

3 Results and Discussion

3.1 *W. ugandensis* extracts prevent the formation of biofilms

Both the acetone and ethanol extracts prevented the formation of biofilms by all the microorganisms tested (Figure 1A). While the hexane extract failed to show activity at 1 mg/mL, inhibition was seen at 4 mg/mL (data not shown). In a study with antimicrobial screening of thirty-seven plant species belonging to 26 families, most of the activity was detected in ethanol and acetone extracts, followed by water extracts, and very little activity was detected in the hexane extracts (Panda et al., 2018). A similar profile is seen in this study where both acetone and ethanol were superior to hexane in extracting bioactive compounds.

We proceeded with the acetone extract for large-scale preparations as it was more potent than the ethanol extract upon 2-fold serial dilution (Supplementary material I). Eloff (1998), described some guidelines that can be used to select appropriate solvents for plant extraction, such as the diversity of inhibitory compounds extracted, the toxicity of the solvent in the bioassay, and the potential health hazard of the extractants.

In traditional medicine, aqueous decoctions and infusions of *W. ugandensis* are common (Nanyingi et al., 2008), suggesting that the active compounds are (at least to some extent) water-soluble. Boiling the powder in water may facilitate the extraction of active compounds (Just et al., 2015), and a large volume of the decoction taken as medicine may compensate for a lower concentration, so that a therapeutically active dose may still be achieved.

We prepared our extracts from (dried) leaves, whereas in African traditional medicine mostly hot decoctions either from bark, roots or leaves are used. Overharvesting of *W. ugandensis* bark was reported to reduce the population of this valuable tree (Maroyi, 2014). From our results, the leaves offer a more sustainable alternative for extracting medicinal compounds of *W. ugandensis* than the barks. Similar conclusions were drawn by Mbwambo and colleagues (2009) and Drewes *et al*., (2001) for *W. salutaris*.

3.2 Bioassay-guided purification of antibiofilm compounds

Fractionation of the acetone extract showed that fractions 3 and 4 (F3: 50% hexane:50% EtOAc; F4: 25% hexane:75% EtOAc) had the most potent activity against developing biofilms (Figure 1B). F3 was further separated on a C18 HPLC column, and tested against developing biofilms. (Supplementary material II). The structures of three active peaks (Figure 2), as well as a major peak devoid of antimicrobial activity were successfully elucidated by NMR as ugandenial A (inactive), warburganal, polygodial and ALA (Liu et al., 2018; Urones et al., 1994; Xu et al., 2009). Ugandenial A, warburganal and polygodial are known sesquiterpenoids from *W. ugandensis*, while ALA is a polyunsaturated fatty acid (PUFA).

According to Wang and colleagues (2015), PUFA constitute the majority of fatty acids in *W. ugandensis* bark and leaf, accounting for 41.34% and 67.76% of their total fatty acids, respectively.

Moreover, ALA is the most abundant PUFA in the leaf, reaching up to 52.77% of the total fatty acids (*ibid*.).

An aliquot of F4 was also analyzed by HPLC using the same chromatography conditions as for F3, and the only peak with bioactivity had the same elution time and UV spectrum as the warburganal peak in F3, and is therefore most likely warburganal. Back-calculated yields based on the weight of warburganal and polygodial obtained from a known volume of F3, showed that warburganal was 7 times more abundant than polygodial, with 1 g of the dried acetone extract yielding 1.24 mg warburganal. This final yield of roughly 0.1% compares favourably with that of 0.05% from *W. salutaris* (Drewes et al., 2001). For F4, the warburganal and polygodial content were estimated based on the area under the curve of the two peaks, compared with those in F3. F4 contained 17% of the amount of warburganal in F3, and 8% of that of polygodial. This leads us to conclude that warburganal accounts for most of the antifungal activity in F3 and F4 (and presumably therefore in the original crude extract).

3.3 Antibiofilm activity of ALA, warburganal and polygodial

Against *S. aureus* USA 300, ALA inhibited completely the growth of both the planktonic and biofilm forms at 25 µg/mL. However, on *C. albicans* planktonic and biofilm cells, the IC_{50} and BIC_{50} values of ALA were above 100 µg/mL. Moreover, no synergy was observed between polygodial or warburganal and ALA on either *Candida* or staphylococcal developing biofilms (data not shown).

C. *albicans* was the most susceptible strain with warburganal being the most potent isolated compound against both developing biofilms and preformed 24 h biofilms of *C. albicans* and the 2 staphylococcal strains (Table 1). Polygodial was active against *Candida* but not against the staphylococcal strains at the highest tested concentration of 100 µg/mL. To the best of our

knowledge, this is the first report highlighting antibiofilm activity of warburganal and polygodial against both developing biofilms and preformed 24 h biofilms.

Higher compound concentrations were required to inhibit preformed 24 h biofilms compared to developing biofilms, possibly due to the formation of extracellular matrix in the former. We observed a ten-fold difference in sensitivity between developing biofilms of *C. glabrata* compared to *C. albicans* upon treatment with warburganal (Table 1). *Candida glabrata* is known to be generally more resistant to antifungals compared to *C. albicans* due to phenotypic and genetic differences (Brunke and Hube, 2013). Moreover, the preformed 24 h biofilms are more resistant to treatment compared to planktonic cells. Similar observations were made by Ramage and colleagues (2001) when 48 h *C. albicans* biofilms were compared to planktonic *C. albicans* cells. Generally, biofilms are observed to be more resistant to antimicrobials compared to planktonic cells (Sardi et al., 2013).

By counting colony-forming units (cfus), we established that warburganal is fungicidal against *C. albicans* cells at 12.5 μg/mL. Polygodial's minimum fungicidal activity on *C. albicans* developing biofilms was >50 μg/mL as a few (~ 5) colonies consistently persisted at this concentration. These may represent a separate population of persister cells. As is evident, planktonic *C. albicans* cells were about 8 times more sensitive to polygodial than their biofilm counterparts (Figure 3). Against *S. aureus*, the cidal concentration of warburganal was above 50 μg/mL (data not shown).

Moreover, by CSLM, we were able to visually confirm the biofilm inhibiting potential of both warburganal and polygodial at low concentrations on developing *C. albicans* biofilms (Figure 4).

3.4 Structure-activity relationship studies

The observed higher potency of warburganal over polygodial could be attributed to the presence of the hydroxyl group at position C9 that distinguishes the two sesquiterpenes. To outline a pharmacophore responsible for the observed activity in polygodial and warburganal, several analogues were tested. As seen in Table 2, the α,β-unsaturated 1,4-dialdehyde in polygodial and warburganal is required for the potent antifungal activity on developing biofilms. Interestingly, drimenol and drimendiol are sesquiterpenoids that have been previously isolated from *W. ugandensis* (Brooks and Draffan, 1969; Drage et al., 2014). They have also been isolated from plants in the Winteraceae and Polygonaceae families (Paza et al., 2013; Prota et al., 2014), but their inhibitory activity against developing and preformed 24 h biofilms has not yet been reported. The hydroxymethyl group at position C-9 in drimenol is required for the observed activity, and two hydroxymethyl groups in drimendiol do not improve the antibiofilm activity.

3.5 Synergy between polygodial/warburganal and clinically used antifungal drugs

Synergy was tested between warburganal/polygodial and 2 azoles: MCZ and FLZ, against developing *C. albicans* biofilms (Table 3). FICI values below 0.5 indicate synergy. The combinations resulted in lower (~ 10-fold) effective concentrations of the isolated compounds and azoles that was required to completely inhibit the developing biofilms, compared to individual compounds alone. This has implications for reducing toxicities (if present), for treatment of polymicrobial infections and for reducing drug resistance development by pathogens (Rybak and McGrath, 1996).

Interestingly, an earlier study on planktonic *C. albicans* cells also showed synergy between miconazole and polygodial (Kubo et al., 2011). Largely dependent on the drug or a given

342 combination, differences in drug efficacy have been observed between planktonic versus biofilm
343 cells, and these are not always predictable (Kuhn et al., 2002). Mechanistic studies of polygodial
344 in *S. cerevisiae* noted that MCZ's mode of action was different from that of polygodial (Yano et
345 al., 1991). Azoles interfere directly with ergosterol biosynthesis, while polygodial appears to affect
346 (*i.a.*) mitochondrial ATPase (Lunde and Kubo, 2000). Since mitochondrial dysfunction also leads
347 to reduced ergosterol levels (Geragthy and Kavanagh, 2003), the observed synergy can be
348 rationalized. Moreover, MCZ induces the ABC transporter Cdr1p in *C. albicans* (Shukla et al.,
349 2003), which removes MCZ from the cytoplasm; but mitochondrial dysfunction reduces Cdr1p
350 activity (Thomas et al., 2013), providing a second potential mechanism for synergy.
351 Little is known about the *in vivo* effects of warburganal, which is surprising since it appears to
352 account for most of the antimicrobial activity of our *W. ugandensis* extracts. However, many
353 studies have focused on polygodial's safety profile. A clinical trial for recurrent vulvovaginal
354 candidiasis used an oral preparation 'K-712' in which 100 mg contains 10 mg of oleoresin from
355 *Pseudowintera (P.) colorata* at 30% (w/w) polygodial, together with trace amounts of *Olea*
356 *europea*. No adverse drug effects were reported in this 2-year trial, which showed comparable
357 efficacy, improved prophylactic effect, and lower relapse rates compared to itraconazole (Chopra
358 et al., 2013). Until their pharmacokinetic profiles are established, it is difficult to estimate the
359 potential of our sesquiterpenoids for systemic infections. But therapeutic areas that could benefit
360 from the antibiofilm properties of polygodial, warburganal, drimenol, drimendiol and ALA include
361 topical preparations for the treatment of vaginal candidiasis, dermal wounds, skin infections by
362 dermatophytes (Lee et al., 1999; Lunde and Kubo, 2000) and mouth rinses for gum and dental
363 infections. Moreover, they can be incorporated into cleaning solutions for medical devices such as
364 prosthetic teeth or in solutions for irrigating catheters.

4 Conclusion

In conclusion, the above results on the antimicrobial activities of warburganal, polygodial and ALA from the acetone extract of *W. ugandensis* not only support the traditional use of the plant in the treatment of fungal and bacterial infections, but also demonstrate the potential of these compounds in the management of both *C. albicans*- and staphylococcal-biofilm infections. Additionally, this report highlights the potencies of drimenol and drimendiol in inhibiting biofilm development; further investigations on these compounds are underway.

Author Contributions

WL, PVD, PK, ML and JM contributed to study conceptualization, design and corrected the manuscript. PK performed the extractions together with LVP and SKP who also revised the manuscript. PK performed the experiments, analysed the data, drafted and corrected the manuscript. AHM, CV and WB performed NMR measurements, analysis and revised the manuscript.

Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Funding

This work was supported by a scholarship from the Interfaculty Council for Development Cooperation programme, KU Leuven, awarded to PK and by the Fund for Scientific Research Flanders (FWO) (WO.009.16N) to PVD; WL largely supported himself.

Acknowledgements

We thank Wuyts J., Kucharíková S., Ma Y., Nackaerts Z., Bouhenna M., Verjans E.T. and Vanden Bosch L., for technical assistance and meaningful discussions. We also thank Prof. Kabuitu S.T for the identification of the plant.

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Figure Legends

Figure 1. A: Inhibition of biofilm formation by crude extracts of *W. ugandensis* tested at 1mg/ml final concentration. Amphotericin B and Vancomycin were used as positive controls for *Candida* and staphylococcal strains respectively. B: Biofilm-inhibitory activity of the 15 fractions

obtained by step-gradient elution of the acetone extract of *W. ugandensis* from a silica gel column, starting concentration 1mg/mL.

Figure 2. Structures of ugandenial A (**1**), warburganal (**2**), polygodial (**3**) and ALA (**4**)

Figure 3. Average log 10 cfu/mL± SEM to establish the minimum fungicidal concentration (MFC) of polygodial and warburganal on *C. albicans* SC5314 cells.

Figure 4. CSLM photographs of *C. albicans* SC5314 developing biofilms showing: A) DMSO-treated cells (control), B) 6.25 µg/mL polygodial-treated cells, and C) 6.25 µg/mL warburganal-treated cells.

Tables

Table 1. $IC_{50,}$ BIC_{50} and BEC_{50} of warburganal and polygodial on planktonic cells, developing biofilms (compounds added after 90 min of adhesion) and preformed 24 h old *Candida* and staphylococcal biofilms. N.A: not active at highest final concentration used of 100 µg/mL.

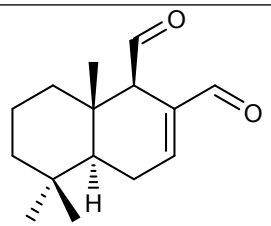
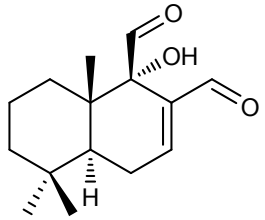
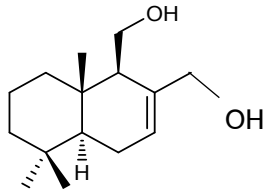
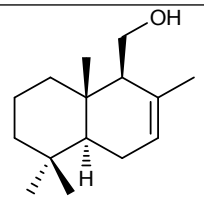
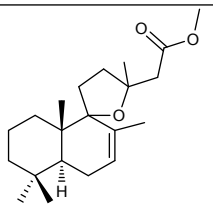
	Warburganal (µg/mL)			**Polygodial (µg/mL)**		
Microorganism	**IC_{50}**	**BIC_{50}**	**BEC_{50}**	**IC_{50}**	**BIC_{50}**	**BEC_{50}**
C. albicans SC5314	4.0±3	4.5±1	16.4±2	4.1±0.1	10.8±5	16.0±5
C. glabrata ATCC 2001	72.0±19	49.1±3	>100	94.1±28	50.6±7	>100
C. glabrata BG2	72.6±22	55. 9±4	>100	>100	61.9±8	>100
S. aureus USA 300	17.5±8	37.9±8	46.2±16	N.A	N.A	N.A
S. epidermidis 1457	15.9±7	38.1±9	35.8±7	N.A	N.A	N.A

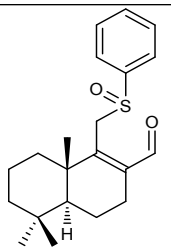
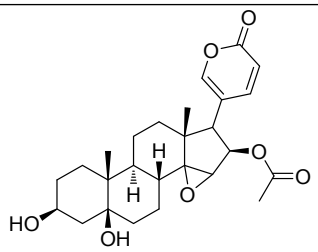
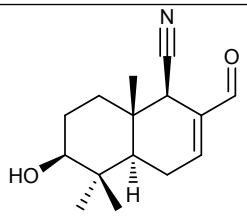
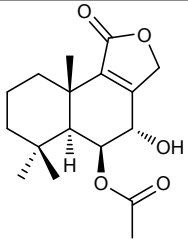
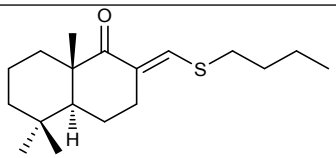
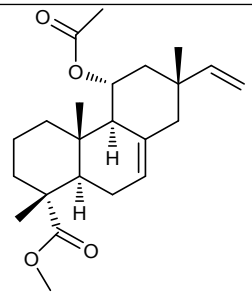
562

563 **Table 2.** BIC_{50} (μg/mL) of analogues of polygodial and warburganal on developing biofilms.

564 NT= not tested. Samples from the Specs® company were in limited supply, and the company

565 had stopped their synthesis.

Structure	Identity	BIC_{50} (μg/ml) *C. albicans*	BIC_{50} (μg/ml) *S. aureus*; *S. epidermidis*
	Polygodial	10.8±5	>100; >100
	Warburganal	4.5±1	37.9±8; 38.1±9
	Drimendiol	25.5±8	65.1±24; 67.1±12
	Drimenol	15.4±7	14.7±2; 16.4±3
	Specs 1	>400	NT

	Specs 2	131.5±6	NT
	Specs 3	>400	NT
	Specs 4	>400	NT
	Specs 5	>400	NT
	Specs 6	>400	NT
	Specs 7	>400	NT

566

567

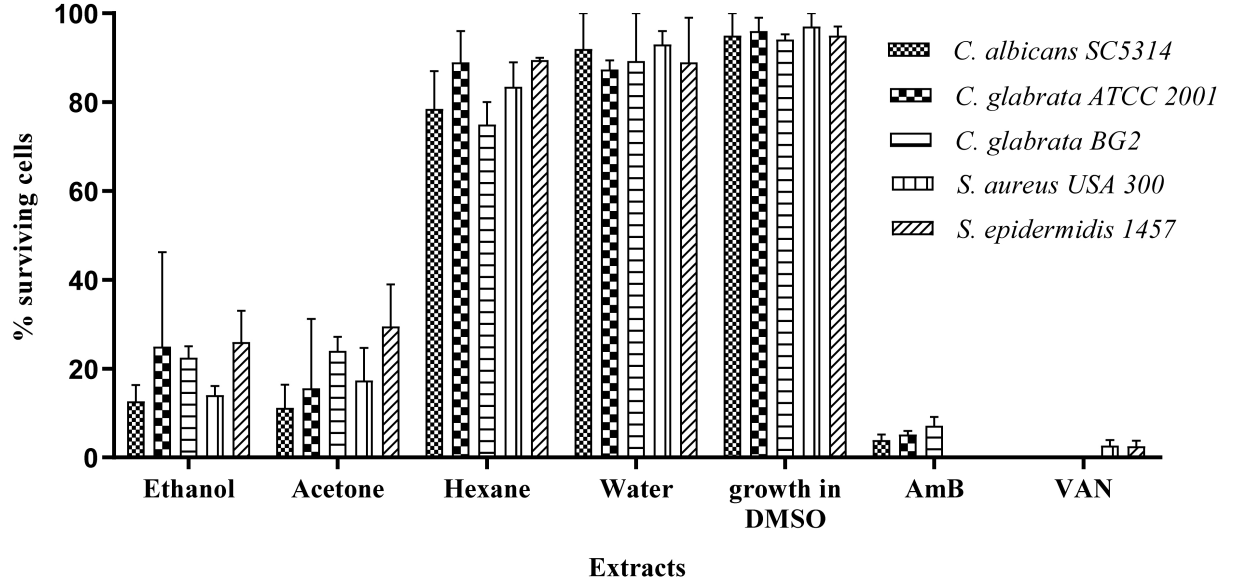
568

569 **Table 3.** Synergistic activities of azoles (miconazole or fluconazole) with warburganal or
570 polygodial against *C. albicans* developing biofilms. BIC_{100} represents the minimum
571 concentration required to completely inhibit the growth of biofilms. W: warburganal, P:
572 polygodial, MCZ: miconazole, FLZ: fluconazole.

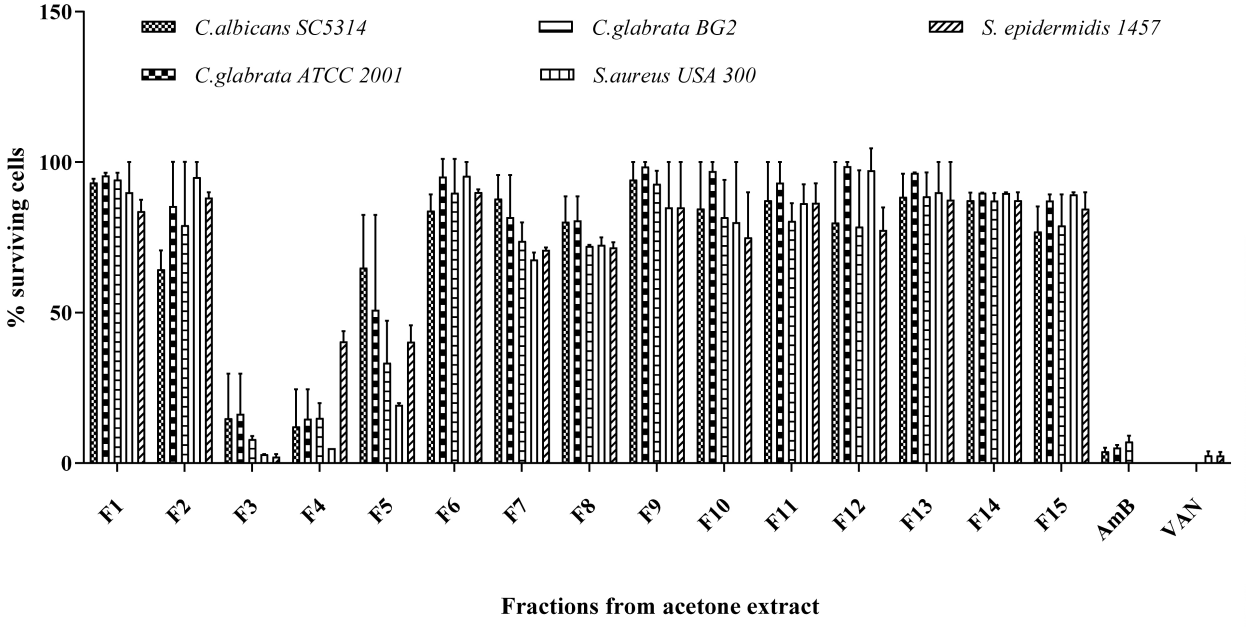
Compounds	BIC_{100} alone (µg/mL)	BIC_{100} combined (µg/mL)		FICI	
		W/MCZ P/MCZ	W/FLZ P/FLZ	W/MCZ P/MCZ	W/FLZ P/FLZ
Warburganal (W)	25	3.13/0.19	3.13/3.3	0.16	<0.25
Polygodial (P)	12.5	1.6/0.05	0.78/1.7	0.13	<0.12
Miconazole (MCZ)	6.13	-		-	
Fluconazole (FLZ)	>26.6	-		-	

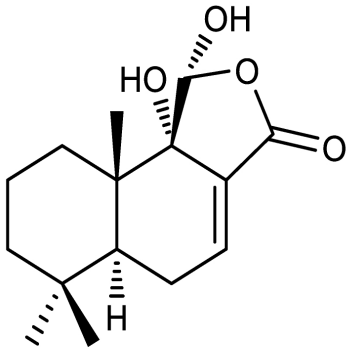
573

A

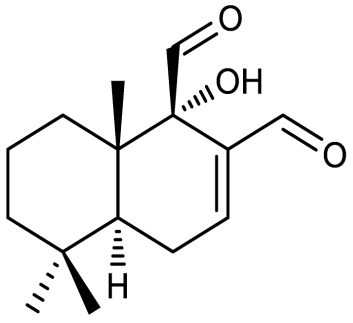

100
80
60
40
20
0
% surviving cells
C. albicans SC5314
C. glabrata ATCC 2001
C. glabrata BG2
S. aureus USA 300
S. epidermidis 1457
Ethanol
Acetone
Hexane
Water
growth in DMSO
AmB
VAN
Extracts

B

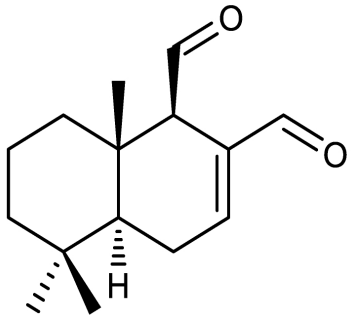

150
100
50
0
% surviving cells
C.albicans SC5314
C.glabrata ATCC 2001
C.glabrata BG2
S.aureus USA 300
S. epidermidis 1457
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
F13
F14
F15
AmB
VAN
Fractions from acetone extract



1



2



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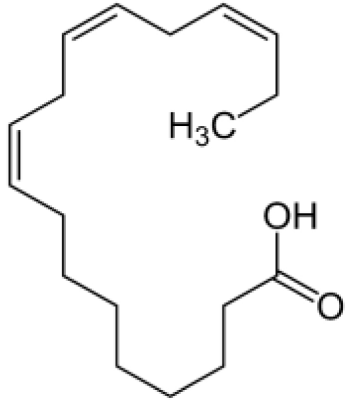
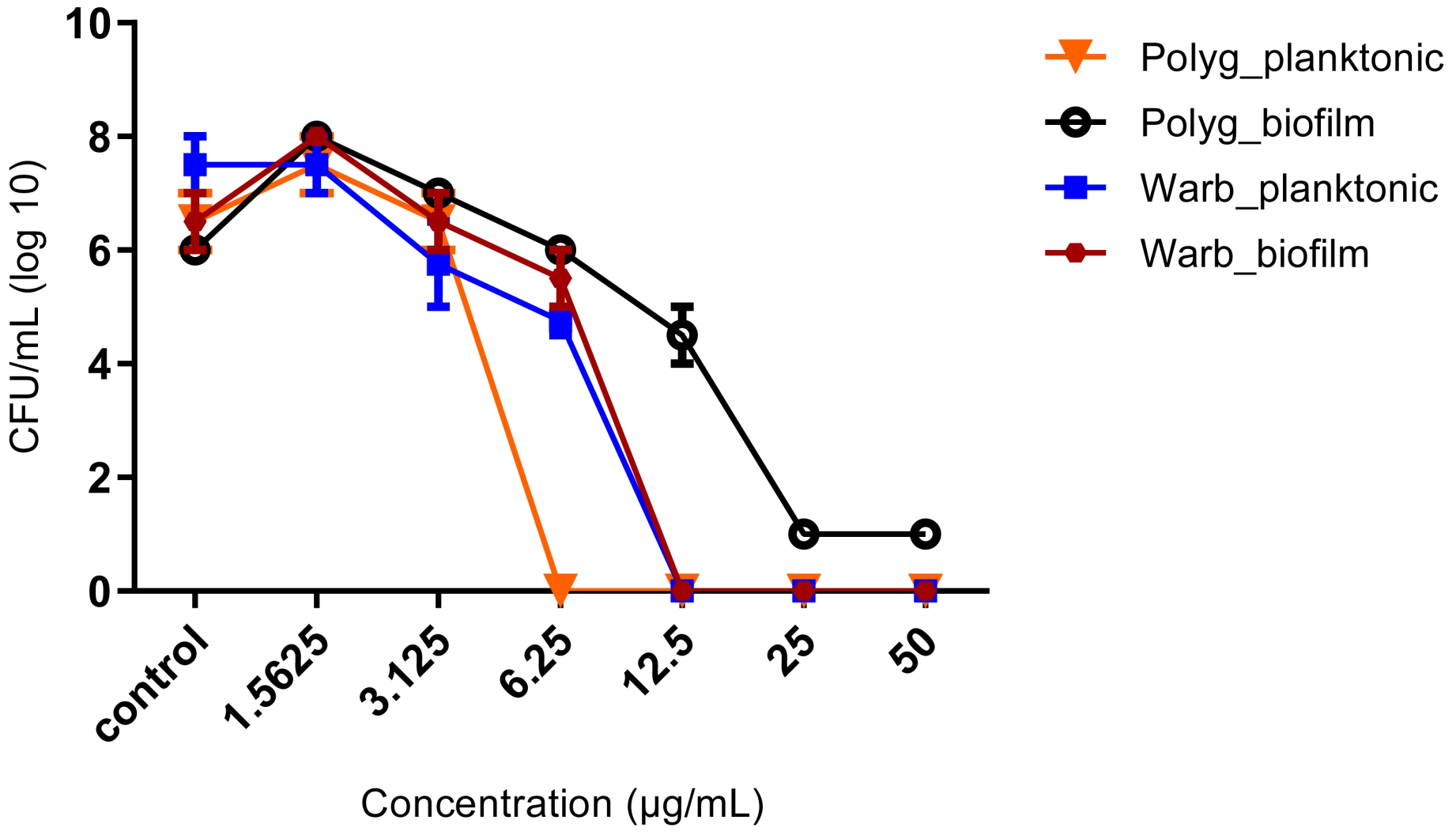
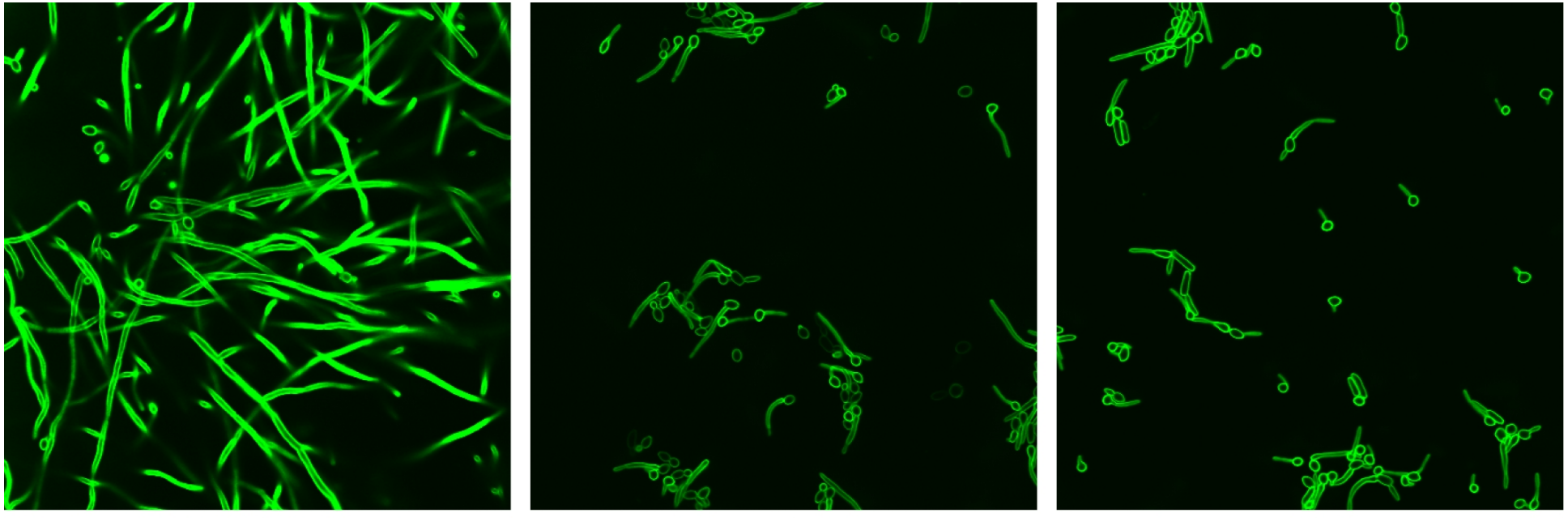


4


10
8
6
4
2
0
CFU/mL (log 10)
control
1.5625
3.125
6.25
12.5
25
50
Concentration (µg/mL)
Polyg_planktonic
Polyg_biofilm
Warb_planktonic
Warb_biofilm

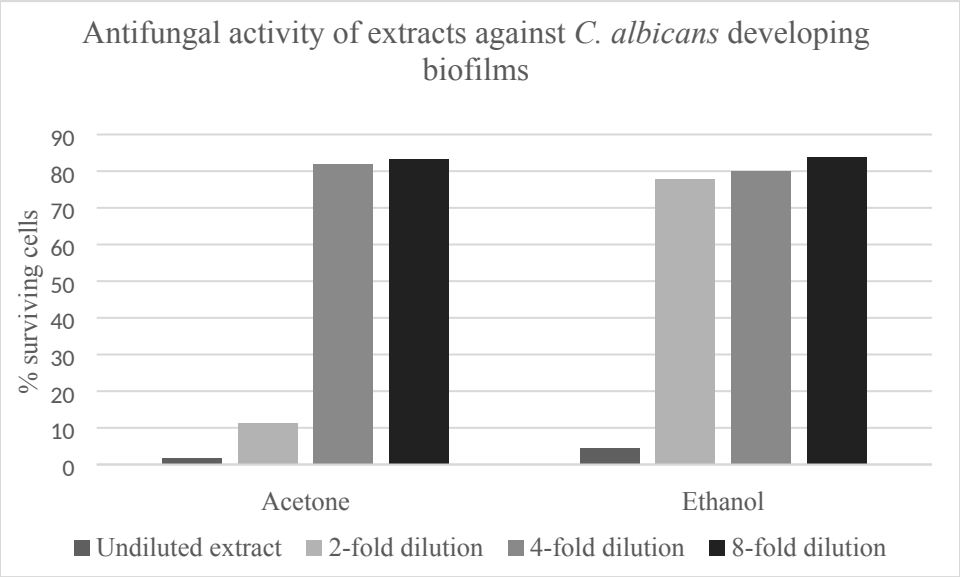


a) b) c)

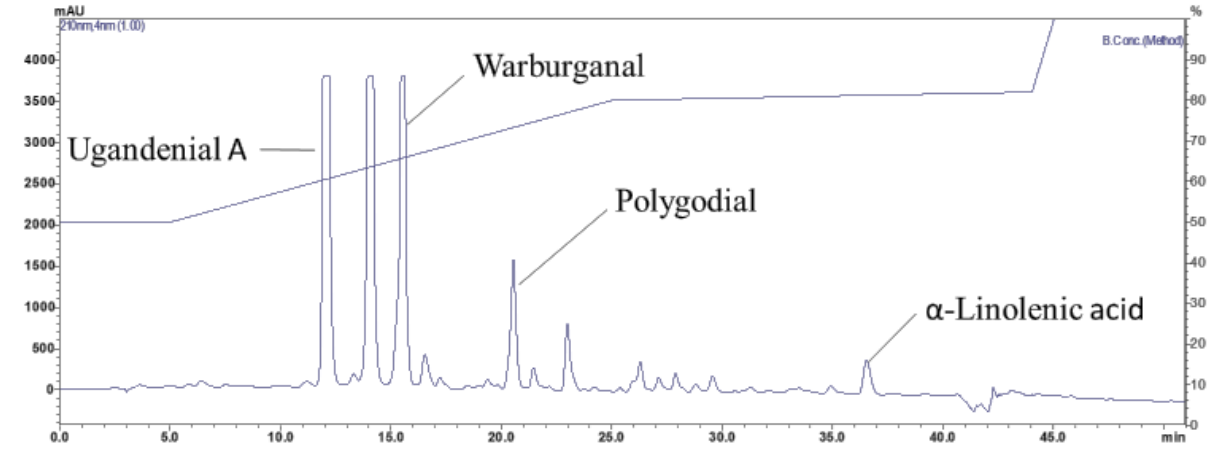
Compounds from *Warburgia ugandensis* Sprague Leaves that inhibit Candida and Staphylococcal biofilms

Purity Kipanga[a,b*], Maoxuan Liu[a], Sujogya Kumar Panda[b], Anh Hung Mai[c], Cedrick Veryser[c], Luc Van Puyvelde[b], Wim M. De Borggraeve[c], Patrick Van Dijck [d,e], Josphat Matasyoh[f] and Walter Luyten[b]

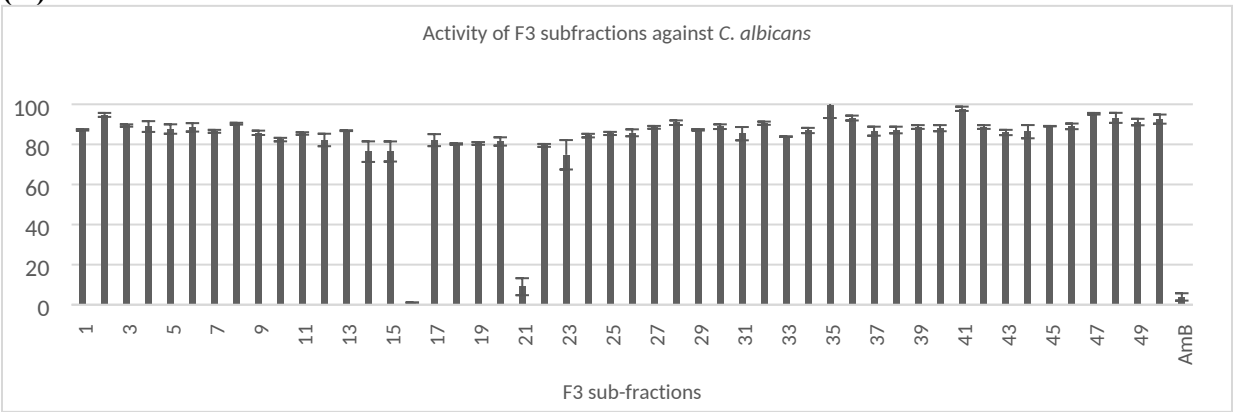
Supplemental material I: 2-fold serial dilution of acetone and ethanol extracts to establish which extract was more active to proceed to large-scale extraction.



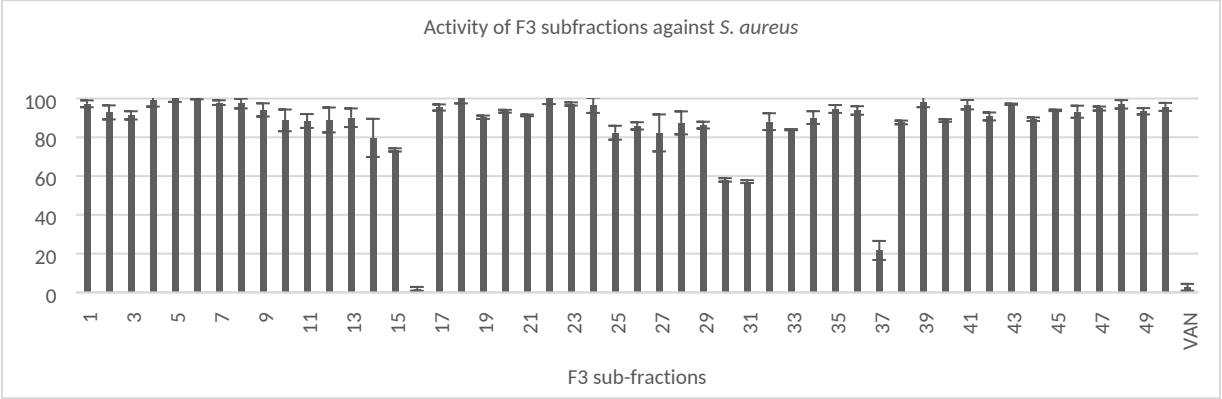
Supplemental material II: Establishing active peaks by comparing activity of fractions collected with peaks in the chromatogram.


mAU
210nm,4nm (1.00)
%
B.Conc.(Method)
Warburganal
Ugandenial A
Polygodial
α-Linolenic acid
min

(A)


Activity of F3 subfractions against C. albicans
F3 sub-fractions
AmB

(B)


Activity of F3 subfractions against S. aureus
F3 sub-fractions
VAN

(C)

A: Chromatogram (210 nm) of F3 resolved on a C18 column showing compounds present in the fraction. A 1 mL dried aliquot of F3 was dissolved in 50% acetonitrile-water, and injected onto a C18 column. The column was eluted by an ACN/water gradient (50-100%; black solid line).

B: Sub-fraction(s) were collected per minute from F3 and tested for activity (% surviving cells) against *C. albicans* developing biofilms

C: Sub-fraction(s) were collected per minute from F3 and tested for activity (% surviving cells) against *S. aureus* developing biofilms