

Polyphenolic Characterization of Aqueous Extract and Comparative Antioxidant and Antibiofilm activities of *Xylopia aethiopica* Fruit Aqueous and Essential Oil Extracts against *Candida* Biofilms

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ABSTRACT

Introduction: The increasing resistance of *Candida* species to conventional antifungals necessitates the search for new bioactive compounds. *Xylopia aethiopica* is a medicinal plant known for its antifungal properties, yet its impact on fungal biofilms remains to be explored. This study characterized the polyphenolic profile of *Xylopia aethiopica* hydroethanolic fruit extract and compared the antibiofilm activity of its aqueous extracts and essential oils against *Candida* biofilms. **Methodes:** Total phenols and flavonoids were quantified, and individual compounds were identified via HPLC-DAD. The antioxidant capacity was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl) test. The antibiofilm potential was evaluated using the crystal violet assay to measure biomass reduction and assess the impact on the yeast-to-hypha transition. **Results:** Analyses revealed a rich profile characterized by high yields of epicatechin, phenolic acids, rosmarinic acid, and resveratrol, which impart significant antioxidant properties. *In vitro* assays demonstrated that among the aqueous extracts, only the hydromethanolic extract (1:2) inhibited biofilms. Conversely, the essential oils exhibited strong dose-dependent efficacy, with hydrodistillation proving superior to steam distillation in restricting the biofilm growth of *C. albicans* and *C. parapsilosis*. **Conclusion:** These findings suggest that *Xylopia aethiopica* is a potent source of antibiofilm agents. The essential oil obtained by hydrodistillation represents a promising alternative for eradicating resistant *Candida* communities, highlighting the crucial impact of the extraction method on bioactivity.

Keywords: HPLC-DAD, individual polyphenols, antibiofilm, crystal violet, *Xylopia aethiopica*.

INTRODUCTION

The significance of medicinal plants has been well-established for a long time. Concurrently, the rise of antibiotic resistance has intensified the focus on discovering alternative therapies and synergistic compounds¹. Aromatic and medicinal plants represent an inexhaustible source of bioactive molecules¹. Among them, polyphenols hold a critical role due to their recognized antioxidant and antimicrobial properties². These secondary metabolites, including flavonoids, are widely studied for their ability to interact with biological structures and inhibit the growth of various pathogens³. Fungal infections, particularly those caused by yeasts of the *Candida* genus, represent a major public health challenge⁴. Although *Candida albicans*, for instance, is a natural commensal of human mucous membranes, it can become a formidable opportunistic pathogen in immunocompromised subjects⁵. One of its primary virulence factors lies in its ability to form structured cellular communities protected by an extracellular matrix⁵, which significantly increases their tolerance to conventional antifungals such as amphotericin B, azoles, echinocandins, and miconazole⁶. In response to the emergence of multi-drug-resistant strains, exploring the chemical reservoir of aromatic plants offers promising

perspectives⁷. Certain secondary metabolites, such as apigenin, Licochalcone A, or glabridin, have already demonstrated the ability to inhibit biofilm formation and the yeast-to-hyphae morphological transition of *Candida*⁸. Microbial contamination of surfaces, facilities, and equipment in the food and hospital sectors represents a significant public health issue⁹. Biofilms are communities of bacteria, fungi, algae, nor protozoa. They typically attach to surfaces and are maintained by the secretion of a protective adhesive matrix, forming a living, dynamic structure that undergoes continuous reorganization. They are ubiquitous in all environments, associated with mineral, aquatic, technological, plant (foliar surfaces), or animal surfaces¹⁰. Fungal biofilms are pathogenic and nosocomial in nature; they can generally cause invasive infections in patients with implanted medical devices (such as catheters or urinary stents), those receiving antibiotic therapy, or those who are immunocompromised. Furthermore, in cases of immune system impairment or underlying pulmonary pathology, they can lead to severe conditions, some of which may be fatal¹¹. Recent studies have demonstrated the antifungal and antibacterial significance of tannins, xylopic acid¹² and β -pinene¹³ present in *Xylopia aethiopica* aqueous and oily extracts. Building upon these findings, this study aims a twofold objective. First, it established a rigorous polyphenolic profile of *Xylopia aethiopica*

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aqueous extracts, an aromatic and medicinal plant of the Annonaceae family¹⁴, by quantifying total phenols and flavonoids and identifying individual polyphenols via HPLC. Second, it evaluated and compared the antioxidant and antibiofilm activity of the plant's aqueous and oily fractions against *Candida* biofilms using a crystal violet colorimetric microtiter plate assay.

MATERIAL AND METHODS

Study setting

Phytochemical and microbiological assays were conducted at the Interdisciplinary Research Platform of King Michael I University of Life Sciences, Timisoara, Romania.

Plant material

The fruits of *Xylopia aethiopica* were harvested in December 2024 in Djokoto, a township within the Kloto 2 commune, located in the Plateaus Region of Togo. This township is situated 120 km northwest of Lomé. The fruits were dried and stored at room temperature under standard cultivation conditions. The coordinates for the cultivation and harvesting site are: Latitude: 7.008135°; Longitude: 0.62979°; Accuracy: 1.8 m. A voucher specimen was identified at the Laboratory of Biology and Plant Ecology of the University of Lomé under No. TOGO15895. To obtain a powder from the dried *Xylopia aethiopica* pods, approximately 2.5 kg of the material was pulverized at the Center for Research and Training on Medicinal Plants (CERFOPLAM) of the University of Lomé.

Biological material

Strain suspensions: ATCC (American Type Culture Collection) (*Candida albicans* (ATCC 10231) and *Candida parapsilopsis* (ATCC 22019). BHI (Brain Heart Infusion) broth. BHI and ATCC strains were used.

Chemicals

Ethanol (96%), methanol (96%), NaCl solution (0.5 µg/mL), Miconazole at 0.5 µg/mL, 0.1% crystal violet solution, saline solution (9% NaCl), DPPH solution.

Extraction materials and equipment

Beakers, plastic containers, graduated cylinders, Erlenmeyer flasks, pipettes, Clevenger-type apparatus (Witeg, Germany), cotton, filter paper, Soxhlet extractor, precision balance (Kern & Sohn, D-72336 Balingen, Germany), UV-VIS spectrophotometer (Specord 205; Analytik Jena AG, Jena, Germany), Shimadzu chromatograph (Model 2010 EV, Kyoto, Japan), ELISA microplate reader (BIORAD PR 1100, Hercules, CA, USA), 96-well microdilution plates, G1 Heidolph semi-automatic rotary evaporator (Germany).

Preparation of hydroalcoholic extracts

Hydroalcoholic maceration was carried out according to the Floares et al.¹⁵ protocol.

Extraction 1:10

A maceration of 2.5 g of dry *X. aethiopica* pod powder was carried out for 48 hours in 70% ethanol and 70% methanol, respectively, at a 1:10 (w/v) ratio. During this maceration period, the mixtures were intermittently agitated. Each macerate was filtered first through hydrophilic cotton and subsequently through Whatman filter paper. Filtrate evaporation was performed using a semi-automatic rotary evaporator set at a temperature of 45 °C. The resulting filtrate was stored at 4°C for further testing. Each chocolate-brown dry extract obtained represented a yield of 1.00±0.02%. (Triplicate extraction).

Extraction 1:2

Twenty-five grams (25 g) of dry *X. aethiopica* pod powder were macerated for 72 hours under intermittent agitation in hydroethanolic and hydromethanolic solvents, respectively. Each solvent was applied at a 1:1 (w/v) ratio. Each macerate was filtered first through hydrophilic cotton and subsequently through Whatman filter paper. Filtrate evaporation was performed using a semi-automatic rotary evaporator set at a temperature of 45 °C. The resulting filtrate was stored at 4°C for further testing. Each chocolate-brown dry extract obtained represented a yield of 1.5±0.01%. (Triplicate extraction).

Essential Oil Extracts

The essential oil was extracted using steam distillation with a Clevenger-type apparatus and hydrodistillation method¹⁶.

The essential oil was extracted using a Clevenger apparatus employing two methods: hydrodistillation and steam distillation. For hydrodistillation, 30 g of plant material was placed in a distillation flask containing 1000 mL of distilled water. The mixture was then boiled for 4 hours. During this process, the vapors produced by boiling were condensed, resulting in two phases; the upper phase contained the essential oil. For steam distillation, 300g of plant material was placed above water, which was heated to produce steam that rose through the material. The steam from boiling water passes through the plant, capturing volatile compounds. This steam, along with the extracted compounds, moves into a condenser where it cools and turns back into liquid (a mixture of essential oil and water). The fluid then flows into a separatory funnel, allowing the essential oil—usually less dense than water—to separate and float on top of the hydrosol (aromatic water). The essential oil was collected, transferred into colored glass vials, and stored at 4°C. The plant oil yields (w/v) were: 2.07 ± 0.07% for hydrodistillation and 0.17 ± 0.01% for steam distillation. (Triplicate extraction).

Determination of Total Polyphenols Content (TPC)

Total polyphenol content (TPC) was determined using the Folin-Ciocalteu method, as described and slightly modified by Floares et al.¹⁵. Each aqueous sample was analyzed in triplicate, and the results were reported as the mean ± SEM in mg GAE/ kg.

Determination of Total Flavonoid Content (TFC)

Following the method described by Floares et al.¹⁵ in their study. The experiment was performed in triplicate, and results were expressed as mean ± SEM, in mg quercetin equivalent (QE) per kg.

Determination of individual polyphenols by HPLC-DAD

Identification and quantification of Polyphenolic Compounds present in ethanolic (1:10) extract of *Xylopia aethiopica* was performed according to the method described by Veloz et al.¹⁷.

Determination of individual polyphenols by LC-MS analysis was performed using a Shimadzu chromatograph. Chromatographic conditions included mobile phases A—acidified water with formic acid (pH 3) (Merck KGaA, Darmstadt, Germany), and B—acetonitrile (Merck KGaA, Darmstadt, Germany) acidified with formic acid (pH 3). The gradient programme consisted of 0.01–20 min 5% B, 20.01–50 min 5–40% B, 40–55 min 40–95% B, and 55–60 min 95% B. The flow rate of solvent is 0.2 mL/min at a temperature of 20 °C. The wavelengths selected for monitoring were 280 nm and 320 nm. The calibration curves were made between 20 and 50 µg/mL. Calibration curves for each analysed compounds are as follows: $f(x) = 6.04661e - 105x - 3.04827$ (gallic acid), $f(x) = 7.36054e - 106x + 3.55894$ (caffeic acid), $f(x) = 3.43873e - 105x + 4.99711$ (epicatechin), $f(x) = 1.45563e - 105x + 3.75512$ (Beta - rezorcilic acid), $f(x) = 4.34018e - 106x + 3.48454$

(cu-maric acid), $f(x) = 8.1887e - 106x + 3.79724$ (ferulic acid), $f(x) = 2.21544e - 105x + 1.3627$ (rosmarinic acid), $f(x) = 4.37174e - 105x + 1.3537$ (resveratrol), and $f(x) = 1.3719e - 105x + 3.97487$ (quercetin). Analytical-grade chemicals were used for all reagents and sol-vents.

Comparative Antioxidant capacity by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Assay

The antioxidant capacity of the essential oils and aqueous extracts was performed according to the method described by Floares et al.¹⁵ (Figure 1).

All samples were tested in triplicate, and the average value was used for analysis. The antioxidant activity was reported as the percentage of radical scavenging activity (RSA), calculated using the following formula:

$$RSA (\%) = \left(\frac{A_c - A_s}{A_c} \right) \times 100 \quad (1)$$

Where:

Ac = absorbance of the control sample

As = absorbance of the extract sample

The IC₅₀, which indicates the concentration required for each extract to achieve 50% inhibition of DPPH, was calculated and expressed in mg/mL.

Antibiofilm assessment

The antibiofilm activity was evaluated in the broth by the microdilution method against different ATCC strains. Strain suspensions and BHI were used as a positive control and Miconazole (0.4 µg/mL) as a negative control. For the values obtained after the reading, the formulas for the percentage of inhibition of the mycelial biofilm (PIBB%) and the percentage of growth of the mycelial biofilm (PCBB%) were used, percentages calculated using the formulas (2), (3):

$$PCBB\% = \left[\frac{OD_{\text{sample}}}{OD_{\text{negative control}}} \right] \times 100 \quad (2)$$

$$PIBB\% = 100 - PCBB\% \quad (3)$$

Where:

DO_{sample}, represents the optical density of the samples and contains a certain concentration of the sample that has been tested, as the average value of the tripled readings at 540 nm. OD_{negative control}, represents the optical density at 540 nm as the average value of triplicate readings for the selected strains selected in BHI⁴.

Statistical analysis

Statistical analyses were performed using the computational software package Prism 6 (Graph Pad Software Inc., San Diego, USA). Polyphenol profile, antioxidant and antibiofilm assays were analyzed using one-way ANOVA; data were represented as Mean ± SEM followed by Tukey's multiple comparisons.

RESULTS AND DISCUSSION

Total Polyphenols Content (TPC) and Total Flavonoids Content (TFC)

Hydroethanolic sample (1:10) exhibited the highest polyphenol concentration at 7364.35 ± 3.31 mg GAE/kg (Figure 2X). Hydroethanolic sample (1:2) exhibited the highest flavonoid concentration at 1522.42 ± 15.97 mg QE/kg (Figure 2Y). The differences in content were highly significant ($p < 0.0001$). Interestingly, hydromethanolic sample (1:2) with the lowest total polyphenol content exhibited the highest flavonoid concentration, suggesting that flavonoids constitute the bulk of its phenolic profile. These results confirm that *Xylopia aethiopica* extracts have a high polyphenol content, particularly flavonoids. Samba et al.¹⁴, identified over 49 compounds. Monoterpenes¹⁹, phenylpropenes²⁰, kauranes²¹, and terpenes²² also were identified.

The observed variations in polyphenol content across the four studied hydroalcoholic extracts indicate that the chemical composition of *Xylopia aethiopica* extracts is strongly dependent on solvent polarity and dilution ratio. This finding is consistent with results obtained by¹⁵. Indeed, the water-ethanol mixture is a widely used solvent in pharmacology due to its low toxicity and high efficiency in extracting a broad range of polar compounds²¹. Conversely, the water-methanol mixture is often considered more effective for the extraction of low-molecular-weight polyphenols¹⁵. Simultaneously, the dilution ratio also influences the chemical composition of an extract. A higher solvent-to-sample ratio generally enables a more efficient extraction of secondary metabolites¹⁵. The hydroethanolic sample (1:10) showed the highest Total Polyphenol Content (TPC). Diluted ethanol appears to be an ideal solvent to solubilize the diverse phenolic compounds present in the dry matter of *Xylopia aethiopica*. However, the hydromethanolic sample (1:10) contained a higher Total Flavonoid Content (TFC). Methanol likely offers greater selectivity in extracting specific flavonoids from *Xylopia aethiopica*. Finally, the hydromethanolic sample (1:2) exhibited a low polyphenol profile, suggesting it was either saturated or resulted in incomplete extraction.

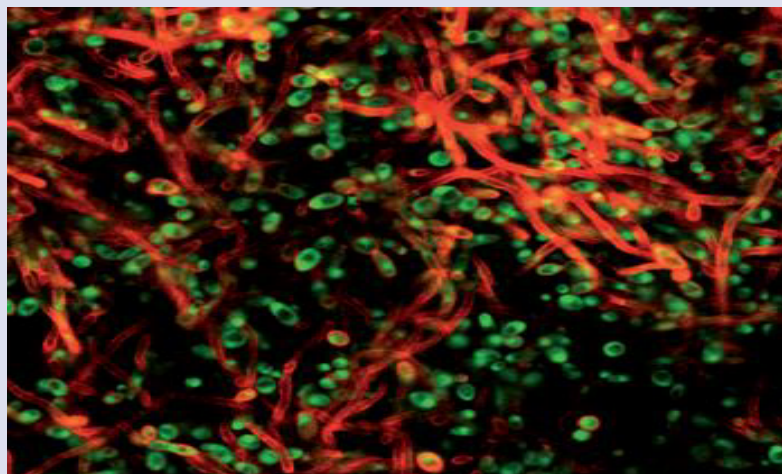


Figure 1. Confocal laser scanning microscopy (CLSM) image of a *Candida albicans* biofilm¹⁸ (Lebeaux et al., 2012)

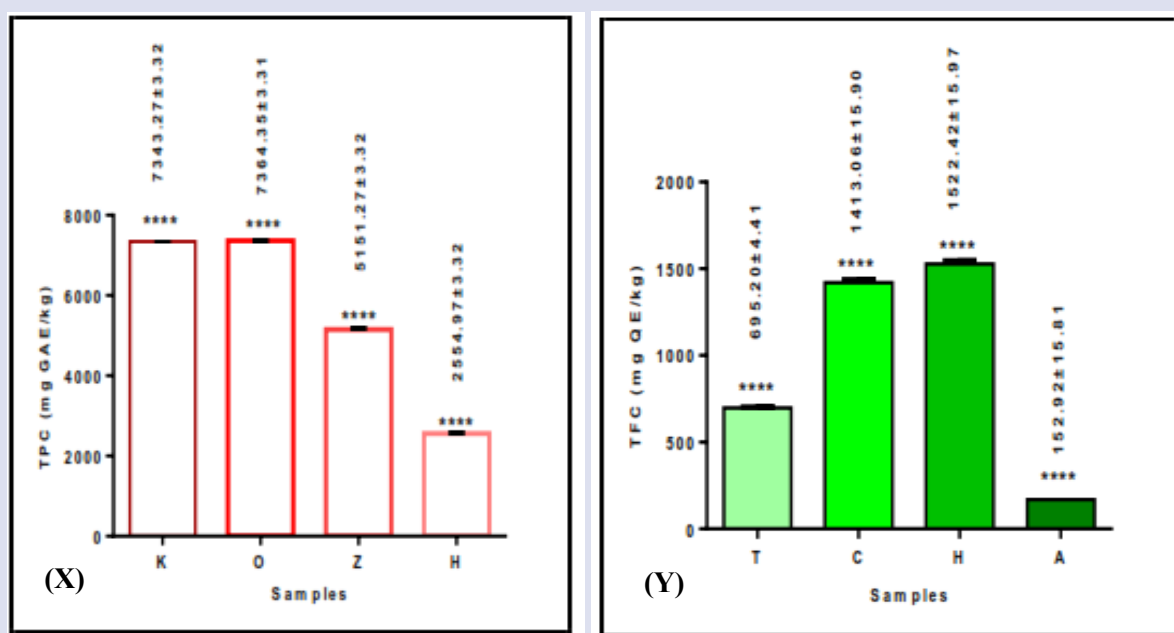


Figure 2. Polyphenols Profile of Hydroalcoholic extracts: (X) Total Polyphenols Content (TPC), (Y) Total Flavonoids Content (TFC). Data are expressed as mean ± SEM (n = 3). Significant differences are indicated by asterisks (****): (p < 0.0001). The Tukey HSD post-hoc analysis established a strict and statistically significant hierarchical order among the evaluated specimens: O > K > Z > H (X); H > C > T > A (Y)

TPC is expressed in mg Gallic acid equivalent/g of extract (mg/GAE/g). TFC is expressed in mg Quercetin equivalent/g of extract (mg/QE/g). K/T. hydroethanolic extract (1:10); O/C. hydromethanolic extract (1:10); Z/H. hydroethanolic extract (1:2); H/A. hydromethanolic extract (1:2).

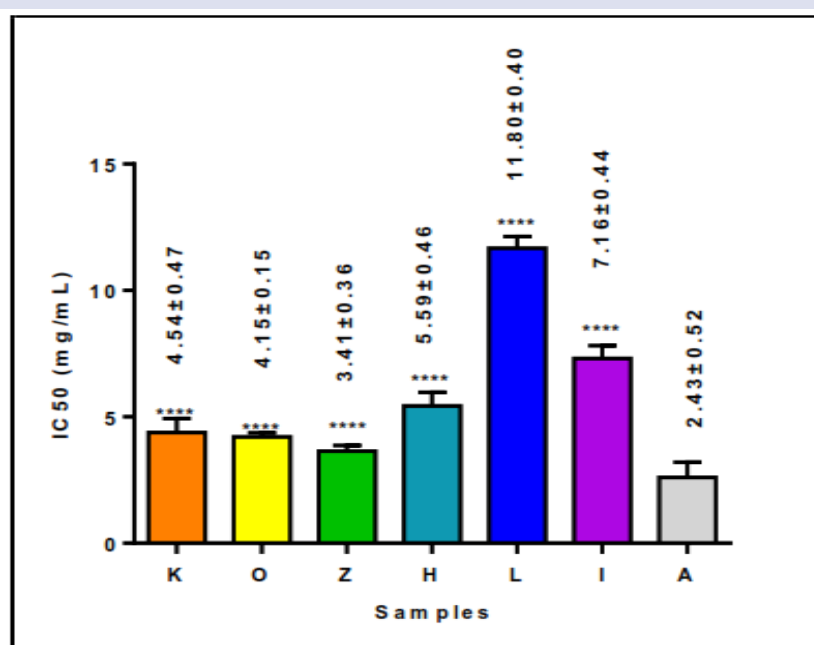


Figure 3. In vitro radical scavenging efficiency (IC₅₀) of X. aethiopica fruits extracts (K, O, Z, H, L, I) compared to Ascorbic acid (A). Data are expressed as mean ± SEM (n = 3). Significant differences are indicated by asterisks (****): (p < 0.0001), compared to Ascorbic acid (A). All remaining extracts (K, O, Z, H, L, and I) exhibited a significantly higher (IC₅₀) compared to A (p < 0.0001).

The established hierarchy of antioxidant potency is ranked as follows: A > Z > O > K > H > I > L.

K. hydroethanolic extract (1:10); O. hydromethanolic extract (1:10); Z. hydroethanolic extract (1:2); H. hydromethanolic extract (1:2); L. essential oil obtained by hydrodistillation; I = essential oil obtained by steam distillation; A. Ascorbic acid (control).

Comparative Antioxidant Capacity

DPPH (2,2-diphenyl-1-picrylhydrazyl) test for antioxidant capacity is a commonly used classical method for different types of extracts¹⁶. The antioxidant capacity of the extracts was expressed as IC₅₀ value and compared with that of ascorbic acid (Figure 3). All species showed antioxidant activities represented by the inhibition of the DPPH radical. Among the six (6) extracts, hydroethanolic extract (1:2) exhibited the most promising biological efficacy (IC₅₀ = 3.41 ± 0.36 mg/mL), closely followed by hydromethanolic extract (1:10) and hydroethanolic extract (1:10) (Figure 3). This pharmacological performance strongly implies a rich presence or a positive synergistic interaction of active principles, such as highly hydroxylated polyphenols capable of neutralizing reactive oxygen species (ROS). Conversely, extract L displayed poor radical affinity or a lack of antioxidant biomolecules, requiring a nearly five-fold higher concentration (IC₅₀ = 11.80 ± 0.40

mg/mL) to achieve the same 50% inhibitory therapeutic endpoint. Biopharmaceutically, hydroethanolic extract (1:2), hydromethanolic (1:10) and hydroethanolic extract (1:10) extracts emerge as prime candidates for the isolation of bioactive lead compounds targeting oxidative stress-induced cytoprotecting. In this study, the antioxidant activity of the essential oils is less effective compared to the aqueous extracts. This finding is highly significant, as the traditional method of utilizing *Xylopia aethiopica* involves maceration²³, which reduces extraction logistics costs. The polyphenolic profile and antioxidant activity of aqueous extracts and essential oils from *Xylopia aethiopica* suggest potential individual polyphenols and biological activities, including anti-candidal and antibiofilm properties. Thus, polyphenols, due to their antioxidant properties and the ability to quench free radicals and reactive oxygen species, exert a beneficial effect on human health, and they are believed to slow down the aging process as well as to be useful in the prevention of the development of many diseases²⁴.

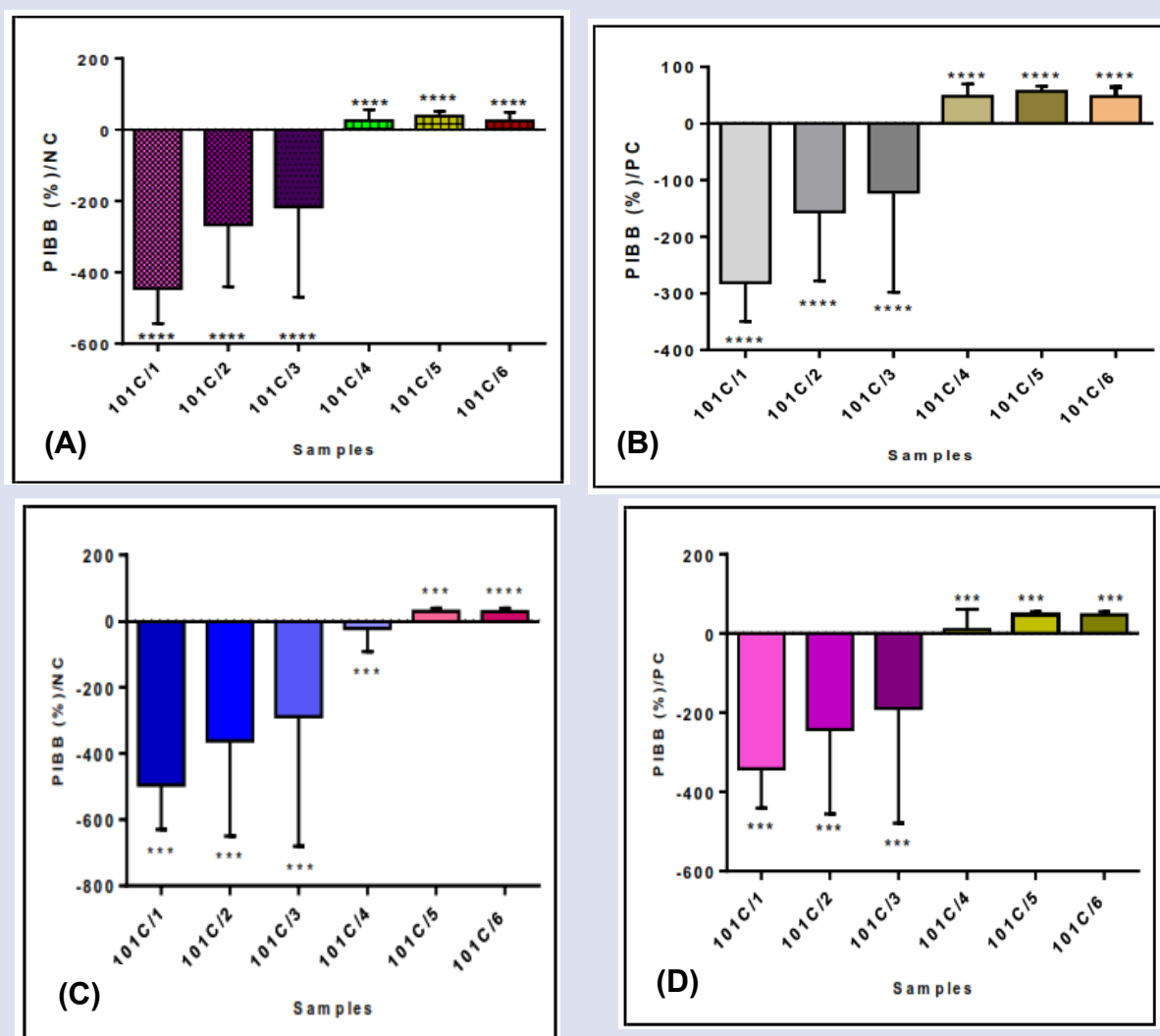


Figure 4. Percentage of Inhibition of Biofilm Biomass (PIBB%/NC) relative to the Negative Control (NC) (A and C) and relative to the Positive Control (PIBB%/PCPC) (B and D) of *X. aethiopica* fruits extracts (101C/1 to 101C/6). Data are expressed as mean ± SEM (n = 3). Significant differences are indicated by asterisks [****, $p < 0.0001$ (A and B); ***, $p < 0.002$ (C and D)].

(A and B): against *C. albicans*. (C and D): against *C. parapsilopsis*. 101C/1: hydroethanolic extract (1:10); 101C/2: hydromethanolic extract (1:10); 101C/3: hydroethanolic extract (1:2); 101C/4: hydromethanolic extract (1:2); 101C/5: essential oil obtained by hydrodistillation; 101C/6: essential oil obtained by steam distillation. Tukey's post-hoc test revealed no statistically significant difference ($p > 0.05$) among the positive inhibition percentages of samples 101C/4, 101C/5, and 101C/6, confirming their effects are statistically equivalent.

In vitro Comparative Antibiofilm Activity and Species Sensitivity

Inhibition of biofilm formation against *Candida* strains

Percentage of Inhibition of Biofilm Biomass relative to the Negative Control (PIBB%/NC) and relative to the Positive Control (PIBB%/PC).

A One-way ANOVA followed by Tukey's multiple comparison test was conducted to evaluate the PIBB/NC and PIBB/PC against both investigated *Candida* species (*C. albicans* and *C. parapsilopsis*) ($p < 0.05$) (Figure 4).

The highly diluted extracts: the hydroethanolic (1:10, 101C/1), the hydromethanolic (1:10, 101C/2), and the hydroethanolic (1:2, 101C/3), consistently exhibited strongly negative Percentage of Inhibition of Biofilm Biomass (PIBB%) values, ranging from (-100%) to nearly (-500%). From a pharmacological standpoint, these negative values indicate a potent induction of biofilm proliferation rather than inhibition. This phenomenon suggests that sub-inhibitory or highly diluted plant extracts might unintentionally provide metabolic substrates (such as residual carbohydrates or minerals) or trigger stress-induced defensive matrix hyper-production.

Conversely, increasing the concentration of the hydromethanolic extract to a 1:2 ratio (101C/4) completely inverted the biological response, yielding true biofilm inhibition. This extract, along with the two volatile fractions the essential oils obtained by hydrodistillation (101C/5) and steam distillation (101C/6) demonstrated positive PIBB% values, establishing them as effective antibiofilm agents.

The findings reveal a distinct heterogeneity in biological activity, depending on both the nature of the extraction matrix and the extraction method employed. Thus, polyphenol and flavonoid contents, as well as the antioxidant activity, of extracts obtained via ultrasound-assisted extraction were superior to those obtained by microwave-assisted and conventional extraction methods¹⁵. Against *C. albicans* (Figures 4A and 4B), both essential oils (101C/5 and 101C/6) achieved robust and highly uniform biofilm eradication, supported by an extremely high level of statistical significance (****, $p < 0.0001$). When tested against *C. parapsilopsis* (Figures 4C and 4D), however, their inhibitory capacity was visually attenuated, and the statistical significance decreased ($p < 0.002$). This indicates a more resilient biofilm architecture in *C. parapsilopsis*. Hydromethanolic extract (1:2, 101C/4) successfully disrupted *C. albicans* biofilms relative to both controls, its efficacy collapsed against *C. parapsilopsis*. In Figure 4C, the PIBB% for 101C/4 approaches the baseline and lacks statistical significance, highlighting a distinct resistance profile unique to *C. parapsilopsis*. The chemical composition of the essential oil extracted from *Xylopia aethiopica* fruits is highly diversified, with a predominance of β -pinene, terpinen-4-ol, 1,8-cineole, isopropylbenzyl alcohol, 3-carene, and santalol²⁵. These volatile compounds likely account for its potent antibiofilm efficacy. Thus, the volatile lipophilic fractions (101C/5 and 101C/6) represent the most promising candidates for anti-candidal biofilm strategies, likely due to the ability of hydrophobic terpenoids and phenolic constituents to destabilize the extracellular polymeric substance (EPS) matrix.

Minimum biofilm inhibitory concentration (MBIC)

The hydroethanolic (1:10, 101C/1), the hydromethanolic (1:10, 101C/2), and the hydroethanolic (1:2, 101C/3) showed no antibiofilm activity. In contrast, hydromethanolic extract (1:2, 101C/4) and both essential oils (101C/5 and 101C/6) demonstrated significant antibiofilm activity compared to the inactive groups (****, $p < 0.0001$) (Figure 5). Tukey's post-hoc test revealed no statistically significant difference ($p > 0.05$) among the three active samples (101C/4, 101C/5, and 101C/6), as their MBIC values are statistically equivalent. The MBIC corresponds to the

concentration at which the biofilm percentage inhibition (%BPI) is 100% or the biofilm percentage growth (%BGI) is 0%⁴. Quantitatively, no strict biofilm Minimum Inhibitory Concentration (MBIC) could be determined within the tested concentration ranges, as the percentage of biofilm inhibition (PIBB%) remained below 100%. *Candida* biofilms are well-known for their extreme phenotypic tolerance to antimicrobial agents, which is frequently 10 to 1.000 times higher than that of planktonic cells. To circumvent this limitation, combination therapy represents a highly promising approach. Interestingly, our previous study demonstrated a potent synergistic effect when these oily extracts were combined with miconazole²³. This strategy successfully restores antifungal efficacy at lower doses, offering a practical solution to overcome biofilm resilience without encountering solubility or toxicity constraints. These results suggest that achieving complete eradication of these biofilms would require essential oil concentrations exceeding 8 mg/mL, or a combination formulation with a reference agent (such as miconazole, used as a reference control). These findings correlate with those of Kozolina et al.²³, who demonstrated both a dose-dependent anti-*Candida* effect and synergy with miconazole. While checkerboard or time-kill assays were not performed in the present work, the synergy between the oily extract and miconazole was previously established and quantified in our earlier study²³. The current results support those findings by confirming the strong cooperative therapeutic potential of this combination.

These findings regarding the antibiofilm effects of essential oils corroborate those of by Kozolina et al.²³, Enoluomen et al.²⁶ and Folorunsho et al.²⁷ who respectively demonstrated anticandidal, antibacterial, and antifungal activities.

The stimulation of biofilm biomass (paradoxical effect) induced by the hydroethanolic and hydromethanolic extracts across the different tested concentrations may be associated with a potential hormetic effect. It is hypothesized that sub-inhibitory concentrations of the extracts might trigger fungal stress response pathways, potentially modulating biofilm-associated regulators such as BCR1 or ALS genes²⁸. However, further molecular and genetic analyses are required to validate these hypothetical mechanisms.

This study has showed that the volatile lipophilic fractions (101C/5 and 101C/6) of *X. aethiopica* represent the most promising candidates for anti-candidal biofilm strategies. Furthermore, the striking differences between extracts 101C/2 and 101C/4 underscore that a critical concentration threshold must be surpassed to overcome *Candida* resistance mechanisms and prevent accidental biofilm stimulation. Exploring metabolic viability (via XTT or CFU counting) or structural details (via SEM/CLSM) represents also an important next step.

Given that the paradoxical effect of hydroethanolic extracts is often attributed to a stress response mechanism at sub-inhibitory doses, profiling their individual polyphenols would be highly valuable. Such effects are typically triggered by phenolic compounds or residual sugars present in the crude extracts at low concentrations (in the mg/L range).

Individual polyphenols profile of hydroethanolic extract

Nine main polyphenols were targeted in the 1:10 hydroethanolic extract by the HPLC-DAD technique, as shown in Fig. 4. Six out of the nine tested polyphenols were identified. In decreasing order of concentration (mg/g), these were: epicatechin, β -resorcylic acid, rosmarinic acid, resveratrol, quercetin, and coumaric acid (Table 1). The epicatechin yield was exceptionally high; a value of 192.35 mg/g indicates that approximately 19.2% of the total dry mass of the *Xylopia aethiopica* pods consists of extractable epicatechin. These results confirm the efficacy of the ethanol solvent (1:10 ratio) combined with the dilution factor. Resorcylic acid, rosmarinic acid, and resveratrol exhibited significant yields for secondary metabolites, ranging between

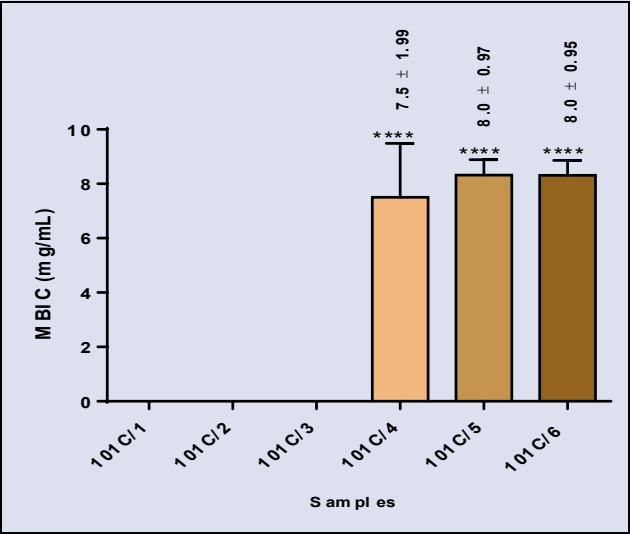


Figure 5. Minimum Biofilm Inhibitory Concentration (MBIC) of *X. aethiopica* fruits extracts (101C/1 to 101C/6), against *C. albicans* and against *C. parapsilopsis*. Data are expressed as mean ± SEM (n = 3). **** indicates a highly significant difference compared to inactive samples ((p < 0.0001), One-way ANOVA followed by Tukey's multiple comparison test)
101C/1: hydroethanolic extract (1:10); 101C/2: hydromethanolic extract (1:10); 101C/3: hydroethanolic extract (1:2); 101C/4: hydromethanolic extract (1:2); 101C/5: essential oil obtained by hydrodistillation; 101C/6: essential oil obtained by steam distillation. Tukey's post-hoc test revealed no statistically significant difference (p > 0.05) among the positive inhibition percentages of samples 101C/4, 101C/5, and 101C/6, confirming their effects are statistically equivalent.

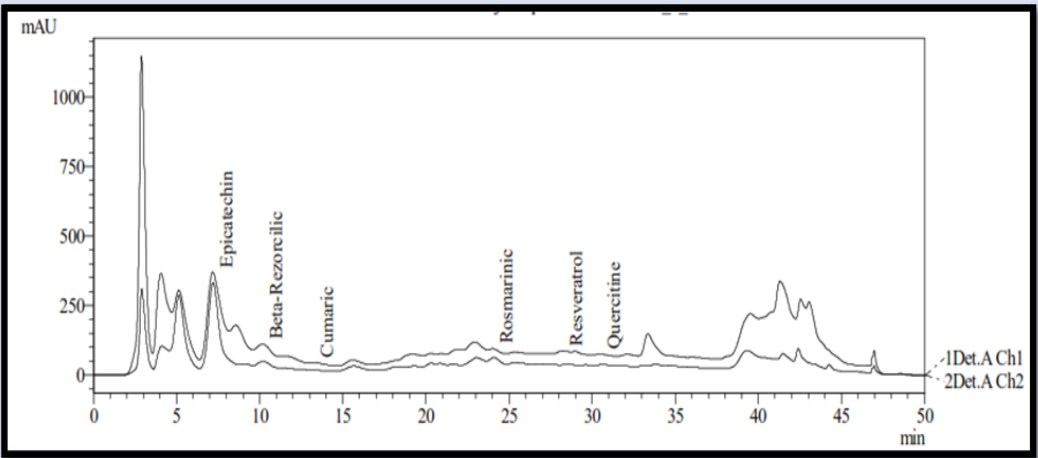


Figure 6. Chromatogram: Six compounds identified in *Xylopia aethiopica* hydroethanolic extract by HPLC-DAD.

Table 1. Quantitative results: Polyphenolics compounds quantified by HPLC-DAD

Compounds	Tr (minutes)	Area (mm²)	Concentration HPLC (µg/mL)	Yield of liquid extract (mg/g dry matter)
Galic	0.000	0	0	0.000
Epicatechin	7.168	19831746	686.957	192.348
Cafeic	0.000	0	0	0.000
Beta-Rezorcilic	10.168	2999606	47.418	13.277
Cumaric	13.205	164407	4.198	1.175
Ferulic	0.000	0	0	0.000
Rosmarinic	24.024	1334266	30.9923	8.277
Resveratrol	28.231	504991	23.431	6.5561
Quercetin	30.490	199006	6.705	1.877

6 and 13 mg/g (Table 1). HPLC-DAD analysis of the ethanolic extract from *Xylopia aethiopica* pods revealed a promising polyphenolic profile, characterized by an outstanding yield of epicatechin (192.35 mg/g) alongside phenolic acids β -resorcylic and rosmarinic acids and resveratrol.

Paradoxical effect (Hormesis) in antibiofilm activity

The paradoxical effect (hormesis) induced by sub-inhibitory doses is clearly linked to the presence of specific polyphenols acting within the targeted concentration range (mg/L or $\mu\text{g/mL}$) (Table 1). Epicatechin orchestrates this profile due to its very high concentration, constituting the primary active matrix of the extract. According to the literature, several molecules fall within the critical low-concentration zone (1 to 50 mg/L) capable of triggering a cellular stress response. Beta-resorcylic acid and rosmarinic acid act as intermediate phenols. Resveratrol is well-known for its hormetic effects and its capacity to mimic stress at low doses. Quercetin and coumaric acid are present in trace amounts, which is ideal for stimulating cellular signaling pathways without causing direct cytotoxicity²⁹. These results (Table 1) demonstrate a clear synergy between a strong epicatechin base and a cocktail of five minor constituents. It is precisely these low doses of resveratrol, quercetin, coumaric, rosmarinic, and beta-resorcylic acids that validate the stress response. These identified compounds are recognized cellular modulators known for their dose-dependent effects³⁰.

CONCLUSION

Qualitative and quantitative analyses demonstrated that *Xylopia aethiopica* aqueous extracts exhibit a high content of polyphenols and flavonoids. Both aqueous extracts and essential oils displayed antioxidant activity. HPLC-DAD analysis performed on the hydroethanolic extract identified six major polyphenols: two flavanols (epicatechin and quercitrin), two phenolic acids (beta-resorcylic acid and coumaric acid), one phenolic acid ester (rosmarinic acid), and one stilbene. *In vitro* evaluation of the antibiofilm efficacy of the extracts, particularly the essential oils, revealed a dose-dependent inhibitory effect against *Candida albicans* and *Candida parapsilopsis*. Since *Candida* biofilms are frequently resistant to conventional antifungals such as miconazole, fluconazole, voriconazole, amphotericin B, and caspofungin, these findings suggest that *Xylopia aethiopica* represents a promising natural source for developing novel alternative antibiofilm agents. So, the oily extract of *Xylopia aethiopica* possesses significant antibiofilm properties and enhances the activity of miconazole in a laboratory setting. While these preliminary findings suggest that *Xylopia aethiopica* could potentially represent an interesting candidate for future development in therapeutics, sanitation, or bio-preservation, further *in vivo* studies and field evaluations are strictly required to confirm its practical efficacy and safety. The paradoxical effect (hormesis) observed with the three aqueous extracts suggests that achieving an antibiofilm effect requires a concentration range that exceeds a critical threshold of low concentrations (1 to 50 mg/L).

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