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The Effect of Torbangun (*Coleus amboinicus* Lour) Leaf Extract on Antibiotic Resistant Bacteria *Escherichia coli pBR322* and Toxicity Tests on *Artemia salina Leach*

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ABSTRACT

Inappropriate antibiotic use can lead to antibiotic resistance, causing many problems in the treatment of *Escherichia coli* infection. One way to avoid resistance is to use the traditional medicine *Coleus amboinicus* Lour, also known as Torbangun in Indonesia. The purpose of this study was to determine the inhibitory ability of 70% ethanol extract of Torbngun leaf against *Escherichia coli pBR322* has plasmid pBR322, which is resistant to antibiotics Ampicillin and Tetracycline based on activity and minimum inhibitory concentration. Extraction was carried out using the maceration method. The presence of inhibition zones indicated antibacterial activity. The appearance of the inhibition zone at the minimum concentration indicated the minimum inhibitory concentration. Following this, the cytotoxic activity was evaluated using the brine shrimp lethality test. Data analysis was performed using an unpaired *t-test* using GraphPad Prism® software. Antibacterial activity tests showed that a 15% torbangun leaf extract concentration had inhibitory power against *Escherichia coli* pBR322. The toxicity test of torbangun leaf extract against Artemia *salina Leach shrimp larvae* showed LD50 results at a concentration of 150 ppm.

1. Introduction

Improper exposure to antibiotics to microorganisms can cause resistance that complicates the healing process, disrupting the composition of normal microflora, extending the patient's treatment period, and even causing death due to treatment failure (Frieri et al., 2017). Escherichia coli usually already forms colonies in the digestive tract of human infants within a few hours after birth and forms a symbiotic commensalism (Sunarti, 2022). Escherichia coli adaptive abilities and certain typical virulence allow it to cause disease in many places in the body; it can even cause disease in immunocompetent individuals (A. Manges, 2016). Clinical syndromes are common due to pathogenic Escherichia coli infections in the gastrointestinal tract, urinary tract, blood vessels, and brain. The main treatment is the use of antibiotics. Unfortunately, improper use of antibiotics can lead to resistance.

Escherichia coli is one of the most reported bacteria resistant to antibiotics. Escherichia coli resistance to third-generation cephalosporin increased from 6.9% to 10.5%, while resistance to fluoroquinolones increased from 22.22% to 24.5% (Struyf & Mertens, 2017). Children, especially infants, suspected of having systemic Escherichia coli infection should usually receive intravenous antibiotic treatment as soon as possible, even before culture results are obtained. About 50% of Escherichia coli strains are resistant to Amoxicillin or Ampicillin, so third-generation aminoglycoside or cephalosporin antibiotics are recommended as empirical therapy (Manges et al., 2019). Cases of bacterial resistance will continue to increase every year, which is the main reason researchers find new antibacterial compounds to overcome the problem, both synthetic and herbal substrate (Rendowaty et al., 2017).

The advantage of herbal-based substrates is that they have proven empirical experience, which means they are safe and do not cause harmful side effects to the human body (Zakaria et al., 2017). Plants with medicinal potential are natural resources that can be used because of the content of bioactive compounds in them. The discovery of new compounds that have antibacterial power against resistant bacteria is significant for treating bacterial infections, especially those already resistant to antibiotics. Coleus amboinicus Lour, or Plectranthus amboinicus Lour- commonly known as torbangun- is a traditional medicinal plant belonging to the Lamiaceae family. This Torbangun plant has been empirically used for generations to treat cough, fever, indigestion and loss of appetite, throat infections, and nasal congestion, and has anti-hyperuricemia activity (Suryowati & Gultom, 2018).

Torbangun, found throughout Indonesia with different names, can grow wildly in loose and barren soil. In northern Sumatera, this plant is called Bangun-bangun, belongs to the group of grass types, and has softwood stems and stalks. The benefits of Torbangun leaf are that the Bataknese widely and traditionally use it to increase breast milk production (Damanik et al., 2006). The results of in vivo tests of Torbangun leaf mixture supplementation can increase milk production in 20 experimental animals (Iwansyah et al., 2017). Phytochemical analysis showed that the ethanol extract of Torbangun leaf contains flavonoid compounds and the main active component that inhibits the activity of the enzyme α-glucosidase. The mechanism of inhibition of flavonoids to the enzyme α-glucosidase is through hydroxylation bonds and substitutions in the β ring. The principle of this inhibition is to produce a delay in the hydrolysis of carbohydrates and absorption of glucose and inhibit the metabolism of sucrose into glucose, so it is beneficial to maintaining health (Phan et al., 2013).

The other study reported that Torbangun has antimicrobial and antioxidant activity (Ashwini & Girish, 2014). Phytochemical analysis shows that ethanol extract of its leaves contains flavonoid compounds (flavonols), and the main active component that inhibits α-glucosidase activity is beneficial for inhibiting the growth and viability of germs (Phan et al., 2013). Although it has been used as an antibacterial agent against Staphylococcus aureus, Bacillus cereus, Escherichia coli, and Yersinia enterocolitica (Nazliniwaty & Laila, 2019; Praveena & Pradeep S, 2012), the use of the leaf of this plant as an antibacterial still limited

to bacteria that are sensitive to antibiotics, while its application to diarrhea-causing pathogens such as Escherichia coli that have resistant plasmids such as pBR322 has never been done.

2. Methods

2.1. Torbangun Leaf Simplisia

Torbangun leaf (Coleus amboinicus Lour) was obtained from the traditional market in Jakarta. The leaf was washed and dried in an oven at 40-50°C for 4-5 days until the moisture content < 10% (weight of simplicial minus the weight of ash, multiplied by 100% (Association of Official Analytical Chemists & Association of Official Agricultural Chemists (US), 2005). The dried simplicial Torbangun leaf is then ground to a powder form measuring 100 mesh.

2.2. Maceration Process

70% ethanol solvent and distilled water were mixed with 100 grams of Torbangun leaf powder in a ratio of 1:5, stirred periodically, and soaked for 24 hours at room temperature. The powder bath was filtered with Whatman No. 40 filter paper and stored in a refrigerator at 10°C to extract the powder maximally. It was macerated with 70% ethanol and distilled water. The second maceration process was carried out for four days. The filtrate obtained was combined and then evaporated with an evaporator at 60°C to produce a crude extract of Torbangun leaf (Elsyana et al., 2016).

2.3. Bacterial Culture

Cultures of Escherichia coli pBR322 bacteria (Flinders University, Adelaide, South Australia) were rejuvenated on Mueller Hinton's agar media with antibiotics Ampicillin and Tetracycline (50µg/mL; 15µg/mL), then taken one colony and put into 10mL of physiological NaCl 0.9%. The bacterial suspension is then vortex and turbidity regulated and equalized with a standard 0.5 McFarland solution so that each mL suspension contains bacteria 1.5 × 10^8 CFU/mL (Mere et al., 2021).

2.4. Antibacterial Activity Test (Method Kirby Bauer)

A total of 15 mL of Luria Bertani agar media containing ampicillin and tetracycline antibiotics (50µg/mL; 15µg/mL) was put into an incubation; each 100µL was taken and dripped on the surface of the media so that Luria Bertani which had solidified on Petri dishes was incubated at 37°C for 24 hours. The number of colonies formed was calculated and compared with the number of colonies at the control. The smallest extract that did not form a colony at dilution showed minimal inhibitory concentrations (Yang et al., 2020).

2.5. Toxicity Test by Method Brine Shrimp Lethality Test (BSLT)

The toxicity activity of Torbangun leaf extract was carried out using the BSLT method, which was described with a slight modification (McLaughlin et al., 1998). Artemia salina Leach were used as test organisms. Shrimp in a sterile petri dish and then allow to solidify. A bacterial suspension that has been adjusted according to McFarland Standard 0.5 as much as 100µL is dripped on the surface of the media and flattened using a sterile glass spreader. Furthermore, each disc paper was given extracts with concentrations of 80%, 65%, 50%, and 25%. The positive control was the antibiotic Kanamycin 30 ppm, while the negative control was 20% DMSO. The amount of material dripped on each disc paper is 20µL. Disc paper that has been dripped with test material is placed on the surface of the media with test bacteria and incubated at 37 °C for 24 hours. Antibacterial activity was determined by measuring the growth inhibition zones formed around the disc after 24 hours (Mere et al., 2021).

2.6. Minimum Inhibitory Concentrations Assay

Escherichia coli pBR322 strains (108 CFU/ml) were incubated with extraction solution at various concentrations in culture media on a 96-well test plate (100µl per well) for 24 hours. The bacteria were incubated with a control medium. After cysts are placed and hatched for 48 hours with a constant oxygen supply at room temperature. Four graded sample concentrations (250, 200, 150, and 75 ppm) were used in the experiment. The experiment was conducted with three repetitions. A test tube containing 50 µL of Tween 80 at 10mL of seawater and 10 live shrimp larvae was used as a negative control. The surviving larvae were counted after 24 hours of sample exposure. A larva is considered dead if it does not move during 10 seconds of observation.

2.7. Analyzes Data

Data analysis was performed using an unpaired t-test using GraphPad Prism® software. P values <0.05 (*), <0.01 (**), and <0.001 (***) are considered significant. The average ± standard deviation (SD) for at least three experiments was calculated independently.

3. Results and Discussion

3.1. Results

A total of 200 g of 70% ethanol extract was obtained from 10 kg of wet Torbangun leaf. The results of the research process showed that Torbangun leaf ethanol extract has an inhibitory power against *Escherichia coli* pBR322, which has resistance to antibiotics at extract concentrations of 15% and continues to increase at concentrations of 25%, 50%, 65%, and 80%. This result is indicated by a growth inhibition zone in antibacterial activity testing with the *Kirby bauer* method shown in Figure 1.

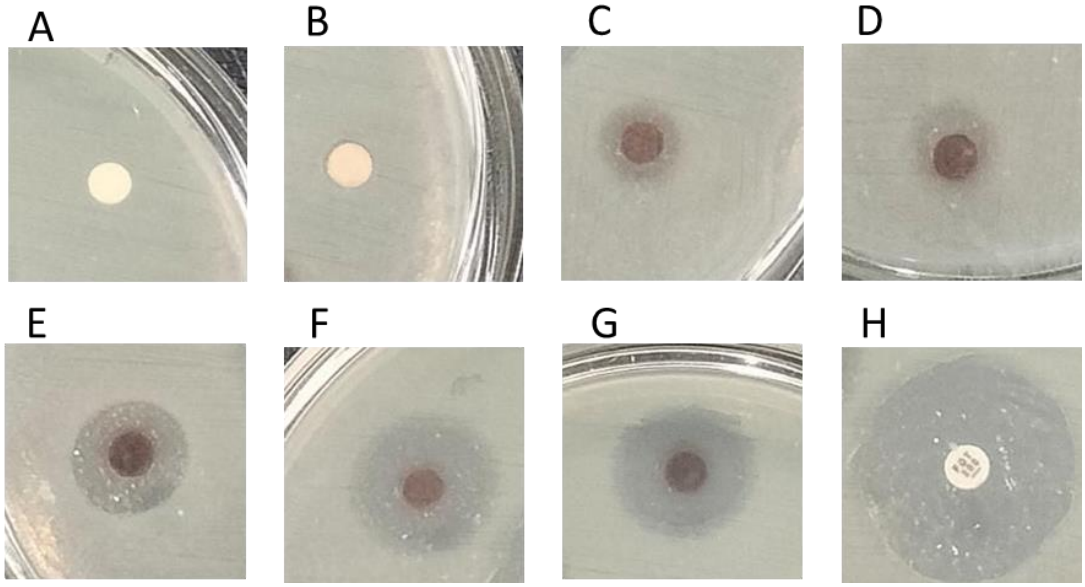

Figure 1 Antibacterial Activity Test

The antibacterial activity of Torbangun leaf extract against antibiotic-resistant bacteria *Escherichia coli* pBR332. A. 0%, B. 10%, C. 15%, D. 25%, E. 50%, F. 65%, G. 80%, H. Kanamycin 30µg.

The analysis of the unpaired antibacterial test (Figure 2) showed an increase in bacterial inhibition with an increase in concentration, with a 15% extract concentration showing a significant difference. This shows that Torbangun leaf ethanol extract has a strong antibacterial ability.

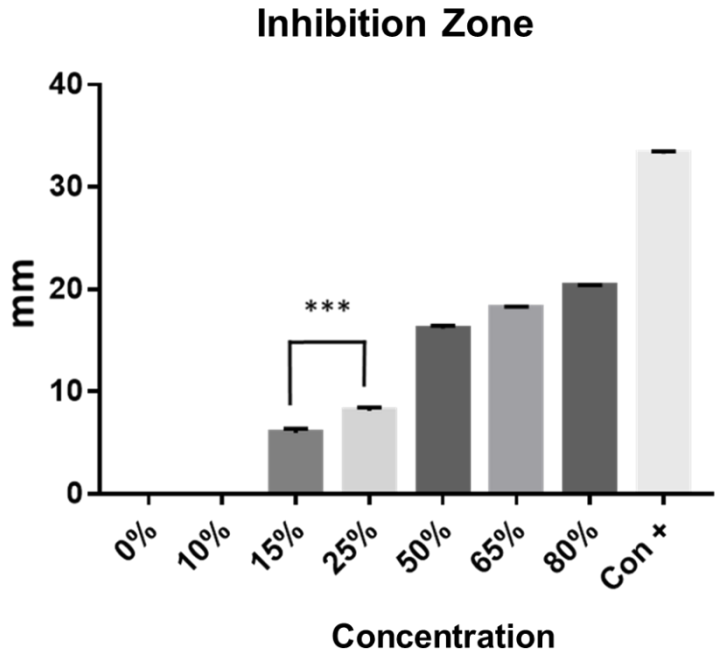

Figure 2 Inhibition Zone of Antibacterial Activity of Torbangun Extract

Analysis of *the unpaired t-test* showed that a concentration of 15% has shown significant antibacterial activity $p < 0.05$ (*), < 0.01 (**), and < 0.001 (***).

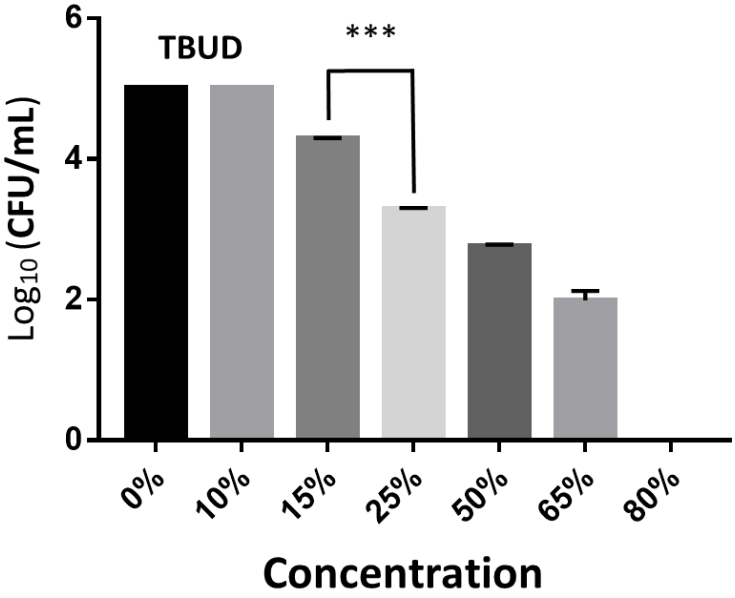

Figure 3 Minimum Inhibitory Concentrations Assay

Torbangun leaf extract (0%, 10%, 15%, 25%, 50%, 65%, 80%) was incubated with *Escherichia coli* pBR322 bacteria for 24 hours, then grown on media, and the number of colonies (Log10 CFU/mL) was counted.

Similar to the results of antibacterial activity tests, in determining the minimum inhibitory concentration of bacteria, it was found that a concentration of 15% inhibited the growth of *Escherichia coli* pBR322 bacteria. The data showed (Figure 3) that the increase in concentration was negatively correlated with the number of bacteria growing on the media. At a concentration of 80%, *Escherichia coli* pBR322 bacteria had no growth.

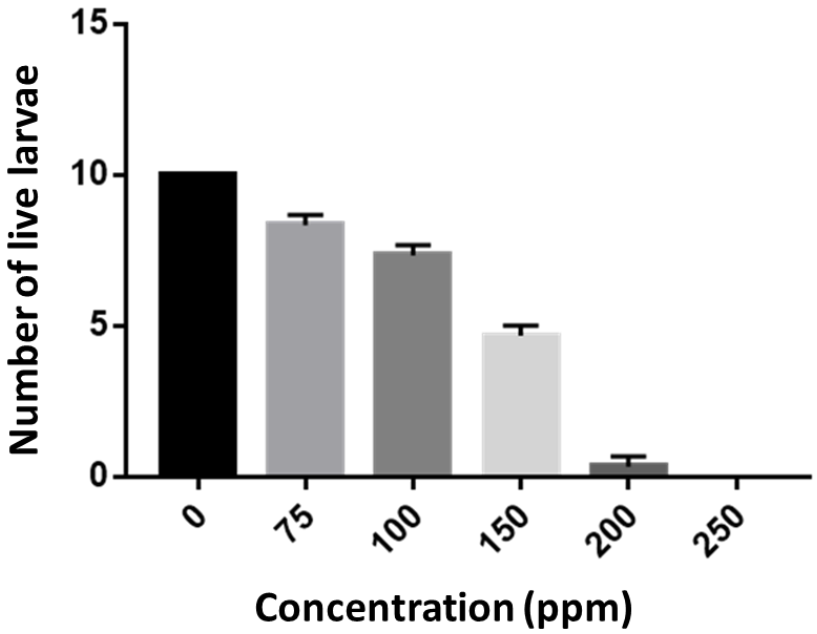

Figure 4 Toxicity test by method of Brine Shrimp Lethality Test (BSLT)

The number of larvae that survived after incubating for 24 hours with Torbangun leaf extract (0, 75, 100, 150, 200, 250 ppm).

The results of statistical tests between concentration and the number of larval deaths showed a significant correlation (Figure 4). Given the amount of initial wet material of Torbangun leaf, the extract used is still very concentrated for testing shrimp larvae, although the results of incubation for 24 hours indicate that the toxic effect is minimal.

Other studies report that Torbangun plants can be used for the treatment of various diseases such as anti-inflammatory, anti-anxiety, lowering blood sugar-cholesterol and uric acid levels, and are non-toxic (Dathar, 2019; Erny Sabrina et al., 2014; Santos et al., 2016). Further research is needed, including animal models with other biochemical tests, that can help to understand Torbangun's antibacterial mechanisms.

Torbangun leaf extract at a concentration of 15% has shown inhibitory activity against the growth of *Escherichia coli* pBR322 bacteria and a mortality rate of 50% of larvae at a concentration of 150 ppm. Further research is necessary to isolate the components of the active compounds of Torbangun leaf and therapeutic mechanisms. Torbangun has enormous prospects in meeting the global demand for cost-effective natural ingredients and safer bioactive molecules in the pharmaceutical and nutraceutical industries.

4. Conclusion

Torbangun leaf extract at a concentration of 15% has shown inhibitory activity against the growth of Escherichia coli pBR322 bacteria and a mortality rate of 50% of larvae at a concentration of 150 ppm. Further research is necessary to isolate the components of the active compounds of Torbangun leaf and therapeutic mechanisms. Torbangun has enormous

prospects in meeting the global demand for cost-effective natural ingredients and safer bioactive molecules in the pharmaceutical and nutraceutical industries.

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