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Activity of Phytochemically Characterized Noomi Basra Extracts Against Planktonic and Biofilm Candida albicans

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Abstract

Understanding how phytochemical composition influences antifungal activity is important for identifying effective plant-derived antimicrobials. Noomi Basra (dried black lime; *Citrus aurantifolia*) is widely used in the Middle East as a plant-derived antimicrobial, yet the relationship between its chemical composition and antimicrobial activity remains unclear. Here, extraction-dependent variation in phenolic and flavonoid content is examined for association with antifungal efficacy against *Candida albicans* in planktonic and biofilm models. Extracts prepared using cold ethanol (CEE), hot water (HWE), and hot hexane (HHE) were tested across concentrations of 0.4–12.8% Noomi Basra, and antifungal activity was quantified as $\log_{10}$ reductions in viable counts. CEE demonstrated significantly greater antifungal activity compared to HWE and HHE, particularly at higher concentrations. A strong dose-dependent effect was observed for CEE in the biofilm model, while a moderate concentration-dependent effect was detected in the planktonic model. In contrast, HWE and HHE showed no significant dose-dependent activity.

Phenolic-rich extraction may be associated with greater antifungal activity and may represent promising candidates for the treatment of *Candida* infections, while flavonoid content exhibited a weaker correlation that was not significant after adjustment. These findings indicate that phenolic-rich ethanolic extracts of Noomi Basra may represent promising candidates for the treatment of *Candida* infections, particularly biofilms.

Introduction

Biofilm formation is a crucial component of the biology of *Candida albicans*, and may be involved in persistence and decreased sensitivity to antifungal treatments [1,2]. Biofilm development is defined by microbial communities embedded in an extracellular matrix (ECM), which promote persistence and antifungal tolerance [3,4]. The ECM works as a cohesive agent, enveloping and gluing cells in biofilms [5]. This can significantly affect the medical management of biofilm-associated infections, sometimes resulting in chronic or recurring disorders that are extremely difficult to control [6–8]. *Candida albicans* is known clinically to be involved in chronic respiratory disease mainly as a persistent airway colonizer which as a result modifies the status of the disease, not as a defined etiology of chronic pneumonia [9,10]. Unfortunately, there is an increase in incidence of antifungal-resistant *C. albicans* due to the widespread use of antifungal agents in healthcare settings. Therefore, identifying alternative anti-candidal medicines is critical [11,12].

Medicinal plants have played a significant role in providing health care to resource-poor populations worldwide [13–15]. During extraction, the choice of solvent is one of the major factors as polarity changes what the plant matrix releases; recent studies show that intermediate or polar solvents can recover more phenolics and flavonoids than non-polar solvents, and those extracts often perform better in antioxidant and antimicrobial assays [16,17]. Phenolic and flavonoid content is important for antimicrobial activity because these compounds are repeatedly linked with stronger inhibition zones, lower MICs, and broader

bioactivity across different plant extracts [18,19]. In practice, this means solvent choice is not just a technical step but a determinant of whether the final extract is phenolic-rich enough to deliver meaningful antimicrobial effects [20,21].

One of the most common traditional remedies in Asia and the Middle East is dried lime, also known as black lime or Noomi Basra (*Citrus aurantifolia* Linn., Rutaceae), which is used mostly to flavor food, but also to produce drinks and for a variety of medicinal uses such as treating lower respiratory tract infections [22,23]. The key lime peel and pulp offer a variety of therapeutic advantages in traditional medicine, such as relief from stomachaches and coughs, as well as anti-diabetic, anti-hypertensive, anti-inflammatory, anti-lipid, anti-platelet, antioxidant, antifungal, and antibacterial activities [24–26].

The high morbidity and mortality of invasive candidiasis, together with *C. albicans*' resistance to antifungal medications, highlight the need for novel treatments to protect humans from *Candida* infections, particularly those involving biofilms. The aim of this study is to evaluate the antifungal and antibiofilm activity of *Citrus aurantifolia* (Noomi Basra) extracts and to investigate the role of phenolic and flavonoid compounds in this activity.

Materials and Methods

Extraction of plant material

Dry black lime was purchased from an Iraqi marketplace and processed to a fine powder using a conventional food grinder prior to preparing the various extracts following the procedures stated in Aibinu et al., 2007; Al-Rubai et al., 2016; and Şeker Karatoprak et al., 2021 [22,25,27]. The hot hexane extract was produced by combining 1250 mL of 80% hexane with 250 g of Iraqi *C. aurantifolia* powder. The extract was produced by running a reflux condenser on low heat (lower than 40°C) for two hours. The hot water extract was made by mixing 800 mL of distilled water with 250 g of powder and heating it in a reflux condenser on low heat (lower than 40°C) for two hours. The cold ethanol extract was produced by mixing 1250 mL of 80% ethanol with 250 g of powder and soaking it in a sealed container for two days, stirring intermittently at room temperature in a dark environment to prevent photolysis of the extracted contents. Extracts were filtered using 8–10 µm Whatman filter paper and a rotary evaporator to remove solvents before lyophilization.

Evaluation of the Total Phenolic and Flavonoid Content

The total phenolic content (TPC) of each extract was analyzed using the Folin-Ciocalteu's technique, while the total flavonoid content (TFC) was obtained using colorimetric methods as described by Abu-Qatouseh et al., 2019 [28]. In brief, the TPC was determined by mixing the extract with Folin-Ciocalteu reagent and sodium carbonate to generate a colored complex while incubating in the dark. The absorbance of the resultant solution was analyzed with a UV-Vis spectrophotometer at a wavelength of

760 nm. The TPC was calculated via comparing the absorbance to a standard curve produced with known amounts of gallic acid. The results were reported as milligrams of gallic acid equivalents per gram of extract (mg GAE/g). The TFC used a colorimetric test using aluminum chloride, in which Al (III) formed a compound with flavonoids, increasing color intensity at a specified wavelength (415 nm). The TFC was calculated by comparing the absorbance to a standard calibration curve, which was frequently generated with catechin or quercetin.

Microbial Strain and Culture Media

The biofilm-related tests were carried out using the reference strain *C. albicans* ATCC 10231, which was obtained from the American Type Culture Collection (ATCC). The yeast strain was kept at -20 ± 2°C in cryovials, then resuspended and sub-cultured in Sabouraud Dextrose Broth SDB (Oxoid, UK) overnight at 35 ± 2°C. The quantity of yeast in the inoculum was measured in three technical replicate wells within the same experimental run using optical density (OD 600 nm). Similarly, the strain's growth was computed using the observed OD, as illustrated below.

The MBEC® Assay: Biofilm Formation

Biofilm production and antifungal sensitivity of yeast biofilms were measured using the modified Minimum Biofilm Eradication Concentration Assay with the 96 well bottom plate (MBEC Assay®). Each microtiter plate lid was fitted with 96 sterile wooden pegs (0.3 x 0.1 x 0.9 cm) instead of the standard polystyrene pegs, with each wooden peg fitting perfectly into a well of the conventional 96 well bottom plate. The bottom portion of each peg extending down into the well served as a surface for yeast to attach, colonize, and produce a homogeneous mature biofilm. The modified MBEC Assay® plate was incubated for 24 ± 2 hours at 35 ± 2°C in an orbital shaker incubator (Lab Companion SI-600R, Korea) at ~ 110 rpm to facilitate yeast biofilm growth on the pegs.

The MBEC® Assay: Antifungal Challenge

The lid with attached pegs was transferred to a newly sterile 96-well plate (SPL, Korea) and rinsed for 30 seconds with 200 µL of sterile PBS. In early studies, the optimal growth conditions for biofilm development were discovered to be about 10^5 -10^7 CFU/peg, allowing for the potential measurement of at least 4 log reductions compared to the untreated controls. After biofilm growth and rinsing, the 96-peg lid was placed in the 96-well challenge plate. The challenge plate had various extract concentrations dissolved in SDB and 0.1% Dimethyl Sulfoxide (DMSO, AZ Chem for Chemicals, Canada). The untreated growth control wells were treated with DMSO and then utilized to compute the $\log_{10}$ reduction. A serial dilution of extract concentrations dissolved in SDB were utilized, including 12.8%, 6.4%, 3.2%, 1.6%, 0.8%, and 0.4% w/v. All test dilutions were done in three technical replicate wells within the same experimental run. The pegged lid was then removed, washed in PBS, and put over another 96-well microtiter plate with

fresh, sterile recovery broth (SDB with 0.1% Tween-80). The recovery plate was sonicated with an ultrasonic cleaner (ULTRAsonik 104x, USA) for 30 ± 1 minutes. The recovered colony forming units per milliliter (CFU/mL) of planktonic cells and the recovered colony forming units per peg (CFU/peg) of adhered biomass for all treatment concentrations were measured via serial dilution and spot plating of each well on Sabouraud Dextrose Agar plates that were incubated for 24 ± 2 hours.

Statistical Analysis

All results were expressed as mean ± SEM and analyzed using GraphPad Prism 9 software (GraphPad Software, San Diego, CA, USA). Two-way ANOVAs were used to evaluate the effects of extract type, concentration, and phenotype (biofilm vs. planktonic). One-way ANOVAs followed by Tukey's multiple comparisons tests were used for normally distributed data. For non-normally distributed data, the Kruskal–Wallis test followed by Dunn's multiple comparisons test was applied. A p-value < 0.05 was considered statistically significant. Additional analyses examining associations between total phenolic content (TPC), total flavonoid content (TFC) and antifungal activity were performed using Python. These included correlation analyses, exploratory stratification of phytochemical content, and mixed-effects modelling to account for clustering by extract.

Results

Total Phenolic and Flavonoid Content

TPC was estimated by gallic acid and expressed as mg gallic acid equivalent (GAE)/g of extract. TFC was expressed as mg Rutin equivalents/g of extract. Samples were analyzed in duplicate. The cold ethanolic extract (CEE) exhibited the highest TPC at 0.18175 mg GAE/g, followed by the hot water extract (HWE) at 0.08875 mg GAE/g, while the hot hexane extract (HHE) showed the lowest TPC at 0.061 mg GAE/g. In contrast, the total flavonoid content (TFC), expressed as mg rutin equivalents (RE)/g of extract, was highest in HHE (0.2005 mg RE/g), followed by CEE (0.13925 mg RE/g) and HWE (0.0905 mg RE/g). Thus, CEE had the highest TPC and demonstrated the greatest antifungal activity, whereas the highest TFC was observed in HHE.

Determination of Antifungal Activity in Planktonic and Biofilm growth modes

The antifungal activity revealed differences depending on the extract type and the fungi growth stage, indicating unique patterns in the planktonic versus biofilm models. In the biofilm model, a two-way ANOVA indicated no significant interaction between extract type and concentration ($F(10,36) = 1.49$, $p = 0.184$). However, a significant impact of concentration was noted ($F(5,36) = 5.03$, $p = 0.001$), demonstrating an increasing concentration-dependent increase in antifungal activity. There was no significant overall effect of extract type ($F(2,36) = 1.21$, $p = 0.312$). Post hoc analysis indicated that at the highest concentration (12.8%), the CEE demonstrated significantly greater antifungal activity in

comparison to both the HHE ($p = 0.017$) and the HWE ($p = 0.002$). However, no significant differences were detected at lower concentrations (Fig. 1A).

There was no significant difference between extract type and concentration in the planktonic model ($F(10,36) = 1.22$, $p = 0.313$), and the impact of concentration was also not statistically significant ($F(5,36) = 1.31$, $p = 0.282$). Nonetheless, a significant main impact of extract type was observed ($F(2,36) = 6.39$, $p = 0.004$), demonstrating differences in antifungal efficacy among the extracts. In line with the biofilm results, CEE showed much higher antifungal action than both HHE ($p = 0.006$) and HWE ($p = 0.008$) at 12.8%. There were no significant differences at lower concentrations (Fig. 1B).

To further assess the impact of fungal growth state, distinct two-way ANOVA tests were performed for each extract. For CEE, there was no significant interaction between concentration and phenotype ($F(5,24) = 1.97$, $p = 0.12$). Nonetheless, both concentration ($F(5,24) = 22.04$, $p < 0.0001$) and phenotype ($F(1,24) = 8.175$, $p = 0.009$) exhibited significant effects, demonstrating a pronounced dose-dependent response and a notable difference in susceptibility between planktonic and biofilm states, with planktonic cells typically demonstrating greater $\log_{10}$ reductions (Fig. 2A).

There was no significant interaction for HHE ($F(5,24) = 0.79$, $p = 0.57$), and the impact of concentration was not statistically significant ($F(5,24) = 2.31$, $p = 0.08$). There was a significant impact of phenotype ($F(1,24) = 6.89$, $p = 0.015$), which means that planktonic and biofilm forms showed differences in how they were affected, with planktonic microorganisms being more readily impacted than biofilms, but there were no significant changes at individual concentrations (Fig. 2B).

Conversely, for HWE, neither the interaction ($F(5,24) = 0.23$, $p = 0.95$) nor the main effects of concentration ($F(5,24) = 0.07$, $p = 0.997$) or phenotype ($F(1,24) = 0.05$, $p = 0.82$) reached statistical significance, suggesting uniformly low antifungal activity across all evaluated models (Fig. 2C).

Overall, these results show that the type of extract and the condition of fungal growth both affect antifungal activity. CEE showed the highest antimicrobial action, especially at higher concentrations.

Dose-Dependent Effects on the Planktonic and Biofilm Models

The impact of extract concentration on antifungal activity was assessed for all extracts in both biofilm and planktonic models. A notable concentration-dependent impact was reported for the CEE ($F(5,12) = 143.7$, $p < 0.0001$). Post hoc analysis indicated that the highest concentration (12.8%) demonstrated significantly greater antifungal activity than all lower concentrations ($p < 0.0001$, Fig. 3A), with significant differences also identified between intermediate and lower concentrations, hence confirming a concentration-dependent trend. Conversely, the HHE exhibited no significant dose-dependent response, as no statistically significant changes were detected across concentrations ($p > 0.05$, Fig. 3B), although slight fluctuations in mean $\log_{10}$ reduction values were measured. The HWE exhibited no significant

concentration impact ($F(5,12) = 0.11$, $p = 0.99$, Fig. 3C), with Tukey's multiple comparisons revealing no significant differences among all evaluated concentrations.

In the planktonic model, concentration-dependent effects differed among extracts. Because the data for CEE was not normally distributed, a Kruskal–Wallis test was used. The test showed that there was a significant difference between the concentrations ($H = 11.97$, $p = 0.04$). Dunn's multiple comparisons test revealed a significant difference between the highest (12.8%) and lowest (0.4%) concentrations ($p = 0.03$, Fig. 4A), indicating a concentration-dependent antifungal effect, though slightly less prominent than in the biofilm model. In contrast, no significant concentration-dependent effects were seen for HHE ($F(5,12) = 1.08$, $p = 0.42$, Fig. 4B) or HWE ($F(5,12) = 0.16$, $p = 0.97$, Fig. 4C) in the planktonic model. Post hoc analysis validated the lack of significant pairwise differences ($p > 0.05$), demonstrating that elevated concentrations of these extracts did not substantially increase antifungal activity.

Overall, these results show that the CEE had a strong dose-dependent antifungal activity, especially in the biofilm model. HHE and HWE, on the other hand, showed little or no concentration-dependent activity in both growth conditions.

Relationship Between Phytochemical Content and Antifungal Activity

Analysis conducted with Python revealed a positive association between phytochemical content and antifungal activity ($\log_{10}$ reduction). Total phenolic content showed a moderate to strong positive correlation with antifungal activity ($r = 0.58$, $p \approx 4.1 \times 10^{-11}$, Fig. 5A), suggesting that higher phenolic levels were associated with greater fungal reduction. In contrast, total flavonoid content demonstrated a weaker, yet statistically significant, correlation ($r = 0.36$, $p \approx 1.1 \times 10^{-4}$, Fig. 5A). Consistent with these findings, binned analysis (low, medium, high) showed a progressive increase in antifungal activity with increasing phenolic content, whereas the trend for flavonoids was less pronounced.

In a mixed-effects model accounting for extract-level clustering, total phenolic content remained significantly associated with antifungal activity ($p < 0.001$, Fig. 6A), while the association with flavonoid content was attenuated and did not reach statistical significance ($p = 0.056$, Fig. 6B). These results suggest that phenolic compounds might be contributing to the observed antifungal efficacy, with flavonoids playing a secondary role.

Discussion

The present study evaluated the antifungal activity of different *Citrus aurantifolia* extracts against *C. albicans* and examined whether their TPC and TFC were associated with antifungal activity. Overall, the findings demonstrated that extraction method influenced both the phytochemical content and antifungal activity of the extracts. Interestingly, the highest TFC was observed in HHE, rather than CEE, whereas CEE showed the highest TPC and greatest antifungal activity. Thus, the observed activity did not parallel

total flavonoid content alone. These findings suggest that the solvent choice may influence the recovery of bioactive compounds and, consequently, the antifungal potential of *Citrus aurantifolia*.

Among the tested extract, CEE was the most effective extract in reducing *C. albicans* viability, with up to about 2 $\log_{10}$ reductions at 12.8% in both planktonic and biofilm phenotypes. However, HWE and HHE caused reductions of less than 1 $\log_{10}$ at all tested concentrations. The increased activity seen with CEE especially at higher doses is consistent with its higher TPC and implies that the extraction dependent changes in phytochemical composition may contribute to antifungal activity [29,30]. These decreases are a sign of antifungal activity but cannot be taken as definite proof of fungicidal action as fungicidal endpoints were not specifically tested in the current experiment. Phenolic antimicrobials act through multiple overlapping mechanisms, including membrane disruption, ROS-driven oxidative damage, DNA injury, enzyme inhibition, and quorum-sensing interference [31–33]. Flavonoids, instead, affect membrane permeabilization, suppress metabolism and protein synthesis, and inhibit virulence and biofilm related pathways [34–36]. Overall, the recent literature supports phenolics and flavonoids as multitarget antimicrobial agents whose activity is often strengthened in combination with antibiotics and may help limit resistance development [37–40].

Although flavonoids have recognized antifungal properties, their activity depends on the chemical structure and composition of individual flavonoids rather than their total concentration alone [41,42]. Consequently, the elevated TFC seen in CEE does not necessarily suggest that flavonoids were independent for its antifungal efficacy. Interestingly, our statistical evaluation revealed that TFC did not serve as an independent predictor of antifungal efficacy, while TPC might be associated with greater antifungal activity [43,44]. This observation agrees with the data that the antifungal action is dependent on the chemical composition and structural features of individual flavonoids rather than their total concentration [45,46]. Furthermore, flavonoid-flavonoid and flavonoid-other phenolic compounds interactions can be synergistic, additive or antagonistic, thus a larger flavonoid concentration does not always represent a stronger antifungal activity [44,47–49].

The extraction solvent likely contributed to these differences. Ethanol has repeatedly been identified as one of the most effective solvents for the extraction of phenolic compounds exhibiting antifungal properties, as it recovers a wide range of moderately polar bioactive substances while maintaining their biological efficacy [50,51]. Conversely, water and hexane extractions selectively retrieve different types of phytochemicals, which may explain the reason these extracts often exhibit less antifungal potency despite having detectable flavonoid levels [50–53]. While this study did not perform detailed chemical characterization; such as LC-MS/MS to identify individual active compounds, the quantitative relationship established here between total phenolics and activity provides a defensible framework for future research. Subsequent work should focus on fractionating these phenolic-rich extracts to identify the individual chemicals responsible for activity and exploring potential synergies with conventional antifungal agents to enhance overall efficacy against Candida biofilms.

Conclusion

This study demonstrates that Noomi Basra (*Citrus aurantifolia*) extracts have antifungal activity against *Candida albicans*, with activity varying according to extract type, concentration, and fungal growth state. Among the assessed extracts, the CEE had the most notable antifungal activity, particularly at higher concentrations. A concentration-dependent effect was observed for CEE in the biofilm model and, to a lesser extent, in the planktonic model, whereas HHE and HWE showed minimal or no concentration-dependent effects. Furthermore, TPC might be associated with greater antifungal activity, whereas TFC showed a weaker and non-significant association after adjustment. Keeping in mind that neither chemical identification of individual compounds in the extracts nor establishing the mechanisms of action were determined. To summarize, CEE was the most active extract of those tested and the results suggest further chemical characterization and fractionation of CEE to identify the chemicals responsible for the antifungal activity and to explore further its potential against *Candida*.

Declarations

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Author Contribution

Author contributions: A.M.O. conceived and designed the study, supervised the work, developed the methodology, provided resources, and contributed to writing and editing the manuscript. L.F.A. contributed to data analysis, manuscript writing, and editing. A.M. contributed to the main data analysis and critically edited the manuscript. M.A.A.N., K.M., L.A., M.B., R.M.A., and S.A. contributed to the experimental work and interpretation of the results. P.N. and L.O. contributed to interpretation of the findings and critically revised the manuscript. All authors reviewed and approved the final manuscript.

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Data Availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

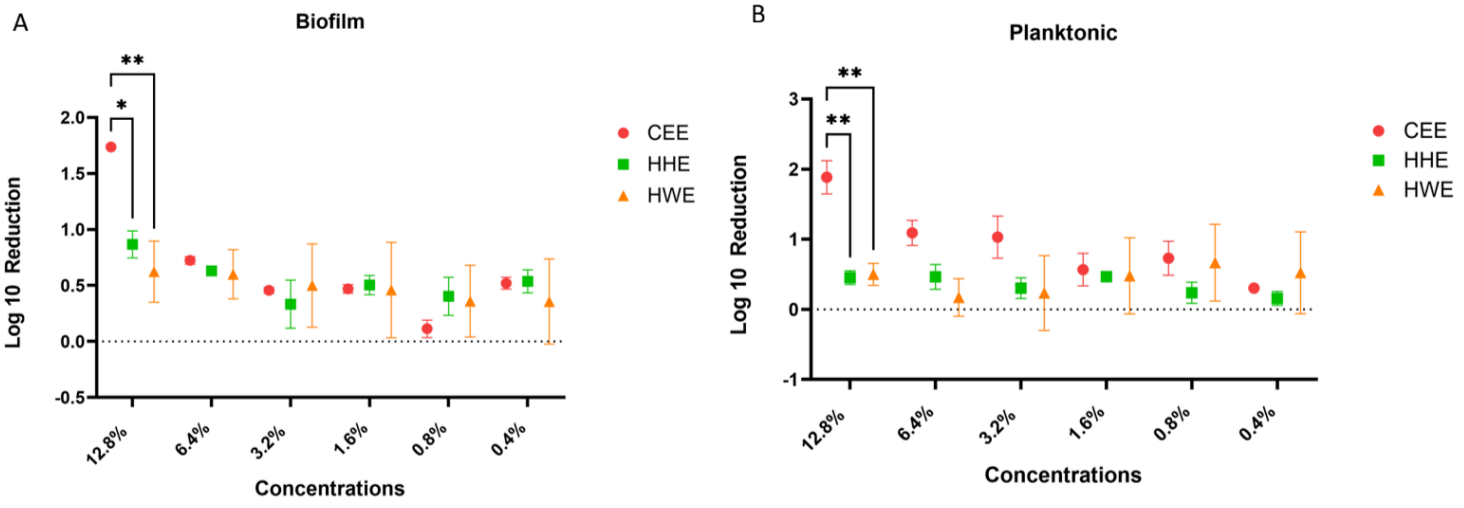


Figure 1

Antifungal activity of *Citrus aurantifolia* extracts against *Candida albicans* in both biofilm and planktonic models. (**A**) Biofilm model showing $\log_{10}$ reduction in viable cells following treatment with CEE, HWE, and

HHE (0.4–12.8%), with a significant concentration-dependent effect (p = 0.001) and greater efficacy of CEE at 12.8% only. (**B**) Planktonic model showing $\log_{10}$ reduction in viable cells following treatment as described above, demonstrating a significant effect of extract type (p = 0.004), with CEE exhibiting the highest activity at 12.8% and no significant differences at lower concentrations.

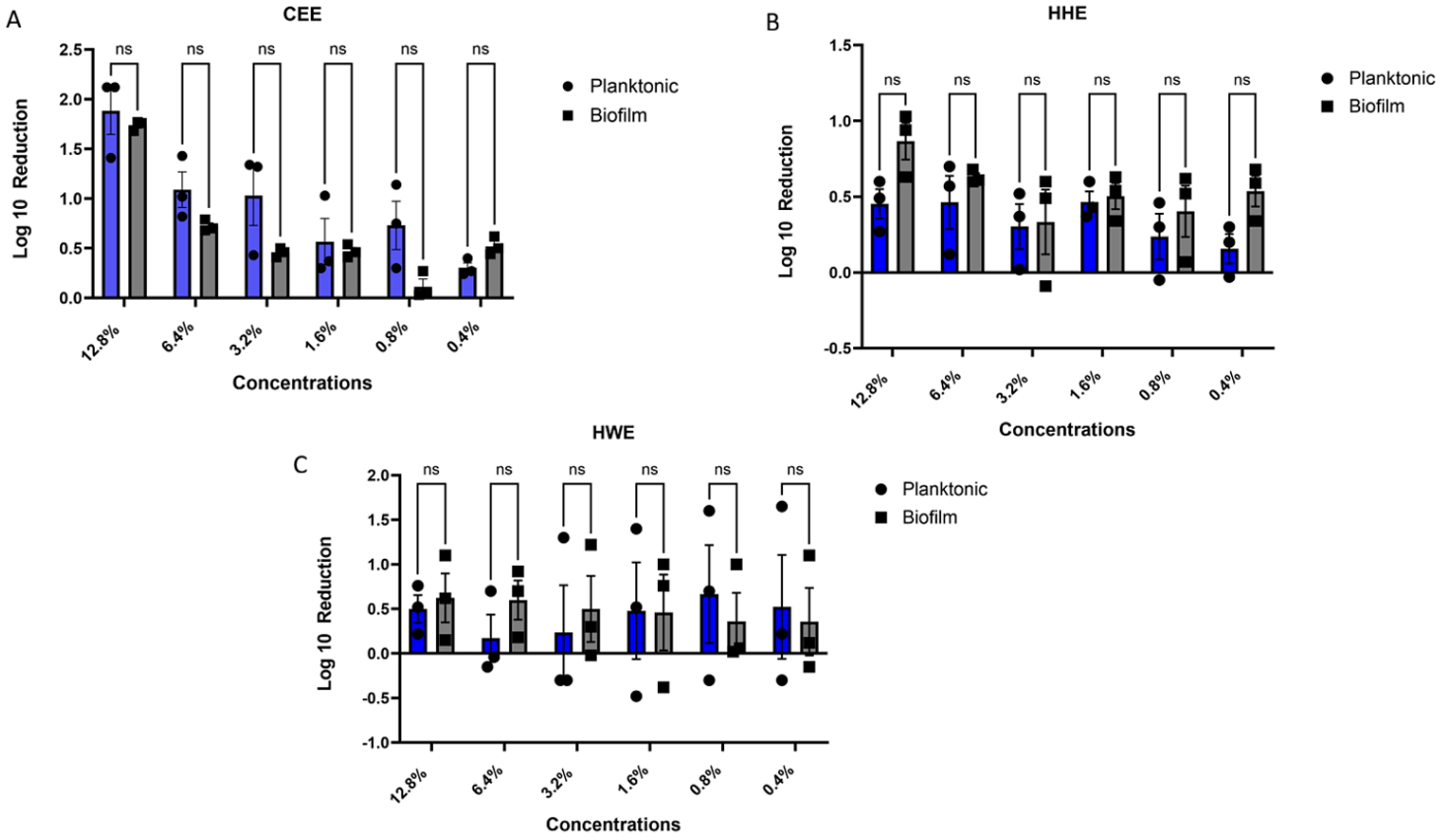


Figure 2

Effect of concentration and phenotype on antifungal activity of individual extracts. (**A**) CEE, (**B**) HHE, and (**C**) HWE showing $\log_{10}$ reductions in *Candida albicans*viability in both biofilm and planktonic models across different concentrations (0.4–12.8%).

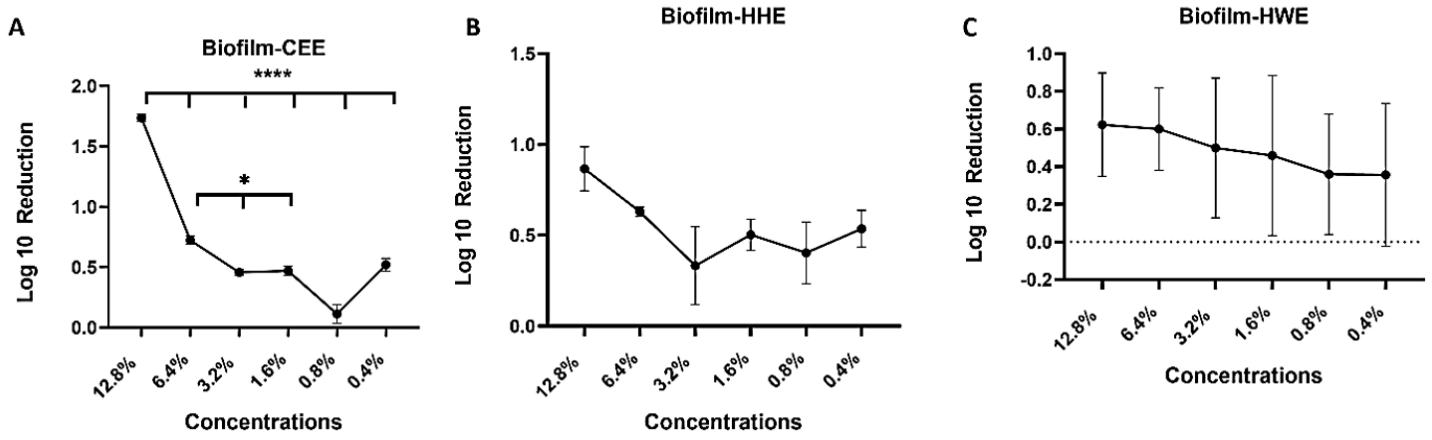


Figure 3

Dose-dependent antifungal activity of *Citrus aurantifolia* extracts in the biofilm model. (**A**) CEE, (**B**) HHE, and (**C**) HWE showing $\log_{10}$ reductions in *Candida albicans* biofilms at different concentrations. CEE exhibited a significant concentration-dependent effect, while HHE and HWE showed no significant dose-dependent response.

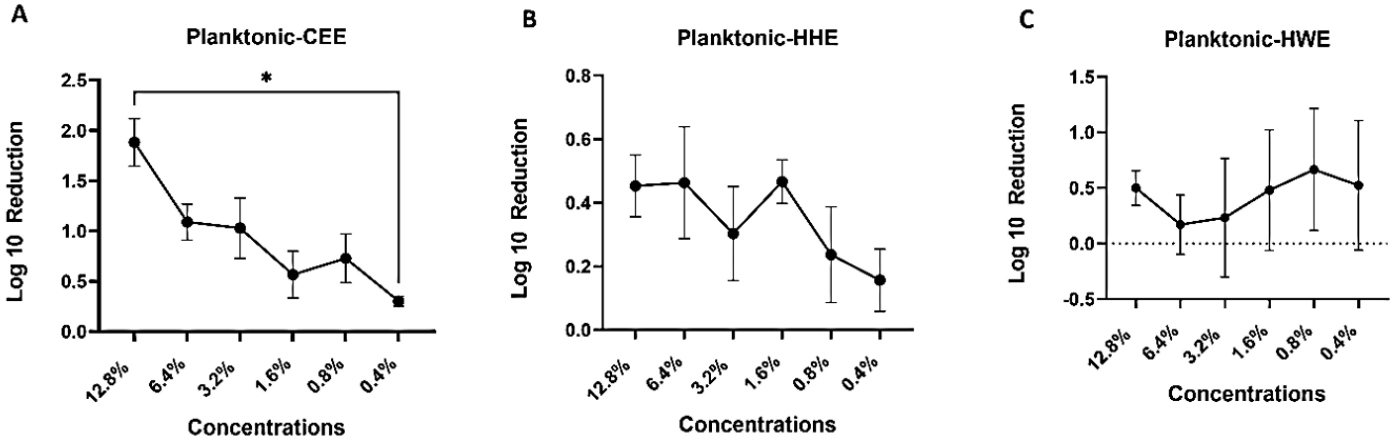


Figure 4

Dose-dependent antifungal activity of *Citrus aurantifolia* extracts in the planktonic model. (**A**) CEE, (**B**) HHE, and (**C**) HWE showing $\log_{10}$ reductions in the planktonic *Candida albicans* model. A significant concentration-dependent effect was observed for CEE, while no significant dose-dependent effects were detected for HHE and HWE.

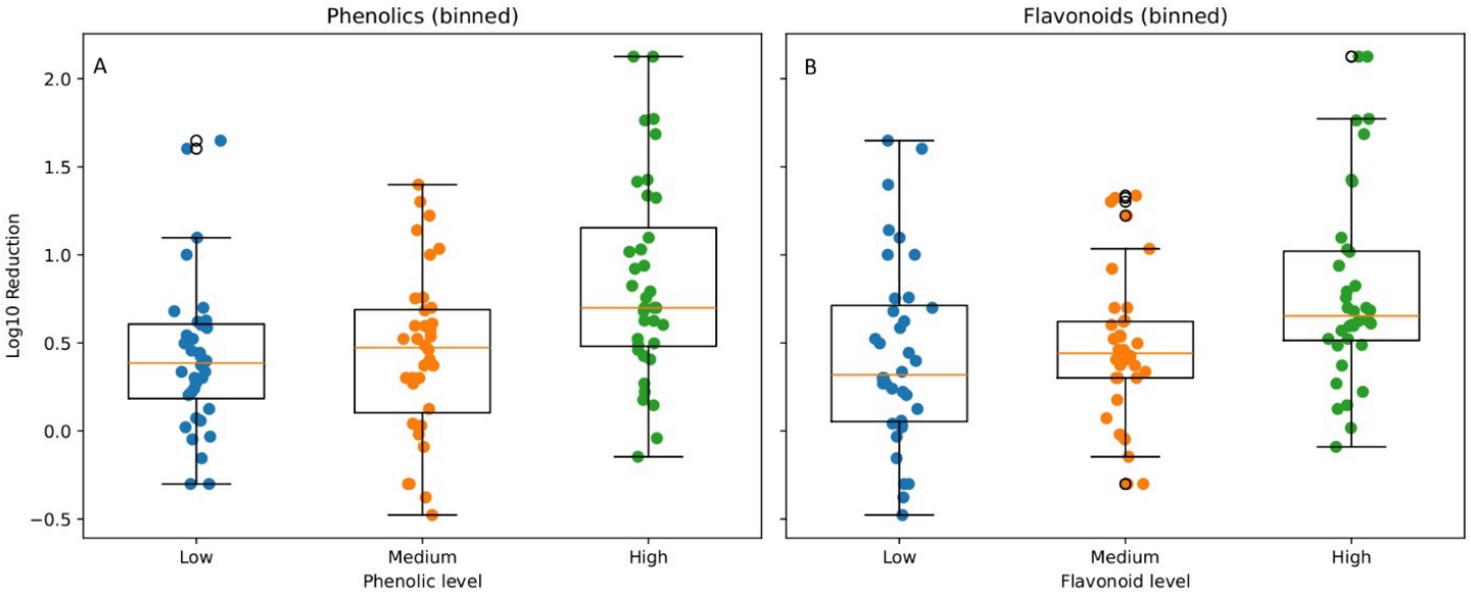


Figure 5

Relationship between (**A**) total phenolic and (**B**) total flavonoid content and antifungal activity against *Candida albicans* (log_{10} reduction). Data are grouped into low, medium, and high levels. Phenolics showed a stronger association (r = 0.58, p < 0.001) than flavonoids (r = 0.36, p < 0.001). Box plots represent median and interquartile ranges with overlaid individual data points.

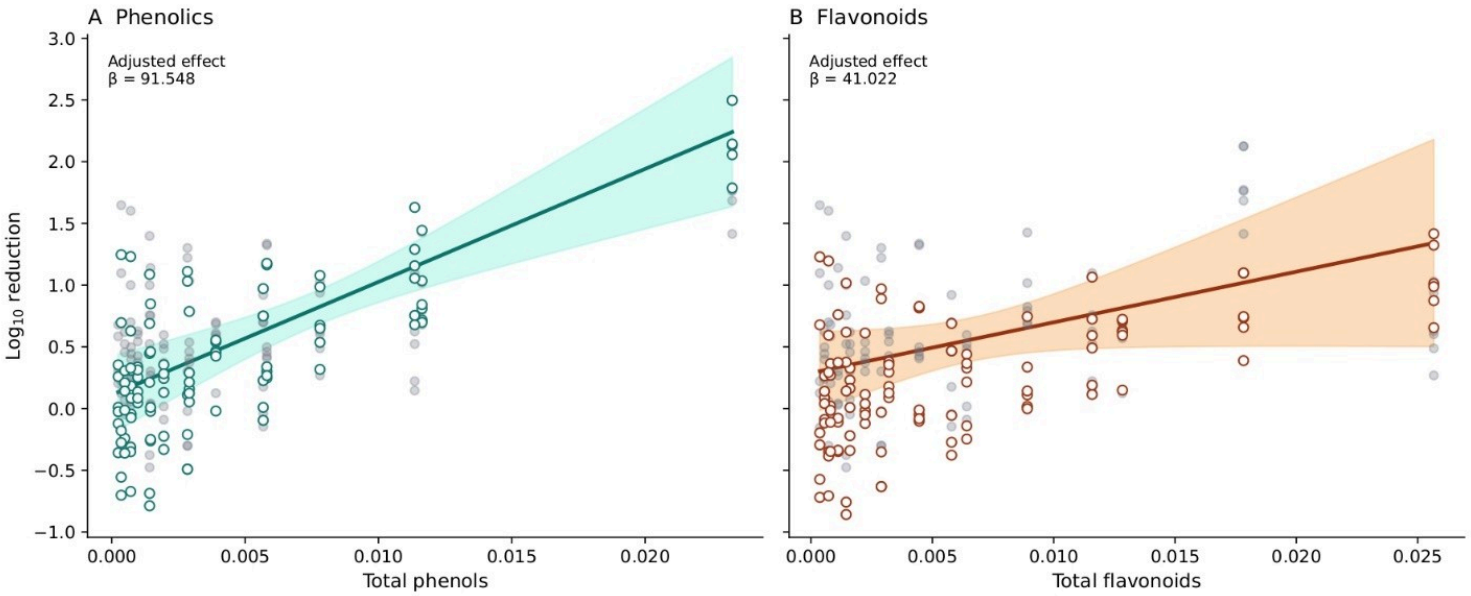


Figure 6

Mixed-effect model accounting for extract-level clustering. Adjusted associations are shown between phytochemical content and antifungal activity (log_{10} reduction). Fitted lines represent regression estimates, with shaded areas indicating 95% confidence intervals. (**A**) Total phenols significantly predict

antifungal activity (adjusted β = 91.548, p < 0.001). (**B**) Total flavonoids show an attenuated, non-significant association (adjusted β = 41.022, p = 0.056).

Supplementary Files

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- SupplementaryFig.png