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RESEARCH ARTICLE

Antifungal Potential and Safety Evaluation of Thai *Piper betle* Leaf Extract and Phenolics Against Animal Pathogenic *Candida* Species

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ABSTRACT

Candida species are opportunistic fungi infecting humans and animals, with increasing incidence of non–*Candida albicans Candida* species (NCACs). This study evaluated the antifungal and antivirulence activities of Thai *Piper betle* leaf extract (Ethanolic *P. betle* extract; EPE) and its primary phenolic compounds, hydroxychavicol and eugenol, against six *Candida* strains isolated from animals. Antifungal efficacy was assessed using broth microdilution to determine minimum inhibitory (MIC) and minimum fungicidal (MFC) concentrations. Anti-virulence activities—biofilm formation, extracellular enzyme activity, and hyphal transition—were evaluated via standard assays. Cytotoxicity was examined in Vero cells using the MTT assay and phase-contrast microscopy. All compounds exhibited antifungal activity, with hydroxychavicol demonstrating the lowest MICs (0.008-0.256mg/mL) and consistent fungicidal activity, followed by EPE (0.016-0.256mg/mL) and eugenol (0.667-1.334mg/mL). Biofilm inhibition occurred only in *C. krusei* WU1, with hydroxychavicol achieving 76.93% reduction at 1/2MIC, followed by eugenol (74.36%) and EPE (69.34%). Enzymatic assays revealed selective inhibition of lipase activity—hydroxychavicol in *C. albicans* and EPE in *C. krusei*—while other enzymes were unaffected. Hyphal formation in *C. albicans* ATCC90028 was markedly suppressed by all compounds, particularly hydroxychavicol. Cytotoxicity profiling revealed that EPE maintained high Vero cell viability (≥98% viability at ≤1MIC; IC_{50}>2MIC), whereas hydroxychavicol and eugenol were cytotoxic at 2MIC but biocompatible at sub-MIC levels. These findings support the potential of *P. betle* extracts, especially hydroxychavicol, which possesses the strongest antifungal potency, while EPE demonstrated preliminary *in vitro* safety as an antifungal agent, supporting their potential as antifungal candidates for veterinary applications targeting *Candida* spp.

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INTRODUCTION

Candidiasis is a substantial opportunistic fungal infection that affects both humans and animals and is caused by a variety of *Candida* species. Traditionally, *Candida albicans* has been the most prevalent causative agent; however, infections caused by non-*Candida albicans* species (NCACs) such as *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* are becoming more prevalent (Pfaller and Diekema,

2007; Swaminathan and Kamath, 2023). NCACs often exhibit intrinsic or acquired resistance to commonly used antifungal agents, making treatment more difficult (Pfaller and Diekema, 2007).

Candidiasis can manifest in two major clinical forms: cutaneous candidiasis and systemic candidiasis. Cutaneous candidiasis typically affects the skin, mucous membranes, and nail beds, presenting as localized infections such as dermatitis, otitis, and mucosal lesions (Howell, 2023). This form is common in animals with underlying predisposing factors such as prolonged antibiotic use, immunosuppression, or skin barrier disruption (Berman and Sudbery, 2002). Systemic candidiasis, in contrast, involves hematogenous dissemination of *Candida* species to internal organs, including the kidneys, liver, spleen, and heart, leading to potentially fatal infections, particularly in immunocompromised hosts (Lionakis and Kontoyiannis, 2003).

In veterinary practice, *Candida* infections are implicated not only in skin and systemic diseases but also in conditions such as fungal mastitis in dairy cattle, leading to reduced milk production and significant economic losses (Krukowski *et al*., 2006). Infections of the oral cavity and esophagus by *Candida* spp., including NCACs, have also been reported in companion animals such as dogs and cats (Sykes, 2013). *Candida* spp. pathogenicity arises from stress adaptability and key virulence traits, including adhesin expression, morphological switching, biofilm formation, and hydrolytic enzyme secretion (Ciurea *et al*., 2020; Pattnaik *et al*., 2021). Biofilms are formed through adhesion, hyphal proliferation, and maturation into a dense matrix of polysaccharides and proteins (extracellular polymeric substances). This structure enhances persistence and confers 30–2,000-fold antifungal resistance through limited drug penetration, altered growth, and specific gene expression, making infections difficult to eradicate and requiring targeted treatments (Kojic and Darouiche, 2004). Moreover, certain *Candida* species, particularly *C. albicans*, exhibit morphological plasticity, switching between yeast, pseudohyphae, and hyphae in response to environmental cues, activating virulence pathways. Blastospores aid dissemination, while hyphae enable tissue invasion, accompanied by changes in gene expression, cell wall structure, and virulence factors (Gow *et al*., 2002; Jacobsen *et al*., 2012). Proteinases and phospholipases are critical extracellular enzymes, facilitating tissue penetration, host invasion, and adherence, thereby enhancing persistence and pathogenic potential (Bravo-Chaucanés *et al*., 2022).

Given these challenges, there is an urgent need for new antifungal agents that are effective against a broad spectrum of *Candida* species, including NCACs. Medicinal plants have been increasingly explored for their antimicrobial properties (Bhalerao *et al*., 2013). *P. betle*, commonly known as betel leaf, is a traditional medicinal plant widely cultivated in Southeast Asia, including Thailand. In particular, *P. betle* varieties from Thailand have been recognized for their potent biological activities. Among them, the variety known locally as *P. betle* var. Tha-Khae from Phatthalung Province in Southern Thailand is traditionally valued for its strong aroma, high phenolic content, and therapeutic properties (Sungkatavat *et al*., 2023).

Phytochemical investigations have revealed that *P. betle* contains active compounds such as hydroxychavicol, eugenol, and chavibetol, which exhibit potent antifungal and antioxidant effects (Nordin *et al*., 2014; Boripun *et al*., 2022). Previous studies have demonstrated that ethanolic extracts of *P. betle* var. Tha-Khae from Phatthalung Province, Thailand, possess bactericidal activity against antibiotic-resistant *Salmonella* spp. isolated from pig farms and avian pathogenic *Escherichia coli* (APEC), indicating its broader antimicrobial potential against bacterial pathogens (Kulnanan *et al*., 2021; Boripun *et al*., 2022). However, there is currently a lack of scientific evidence supporting the antifungal activity of *P. betle* var. Tha-Khae, particularly in veterinary applications. In contrast, studies on other *P. betle* varieties have reported significant antifungal properties, demonstrating the ability of *P. betle* extracts to inhibit the growth of both *C. albicans* and NCACs, disrupt biofilm formation, impair adhesion, and suppress hyphal development—key virulence factors in candidiasis (Ali *et al*., 2010; Ali *et al*., 2016; Sivareddy *et al*., 2019; Phumat *et al*., 2020; Nayaka *et al*., 2021; Selvaraj *et al*., 2022). Despite these promising findings, scientific evidence supporting the antifungal efficacy of Thai *P. betle* leaf extracts, particularly from the Tha-Khae variety, against *Candida* species isolated from animal infections remains limited. Therefore, this study aims to investigate the inhibitory effects of Thai *P. betle* leaf extracts on *Candida* spp., assess their impact on key virulence factors, and evaluate their *in vitro* cytotoxicity, thereby highlighting their potential applications in veterinary medicine.

MATERIALS AND METHODS

Ethical approval: All procedures were approved by the Walailak University Institutional Biosafety Committee (WU-IBC-67-041).

Test organisms: Six *Candida* strains were tested: one reference strain (*C. albicans* ATCC90028) and five clinical isolates (*C. albicans* WU3, *C. krusei* WU1, *C. glabrata* WU1, *C. parapsilosis* WU2, and *C. tropicalis* WU1) obtained from the Veterinary Mycology Laboratory, Walailak University. Species identification was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Schulthess *et al*., 2014).

Preparation of extracts: Mature *P. betle* leaves (Tha-Khae variety) were collected from Phatthalung Province, Southern Thailand. Leaves were washed, dried at 40°C for 3d, and powdered. Ethanolic extract (EPE) was obtained by macerating 50g of powder in 200mL of 95% ethanol for 7d, filtering, and concentrating under reduced pressure. Residue was dissolved in DMSO and stored at 4°C. Hydroxychavicol (≥98%, TCI, Japan) and eugenol (≥98%, Sigma-Aldrich, USA) were purchased as analytical-grade pure standards.

Preliminary antifungal activity testing: EPE and hydroxychavicol were tested at 1mg/mL, eugenol at 10.67mg/mL, and amphotericin B at 10µg/mL. *Candida* suspensions (1×10^6cells/mL) were incubated with test

extracts in 96-well plates (triplicates) at 37°C for 24h. Resazurin (0.018%, wt/vol) was added; blue color indicated growth inhibition.

Minimum inhibitory (MIC) and minimum fungicidal (MFC) concentration determination: MICs were determined according to CLSI-M27 (CLSI, 2017). Serial dilutions of EPE (0.004–2.048mg/mL), hydroxychavicol (0.001–0.512mg/mL), and eugenol (0.010–5.335mg/mL) were tested with *Candida* suspensions in triplicate. MIC was the lowest concentration yielding a blue color after 24h. For MFC, 10µL from inhibited wells were spot-inoculated on Sabouraud Dextrose Agar, incubated at 37°C for 24h; MFC was the lowest concentration with no colony growth (Jeenkeawpieam *et al*., 2023).

Enzymatic activity inhibition: *Candida* isolates were screened for extracellular enzymes. *C. albicans* ATCC90028 produced proteinase, lipase, and haemolysin, while *C. krusei* WU1 produced lipase only. Proteinase inhibition was tested on skim milk agar using 5µL of pre-treated suspensions (1/2–1/8MIC) and observing lysis zones after 1–3d. Lipase was examined on egg yolk agar by detecting opaque halos; haemolysin on sheep blood agar by hemolytic zones (dos Santos and Marin, 2005; Kadir *et al*., 2007). The enzyme activity was measured by dividing the diameter of the colony plus the zone by the diameter of the colony.

Inhibition of pseudohyphae and true hyphae formation: Treated *Candida* suspensions were stained with lactophenol cotton blue and examined microscopically for morphological changes.

Inhibition of biofilm formation: Biofilms were assessed following Shin *et al*. (2002) with modifications. Biofilms were induced in Sabouraud Dextrose Broth containing 8% glucose at 35°C for 24h. *Candida* suspensions (1×10^6cells/mL) were added in triplicate to sterile, flat-bottom polystyrene 96-well microplates containing the test extracts. After 48h at 35°C, wells were washed three times with phosphate-buffered saline (PBS, pH 7.2). Biofilms were stained with 0.1% (wt/vol) crystal violet, solubilized in DMSO, and quantified by absorbance at 570nm.

***In vitro* safety evaluation of *P. betle* leaf extracts:** Vero cells (ECACC 84113001) were cultured in DMEM with 10% fetal bovine serum and 1% antibiotics. Cells (1.5×10^4cells/100µL/well) were seeded in 96-well plates and incubated for 24h at 37°C, 5% CO_2. Test extracts at 1/8–2MIC, using the MIC of *C. albicans* ATCC90028 as reference. After 24h, medium was replaced with MTT solution (0.5mg/mL, wt/vol) for 4h. Formazan crystals were dissolved in DMSO. Absorbance was measured at 570nm with background subtraction at 650nm. Cell viability (%) was calculated as $(AB_t/AB_{neg})\times100$, where AB_t and AB_{neg} are absorbance values of treated and negative control cells (2% DMSO) (Freshney, 2015).

Statistical analysis: Data were analyzed by one-way ANOVA with Tukey's HSD post hoc test (Jamovi v2.6.44). All experiments were in triplicate, results are mean ± SE, and significance was set at $P<0.05$.

RESULTS

Preliminary antifungal activity of *P. betle* leaf extracts: Preliminary antifungal testing revealed that EPE and hydroxychavicol at a concentration of 1mg/mL, along with eugenol at 10.67mg/mL, effectively inhibited the growth of all tested *Candida* species. No antifungal activity was observed in the solvent control (2% DMSO), confirming that the observed inhibition was attributable solely to the active constituents of the extracts (Fig. 1).

Determination of MIC and MFC values of *P. betle* extracts determined by broth microdilution method: The antifungal activity of *P. betle* extracts and their major constituents was evaluated against multiple *Candida* strains. MIC and MFC values were determined following the CLSI guideline. Among the tested agents, hydroxychavicol exhibited the strongest antifungal activity, with MIC values ranging from 0.008–0.256mg/mL across all tested strains, indicating superior potency compared to the other compounds. EPE demonstrated comparable inhibitory effects, with MIC between 0.016 and 0.256mg/mL, whereas eugenol required higher concentrations (MIC: 0.667–1.334mg/mL), reflecting moderate efficacy. In terms of fungicidal activity, hydroxychavicol showed MFC values closely matching its MIC values, suggesting potent fungicidal properties. EPE also exhibited relatively low MFC values, although slightly higher than its corresponding MIC values in certain strains. Amphotericin B, used as a standard antifungal agent, displayed potent inhibitory activity with very low MIC values; however, its MFC values were notably higher in some clinical isolates, reflecting potential fungistatic behavior under the test conditions. The antifungal activity of EPE, hydroxychavicol, and eugenol was species-specific among six *Candida* isolates. *C. krusei* WU1 showed the highest susceptibility, especially to hydroxychavicol (MIC 0.032mg/mL), while *C. glabrata* WU1 showed the lowest MIC overall (MIC 0.008mg/mL), though its MFC was higher, suggesting fungistatic behavior at lower doses. *C. albicans* strains (ATCC90028 and WU3) showed moderate sensitivity, with both EPE and hydroxychavicol displaying similar MICs (0.256mg/mL). *C. tropicalis* WU1 and *C. parapsilosis* WU2 were less responsive, with higher MFC values required for eradication. Overall, hydroxychavicol displayed the most consistent and potent antifungal and fungicidal activity, followed by EPE and eugenol. The efficacy of all tested agents varied among *Candida* species, emphasizing the importance of strain-specific evaluations (Fig. 2 and Table 1).

Inhibition of biofilm formation: The biofilm-inhibitory activity of EPE, hydroxychavicol, and eugenol was assessed against six *Candida* species isolated from animals using both qualitative (crystal violet staining; Fig. 3) and quantitative (OD570nm measurement; Fig. 4) methods. Among all species tested, only *C. krusei* WU1 exhibited a dose-dependent reduction in biofilm biomass in response to all three compounds. At 1/2MIC, hydroxychavicol showed the highest inhibition (76.93%), followed by eugenol (74.36%) and EPE (69.34%). This trend continued at 1/4MIC, with hydroxychavicol and EPE maintaining

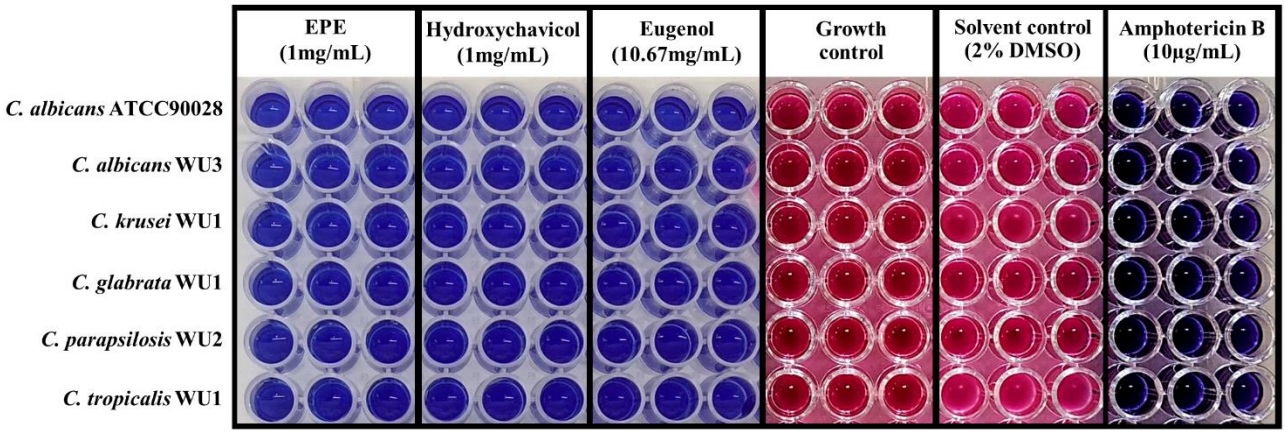

Fig. 1: Screening of anti-*Candida* activity of ethanolic *Piper betle* extract (EPE) and hydroxychavicol at 1mg/mL, and eugenol at 10.67mg/mL, by the broth microdilution method. Growth inhibition was assessed by resazurin colorimetric assay: blue color indicates effective inhibition of fungal growth, while a color change to pink indicates fungal viability and lack of inhibition.

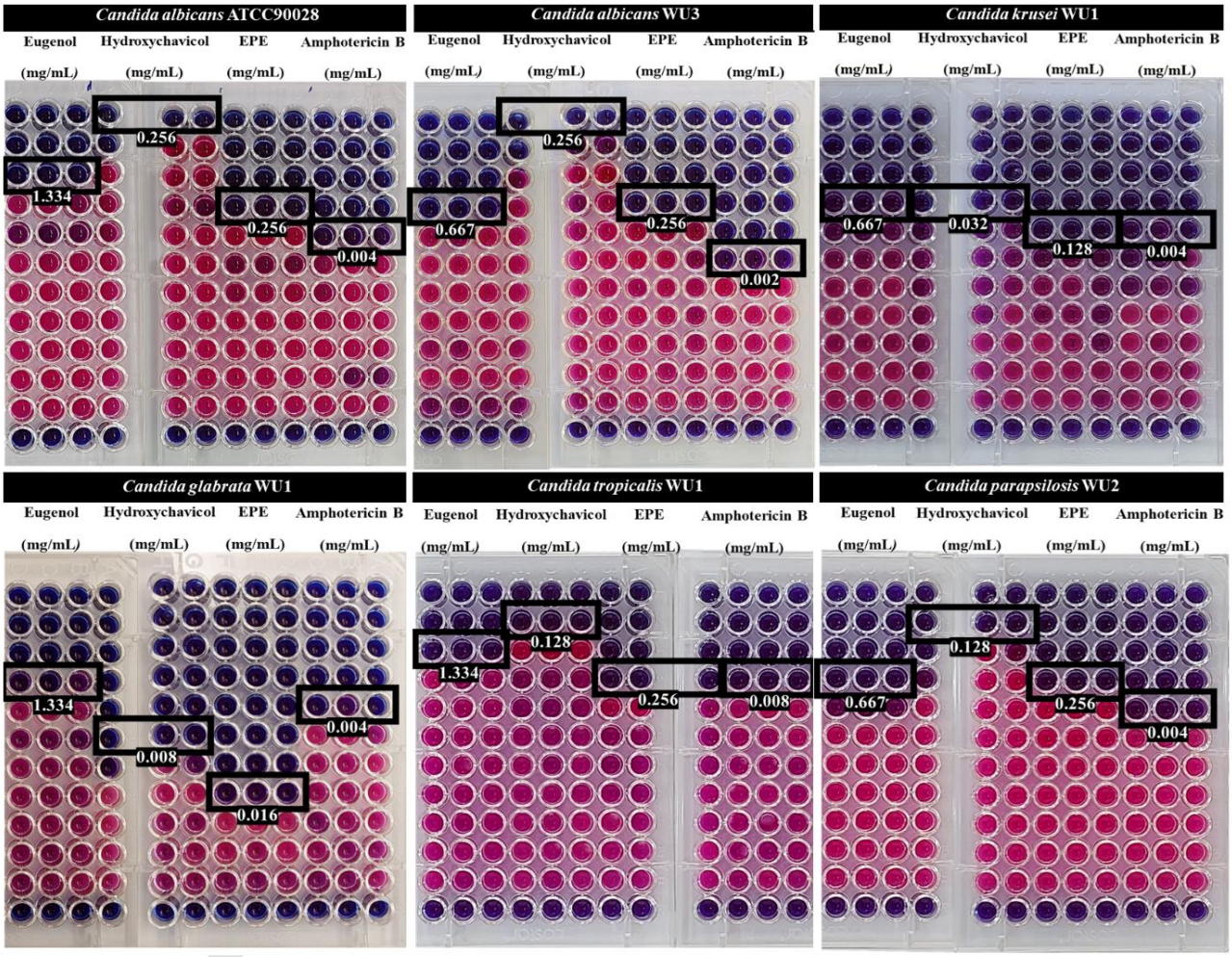

Fig. 2: Fungal species-specific analysis of MIC values of *Piper betle* extracts. MIC determination for amphotericin B, ethanolic *P. betle* extract (EPE), hydroxychavicol, and eugenol against six *Candida* species by the broth microdilution method. Fungal viability was assessed by resazurin assay, where blue indicates growth inhibition and pink indicates active fungal growth. Black rectangles highlight the MIC endpoint concentrations for each tested compound.

Table 1: MIC and MFC values of *Piper betle* extracts and amphotericin B against *Candida* species

Test Substance	MIC/MFC (mg/mL)					
	C. albicans ATCC90028	*C. albicans* WU3	*C. krusei* WU1	*C. glabrata* WU1	*C. tropicalis* WU1	*C. parapsilosis* WU2
Amphotericin B	0.004/0.064	0.002/0.016	0.004/>0.064	0.004/>0.064	0.008/0.064	0.004/0.064
EPE	0.256/0.512	0.256/0.512	0.128/0.128	0.016/1.024	0.256/1.024	0.256/0.512
Hydroxychavicol	0.256/0.256	0.256/>0.256	0.032/0.064	0.008/0.128	0.128/>0.256	0.128/0.256
Eugenol	1.334/2.668	0.667/2.668	0.667/2.668	1.334/5.335	1.334/2.668	0.667/2.668

Abbreviations: EPE; Ethanolic *Piper betle* extract.

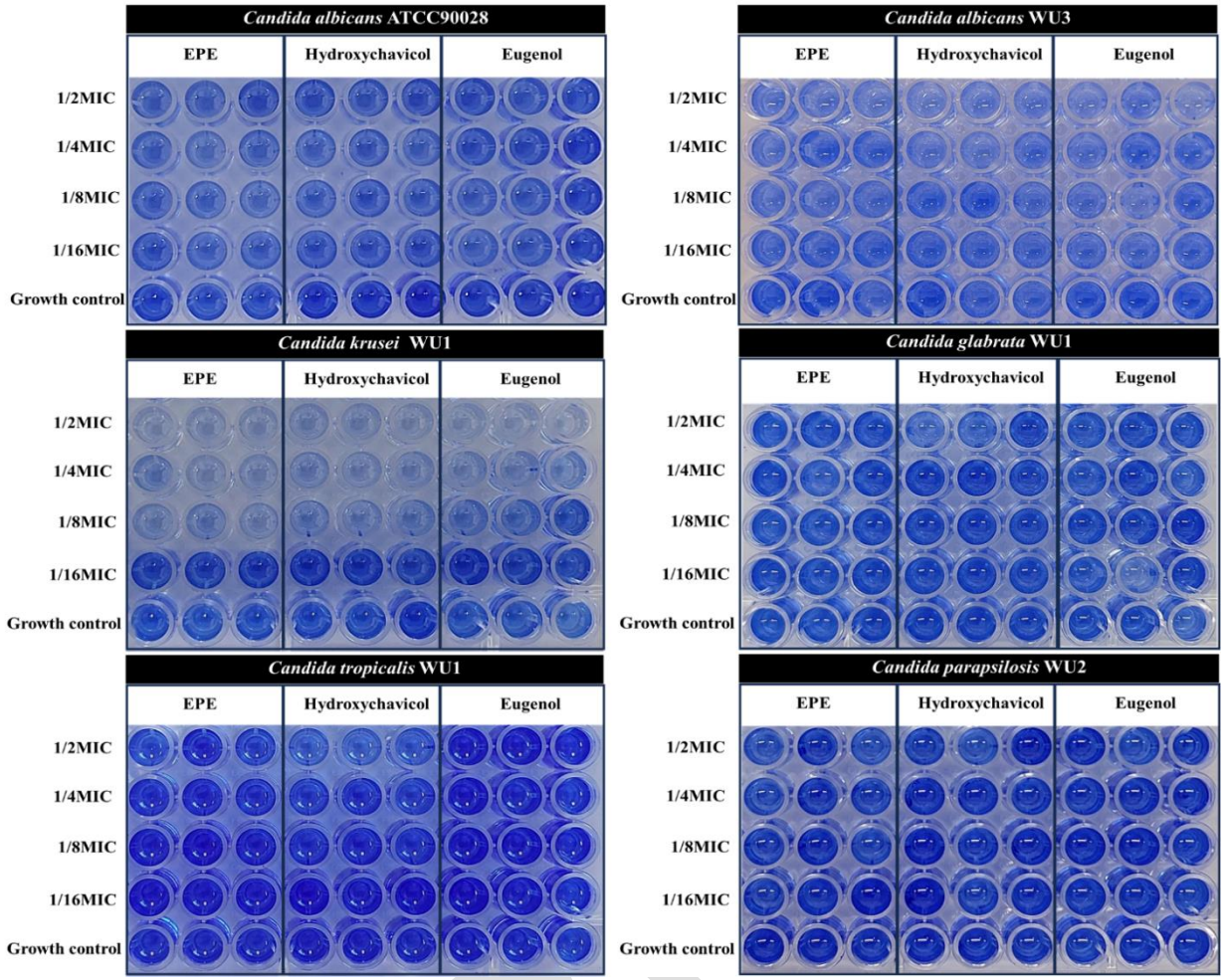

Fig. 3: Biofilm formation by six *Candida* species following treatment with ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol at sub-inhibitory concentrations (1/2, 1/4, 1/8, and 1/16MIC). Biofilm biomass was assessed by crystal violet staining after 48h incubation.

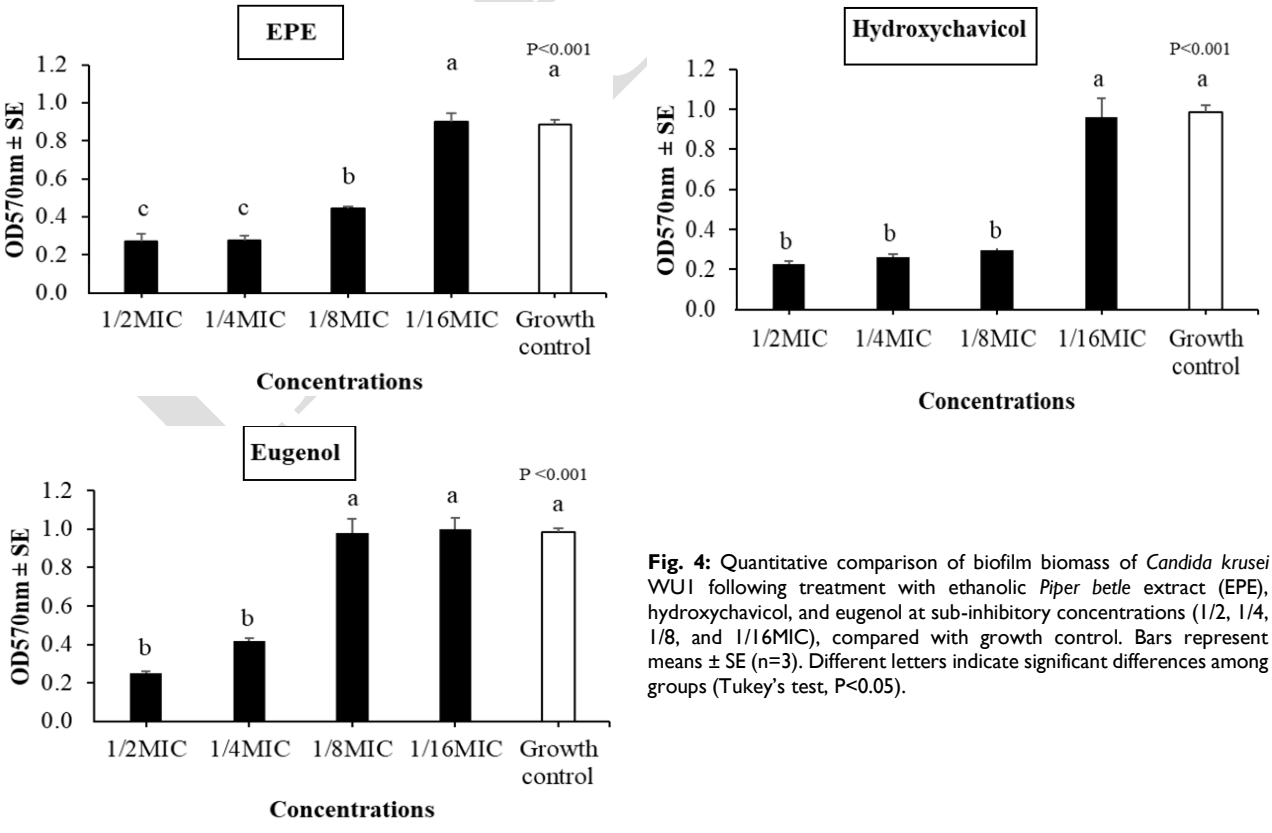

Fig. 4: Quantitative comparison of biofilm biomass of *Candida krusei* WU1 following treatment with ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol at sub-inhibitory concentrations (1/2, 1/4, 1/8, and 1/16MIC), compared with growth control. Bars represent means ± SE (n=3). Different letters indicate significant differences among groups (Tukey's test, P<0.05).

strong inhibitory effects (73.31 and 69.08%, respectively), while eugenol demonstrated moderate activity (57.55%). At 1/8MIC, hydroxychavicol and EPE still showed inhibition (69.73 and 49.73%), whereas eugenol's effect diminished dramatically (0.65%). None of the compounds inhibited biofilm formation at 1/16MIC, and EPE and eugenol even appeared to promote biofilm growth slightly. Statistical analysis confirmed significant inhibition ($P<0.05$) at 1/2 and 1/4MIC, with EPE and hydroxychavicol remaining effective at 1/8MIC. No substantial biofilm inhibition was observed in *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis*, highlighting the species-specific nature of biofilm susceptibility and the need for targeted antifungal strategies against *C. krusei*.

Effect of *P. betle* extracts on enzymatic activities: The inhibitory effects of EPE, hydroxychavicol, and eugenol on key extracellular enzymatic activities, which are virulence factors contributing to host tissue invasion and infection persistence in *Candida* spp., were investigated. *C. albicans* ATCC90028 exhibited proteinase, haemolysin, and lipase activities, whereas *C. krusei* WU1 showed lipase activity only. These two strains were selected for further enzymatic inhibition assays using substrate-specific agar media. Fungal cells were treated with sub-inhibitory concentrations of the compounds (1/2, 1/4, and 1/8MIC). In *C. albicans*, hydroxychavicol at 1/2MIC significantly reduced lipase activity (8.37% inhibition) ($P<0.05$). None of the compounds affected haemolysin and proteinase activities compared with the *C. albicans* growth control. In *C. krusei*, only EPE at 1/2MIC significantly lowered lipase activity (8.75% inhibition) ($P<0.05$). In contrast, eugenol did not inhibit enzymatic activities in either fungal strain. These findings suggest that, under the tested *in vitro* conditions, *P. betle* and its phenolic constituents can attenuate specific virulence traits in a concentration- and species-dependent manner, potentially reducing pathogenic potential without directly inhibiting fungal growth (Fig. 5 and Table 2).

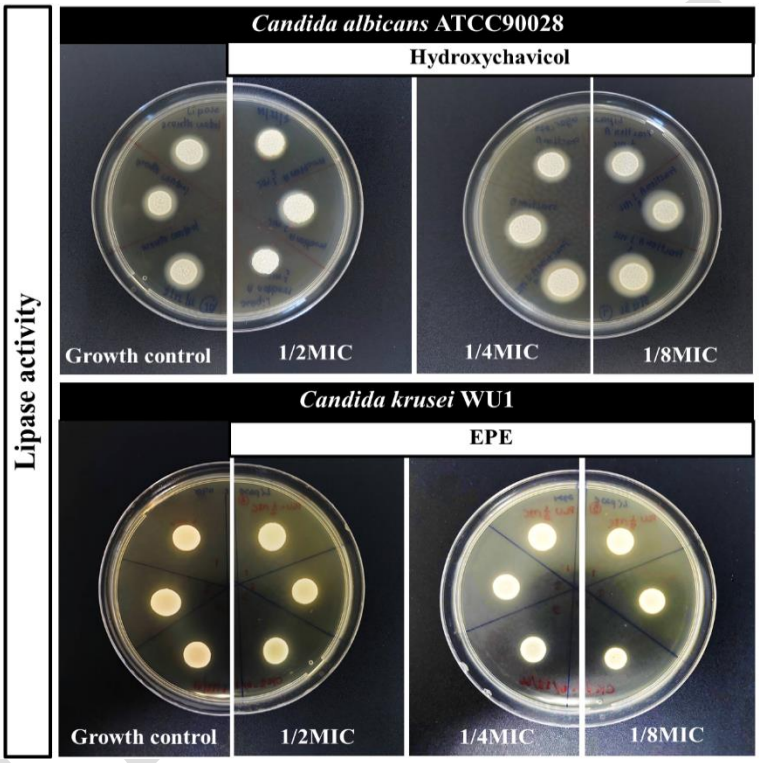

Fig. 5: Effect *Piper betle* extracts on extracellular enzyme production of *Candida* spp. Hydroxychavicol reduced lipase activity of *C. albicans* ATCC90028, and EPE inhibited lipase activity of *C. krusei* WU1 in a concentration of 1/2MIC compared with the growth control.

Table 2: Effect of ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol at sub-MIC concentrations on extracellular enzyme activities of *Candida albicans* ATCC90028 and *Candida krusei* WU1

Treatments	MICs	*Candida albicans* ATCC90028			*Candida krusei* WU1
		Proteinase activity	Haemolysin activity	Lipase activity	Lipase activity
		(Mean ± SE)	(Mean ± SE)	(Mean ± SE)	(Mean ± SE)
Cell control	0	1.374±0.022^{ns}	1.191±0.007^{ns}	1.625±0.021^{a}	1.291±0.007^{a}
EPE	1/2	1.342±0.015^{ns}	1.212±0.022^{ns}	1.567±0.041^{ab}	1.178±0.009^{b}
	1/4	1.345±0.029^{ns}	1.231±0.022^{ns}	1.590±0.034^{ab}	1.224±0.013^{ab}
	1/8	1.342±0.017^{ns}	1.221±0.031^{ns}	1.673±0.043^{a}	1.246±0.006^{ab}
Hydroxychavicol	1/2	1.401±0.057^{ns}	1.198±0.022^{ns}	1.489±0.031^{b}	1.204±0.017^{ab}
	1/4	1.381±0.024^{ns}	1.184±0.009^{ns}	1.634±0.018^{a}	1.208±0.012^{ab}
	1/8	1.355±0.021^{ns}	1.212±0.019^{ns}	1.619±0.026^{a}	1.226±0.025^{ab}
Eugenol	1/2	1.268±0.020^{ns}	1.209±0.013^{ns}	1.612±0.023^{a}	1.209±0.013^{ab}
	1/4	1.317±0.016^{ns}	1.188±0.007^{ns}	1.622±0.019^{a}	1.211±0.015^{ab}
	1/8	1.318±0.021^{ns}	1.619±0.019^{ns}	1.622±0.005^{a}	1.230±0.047^{ab}

Means in the same column within each classification bearing different letters are significantly different by Tukey's test ($P<0.05$). Abbreviations: SE; standard error, ns; non-significant, MICs; Minimal inhibitory concentration.

Inhibition of pseudohyphae and true hyphae formation: The effect of EPE, hydroxychavicol, and eugenol on hyphal transition in *C. albicans* ATCC90028 was examined under light microscopy following staining with lactophenol cotton blue. Among the six tested *Candida* species, only *C. albicans* ATCC90028 demonstrated the ability to form both pseudohyphae and true hyphae under hyphal-inducing conditions. In the untreated growth control, extensive hyphal networks and long true hyphae were observed, indicating strong morphogenetic transition (Fig. 6, top row). At 1/2MIC, all three compounds inhibited hyphal formation. Cells appeared as isolated yeast forms with no visible filamentation. This suggests a complete suppression of the yeast-to-hyphae transition at this concentration. At 1/4MIC, EPE and hydroxychavicol continued to prevent filamentation, showing mainly yeast-phase morphology. In contrast, eugenol-treated cells began to exhibit short hyphal projections and branching pseudohyphae. At 1/8MIC, filamentation began to re-emerge in all treatments. EPE and hydroxychavicol showed rare hyphal forms, whereas eugenol treatment resulted in longer and more frequent pseudohyphal structures, resembling the growth control. These findings indicate that all tested compounds inhibited hyphal transition in *C. albicans* ATCC90028 in a dose-dependent manner. Hydroxychavicol showed the most pronounced inhibitory effect, followed by EPE. Eugenol exhibited weaker suppression at lower concentrations, consistent with its concentration-dependent antifungal profile.

Cytotoxicity evaluation on Vero cells: The cytotoxic potential of EPE, hydroxychavicol, and eugenol was evaluated on normal kidney epithelial cells (Vero cells) using the MTT assay and phase-contrast microscopy. Cells were treated with five concentrations (1/8–2MIC). The MIC reference value was based on *C. albicans* ATCC90028, a standard, well-characterized strain widely used in antifungal susceptibility testing and included in all

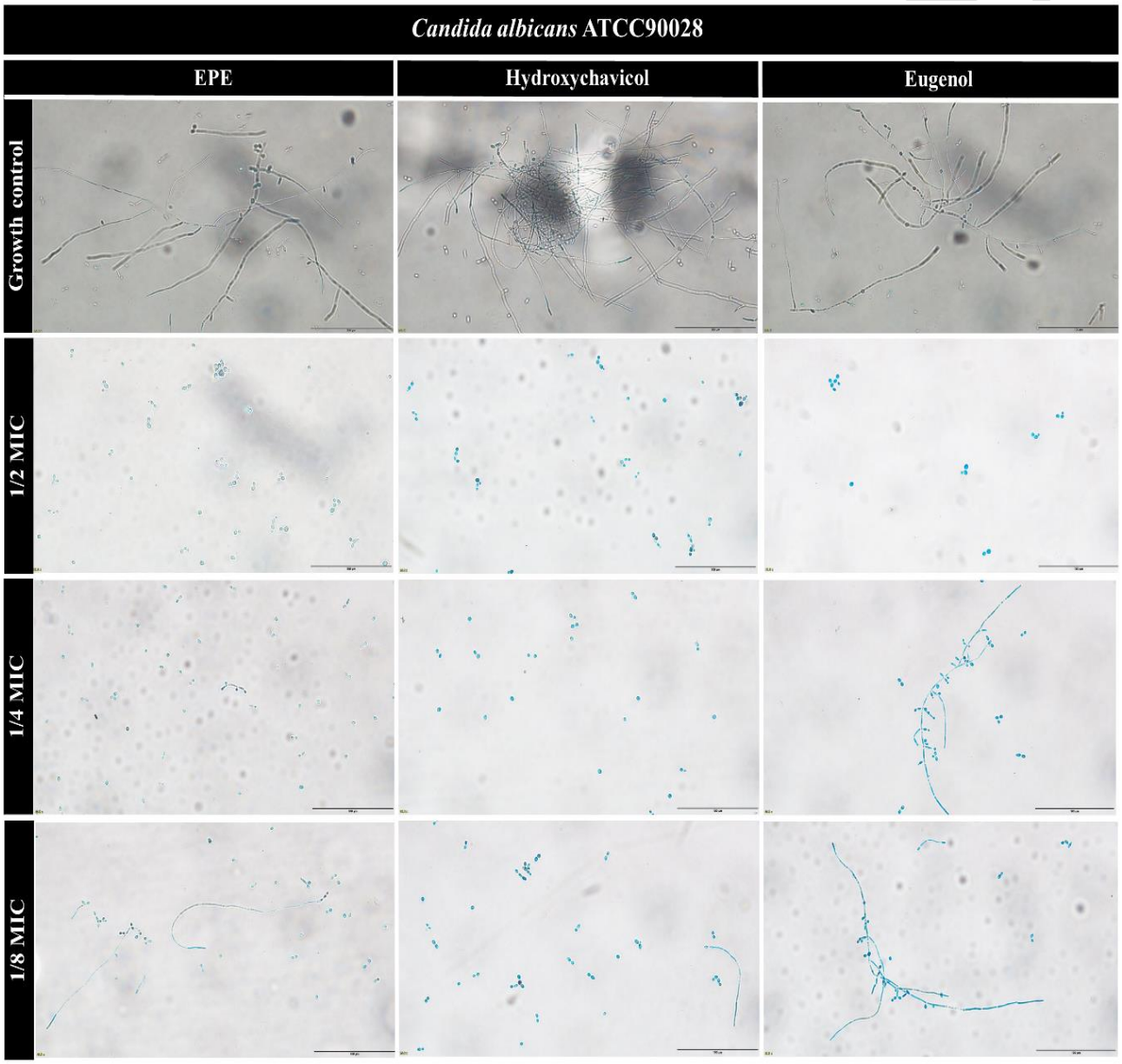

Fig. 6: Microscopic evaluation (objective lens 40×, scale bar = 100 µm) of *Candida albicans* ATCC90028 morphology following treatment with ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol at sub-inhibitory concentrations.

bioassays of this study. This concentration range was selected to encompass the MIC values of all six *Candida* strains tested, ensuring that the cytotoxicity assessment reflected the therapeutic range relevant to the entire panel of fungal isolates. Cell viability was expressed as an OD570nm-OD650nm value relative to the solvent control (2% DMSO). The results are presented in Fig. 7. At the highest tested concentration (2MIC), EPE exhibited moderate cytotoxicity, with cell viability reduced to 29.77%. In contrast, hydroxychavicol and eugenol caused severe cytotoxicity at this concentration, reducing viability to 2.27 and 2.01%, respectively. Notably, EPE maintained high biocompatibility at all concentrations below 2MIC, with cell viability consistently exceeding 98%, indicating minimal toxicity. At 1MIC, hydroxychavicol caused partial cytotoxicity (39.91%), while eugenol-treated cells showed 71.13% viability, suggesting moderate concentration-dependent toxicity. By contrast, EPE-treated cells displayed 107.27% viability, indicating no detectable adverse effects and potential proliferative support. At 1/2MIC and lower, all three compounds showed cell viabilities exceeding 95%, suggesting that these sub-MIC concentrations are generally non-toxic to Vero cells. The estimated IC_{50} values suggest EPE: No IC_{50} observed within the tested range (IC_{50}>2MIC); Hydroxychavicol: IC_{50} estimated between 1MIC and 2MIC; and Eugenol: IC_{50} approximated near 1MIC. These quantitative findings were corroborated by phase-contrast microscopy (Fig. 8). Cells treated with hydroxychavicol and eugenol at 2MIC displayed marked morphological alterations, including cell rounding, detachment, and monolayer disruption. At 1MIC, hydroxychavicol-treated cells remained largely non-adherent, whereas eugenol-treated cells began to exhibit partial restoration of normal epithelial morphology. In contrast, EPE-treated cells retained elongated and polygonal morphology across all concentrations, including 2MIC, although a partial loss of confluence was noted at the highest dose. These results indicate that while hydroxychavicol and eugenol possess significant cytotoxicity ($P<0.05$) at or above MIC, their use at sub-inhibitory concentrations could be considered safe *in vitro*. Conversely, EPE demonstrates a favorable cytotoxicity profile, making it a promising candidate for further development in antifungal applications with minimal host toxicity.

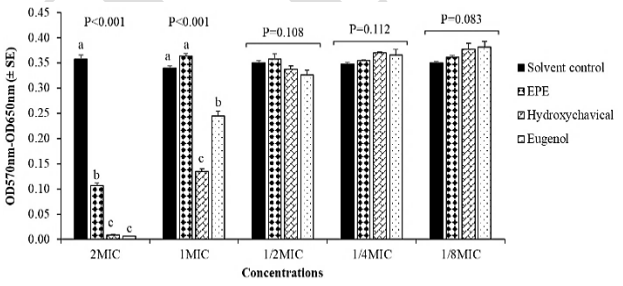

Fig. 7: Cytotoxicity profile of ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol against Vero cells at varying concentrations (2, 1, 1/2, 1/4, and 1/8MIC). Cell viability was assessed using the MTT assay after 24h of compound exposure. Bars represent the mean OD570nm-OD650nm relative to the solvent control (2% DMSO), with bars represent means ± SE (n=3). Different letters indicate significant differences among groups (Tukey's test, $P<0.05$).

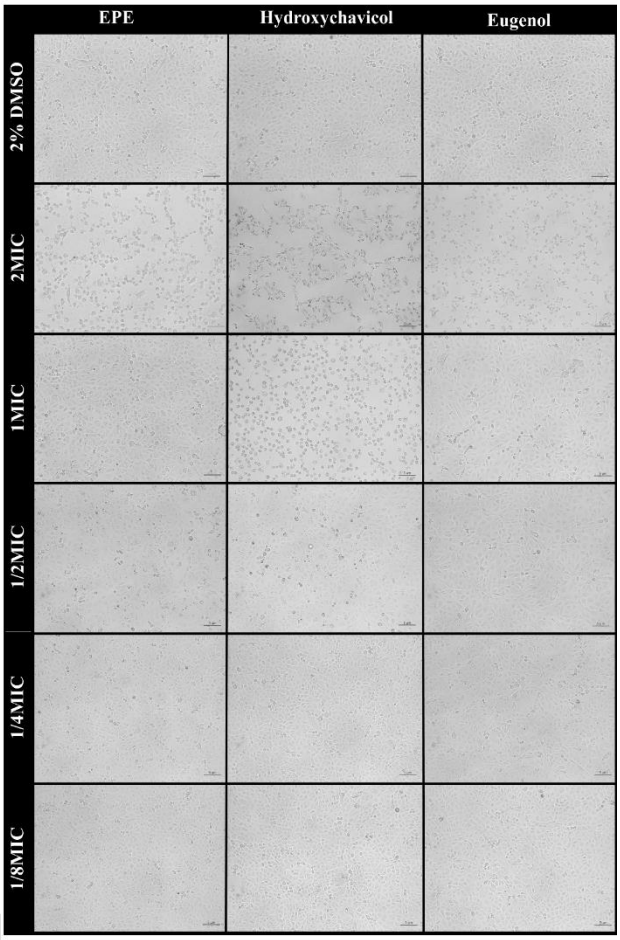

Fig. 8: Phase-contrast microscopic images of Vero cells treated with ethanolic *Piper betle* extract (EPE), hydroxychavicol, and eugenol at 2, 1, 1/2, 1/4, and 1/8MIC after 24h of incubation. The 2% DMSO control group retained normal cell morphology. Images captured at 10× objective lens; scale bar = 5µm.

DISCUSSION

This study demonstrates the antifungal and antivirulence potential of EPE, hydroxychavicol, and eugenol against *Candida* species isolated from animals. Among the tested agents, hydroxychavicol exhibited the broadest and most consistent antifungal activity, with MIC values ranging from 0.008 to 0.256mg/mL, followed by EPE and eugenol. These findings are consistent with previous reports showing that phenolic compounds such as hydroxychavicol possess potent antifungal activity against a wide range of fungal pathogens (Ali *et al*., 2010; Ali *et al*., 2016; Sivareddy *et al*., 2019; Phumat *et al*., 2020; Nayaka *et al*., 2021; Selvaraj *et al*., 2022).

The antifungal effects were species-specific. *C. krusei* WU1 and *C. glabrata* WU1 demonstrated the highest susceptibility to hydroxychavicol, while *C. tropicalis* WU1 and *C. parapsilosis* WU2 were more resistant and required higher concentrations for fungicidal activity. This variability is likely due to differences in membrane composition, efflux pump activity, and metabolic adaptability among *Candida* species (Amann *et al*., 2025). Moreover, the earlier findings that hydroxychavicol alters the cell membrane structure, resulting in the disruption of the permeability barrier of *C. albicans* membrane structures (Ali et al., 2010).

The present study demonstrates that EPE and its phenolic constituents, hydroxychavicol and eugenol, attenuate specific virulence traits of *Candida* spp. in a species- and concentration-dependent manner. The observed antivirulence mechanisms include inhibition of biofilm formation, suppression of selected extracellular enzymes, and prevention of yeast-to-hyphae transition.

Biofilm inhibition was observed only in *C. krusei* WU1, suggesting that the biofilm-disrupting effects of these compounds are also species-dependent. Hydroxychavicol achieved nearly 77% inhibition at 1/2MIC, while eugenol and EPE also showed moderate activity. The absence of inhibition in other species underscores the resilience of biofilms and the importance of identifying strain-specific strategies to target these protective structures (Kojic and Darouiche, 2004; Amann *et al*., 2025). For example, Bravo-Chaucanés *et al*. (2023) showed piperine from *P. nigrum* suppressed *C. albicans* biofilms, while Nayaka *et al*. (2021) highlighted *P. betle*'s ability to reduce microbial adhesion via interference with cell-surface hydrophobicity. Our results suggest that *C. krusei* is particularly susceptible to biofilm disruption by *P. betle* phenolics, indicating differences in cell surface composition and extracellular matrix properties between species that influence responsiveness to phenolic compounds.

Extracellular enzyme inhibition was modest and enzyme-specific. Hydroxychavicol reduced lipase activity in *C. albicans* ATCC90028, and EPE reduced lipase activity in *C. krusei* WU1, both at 1/2MIC. No compound inhibited proteinase or haemolysin production. This outcome contrasts with Sivareddy *et al*. (2019), who reported that *P. betle* extracts inhibited secreted aspartyl proteinases in *C. albicans*. The discrepancy may be due to differences in extraction methods, fungal strains, assay sensitivity, and the phenolic profiles of the extracts tested. In our study, Thai *P. betle* var. Tha-Khae phenolics did not modulate proteinase activity under the tested conditions, suggesting that their antivirulence action is directed toward specific enzymatic targets, such as lipase, rather than broadly affecting all extracellular enzymes. Phumat *et al*. (2020) found that 4-allylpyrocatechol (a hydroxychavicol derivative) from *P. betle* inhibited *C. albicans*, but its specific effects on extracellular enzymes were not quantified. Our results confirm that lipase activity is a target of *P. betle* phenolics from the Tha-Khae variety, Thailand. The present findings suggest that *P. betle* phenolics act on specific extracellular enzymes rather than exerting broad-spectrum enzyme suppression, highlighting the importance of targeted virulence assays. The limited inhibition of proteinase and haemolysin observed here may also be due to the use of sub-inhibitory concentrations and relatively short exposure times, which might be insufficient to disrupt enzyme biosynthesis or secretion (Mores *et al*., 2009).

The inhibition of hyphal and pseudohyphal formation in *C. albicans* ATCC90028 is noteworthy. All three compounds suppressed filamentation in a concentration-dependent manner, with hydroxychavicol demonstrating the strongest effect. As the yeast-to-hyphae transition is a major virulence factor in candidiasis, the ability to interfere with this process highlights the therapeutic potential of hydroxychavicol and related compounds (Ali *et al*., 2016). This mirrors the morphogenesis-inhibiting effect of piperine reported by Bravo-Chaucanés *et al*. (2023) and the filamentation suppression described for *P. betle* extracts in Nayaka *et al*. (2021). Given that morphogenesis is critical for tissue invasion and immune evasion, the potent inhibition observed here underscores hydroxychavicol's therapeutic potential. For example, Bar-Yosef *et al*. (2017) demonstrated that certain inhibitors prevent the yeast-to-hypha transition by inhibiting endocytosis, without necessarily influencing extracellular enzyme activity. Moreover, the robust suppression of hyphal development observed here suggests that morphogenetic regulation is a particularly sensitive target for *P. betle* phenolics, likely due to interference with signaling pathways such as cAMP-PKA or MAPK cascades that govern filamentation (Bravo-Chaucanés *et al*., 2023).

Cytotoxicity profiling revealed that hydroxychavicol and eugenol were highly toxic to Vero cells at 2MIC, reducing viability to below 3%. However, both compounds showed improved *in vitro* safety at sub-inhibitory concentrations, with cell viability exceeding 100% at 1/8MIC. EPE demonstrated the most favorable profile, maintaining over 98% viability at all tested concentrations below 1MIC. This improvement may be attributed to the synergistic or buffering effects of multiple phytochemicals present in the crude extract (Park *et al*., 2009).

Taken together, the results indicate that *P. betle* extract and its phenolic constituents hold promise as natural, *in vitro* safe antifungal and antivirulence agents for veterinary use. However, their effectiveness varies by species, and further *in vivo* studies are recommended to support their use in veterinary applications.

Conclusions: This study highlights the antifungal and antivirulence potential of Thai *P. betle* extract (EPE), hydroxychavicol, and eugenol against *Candida* species isolated from animals. Hydroxychavicol demonstrated the most potent and broad-spectrum antifungal activity, including low MIC and MFC values, inhibition of hyphal formation, strong anti-biofilm effects against *C. krusei*, and lipase activity, particularly in *C. albicans.* EPE exhibited moderate antifungal efficacy, inhibited lipase activity in *C. krusei*, and demonstrated high *in vitro* biocompatibility. Eugenol also showed inhibitory effects, but greater cytotoxicity at higher concentrations. The inhibitory effects on extracellular enzymes were modest and species-specific, with no impact on proteinase or haemolysin activity. Additionally, hydroxychavicol and eugenol displayed cytotoxicity at or near their MIC values, indicating that therapeutic use would require careful dose optimization or formulation strategies to reduce toxicity. Taken together, these findings support the potential application of *P. betle*, Tha-Khae variety, constituents, particularly hydroxychavicol and EPE, which demonstrated preliminary safety as an antifungal agent *in vitro*. Nevertheless, further *in vivo* research and formulation development are necessary to verify the efficacy, safety, and practicality of the product for clinical use in animals.

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REFERENCES

Ali I, Khan FG, Suri KA, *et al*., 2010. *In vitro* antifungal activity of hydroxychavicol isolated from *Piper betle* L. Ann Clin Microbiol Antimicrob 9:7.

Ali I, Satti N, Dutt P, *et al*., 2016. Hydroxychavicol: A phytochemical targeting cutaneous fungal infections. Sci Rep 6:37867.

Amann V, Kissmann AK, Firacative C, *et al*., 2025. Biofilm-associated candidiasis: pathogenesis, prevalence, challenges and therapeutic options. Pharm 18:460.

Bar-Yosef H, Vivanco Gonzalez N, Ben-Aroya S, et al., 2017. Chemical inhibitors of *Candida albicans* hyphal morphogenesis target endocytosis. Sci Rep 7:5692.

Berman J and Sudbery PE, 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. Nat Rev Genet 3:918–30.

Bhalerao S, Verma D, Gavankar R, *et al*., 2013. Phytochemistry, pharmacological profile, and therapeutic uses of *Piper betle* Linn. – an overview. Res Rev J Pharmacogn Phytochem 1:10–9.

Boripun R, Paopradit P, Prampramote J, *et al*., 2022. Bactericidal activity of *Piper betle* L. extract against antibiotic resistant *Salmonella* spp. isolated from pig farms in Southern Thailand. Vet Integr Sci 20:557–69.

Bravo-Chaucanés CP, Vargas-Casanova Y, Chitiva-Chitiva LC, *et al*., 2022. Evaluation of anti-*Candida* potential of *Piper nigrum* extract in inhibiting growth, yeast-hyphal transition, virulent enzymes, and biofilm formation. J Fungi 8:784.

Ciurea CN, Kosovski IB, Mare AD, et al., 2020. *Candida* and candidiasis – opportunism versus pathogenicity: a review of the virulence traits. Microorganisms 8:857.

CLSI. 2017. Reference method for broth dilution antifungal susceptibility testing of yeasts (CLSI standard M27), 4th Ed. Clinical and Laboratory Standards Institute, Wayne, PA, USA.

dos Santos RC and Marin JM, 2005. Isolation of *Candida* spp. from mastitic bovine milk in Brazil. Mycopathologia 159:251–3.

Freshney RI, 2015. Culture of animal cells: a manual of basic technique and specialized applications. 7th Ed, Wiley-Blackwell, Hoboken, NJ, USA.

Gow NAR, Brown AJP and Odds FC, 2002. Fungal morphogenesis and host invasion. Curr Opin Microbiol 5:366–71.

Howell SA, 2023. Dermatopathology and the diagnosis of fungal infections. Br J Biomed Sci 80:11314.

Jacobsen ID, Wilson D, Wächtler B, et al., 2012. *Candida albicans* dimorphism as a therapeutic target. Expert Rev Anti Infect Ther 10:85–93.

Jeenkeawpieam J, Rodjan P, Roytrakul S, et al., 2023. Antifungal activity of protein hydrolysates from Thai Phatthalung Sangyod rice (*Oryza sativa* L.) seeds. Vet World 16:1018–28.

Kadir T, Gumru B, Can B, et al., 2007. Phospholipase activity of *Candida albicans* isolates from patients with denture stomatitis: the influence of chlorhexidine gluconate on phospholipase production. Arch Oral Biol 52:691–6.

Kojic EM and Darouiche RO, 2004. *Candida* infections of medical devices. Clin Microbiol Rev 17:255–67.

Krukowski H, Lisowski A, Rózański P, et al., 2006. Yeasts and algae isolated from cows with mastitis in the south-eastern part of Poland. Pol J Vet Sci 9:181–4.

Kulnanan P, Chuprom J, Thomrongsuwannakij T, et al., 2021. Antibacterial, antibiofilm, and anti-adhesion activities of *Piper betle* leaf extract against avian pathogenic *Escherichia coli*. Arch Microbiol 204:49.

Lionakis MS and Kontoyiannis DP, 2003. Glucocorticoids and invasive fungal infections. Lancet 362:1828–38.

Mores A, Souza R, Cavalca L, et al., 2009. Enhancement of secretory aspartyl protease production in biofilms of *Candida albicans* exposed to sub-inhibitory concentrations of fluconazole. Mycoses 54:195–201.

Nayaka NMDMW, Sasadara M, Sanjaya D, et al., 2021. *Piper betle* (L): Recent review of antibacterial and antifungal properties, safety profiles, and commercial applications. Molecules 26:2321.

Nordin F, Wan Harun HA, Razak FA, et al., 2014. Growth inhibitory response and ultrastructural modification of oral-associated candidal reference strains (ATCC) by *Piper betle* L. extract. Int J Oral Sci 6:15.

Park H, Myers CL, Sheppard DC, et al., 2009. Role of the fungal-specific subunits of the mitochondrial cytochrome bc1 complex in *Candida albicans* pathogenesis. PLoS Pathog 5:e1000350.

Pattnaik S, Maharana L and Sethi M, 2021. Pathogenicity mechanism of *Candida albicans*. In: Advances in *Candida albicans* (IntechOpen, ed). IntechOpen, London, UK, pp:1–20.

Pfaller MA and Diekema DJ, 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20:133–63.

Phumat P, Khongkhunthian S, Wanachantararak P, et al., 2020. Comparative inhibitory effects of 4-allylpyrocatechol isolated from *Piper betle* on *Streptococcus intermedius, Streptococcus mutans,* and *Candida albicans*. Arch Oral Biol 113:104690.

Schulthess B, Bloemberg GV and Zbinden R, 2014. Bacterial and fungal identification by MALDI-TOF MS in clinical microbiology. FEMS Microbiol Rev 38:253–71.

Selvaraj GK, Wilson J, Kanagaraj N, *et al*., 2022. Enhanced antifungal activity of *Piper betle* against candidiasis infection causing *Candida albicans* and *in silico* analysis with its virulent protein. Biomed Biotechnol Res J 6:73–80.

Shin JH, Kee SJ, Shin MG, *et al*., 2002. Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources. J Clin Microbiol 40:1244–8.

Sivareddy B, Reginald BA, Sireesha D, *et al*., 2019. Antifungal activity of solvent extracts of *Piper betle* and *Ocimum sanctum* Linn on *Candida albicans*: an *in vitro* comparative study. J Oral Maxillofac Pathol 23:333–7.

Sungkatavat P, Khongkhai H, Kanchana W, *et al*., 2023. *Piper betle* extract and its application in bovine teat dipping solution inhibit and eliminate biofilms in bovine mastitis-inducing staphylococci. Vet World 16:2135–42.

Swaminathan R and Kamath N, 2023. *Candida* – an emerging and re-emerging pathogen. IP Int J Med Microbiol Trop Dis 9:6–9.

Sykes JE, 2013. Candidiasis. In: Canine and Feline Infectious Diseases (Sykes JE, ed). Saunders, St. Louis, USA, pp:653–9.