Inhibition of Bilimbi Leaf Extract (*Averrhoa bilimbi* Linn.) Against the Growth of *Candida albicans* ATCC 10231

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**ABSTRACT**

*Candida albicans* ATCC 10231 is a microflora in the female genital tract that can cause candidiasis/vaginal discharge. Natural treatment without side effects can use bilimbi leaves (*Averrhoa bilimbi* Linn.). This plant contains flavonoids, alkaloids, saponins, and tannins which can inhibit *C. albicans* ATCC 10231. This study aimed to determine the potential of bilimbi leaf extract in inhibiting the growth of *C. albicans* ATCC 10231. The maceration method using ethanol as a solvent was used to obtain the extract of bilimbi leaves. The antifungal ability of the extract was tested using the agar well diffusion method. The extraction yield was 7.69% and was able to inhibit *C. albicans* ATCC 10231 at a concentration of 80% and 100% with an antimicrobial index of 0.66 and 0.95, respectively, with weak and moderate inhibition categories. These results are new information about using bilimbi leaves to inhibit the growth of *C. albicans* ATCC 10231 so that the public can use it as an alternative treatment for vaginal discharge.

**Introduction**

Candidiasis or vaginal discharge is a case of infection that affects women. At some point during their life, 75% of women will experience attacks of candidiasis vaginalis, and as many as 10-20% are asymptomatic carriers (Mandal *et al.*, 2004). Vaginal discharge in the reproductive organs can be found in normal (physiological) conditions and symptoms that arise due to a disorder that must be treated (pathological) (Clayton, 2008). *Candida albicans* causes leucorrhoea or vaginal discharge. Leucorrhoea can be overcome by administering drugs that contain antimicrobial compounds either directly (orally) or used as external drugs (Ratnah *et al.*, 2018). Until now, azole drug compounds such as nystatin, clotrimazole, and miconazole are the main antifungal agents for treating vaginal discharge or candidiasis vaginalis (Monalisa *et al.*, 2012). However, the use of these antifungal drugs has various limitations, such as a narrow spectrum of antifungals, low tissue penetration rates, side effects in allergies, irritation, and nausea (Deza, 2010), and the emergence of fungal resistance (Setyowati *et al.*, 2013).

Microbial resistance to antibiotics or antifungals is a growing global problem in developing and developed countries. Therefore, an alternative treatment is needed in the form of compounds produced by plants in plant secondary metabolites as a source of medicinal raw materials. Secondary
metabolites have been shown to work as antibacterial and antifungal, including alkaloids, tannins, polyphenols, and derivatives. Plants that can produce antimicrobials are Bilimbi (*Averrhoa bilimbi* Linn.). The fruit and leaves of bilimbi are often used as traditional medicinal plants (Hasim et al., 2019). Bilimbi leaves contain phenolic compounds, alkaloids, flavonoids, saponins, triterpenoids, and tannins (Faharani, 2009; Valsan & Raphael, 2016) as an anti-inflammatory, antioxidant, anti-inflammatory (Liantari, 2014), and antimicrobial compounds (Abraham, 2016; Valsan & Raphael, 2016). The high content of secondary metabolic compounds causes bilimbi to be widely used to treat various diseases such as diseases caused by bacteria and fungi, inflammation, cancer, canker sores (Bhaskar & Shantaram, 2014; Kumar et al., 2013), acne, ulcers, arthritis, disorders, intestines, and cough (Anitha et al., 2011).

The effectiveness of bilimbi leaf extract against *C. albicans* has been reported to inhibit the growth of *C. albicans* in the mouth (Sari & Suryani, 2014) and dental/orthodontic removable (Puspitasari & Ardiansyah, 2017). However, it has not been tested against *Candida albicans* is the cause of vaginal discharge in organ reproduction, namely *C. albicans* ATCC 10231. *C. albicans* ATCC 10231 is a strain that causes vulvovaginal candidiasis (Liao et al., 2017; Muñoz et al., 2017), which has experienced flucconazole resistance last few decades (Tovar et al., 2014). Therefore, this study aims to determine the inhibitory ability of bilimbi leaf extract to the growth of *C. albicans* ATCC 10231, which causes vaginal discharge (vulvovaginal candidiasis).

**Materials and Methods**

The materials used in this study were bilimbi leaves (*Averrhoa bilimbi* Linn.) from Sukarame, Lampung, Indonesia and *C. albicans* ATCC 10231 purchased from IPB Culture Collection (IPBCC) grown on Saboraud Dextrose Agar (SDA) media (Merck 1.05438.0500) incubated at 30 °C for 24 hours. The culture that grows is a microbial culture that will be used as the next test organism.

A total of 500 grams of bilimbi leaves were dried in the oven at 37 °C for 4-5 hours. The dried bilimbi leaves were then crushed into simplicia, the results obtained were used as samples. Bilimbi leaf powder soaked in 70% ethanol (1:10), for 3 x 24 hours in a shaking incubator (IS-RSDAA Bench-top Incubator Shaker 20A00G539). Then the bilimbi leaf extract solution is filtered. The extract of bilimbi leaves was concentrated using a rotary evaporator (RE-201D) (Ibrahim et al., 2014). The concentrated extract obtained was used for the antifungal test with a concentration of 20%, 40%, 60%, 80%, and 100% and positive (nystatin (100mg/ml)) and negative (sterile distilled water) controls as a comparison.

The ethanol extract of bilimbi leaves was tested using Agustina et al., 2017, method for phytochemical characteristics. Phytochemical compounds that were identified qualitatively were alkaloids, flavonoids, tannins, and steroids with specific reagents. This study is an experimental laboratory study examining the effect of ethanol extract of bilimbi leaves (*A. bilimbi* Linn.) On the inhibition zone diameter of *C. albicans* ATCC 10231 in vitro. The method used in this research is the modified Kirby Bauer method, namely using perforating the Saboraud Dextrose Agar (SDA) medium, which has been inoculated with *C. albicans* ATCC10231 so that it is in the form of a well. The holes formed were dripped with 100 µl of bilimbi leaf extract with various concentrations then incubated at 25 °C for 24 hours. The diameter of the resistance zone formed is measured with a ruler to determine the antifungal effectiveness. The resistance zone measurement is done by measuring the diameter of the clear zone area around the well. The inhibition zone diameter is the diameter that is not covered by bacteria around the well minus the diameter of the well (Nassar et al., 2019).

\[
\text{Antimicrobial Index} = \frac{\text{Diameter of clear zone} - \text{Diameter of well}}{\text{Diameter of well}}
\]

**Results and Discussion**

Based on bilimbi leaf extraction results using 70% ethanol, the yield was 7.69% (Table 1). The yield produced is small, so that
to produce ethanol extract of bilimbi leaves requires a large number of leaves. The raw material used in this study was the leaves of the bilimbi plant (A. bilimbi L.). The selected bilimbi leaves were extracted with ethanol using the maceration method to extract all chemical components in the cell cavity containing the active compound. The yield of the condensed ethanol extract obtained after the evaporation process was 7.69%. This means that after going through the extraction process, bilimbi leaves lost weight of 92.31%. The yield is the percentage of the raw material used or utilized by the total raw material. The higher the yield value indicates that the raw material has a more significant opportunity to be utilized (Kusumaningtyas et al., 2008).

### Table 1. The yield percentage of crude ethanol extract of bilimbi leaves

<table>
<thead>
<tr>
<th>Initial weight of the sample (g)</th>
<th>Weight ethanol extract (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>5</td>
<td>7.69</td>
</tr>
</tbody>
</table>

Besides, the yield percentage informs about the solubility of a compound present in plant samples. The solubility of the compound depends on the solvent used. Polar compounds in plants will dissolve more easily in polar solvents (Okiei et al., 2011). The yield of bilimbi leaf extract in 7.69% ethanol solvent indicates that the polar compounds contained therein can dissolve well. The same thing was also reported by Soedirga & Parhusip (2019), the yield of bilimbi leaf extract with ethanol solvent was 10.74%, while with non-polar solvents in the form of ethyl acetate and hexane, respectively 3.92% and 1.98%. This showed that the compounds contained in the leaf extract are more polar than non-polar.

The test results of the phytochemical screening of ethanol extract of leaves of bilimbi indicate several secondary metabolites, such as tannins, flavonoids, alkaloids, saponins, and steroids, as shown in Table 2.

The ethanol extract of bilimbi leaves was tested for phytochemical screening. Phytochemical screening is the initial stage to detect secondary metabolite compounds in the plant samples studied (Simaremere, 2014). The qualitative test results show that the ethanol extract of bilimbi leaves contains tannins, flavonoids, alkaloids, phenolics, saponins, and steroids. The presence of tannins, flavonoids, alkaloids, saponins, and steroids in bilimbi leaf extract has the same information reported by (Hasim et al., 2019). The components of flavonoids, alkaloids, phenolics, and tannins act as natural antimicrobials produced by plants (Ahmad et al., 2015; Cushnie & Lamb, 2005; Valsan & Raphael, 2016) and are mostly found in plants (Yordi et al., 2012).

### Table 2. The content of secondary metabolites compounds of crude ethanol extract of bilimbi leaves

<table>
<thead>
<tr>
<th>Phytochemical compounds</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>++</td>
</tr>
<tr>
<td>• Meyer</td>
<td>++</td>
</tr>
<tr>
<td>• Wagner</td>
<td>++</td>
</tr>
<tr>
<td>• Dragedorf</td>
<td>+++</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+++</td>
</tr>
<tr>
<td>Tanin</td>
<td>+++</td>
</tr>
<tr>
<td>Fenolic</td>
<td>+++</td>
</tr>
<tr>
<td>Saponins</td>
<td>++</td>
</tr>
<tr>
<td>Steroid</td>
<td>++</td>
</tr>
</tbody>
</table>

Besides, in the ethanol extract of bilimbi leaves, saponins were also found. Saponins are surfactants that can bind to the fat layer on the cell membrane of C. albicans and cause interference with cell membrane permeability so that the diffusion of materials or substances needed by fungi is disrupted; as a result, the cells swell and lysis (Utami & Puspaningtyan, 2013). In addition to these compounds, sulfur, formic acid, calcium oxalate, and potassium citrate are found in the ethanol extract of bilimbi leaves (Wijayanti & Safitri, 2018).

The condensed ethanol extract of bilimbi leaves was obtained then tested for the effectiveness of C. albicans ATCC 10231 using the agar well diffusion method, which is the standard for antimicrobial testing. Based on the measurement of the antimicrobial index against C. albicans ATCC 10231 using the well diffusion method, the crude ethanol extract of bilimbi leaves was able to inhibit the growth of C. albicans ATCC 10231 by 3.96 mm with an Antimicrobial Index (AMI)
The inhibitory capacity of the two extract concentrations was categorized as moderate and weak. Meanwhile, the positive control of nystatin had greater effectiveness against the test organism than the treatment, namely 15.12 mm and an IAM of 2.52 (strong category) (Table 3). Negative control using aquadest showed no inhibition against the type of test organism.

The ability of bilimbi leaf extract to inhibit *C. albicans* ATCC 10231 was also influenced by the type of leaf taken. The young leaves have better effectiveness, demonstrated by the inhibitory concentration of 20% (Sari & Suryani, 2014). The young leaves of bilimbi (*A. bilimbi* L.) have higher tannins levels than the old leaves. In contrast, in this study, the leaves used were all on the petiole without separating old and young leaves. This caused the tannin levels obtained quantitatively to inhibit *C. albicans* ATCC 10231 at low extract concentrations. In inhibition of *C. albicans*, tannins will bind to fungal cell walls (Kusumaningtyas *et al.*, 2008) of *C. albicans* that consisting of glucan (Suryanto *et al.*, 2014) will inhibit protease activation and direct inactivation.

Apart from the types of leaves, the extraction method also greatly affects the concentration being tested. This is following previous research conducted by Cahyono (2007), where the more significant the concentration of bilimbi leaf juice, the fewer microbial colonies formed.

### Table 3. Inhibition of ethanol extract of bilimbi leaves against *C. albicans* ATCC 10231

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Clear Zone Diameter (mm)</th>
<th>Well Diameter (mm)</th>
<th>Inhibition Zone Diameter (mm) + SD</th>
<th>Antimicrobial Index + SD</th>
<th>Inhibition Category*</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>0.00</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>none</td>
</tr>
<tr>
<td>40</td>
<td>0.00</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>none</td>
</tr>
<tr>
<td>60</td>
<td>0.00</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>none</td>
</tr>
<tr>
<td>80</td>
<td>9.96</td>
<td>6.00</td>
<td>3.96 ± 0.005</td>
<td>0.66 ± 0.00</td>
<td>weak</td>
</tr>
<tr>
<td>100</td>
<td>11.70</td>
<td>6.00</td>
<td>5.70 ± 0.03</td>
<td>0.95 ± 0.005</td>
<td>moderate</td>
</tr>
<tr>
<td>Control (+)</td>
<td>21.12</td>
<td>6.00</td>
<td>15.12 ± 0.005</td>
<td>2.52 ± 0.00</td>
<td>strong</td>
</tr>
<tr>
<td>Control (-)</td>
<td>0.00</td>
<td>6.00</td>
<td>0.00</td>
<td>0.00</td>
<td>none</td>
</tr>
</tbody>
</table>

*According to (Davis & Stout, 1971)

The categories for inhibitory activity based on the inhibition zone diameter are as follows:
1. The zone of inhibition of 20 mm or more is categorized as very strong.
2. The 11-20 mm zone of inhibition is categorized as strong.
3. The 5-10 mm zone of inhibition is categorized as moderate.
4. The inhibition zone of 5 m or less is categorized as weak.
extract's improvement. The maceration method (without heating) used in this study is not optimal for obtaining a good quality extract. The heating method was thought to improve the solubility of the extract. This is due to the chemical properties of tannins, which have high solubility in hot water. Tannins heated to (98.89 °C-101.67 °C) break down into pyrogallol, phloroglucinol, and pyrocatechol called hydrolyzed tannins. Tannins in hydrolyzed form have a strong antimicrobial inhibitory effect (Irianty & Yenti, 2014). Pyrogallol is a compound that is widely reported to have candidacidal and fungicidal activity. This activity is triggered by three groups of hydroxyl structures that affect C. albicans cell walls and membranes (Yanti et al., 2016).

Besides, the type of Candida used in the test is C. albicans ATCC 10231, which has experienced resistance to various antifungal compounds, including Anidulafungin, Voriconazole, Itraconazole, and Fluconazole (ATCC, 2017). Candida albicans is a fungus that has experienced increased resistance to fluconazole in recent decades. Various studies have been conducted to find new compounds or new methods to reduce the resistance level of C. albicans, one of which is "Click Chemistry" or testing of various new compounds against resistant C. albicans (Tovar et al., 2014). This shows that this research can support the search for the potential of new compounds from plants to inhibit C. albicans whose resistance levels are increasing.

Conclusion
The ethanol extract of bilimbi leaves contains flavonoids, phenolics, tannins, alkaloids, saponins, and steroids, which are useful as antimicrobials in inhibiting the growth of C. albicans ATCC 10231. The inhibition of ethanol extract of bilimbi leaves against C. albicans ATCC 10231 was detected at a concentration of 80% and 100%, respectively 3.96 mm (weak category) and 5.70 mm (moderate category). Inhibition activity is not optimal due to the content of metabolite compounds, one of which is tannins obtained from leaf species, the extraction method is not yet optimal. Besides, the type of C. albicans used was a strain that had resistance to various types of antifungals. However, these results provide new information that bilimbi leaves can be used as an alternative treatment for C. albicans, which causes the vaginal discharge

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