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Anti-biofilm and anti-quorum sensing activities of extract, fractions and compounds from the leaves of *Cassia alata* L. against yeast pathogens

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ABSTRACT

Background: *Cassia alata* or *Senna alata,* also known as "ringworm bush" because of its very effective fungicidal properties, is commonly used in African traditional medicine to treat fungal infections. Despite extensive phytochemical and pharmacological studies previously reported on *C. alata*, the antibiofilm activity against pathogenic yeast as well as the related anti-quorum sensing mechanism of some active constituents has not yet been elucidated. The aim of the study was to isolate the bioactive constituents from the methanol extract of the leaves of *C. alata* (CAExt) using antibiofilm-guided fractionation against yeast fungal pathogens and then to investigate the anti-quorum sensing activity of the active constituents by assessing their ability to inhibit violacein production in *Chromobacterium violaceum*.

Methods: Chromatographic methods were used to isolate the constituents of CAExt, and spectroscopic methods were used to elucidate the chemical structures of the isolated compounds. The broth microdilution assay was used to evaluate the antifungal activity against *Candida albicans* and *C. parapsilosis*, while crystal violet staining was used for the inhibition of biofilm formation and the disruption of preformed biofilm. The biosensor strain *C. violaceum* ATCC 12472 was used to investigate the anti-quorum sensing activity of the most active constituents.

Results: The crude extract exhibited biofilm inhibition and eradication activities against the tested pathogenic yeast. The biofilm inhibition percentages ranged from 53.22 % to 75.38 %, while the biofilm eradication percentages ranged from 23.21 % to 64.25 %. The ethyl acetate fraction demonstrated high biofilm inhibition and eradication activities against the tested microorganisms. The biofilm inhibition percentages ranged from 58.19 % to 79.30 %, while the biofilm eradication percentages ranged from 34.105 % to 69.54 %. The purification of subfractions led to the identification of six compounds: stigmasterol **(1)**, sitosterol **(2)**, lupeol **(3)**, emodin **(4)**, kaempferol **(5)** and stigmasterol glycoside **(6)**, two of which (**4** and **5**) showed potent biofilm inhibition and eradication activities. Both compounds demonstrated significantly lower $MBIC_{50}$ values of 70.81 μg/mL and 65.65 μg/mL against *Candida albicans* and $MBEC_{50}$ values of 63.65 μg/mL and 82.66, respectively, against *C. albicans* and *C. parapsilosis*. The crude extract and compounds **(4)** and **(5)** also demonstrated quorum sensing inhibitory activity, as indicated by the MQSIC value of 1024 μg/mL for the crude extract and 128 μg/mL for the two compounds. Moreover, compounds **(4)** and **(5)** displayed significant inhibitory effects on violacein production, as indicated by their low IC_{50} values of 28.08 μg/mL and 26.44 μg/mL, respectively.

Conclusions: Data obtained in this study not only support the traditional use of *C. alata* in the treatment of fungal infections but also reveal *C. alata* extract, as well as the two isolated bioactive compounds emodin **(4)** and

List of abbreviations: ANOVA, analysis of variance; CAE, *Cassia alata* ethyl acetate fraction; CAExt, methanol extract of the leaves of *C. alata*; CAH, *Cassia alata* hexane fraction; COSY, Correlation Spectroscopy; DMSO, dimethyl sulfoxide; EI-MS, Electronic Impact- Mass Spectrum; EtOAc, ethyl acetate; HSQC, Heteronuclear Single Quantum Correlation; Hz, Hertz; IC_{50}, inhibitory concentration 50 %; MeOH, methanol; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; $MBIC_{50}$, minimum biofilm inhibitory concentration; $MBEC_{50}$, minimum biofilm eradicating concentration; MQSIC, minimum quorum sensing inhibitory concentration; NMR, nuclear magnetic resonance; OD, optical density; PBS, phosphate-buffered saline; RA, reference antifungal; SD, standard deviation; SDA, Sabouraud dextrose agar; SDB, Sabouraud dextrose broth; TLC, Thin layer chromatography; TMS, tetramethylsilane; UV, ultraviolet.

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kaempferol **(5),** as a potential source for developing antibiofilm alternative agents against biofilm-associated yeast infections.
List of compounds studied: stigmasterol **(1)**, sitosterol **(2)**, lupeol **(3)**, emodin **(4)**, kaempferol **(5)**, stigmasterol glycoside **(6)**

1. Introduction

Fungal infections have become a significant global health concern, particularly in individuals with compromised immune systems (Mendonça et al., 2022). Yeast fungal infections such as candidiasis range from mild mucosal infections, such as oral thrush and vaginal yeast infections, to severe systemic cryptococcosis, which can be life-threatening (Vázquez-González et al., 2013). The rise in drug-resistant yeast strains and the ability of these pathogens to form biofilms enhance their virulence and resistance to conventional antifungal therapies and further complicate treatment. Biofilms, structured communities of bacteria, fungi and other microorganisms, have been identified as major contributors to antibiotic resistance (Kaur and Nobile, 2023). These resilient biofilms are notoriously difficult to eradicate due to their protective matrix, rendering conventional antimicrobial agents less effective (Bi et al., 2021).

Yeast biofilms pose a significant challenge in clinical settings, as they can form on medical devices, such as catheters, implants and prosthetics, leading to persistent and difficult-to-treat infections (Cavalheiro and Teixeira, 2018). In addition to biofilm formation, quorum sensing is another important aspect of yeast pathogenicity. Quorum sensing is a cell-to-cell communication system utilized by many microorganisms, including *Candida* and *Cryptococcus* species, to coordinate and regulate various physiological processes, including biofilm formation, virulence factor production and gene expression (Mehmood et al., 2019; Tian et al., 2018). The resistance of yeast biofilms to conventional antifungal agents further emphasizes the need to explore alternative sources of antifungal agents. The discovery of new therapeutic agents that can disperse biofilms and disrupt quorum sensing mechanisms could provide a promising strategy to attenuate microbial infections.

Natural products derived from plants have long been recognized as valuable sources of bioactive compounds with diverse biological activities, including antifungal properties (Melander et al., 2020). The process of bioassay-guided fractionation and isolation involves a systematic approach to isolate and identify bioactive compounds in plant extracts. By employing this approach, researchers can obtain purified bioactive compounds with enhanced efficacy and reduced side effects, paving the way for potential therapeutic applications (Brusotti et al., 2014).

Cassia alata L. (synonym *Senna alata* (L.) Roxb.), commonly known as the candle bush or ringworm shrub, is a tropical plant belonging to the Fabaceae family. Traditionally, *C. alata* has been used in various folk medicinal practices across different regions of the world, particularly in tropical countries such as India, Brazil, Nigeria, and Cameroon, for its numerous therapeutic benefits (Yon et al., 2023). The plant has been recorded to be used in traditional medicine in various areas of the world for treating skin diseases (Ajibesin et al., 2008) and fungal diseases, including ringworm and pityriasis versicolor (Yon et al., 2023).

The leaves of *C. alata* have gained attention due to their remarkable medicinal properties, including antibacterial, antifungal, antiviral and anti-inflammatory activities (Yon et al., 2023). Several studies have reported the antifungal activity of crude extracts obtained from different parts of *C. alata* against various pathogenic fungi, including *Candida* species (Palanichamy and Nagarajan, 1990; Rekha et al., 2017). These properties have been attributed to the presence of secondary metabolites such as anthraquinones, flavonoids, tannins, and phenolic compounds. Amongst these constituents, emodin and kaempferol have emerged as potential bioactive compounds with antibacterial and anti-inflammatory properties (Kim and Park, 2020; Stompor–gorący, 2021). It is noteworthy that several studies showed promising evidence for the safe use of extract of *C. alata* (Eziuche et al., 2016; Ugbogu AE et al., 2016). In addition, Roy et al. (2016) and Senou (2022) reported that the leaf extract of *C. alata* had no acute or subchronic oral toxicity in Wistar rats at doses of 1000, 2000 and 3000 mg/kg body weight through oral administration daily (Roy et al., 2016; Senou et al., 2022). Despite extensive phytochemical and pharmacological studies previously reported on *C. alata*, the antibiofilm activity against pathogenic yeast as well as the related antibiofilm mechanism of action have not yet been elucidated. Moreover, the specific bioactive constituents underlying the potential antibiofilm activity remain unknown. Therefore, in this study, our objective was to identify and isolate bioactive constituents from the leaves of *C. alata* that exhibit potent activity against *Candida* species and *Cryptococcus neoformans* biofilms and then assess the effect of the active compounds on quorum sensing in *Chromobacterium violaceum*.

2. Materials and methods

2.1. General procedures

Ultraviolet spectra were recorded on a Hitachi UV 3200 spectrophotometer in MeOH. EI-MS (Electronic Impact- Mass Spectra) were recorded on a Finnigan MAT 95 spectrometer (70 ev) with perfluorokerosene as reference substance for HR-EI-MS (High Resolution-Electrospray ionization-Mass Spectra) were measured on Agilent Technologies, Santa Clara, CA, USA). The 1H and 13C—NMR spectra were recorded at 500, 400, 125 and 100 MHz on Bruker AMX 600 NMR spectrometers. Homonuclear 1H connectivity was determined using the COSY (Correlation Spectroscopy experiment. 1H/13C one bond connectivity was determined using HSQC (Heteronuclear Single Quantum Correlation) gradient pulse factor selection. Chemical shifts are reported in δ ppm using TMS as an internal standard, and coupling constants (J) were measured in Hz. Column chromatography was carried out with silica gel (70–230 mesh, Merck). Thin layer chromatography (TLC) was performed on Merck precoated silica gel 60 F254 aluminium-backed plates, and spots were revealed using ceric sulfate spray reagent, UV lamp, and iodine vapour. All other substances, if otherwise not specified, were purchased from Sigma−Aldrich (Germany). All reagents used were of analytical grade.

2.2. Plant material

The leaves of *C. alata* were collected near the city of Yabassi, Littoral Region of Cameroon, in March 2019. The plant was identified at the Cameroon National Herbarium, Yaounde-Cameroon, with the Letouzey Rene specimen of the Herbarium collection N° 45,146-HNC by Mr. Victor Nana (plant taxonomist).

2.3. Extraction and isolation

The air-dried, ground leaves of *C. alata* (1.5 kg) were macerated in methanol (10 L) for 72 h at room temperature. The filtrate was then evaporated under reduced pressure, yielding a green−brown crude extract (85.2 g) (CAExt). Part of this crude extract (80 g) was successively extracted with equal volumes (1 L) of hexane and ethyl acetate (EtOAc), yielding the hexane fraction (CAH) (8.4 g) and ethyl acetate extract fraction (CAE) (50.9 g), respectively, after concentration to dryness. The latter was separately subjected to silica column chromatography over silica gel. The elution was carried out with a mixture of n-hexane-EtOAc (2/5), n-hexane-EtOAc (4/5), n-hexane-EtOAc (3/5), n-

hexane-EtOAc (1/1), and n-hexane-EtOAc (1/3) in increasing polarity, resulting in 5 major subfractions, namely, CAEa, CAEb, CAEc, CAEd, and CAEe. Subfraction CAEa (8.60 g) was composed of subfractions 1–50 and eluted with an isocratic system of n-hexane-EtOAc (9/1), and fractions of 100 mL were collected to yield a mixture of stigmasterol **(1)** and sitosterol **(2)** (20.17 mg) and lupeol **(3)** (4.9 mg). Subfraction CAEb (7.70 g) was composed of subfractions 51–100 and eluted with n-hexane-EtOAc (7/3). Fractions of 100 ml were collected to give a mixture of stigmasterol and sitosterol with a total mass of 30.4 mg, emodin **(4)** (39.2 mg) and chlorophyll. Subfraction CAEc (5.2 g) resulting from subfractions 101–150 and eluted with n-hexane-EtOAc (6/4); fractions of 100 ml were collected to give kaempferol **(5)** (58.7 mg). Subfraction CAEd (7.94 g) was composed of subfractions 151–175 and eluted with n-hexane-EtOAc (3/7); fractions of 100 ml were collected to give only chlorophyll. Subfraction CAEe (4.4 g) resulting from subfractions 176–200 was eluted with n-hexane-EtOAc (2/8); fractions of 100 ml were collected to give stigmasterol glycoside (**6**) (24.9 mg).

2.3.1. Mixture of stigmasterol (1) and sitosterol (2)

(20.17 mg).White amorphous powder. **UV** λ max (MeOH), nm: 189. **^{1}H NMR** ($CDCl_3$, 500 MHz): 1.15 (1H, m, H-1), 1.44 (11H, m, H-2, H-8, H-9, H-11, H-12, H-14), 3.52 (1H, m, H-3), 1.98 (2H, m, H-4), 5.35 (1H, m, H-6), 1.85 (2H, m, H7), 1.54 (4H, m, H-15, H-16), 1.53 (1H, m, H-17), 0.68 (3H, s, H-18), 1.02 (3H, s, H-19), 2.27 (1H, m, H-20), 0.87 (3H, d, $J = 6.3$ Hz, H-21), 5.13 (1H, dd, $J = 12.0$, 8.0 Hz, H-22), 5.03 (1H, dd, $J = 12.0$, 8.0 Hz, H-23), 2.22 (1H, m, H-24), 1.85 (1H, s, H-25), 0.85 (3H, d, $J = 6.8$ Hz, H-26), 0.82 (3H, d, $J = 6.8$ Hz, H-27), 1.25 (3H, m, H-28), 0.82 (3H, d, $J = 6.9$ Hz, H-29); **^{13}C NMR** ($CDCl_3$, 125 MHz): 140.8 (C-5), 138.3 (C-22), 129.3 (C-23), 121.7 (C-6), 71.8 (C-3), 56.9 (C-14, C-17), 51.3 (C-24), 50.2 (C-9), 42.4 (C-4, C-13), 40.5 (C-20), 39.8 (C-12), 37.3 (C-1), 36.6 (C-10), 32.0 (C-7), 31.9 (C-8), 31.7 (C-2), 31.2 (C-25), 28.9 (C-16), 25.4 (C-28), 24.4 (C-15), 21.2 (C-26, C-27), 21.1 (C-11, C-21), 19.4 (C-19), 12.2 (C-18, C-29).

2.3.2. Lupeol (3)

(4.9 mg, mp: 160–162 °C) White fibre, **UV** λ max (MeOH), nm: 185. **^{1}H NMR** ($CDCl_3$, 500 MHz): 0.91 (1H, m, H-1a), 1.66 (1H, m, H-1b), 1.61 (1H, m, H-2a), 1.57 (1H, m, H-2b), 3.18 (1H, dd, $J = 11.4$, 5.3 Hz, H-3), 0.70 (1H, m, H-5), 1.52 (1H, m, H-6a), 1.41 (1H,m, H-6b), 1.40 (2H, m, H-7), 1.26 (1H, m, H-9), 1.42 (1H, m, H-11a), 1.22 (1H, m, H-11b), 1.05 (1H, m, H-12a), 1.66 (1H, m, H-12b), 1.65 (1H, m, H-13), 0.99 (1H,m, H-15a), 1.69 (1H, m, H-15b), 1.37 (1H, m, H-16a), 1.46 (1H, m, H-16b), 1.38 (1H, m, H-18), 2.39 (1H, m, H-19), 1.32 (1H, m, H-21a), 1.91 (1H, m, H-21b), 1.19 (1H, m, H-22a), 1.37 (1H, m, H-22b), 0.98 (3H, s, H-23), 0.77 (3H, s, H-24), 0.84 (3H, s, H-25), 1.05 (3H, s, H-26), 0.95 (3H, s, H-27), 0.80 (3H, s, H-28), 4.55 (1H, d, $J = 2.4$ Hz, H-29a), 4.70 (1H, $J = 2.4$ Hz, H-29b), 1.70 (3H, s, H-30); **^{13}C NMR** ($CDCl_3$, 125 MHz): 151.6 (C-20), 109.3 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 48.3 (C-19), 48.0 (C-18), 43.0 (C-17), 42.8 (C-14), 40.8 (C-8), 40.0 (C-22), 38.8 (C-4), 38.7 (C-1), 28.0 (C-13), 37.1 (C- 10), 35.6 (C-16), 34.3 (C-7), 29.7 (C-21), 28.0 (C-23), 27.4 (C-2, C-15), 25.1 (C-12), 20.9 (C-11), 19.3 (C- 30)18.3 (C-6), 18.0 (C-28), 16.1 (C-26), 15.9 (C-25), 15.3 (C-24), 14.5 (C-27).

2.3.3. Emodin (4)

(39.2 mg, mp: 258–260 °C). Yellow powder, UV λ_{max} (MeOH), nm: 252, 262, 285. 1H NMR (500 MHz, C2D6CO, ppm) **δ** 12.25 (1H, s, C1-OH), 12.06 (1H, s, C8-OH) δ 7.71 (^{1}H, d, $J = 1.8$ Hz, H-4), 7.35 (1H, d, $J = 2.5$ Hz, H-7), 7.21 (^{1}H, d, $J = 1.8$ Hz, H-2), 6.68 (^{1}H, d, $J = 2.5$ Hz, H-5), 2.50 (3H, s, CH_3). ^{13}C NMR (125 MHz, CD3OD, ppm) δ: 183.7 (C-9), 179.5 (C-10), 107.9 (C-7), 21.7 (-CH3); HR-ESI-MS *m/z* 270.0607 of $(M + H)+$ (calculated for $C_{15}H_{10}O_5$, 270.0628 (Santos et al., 2008).

2.3.4. Kaempferol (5)

(45.7 mg, mp: 276–278 °C). Yellow powder, UV λ_{max} (MeOH), nm: 272, 395. 1H NMR (500 MHz, CD4O, ppm) **δ** 6.20 (1H, d, 2.3 Hz, H6), 6.41 (1H, d, 2.3 Hz, H8) δ 8.01 (**^{1}**H, d, $J = 8.8$ Hz, H-2′), 6.97 (1H, d, $J = 8.5$ Hz, H-3′), 6.97 (^{1}H, d, $J = 8.5$ Hz, H-5′), 8.01 (^{1}H, d, $J = 8.5$ Hz, H-6′). ^{13}C NMR (125 MHz, CD3OD, ppm) δ: 98.2 (C-6), 93.5 (C-8), 129.5 (C-2′), 115.4 (C-5′); 129.5 (C-6′) HR-ESI-MS *m/z* 286.23502 of $(M + H)+$ (calculated for $C_{15}H_{10}O_6$, 286.234 (Santos et al., 2008).

2.3.5. Stigmasterol 3-O-β-d-glucopyranoside (6)

(24.9 mg). White amorphous powder**. UV** λ max (MeOH), nm: 189, 205. **^{1}H NMR** (DMSO-d6, 500 MHz): 1.24 (2H, m, H-1), 1.39 (2H, m, H-2), 3.47 (1H, m, H-3), 1.93 (2H, m, H-4), 5.32 (2H, m, H-6), 1.80 (2H, m, H-7), 1.50 (6H, m, H-8, H-9, H-11), H-12), 1.15 (1H, m, H-14), 1.80 (4H, m, H-15, H-16), 1.24 (1H, m, H-17), 0.82 (3H, s, H-18), 0.95 (3H, s, H-19), 2.12 (1H, m, H-20), 0.90 (3H, d, $J = 6.4$ Hz, H-21), 5.16 (1H, dd, $J = 12.1$, 8.2 Hz, H-22), 5.03 (1H, dd, $J = 12.1$, 8.2 Hz, H-23), 1.50 (4H, m, H-24, H-26), 1.15 (2H, m, H-25), 1.63 (1H, m, H-27), 0.76 (3H, m, H-28), 0.99 (3H, d, $J = 6.9$ Hz, H-29), 4.22 (1H, d, $J = 6.5$ Hz, H-1′), 2.90 (1H, t, $J = 7.0$ Hz, H-2′), 3.12 (1H, d, $J = 7.5$ Hz, H-3′), 3.06 (1H, m, H-4′), 3.10 (1H, m, H-5′), 3.44 (1H, m, H-6′a), 3.67 (1H, m, H-6′b); **^{13}C NMR** (DMSO-d6, 125 MHz): 141.0 (C-5), 138.8 (C- 22), 129.6 (C-23), 121.9 (C-6), 102.6 (C-1′), 78.6 (C-3), 78.4 (C-3′), 78.2 (C-5′), 75.4 (C-2′), 71.8 (C-4′), 63.0 (C- 6′), 57.0 (C-14), 56.2 (C-17), 51.5 (C-24), 50.4 (C-9), 42.4 (C-4, C-13), 40.8 (C-20), 39.4 (C-12), 37.5 (C-1), 37.0 (C-10), 32.2 (C-2, C-7), 32.1 (C-8, C-25), 29.3 (C-16), 25.7 (C-28), 24.6 (C-15), 21.3 (C-21), 19.5 (C-11, C- 26), 19.3 (C-27), 19.2 (C-19), 12.5 (C-29), 12.2 (C-18).

2.4. Antimicrobial susceptibility and antibiofilm assays

2.4.1. Microbial strains and growth conditions

Strains of *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, *Candida tropicalis* ATCC 13803, *Cryptococcus neoformans* ATCC 14116, and *Chromobacterium violaceum* ATCC 12472 from the American Type Culture Collection (ATCC) were used. Fungi were maintained in Sabouraud dextrose agar (SDA) at 37 °C, while *Chromobacterium violaceum* strain was maintained in Luria–Bertani (LB) agar at 30 °C.

2.4.2. Determination of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC)

MIC and MFC values were determined by the broth microdilution method using Sabouraud dextrose broth (SDB). Stock solutions of extract, fractions, compounds, and reference antifungal were prepared in 100 % dimethyl sulfoxide (DMSO; Sigma), and twofold serial dilutions were prepared in media in amounts of 100 µL per well in a 96-well plate. Then, 100 µL of a yeast suspension was added to each well of the plate except those of the sterility control, resulting in a final inoculum of 1.5×10^4 CFU/mL. The final concentration of samples ranged from 8 to 1024 µg/mL for extract and fractions and from 0.125 to 256 µg/mL for compounds and the reference antifungal (RA). The final concentration of DMSO was lower than 2.5 % and does not affect the microbial growth. The medium without the agents was used as a growth control, and the blank control contained only the medium. Amphotericin B (the RA) served as positive control. The microtitre plates were incubated at 37 °C for 24 h (48 h for *C. neoformans*). The assay was repeated three times in triplicate. The MIC of the samples was detected following the addition (40 µL) of 0.2 mg/mL *p*-iodonitrotetrazolium chloride and incubation at 37 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink colour. MIC was defined as the lowest sample concentration that prevented this change and exhibited complete inhibition of yeast growth.

The MFC was determined by adding 50 µL of the suspensions from the wells, which did not show any growth after incubation during MIC assays, to 150 µL of fresh SDB. These suspensions were incubated at 37 °C for 48 h (72 h for *C. neoformans*). The MFC was determined as the lowest concentration of sample that completely inhibited the growth of yeast.

2.4.3. Determination of the median minimum biofilm inhibitory concentration ($MBIC_{50}$) and the median minimum biofilm eradicating concentration ($MBEC_{50}$)

In a preliminary experiment, all the samples were tested at their MIC concentration, and those with more than 50 % biofilm inhibition/eradication were selected for a dose–response assay to determine the $MBIC_{50}$ and $MBEC_{50}$ values. This was done by the broth microdilution method as previously described (Bisso et al., 2023). Briefly, 100 µL of SDB supplemented with 2 % glucose containing the samples was introduced into the first wells followed by a serial twofold dilution. Subsequently, 100 µL of yeast suspension was added to all wells except those of the sterility control, resulting in a final inoculum of 1.5×10^4 CFU/mL, followed by incubation at 37 °C for 24 h. After incubation, the plate was washed three times with phosphate-buffered saline (PBS; pH 7.2) to remove nonadherent yeast cells. Wells containing SDB without yeast served as the negative control. The remaining yeast cells that attached to the well surface were considered a true biofilm. Then, the plates were stained with 0.1 % crystal violet solution for 20 min at room temperature. After staining, the plates were washed three times with PBS. Then, the plates were air-dried and destained with 150 µL of 95 % ethanol (v/v) for 30 min. Finally, the optical density was measured at 590 nm using a microplate reader (BioTek Epoch Microplate Spectrophotometer). Untreated wells and wells containing broth only were used as positive and blank controls, respectively, and the percentage of biofilm inhibition was calculated by using the following formula:

$$\%\ \text{inhibition} = 100 - \left[\left(OD_{sample} - OD_{blank}\right) / \left(OD_{control} - OD_{blank}\right) \times 100\right]$$

The $MBEC_{50}$ was determined under the same conditions as the $MBIC_{50}$, with the only difference being that the biofilm was allowed to form for 24 h before treatment with the samples.

Median $MBIC_{50}$ and $MBEC_{50}$ values were defined as the concentration inhibiting 50 % of biofilm formation and preformed biofilm, respectively. This was calculated by plotting the percentage of inhibition or eradication versus the concentrations using GraphPad Prism software. Samples were tested in triplicate, and experiments were repeated three times.

2.5. Anti-Quorum sensing assay

2.5.1. Inhibition of violacein production

The inhibition of violacein production was performed according to a previously described method (Ahmad et al., 2015) and miniaturized in a 48-well microplate. This was achieved by transferring 1000 µL of samples into the first well of a 48-well microplate, followed by a serial twofold dilution in LB broth. Then, 500 µL of *C. violaceum* inoculum standardized at 3×10^6 CFU/mL was added to all wells except those of the sterility control to obtain a final concentration of 64–2048 µg/mL for extract and fractions and 16–512 µg/mL for compounds and reference compound vanillin (Choo et al., 2006). Plates were properly sealed with parafilm and incubated in an orbital shaker (140 rpm) at 30 °C for 24 h. The MIC was defined as the minimum concentration inhibiting visible bacterial growth and therefore preventing the production of purple pigmentation. The minimum quorum sensing inhibitory concentration (MQSIC) was defined as the lowest sample concentration allowing bacterial growth (shown by turbidity) without the visible production of purple pigmentation.

2.5.2. Quantification of violacein

The inhibitory effect of selected samples on violacein pigment production was further quantified using a spectrophotometric method. After MIC and MQSIC data were collected, the plates were centrifuged at 4000 rpm for 20 min, and the supernatant was discarded. Then, the bacterial pellet was resuspended in 1 mL of DMSO, and the plates were further left in an orbital shaker for 10–15 min. Then, 200 µL of the supernatant was transferred into a 96-well microplate, and the optical density was measured at 595 nm. The percentage of violacein inhibition was calculated using the following formula:

$$\%\text{violacein inhibition} = 100 - \left[\left(OD_{sample} - OD_{blank}\right) / \left(OD_{control} - OD_{blank}\right) \times 100\right].$$

The IC_{50} value of the samples was defined as the concentration inhibiting 50 % of violacein production. This was calculated by plotting the percentage of violacein production versus the concentrations using GraphPad Prism software. Samples were tested in triplicate, and each experiment was repeated three times.

2.6. Statistical analysis

The data are presented as the mean ± standard deviation (SD) of three independent experiments. Statistical differences between the IC_{50} values inhibiting the violacein production of samples and the reference compound (vanillin) were assessed by two-way ANOVA followed by Sidak's multiple comparisons test in GraphPad Prism software.

3. Results and discussion

3.1. Bioguided fractionation of Cassia alata extract

Bioguided fractionation and isolation is a process used to identify and isolate bioactive compounds from complex mixtures, such as plant extracts (Abubakar and Haque, 2020). In this study, an in vitro bioguided approach was applied to identify potential molecules with antibiofilm activity. The methanol extract of the leaves of *Cassia alata* was successively extracted with hexane and ethyl acetate, generating two different fractions: hexane and ethyl acetate fractions. The minimum inhibitory concentrations (MICs) were determined against four clinically relevant yeast including *Candida albicans, Candida parapsilosis, Candida tropicalis, Cryptococcus neoformans* (Table 1). The crude extract and the ethyl acetate fraction had the lowest MIC of 128 µg/mL. It's noteworthy that *Cryptococcus neoformans* was the less sensitive of the five tested fungal strains. The antifungal activity of various extracts and isolated compounds from *Cassia alata* has been reported (Waris et al., 2021). However, the main purpose of the antifungal activity study in this work was to further use MIC values at a single concentration for the determination of the percentage of biofilm inhibition and biofilm eradication, which then served as a basis for bioguided fractionation. The crude extract (CAExt) exhibited biofilm inhibition percentages ranging from 36.43 % to 75.38 %, while the biofilm eradication percentages ranged from 23.21 % to 64.25 % (Table 2). Compared to the hexane fraction (CAH), the ethyl acetate fraction (CAE) was the most promising, demonstrating higher biofilm inhibition and eradication activities against the tested microorganisms. The biofilm inhibition percentages ranged from 41.67 % to 79.30 %, while the biofilm eradication percentages ranged from 34.10 % to 69.54 %. Some negative percentage values of biofilm eradication were obtained against *Cryptococcus neoformans*, indicating the potential of compounds to enhance biofilm formation. Overall, the results indicated that the biofilm eradication activity appears to be more difficult to achieve than the biofilm inhibition. *Cassia alata* extract is well known for its broad spectrum of biological activities; however, the antibiofilm activity of *Cassia* species remains relatively unexplored. Although the inhibitory effect of the methanol extract of *Cassia spectabilis* leaves was recently evaluated against biofilm-forming *C. albicans* (Torex and Sasidharan, 2011), to the best of our knowledge, the anti-biofilm activity of *Cassia alata* leaf extract against *Candida* is reported here for the first time. The ethyl acetate fraction, which appeared to be the most promising fraction, was then selected for subsequent phytochemical analysis for compound isolation.

Table 1
The minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of extract, fractions and compounds from the leaves of *Cassia alata*. (μg/mL).

Samples	Microorganisms							
	Candida albicans		*Candida parapsilosis*		*Candida tropicalis*		*Cryptococcus neoformans*	
	MIC	MFC	MIC	MFC	MIC	MBC	MIC	MBC
CAExt	**128**	1024	512	1024	128	1024	512	–
CAH	512	–	–	nd	1024	–	–	nd
CAE	128	1024	256	512	128	1024	512	–
CAEa	–	512	1024	1024	512	–	1024	–
CAEb	**128**	512	128	512	**64**	1024	1024	–
CAEc	**128**	1024	256	1024	128	1024	256	1024
CAEd	1024	–	512	–	512	1024	1024	–
CAEe	512	1024	1024	–	–	nd	–	nd
(1) & (2)	–	nd	256	128	256	–	–	–
(3)	256	–	128	–	128	nd	–	–
(4)	**32**	128	**64**	256	**64**	256	128	–
(5)	**64**	256	128	–	**64**	128	128	256
(6)	–	nd	256	–	–	nd	256	–
Am B	2	16	1	16	2	32	8	64

- = >1024 μg/mL for extract and fractions and >256 μg/mL for compounds. SA Ext: crude extract; SAH: hexane fraction; SAE: ethyl acetate fraction; CAEa, CAEb, CAEc, CAEd, CAEe: subfractions from ethyl acetate fraction; (1&2)-(6): isolated compounds, AmB: amphotericin B.

Table 2
Percentage of biofilm inhibition and biofilm eradication of extract, fractions and compounds from the leaves of *Cassia alata*.

Samples	Biofilm formation inhibition (%)				Biofilm eradication (%)			
	Candida albicans	*Candida parapsilosis*	*Candida tropicalis*	*Cryptococcus neoformans*	*Candida albicans*	*Candida parapsilosis*	*Candida tropicalis*	*Cryptococcus neoformans*
CAExt	75.38±7.54	54.05±3,43	53.22±3.45	36.43±1.32	64.25±3.43	62.11±5.66	23.21±2.23	35.42±3.44
CAH	28.54±2.54	19.34±1.45	22.55±2.22	15.54±2.96	9.42±1.76	−4.66±0.54	13.09±1.22	−7.14±0.57
CAE	79.30±5.98	60.48±4.65	58.19±3.65	41.67±2.54	66.65±4.54	69.54±5.65	53.12±3.25	34.10±2.85
CAEa	10.53±1.76	18.30±2.86	20.50±1.76	22.67±0.33	−13.98±0.22	15.12±1.34	−9.43±1.05	−21.33±2.21
CAEb	72.32±8.09	68.43±3.76	53.16±3.98	48.73±4.09	61.46±5.21	58.89±3.33	43.34±3.21	40.16±4.94
CAEc	78.84±7.88	60.04±2.22	57.09±2.17	41.27±2.76	58.93±2.87	50.12±3.25	38.41±2.04	44.07±3.29
CAEd	18.29±2.76	10.88±2.98	14.15±2.87	27.35±1.25	9.24±0.55	15.90±0.45	12.48±1.05	14.32±1.73
CAEe	19.51±2.12	28.56±1.43	14.50±1.30	19.74±1.04	2.45±0.08	8.54±1.23	20.36±2.43	22.66±2.54
(1) & (2)	28.55±3,87	16.18±1.33	24.43±1.23	15.57±2.76	15.48±1.15	24.34±1.27	17.32±0.78	−8.56±0.34
(3)	44.34±4.98	43.09±2.54	48.78±2.55	49.20±2.55	30.21±2.10	36.90±2.75	20.56±1.03	−11.32±1.92
(4)	77.03±5.87	69.55±5.65	74.08±4.67	58.57±2.76	54.55±2.74	59.45±3.59	48.19±2.49	45.54±2.19
(5)	85.43±5.65	73.44±3.87	68.23±5.23	59.78±3.54	62.05±4.16	52.72±2.21	58.42±5.72	44.06±3.06
(6)	23.26±1.54	12.21±0.55	22.52±1.34	9.32±0.65	12.43±0.75	14.25±1.08	12.43±1.03	24.15±3.82
AmB	82.43±6.54	91.78±6.21	80.49±5.05	90.51±8.77	62.27±6.30	74.25±5.43	75.76±5.55	74.89±8.53

Samples were tested at their MIC concentration or at 1024 μg/mL for extracts and fractions and at 256 μg/mL for compounds that did not show MIC values. CA Ext: crude extract; CAH: hexane fraction; CAE: ethyl acetate fraction; CAEa, CAEb, CAEc, CAEd, CAEe: subfractions from the ethyl acetate fraction; (1&2)-(6): isolated compounds, AmB: amphotericin B.

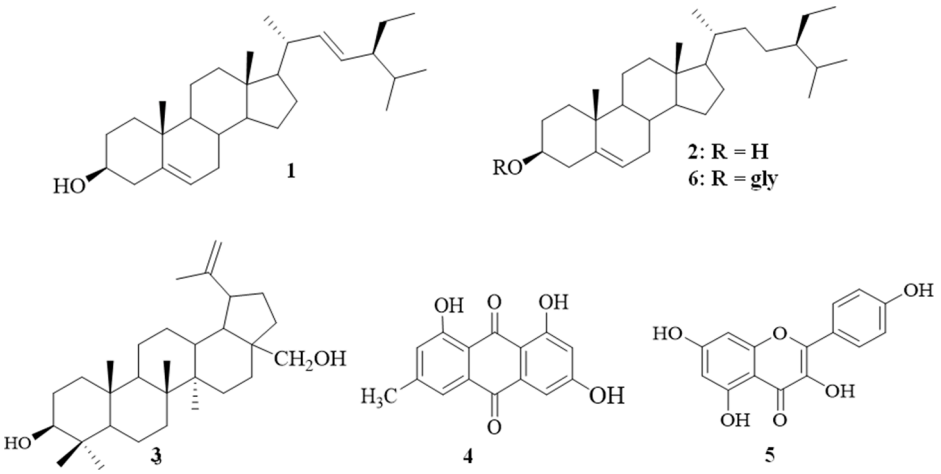

Fig. 1. Chemical structure of isolated compounds: stigmasterol **(1)** and β-sitosterol **(2)**, lupeol **(3)**, emodin **(4)**, kaempferol **(5)**, and sitosterol glycoside **(6)**.

3.2. Compound isolation and structural elucidation

The structures of the isolated compounds isolated from the ethyl acetate fraction were elucidated based on the spectroscopic data (ESI-MS, ^{1}H, ^{13}C, HSQC, and HMBC) and by comparison of the data with those reported in the literature. The present work permits us to afford six known compounds (Fig. 1) by comparison with the reported data; the known compounds were identified as a mixture of stigmasterol **(1)** and β-sitosterol **(2)** (De Nkainsa et al., 2020), lupeol **(3)** (Castilho and Kaplan, 2008), emodin **(4)** (Santos et al., 2008), kaempferol **(5)** (Tram et al., 2017), and sitosterol glycoside **(6)** (De Nkainsa et al., 2020).

3.3. Antibiofilm activity of subfractions and isolated compounds

Table 2 also provides the results of the percentage of biofilm inhibition and biofilm eradication of the subfractions derived from the ethyl acetate fraction. Subfractions show varying biofilm inhibition and eradication activities. The purification of these subfractions led to the identification of the six previously mentioned compounds **(1&2)-(6)**. The isolated compounds show biofilm inhibition and eradication activities to various extents. The biological activities of secondary metabolites from *Cassia alata* are well documented (Fatmawati et al., 2020). Compounds **(4)** and **(5)** demonstrated the highest biofilm inhibition and eradication with more than 50 % inhibition against almost all the tested fungal pathogens. The biofilm inhibition percentage ranged from 59.78 % to 85.43 % and from 58.57 % to 77.03 %, respectively. Compound **(5)** was the most potent against the biofilm of *Candida albicans,* with 85.43 % biofilm inhibition and 62.05 % biofilm eradication.

All samples that showed a percentage of biofilm inhibition or eradication >50 % were further investigated in a dose–response experiment to determine the minimum concentration inhibiting 50 % of the biofilm formation ($MBIC_{50}$) and the minimum concentration disrupting 50 % of the preformed biofilm ($MBEC_{50}$). As shown in Table 3, the extract, selected fractions, and compounds showed $MBIC_{50}$ values ranging from 19.43 to 64.07 μg/mL against *C. albicans*, from 31.86 to 182.76 μg/mL against *C. parapsilosis* and from 32.18 to 125.45 μg/mL against *C. tropicalis*. The $MBEC_{50}$ values ranged from 23.81 to 193.23 μg/mL against *Candida* species. *Cassia alata* extracts have been reported to inhibit bacterial biofilm formation in *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (Saito et al., 2012), but studies reporting the antibiofilm activity of *Cassia* species are very scarce. Yeast biofilms pose a significant challenge in clinical settings (Cavalheiro and Teixeira, 2018); therefore, the antibiofilm results reported here constitute great promise for the discovery of novel antifungal agents targeting biofilm formation.

Isolated compounds **(4)** and **(5)** exhibit the most potent biofilm inhibitory and eradicating activity amongst the tested samples. Both compounds demonstrated significantly lower $MBIC_{50}$ values of 19.43 μg/mL and 22.66 μg/mL and $MBEC_{50}$ values of 23.81 μg/mL and 29.65, respectively, against *C. albicans*. The results obtained with compound **(4)** (emodin) are not surprising since emodin is a natural anthraquinone derivative with a wide range of pharmacological activities (Semwal et al., 2021). Although this study is the first report on the antibiofilm potential of compound **4** against yeast pathogens, it was previously found to have multiple pathways of antibiofilm action against *Staphylococcus aureus* biofilm, such as interaction with the initial adhesion stage of biofilm development and extracellular protein production (Đukanović et al., 2022; Xiang et al., 2017). Compound **(5)** (kaempferol) is a flavonoid that is a group of plant-derived compounds exhibiting a large number of health-related effects (Kim and Park, 2020). It was previously reported to inhibit the primary attachment phase of biofilm formation of *Staphylococcus aureus* (Ming et al., 2017) and affect the biomass as well as the biofilm structure of the *C. parapsilosis* complex (Rocha et al., 2019).

Overall, the results indicate that the methanol extract of *Cassia alata* leaves exhibited promising biofilm inhibitory activity against *Candida* species yeast pathogens. Additionally, two isolated compounds **(4)** and **(5)** also showed potent biofilm inhibitory and eradicating activities. However, amphotericin B remains the most effective in terms of biofilm inhibition and eradication, as it demonstrates the respective lowest $MBIC_{50}$ and $MBEC_{50}$ values of 0.88 μg/mL and 0.87 μg/mL against *C. parapsilosis.* It is worth noting that none of the tested samples showed activity against the preformed biofilm of *C. neoformans*. This result could be supported by previous findings that preventing biofilm formation usually requires less effort than inhibition and eradication of the biofilm (Yin et al., 2021). Moreover, biofilm formation by *C. neoformans* is an extremely complex and intricate process, probably due to the presence of a polysaccharide capsule, which is essential for biofilm formation and pathogenesis (Aslanyan et al., 2017).

Table 3
$MBIC_{50}$ and $MBEC_{50}$ values (μg/mL) of the most active samples from the leaves of *Cassia alata* against yeast pathogen strains.

Samples	$MBIC_{50}$ (μg/mL)			
	C. albicans	*C. parapsilosis*	*C. tropicalis*	*C. neoformans*
CAExt	81.45±7.21	100.36±9.33	112.02±10.62	nd
CAE	59.11±6.09	182.76±12.09	125.45±10.55	nd
CAEb	53.12±5.21	93.31±8.56	123.18±9.92	nd
CAEc	64.07±5.34	99.22±9.34	111.09±10.88	nd
(4)	19.43±2.03	31.86±3.65	40.44±3.97	107.87±8.45
(5)	22.62±2.45	43.66±5.34	32.18±2.28	104.63±9.20
AmB	1.22±0.22	0.88±0.07	1.01±0.05	3.28±1.04
	$MBEC_{50}$ (μg/mL)			
CAExt	91.52±9.25	121.76±11.32	nd	nd
CAE	88.08±9.16	193.23±15.07	109.99±8.77	nd
CAEb	84.87±7.43	102.98±10.29	nd	nd
CAEc	70.09±7.23	118.56±9.57	nd	nd
(4)	23.81±3.08	42.31±4.62	nd	nd
(5)	29.65±2.86	58.09±5.02	45.25±3.32	nd
AmB	1.95±0.15	0.87.25±0.04	1.06±0.18	5.94±0.87

nd = not determined, CA Ext: crude extract; CAE: ethyl acetate fraction; CAEb, CAEc, subfractions from the ethyl acetate fraction. AmB: amphotericin B.

3.4. Anti-quorum sensing activity of the extract and the most active isolated compounds

Quorum sensing (QS), a cell-to-cell communication system used by *Candida* species, plays a vital role in biofilm formation and virulence (Kovács and Majoros, 2020). Inhibition of QS is therefore being considered as a new target for antimicrobial therapy. Therefore, investigating the impact of *Cassia alata* extracts and bioactive constituents on QS may provide valuable strategies to disrupt *Candida* biofilms and mitigate their pathogenicity. As the production of the hallmark purple pigment violacein is a well-studied trait controlled by QS in *C. violaceum*, violacein production and inhibition by *Chromobacterium violaceum* has been exploited for the detection of QS activity (McClean et al., 1997).

Table 4
Inhibition of violacein production in *Chromobacterium violaceum* by extract and the most active compounds from the leaves of *Cassia alata*.

Samples	MIC (μg/mL)	MQSIC (μg/mL)	IC_{50} (μg/mL)
CAExt	2048	1024	374.29±13.54****
(4)	512	128	28.08±3.07***
(5)	256	128	26.44±2.23***
Gentamicin	256	64	23.12±2.12****
Vanillin	512	256	54.24±4.87

MQSIC: minimum quorum sensing inhibitory concentration, MIC: minimum inhibitory concentration, IC_{50}: Cconcentration inhibiting 50 % of violacein production. Statistical analysis was performed with Sidak's multiple comparisons test using two-way ANOVA;*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

Table 4 presents the results of the inhibition of violacein production activity in *C. violaceum* by the extract and the most active compounds emodin and kaempferol. All the tested samples showed QS inhibitory activity, as indicated by the minimum quorum sensing inhibitory concentration (MQSIC) value of 1024 μg/mL for the crude extract and 128 μg/mL for compounds. The tested extract and compounds **(4)** and **(5)** also demonstrated MIC values of 2048 μg/mL, 512 μg/mL and 256 μg/mL, respectively, against *C. violaceum,* indicating the ability of the samples to inhibit violacein production without affecting the growth of the bacteria. These results are similar to those obtained by Rekha et al., who reported for the first time the anti-QS activity of *C. alata* using *C. violaceum* and *P. aeruginosa* (Rekha et al., 2017). Compounds **(4)** and **(5)** displayed significant inhibitory effects on violacein production, as indicated by their low IC_{50} values of 28.08 μg/mL and 26.44 μg/mL, respectively. Vanillin, a reference substance for QS inhibition, was included for comparison and showed higher IC_{50} values (54.24 μg/mL) than the isolated compounds. It was previously found that compound **(4)** could be used as a potential QS inhibitor for the control of biofilm formation and growth of *Pseudomonas aeruginosa* (Ding et al., 2011). This result suggests that *C. alata* extract and its active compounds could penetrate the biofilm and interfere with fungal intercellular communication, by which the QS system of the tested *Candida* species is disturbed. Inhibiting quorum sensing can be an effective strategy to disrupt fungal communication and potentially control biofilm formation.

4. Conclusion

Using a bioassay-guided fractionation approach, two bioactive antibiofilm compounds were isolated from the methanol extract of the leaves of *C. alata*. The isolated compounds exhibited promising biofilm inhibitory and eradicating activities against *Candida* species pathogens, with the anti-quorum sensing ability reflected as the potential to interfere with violacein production in *C. violaceum*. These findings not only support the traditional use of *C. alata* in the treatment of fungal infections but also reveal *C. alata* extract, as well as emodin and kaempferol, as potential sources for developing alternative antibiofilm agents against biofilm-associated yeast infections.

CRediT authorship contribution statement

Jean Paul Dzoyem: Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simplice Chimi Fotso:** Writing – original draft, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Jean Duplex Wansi:** Writing – review & editing. **Bellier Tabenkoueng:** Writing – review & editing, Validation, Formal analysis, Data curation. **Willifred Dongmo Tekapi Tsopgni:** Writing – review & editing, Validation, Investigation, Formal analysis, Data curation. **Flavien Aristide Alfred Toze:** Writing – review & editing, Validation, Formal analysis, Data curation, Conceptualization. **Lyndy Joy McGaw:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jean Paul Dzoyem reports financial support was provided by German Academic Exchange Service. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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