

ANTIMICROBIAL AND ANTIBIOFILM PROPERTIES OF *Combretum platypetalum* subsp. *oatesii* (Rolfe) Exell (*Combretaceae*) EXTRACTS

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ABSTRACT

Combretum species exhibit potent antibacterial and antifungal properties, but limited studies exist for most species. This study reports for the first time the *in vitro* antimicrobial and antibiofilm activities of *Combretum platypetalum* leaf, stem, and root. Fungi and bacterial strains implicated in the causes of some of the conditions to which *C. platypetalum* is traditionally applied were evaluated by Agar-well diffusion and micro-dilution methods, and their biofilm inhibition capacities were investigated. The highest zone of fungal inhibition of 22.70 ± 2.08 mm against *C. albicans* and the highest zone of bacterial inhibition of 21.70 ± 2.08 mm against *S. aureus* were observed. The minimum inhibitory concentration (MIC) ranged between 0.0156 mg/mL and 8.0000 mg/mL for fungi and 0.3906 mg/mL and 8.0000 mg/mL for bacteria at $P = 0.0002$. The best minimum fungicidal concentrations (MFCs), at $P = 0.003$, were observed at 0.0630 mg/mL for the leaf against *C. glabrata* and *C. neoformans* and 0.5 mg/mL for the root against *C. neoformans*, respectively. The highest biofilm inhibition of 40.61%, 36.04%, and 23.85% was observed in the stem, root, and leaf extracts against *C. glabrata*. Chloroform leaf extract reveals β -sitosterol as the active antifungal and antibacterial agent. Computational antifungal activity and molecular docking analysis of the structure versus target (sterol 14 α -demethylase) show a binding affinity for druggability. The leaves and roots are the most potent parts of the plant, with leaf extracts showing non-synergistic effects. Bioguided isolation is recommended to fully explore the species' evidence-based therapeutic potential.

Keywords: Antibacterial, Antibiofilm, Antifungal, Bioactivity, *Combretum platypetalum*

INTRODUCTION

The unabated rise in antimicrobial resistance is a recognized crisis in global health [1]. This increasing trend in resistance to commonly licensed antimicrobials has rendered some diseases difficult to treat or untreatable. This problem is exacerbated by the ability of multi-drug resistant pathogens to form biofilms, an adherent group of microbial communities surrounded by an exopolysaccharide

matrix [2]. It is estimated that up to 75% of antimicrobial-resistant infections involve biofilm formation [3]. Antibiotic resistance within a biofilm is due to several factors, such as decreased permeability of the matrix, exchange of genetic information, expression of antibiotic efflux pumps, and enhanced activity of antibiotic-modifying enzymes [4]. The alarming rise in antimicrobial resistance has necessitated the search for alternatives to conventional antimicrobial therapy.

In this context, plants produce various chemical products with biological and pharmacological activities in response to biotic and abiotic stress as well as during their growth and development [5]. These natural products have antibiofilm properties [6], antimicrobial, antioxidant, anticancer, and anti-inflammatory properties [7] among others. More crucially, they have also been shown to alleviate antimicrobial resistance associated with biofilm formation [8]. Plant-derived natural products have attracted increased scientific attention because they are generally regarded as safe [9], with minimal side effects, and also impart positive benefits to human health.

Combretum platypetalum subsp. *oatesii* (Rolfe) Exell is an African multi-stemmed dwarf shrub belonging to the *Combretum* genus of the *Combretaceae* family [10]. It has been utilized in African folk medicine to treat ailments such as pneumonia, diarrhea, menstrual pains, and earaches [11]. However, the plant part used in traditional practice is not explicitly stated in the literature [11-13]. Scientifically, *C. platypetalum* has been reported to display different biological effects such as antioxidant [13], control of *Mycobacterium tuberculosis* [12], anti-inflammatory effect [14], synergistic cytotoxic effect [15], and prostaglandin-synthesis inhibition [15]. Unfortunately, all reported scientific studies were conducted utilizing leaves, despite the potential of stems and roots as applied in traditional African treatments and natural product drug discovery programs [16], especially for the *Combretum* genus. The therapeutic potential of *C. platypetalum* has been poorly explored, especially in the context of the rise in multi-drug-resistant pathogens. In this study, we report for the first time a detailed investigation of the antibacterial,

antifungal, and antibiofilm formation properties of leaf, stem, and root extracts of *C. platypetalum*.

MATERIALS AND METHODS

Collection and identification of plant material

C. platypetalum leaf, stem, and root specimen were collected as described by Umoh *et al.* [16] and authenticated at the Forestry Research Institute of Nigeria (FRIN) with a deposit of herbarium samples as file number FHI 109750.

Extractions from C. platypetalum

The extraction procedures (method, extraction solvents, and extraction yields of the extracts) that were under examination as published by Umoh *et al.* [16] were adopted without any modification. Additionally, 30 g of the pulverized leaves were extracted by decoction.

Microorganisms and growth conditions

Microorganisms were obtained from the Department of Microbiology, University of Botswana. The bacteria were *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 13315, while the fungi (yeasts) were *Cryptococcus neoformans* ATCC 208821, *Candida albicans* ATCC 10231, and *Candida glabrata* ATCC 2001. The organisms were streaked onto Luria Bertani (LB) agar (Merck, Darmstadt, Germany) for bacteria and yeast extract peptone dextrose (YPD) agar (Merck) for fungi and incubated for 24 hours at 37 °C for bacteria and 72 hours at 37 °C for fungi to obtain pure isolates. Isolated organisms were subcultured and thereafter maintained in LB broth for bacteria and YPD broth

for fungi for 72 hours on a Gallenkamp orbital shaker at 150 rpm. Each actively growing culture was standardized at 0.6 and 1.8 McFarland standards for bacteria and fungi, respectively, using a DensiCHEK (bioMerieux, Hazelwood, Missouri, USA) [17].

Optimization of the assay

Streptomycin sulfate and fluconazole (both obtained from Merck) were first investigated for the best concentrations for the assays, which led to the use of 0.8577 μ M and 0.1 mM as positive controls for bacteria and fungi, respectively. Different concentrations ranging from 25 mg/mL to 100 mg/mL of the extracts were used, and all extracts were found to produce optimal activity at 25 mg/mL, which informed the choice of a 25 mg/mL concentration for the comparative study of the extracts by the agar-well diffusion method. All plant extracts were prepared in distilled water or Tween 80 and subjected to antibacterial, antifungal, and antibiofilm formation assays. Solvents used in the preparation of the plant extracts were used as negative controls in all the assays.

Agar-well diffusion assay

This was carried out according to the method described in Balouiri *et al.* [18] with slight modifications as follows: each plate with nutrient agar was aseptically inoculated with 0.1 mL of the respective standardised organism population. A cork borer of 6 mm diameter was used to bore the wells on the agar, and prepared plant extracts of 50 μ L were introduced. The inoculated plates with the plant extracts and positive control were kept at 4 °C for at least 3 hours to allow proper diffusion into the

agar; thereafter, they were incubated at 37 °C for 24 hours.

Determination of MICs, MFCs, and minimum bactericidal concentrations (MBCs) by micro-dilution method

The MICs and MBCs were determined according to the methods described by Hendiani *et al.* [19], while the MFCs were determined in a similar way to the MBCs with some modifications as follows: Different concentrations of the plant extracts, ranging from 25.0000 mg/mL to 0.0488 mg/mL, were prepared by serial dilutions. The last two columns of a 96-well microplate were used as positive and negative controls, respectively. Sterile plates were used throughout the assay, and 125 μ L of the respective nutrient broth (Merck) was introduced to each well under sterile conditions. The plant extracts were then introduced (125 μ L) to each well, respectively, and properly homogenized with the nutrient broth using the multichannel micropipette. The positive and negative controls were introduced to their respective wells, followed by 10 μ L of the respective organisms in each well. All samples were incubated for 24 hours at 37 °C. The addition of 50 μ L of 0.20 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) obtained from Sigma-Aldrich (Co., 3050 Spruce Street, St. Louis, MO 63103, USA) to each well after incubation detected the growth of the microorganism.

Biofilm formation and antibiofilm properties of *C. platypetalum* extracts

This was done according to the method described in Mombeshora *et al.* [20], with some modifications. Microbial cultures standardized as above were utilized. An aliquot of 200 μ L of each organism was

cultured without shaking at 37 °C in their appropriate media. The respective NBs without cells were used as negative controls. After 72 hours of incubation, nonadherent cells were removed by washing each sample three times in sterile phosphate-buffered saline (PBS). Plates were first inverted on absorbent paper for 15 minutes and then fixed at 60 °C for 1 hour. The biofilms produced were stained with 200 μ L of 0.1% crystal violet solution. The plates were incubated at room temperature for 15 minutes, and thereafter, the stained samples were washed three times with PBS. The biofilms produced were de-stained and quantified by solubilizing them with 200 μ L of 95% ethanol. Absorbance readings were taken at 510 nm using a Multiskan FC (Thermo Fisher Scientific, Waltham, MA, USA) microplate reader. A uniform mixture of 200 μ L of each plant extract (8 mg/mL) and standardized culture, respectively, was cultured without shaking at 37 °C in their appropriate media for 24 and 72 hours, respectively, for bacteria and fungi. The respective broths without cells were used as negative controls, and 200 μ L of streptomycin sulfate (0.8577 μ M) and fluconazole (0.1000 mM) were used as positive controls for bacteria and fungi, respectively. The procedure described above for the formation of biofilm was repeated. The percentage of biofilm inhibition was evaluated as described by Tamfu *et al.* [21]. The experiment was repeated four times, and mean absorbance values were used to calculate the percentage of biofilm inhibition.

Isolation and identification of the antifungal and antibacterial compound from the leaf extract

Repeated chromatographic separation by gradient and isocratic elution of the chloroform extract of the

leaf, obtained under Soxhlet conditions, was carried out using silica gel 60 (0.0400–0.0630 mm). Identification of the bioactive compound isolated was done by spectroscopic analysis of 1D and 2D NMR spectroscopic data obtained from a Bruker Ascend 500 and 126 MHz for proton and carbon, respectively (Bruker, Switzerland AG, Industriestrasse 26, CH-8117 Fallanden). The deuterated solvent was used for the analysis and TMS as a reference.

Computational and molecular docking studies of the isolated compound

The molecular docking studies of the compound and the positive control were performed using the AutoDock software against the active sites of the protein of 14 α -demethylase of *Candida Albicans* (PDB: 5TZ1). All the geometries of the ligand and its metal complexes used for the docking calculations were optimized using DFT calculations and converted to PDB files using the Gaussview software, whereas the PDB file for 14 α -demethylase of *Candida Albicans* was downloaded from the Protein Data Bank (PDB: 5TZ1). The co-crystallized substrate and water molecules were removed from the receptor using MGL 1.5.6 software. After cleaning the protein, only polar hydrogens were added, along with the Kollman charges. The grid box was constructed using 100, 100, and 100 pointing in the x, y, and z dimensions, respectively, with a grid point spacing of 0.3750 Å. The center grid box was set at 62.5940, 66.7740, and 2.6880 Å for the x, y, and z centers, respectively. The total number of genetic algorithms was set at 100, which generated one hundred different conformations for each of the molecules.

The conformers with the lowest binding free energies were used for the visualization of the interactions between the active amino acids and the molecule using the Discovery Studio software.

Statistical analyses

The antibacterial and antifungal experiments were conducted in triplicate and statistically evaluated using univariate statistics on an Excel page and by supervised multivariate classification and pattern recognition using partial least squares (PLS) regression analysis and non-supervised principal component analysis (PCA) chemometric tools with Minitab, version 19. The antibiofilm data were analyzed using univariate statistics on an Excel page. All statistical analysis data presented were significant at $P < 0.05$.

RESULTS AND DISCUSSION

The rise in antimicrobial resistance globally has heightened the search for reliable and inexpensive therapeutics from medicinal plants. Hence, this study investigated *C. platypetalum* and validated its use in traditional African medicine. It is crucial to note here that, traditionally, extracts have been produced by either soaking pulverised plant materials in alcoholic beverages or by soaking the plant materials in water followed by boiling. These conditions were mimicked in this study by maceration and decoction and thereafter investigated for constructive comparison. *C. platypetalum* displayed significant antifungal and antibacterial activities. The highest zones of inhibition of 22.7000 ± 2.08 mm (Figure 1) were

observed against *C. albicans* for acetone leaf extracts for both maceration and Soxhlet extraction methods ($P = 0.016$). These results suggest the thermal stability of the bioactive phytochemical constituents and agree with previous findings [16, 22].

The stem extracts appeared to be the least active part of the plant, with *C. glabrata* being the most susceptible of the fungi strains. Zones of inhibition of 20.3000 ± 0.58 and 18.3000 ± 0.58 mm and MIC values of 8 and 4 mg/mL were observed for DCM-MeOH and MeOH stem extracts, respectively, against *C. glabrata* (Figure 1). The root extracts exhibited zones of inhibition between 11.3000 ± 1.15 and 21.0000 ± 1.00 mm (samples O and P in Figure 1) and showed MICs of 0.0156-0.5000 mg/mL (samples O and P in Table 1) for the three fungi species that were investigated. The three fungi were most susceptible to the MeOH extract for the root. The best antifungal MICs of 0.0156 mg/mL were observed for the leaf and root extracts against *C. albicans*, *C. neoformans*, and *C. glabrata* at $P = 0.0002$ (Table 1). The best MFCs at $P = 0.003$ were observed at 0.063 mg/mL for the leaf against *C. neoformans* and *C. glabrata* and 0.5000 mg/mL for the root against *C. neoformans*, respectively. The observed MICs for the root against fungi pathogens were comparatively lower than the positive control (fluconazole), and the results therefore suggest the potential of *C. platypetalum* extracts against antifungal-resistant pathogen. To the best of our knowledge, this study is the first to report on the biological investigation of *C. platypetalum* root extracts.

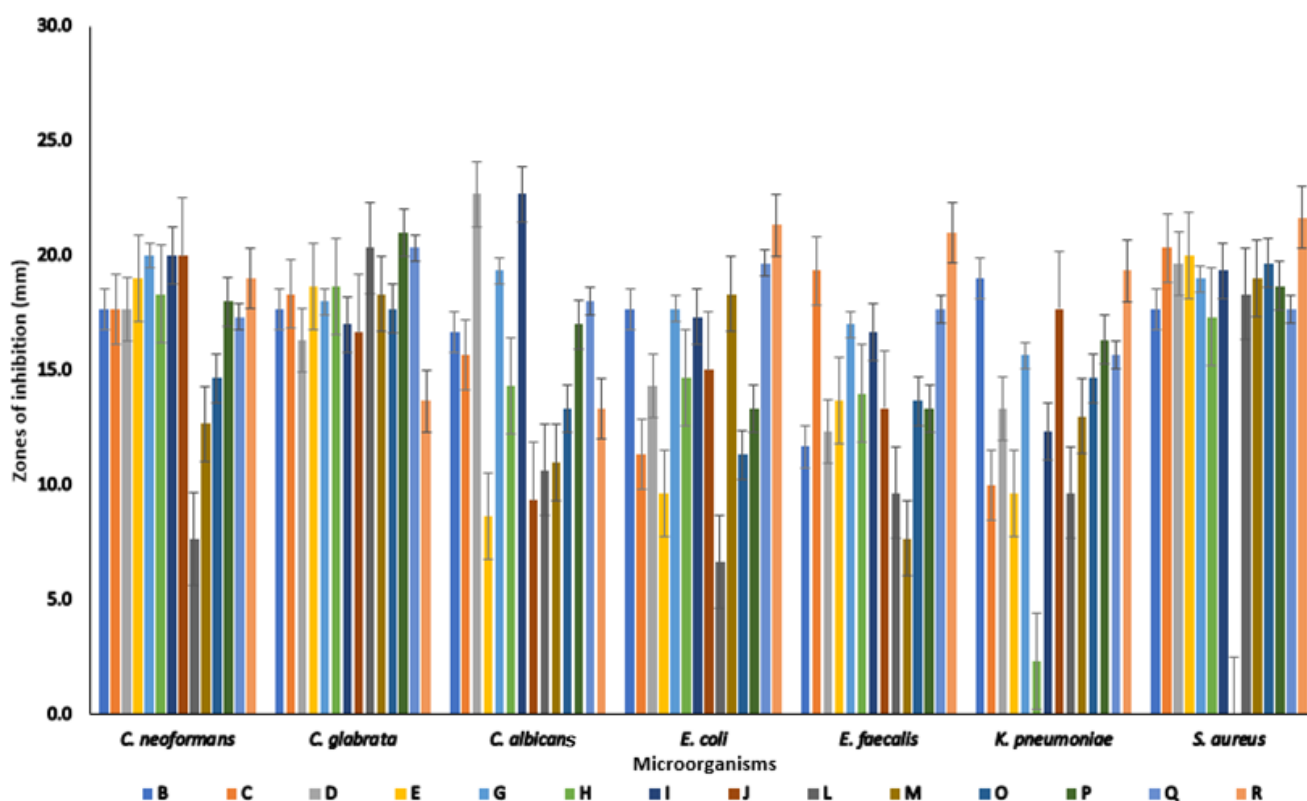


Figure 1: Zones of inhibition (mm) of *C. platyptalum* using 25 mg/mL. Values are the mean of triplicates run for each sample. Error bars indicate the standard deviation (SD±) values for each sample ($P = 0.016$). Seven microorganisms (X-axis) were under investigation against fourteen (14) antimicrobial agents (samples B, C, D, E, G, H, I, J, L, M, O, P, Q and R). The height of the bars represents the zones of inhibition (mm) (Y-axis) displayed by the respective antimicrobial agents

Decocted *C. platyptalum* leaves showed a strong antibacterial property, with zones of inhibition ranging from 19.3000 ± 1.15 to 21.7000 ± 2.08 mm against Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria species. No significant differences in antibacterial activity were observed between Gram-positive and Gram-negative bacteria. The best antibacterial MICs of 0.3906 mg/mL and 2.0000 mg/mL were also observed in the decoction of the leaf against *E. faecalis* and *S. aureus* and in the methanol extract of the root against *E. coli*, respectively. *E. coli* has been implicated in the causes of some diarrhea [23], urinary tract infections [24], respiratory illnesses [25], and

pneumonia [26], among others, which are some of the main traditional applications of *C. platyptalum*. Further, the stem and root extracts exhibited zones of inhibition of 6.6000 ± 0.58 to 19.0000 ± 1.73 mm and 11.3000 ± 1.15 to 21.0000 ± 1.00 mm, respectively. The MICs ranged between 0.0156 mg/mL and 8.0000 mg/mL for fungi and 0.3906 to 8.0000 mg/mL for bacteria. Most of the samples exhibited MBCs at $P = 0.003$ around 8.0000 mg/mL against *E. coli*, *E. faecalis*, *S. aureus*, and *K. pneumoniae* (Table 2). Due to the global rise of antimicrobial resistance [27], *C. platyptalum* extracts bioactivity revealed in the present study brings to the fore alternative therapies against multi-drug-resistant bacteria and fungi.

Table 1: Determination of the MICs (mg/mL) of *C. platypetalum* ($P = 0.0002$)

Sample	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. neoformans</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>
B	0.0156	0.0156	0.0156	1.0000	1.0000	1.0000	4.0000
C	0.0156	0.0630	0.0630	0.7813	8.0000	3.1250	0.7813
D	0.0156	0.0156	0.0156	4.0000	4.0000	4.0000	2.0000
E	0.0625	0.0156	0.1250	8.0000	8.0000	8.0000	8.0000
G	0.0625	0.0625	0.0313	1.0000	2.0000	1.0000	2.0000
H	0.0156	0.0156	0.0156	4.0000	4.0000	4.0000	4.0000
I	0.0156	0.0156	0.0156	2.0000	2.0000	2.0000	4.0000
J	4.0000	4.0000	4.0000	8.0000	8.0000	8.0000	8.0000
L	1.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000
M	4.0000	4.0000	4.0000	8.0000	8.0000	8.0000	8.0000
O	0.0156	0.1250	0.0156	8.0000	8.0000	8.0000	8.0000
P	0.0625	0.0625	0.5000	2.0000	8.0000	4.0000	4.0000
Q	0.0488	0.0488	0.0488	0.7813	0.7813	3.1250	3.1250
R	0.0488	0.09766	0.0488	0.7813	0.3906	0.3906	3.1250

B: Chloroform extract leaves by maceration; C: Ethyl acetate extract leaves by maceration; D: Acetone extract leaves by maceration; E: Methanol extract leaves of maceration and Soxhlet mixed; G: Chloroform extract leaves by Soxhlet; H: Ethyl acetate extract leaves by Soxhlet; I: Acetone extract leaves by Soxhlet; J: Aqueous methanol extract leaves; L: Dichloromethane : Methanol (1:1) extract stem; M: Methanol extract stem; O: Dichloromethane : Methanol (1:1) extract root; P: Methanol extract root; Q: methanol total extraction by maceration of the leaves; R: Decoction of leaves

Table 2: MBCs and MFCs (mg/mL) of the *C. platypetalum* ($P = 0.003$)

Sample	<i>C. albicans</i>	<i>C. glabrata</i>	<i>C. neoformans</i>	<i>E. coli</i>	<i>E. faecalis</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>
B	8.0000	4.0000	2.0000	8.0000	8.0000	4.0000	8.0000
C	0.1250	0.0630	0.0630	8.0000	8.0000	8.0000	8.0000
D	8.0000	2.0000	4.0000	8.0000	8.0000	8.0000	8.0000
E	8.0000	4.0000	2.0000	8.0000	8.0000	8.0000	8.0000
G	8.0000	8.0000	4.0000	8.0000	8.0000	1.0000	2.0000
H	8.0000	2.0000	2.0000	8.0000	8.0000	4.0000	8.0000
I	8.0000	8.0000	4.0000	8.0000	8.0000	8.0000	8.0000
J	8.0000	8.0000	4.0000	8.0000	8.0000	8.0000	8.0000
L	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000	8.0000
M	8.0000	8.0000	4.0000	8.0000	8.0000	8.0000	8.0000
O	4.0000	4.0000	4.0000	8.0000	8.0000	8.0000	8.0000
P	4.0000	4.0000	0.5000	8.0000	8.0000	4.0000	4.0000
Q	25.0000	25.0000	25.0000	6.2500	6.2500	6.2500	3.1250
R	25.0000	25.0000	25.0000	3.1250	6.2500	6.2500	3.1250

B: Chloroform extract leaves by maceration; C: Ethyl acetate extract leaves by maceration; D: Acetone extract leaves by maceration; E: Methanol extract leaves of maceration and Soxhlet mixed; G: Chloroform extract leaves by Soxhlet; H: Ethyl acetate extract leaves by Soxhlet; I: Acetone extract leaves by Soxhlet; J: Aqueous methanol extract leaves; L: Dichloromethane : Methanol (1:1) extract stem; M: Methanol extract stem; O: Dichloromethane : Methanol (1:1) extract root; P: Methanol extract root; Q: methanol total extraction by maceration of the leaves; R: Decoction of leaves

The highest biofilm inhibition of 40.61%, 36.04%, and 23.85% was observed in stem, root, and leaf extracts, respectively, of the methanol-composed solvents against *C. glabrata*. The statistical conclusions on biofilm formation inhibition by the

extracts are presented. The very polar extract of the decoction inhibited biofilm formation of some bacterial strains (*S. aureus*, *E. faecalis*, and *E. coli* by 21.15%, 2.70%, and 5.53%, respectively) only (Figure 2). The methanol extracts for the leaves,

stem, and root of *C. platypetalum* showed comparably moderate biofilm inhibition potentials (Figure 2). The highest biofilm inhibition (40.61%) was observed in the methanol extract of the stem against *C. glabrata*, an emerging antifungal-resistant yeast pathogen known to form biofilms [28]. Extracts of methanol stem and root and decoctions of leaves also showed antibiofilm capacity against *S. aureus*, an established Gram-positive bacterial pathogen that causes nosocomial and community-acquired infections [29]. Methicillin-resistant *Staphylococcus aureus* (MRSA) strains are particularly virulent with

biofilm formation, a recognized mediator of pathogenicity and antibiotic resistance [30]. The chloroform extract of the leaves by maceration displayed slight biofilm inhibition against *C. albicans* (Figure 2). A recently reported metabolomic study [16] indicated thermolabile phytochemical constituents that are unique to this chloroform extract of the leaves compared to the leaf extract obtained by the Soxhlet extraction method. Taken together, these results indicate that *C. platypetalum* extracts may provide a promising control avenue against multi-drug-resistant pathogens that are spreading globally.

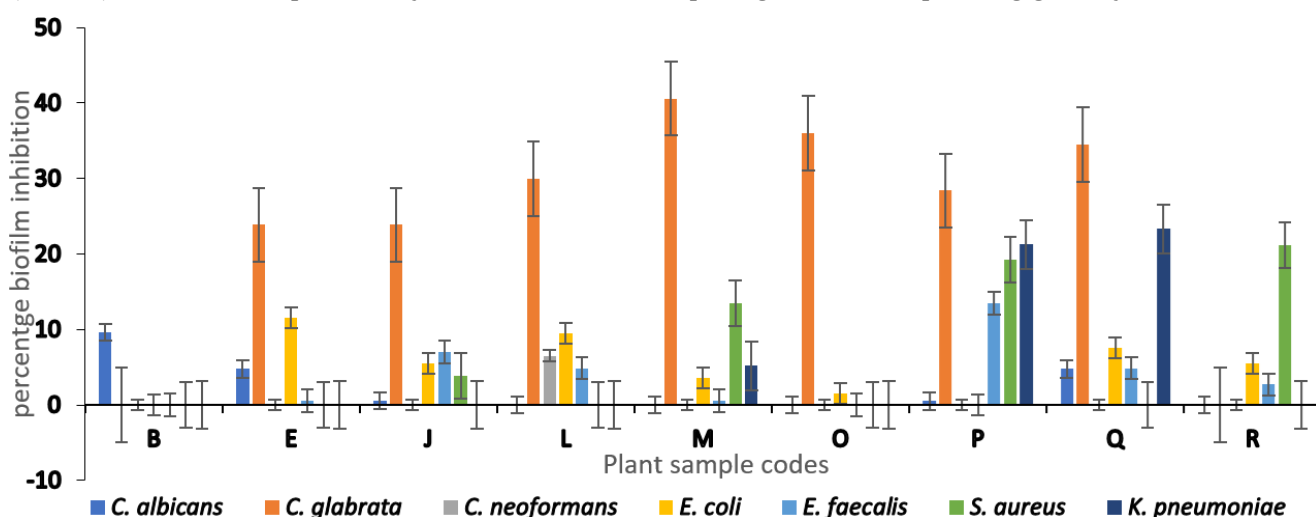


Figure 2: Percentage biofilm inhibition ($P < 0.05$) of the plant extracts against microorganisms under investigation. Only samples out of the 14 antimicrobial agents (X-axis) under investigation that displayed an antibiofilm effect against one or more microorganisms were captured in this plot. Some samples displayed antibiofilm effects against only a few microorganisms, indicated by the heights of the bars in the Y-axis as percentage biofilm inhibition, while the microorganisms without bars [zero (0) mark] indicate that they were not susceptible to the respective samples

We also compared statistically the antimicrobial activity and antibiofilm formation capacity of different extracts of *C. platypetalum*. Using Pearson correlation, it was observed that biofilm inhibition activity for the different extracts correlated weakly with their antimicrobial effects (Figure A.1). This also implies the possibility of observing antimicrobial activity but not necessarily a corresponding antibiofilm effect, and vice versa.

The poor or no biofilm inhibition observed could be due to the poor stability of the antimicrobial coatings [31] and their inability to interfere with quorum sensing or disperse biofilms from the onset [31]. The results from this study suggest a different antimicrobial mechanism of action of the extracts other than the inhibition of the biofilm formation pathway [32].

The antimicrobial properties of *C. platypetalum* extracts were analysed using chemometric tools and matrix plot correlation (Figure 3). It can be observed that the activity of the leaf extracts lies in the mid-polar and slightly polar regions, which agrees with previous findings [15, 33, 34]. This present study on pre-isolation analyses of *C. platypetalum* extracts showed that the least activity was observed in the stem. However, bio-guided or structure-directed isolation will corroborate these findings. The results were also analyzed by a supervised multivariate classification and pattern recognition (Figure 3) output from partial least squares (PLS) at a *P*-value of 0.003 and a non-supervised principal component analysis (PCA) on Minitab 19 statistical software, where both PLS and PCA methods produced comparable results. The plant displayed a non-synergistic biological response with pattern recognition for poor active samples (samples of

DCM-MeOH of the stem, MeOH of the stem, and MeOH of total extraction by maceration of the leaves, respectively) indicated with a black dash-type broken line circle in the PLS scores plot of Figures 3a and c and a corresponding reflection in the PLS loadings plot of Figure 3b. The scores plot enabled us to find groupings of similar samples. Samples outside the two circles in Figures 3a to c showed activity for either bacteria or fungi, respectively, in both the scores and loadings plots. Samples with distributed and pronounced antifungal and antibacterial activity (samples of chloroform by maceration, ethyl acetate by maceration, a mixture of methanol extract of Soxhlet and maceration, chloroform extract of the leaves, DCM-MeOH of the roots, and MeOH extract of the root) are captured with the red circle dash-type broken line in Figures 3a and c and reflected in b.

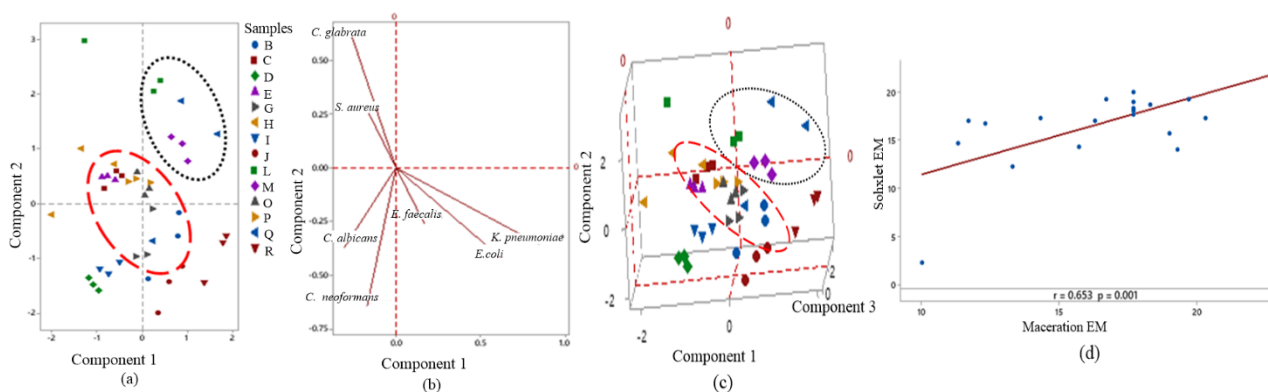


Figure 3: Partial Least Squares (a) scores plot (b) loadings plot (c) a 3D view of the scores plot with the groupings of the different extracts relative to the antimicrobial response of microorganisms investigated. Samples with poor activities are indicated with the black dash-type broken line circle in 4a and c and a corresponding reflection in the PLS loadings plot in 4b. Samples outside the two circles showed activity for either bacteria or fungi, respectively, in the scores and were reflected in the loading plots. Samples with distributed and pronounced antifungal and antibacterial activity are indicated with the red circle dash-type broken line in 4a and reflected in b. (d) matrix plot of the correlation between maceration and Soxhlet extraction methods on the biological response of microorganisms ($P < 0.05$)

During the extraction with Soxhlet, acetone leaf extract produced some needle-shaped crystals and displayed the highest antifungal activity against *C. albicans* and other yeasts (*C. glabrata* and *C. neoformans*), but with comparably moderate antibacterial activities against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumoniae*. The observed activity is in agreement with previous findings [33, 35] for the acetone extract of *C. platypetalum* leaves. In a similar study, the ethanol extract of *C. platypetalum* leaves showed antimycobacterial effects against *Mycobacterium smegmatis* and *Mycobacterium aurum* at MICs of 63 and 125 µg/mL and MBCs of 250 and 500 µg/mL, respectively [12]. The present findings further suggest a non-synergistic effect, as the extraction protocol presents a selective approach [36] and reduces the rigor of the isolation of possible active compound(s).

Chloroform and acetone extracts from maceration and Soxhlet extraction methods, respectively, are comparable for antimicrobial activity against

Cryptococcus neoformans, *Candida glabrata*, *Candida albicans*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* (Figure 4). This implies that the phytochemical constituents responsible for the antimicrobial activity are thermostable, and any of these methods could be adopted for the extraction of the constituents. A correlation plot (Figure 3d) and comparative bar chart (Figure 4) between the biological response for maceration and Soxhlet extraction methods indicate that both methods linearly correlate ($r = 0.653$; $P = 0.001$) and are comparable. These are in strong agreement with the metabolomic report on the thermal stability of the phytochemical constituents of the leaves, with a correlation of $r=0.782$ at $p = 0.000$ [16]. However, there is the possibility of slight thermal decomposition of phytochemical constituents in ethyl acetate extract from Soxhlet extraction methods, leading to a slight variation in antimicrobial and antibiofilm formation activities compared to ethyl acetate extract from maceration (Figure 4).

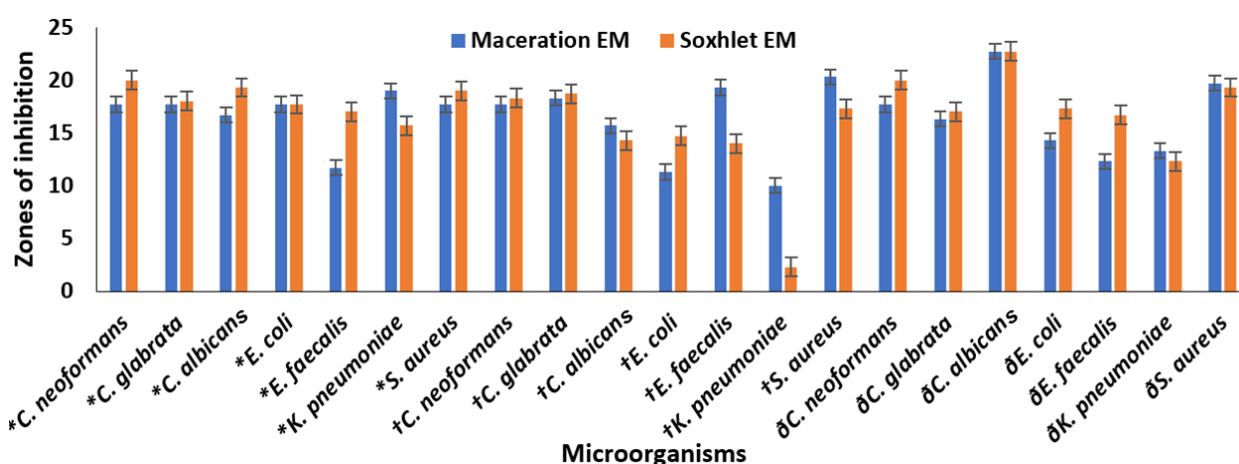


Figure 4: Comparative bar chart ($P < 0.05$) of biological response between samples from maceration and Soxhlet extraction methods (*: Chloroform extract - samples B and G, †: Ethyl acetate - extract samples C and H, δ: Acetone extract - samples D and I), respectively

Among the bacterial species, the antimicrobial activity against *K. pneumoniae* is a significant finding because of the rise in isolates producing beta-lactamase and carbapenemase [37]. *C. platypetalum* was more efficacious against fungi than bacteria, with the MIC for most fungi averaging at 0.0156 mg/mL and the best fungicidal concentration of 0.063 mg/mL observed in the ethyl acetate extract of the maceration extraction method against *C. neoformans* and *C. glabrata*, respectively (Tables 1 and 2). The mechanism of action of the extracts was not investigated in the present study. However, untargeted GC-MS metabolomics studies of the plant extracts [16] have identified scandenone, cholest-4-en-3-one, fucosterol, stigmasterol, (5 α)-stigmasterane-3,6-dione, cis-vaccenic acid, cis-13-octadecenoic acid, oleic acid, pyrogallol, n-hexadecanoic acid, octadecanoic acid, (E)-9-octadecenoic acid, tetradecanoic acid, (Z, Z)-9, 12-octadecadienoic acid, lasiodiplodin, farnesyl acetate, and farnesol as the volatile and semi-volatile bioactive constituents of the plant extracts that may account for the observed antimicrobial activity of the extracts. In the later study [16], ninety-seven (97) volatile and semi-volatile

phytoconstituents were identified in the plant leaves, stems, and roots. These present a strong interest in a concerted effort to adopt the bioguided isolation approach in the isolation and biological investigation of both the volatile and non-volatile constituents of the plant's antimicrobial principles. In the present study, repeated chromatographic separation led to the isolation of 1.8 mg of antifungal and antibacterial [38] compound (**1**). Compound (**1**) was isolated as a white crystal. Its spectroscopic data (**Figure B.1 a/b and Table A1**) was consistent with previously published data for the known β sitosterol [39]. The computed docking score inhibition constant (K_i) values of 4.87 nM (Figure 5 and **Table B1**) were further recorded for compound (**1**) against *C. albicans* (PDB: 5TZ1) species as a reference fungal pathogen. Compound (**1**) shares strong structural features with ergosterol and some ergosterol synthetic intermediates but is without the double bond between C7 and C8 of ring B in ergosterol and differs markedly in their side chains at C17.

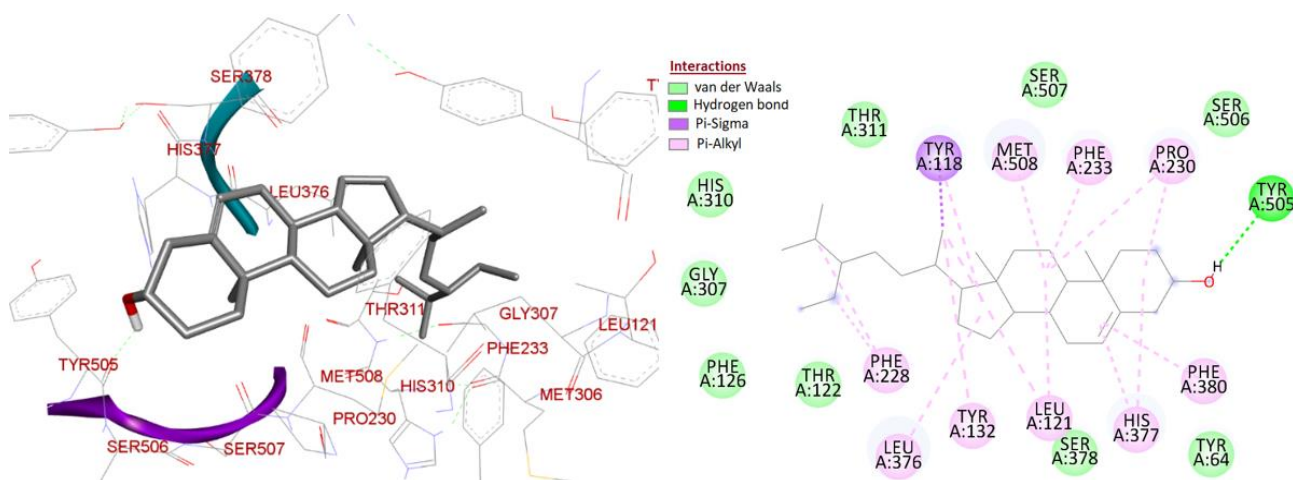


Figure 5: Molecular docking and the corresponding residual amino acid interactions of compound **1** against sterol 14 α - demethylase of *Candida albicans* (PDB: 5TZ1); Binding energy of -11.34 kcal/mol and inhibition constant (K_i) of 4.87 nM

In comparison with lanosterol, compound (**1**) lacks the three methyl groups at C4, C4, and C14 of lanosterol. The position of the double bonds in ring B of lanosterol is between carbons C8 and C9. Both also differ slightly in the C17 side chains. In particular, ergosterol, cholesterol, and β -sitosterol are eukaryotic sterols in fungi, humans, and plants [40], respectively. Hence, it justifies the molecular binding affinity outcome observed. From the molecular docking study (Figure 5), compound (**1**) is expected to exhibit its antifungal activity through the interruption of the ergosterol biosynthetic pathway. The antimicrobial mechanisms of action of the previously identified compounds [16] could either be due to protein synthesis machinery, cell wall disruption, or inhibition of enzymes required for nucleic acid synthesis in the microorganisms and should be subsequently investigated for a possible guide to the development of phytopharmaceuticals from the species. This is the first study to present the antibiofilm formation capacity of *C. platypetalum* extracts. The reported antimicrobial and antibiofilm formation properties of *C. platypetalum* extracts in this study widen the pool of possible sources of therapeutic agents against pathogenic microorganisms in light of the rising global crisis of antimicrobial resistance.

This study is the first to uncover the therapeutic potential of the root of *C. platypetalum* as well as elucidate the comparative biological activity similarities between maceration and Soxhlet extraction methods for the leaves. The leaves and roots of *C. platypetalum* were found to be the most potent parts of the plant. The activity of the leaf

extracts displayed non-synergistic effects as analysed by univariate statistics and the multivariate PLS chemometric tool. The acetone leaf extract proved very active in present and previous studies with thermostable bioactive phytochemical constituents for investigated microorganisms. Bioguided isolation of the extracts is strongly recommended to completely explore the therapeutic potentials of the species. The findings of the present study corroborate the traditional uses and project *C. platypetalum* as a promising source of evidence-based medicinal leads.

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COMPETING INTERESTS

The authors declare that they have no conflict of interest.

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ETHICAL APPROVAL

Not applicable

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