

Effectiveness Test and Phytochemical Screening of Ethanol Extract of Cashew Leaf (*Anacardium occidentale* Linn.) against *Bacillus cereus*

Aziizah Nabiilah Putri Hanafi¹, Ni Luh Suriani^{1*}, Anak Agung Ketut Darmadi¹, Ting Seng Ho,² Gusti Ayu Mayani Kristina Dewi³

¹Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University Bali 80361 – Indonesia

²Back2nature organic farm, Kuala Pilah, Malaysia

³Department of Animal Husbandry Undergraduate Study Program, Faculty of Animal Husbandry, Udayana University, Bali 80361–Indonesia

Corresponding Author: Ni Luh Suriani; niluhsuriani@unud.ac.id

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ABSTRACT

Indonesia is a tropical country that has very abundant biodiversity and great potential to explore its benefits in the health sector, including as a source of natural antibacterial ingredients. One of the potential plants is cashew (*Anacardium occidentale* L.), which is known to be able to grow in an environment with a dry climate, the leaves have long been used in traditional medicine and have antibacterial activity. This study aims to assess the Minimum Inhibitory Concentration (MIC) value and effectiveness of cashew leaf ethanol extract against *Bacillus cereus* bacteria, as well as the identification of phytochemical compound groups. The methods used include well diffusion for MIC test from 1%-0.025% concentration and effectiveness test from 5%-20% concentration, as well as qualitative phytochemical screening to identify active compound groups in the extract. The results showed the MIC value obtained at a concentration of 0.05% inhibition zone of 5.93 mm. The antibacterial effectiveness test of 5-20% concentration increased as the concentration increased. Phytochemical screening identified the presence of alkaloids, phenolics, flavonoids, tannins, saponins, steroids, and terpenoids. These results indicate the potential of cashew leaf extract as a natural antibacterial agent against *B. cereus*.

1. Introduction

Indonesia is a tropical country that has very abundant biodiversity and has the potential to be explored for its benefits as a natural ingredient with natural antibacterial activity. One plant that has economic and medical value is cashew (*Anacardium occidentale* L.), which is able to grow optimally in dry climates. All parts of this plant, especially cashew leaves, have long been utilized in traditional medicine due to their bioactive compounds (Saenab et al., 2016).

The presence of secondary metabolite compounds in plants, such as alkaloids, flavonoids, tannins, and saponins, plays an important role in the plant's defense system against pathogenic microorganisms. These compounds not only serve as natural plant protectors, but also exhibit antimicrobial activity. The content of secondary metabolites in plants is influenced by environmental factors such as temperature, light intensity, humidity, wind, and soil conditions. Plants that grow in extreme environments tend to produce higher levels of secondary metabolites than plants that grow in normal conditions (Qodri et al., 2023).

One of the pathogenic bacteria that often contaminate food products is *Bacillus cereus*, a Gram-positive, spore-forming bacterium that is a facultative anaerobe. The ability of *B. cereus* to form spores makes it resistant to various extreme environmental conditions, such as drought, heat, radiation, and low pH environments. *B. cereus* is known to cause food poisoning, with symptoms such as nausea, vomiting, and diarrhea. Its enzymatic activity causes sensory damage to food products, such as milk clumping and the appearance of abnormal textures in dairy products (Jessberger et al., 2020).

Previous studies have shown that ethanol extract of cashew leaves has potential as an antibacterial. Agustín (2023) reported that ethanol extract of cashew leaves was able to inhibit the growth of *Staphylococcus aureus*, while the research of Astuty et al. (2022) showed antibacterial activity against *Escherichia coli*. Based on this background, this study aims to evaluate the MIC value and effectiveness of cashew leaf ethanol extract against *B. cereus*, as well as the identification of phytochemical compound content.

2. Methodology

2.1 Time and place of research

This research was conducted from November 2024 to January 2025. Cashew leaf samples were obtained from Ban Village, Kubu District, Karangasem Regency, Bali Province. The process of making cashew leaf extracts and bacterial inhibition tests were carried out at the Microbiology Laboratory, Biology Study Program, Faculty of Mathematics and Natural Sciences, Udayana University. Phytochemical screening test was carried out at the Integrated Laboratory, FMIPA, Udayana University. *Bacillus cereus* bacterial isolates were obtained from Gadjah Mada University, Yogyakarta, Indonesia.

2.2 Methods

This study used a completely randomized design (CRD) for antibacterial effectiveness test and Minimum Inhibitory Concentration (MIC) test. The effectiveness test consisted of 6 treatments, namely negative control (P1), 5% concentration (P2), 10% (P3), 15% (P4), 20% (P5), and positive control (P6), each treatment was carried out as many as four replicates. In the MIC test, 6 levels of extract concentration were used, namely 1%, 0.5%, 0.25%, 0.1%, 0.05%, and 0.025%, each with four replicates. Qualitative phytochemical screening of alkaloid, phenolic, flavonoid, tannin, saponin, steroid, and terpenoid compound groups.

Preparation of Cashew Leaves Extract

Samples of cashew leaves (*Anacardium occidentale* L.) that have been finely macerated using 96% ethanol in a ratio of 1:2 for 3 days, then filtered. The maceration process was repeated 3 times. The extracts obtained were combined into one and then evaporated with a rotary evaporator at a temperature of 45 ° C (boiling point of ethanol) so that a thick ethanol extract was obtained. The extract obtained was then weighed and the extract yield was calculated using the following formula (Gustiana et al., 2022):

$$\% \text{Rendemen} = \frac{\text{Weight of thick extract (gram)}}{\text{Weight of initial simplisia (gram)}} \times 100\%$$

Then the thick extract was dissolved with 96% ethanol solvent to obtain concentrations of 20%, 15%, 10%, and 5%. The dilution formula, as follows:

$$M1.V1 = M2.V2$$

Description:

M1 : Concentration before dilution

M2: Concentration after dilution

V1: Volume before dilution

V2: Volume after dilution

Phytochemical Screening of Cashew Leaf Extracts

1. Alkaloids

A total of 1 mL of extract was put into a test tube, then added 1 mL of HCl 2N and a little aquadest. The mixture was heated on a hotplate to boil, then allowed to stand for 2 minutes and cooled, after cooling, the filtrate was added 2 drops of Mayer reagent and allowed to stand for 5-7 minutes. A positive reaction to alkaloids is indicated by the formation of a white or yellow precipitate (Susetyarini and Nurrohman, 2022).

2. Phenolic

A total of 1 mL of extract was put into a test tube, then 3 drops of 1% FeCl₃ solution were added. A positive reaction to phenolic compounds is indicated by the formation of green, blue, or purple color (Astuty et al., 2022).

3. Flavonoid

A total of 1 mL of extract was put into a test tube, then 1 mL of concentrated HCl and magnesium powder (Mg) was added. A positive reaction is characterized by the formation of a red, yellow, or green color, which indicates the presence of flavonoid compounds (Astuty et al., 2022).

4. Tannin

The ethanol extract of cashew leaves was heated with 10 mL of distilled water for 3 minutes, then cooled. After that, 3-5 drops of FeCl₃ solution were added. A positive reaction is characterized by the

formation of a blue black or green black color, which indicates the presence of tannin compounds (Astuty et al., 2022).

5. Saponins

A total of 1 mL of extract was put into a test tube, then 10 mL of hot water was added. The mixture was cooled, then shaken vigorously for 10 seconds. The formation of a stable white froth of 1-10 cm in height that does not disappear within 10 minutes indicates a positive reaction. The addition of 1 drop of 2N HCl is done to confirm the results; if the foam persists, then the extract contains saponins (Astuty et al., 2022).

6. Steroid

A total of 1 mL of extract was mixed with 1 mL of chloroform in a test tube, then 1 mL of concentrated sulfuric acid (H₂SO₄) was added slowly through the tube wall. A positive reaction is characterized by the formation of a blue or green ring on the border of two solvents, which indicates the presence of steroid compounds (Fajriaty et al., 2017).

7. Terpenoids

A total of 1 mL of extract was put into a test tube, then 1 mL of chloroform and 1 mL of anhydrous acetic acid were added respectively. Furthermore, 1 mL of concentrated sulfuric acid (H₂SO₄) was added slowly through the tube wall. A positive reaction to terpenoids is indicated by the formation of a reddish brown ring on the border of two solvents (Fajriaty et al., 2017).

***Bacillus cereus* Bacteria Rejuvenation**

Bacillus cereus bacteria used in this study were obtained from pure isolates of Gajah Mada University (UGM) with strain number FNCC 0057. Bacterial rejuvenation was carried out by inoculating 1 Ose of *B. cereus* culture into Nutrient Agar (NA) tilted media. The media containing bacterial culture was then incubated at 37°C for 24 hours. Colonies that grew on the NA tilted media were taken with an Ose needle, inoculated into Nutrient Broth (NB) media in a test tube in a zigzag manner. The test tube was covered with cotton and plastic wrap, incubated at 37°C for 24 hours (Rosmania and Yanti, 2020).

Preparation of *Bacillus cereus* Bacteria Suspension

Preparation of bacterial suspensions was carried out by taking 1 Ose of *B. cereus* bacterial colonies on tilted NA media, then suspended into a bottle containing 25 mL of Nutrient Broth (NB) media and incubated for 24 hours at 37°C. The bacterial suspension was diluted with sterile 0.9% NaCl until the turbidity was equivalent to 0.5% McFarland standard solution which is equivalent to a cell density of 1.5×10⁸ CFU/mL (Aviany and Pujjianto, 2020).

Minimum Inhibitory Concentration (MIC) Test of Cashew Leaf Extract

MIC testing was carried out using the well diffusion method on Nutrient Agar (NA) media. A total of 15 mL of NA media was poured into a sterile Petri dish. *Bacillus cereus* suspension that has been adjusted to 0.5 McFarland turbidity standard was inoculated evenly using sterile cotton using the streak method. Well holes of 6 mm diameter and 3 mm depth were made using a sterile cork borer. Treatments consisted of six concentrations of cashew leaf ethanol extract, namely 1%, 0.5%, 0.25%, 0.1%, 0.05%, and 0.025% (b/v). Each well was filled with 20 µL of extract using a micropipette. One Petri dish contained four replicates. All dishes were incubated at 37°C for 24 hours. The zone of inhibition formed was observed and measured using a ruler with a cm size (Astuty et al., 2022).

Antibacterial Effectiveness Test of Cashew Leaf Extract

Nutrient Agar (NA) media as much as 15 mL was poured into a sterile Petri dish. *Bacillus cereus* suspension was inoculated on the surface of the media using the streak method with sterile cotton. Diffusion wells were made using a sterile cork borer tip, each well was filled with 20 µL of cashew

leaf extract using a micropipette with four extract concentrations, namely 5%, 10%, 15%, and 20% (b/v), as well as negative control (96% ethanol) and positive control (Chloramphenicol 1%). Each treatment was carried out in four replicates in each well. NA media containing the treatments were incubated at 37°C for 24 hours. The zone of inhibition formed was observed and measured using a ruler with a cm size (Astuty et al., 2022).

Calculation Zone of Inhibition Diameter Extracts

The clear zone formed was calculated by measuring the length of the vertical, horizontal, diagonal 1, and diagonal 2 diameters, then the four diameters were summed and divided by 4 to obtain the diameter of the inhibition zone. The diameter of the clear zone is used as a determinant of the ability of the inhibitory level of the extract against a bacterium. (Astuty et al., 2022).

Table 1. Inhibition Zone Diameter Category

Diameter	Strength of Inhibition Zone
≤ 5 mm	Very weak inhibition
5-10 mm	Weak inhibition
10-20 mm	Moderate inhibition
20-30 mm	Strong inhibition
≥ 30 mm	Very strong inhibition

2.3 Data analysis

The data obtained from this study are qualitative and quantitative data. Qualitative data includes phytochemical tests to prove the presence of alkaloid, phenolic, flavonoid, tannin, saponin, steroid, and terpenoid compounds, while quantitative data includes MIC and effectiveness tests. Quantitative data were analyzed statistically using Analysis of Variance (ANOVA) at the 5% level with SPSS application. The ANOVA test was continued using the Duncan Multiple Range Test to determine differences between treatments.

3. Result and Discussion

3.1 Result

MIC Test of Ethanol Extract of Cashew Leaf

The results of the inhibition zone around the diffusion wells during the observation time of 24 hours can be seen in the following table.

Table 2. MIC Test of Ethanol Extract of Cashew Leaf

No	Concentration	N	Inhibition Zone (mm)	Category
1	1%	4	11,68 ± 1,39 ^a	Moderate inhibition
2	0,5%	4	8,18 ± 2,06 ^b	Weak inhibition
3	0,3%	4	7,12 ± 0,32 ^b	Weak inhibition
4	0,1%	4	6,50 ± 0,40 ^b	Weak inhibition
5	0,05%	4	5,93 ± 0,65 ^b	Weak inhibition
6	0,025%	4	0,00 ± 0,00 ^c	No inhibition

Note: The values in Table 2 standard deviation are the average of 4 replicates with values followed by the same letter in the same column are means that are not significantly different ($p > 0.05$) based on Duncan's multiple range test, after Anova analysis of variance.

Table 2 shows that the concentrations of 1%, 0.5%, 0.3%, 0.1% and 0.05% of the ethanol extract of cashew leaves can still inhibit the growth of bacteria *B. cereus*, with an average inhibition zone diameter of 11.68 mm, 8.18 mm, 7.12 mm, 6.50 mm, and 5.93 mm, respectively, while at the smallest concentration of MIC testing, 0.025%, there was no visible antibacterial activity with no inhibition zone formed around the diffusion wells, therefore, the MIC value was obtained at a concentration of 0.05%, with an inhibition zone diameter of 5.43 mm.

Effectiveness Test of Cashew Leaf Ethanol Extract

The results of the inhibition zone around the diffusion wells during the observation time of 24 hours can be seen in the following table.

Table 3. Effectiveness Test of Cashew Leaf Ethanol Extract

No	Application	N	Inhibition Zone (mm)	Category
1	Negative control	4	0,00 ± 0,00 ^d	No inhibition
2	5%	4	12,87 ± 0,92 ^{bc}	Moderate inhibition
3	10%	4	12,87 ± 0,43 ^{bc}	Moderate inhibition
4	15%	4	14,81 ± 0,55 ^b	Moderate inhibition
5	20%	4	16,56 ± 0,68 ^a	Moderate inhibition
6	Positive control	4	14,00 ± 1,41 ^b	Moderate inhibition

Note: The values in Table 3 standard deviation are the average of 4 replicates with values followed by the same letter in the same column are means that are not significantly different ($p > 0.05$) based on Duncan's multiple range test, after Anova analysis of variance.

Table 3 shows that the treatment of ethanol extract of cashew leaves at concentrations of 5%, 10%, 15%, 20%, as well as the positive control produced inhibition zone diameters that were significantly different compared to the negative control. The diameter of the inhibition zone was 12.87 mm (5% and 10%), 14.81 mm (15%), 16.56 mm (20%), and 14 mm (positive control), respectively. Based on statistical tests, there was no significant difference between the 5%, 10%, 15%, and positive control concentrations, while the 20% concentration showed a significant difference compared to the four treatments.

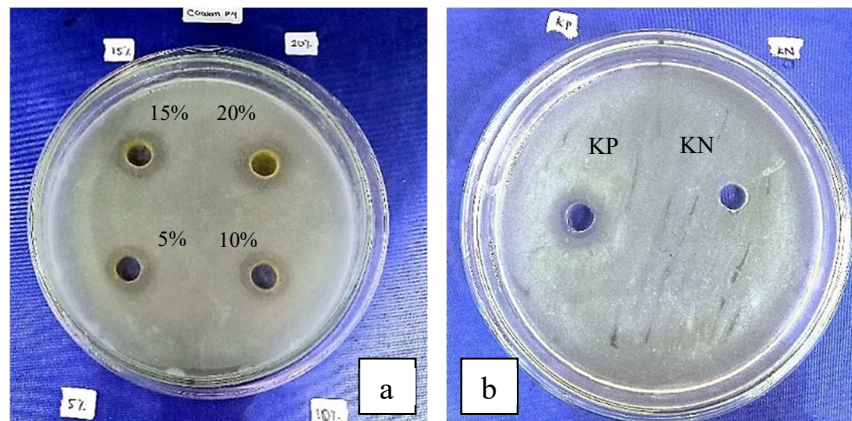


Figure 1: Effectiveness test results of cashew leaf ethanol extract against *Bacillus cereus*: (a) concentration power of 5%, 10%, 15%, and 20%; (b) KP = Positive Control and KN = Negative Control.

Based on Figure 1, the higher the concentration used, the wider the inhibition zone formed.

Phytochemical Screening of Ethanol Extract of Cashew Leaf

The groups of compounds present in the ethanol extract of cashew leaves were identified using the phytochemical screening test.

Table 4. Phytochemical Test Results

Compound Class	Reagent	Result	Conclusion
Alkaloids	Mayer's reagent	Yellow precipitate	+
Phenolic	1% FeCl ₃	Green coloration	+
Flavonoid	Mg + HCl	Dark red coloration	+
Tannin	1% FeCl ₃	Green coloration	+
Saponins	Distilled water + 2N HCl	Persistent foam	+
Steroid	H ₂ SO ₄	Reddish-brown ring	+
Terpenoids	Acetic anhydride + H ₂ SO ₄	Green ring	+

Note:

+ = extract contains compounds

- = extract does not contain compounds

The results of the phytochemical test of cashew (*A. occidentale*) leaf extract showed that cashew leaf extract contains alkaloids, phenolics, flavonoids, tannins, saponins, steroids, and terpenoids (Table 4).

3.2 Discussion

Preparation of Ethanol Extract of Cashew Leaf

The solvent used in this study is ethanol which has polar properties. The compounds contained in cashew leaf extracts are generally compounds that dissolve in polar solvents (Yusvantika, 2021). The polarity of the solvent used in the extraction process must be similar or very close to the polarity of the extracted active ingredient, according to the principle of "like dissolves like.". According to Prayoga et al. (2019), each type of solvent has a different level of polarity and solvents with different concentrations also show different polarities. The more similar the polarity of the solvent is to the polarity of the substance contained in the extraction material, the more components of the substance can be extracted, so as to produce a larger zone of bacterial inhibition.

MIC Test of Ethanol Extract of Cashew Leaf

The Minimum Inhibitory Concentration (MIC) test results show that the ethanol extract of cashew leaves has an MIC value of 0.05%, with an average inhibition zone diameter of 5.93 mm. The results of this study are in line with the research of Soleha (2015), which explains that the larger the diameter of the inhibition zone, the smaller the MIC concentration required. This study shows that *B. cereus* is a Gram-positive bacterium that tends to be more sensitive to antibacterial compounds due to the simpler cell wall structure of Gram-positive bacteria compared to Gram-negative bacteria. The cell wall of Gram-positive bacteria is dominated by peptidoglycan, which is the target of many antibacterial compounds, so Gram-positive bacteria such as *B. cereus* become more susceptible to antibacterial compounds, which is reflected in lower MIC values (Sumaryati and Sudiyono, 2015).

Effectiveness Test of Cashew Leaf Ethanol Extract

The results of testing the effectiveness of cashew leaf ethanol extract against the growth of *B. cereus* bacteria showed that cashew leaf ethanol extract at all concentrations tested was able to inhibit the growth of *B. cereus* bacteria. At concentrations of 10%, 15%, and 20% showed variations in the ability to inhibit the growth of *B. cereus*, with an average diameter of 12.87 mm, 14.81 mm, and 16.56 mm, respectively, included in the strong inhibition category. Inhibition against the growth of *B. cereus* bacteria increases with the increase in the concentration of cashew leaf extract used (Yuliati, 2016). The selection of Chloramphenicol as a positive control is because Chloramphenicol works by disrupting the binding of amino acids to the peptide chain by inhibiting the activity of the enzyme peptidyl transferase, resulting in inhibition of protein synthesis and reducing the formation of energy and bacterial structures, which causes inhibition of bacterial development (Santri et al., 2020). Based on this study, the average diameter of the inhibition zone produced by Chloramphenicol 1% as a positive control against *B. cereus* was 14 mm, which indicates that *B. cereus* is intermediate to Chloramphenicol.

Phytochemical Screening of Ethanol Extract of Cashew Leaf

Based on the results of phytochemical tests addressed in Table 6, it can be seen that the ethanol extract of cashew leaves (*A. occidentale*) contains secondary metabolite compounds such as alkaloid compounds, phenolics, flavonoids, tannins, saponins, steroids, and terpenoids. Tests on the content of alkaloid compounds show that the ethanol extract of cashew leaves contains alkaloid compounds, as evidenced by the positive reaction to Meyer's reagent which produces a yellow precipitate. According to Khafid et al. (2023), the precipitate is formed through the complexation of K^+ ions from the potassium tetraiodomercurate (II) reagent with nitrogen atoms from alkaloids, which act as electron pair donors. Alkaloids function as antibacterial compounds with a mechanism of action that changes the structure of peptidoglycan in the bacterial cell wall, so that the cell wall layer is not formed perfectly and leads to the death of bacterial cells (Ramadhani et al., 2022).

The green color change in the extract after the addition of 1% $FeCl_3$ is caused by the formation of complex compounds between phenolic compounds and Fe^{3+} ions (Siahaan et al., 2021). Phenol compounds have the ability to bind to proteins through hydrogen bonds, which can cause damage to protein structures. This damage can interfere with the function of selective permeability, active transport, and control of protein composition in bacterial cells due to instability in the cell wall and cytoplasmic membrane (Rostikawati and Supratman, 2021).

Testing the content of flavonoid compounds in the ethanol extract of cashew leaves showed positive results with a change in color to dark red after the addition of concentrated HCl and magnesium powder, which reduces the benzopyrone nucleus in the flavonoid structure. Flavonoids are antibacterial compounds with a broad spectrum of activity that work by interfering with the bacterial immune system. Flavonoids have three main mechanisms in their antibacterial activity, namely disrupting energy metabolism, damaging cell membrane function, and inhibiting nucleic acid synthesis (Ramadhani et al., 2022).

Testing the content of saponin compounds in the ethanol extract of cashew leaves showed positive results, which were characterized by the formation of foam around the test tube. This froth formation process occurs when the saponin is shaken, where the hydrophilic group binds to water, while the hydrophobic group binds to air. Saponin has properties as a surfactant with a structure similar to polar, so that the fat in the cell membrane will break, which results in disruption of cell membrane

permeability, as an antibacterial, saponin can damage the bacterial cell membrane, which leads to damage to the components in the bacteria (Yulia et al., 2023).

Testing the content of steroid and terpenoid compounds in the ethanol extract of cashew leaves showed positive results. The steroid test is characterized by the formation of a green ring at the boundary between two solvents, while the terpenoid test shows the formation of a reddish brown ring at the boundary of two solvents. The terpenoid test uses Liebermann-Burchard reagent (anhydrous acetic acid-H₂SO₄), the purpose of adding Liebermann-Burchard is to form acetyl derivatives from the acetylation reaction of the OH group to form a reddish brown ring (Habibi et al., 2018). In the steroid test, the oxidation of H₂SO₄ results in the formation of a conjugated double bond which causes a green color change at the border of two solvents (Fajriaty et al., 2018).

4. Conclusion

The Minimum Inhibitory Concentration (MIC) value of cashew leaf ethanol extract against *Bacillus cereus* bacteria is at a concentration of 0.05% with an average inhibition zone diameter of 5.93 mm. The ethanol extract of cashew leaves effectively inhibited the growth of *B. cereus* bacteria at concentrations of 5%, 10%, 15%, and 20% with an average inhibition diameter of 12.87 mm, 12.87 mm, 14.81 mm, and 16.56 mm. In addition, the ethanol extract of cashew leaves contains phytochemical compounds, namely alkaloids, phenolics, flavonoids, tannins, saponins, steroids, and terpenoids.

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