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Ethanolic Extract of Butterfly Pea Flower (*Clitoria ternatea* L.) as an Antifungal and Antibiofilm Agent Against *Candida albicans* ATCC 10231

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ABSTRACT

Candida albicans is the leading cause of fungal infections worldwide, with mortality rates reaching up to 60%. Biofilm formation by C. albicans makes infections more difficult to treat and increases resistance to antifungal agents. The ethanolic extract of butterfly pea flower (Clitoria ternatea L.) has potential as an antifungal and antibiofilm agent, making it a promising alternative treatment. This study aimed to determine the antifungal and antibiofilm activity of butterfly pea flower ethanolic extract against C. albicans ATCC 10231. The research employed a true experimental method with a post-test only control group design. The antifungal assay used the microdilution method to determine the Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC), while the antibiofilm assay used the Microtiter Plate Assay to determine $MBIC_{50}$ and $MBRC_{50}$. The concentrations tested were 5, 10, 20, 40, and 80 mg/mL, with fluconazole 6.25 µg/mL as the positive control. The results showed an MFC value at 20 mg/mL. The $MBIC_{50}$ and $MBRC_{50}$ values were both at 10 mg/mL, with inhibition and reduction percentages of 72.28% and 65.18%, respectively. The ethanolic extract of butterfly pea flower exhibited better antifungal and antibiofilm activity than the positive control fluconazole at 6.25 µg/mL. The ethanolic extract of butterfly pea flower has the potential to be developed as an antifungal and antibiofilm agent.

1. INTRODUCTION

Invasive fungal infections are a growing global health problem, driven by an expanding population of immunocompromised patients, the widespread use of broad-spectrum antibiotics, and the increasing use of invasive medical procedures. One factor that aggravates the management of these infections is the ability of microorganisms to form biofilms. A biofilm is a complex community of microorganisms—both bacteria and fungi—enclosed within an extracellular matrix and attached to biotic or abiotic surfaces (Sauer et al., 2022). Biofilm formation by microorganisms is closely associated with chronic infection, treatment failure, and increased antimicrobial resistance. Antimicrobial resistance reduces therapeutic effectiveness and is accompanied by increased morbidity and mortality. The high rate of irrational antimicrobial drug consumption, together with the capacity for biofilm formation, are two mutually reinforcing factors driving the global rate of antimicrobial resistance.

One of the clinically important biofilm-forming pathogens is the fungus *Candida albicans* (*C. albicans*) (Ponde et al., 2021). *Candida albicans* is the most common cause of fungal infection and is responsible for approximately 70% of fungal infections worldwide. This fungus is a commensal member of the oral cavity, gastrointestinal tract, and urogenital tract, but it can become an opportunistic pathogen when the balance of the microbiota is disrupted or host

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immunity declines. *Candida albicans* infections can manifest as anything from mild mucocutaneous candidiasis to life-threatening systemic candidemia. Candidemia is even recorded as the fourth most common cause of bloodstream infection, with a mortality rate of >40% in the United States and Europe, while in the Asia-Pacific region the mortality rate of candidemia in intensive care units (ICUs) reaches 60% (Atiencia-Carrera et al., 2022; Vitális et al., 2020).

This high mortality rate is largely attributable to the virulence factors of *C. albicans*, particularly its ability to form biofilms (Dewi et al., 2020). Biofilms protect fungal cells from the host immune system and from exposure to antifungal drugs through several mechanisms: impaired drug penetration into the extracellular matrix, phenotypic changes of cells within the biofilm, the presence of drug-tolerant *persister* cells, and increased expression of efflux pumps (Dewi et al., 2020; Hamzah et al., 2019). As a result, managing biofilm-associated infections requires drug concentrations 10–1,000 times higher than those needed to kill ordinary planktonic cells (Nyoman et al., 2023). This condition further drives the rise of antimicrobial resistance and ultimately increases mortality. Several studies have reported that *C. albicans* is resistant to various classes of antifungal agents, including the azoles (voriconazole), the polyenes (amphotericin B), and flucytosine (Hossain et al., 2022).

Clinically, the ability of *C. albicans* to form biofilms on mucosal surfaces and on medical devices such as catheters, dental prostheses, and implants makes it a major source of persistent infection, recurrence, and treatment failure, which in turn leads to prolonged hospitalization, higher treatment costs, and increased mortality (Vitális et al., 2020). These clinical consequences make the *C. albicans* biofilm an important target in the development of new antifungal agents. To obtain valid and comparable results, evaluation of antifungal candidates ideally uses a standardized reference strain. This study selected *C. albicans* ATCC 10231 because it is a reference (quality-control) strain that is widely used in antifungal susceptibility testing and biofilm formation studies, forms biofilm consistently and reproducibly, and allows the results of this study to be compared directly with those of other laboratories and studies.

To date, no antifungal agent is both truly effective and safe for treating biofilm-associated fungal infections, so there is a need to search for new sources of compounds that are more effective, safe, and non-toxic. The use of natural materials, particularly medicinal plants, is one promising strategy because of their rich secondary metabolite content. Various plant extracts have been reported to have antifungal activity against *C. albicans*, both by inhibiting cell growth and by inhibiting biofilm formation.

Butterfly pea flower (*Clitoria ternatea* L.) is one of the medicinal plants with potential as an antifungal and antibiofilm agent because of its bioactive compound content (Widjajanti, 2023). Butterfly pea flower contains bioactive components such as flavonoids, tannins, saponins, terpenoids, alkaloids, and anthocyanins, which are known to have a range of pharmacological activities, including antioxidant, antidiabetic, antihistamine, anti-inflammatory, analgesic, anticancer, and antimicrobial effects (Li et al., 2022; Sholihatunnisa and Ramadhania, 2024). Flavonoids have been reported to have significant antifungal and antibiofilm activity against *C. albicans* through inhibition of ergosterol synthesis and induction of oxidative stress, whereas terpenoids show antifungal activity against *C. albicans* in in vivo models (Dantas et al., 2025). Several studies have also demonstrated the effectiveness of butterfly pea flower extract as an antibiofilm agent against bacteria such as *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, and *Bacillus cereus* (Dantas et al., 2025; Jeyaraj et al., 2022; Novicadlitha et al., 2025; Satria et al., 2022).

Several of the main compounds in butterfly pea flower are known to have mechanisms directly relevant to *C. albicans*. Flavonoids inhibit ergosterol synthesis—the principal sterol of the fungal cell membrane—and trigger the formation of reactive oxygen species (ROS), while also downregulating the expression of adhesin genes (for example, *YWP1*) and inhibiting quorum sensing, which plays a role in the early stage of biofilm formation. The lipophilic saponins and terpenoids disrupt the integrity and permeability of the cell membrane, causing leakage of cell contents, while tannins interfere with the formation of the biofilm extracellular matrix and bind to cell-wall proteins. By combining membrane disruption, inhibition of ergosterol synthesis, reduced adhesion, and disruption of the biofilm matrix, this combination of compounds has the

potential to suppress *C. albicans* in both the planktonic and biofilm phases ((Aboody and Mickymaray, 2020; Nyoman et al., 2023; Salinas et al., 2022).

Nevertheless, most of the available evidence for the activity of butterfly pea flower against *C. albicans* is still qualitative (generally using the disc diffusion method) and limited to the inhibition of planktonic cells. Quantitative data that evaluate activity against planktonic cells (MIC and MFC) together with activity against biofilms ($MBIC_{50}$ and $MBRC_{50}$) within a single study are still rarely reported, so this study is expected to help fill that gap.

Interestingly, the direction of the effect of butterfly pea flower extract concentration on microbial inhibition is still inconsistent across studies. Some studies report that the higher the extract concentration, the greater the inhibition, as in tests against *S. aureus* and *S. mutans* that used concentrations of 12.5, 25, 50, and 100 mg/mL (Nabila et al., 2022). Conversely, another study that used an aqueous butterfly pea flower extract at concentrations of 50%, 75%, and 100% against *C. albicans* with the disc diffusion method actually showed decreasing inhibition at higher concentrations (Widjajanti, 2023). In addition, studies on the activity of butterfly pea flower extract as both an antifungal and antibiofilm agent against *C. albicans*, particularly using dilution methods that can quantitatively determine MIC, MFC, $MBIC_{50}$, and $MBRC_{50}$ values, are still very limited. Therefore, this study aimed to determine the activity of butterfly pea flower ethanolic extract (*Clitoria ternatea* L.) as an antifungal and antibiofilm agent against *C. albicans* ATCC 10231. The novelty of this study lies in the simultaneous evaluation of antifungal (MIC and MFC) and antibiofilm ($MBIC_{50}$ and $MBRC_{50}$) activity of butterfly pea flower ethanolic extract against *C. albicans* using the microdilution method and the Microtiter Plate Assay, which has not been widely reported previously.

2. METHOD

This was a true experimental laboratory study using a post-test only control group design that compared a control group and a treatment group. The control group consisted of a negative control (fungal suspension without treatment), a positive control (fluconazole 6.25 µg/mL), a medium control (RPMI 1640 + 5% glucose), and a solvent control (5% DMSO), while the treatment group consisted of butterfly pea flower extract at concentrations of 5, 10, 20, 40, and 80 mg/mL.

The study subject was a culture of *Candida albicans* ATCC 10231 from the collection of the Microbiology Laboratory, Faculty of Medicine, Universitas Jenderal Soedirman. The main material was butterfly pea flower ethanolic extract (*Clitoria ternatea* L.), which had undergone phytochemical screening and contained flavonoids, tannins, alkaloids, terpenoids, and saponins. Other materials and reagents included Sabouraud Dextrose Agar (SDA), RPMI 1640 medium, glucose, sterile 0.9% NaCl, dimethyl sulfoxide (DMSO), fluconazole, 96% methanol, 96% ethanol, 1% crystal violet, methylene blue, phosphate-buffered saline (PBS), and sterile distilled water. The main equipment used included a rotary evaporator, 96-well microplates, an ELISA reader, an incubator, a colony counter, and a microscope.

Butterfly pea flowers were obtained from a supplier in Masaran, Sragen, Central Java, and a determination test was performed to confirm the plant identity. Extraction was carried out by maceration using 70% ethanol at the Pharmacology Laboratory, Faculty of Medicine, Universitas Jenderal Soedirman. A total of 11,400 g of fresh butterfly pea flowers was dried and ground, then macerated with 70% ethanol for 3 × 24 h with periodic stirring. The filtrate was collected and evaporated using a rotary evaporator at 40–50 °C to obtain 135 g of thick extract (yield 1.18%) (Jaafar et al., 2020). The extract was stored in a closed container at 4 °C until use.

C. albicans ATCC 10231 isolates were grown on SDA and incubated at 37 °C for 24 h. The colonies that grew were identified based on colony and cell morphology. Cell morphology identification was performed by methylene blue staining and microscopic observation to confirm the fungal cell shape and the presence of budding yeast. The fungal suspension was prepared by dissolving colonies in sterile 0.9% NaCl until reaching a turbidity equivalent to a 0.5 McFarland standard (equivalent to ± 10^8 CFU/mL).

The biofilm-forming ability of *C. albicans* ATCC 10231 was tested using the Microtiter Plate Assay. A fungal suspension (100 µL, 10^6 CFU/mL) was added to a 96-well microplate containing

100 µL of RPMI 1640 + 5% glucose medium, then incubated at 37 °C for 24 h. After incubation, the wells were rinsed with PBS to remove planktonic cells, fixed with 96% methanol for 15 min, and stained with 1% crystal violet for 15 min. The bound crystal violet was dissolved with 96% ethanol, and the optical density (OD) was measured at a wavelength of 620 nm using an ELISA reader. Biofilm formation categories were determined using the formula OD_c = mean OD of the medium control + (3 × standard deviation of the medium control), with categories: OD ≤ OD_c (no biofilm production); OD_c < OD ≤ 2×OD_c (weak); 2×OD_c < OD ≤ 4×OD_c (moderate); and 4×OD_c < OD (strong) (Kırmusaoğlu, 2019).

The MIC and MFC tests were performed using the microbroth dilution method according to Clinical and Laboratory Standards Institute (CLSI) standards (Cuenca-Estrella and Rodriguez-Tudela, 2010). The extract was dissolved in 5% DMSO to prepare a 160 mg/mL stock. A 100 µL aliquot of extract at various concentrations (5, 10, 20, 40, and 80 mg/mL) was mixed with 100 µL of fungal suspension (10^6 CFU/mL) in RPMI 1640 + 5% glucose medium in a 96-well microplate, together with the positive, negative, solvent, and medium controls, then incubated at 37 °C for 24 h. The MIC value was determined from the lowest concentration showing clarity. For the MFC, 10 µL of suspension from the clear wells was cultured on SDA using the spread plate method and incubated at 37 °C for 24 h (Setiawati et al., 2017). Colonies were counted with a colony counter. The percentage of viability = (number of treatment colonies / number of negative control colonies) × 100%, and the percentage of inhibition = 100% − percentage of viability. The MFC value was set at the concentration with viability <1% and inhibition >99%.

The $MBIC_{50}$ test determined the minimum concentration that inhibited 50% of biofilm formation using the Microtiter Plate Assay (Nuryastuti et al., 2018). The fungal suspension was mixed with extract at various concentrations in RPMI 1640 + 5% glucose medium and incubated at 37 °C for 24 h to allow biofilm formation, then rinsed with PBS, fixed with 96% methanol, and stained with 1% crystal violet. After dissolving with 96% ethanol, the OD was measured at 620 nm. The percentage of biofilm inhibition = [(OD of negative control − OD of sample) / (OD of negative control − OD of blank)] × 100%. The $MBIC_{50}$ value is the lowest concentration that inhibits ≥50% of biofilm formation.

The $MBRC_{50}$ test determined the minimum concentration that reduced 50% of preformed biofilm (Setiawati et al., 2024). *C. albicans* biofilm was first grown for 24 h at 37 °C, then rinsed with PBS and treated with extract at various concentrations, and incubated again at 37 °C for 24 h. The remaining biofilm was rinsed, fixed, stained with crystal violet, and the OD was measured at 620 nm. The percentage of biofilm reduction = [(OD of negative control − OD of sample) / (OD of negative control − OD of blank)] × 100%. The $MBRC_{50}$ value is the lowest concentration that reduces ≥50% of biofilm.

Data were analyzed using the Statistical Product and Service Solutions (SPSS) version 27. Homogeneity was tested using Levene's test, while normality was tested using the Shapiro–Wilk test because the sample size was <30. The $MBIC_{50}$ data were normally distributed but had non-homogeneous variance, so the Welch ANOVA was used, followed by the Games–Howell post hoc test. The $MBRC_{50}$ data were not normally distributed, so the non-parametric Kruskal–Wallis test was used, followed by Dunn's post hoc test. A significant difference was set at $p \leq 0.05$.

3. RESULT AND DISCUSSION

Result

Identification of Candida albicans ATCC 10231

Colony morphology identification showed cream-white colonies with a smooth surface on SDA (Figure 1A), while cell morphology identification using methylene blue revealed oval-shaped fungal cells with budding yeast (Figure 1B), which is characteristic of *C. albicans*.

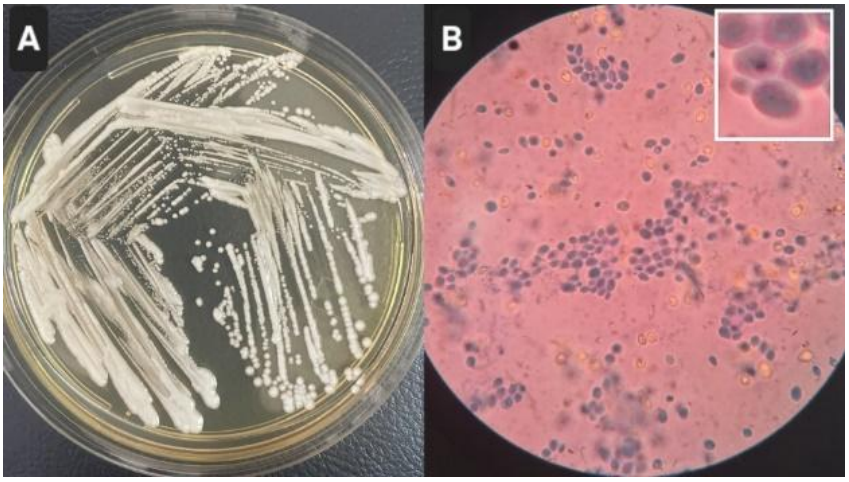

Figure 1. Morphological identification of *Candida albicans* ATCC 10231. (A) Colony morphology on SDA; (B) Cell morphology with methylene blue staining.

Biofilm Formation by Candida albicans ATCC 10231

Candida albicans biofilm was grown on RPMI 1640 + 5% glucose medium for 24 h at 37 °C, and the optical density (OD) was then read at a wavelength of 620 nm. The OD values of biofilm formation are presented in Table I.

Table I. OD values of biofilm formation by *Candida albicans* ATCC 10231

Well	Mean ± SD
C. albicans ATCC 10231	1.07 ± 0.39
Medium control	0.21 ± 0.06
Blank	0.04 ± 0.00

The biofilm-forming ability was determined by comparing the OD of the fungal isolate with the OD_c value. Based on the calculation, OD_c = 0.39, so *C. albicans* ATCC 10231 was classified as a moderate biofilm former. This is consistent with other studies reporting that growing *C. albicans* biofilm at a lower temperature (25 °C) with a longer incubation time (48–72 h) produces greater biofilm formation.

Minimum Inhibitory Concentration (MIC) of Butterfly Pea Flower Extract

MIC determination was performed using the microbroth dilution method by observing the turbidity of each well (Figure 2). The MIC was determined from the lowest concentration that appeared clear.

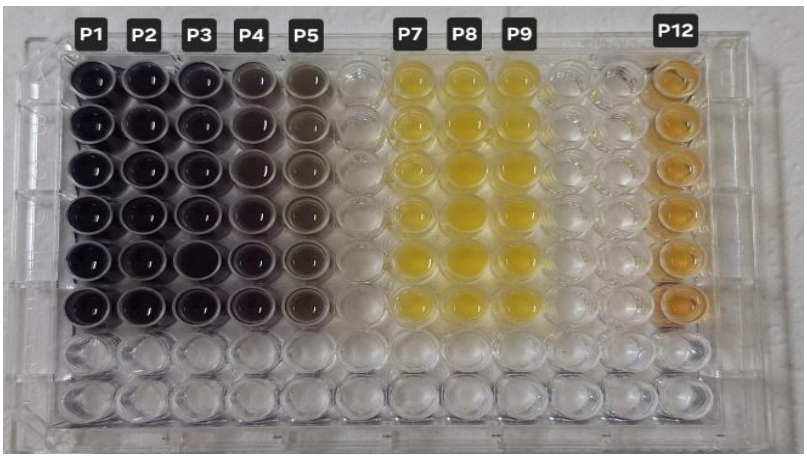

Figure 2. MIC test of butterfly pea flower extract in a 96-well microplate. P1: extract 80 mg/mL; P2: 40 mg/mL; P3: 20 mg/mL; P4: 10 mg/mL; P5: 5 mg/mL; P7: positive control (fluconazole 6.25 µg/mL); P8: negative control; P9: solvent control (5% DMSO); P12: medium control (RPMI 1640 + 5% glucose).

Based on Figure 2, the clarity of the wells could not be determined visually because all concentrations appeared turbid. This was due to the intense color of the extract, which made it difficult to assess the turbidity level of each concentration, so the MIC value could not be established visually in this study.

Minimum Fungicidal Concentration (MFC) of Butterfly Pea Flower Extract

The results of the MFC test of butterfly pea flower extract against *C. albicans* ATCC 10231 are presented in Table II. The MFC value was found at a concentration of 20 mg/mL, with 0.91% viability and 99.09% inhibition. The 5 mg/mL concentration showed 98.79% inhibition and the 10 mg/mL concentration showed 80.36%, while the positive control fluconazole 6.25 µg/mL showed 64.05%. However, at higher concentrations (40 and 80 mg/mL) more colony growth was observed than in the negative control, with viabilities of 173.72% and 215.41%, respectively.

Table 2. Killing percentage of butterfly pea flower extract against *Candida albicans* ATCC 10231

Group	Colony count	Viability (%)	Killing (%)
80 mg/mL	713	215.41	−115.41
40 mg/mL	575	173.72	−73.72
20 mg/mL	3	0.91	99.09
10 mg/mL	65	19.64	80.36
5 mg/mL	4	1.21	98.79
Positive control (fluconazole 6.25 µg/mL)	119	35.95	64.05
Negative control	331	100.00	0.00

The MFC value is determined from a viability of <1% and inhibition of >99%. Accordingly, the MFC of butterfly pea flower extract in this study was at a concentration of 20 mg/mL. The fungicidal activity of the extract at this concentration was even better than that of the positive control fluconazole 6.25 µg/mL, which inhibited only 64.05%.

$MBIC_{50}$ of Butterfly Pea Flower Extract Against Biofilm Formation

The $MBIC_{50}$ test results showed that the lowest extract concentration that inhibited ≥50% of biofilm formation was 10 mg/mL, with an inhibition percentage of 72.28% (Table III and Figure 3). The highest inhibition was found at 20 mg/mL (74.48%). However, at higher concentrations (40 and 80 mg/mL) the inhibition percentage decreased to 53.38% and 47.94%. The positive control fluconazole 6.25 µg/mL inhibited 64.97%. Statistical analysis showed a significant difference between the 10 mg/mL extract and the negative control ($p < 0.05$).

Table 3. OD values of the activity of butterfly pea flower extract against biofilm formation by *C. albicans* ATCC 10231

Group	Mean OD ± SD	Biofilm inhibition (%)
80 mg/mL	1.30 ± 0.10	47.94
40 mg/mL	1.17 ± 0.15	53.38
20 mg/mL	0.66 ± 0.19	74.48
10 mg/mL	0.71 ± 0.16	72.28
5 mg/mL	1.89 ± 0.21	23.31
Positive control (fluconazole 6.25 µg/mL)	0.89 ± 0.04	64.97
Negative control	2.46 ± 0.12	0.00

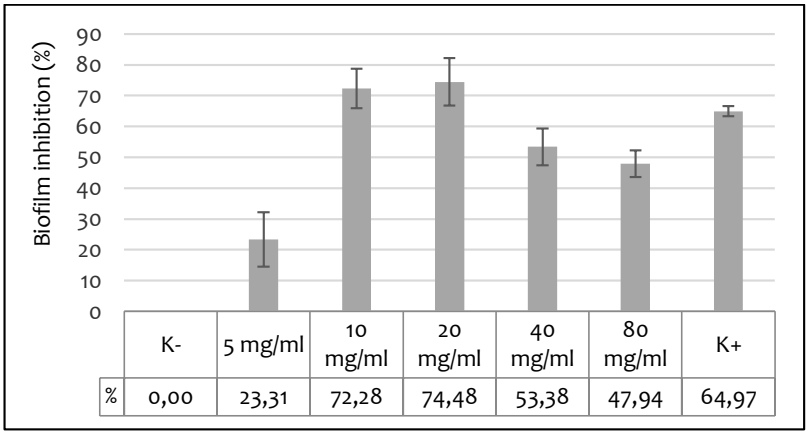

Figure 3. Percentage of biofilm inhibition of *Candida albicans* ATCC 10231 by butterfly pea flower extract (K-: negative control; K+: positive control, fluconazole 6.25 µg/mL).

Inhibition of biofilm formation by the 10 mg/mL butterfly pea flower extract was 72.28%, better than the positive control fluconazole 6.25 µg/mL (64.97%), confirming the potential of the extract as an antibiofilm agent.

$MBRC_{50}$ of Butterfly Pea Flower Extract Against Biofilm

The $MBRC_{50}$ test results showed that the lowest extract concentration that reduced ≥50% of preformed biofilm was 10 mg/mL, with a reduction percentage of 65.18% (Table IV and Figure 4). This concentration also showed the highest reduction. At higher concentrations (20, 40, and 80 mg/mL) the reduction percentage decreased to 36.51%, 42.20%, and 14.47%. The positive control fluconazole 6.25 µg/mL reduced 57.52%. Statistical analysis showed a significant difference between the 10 mg/mL extract and the negative control ($p < 0.05$).

Table 4. Biofilm reduction percentage of butterfly pea flower extract against *C. albicans* ATCC 10231

Group	Mean OD ± SD	Biofilm reduction (%)
80 mg/mL	0.66 ± 0.04	14.47
40 mg/mL	0.46 ± 0.07	42.20
20 mg/mL	0.50 ± 0.04	36.51
10 mg/mL	0.29 ± 0.04	65.18
5 mg/mL	0.54 ± 0.03	31.60
Positive control (fluconazole 6.25 µg/mL)	0.35 ± 0.04	57.52
Negative control	0.77 ± 0.25	0.00

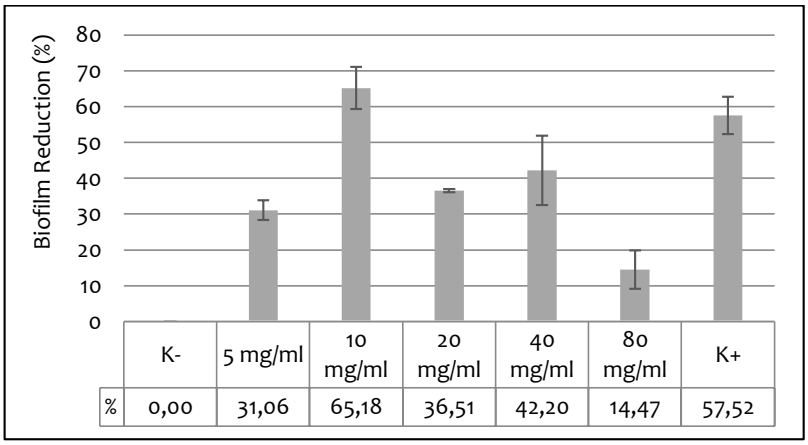

Figure 4. Percentage of biofilm reduction of *Candida albicans* ATCC 10231 by butterfly pea flower extract (NC: negative control; PC: positive control, fluconazole 6.25 µg/mL).

Biofilm reduction by the 10 mg/mL butterfly pea flower extract was 65.18%, also better than the positive control fluconazole 6.25 µg/mL (57.52%).

Bivariate Analysis of Biofilm Inhibition Activity

The biofilm inhibition percentage data were tested for normality (Shapiro–Wilk) and homogeneity (Levene's test). The data were normally distributed but not homogeneous, so the Welch ANOVA was used, yielding p = 0.000 (p ≤ 0.05), indicating a significant difference between groups, followed by the Games–Howell post hoc test (Table V).

Table 5. Results of the Games–Howell post hoc test for butterfly pea flower extract

Conc. (mg/mL)	80	40	20	10	5	PC	NC
80	–	0.894	0.084	0.061	0.149	0.074	0.002*
40	0.894	–	0.166	0.159	0.085	0.326	0.003*
20	0.084	0.166	–	1.000	0.016*	0.606	0.003*
10	0.061	0.159	1.000	–	0.018*	0.661	0.001*
5	0.149	0.085	0.016*	0.018*	–	0.062	0.167
PC	0.074	0.326	0.606	0.661	0.062	–	0.004*
NC	0.002*	0.003*	0.003*	0.001*	0.167	0.004*	–

*Note: * = p ≤ 0.05; PC = positive control (fluconazole 6.25 µg/mL); NC = negative control.*

Bivariate Analysis of Biofilm Reduction Activity

The biofilm reduction percentage data were tested using the Shapiro–Wilk test and yielded p ≤ 0.05 for the 10, 40, and 80 mg/mL groups, so the data were not normally distributed. Therefore, the non-parametric Kruskal–Wallis test was used, yielding p = 0.003 (p < 0.05), indicating a significant difference between groups, followed by Dunn's post hoc test (Table VI).

Table 6. Results of Dunn's post hoc test for butterfly pea flower extract

Conc. (mg/mL)	80	40	20	10	5	PC	NC
80	–	0.122	0.207	0.003*	0.439	0.012*	1.000
40	0.122	–	0.777	0.149	0.439	0.327	0.122
20	0.207	0.777	–	0.084	0.624	0.207	0.207
10	0.003*	0.149	0.084	–	0.027*	0.643	0.003*
5	0.439	0.439	0.624	0.027*	–	0.080	0.439
PC	0.012*	0.327	0.207	0.643	0.080	–	0.012*
NC	1.000	0.122	0.207	0.003*	0.439	0.012*	–

*Note: * = p ≤ 0.05; PC = positive control (fluconazole 6.25 µg/mL); NC = negative control.*

Discussion

This study showed that butterfly pea flower ethanolic extract (*Clitoria ternatea* L.) has antifungal activity with an MFC value of 20 mg/mL, as well as antibiofilm activity with $MBIC_{50}$ and $MBRC_{50}$ values of 10 mg/mL each against *C. albicans* ATCC 10231. Interestingly, at these optimal concentrations the extract showed better activity than the positive control fluconazole 6.25 µg/mL, in terms of both antifungal and antibiofilm effects. These findings are consistent with previous studies reporting the antimicrobial potential of butterfly pea flower extract against various microorganisms (Jeyaraj et al., 2022; Nabila et al., 2022; Novicadlitha et al., 2025; Nyoman et al., 2023; Satria et al., 2022).

The antifungal activity of butterfly pea flower extract is mainly due to its bioactive compound content, namely flavonoids, tannins, saponins, terpenoids, and alkaloids (Jaafar et al., 2020; Nurcholis et al., 2023; Rahma et al., 2024). Flavonoids exert an antifungal mechanism by inhibiting ergosterol synthesis—an important component of the fungal cell membrane—as well as inducing the formation of reactive oxygen species (ROS) that trigger fungal cell apoptosis

(Aboody and Mickymaray, 2020). Alkaloids denature membrane proteins, causing changes in permeability and leakage of cell contents. Saponins, through their monosaccharide groups, disrupt cell membrane stability, increase permeability, and induce cell lysis (Chen et al., 2023; Shakeel et al., 2025). Tannins disrupt the lipid structure and amino acids of the fungal cell wall, while terpenoids, with their lipophilic properties, interact directly with the lipid components of the fungal cell membrane and cause structural damage (Moreira et al., 2024). This combination of compounds with different targets is thought to produce additive to synergistic effects, so that the extract can be more effective than fluconazole, which acts on a single target (inhibition of the enzyme lanosterol 14α-demethylase in the ergosterol synthesis pathway).

Although butterfly pea flower extract in this study showed "better" antifungal and antibiofilm activity than fluconazole, this remains contextual and cannot be interpreted as absolute superiority. Butterfly pea flower extract is a multi-component mixture tested in mg/mL units, whereas fluconazole is a single pure compound tested at 6.25 µg/mL, so the two differ in absolute concentration, purity, and mechanism of action. Fluconazole acts on a single target, namely inhibition of the enzyme lanosterol 14α-demethylase in the ergosterol synthesis pathway (Varfa, 2026), whereas the extract works through many targets simultaneously (membrane disruption, ergosterol inhibition, ROS induction, and inhibition of adhesion and quorum sensing). This difference in mechanism is thought to produce additive to synergistic effects, so that under the same test conditions the extract shows higher percentages of biofilm inhibition and reduction. Therefore, this comparison should be regarded as an indicator of preliminary potential rather than direct pharmacological equivalence, and further confirmation requires testing on a potency-equivalent basis (for example, comparison based on the respective MIC values).

In addition, each parameter used has a different biological meaning. MIC (Minimum Inhibitory Concentration) describes the lowest concentration that can inhibit the growth of planktonic cells, while MFC (Minimum Fungicidal Concentration) indicates the lowest fungicidal concentration. $MBIC_{50}$ (Minimum Biofilm Inhibitory Concentration) reflects the ability to prevent ≥50% of new biofilm formation, while $MBRC_{50}$ (Minimum Biofilm Reduction Concentration) describes the ability to disperse or reduce ≥50% of mature, preformed biofilm. Activity against planktonic cells (MIC/MFC) is not always aligned with activity against biofilms ($MBIC_{50}$/$MBRC_{50}$), because cells within a biofilm are protected by the extracellular matrix, undergo phenotypic changes, and include drug-tolerant persister cells. In this study, the effective antibiofilm concentration (10 mg/mL), which was lower than the fungicidal value (MFC 20 mg/mL), indicates that the extract can disrupt the process of biofilm formation and maturation at sub-fungicidal concentrations, namely through inhibition of adhesion and quorum sensing rather than solely through cell killing.

Compared with other studies, these results show comparable activity while also extending previous findings. Some studies reported the antibiofilm activity of butterfly pea flower extract against bacteria such as *S. aureus* and *P. aeruginosa* (Besan, 2023; Jeyaraj et al., 2022). This study extends those findings by demonstrating similar activity against the fungus *C. albicans*. Conversely, a study using an aqueous butterfly pea flower extract reported decreasing inhibition at higher concentrations (Widjajanti, 2023), a pattern also observed in this study at the 40 and 80 mg/mL concentrations. Differences in extraction solvent (ethanol vs. water) likely also influence the profile of extracted compounds and the magnitude of activity.

To place these findings in a broader context, the results were compared directly with several previous studies based on solvent type, concentration range, test strain or organism, testing method, and the parameters used (Table VII). This comparison shows that differences in solvent (ethanol vs. water), method (dilution vs. disc diffusion), and parameters (quantitative MIC/MFC/$MBIC_{50}$/$MBRC_{50}$ values vs. inhibition zone) strongly affect the results obtained. This also confirms the novelty of this study, which evaluated antifungal and antibiofilm activity quantitatively and simultaneously on the reference strain *C. albicans* ATCC 10231.

Table 7. Comparison of this study with previous studies on the activity of butterfly pea flower (*Clitoria ternatea* L.)

Study	Solvent	Concentration	Test organism	Method	Main parameter & result
This study	70% ethanol	5–80 mg/mL	*C. albicans* ATCC 10231	Microdilution & Microtiter Plate Assay	MFC 20 mg/mL; $MBIC_{50}$ & $MBRC_{50}$ 10 mg/mL
Widjajanti (2023)	Water	50, 75, 100%	*C. albicans*	Disc diffusion	Inhibition zone; inhibition decreased at high concentration
Nabila et al. (2022)	Ethanol	12.5–100 mg/mL	*S. aureus*, *S. mutans*	Diffusion/dilution	Inhibition zone increased with concentration
Jeyaraj et al. (2022)	Anthocyanin-rich fraction	Varied	*P. aeruginosa*	Microtiter Plate Assay	Positive antibiofilm activity

The antibiofilm mechanism of butterfly pea flower extract against *C. albicans* involves several pathways. Flavonoids affect the expression of the *YWP1* gene, one of the adhesin molecules that plays an important role in the early stage of biofilm formation, and bind to cell-surface adhesin proteins, thereby reducing cell-to-cell adhesion of the fungus (Aboody and Mickymaray, 2020). Flavonoids also inhibit quorum sensing, the intercellular communication system essential for biofilm formation (Paczkowski et al., 2017). Saponins inhibit adhesin enzymes and reduce polymer components in the extracellular matrix, thereby weakening biofilm integrity. Tannins disrupt the formation of the biofilm extracellular matrix (Villanueva et al., 2023), while terpenoids interfere with H^+-ATPase pump activity by disrupting the electron transport complex (ETC) that is important for regulating intracellular fungal pH (Salinas et al., 2022). Alkaloids inhibit adhesion and quorum sensing (Zhou et al., 2025). This diversity of mechanisms explains why the extract can both inhibit and reduce biofilm—two biologically distinct processes—at relatively low concentrations.

An interesting phenomenon in this study was the decrease in antifungal and antibiofilm activity at higher extract concentrations (40 and 80 mg/mL), even accompanied by an increase in colony growth exceeding the negative control. This phenomenon can be explained by several mechanisms. First, the stress caused by high extract concentrations may induce fungal adaptive responses, including increased expression of resistance- and stress-related genes (Lara-Martínez et al., 2025). Second, certain components in the extract may exert a hormetic effect, namely stimulation of growth at high doses after showing toxic effects at low doses (Calabrese et al., 2026). Third, the high viscosity of the extract solution may inhibit the penetration of active compounds into fungal cells while also affecting the accuracy of OD readings because of the darker, more concentrated solution. Fourth, at high concentrations the intensely colored extract may precipitate and adhere to the bottom of the wells, being read as an artificially increased OD. This combination of biological factors and methodological artifacts underscores the importance of careful interpretation of results at high concentrations, and further research is needed to clarify the precise mechanism.

The clinical implications of this study are considerable. Butterfly pea flower extract shows potential as an alternative therapy for *C. albicans* infections, especially in an era of increasing antifungal resistance. The demonstrated antibiofilm activity is highly clinically relevant because *C. albicans* biofilm is a major cause of conventional antifungal treatment failure and increased mortality in candidemia patients (Vitális et al., 2020). The optimal extract concentration (10–20 mg/mL) that is effective as an antibiofilm agent is relatively low compared with the fluconazole requirement for biofilm eradication (which can reach 10–1,000 times the planktonic dose), suggesting a favorable pharmacoeconomic potential. These findings open opportunities for developing topical or systemic formulations based on butterfly pea flower extract for the management of mucocutaneous candidiasis, oral candidiasis in immunosuppressed patients, or as adjuvant therapy in candidemia.

This study has several limitations. First, MIC could not be determined visually because the dark color of the extract interfered with turbidity observation—a common limitation of intensely colored plant extracts. Second, the study used only a single strain, *C. albicans* ATCC 10231, so results may differ for clinical strains with varying resistance profiles. Third, the study was in vitro, so the results need to be validated through in vivo studies to evaluate the effectiveness, safety, and pharmacokinetics of the extract in complex biological systems. Fourth, the biofilm culture conditions (37 °C for 24 h) may produce biofilm with lower maturity than culture at a lower temperature (25 °C) for a longer time (48–72 h).

For future research, several recommendations can be considered: (1) isolation and identification of the specific bioactive compounds responsible for the antifungal and antibiofilm activity; (2) testing combinations of butterfly pea flower extract with conventional antifungals to evaluate potential synergistic effects; (3) testing against various clinical *C. albicans* strains with different resistance profiles; (4) in vivo studies to evaluate effectiveness, toxicity, and pharmacokinetics; (5) optimization of the extraction method to increase the concentration of active compounds while minimizing components that may cause hormetic effects at high concentrations; and (6) development of a stable and effective formulation for clinical application.

4. CONCLUSION

Butterfly pea flower ethanolic extract (*Clitoria ternatea* L.) has antifungal activity against *Candida albicans* ATCC 10231, with an MFC value at 20 mg/mL, and antibiofilm activity with $MBIC_{50}$ and $MBRC_{50}$ values at 10 mg/mL each. At these optimal concentrations, the activity of the extract was better than that of the positive control fluconazole 6.25 µg/mL. These findings indicate the potential of butterfly pea flower extract as an alternative therapy for *C. albicans* infections, particularly in addressing biofilms that are resistant to conventional antifungals. Further research is needed—including isolation of active compounds, synergism testing, and in vivo and clinical studies—before the extract can be applied clinically.

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