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Examination of *Candida albicans* strains from South Africa for the production of gliotoxin and other cytotoxic secondary metabolites

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Candida **species cause superficial mycosis and more serious systemic infections. The virulence factors of *Candida* are their adherence and hyphae formation ability and the production of hydrolytic enzymes. In addition, they produce secondary metabolites which are also implicated in the pathogenesis. Literature on the production of gliotoxin, one of the metabolites by *Candida* spp. is controversial. The aim of this study was to examine production of gliotoxin and related secondary metabolites by *C. albicans* strains isolated from the oral cavities of patients with oral infections. Seventeen strains of *Candida albicans* were obtained and were grown in Eagle's minimal essential medium supplemented with 10% glucose and 5% foetal bovine serum for 10 and 30 days. Extraction of culture fluids and biomass was done using dichloromethane and examined using a two dimensional thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) with time of flight mass spectrometry (MS). Fifteen strains of *C. albicans* (88%) produced secondary metabolites with a molecular weight of 452 which was nearest to that of gliotoxin standard with a molecular weight of 326. Three major metabolites were isolated and identified. They were eburicol produced by 10 strains, Tryptophol produced by 10 strains and dehydro eburical produced by 12 strains of *C. albicans*. In conclusion, the strains of *C. albicans* isolated from the patients with oral infections did not produce gliotoxin. However, they produced tryptophol which is known to be produced by *C. albicans,* and they also produced eburicol and dehydro eburical which are intermediate compounds in the metabolic pathways to ergosterol. Further research is required to establish their role in the pathogenesis of this organism.**

Key words: *Candida albicans*; secondary metabolites; gliotoxin; tryptophol; dehydro eburical.

INTRODUCTION

Candida species are medically important unicellular fungi that cause superficial mycosis and more serious systemic infections. In addition, they have been described, in the past 20 years, as the most important emerging nosocomial pathogens (Pasqualotto et al., 2005) and can lead to long hospitalisation with high mortality rates (Lee

et al., 2010). Although many *Candida* spp., such as *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, *Candida krusei*, *Candida kefyr*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida stellatoidea* and *Candida dubliensis* are known to be pathogenic, *C. albicans* is the predominant species that causes infections in HIV positive and cancer patients, patients with diabetes and organ transplants, and hospitalised individuals. The virulence factors of *C. albicans* responsible for the development of infection are their ability to adhere, form hyphae and biofilm, and produce hydrolytic enzymes. Secondary metabolites produced by some eukaryotic microorganisms, such as fungi, are also known to contribute towards the pathogenesis of these organisms. However, the secondary metabolites formed by *Candida* spp. have not been fully explored. Some of the metabolites produced by *Candida* spp. are Ergost-8-en-3β ol, Ergosta-8,22-dien-3β-ol, Lanosta-8,24-dien-3β-ol, 24-methyl-24,25-dihydrolanosterol, 4α methylzymosterol and Obstusifoliol (Turner and Aldrich, 1983).

Gliotoxin (GLT) is an immunosuppressive mycotoxin that is produced by various fungi, such as *Aspergillus fumigatus*. A GLT-like substance was isolated from *C. albicans* by Shah and Larsen (1991) implying that GLT might be involved in *Candida* infections. Subsequently, the researchers detected "GLT-like" metabolites from a female genital tract together with a *C. albicans* isolate that may have produced the metabolite (Shah et al., 1995). They also showed that at 1 µg/ml, GLT impaired the ability of human polymorphonuclear neutrophils (PMN) to destroy invading foreign cells without affecting their viability (Shah et al., 1998). These results supported the implication of GLT in the pathogenesis of *C. albicans*. However, a subsequent study conducted by Kupfahl et al. (2007) could not show evidence of GLT production by *C. albicans*. Therefore, the aim of this study was to examine production of GLT and related secondary metabolites by *C. albicans* strains isolated from the oral cavities of patients with oral infections.

MATERIALS AND METHODS

Cultures

Seventeen strains of *C. albicans* obtained from the Department of Oral Biological Sciences, University of The Witwatersrand were cultured on Sabouraud Dextrose Agar (SDA) and incubated for 3 days at 32°C. Ethical clearance was obtained for the isolation of these strains. The colonies of *C. albicans* strains were inoculated aseptically into 100 ml of Eagles minimal essential medium supplemented with 5% foetal bovine serum (FBS) and 10% glucose(pH 7.0), and incubated for 30 days at 29°C shaking at 120 rpm. Cultures were centrifuged at 4000 rpm for 10 min. The biomass and the culture fluids of the 17 strains were freeze dried at -20°C. The resulting powders were stored at -20°C and used for the extraction. Acetone was used as a solvent for the extraction.

Extraction of metabolites and potential mycotoxins

One hundred millilitres of acetone was added to the freeze-dried biomass and filtrates, shaken for 1 min at 120 rpm and was filtered through Whatman number 1 filter paper. The filtrates were evaporated and the residues were collected. Similarly, second extraction was performed using dichloromethane (DCM) and the dried extracts were stored at -20°C in amber vials for further use.

Detection of metabolites and potential mycotoxins using thin layer chromatography (TLC)

Two-dimensional thin layer chromatography was performed on the acetone extracts of freeze-dried culture fluids and biomass using dichloromethane:ethyl acetate:propan-2-ol (90:5:5 v/v/v) for the first dimension and toluene:ethyl acetate:formic acid (6:3:1 v/v/v) for the second dimension (Patterson and Roberts, 1979). Plates were dried and viewed under ultra-violet light at 366 and 254 nm. The plates were also treated with seven spray reagents, one plate per spray: Pauly's; *p*-anisaldehyde; 2,4-dinitrophenyl hydrazine; 5% silver nitrate in 70% ethanol; 0.5% ferric chloride; 0.4 M fluorescamine and Ehrlich's reagent. The ARS-sprayed plates were heated to 120°C for 2 min. A GLT standard was prepared in DCM (0.5 mg/ml), and 20 µl was spotted on a two-dimensional plate and developed in DEP and TEF as per the standard protocol.

Extraction of GLT and other potential mycotoxins

Strains of *C. albicans* were grown in Eagles minimal essential medium, supplemented with 5% FBS and 10% glucose (10 GEMEM) at a pH of 7.0 in two batches. One batch was incubated for 10 days and the second batch for 30 days. The extraction was performed as described by Shah and Larsen (1991). Briefly, the cultures were centrifuged at 4000 rpm for 10 min and 50 ml of iso-octane was added and recentrifuged. Two rounds of extractions were performed using DCM and the bottom layer of DCM was passed through a bed of anhydrous sodium sulphate on a Whatman number 1 filter paper and the filtrates were collected which was dried, and stored at -20°C in amber vials.

For the GLT standard, 100 ml of freshly prepared 10 GEMEM was mixed with 20 ng/ml of GLT, and extracted with 50 ml of 2x DCM and treated as for the culture fluid extracts. The residue was stored at -20°C for further use.

Detection of GLT

DCM extracts were reconstituted in 200 µl of DCM and 2D-TLC was performed as described previously. A chromatogram of GLT standard was also produced. The plates were then sprayed with 5% silver nitrate reagent.

Purification of metabolites

The extracts that were positive for major metabolites were purified

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Table 1. A summary of the thin layer chromatographic properties of metabolites obtained from cultures of *Candida albicans.*

Strain (*n*=17)	Metabolite	Rf value	Comment
C. albicans (10)	AFC1	50:68	UV+/ARS+/UVA+
C. albicans (10)	AFC2	50:45	UVA+/ER+/$FeCl_3$+/ARS+
C. albicans (12)	AFC3	34:45	UVA+/2,4DNPH+

UV: Ultra-violet; UVA:ultra violet absorb; ER: Ehrlich's; $FeCl_3$: ferric chloride reagent; ARS: anisaldehyde reagent; 2,4-DNPH: 2,4-dinitrophenyl hydrazine reagent; YG: yellow-green.

using preparative TLC plates. After air drying overnight, the TLC plates were baked overnight at 230°C. The acetone extract was streaked on the surface of preparative plates, which were then developed in DEP. The dried plates were examined under UV light to locate the separated bands of metabolites, which were then separately scraped off the plates. The various bands were pooled in beakers to which 50 ml of acetone was added. After mixing, the slurry was filtered through a sintered glass funnel (porosity 3) and the residue was washed with acetone. The acetone solution was then evaporated in a Buchi rotary evaporator and the residue was dissolved in 4 ml of acetone and transferred to 4 ml amber vials (Anatech SA). The purity of the metabolite fractions were tested by spotting 20 µl of the extract on 2D-TLC plates developed by the standard protocol. The purification process, using preparative TLC, was repeated until a single spot was obtained on the test plate. Test plates of the purified products were prepared, viewed under UV light and sprayed individually with various spray reagents (viz, ARS, ER and $FeCl_3$) to confirm their relationship with the metabolites in the original crude extract. The purified metabolites were weighed on an analytical balance and then stored in a freezer for further use.

Detection of GLT using liquid chromatography mass spectrometry/mass spectrometry (LC/MS/MS)

Dichloromethane extracts were reconstituted in 100 µl of acetonitrile and filtered through a 0.22 µm nylon filter disc connected to a 3 ml luer lock syringe and the filtrates were collected in seventeen 1 ml clear vials capped with crampable septated caps. Three concentrations of the GLT standards 0.1, 1.0 and 20 ng/ml were prepared from the 100 µg/ml of a GLT standard (Sigma-Aldrich) and transferred into 1 ml vials and also capped. The vials were loaded into the sample tray of a Waters Synapt G2 high-definition mass spectrometer (HDMS) LC/MS (MICROSEP South Africa) equipped with a Waters BEH C18 column, with the capillary voltage set at 3 kV and the cone voltage at 20 V. Twenty microliters of the GLT standards were injected to establish a chromatogram and a standard curve. Then 20 µl of each of the 34 extracts were injected into the instrument, with the mobile phase flow rate set at 0.4 ml/min, using a solvent gradient water and acetonitrile mixed with 1% formic acid. The run was set at 15 min at a pressure of 15000 psi. The mass peak of the GLT and of the extracts were further analysed using the MS/MS detector, with the trap energy collisions ramping from 20 to 60 V, and the resultant data being interpreted with Waters MassLynx elemental composition software.

RESULTS AND DISCUSSION

Detection of metabolites using TLC

In a preliminary investigation, the seventeen cultures of *C. albicans* were grown in 10 GEMEM and extracted with acetone. Acetone extraction gave the best representative results, with three principal metabolites being separated and detected by anisaldehyde spray reagent (ARS) with heat. These metabolites were labelled AFC1, AFC2 and AFC3 for convenience. The chromatographic characteristics are given in Table 1. The Rf values using the standard solvent systems DEP: TEF were AFC1 58:78, AFC2 58:67 and AFC3 38:51.

p-Anisaldehyde reagent is suitable for the detection of a range of compounds, such as, sugars, steroids, terpenes and nucleophiles (e.g., amines), or compounds bearing these functionalities. The colours developed with ARS can be linked to a chemical type, that is, green indicates allylic alcohols; violet, phenolic molecules; blue/red, amines, aldehydes, ketones, carbohydrates and esters like alkylphthalates; and brown, diterpenes (Paul et al., 2011). Although not all strains produced the three major metabolites, AFC1, AFC2 and AFC3, all 17 strains (8 strains produced 3; 4 strains produced 2; 3 strains produced 1 and 2 strains produced none) (Table 1) were further screened for GLT.

Metabolite AFC1

Compound 1 (AFC1) was the least polar of the metabolites, due to its higher retention factor (Rf) on the TLC system used (Table 1). AFC1 also had fluorescent properties under UV which indicated that it was aromatic or conjugated unsaturated compounds. AFC 1 was purified and investigated further with HPLC/MS.

On LC/MS, the main metabolite produced a $M+H^+$ of 441 (Figure 1) which means that the correct molecular weight was 440, when the proton is added to ionise the molecule for MS. In addition, other peaks with similar RTs gave the same mass-to-ion ratio (m/z). Because of the low polarity and staining reaction with ARS, indicating a higher terpenoid, and lack of staining with other reagents, a search was made for terpenoids with a molecular weight of 440. 24-methylene dihydrolanosterol, with a molecular formula of $C_{31}H_{52}O$ (MWt 440) is a C-31 triterpene, was reported as being formed by *Candida* spp. (Abe et al., 1993). Lanosterol is the original triterpene for ergosterol biosynthesis (Figure 2) that is found in nearly all fungal and yeast biomembranes (Abe et al., 1993).

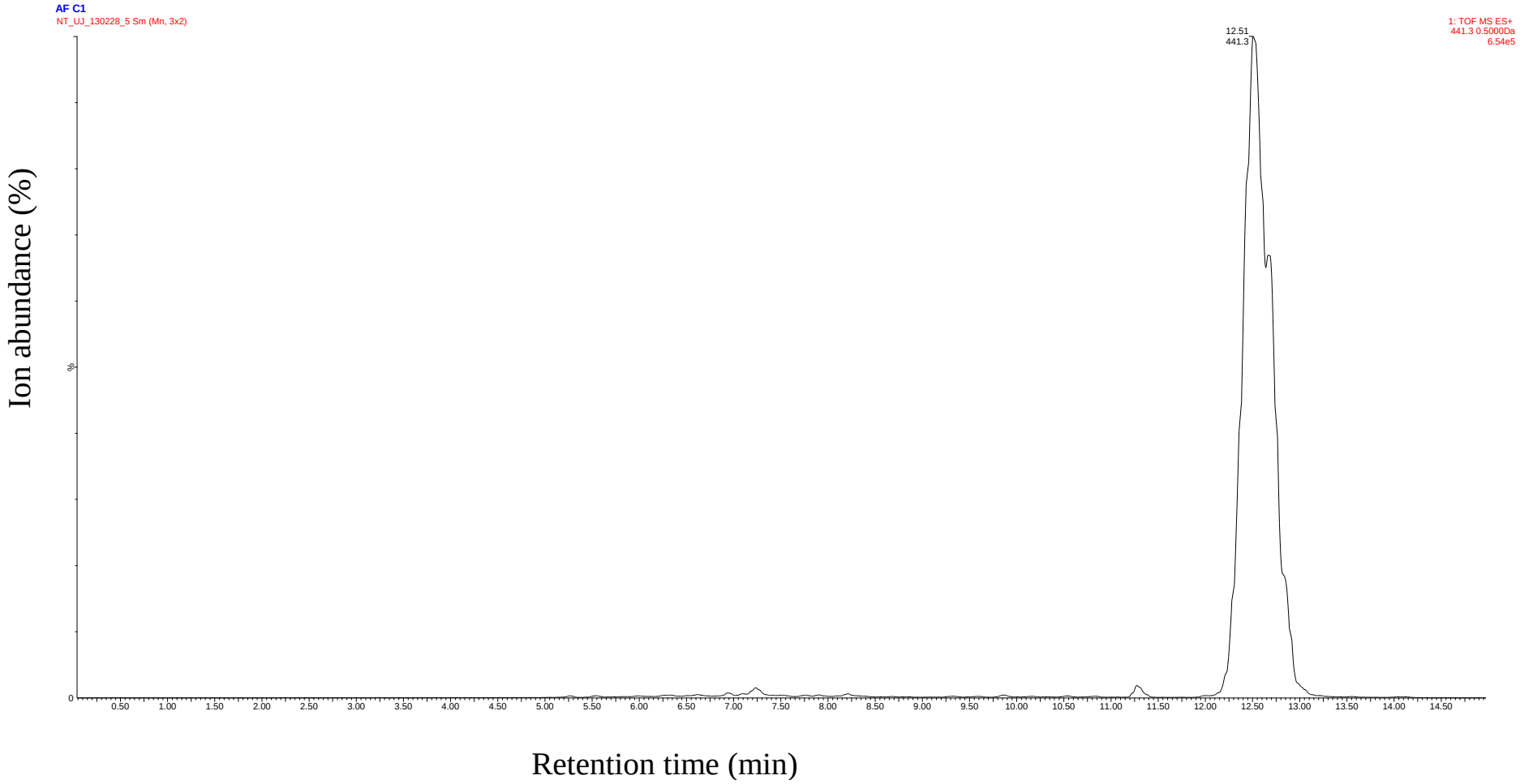
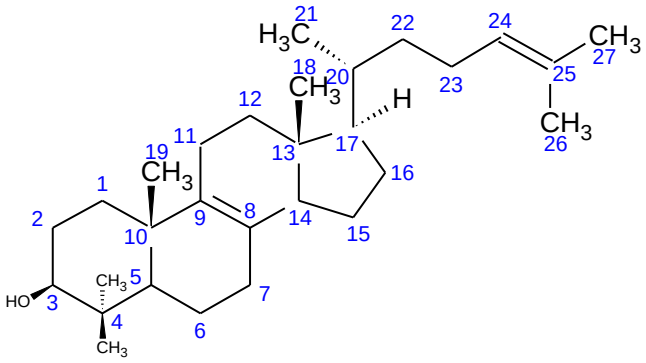


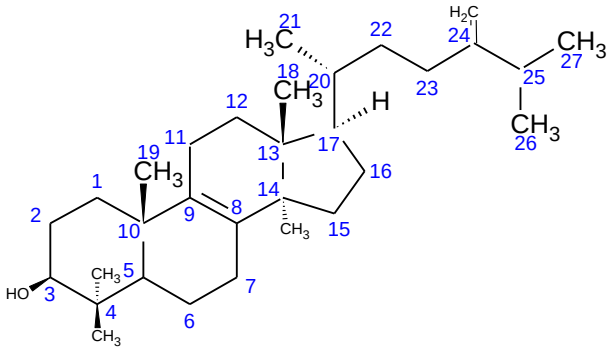
Figure 1. A chromatogram of LC-MS of the AFC1 metabolite isolated from *C. albicans* acetone extracts, showing a mass-to-charge ratio of 441 [$M+H^+$]. $M+H^+$ = the molecule-proton complex.

Position 24 has to be methylated to form 24-methylene dihydrolanosterol from lanosterol. The double bond between positions 24 and 25 then rearranges between position 24 and methyl, to make methylene, thus forming the required structure. This metabolite is thought to be an intermediate in the metabolic pathway to ergosterol (Akhtar et al., 1966), and has been named eburicol (Figure 2).

Eburicol is one group of sterols produced by *C. albicans* and it accumulates in high levels when wild-type strains of *C. albicans* are treated with nitrosoguanidine (Subden et al., 1977). Eburicol is an ergosterol derivative found in the cell membrane of most fungi. There are antifungal



Lanosterol



Eburicol

Figure 2. Structure of Lanosterol and identified Eburicol (AFC1).

agents such as the azoles, that is, imidazole and triazole which can interfere with the biosynthesis of eburicol. They do this by inhibiting the expression of the enzyme cytochrome P450 eburicol 14α-demethylase, which is encoded by the gene CYP51 (Bean et al., 2009).

Metabolite AFC2

Metabolite AFC2 was less polar than AFC1 with a lower Rf on TLC (Table 1). It produced a tan spot with $FeCl_3$ and showed no reaction with PR. As AFC2 gave a red-violet spot with ARS, an amine, aldehyde, ketone, carbohydrate were inferred to be part of its structure. The absence of a reaction with 2,4-Dinitrophenylhydrazine (2,4 DNPH) eliminated aldehydes and ketones. Thus, the purple colour indicates that it was possibly an amine which was supported by its reaction with ER. An examination of the literature (Kosalec et al., 2008) revealed a metabolite with a secondary amine moiety, that is, L-tryptophol (L-TOL).

Thin-layer chromatographs of AFC2 and tryptophol (TOL) standard were compared using various spray reagents. Tryptophol produced the same reaction with ARS reagent as AFC2 at the same Rf values on the chromatogram, as well as a tan spot with the $FeCl_3$. Ehrlich's reagent is useful for the identification of indole groups (Ehmann, 1977). Furthermore, both AFC2 and TOL produced a purple colour indicating the presence of this moiety. In addition, FSC, which causes amine-containing metabolites to fluoresce, produced a positive reaction with both AFC2 and TOL (Klein et al., 1974). It was thus concluded that AFC2 was TOL, and this was further supported by spectroscopy (Figures 3 and 4). Tryptophol is thought to be involved in the development of sleeping sickness caused by *Trypanosma brucei rhodesiense* (Odiit et al., 1997). It is also known to be produced by strains of *C. albicans* and its toxicity has been established (Kosalec et al., 2008, 2011). This study confirms that *C. albicans* produces TOL, as 10 out of the 17 isolates produced this compound (Table 1). This is probably an important finding, as it may prove that TOL has a role in invasive yeast infections, as also implicated in sleeping sickness (Chen and Fink, 2006).

Metabolite AFC3

Compound 3 produced a brown purple spot on treatment with ARS, which indicated it to be a diterpene or similar molecule, or an aldehyde because of the colour. Its lower Rf value than AFC1 and AFC2, indicated that it was more polar, suggesting the presence of more polar functional groups. This was confirmed by its reaction with 2,4 DNPH, which produced a green spot. It seemed more likely that this was an aldehyde group rather than a ketone, as the latter generally produce yellow-brown colours, which is in agreement with the ARS reagent. It was speculated that the compound was a triterpene/sterol derivative containing a carbonyl group, either as an aldehyde, ring or side chain ketone. Thus the sterol aldehyde intermediate is a possible candidate that may be involved in the 14α demethylation of lanosterol (Fischer et al., 1991); a ring ketone in 4 demethylation pathway, as zymosterone derivatives (Pierson et al., 2004); and a ketone side chain derivative such as senexdione (Batta and Rangaswami, 1975). This was investigated further using spectroscopic means.

Its molecular weight of 452 was in the range for known sterols (Figure 5), but higher than the other two metabolites (Table 1). The molecular weight of 452 is consistent with the formula $C_{31}H_{48}O_2$ and corresponds to a metabolite derived from eburical (Anderson and Epstein, 1971), another C31 triterpenoid related to metabolites in the biosynthetic pathway of conversion of lanosterol to ergosterol, which is also found in *C.*

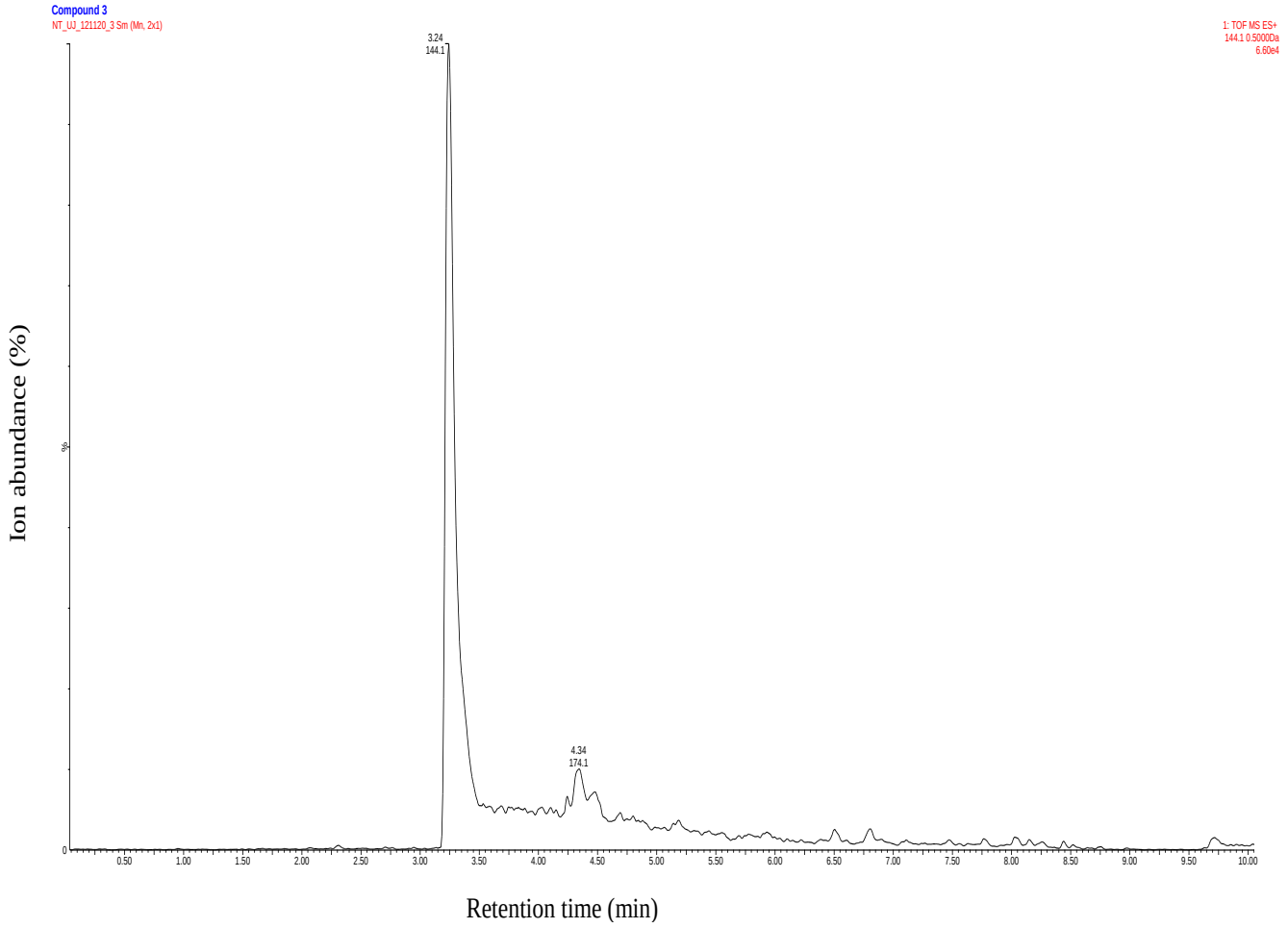


Figure 3. A chromatogram of LC-MS of the metabolite AFC2. The peak with mass to ion ratio of 144.1 [$M+H^+$] at the retention time of 3.24 min, the indole ethyl carbonium ion derived from tryptophol. $M+H^+$: Molecule-proton complex.

albicans. Eburicol has a molecular weight of 440 and a molecular formula of $C_{31}H_{52}O$. The metabolite AFC3 had 12 units more than eburicol, which suggest it has either an extra carbon or oxygen with 2 double bonds. These could be either at the C-22 position or possibly there could be a rearrangement of the double bonds in lanosterol position 7 – > 8; and 9 - > 11, that is, a variation found in other triterpenoids, e.g., dehydroeburicoic acid (Anderson and Epstein, 1971). Thus, it is likely that the metabolite AFC3 is dehydro eburical (Figure 6), with the extra double bond being conjugated with others found in the eburical structure. It has an aldehyde group at position 20, which explains the reaction of AFC3 with 2,4 DNPH reagent on TLC plates (Table 1), as well as its higher polarity than the other two metabolites. It is in effect an aldehyde derivative of AFC1 (Turner and Aldridge, 1983).

Gliotoxin

GLT was not found in the cultures of any of the *C. albicans* strains extracted with acetone, ethanol or dichloromethane. This is contradictory to the studies of Shah and Larsen (1991), but in agreement with Kupfahl et al. (2007), who also found a lack of intra- and extracellular GLT production by 100 *Candida* isolates. These strains were isolated from invasive fungal infections. GLT produced by *Aspergillus fumigatus* has been listed as one of the virulence factors in the pathogenesis of this filamentous fungus. It can induce cell death in the majority of innate immune cells (Morton et al., 2012). It causes immunosuppression, has antithrombotic properties (Berting et al., 2010) and blocks mast cell activation (Niide et al., 2006). However, results in this study have shown that the strains of *C. albicans* isolated from the oral cavities of South African patients did not produce GLT. Further research is required to detect the expression of GLT genes during infection. Often, virulence genes are expressed during infection and the products are detected.

In conclusion, three secondary metabolites were identified from 15 strains of *C. albicans* isolated from the

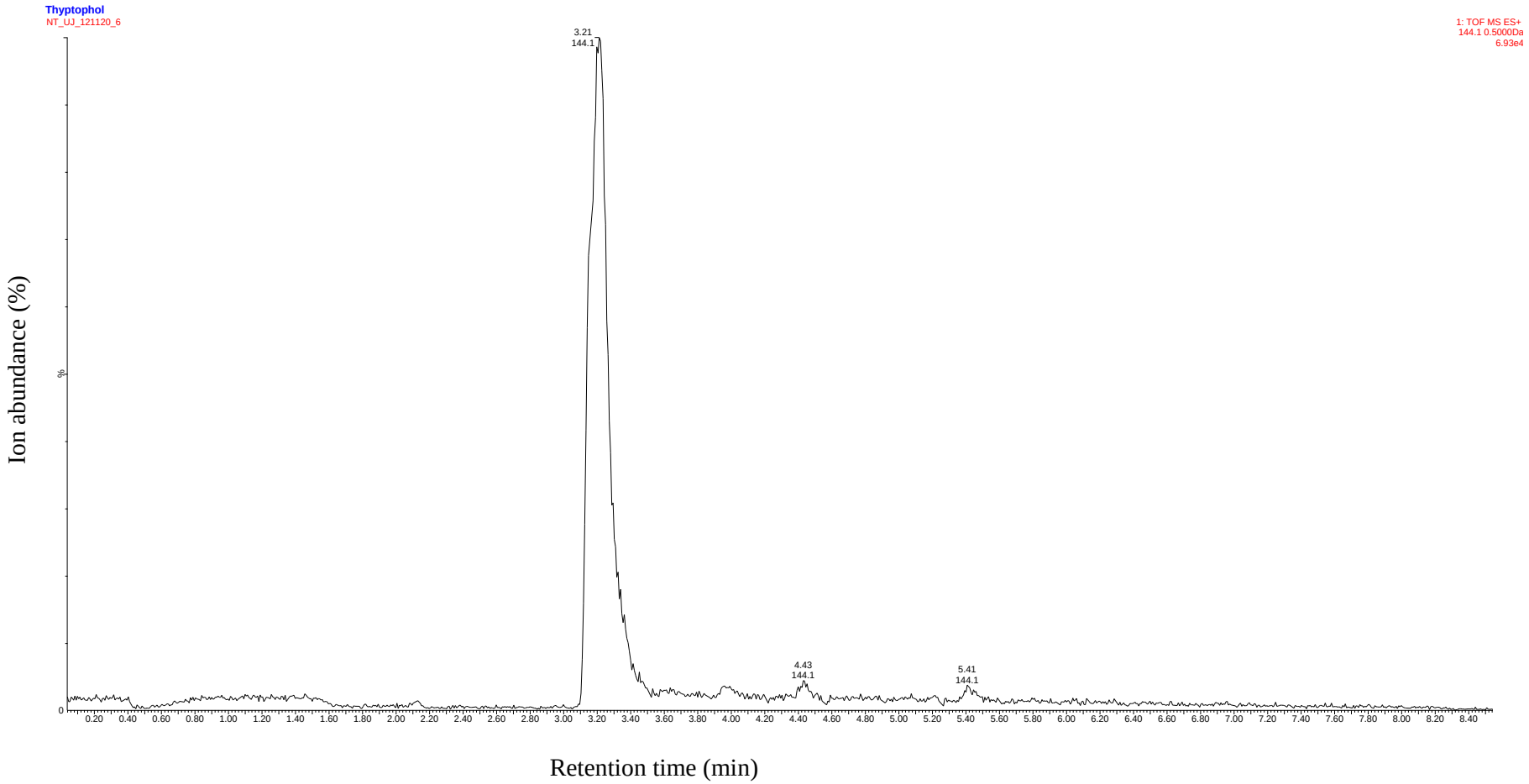


Figure 4. A chromatogram of LC-MS of the tryptophol standard. The important peak, with a mass to charge ratio of 144.1 (arrow), is the ion peak of an indole ethyl carbonium ion, which is the fragmentation of tryptophol.

oral cavities of patients with oral infections. They were identified as tryptophol, which is known to have toxigenic potential, and eburicol and dehydro eburical which are intermediate compounds in the metabolic pathway to ergosterol. GLT was not detected in any of the test strains of *C. albicans,* however, further research can be conducted to detect the gene expression during infection in an animal model.

Conflict of Interests

The authors have not declared any conflict of interests.

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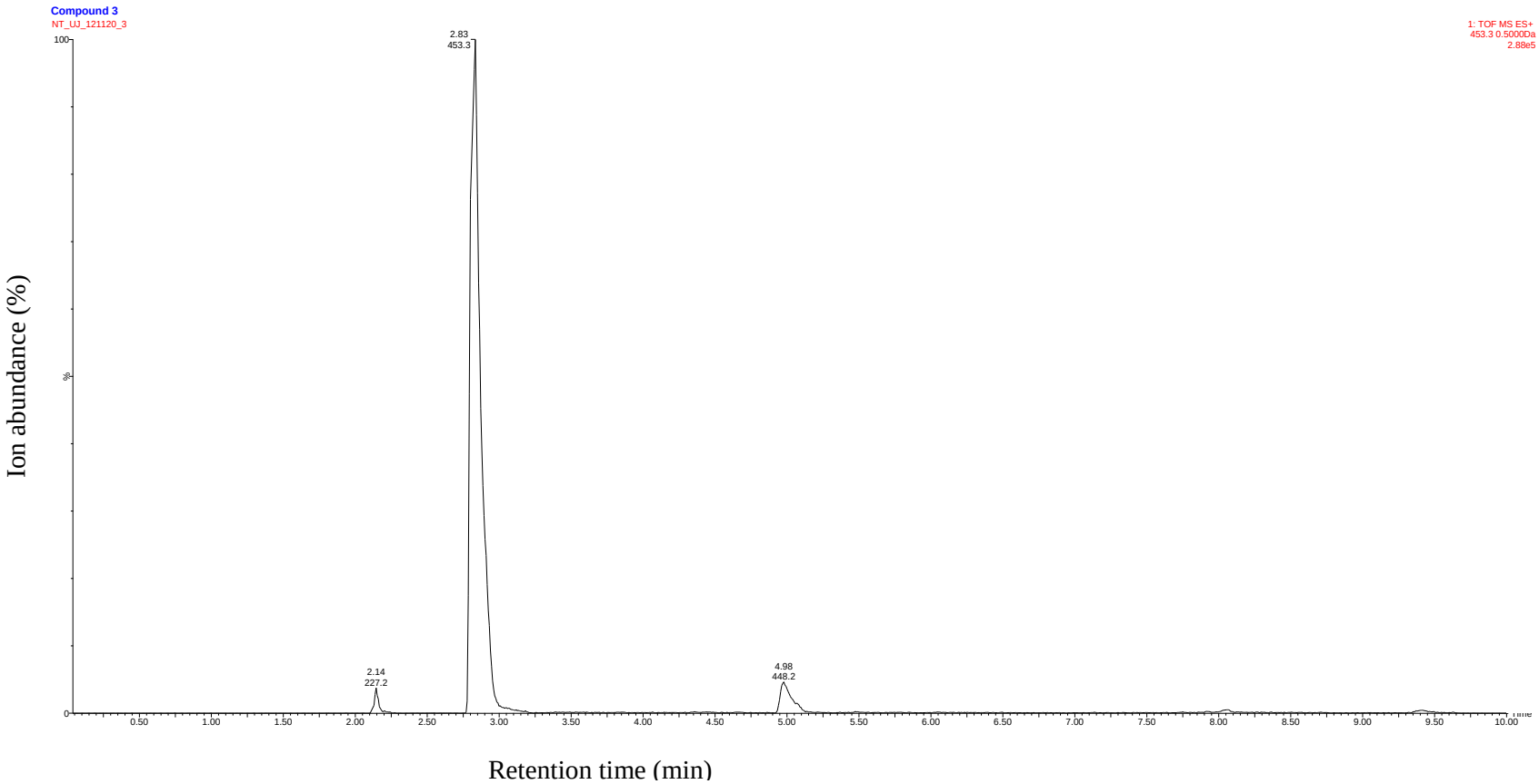

Figure 5. A chromatogram of LC-MS of AFC3 metabolite an important peak with a mass-to-charge ratio of 453 ($M+H^+$). $M+H^+$: Molecule-proton complex.

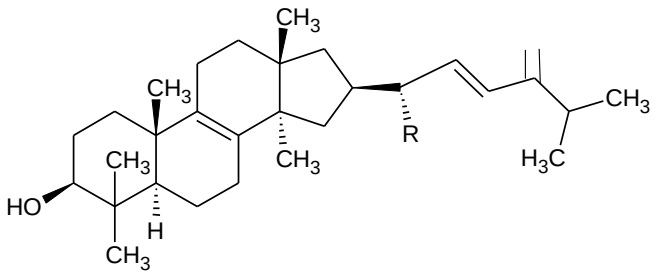

Figure 6. Suggested structure for Dehydro eburical R = CHO.

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