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Original article

Antimicrobial activity of yellow tabebuia flower extract (*Tabebuia aurea*) against drug-resistant microbes

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Abstract
Antimicrobial resistance is an urgent global challenge in treating infections. Aminoglycosid-2“-phosphotransferase-IIa (APH(2”)-IIa) and aspartic proteinases (Saps), respectively, are enzymes in bacteria and fungi that are responsible for the development of virulence and antimicrobial resistance mechanisms. *Tabebuia aurea*, known for its yellow flowers, has therapeutic potential due to the high concentration of active compounds such as stigmast-5-en-3-ol, kaempferol-3-O-rutinoside, rutin, and others. This study evaluated the antimicrobial activity of yellow tabebuia flower extract against drug-resistant microbes *in silico* and in vitro. Molecular docking was performed between APH (2”)-IIa and Saps with active compounds from tabebuia. Yellow tabebuia flower extract was obtained by sonication extraction method with 96% ethanol. The antimicrobial activity was tested using agar-well diffusion and agar dilution tests (0-500 mg/mL extract concentration) to determine the minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) against 6 resistant pathogens including *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans,* and *Candida albicans*. All selected active compounds in yellow tabebuia flower could bind to APH(2")-IIa and Saps. Among the bond between active compounds and microbial proteins, stigmast-5-en-3-ol - APH(2")-IIa and rutin – Saps complexes produced the strongest bond, with affinity energy lower than the positive control, streptomycin and pepstatin, respectively. Yellow tabebuia flower extract showed antimicrobial activity against all microbes tested, with the greatest zone of inhibition on *P. aeruginosa*. The MIC values obtained varied, with a bactericidal effect against *S. aureus* (MBC 500 mg/mL). This study proved the potential of the yellow tabebuia flower as an alternative to overcome antimicrobial resistance.

Keywords: Aminoglycosid-2“-phosphotransferase-Iia, antimicrobial resistance, aspartic proteinase, *Tabebuia aurea* flower
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Introduction

Tabebuia aurea is known for the beauty of its yellow flowers and its therapeutic potential (El-hawary et al., 2021). The stem parts have been used traditionally to treat snake bites and as an anti-inflammatory (Reis et al., 2014), against influenza and anticancer (Xisto et al., 2004). In recent years, the focus of research has shifted to the medical benefits of this plant, mainly related to its content of active compounds that have significant biological effects. Tabebuia flowers have been reported to contain various active compounds such as flavonoids, alkaloids, and saponins, thus having potential as promising antimicrobial agents (Sobiyana et al., 2019).

The growing problem of antimicrobial resistance has led to an urgent need to find new alternatives in the treatment of infections (Khare et al., 2021). Resistance to conventional antibiotics has led to the emergence of difficult-to-treat pathogens and exacerbated public health challenges (Park et al., 2024). Therefore, the identification of antimicrobial compounds from natural sources such as yellow tabebuia flower becomes very important. Phytochemicals demonstrate promising efficacy in addressing the development of resistance in bacterial pathogens and in combating bacterial infections (Khameneh et al., 2021).

Research on antimicrobial activity generally involves in vitro antimicrobial tests to evaluate the effectiveness of plant extracts against various pathogens (Kebede & Shibeshi, 2022; Rahayu et al., 2023). However, to understand the mechanism of action of active compounds in-depth, molecular docking approaches are highly relevant. This technique allows the modeling of interactions between active compounds and target biomolecules at the molecular level, providing insight into the potential of such compounds to inhibit pathogens (Wijayanti et al., 2021).

Two important biomolecular targets in the context of antimicrobial resistance are the enzymes Aminoglycosid-2“-phosphotransferase-IIa (APH(2”)-IIa) and aspartic proteinases (Saps). APH(2")-IIa is an enzyme involved in the mechanism of resistance to aminoglycoside antibiotics, while Saps play an important role in the pathogenesis of fungal and bacterial infections (Sulistyowaty et al., 2023). In this study, *in silico* techniques such as molecular docking was used to predict how active compounds from the yellow tabebuia flower bind to these enzymes, providing valuable information on their potential to overcome antimicrobial resistance.

In the in vitro test, 6 microbes known to be resistant to antimicrobial drugs were used including *Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus mutans,* and *Candida albicans*. The selected microbes represent

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Gram-positive and negative bacteria with rod and cocci forms, and represent the fungi group. The WHO has identified *P. aeruginosa, E. coli,* and *S. aureus* as critical antibiotic-resistant priority pathogens due to the urgent need for new antibiotics (Thakur et al., 2024). *Bacillus cereus* is a gram-positive, rod-shaped, toxin-producing bacterium that is resistant to several types of antibiotics (Fiedler et al., 2019). *Escherichia coli,* is a multidrug-resistant microbe that is a rod-shaped, gram-negative bacterium (Poirel et al., 2018). *Staphylococcus aureus* and *Streptococcus mutans* are gram-positive, cocci-shaped bacteria that are also known as multi-drug resistant bacteria (Li et al., 2022). *Candida albicans* develops resistance to prolonged exposure to antifungals by forming biofilms (Costa-de-Oliveira & Rodrigues, 2020). Infections caused by multi-resistant micro-organisms are leading to increased mortality in patients (Park et al., 2024).

By combining in vitro and *in silico* antimicrobial studies, this work aims to explore and understand the potential of yellow tabebuia as a source of antimicrobial active compounds. This study not only assesses the effectiveness of compounds in inhibiting pathogens, but also provides insight into the mechanism of action of these compounds through molecular interactions with target biomolecules such as APH(2")-IIa and Saps. Through this approach, we hope to find innovative therapeutic alternatives to overcome the growing challenge of antimicrobial resistance.

Methods

Simplicia preparation and extraction of tabebuia flower

Yellow tabebuia flower that have bloomed are washed with running water and then drained. The yellow tabebuia flower were dried in the sun and covered with a black cloth until the moisture content was ≤ 10%. The extraction of yellow tabebuia flower was performed by sonication (Ranjha et al., 2021). A total of 150 grams of yellow tabebuia flower simplicia powder was put into an erlenmeyer, and then 96% pro-analyst ethanol solvent was added in a ratio of 1:10. The sonication time was set at a frequency of 40 kHz for 30 minutes at room temperature—the filtration process of the extraction results using Whatman paper number 1 in vacuum. The filtrate was evaporated on a rotary evaporator at 60°C, then on a porcelain cup in a water bath at 60°C until a concentrated extract was obtained.

Phytochemical screening

The color reaction method was used for phytochemical screening, including alkaloids, flavonoids, phenolics, tannins, saponins, and terpenoids (Rahimah et al., 2019; Wijayanti et al., 2024). Alkaloids were detected by mixing extract with distilled water and HCl 2 N in a 9:1 ratio before being heated in a water bath for 2 minutes. The mixture was chilled and filtered before being treated with three separate reagents: Mayer, Dragendorf, and Wagner. Alkaloids can be identified as white precipitate (Mayer), reddish brown precipitate (Dragendorf), or brown precipitate (Wagner). Flavonoids were determined by combining the extract with magnesium powder and concentrated HCl. The presence of red, orange, or yellow color indicates positive flavonoid test results. Phenolics were identified by adding 10% $FeCl_3$. Positive phenolics were indicated by green to slightly blackish color. To detect tannins, the extract was mixed with a 0.1% $FeCl_3$ solution. The appearance of dark blue and blackish green colors indicated the presence of tannin. Saponins were detected by dissolving the extract in hot water and shaking it for 10 minutes before adding 2 N HCl. A stable foam shows the presence of saponins. Terpenoids were determined with the Lieberman-Burchard reagent. Positive terpenoids produce red or violet colors.

***In silico* Analysis**

Design

In silico analysis used a molecular docking between the active compounds found in *T. aurea* against the bacterial enzyme, Aminoglycosid-2”-phosphotransferase-IIa enzyme (APH (2”)-IIa, PDB ID 3hav) and fungal enzyme, aspartic proteinases (Saps, PDB ID 2qzx). The amino acid sequences that make up these enzymes were obtained from The Research Collaboratory for Structural Bioinformatics Protein Data Bank database (https://www.rcsb.org). The three-dimension structure of protein was downloaded in ID 3hav and ID 2qzx.

Preparation of Ligand Compounds

The ligands in this study were compounds found in Tabebuia while the native ligands are streptomycin and pepstatin. The compounds used as ligands (Table 1) were selected from the reference tabebuia flower (Irfansyah et al., 2023) and tabebuia leaf (Mahmoud et al., 2024), which are several compounds that have high concentrations and already have structures in PubChem. The ligands were then interacted with a receptor, and their chemical structures were obtained by accessing PubChem. The three-dimensional structure of the compounds of Tabebuia flower extract were obtained from the PubChem Open Chemistry Database. The three-structure of various compounds in the *.sdf file format was then converted into *.pdb files using Avodgadro software ver. 1.2.0 (Chandel et al., 2021).

Table 1. Selected tabebuia compounds as ligands

Active compounds	Compound ID
Flower part	
1-Docosanol	CID12620
Pyrrolidine	CID31268
Nonacos-1-ene	CID156989
Stigmast-5-en-3-ol	CID22012
Tricosane	CID12534
Leaves part	
Astragalin	CID5282102
Isoquercetin	CID5280804
Kaempferol-3-O-rutinoside	CID5318767
Quercetin-3-sambubioside	CID5487635
Rehmaglutin D	CID5320906
Rutin	CID5280805

Preparation of Receptors

The receptors used are bacterial (3hav) and fungal (2qzx) enzymes obtained from PDB. The receptors are in the form of complex macromolecules that need to be separated from their ligands. The process of separating unneeded ligand residues aims to avoid interfering with the docking process that may affect the final result. The 3D structures of bacterial (id: 3hav) and fungi (id: 2qzx) enzymes were searched by accessing (https://www.rcsb.org/) and then downloaded in (*.pdb) format. Water molecules and residues that do not play a role in the enzyme structure were removed. The enzyme structure was separated with its natural ligand using Discovery Studio and the results were saved in (*.pdb) format. The protein was added with hydrogen ions and charge, converted in the form of (*.pdbqt) using AutoDock Tools.

Molecular Docking of Ligand with Target Protein

Ligands and target proteins were docked using Autodock Vina version 1.2.3 (Macalalad & Gonzales III, 2023). The target protein and ligand are opened in ADT. The Grid Box is sized on the active side of the enzyme (size center x, y, z). Furthermore, a notepad file (*grid.txt) was created containing center_x, center_y, and center_z coordinates. Grid box was validated by re-docking native ligand. Test ligands were docked with bacterial and fungal enzymes using Autodock Vina version 1.2.3 run using a terminal. The result of docking between protein and ligand in the form of bond affinity value was then analyzed.

Visualization and Energy Analysis of Docking Results

Protein and ligand interactions from the docking results were then analyzed and visualized using BIOVIA Discovery Studio software (Wijayanti et al., 2022). The results were analyzed by determining the ligand conformation that has the best binding affinity value and analyzing the binding interaction based on its amino acid residues in 2D and 3D. The binding affinity value was determined based on the most negative value and compared with its native ligand.

In Vitro Antimicrobial Assay

Preparation of microbial suspensions

A total of 1 oose of test microbes from the culture stock was inoculated into each selective medium and incubated at 37°C for 24 hours. The medium used were mannitol egg yolk (MYP) agar for *B. cereus*, eosin methylene blue (EMB) agar for *E. coli*, MacConkey agar for *P. aeruginosa*, mannitol salt agar (MSA) for *S. aureus*, blood agar for *S. mutans*, and Sabouraud dextrose agar (SDA) for *C. albicans*.

The 24-hour microbes were each inoculated into 30 mL of Nutrient Broth media (for bacteria) and Sabouraud Dextrose Broth media (for fungi). The suspension was placed in an orbital shaker and incubated for 24 hours at 120 rpm at room temperature. After 24 hours, for bacteria, the % transmittance value was measured using a UV-Vis spectrophotometer at a wavelength of 625 nm until a % transmittance value of 25 was obtained. While for fungi, the % transmittance value was measured using a UV-Vis spectrophotometer at a wavelength of 530 nm until a transmittance of 90% was obtained (Sanuddin et al., 2024).

Agar well diffusion assay

In a Petri dish, 1 mL of the microbial suspension was placed. Mueller Hinton agar media (for bacteria) and Sabouraud dextrose agar media (for fungi) were then added using the pour plate method. Solidified media containing bacteria are perforated in the center with a cork borer, then filled with 0.1 mL of yellow tabebuia flower extract and incubated at 37°C for 24 hours. After 24 hours, the diameter of the inhibition zone was measured with a caliper (Kebede & Shibeshi, 2022).

Agar dilution assay

Yellow tabebuia flower extract was diluted with 10% DMSO into several concentrations including 100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, and 500 mg/mL. Each diluted extract was placed in a Petri dish, and then bacterial suspension and selective media were added and incubated at 37°C for 24 hours. The growing microbial colonies were counted. The MIC value was determined based on the number of microbes that were significantly different from the bacterial control suspension. The MBC/MFC value was determined at the lowest concentration where there was no growth of bacterial colonies (Schumacher et al., 2018).

Data analysis

The *in silico* data and phytochemical screening were analyzed qualitatively. Inhibition zone diameters were compared between microbes by ANOVA and Tukey's HSD using GraphPad Prism 8. The same analysis was used to compare the number of bacterial colonies on agar dilution results.This study consisted of 3 different media treatments namely MS (P1), MS with the addition of $NBsO_2$ (P2) and $NBsO_2$ (P3) then replicated 8 times so that there were 24 experimental units. The observation parameters consisted of PLB weight (g), PLB height (cm), number of live PLB (%), PLB color, root emergence, root length, shoot emergence, number of new shoots, embryo emergence, and number of embryos. So that data analysis is done in two ways, namely qualitative data analysis and quantitative data analysis. Qualitative data analysis is done by reading and processing the study result and then presented in the form of tables, figures and graphs. Meanwhile, quantitative data analysis was carried out by processing the observation data using normality test, homogeneity test, and multivariate test. If it is known that there is a real or very real difference, it will be continued with a univariate comparison test and further tests using the games-howell test and the bonferroni test.

Results and Discussion

Yellow tabebuia flower were extracted by sonication method using ethanol solvent, resulting in a yield of 13.54%. The extract of yellow tabebuia flower showed the presence of secondary metabolites including alkaloids, flavonoids, phenolics, tannins, and terpenoids, while saponins were not detected, as shown in Figure 1. Differing from the previous research using yellow tabebuia flower from India, the ethanolic extract of yellow tabebuia flower was also found to be positive for

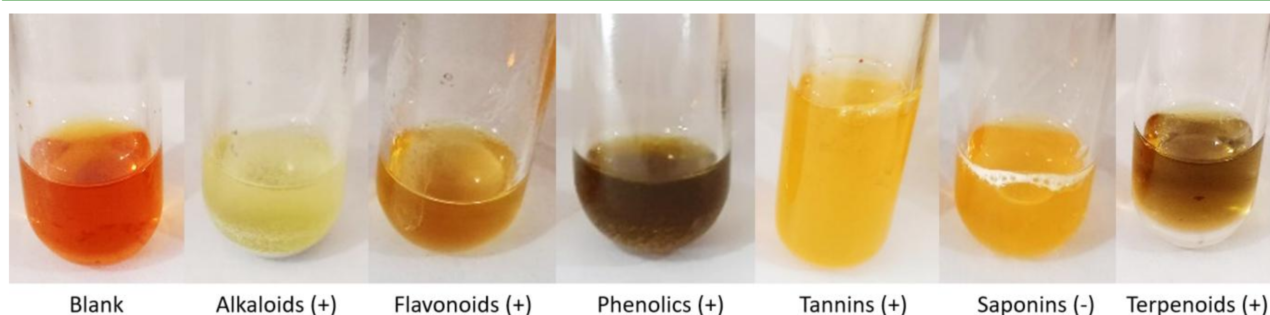


Figure 1. Phytochemical screening of tabebuia flower extract, (+): present, (-): absent.

saponins (Sobiyana et al., 2019). Differences in geography, climate, cultivation methods, harvesting methods, and times, as well as post-harvest treatment (drying, storage), can cause differences in the amount of secondary metabolite content produced from plants. Geographical conditions such as temperature, light intensity, rainfall, humidity, altitude, and soil type have different effects on the phytochemical content of plants (Ghasemzadeh et al., 2018).

Earlier studies found pyrrolidine, classified as an alkaloid, and stigmast-5-en-3-ol, classified as a sterol, in flower parts (Irfansyah et al., 2023). While the leaves contain flavonoids such as astragalin, isoquercetin, kaempferol-3-O-rutinoside, quercetin-3-sambubioside, and rutin, as also rehmaglutin D, which is classified as a terpenoid (Mahmoud et al., 2024). These compounds were evaluated *in silico* for their potential as antimicrobials. The active compounds selected were not only in the flower but also in the leaves with the assumption that different parts of the same plant generally have similar profiles of active compounds, but different levels (Ayoade et al., 2019). Active compounds in tabebuia were docked with proteins in microbes responsible for antimicrobial resistance, namely APH(2")-IIa in bacteria and Saps in fungi. For comparison, native ligands were streptomycin for APH(2")-IIa and pepstatin for Saps (Sulistyowaty et al., 2023).

The docking results can be observed in Figure 2, and the binding affinity can be seen in Table 2. Some compounds showed lower binding affinity values than the native ligand (indicated by bold numbers). In the docking between the bacterial protein, APH(2")-IIa, with compounds on tabebuia, the stigmast-5-en-3-ol, isoquercetin, kaempferol-3-O-rutinosid, quercetin-3-sambubioside, and rutin have lower binding affinity than streptomycin. Stigmast-5-en-3-ol also has a lower binding affinity than pepstatin in binding to the fungal protein, Saps. Besides these compounds, astragalin, isoquercetin, kaempferol-3-O-rutinoside, quercetin-3-sambubioside, and rutin also had lower binding affinity than pepstatin. These findings show the potential of these compounds as inhibitors of microbial proteins in degrading antimicrobial compounds, and prove one of the antimicrobial mechanisms of tabebuia flower, preventing microbes from becoming resistant to antimicrobials.

Table 2. Binding Affinity of Tabebuia Flower Active Compounds Compared to Native Ligands

Ligand Compounds toward	Binding Affinity (kcal/ mol)	
	APH (2”)-IIa (3hav)	Saps (2qzx)
Streptomycin	**-7.699**	
Pepstatin		**-5.025**
1-Docosanol	-5.233	-3.332
Pyrrolidine	-3.347	-2.483
Nonacos-1-ene	-4.633	-3.489
Stigmast-5-en-3-ol	**-9.387**	**-5.911**
Tricosane	-4.330	-2.739
Astragalin	-7.494	**-5.861**
Isoquercetin	**-8.199**	**-5.642**
Kaempferol-3-O-rutinoside	**-8.960**	**-5.829**
Quercetin-3-sambubioside	**-8.670**	**-6.698**
Rehmaglutin D	-5.622	-3.806
Rutin	**-8.957**	**-7.199**

Note: The bold numbers indicate the binding affinity value of the native ligand and the binding affinity value of the active compound which is lower than the native ligand.

The antimicrobial activity of tabebuia flowers was also confirmed through in vitro studies against several pathogenic microbes that are generally resistant to antibacterials and antifungals. The selected microbes represent gram-negative and positive, rod and cocci cell forms, and also fungi. The antimicrobial test was carried out by agar-well diffusion method followed by agar dilution to determine the MIC and MBC/MFC values. Figure 3 shows the antimicrobial activity of tabebuia flower extract against 6 pathogenic microbes. Antimicrobial activity is indicated by the diameter of the inhibition zone (Mohamed et al., 2020). The greater the activity of an antimicrobial material, the wider the zone of inhibition against bacterial growth (Bubonja-šonje et al., 2020). Tabebuia flower extract showed the greatest inhibitory activity against *P. aeruginosa* followed by *B. cereus* with significantly different inhibition zone diameters of 47.47 mm and 28.23 mm, respectively. This value is also significantly different from the diameter of the inhibition zone against other test microbes. The diameter of the inhibition zone against the other 4 test microbes was not significantly different from each other. This indicates that *P. aeruginosa* is most susceptible to tabebuia flower extract. While *S. mutans, S. aureus, E. coli,* and *C. albicans* were stronger than the other test microbes.

APH (2”)-IIa with streptomycin
Binding Affinity= -7.699 kcal/mol

APH (2”)-IIa with Stigmast-5-en-3-ol
Binding Affinity= -9.387 kcal/mol

Saps with pepstatin
Binding Affinity= -5.025 kcal/mol

Saps with Rutin
Binding Affinity= -7.199 kcal/mol

Figure 2. Visualization of Binding Poses in 3D and 2D

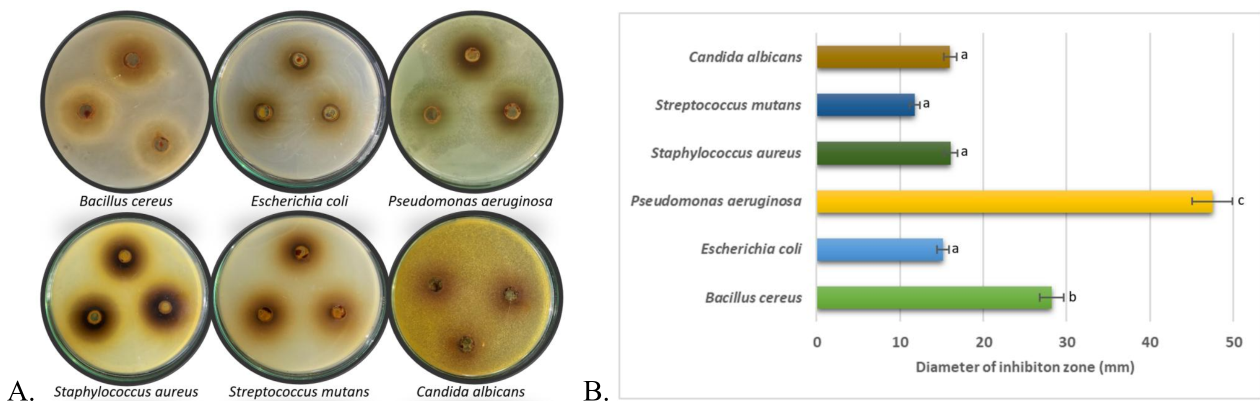
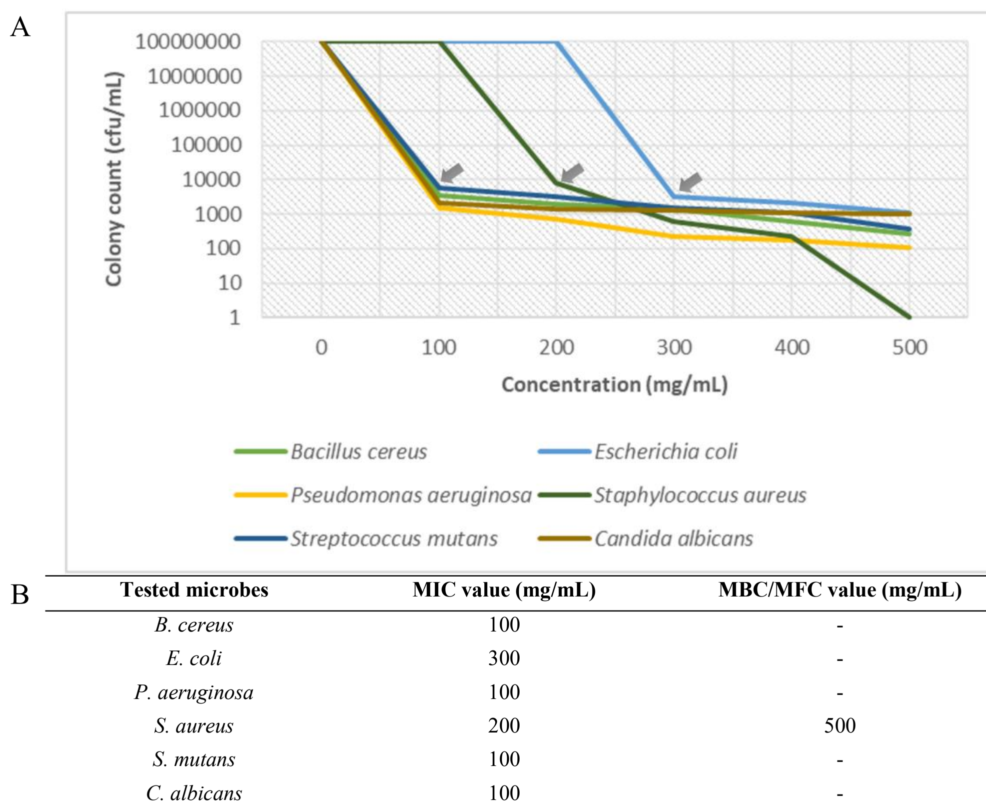


Figure 3. Growth inhibition of some microbes by yellow tabebuia flower extracts, A. Inhibition zone by agar well-diffusion method, B. Bar diagram of inhibition zone diameter. The different letters indicated significant difference (Tukey HSD, $P < 0.05$).

A



B

Tested microbes	MIC value (mg/mL)	MBC/MFC value (mg/mL)
B. cereus	100	-
E. coli	300	-
P. aeruginosa	100	-
S. aureus	200	500
S. mutans	100	-
C. albicans	100	-

Figure 4. Growth inhibition of some microbes by yellow tabebuia flower extracts at different concentrations, A. Colonies growth curve of some tested microbes. The arrow on the curve indicates the MIC value, B. Summary of MIC and MBC/MFC value.

Since the extract had antimicrobial activity against all test microbes, all microbes were used in further tests to determine the MIC and MBC/MFC values using the agar dilution method. Microbial colonies that grew after being exposed to tabebuia flower extracts with various concentrations (0-500 mg/mL) were counted and then plotted on a graph. Figure 4 shows the inhibition of microbial growth in the presence of tabebuia flower extract. The higher the concentration of extract exposed to microbes, the fewer the number of microbes that grow. This shows that the higher the concentration of the extract, the more phytochemical compounds that act as antibacterials that can inhibit bacterial growth. The antibacterial properties of phytochemicals pertain to their chemical interference with the function or synthesis of essential components, as well as their ability to circumvent mechanisms of antibacterial resistance (Khameneh et al., 2021). The concentration of tabebuia flower extract that decreased the number of microbial colonies significantly (tested by ANOVA and Post hoc Tukey) was determined as the MIC value. While the MBC/MFC value is determined based on the concentration of the extract that results in no microbial growth, or the number of colonies is zero.

Figure 4 shows that at a concentration of 100 mg/mL, yellow tabebuia flower extract was able to inhibit *B. cereus, P. aeruginosa, S. mutans,* and *C. albicans*. Meanwhile, to inhibit other microbes, higher concentrations are needed, 200 mg/mL for *S. aureus*, and 300 mg/mL for *E. coli*. The antimicrobial activity of yellow tabebuia flower extract was bacteriostatic against *B. cereus, E. coli, P. aeruginosa, S. mutans,* and *C. albicans* because it was unable to kill the test microbes at the highest concentration used. In general, the MBC/MFC value can be obtained by increasing the concentration of the extract used. However, in this study, the use of extract concentrations higher than the MIC value did not show significant inhibition of microbial growth. Therefore, increasing the concentration of extracts even further was not considered to have a significant effect. Yellow tabebuia flower extract only showed bactericidal activity against *S. aureus* at a KBM of 500 mg/mL.

The antimicrobial activity of the yellow tabebuia flower extract is supported by the content of phytochemicals, each of which has a different mechanism of action. Phenolics shrink the size of cells and form pores in the cells, leading to cell lysis (Wijayanti et al., 2021). Flavonoids denature proteins and nucleic acids, disrupting the physiological functions of microbes. Tannins disrupt cell permeability and inhibit cell wall synthesis, causing death. Alkaloids inhibit peptidoglycan synthesis of bacterial cell walls and inhibit ergosterol synthesis in fungi, causing death. Terpenoids reduce cell permeability resulting in nutrient deprivation and death (Novitasari & Wijayanti, 2018; Sopandani et al., 2020; Wijayanti & Susilowati, 2017).

These findings confirm the potential of yellow tabebuia flower as antimicrobial agent *in silico* and in vitro. Yellow tabebuia flower extract has a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi. The active

compounds in yellow tabebuia flower support their role as antimicrobials by inhibiting the growth of microbial colonies. In silico, the compounds were also able to bind to microbial enzymes, APH(2")-IIa and Saps, which are involved in antimicrobial resistance mechanisms. APH(2")-IIa is an enzyme produced by Gram positive and some Gram negative bacteria to degrade aminoglycosides (Smith et al., 2017). Meanwhile, Saps plays a role in candida virulence by assisting protein degradation and attachment to the cell mucosa (Hamid et al., 2018). Both enzymes increase microbial virulence to become resistant to antimicrobials (Sulistyowaty et al., 2023). The alleged mechanism that occurs is that when microbes are exposed to the active compounds of yellow tabebuia flower, the enzymes that play a role in the resistance mechanism are inhibited. This causes microbes to become more susceptible to antimicrobials so that their growth is inhibited. Therefore, tabebuia flowers offer an alternative as a natural antimicrobial that can contribute to overcoming the problem of antimicrobial resistance. However, further research is required to thoroughly evaluate its efficacy and ensure its use to overcome or even reduce resistance to conventional antibiotics.

Conclusion

Yellow tabebuia flower extract demonstrated an antimicrobial effect against 6 multi-drug resistant microbes including *B. cereus, E. coli, P. aeruginosa, S. aureus, S. mutans,* and *C. albicans.* The yellow tabebuia flower extract was deadly to *S. aureus* at an MBC of 500 mg/mL. An *in silico* study confirmed the mechanisms of tabebuia flower active compounds to prevent antimicrobial resistance.

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