JKPK (JURNAL KIMIA DAN PENDIDIKAN KIMIA), Vol. 9, No.1, 2024 pp. 50-68
Chemistry Education Study Program, Universitas Sebelas Maret ISSN 2503-4146
https://jurnal.uns.ac.id/jkpk ISSN 2503-4154 (online)

PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL ACTIVITY OF ETHANOLIC EXTRACTS FROM *Delonix regia* AGAINST LABORATORY STRAINS OF DIARRHEAL BACTERIA

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ARTICLE INFO

Keywords:
Delonix regia;
phytochemical;
FTIR;
LC-MS/MS;
antibacterial

Article History:
Received: 2024-02-27
Accepted: 2024-04-20
Published: 2024-04-23

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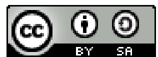
Email: a.safitri@ub.ac.id
doi:10.20961/jkpk.v9i1.85374



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ABSTRACT

The present study evaluates the antibacterial efficacy of ethanolic extracts from *Delonix regia* leaves against diarrhea-inducing bacteria *Escherichia coli* and *Salmonella typhimurium*. Preliminary phytochemical screening revealed that *D. regia* leaves comprise flavonoids, alkaloids, saponins, tannins, phenolics, and terpenoids. Fourier Transform Infrared Spectroscopy (FTIR) analysis identified various functional groups in the *D. regia* leaf extract, including O-H, C-H, C=O, C=C, C-C, C-O, and C-O-C. Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS) analysis further confirmed the presence of 16 distinct compounds comprising amino acids, alkaloids, phenolics, flavonoids, terpenoids, anthraquinones, n-acyl pyrrolidines, and fatty acids. The disc diffusion method (Kirby-Bauer) was employed for the antibacterial tests. The extracts of *D. regia* leaves at concentrations of 25%, 50%, 75%, and 100% generated inhibition zones measuring 5.26 mm, 6.37 mm, 7.27 mm, and 10.18 mm against *E. coli*, and 5.05 mm, 7.01 mm, 8.77 mm, and 10.04 mm against *S. typhimurium*, respectively. The commercial antibiotic ciprofloxacin (a positive control) produced inhibition zones of 30.02 mm for *E. coli* and 28 mm for *S. typhimurium*. The negative control, consisting of 10% ethanol, showed no inhibitory effect on bacterial growth. These findings indicate that the ethanolic extract of *D. regia* leaves possesses antibacterial properties against *E. coli* and *S. typhimurium*. It is likely that secondary metabolite compounds, such as flavonoids and phenolics, contribute significantly to the observed antibacterial activity

How to cite: Y. S. Senge, I. Mustafa, A. Safitri, , "Phytochemical Screening and Antibacterial Activity of Ethanolic Extracts from *Delonix regia* Against Laboratory Strains of Diarrheal Bacteria," *Jurnal Kimia dan Pendidikan Kimia (JKPK),* vol. 9, no. 1, pp. 50-68, 2024. Available: http://dx.doi.org/10.20961/jkpk.v9i1.85374

INTRODUCTION

Indonesia is abundantly endowed with natural resources, supporting the growth of nearly all plant types [1]. It is estimated that there are approximately 90,000 plant species in Indonesia, of which 9,600 are recognized for their medicinal properties. About 300 of these species are utilized by the traditional medicine sector as raw materials for remedies [2]. According to the World Health Organization (WHO), 80% of populations in developing countries rely on medicinal plants for traditional medicine practices [3], and Indonesia is prominent among these nations.

One notable example of a medicinal plant used in traditional treatments in Indonesia is *Delonix regia*.

Delonix regia, commonly known as flamboyant, is a flowering legume within the Fabaceae family and the Caesalpinioideae subfamily, originally from Madagascar [4]. *D. regia* plants are prevalent across most subtropical and tropical regions [5]. In East Nusa Tenggara (NTT), particularly in Kupang City, flamboyants are common along roadsides, city parks, residential yards, and forests. Despite their widespread presence, public awareness of the health benefits of *D. regia* plants could be improved, leading to underutilization in medicinal applications. These plants are commonly regarded as roadside vegetation providing shade or used as ornamentals due to their visually appealing flowers. However, existing research substantiates the medicinal potential of *D. regia*, with various plant parts such as leaves, flowers, bark, stems, and seeds effectively treating various ailments [4].

Delonix regia (D. regia) flowers are utilized in treating various ailments, including constipation, inflammation, rheumatoid arthritis, diabetes, pneumonia, and malaria, and serve roles as antioxidants, anticancer, and antibacterial agents [6, 7]. The leaves are recognized for their cardioprotective potential, preventing or repairing heart damage in ischemic heart disease (IHD), and exhibiting significant antioxidant and anti-inflammatory properties [8, 9, 10]. The stem and bark of *D. regia* demonstrate antibacterial, antifungal, and antioxidant activities [11], with the bark specifically noted for its antimalarial activity [12]. The seeds, roots, stems, and leaves are all documented to exhibit antibacterial activities [13, 14, 15]. The phytochemical content of *D. regia* is extensive; its flower extract comprises flavonoids, terpenoid saponins, phenolic compounds, carbohydrates, tannins, and glycosides [15]. Similarly, the plant's leaves, roots, and stems contain a blend of flavonoids, saponins, steroids, alkaloids, and terpenoids [12], with the leaf extract specifically rich in flavonoids, alkaloids, tannins, saponins, phenolics, and glycosides [10].

Previous studies have shown that the ethanol extract of *D. regia* leaves inhibited the growth of *Staphylococcus aureus* and *Klebsiella* bacteria, achieving inhibition zones of 12 mm and 19 mm, respectively [16]. Further research by Gautam and Sumet [17] revealed that the methanol extract of *D. regia* leaves, rich in flavonoid content, effectively inhibited the growth of *S. aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, with inhibition zones measuring 25.00 mm, 23.67 mm, 19.67 mm, and 24.67 mm at a concentration of 400 µg, respectively. These antibacterial properties suggest that *D. regia* leaves could be a viable alternative for combating diseases caused by bacteria, such as diarrhea. Diarrhea, a prevalent condition caused by infections in the human digestive tract [18], involves multiple biological agents, including viruses and bacteria, and is a significant cause of mortality, especially among children [19]. The use of *D. regia* leaves in treating pathogenic bacteria responsible for infections in the

digestive tract, particularly *Escherichia coli* and *Salmonella typhimurium*, remains underexplored and merits further research.

Salmonella enterica serovar typhimurium is a major enteric pathogen infecting humans and animals. Infection occurs through consuming contaminated food or drink, enabling the pathogen to penetrate the intestinal epithelium and instigate gastrointestinal infections [20]. Symptoms typically include diarrhea, abdominal pain, nausea, vomiting, acute fever, and diabetes [21]. Additionally, *E. coli* is notably the most frequent bacterial cause of diarrhea in children under five, primarily transmitted through ingesting contaminated food and drinks, exposure to unsanitary environmental conditions, or improper handling of animal products [22].

In developing countries like Indonesia, diarrhea remains a significant health challenge due to its role in causing malnutrition and mortality [23]. One common treatment approach involves using antibiotics to manage diarrhea resulting from bacterial infections. However, the rise of antibiotic resistance poses a severe threat to global health, necessitating the discovery of new antibacterial agents. This issue is particularly acute with gram-negative bacteria, which exhibit greater resistance due to their complex cell wall structures than gram-positive bacteria. Consequently, there is an urgent need for novel antibacterial substances that can effectively combat these resistant strains. Natural sources, such as plant extracts, offer promising alternatives for developing such agents [24].

Given this context, this study aims to evaluate the antibacterial efficacy of *Delonix regia* (D. regia) leaf extract against pathogenic bacterial strains that cause diarrhea, specifically *Escherichia coli* (E. coli) and *Salmonella typhimurium* (S. typhimurium). Furthermore, this research will include the identification and detailed characterization of the chemical constituents in the ethanol extract of *D. regia* leaves, which may underpin its biological activity as an antibacterial agent. The methods employed for characterization will involve qualitative phytochemical screening and analytical techniques such as Fourier Transform Infrared Spectroscopy (FTIR) and Liquid Chromatography-Mass Spectrometry/Mass Spectrometry (LC-MS/MS). These techniques will be used to analyze the structural properties and identify the metabolite profile of the ethanol extract. The findings could highlight *D. regia* leaves as a valuable source of new antibacterial compounds, particularly for treating infections caused by pathogenic bacteria linked to diarrhea

METHODS

1. Materials and Tools

Delonix regia leaves were collected from Pukdale village in Kupang City, East Nusa Tenggara (NTT), Indonesia. Selection criteria for the leaves included maturity and freshness, and the collection was conducted in the afternoon to avoid high sunlight intensity and ambient temperature. The chemicals used in this study included ethanol (96%), distilled water, Mayer's reagent, Dragendorf's reagent, Bouchardat's reagent, concentrated HCl, Mg powder, $FeCl_3$, 1%

$FeCl_3$, 0.9% NaCl, nutrient agar (Oxoid), blank disks (Oxoid), and antibiotic disks (ciprofloxacin). The bacterial cultures involved were *Salmonella typhimurium* and *Escherichia coli*.

Instrumentation for the research included a UV-VIS spectrophotometer (Shimadzu), a Fourier-transform infrared (FTIR) spectrophotometer type SHIMADZU 8400S, and liquid chromatography-tandem mass spectrometry (LC-MS/MS). High-resolution mass spectrometry analysis was performed using an ultra-performance liquid chromatography (UPLC) system, specifically the ACQUITY UPLC® H-Class System (Waters, USA) with MS Xevo G2-S QTof (Waters, USA). The analyses employed a C18 column (1.8 μm, 2.1x100 mm, ACQUITY UPLC® HSS, Waters, USA) at a room temperature of approximately 25°C and a column temperature of 50°C.

2. Extraction of *D. regia* Leaves

The leaves of *Delonix regia* were harvested, washed under running water, and left to air dry for five days while being shielded from direct sunlight. Once dried, the leaves were ground into a fine powder using a blender.

The extraction of *D. regia* leaves employed the maceration method, using a ratio of 1:4 (leaf powder to solvent). Specifically, with periodic stirring, 250 g of D. regia leaf powder was macerated in 1000 mL of 96% ethanol for three consecutive 24-hour periods. After each period, the mixture was filtered using filter paper and compressed with a hydraulic press to extract the liquid. The filtrate was then evaporated using a water bath set at 70°C to remove the ethanol, resulting in a concentrated extract. The extract was weighed and stored in vials for further analysis. The yield percentage was calculated using the following formula:

$$\% \text{ Yield} = \frac{\text{Extract weight}}{\text{Sample weight}} \times 100\ \% \qquad (1)$$

This formula helps determine the efficiency of the extraction process in terms of the amount of extract obtained relative to the initial weight of the plant material used.

3. Phytochemical Screening of Ethanolic Extract of *D. regia* Leaves

Phytochemical screening was conducted to identify and confirm the presence of bioactive compounds in the ethanolic extract of *Delonix regia* leaves. This process is crucial for understanding the potential therapeutic properties of the extract. A prepared solution of 250 mg of the *D. regia* leaf extract in 50 mL of 96% ethanol served as the basis for various qualitative tests to detect specific classes of phytochemicals.

a. Flavonoids

To test for flavonoids, a sample of the *D. regia* leaf extract solution (1 mL) was heated for 5 minutes to enhance the reactivity of potential flavonoids. After heating, 1 mL of hydrochloric acid (HCl) and a small quantity (0.05 mg) of magnesium powder were added to the solution. The presence of flavonoids is typically indicated by the development of red, yellow, or orange colors in the reaction mixture. These color changes result from the complexation of the flavonoids with the added magnesium, forming colorful complexes characteristic of this phytochemical group [24].

b. Alkaloids

For alkaloid detection, 250 mg of the *D. regia* leaves extract was mixed with 1 mL of 2N hydrochloric acid and 9 mL of distilled water and then heated for 2 minutes to activate the alkaloids. After cooling, the mixture was filtered, and 1 mL of the filtrate was transferred into three separate test tubes for testing with different chemical reagents. The first tube received a few drops of Mayer's reagent, typically forming a white or yellow precipitate if alkaloids are present. The second tube was treated with Bouchardat's reagent, which resulted in a brown-to-black precipitate, and the third tube received Dragendorff's reagent, forming a yellow-orange precipitate. The test is considered positive for alkaloids if at least two reagents form a precipitate, indicating a substantial presence of alkaloid compounds in the extract [25, 26].

c. Saponins

The saponin content was assessed by adding 1 mL of the *D. regia* leaves extract solution to 5 mL of distilled water, followed by vigorous shaking for 10 seconds. The presence of saponins is evidenced by forming a stable foam layer measuring 1 to 10 cm in height that persists for approximately 10 minutes. This foam formation occurs due to the soap-like properties of saponins, which lower the surface tension of the liquid. The foam's stability, even after adding 2N hydrochloric acid, which does not cause the foam to dissipate, further confirms the presence of saponins in the extract [27].

d. Tannins

To test for tannins in the *Delonix regia* leaves extract, 1 mL of the extract solution was treated with 1% ferric chloride ($FeCl_3$) solution. The presence of tannins is typically indicated by the formation of green, blue, or black coloration in the solution. These color changes occur due to the complexation between tannins and the ferric ions, demonstrating the ability of tannins to bind and precipitate metals, a property that contributes to their astringency and potential health benefits [28].

e. Phenolics

To detect phenolic compounds, 1 mL of the *D. regia* leaves extract solution was mixed with a few drops of $FeCl_3$. The presence of phenolics is confirmed by the appearance of colors ranging from green, red, purple, blue, dark blue, and bluish-black to dark green. These color variations result from forming phenolic-Fe(III) complexes, which differ based on the specific types of phenolic compounds present in the extract. The complexation reflects phenolic compounds' antioxidative and metal-chelating capacities [29].

f. Terpenoids

To assess the presence of terpenoids, 2 mL of the *D. regia* leaves extract solution was mixed with 2 mL of chloroform, followed by adding a few drops of concentrated sulfuric acid (H_2SO_4). The development of a reddish-brown precipitate upon this addition is indicative of terpenoids. This color change typically results from the acid-based reaction between terpenoids and sulfuric acid, reflecting the structural

rearrangements and complexation within the terpenoid molecules [15].

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4. Characterization of the Ethanolic Extract of *D. regia* Leaves

a. FTIR (Fourier Transform Infrared Spectroscopy)

The ethanolic extract of *Delonix regia* leaves was characterized using FTIR to identify the functional groups present in the extract. For this analysis, the extract was mixed with potassium bromide (KBr) and compressed into a circular pellet with a thickness of 1 mm. This pellet was then placed in the sample holder of the FTIR spectrometer. The analysis was conducted over a wavenumber range of 4000-400 cm^{-1}. The resulting FTIR spectrum provided detailed information about the types of chemical bonds and functional groups within the sample, such as hydroxyl, carbonyl, and aromatic groups, which indicate the compound classes present in the extract.

b. LC-MS/MS (Liquid Chromatography-Mass Spectrometry/Mass Spectrometry)

For the LC-MS/MS analysis, 10 mg of *D. regia* leaf extract was dissolved in 10 mL of methanol. A microsyringe was used to draw 5 μL of this solution, which was injected into the sample area and UPLC column. The liquid chromatography analysis employed a mobile water phase with 5 mM ammonium formate (A) and acetonitrile with 0.05% formic acid (B). The flow rate was maintained at 0.2 mL/min over 23 minutes, with an injection volume of 5 μL. Mass spectrometry was analyzed using an electrospray ionization (ESI) source operating in positive ionization mode across a mass range of 50–1200 m/z. The temperatures for the source and the desolvation processes were set at 100°C and 350°C, respectively. The chromatograms and mass spectra obtained from UPLC-MS/MS were analyzed using MassLynx Version 4.1 software. Data were recorded, including each detected peak's peak areas and m/z values. These data were then cross-referenced and interpreted using various chemical databases such as PubChem, ChemSpider, HMDB, and CFM-ID to identify the chemical constituents present in the extract [30, 31].

5. Antibacterial Activity Test

The medium used in this study was nutrient agar (NA). Initially, 7 g of NA powder was added to an Erlenmeyer flask, dissolved in 250 mL of distilled water, and heated with constant stirring until the solution was homogeneous and boiling. Then, 7 mL of the solution was transferred to a sterile test tube, sealed with cotton, wrapped in aluminum foil, and sterilized using an autoclave at 15 psi and 121 °C for 15 minutes. After sterilization, the test tube was inclined to allow the media to solidify in a slanted position, which was then used to rejuvenate the test bacteria.

a. Subculture of Test Bacteria

Pure isolates of *Salmonella typhimurium* and *Escherichia coli* were each transferred using a sterile loop needle and streaked aseptically onto the solid slanted agar media in a zig-zag pattern. The cultures were then incubated for 24 hours at 37 °C.

b.Preparation of Test Bacterial Suspension

The first step in preparing the bacterial suspension involved creating a 0.9% NaCl solution. This was done by

dissolving 0.9 g of NaCl in a 100 mL volumetric flask filled with distilled water. Af.ter homogenization, 9 mL of this solution was transferred to a test tube and sterilized in an autoclave at 15 psi and 121 °C for 15 minutes. In the second step, *Salmonella typhimurium* and *E. coli* bacteria, previously rejuvenated on nutrient agar, were taken using a sterile loop and transferred into a test tube containing 9 mL of the sterile 0.9% NaCl solution. The suspension was homogenized using a vortex. The optical density (OD) was then measured at 600 nm using a UV-vis spectrophotometer, aiming for a cell density value 0.6, equivalent to approximately 10^6 CFU/mL.

c. Antibacterial Activity Test

The antibacterial activity was assessed using the disc diffusion method (Kirby Bauer) [32]. Concentrations of *D. regia* leaves extract tested were 25%, 50%, 75%, and 100%. Initially, 5 g of *D. regia* leaves extract was dissolved in 5 mL of 10% ethanol to prepare the 100% concentration solution. This solution was then serially diluted to achieve 25%, 50%, and 75% concentrations. Approximately 15–20 mL of nutrient agar was poured into each sterile petri dish and allowed to solidify. Each bacterial suspension of *Salmonella typhimurium* and *E. coli* was then evenly swabbed over the surface of the solidified NA using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were soaked in each concentration of the *D. regia* leaves extract solution for 30 minutes, placed onto the agar surface using sterile tweezers, and incubated at 37 °C for 24 hours. A sterile paper disc soaked in 10% ethanol served as the negative control, while a disc impregnated with ciprofloxacin acted as the positive control. Post-incubation, the diameter of the inhibition zones formed around the discs was measured using a caliper and recorded in millimeters (mm), subtracting the diameter of the disc (6 mm) to account for the actual zone of inhibition. The experiment was conducted in triplicate [33].

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6. Statistical Analyses

Data analysis was conducted using the IBM SPSS software, version 29.0. Initially, a normality test was applied using the Kolmogorov-Smirnov method to determine the data distribution. If the data were normally distributed, a One-Way ANOVA was utilized to assess the significance of the differences among treatments, setting the confidence level at 95% ($\alpha = 0.05$). After the ANOVA, the Tukey HSD (Honestly Significant Difference) post-hoc test was performed to pinpoint the differences between each treatment group. Experimental results are expressed as mean ± Standard Deviation (SD). Statistical significance was established at p-values less than 0.05, indicating meaningful differences between the experimental groups. This approach ensures a rigorous data analysis, providing a reliable basis for interpreting the efficacy of the treatments under study.

RESULTS AND DISCUSSION

1. Phytochemical Screening Results of Ethanolic Extract of *D. regia* Leaf

Delonix regia leaves were extracted using the maceration method with 96% ethanol as the solvent. Ethanol is widely regarded as a universal solvent due to its ability to dissolve almost all secondary metabolite compounds and its relatively low

toxicity compared to other organic solvents. From the maceration process, 18.88 g of a dark green ethanolic extract was obtained from *D. regia* leaves, yielding 7.55%.

The extract was subjected to preliminary qualitative phytochemical screening to analyze its content of secondary metabolites. The compounds analyzed included flavonoids, alkaloids, saponins, phenolics, tannins, and terpenoids. Color changes indicated the presence of these compounds upon reaction with specific standard reagents, a common method for identifying such metabolites. Understanding the phytochemical components present in the extracts is crucial for elucidating the biological and pharmacological effects of the plant [34]. The observed color changes in Table 1 confirmed flavonoids, alkaloids, saponins, phenolics, tannins, and terpenoids in the ethanolic extract of *D. regia* leaves. These results are consistent with previous studies, which identified flavonoid, alkaloid, saponin, phenolic, tannin, and terpenoid compounds in *D. regia* leaf extracts [35, 36].

Table 1. Phytochemical qualitative screening results of ethanolic extract of *D. regia* leaves

No	Secondary Metabolites	Observation	Result
1	Flavonoids		+
2	Alkaloids		+
3	Saponins		+
4	Tannin		+
5	Phenolic		+
6	Terpenoids		+

Description: (+) = Contains these phytochemicals,
(–) = Does not contain these phytochemicals.

Fourier-transform infrared spectroscopy (FTIR) was utilized to characterize further the *Delonix regia* leaves extract by identifying the functional groups present. The FTIR analysis, conducted over a wavenumber range of 4000-400 cm^{-1}, revealed several absorption peaks, each corresponding to different functional groups, as depicted in Figure 1 and detailed in Table 1.

The spectrum displayed a significant peak at 3283.14 cm^{-1}, indicative of an alcohol or phenol hydroxyl group (O-H). Peaks at 2922.31 cm^{-1} and 2852.42 cm^{-1} were associated with aliphatic C-H functional groups. A peak suggested the presence of aliphatic aldehydes at 1737.13 cm^{-1}, characteristic of the C=O group. The absorption peaks at 1607.34 cm^{-1} and 1516.06 cm^{-1} were attributed to alkene groups (C=C). A peak at 1446.18 cm^{-1} indicated the presence of an aromatic ring (C-C), while peaks at 1239.38 cm^{-1} and 1041.13 cm^{-1} suggested the presence of alcohol (C-O) and ether (C-O-C), respectively. Additionally, the absorption wavelengths at 832.91 cm^{-1} and 717.38 cm^{-1} were consistent with aromatic rings (C-H).

The spectral interpretation confirms the presence of phenol hydroxyl groups, alkanes, aldehydes, alkenes, aromatic rings, alcohols, and ethers in the *D. regia* leaves extract. Notably, the characteristic absorption band at 3283.14 cm^{-1}, associated with O-H stretching, underscores the presence of hydroxyl groups, a hallmark of phenolic compounds. This suggests that the ethanolic extract of *D. regia* leaves is rich in phenolic compounds [37], underscoring its potential therapeutic properties.

While Fourier-transform infrared spectroscopy (FTIR) provides valuable insights into the functional groups present in the *Delonix regia* leaves extract, it alone cannot sufficiently detail the specific compound content within the extract. Therefore, further analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS) is imperative. LC-MS/MS facilitates a more comprehensive characterization, offering intricate details regarding the composition, structure, and identification of compounds within the extract. According to the chromatogram results depicted in Figure 2, 16 primary compounds have been identified in the *D. regia* leaves extract. These compounds were further analyzed using several databases, including PubChem, ChemSpider, HMDB, and CFM-ID, which revealed their chemical nature as amino acids, alkaloids, phenolics, flavonoids, terpenoids, anthraquinones, n-acylpyrrolidines, and fatty acids, as listed in Table 3. The polyphenol group, particularly flavonoids and phenolics, emerged as the most dominant secondary metabolites, indicating their prevalence in the extract as evidenced by the metabolite data in Table 3. This detailed analysis underscores the complex biochemical composition of the *D. regia* leaves extract, highlighting its potential for various pharmacological applications.

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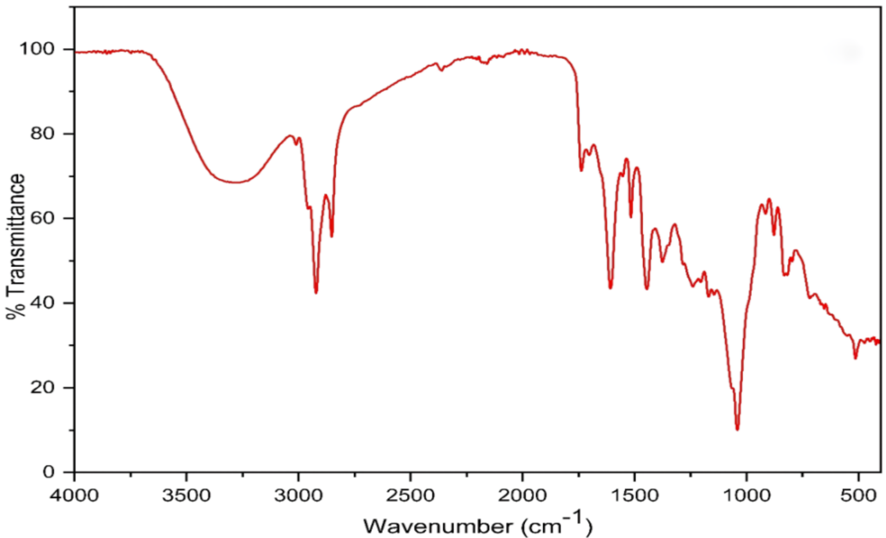

Figure 1. FTIR spectrum of *D. regia* leaves extract

Table 2. Interpretation results of the FTIR spectrum of *D. regia* leaves extract

No	Wavenumber (cm^{-1})	Functional Group	Compound Class
1	3283.14	O-H Stretching	Phenols
2	2922.31 and 2852.42	C-H Stretching	Alkanes
3	1737.13	C=O Stretching	Aldehyde
4	1607.34 and 1516.06	C=C Stretching	Alkene
5	1446.18	C-C Stretching	Aromatic
6	1239.38	C-O Stretching	Alcohol
7	1041.13	C-O-C Stretching	Ethers
8	832.91 and 717.38	C-H Stretching	Aromatic

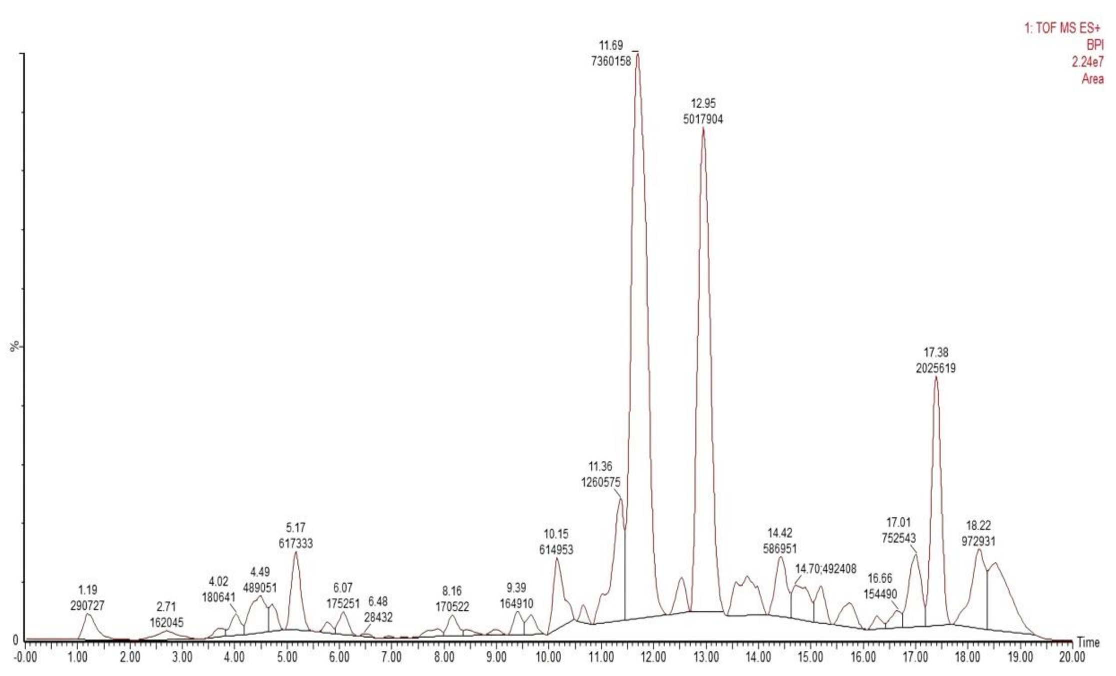

Figure 2. UPLC-QToFMS/MS chromatogram of *D. regia* leaves extract

Table 3. Data from UPLC-QtoFMS/MS chromatogram interpretation results of *D.regia* leaves extract

No	Retention Time (RT)	Peak Area (%)	Formula	Measured mass (m/z)	Name Compound	Group
1	1.19	1.15	$C_4H_{10}N_3O_2$	132.0822	3-Guanidinopropanoic acid	Amino acid
2	2.71	0.64	$C_8H_{10}N$	120.0821	2,3-Dihydro-1H-indole	Alkaloids
3	4.02	0.72	$C_9H_7O_2$	147.0451	Coumarin	Phenolic
4	4.49	1.94	$C_{27}H_{31}O_{15}$	595.1683	**Kaempferol 3-rhamno-glucoside**	Flavonoids
5	5.17	2.45	$C_{15}H_{11}O_6$	287.0556	Kaempferol	Flavanoids
6	6.07	0.70	$C_{13}H_{21}O$	193.1595	Beta-Ionone	Terpenoids
7	6.48	0.11	$C_{15}H_{17}O_7$	309.0969	Hydroisoflavone B	Flavonoids
8	8.16	0.68	$C_{22}H_{25}O_9$	433.1506	3-Methoxynobiletin	Flavanoids
9	9.39	0.65	$C_{20}H_{19}O_9$	403.1039	**5-Hydroxy-3,3',7,8-tetramethoxy-4',5'-methylenedioxyflavone**	Flavanoids
10	10.15	2.44	$C_{19}H_{19}O_7$	359.1135	Retusin (flavonol)	Flavanoids
11	11.36	5.01	$C_{18}H_{29}O$	261.2215	Laurophenone	Phenylpropanoid
12	11.69	29.23	$C_{21}H_{42}NO$	324.3276	1-(14-methylhexadecanoyl) pyrrolidine	N-acylpyrrolidines
13	12.95	19.93	$C_{23}H_{46}NO$	352.3590	Nonadecanoic acid	Fatty acid
14	14.70	1.95	$C_{17}H_{27}O_2$	263.2013	3-Methoxy-5-pentyl-2-prenylphenol	Phenolic
15	17.01	2.99	$C_{10}H_{13}O_2$	165.0918	**Eugenol**	Phenolic
16	17.38	8.04	$C_{40}H_{55}$	551.4245	Echinenone	Carotenoids

2. Antibacterial Activity

Antibacterials are substances capable of combating pathogenic bacteria by either killing them or inhibiting their metabolic activity, thus mitigating their deleterious effects within a biological environment [38]. In this study, *Delonix regia* leaf extract was tested at various concentrations—25%, 50%, 75%, and 100%—to determine the most effective concentration for inhibiting the growth of *Escherichia coli* and *Salmonella typhimurium*. The efficacy of each concentration was assessed by comparing the diameter of the inhibition zones formed around the paper discs soaked in the extract solutions against those formed around a disc soaked in the positive control, ciprofloxacin. Additionally, a 10% ethanol solution served as the negative control to evaluate any potential antibacterial effects of the solvent on the growth of *E. coli* and *S. typhimurium*.

The results indicated that the *D. regia* leaf extract effectively inhibited the growth of both *E. coli* and *S. typhimurium*, as evidenced by the clear zones of inhibition observed around the paper discs. The data on the average diameters of these inhibition zones for each concentration are detailed in Table 4 (Figures 3 and 4). This data supports the hypothesis that *D. regia* leaf extract possesses significant antibacterial properties and is effective across various concentrations against these specific bacterial strains.

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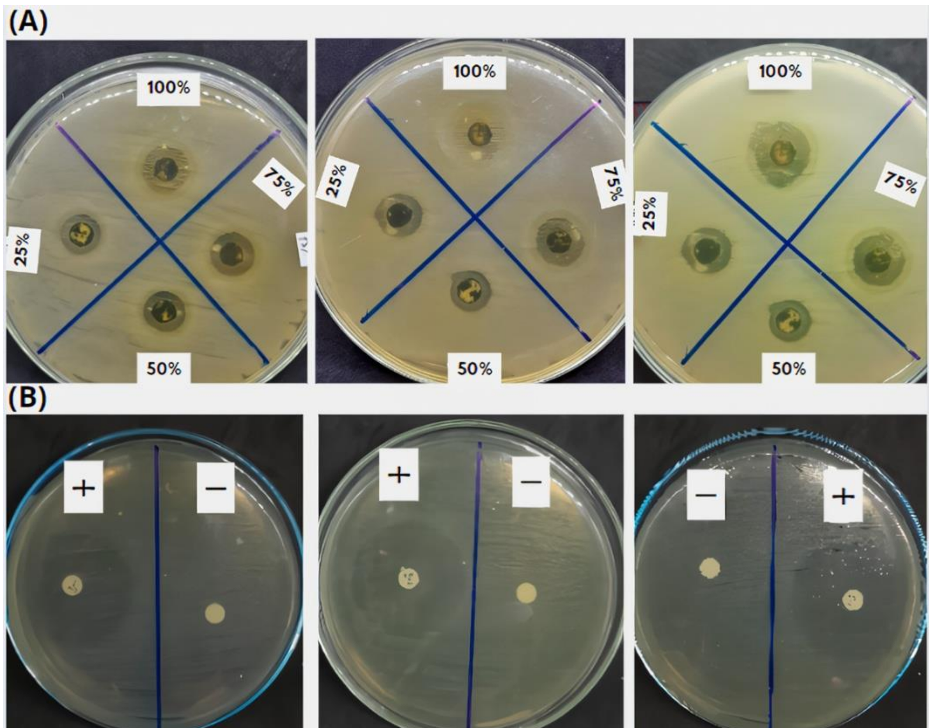

Figure 3. Antibacterial activity results in test of *D. regia* leaves ethanolic extract against *E. coli* (A). Concentrations 100%, 75%, 50%, and 25%, replication 1, 2, and 3; (B) Control + (ciprofloxacin) and control – (10% ethanol), replication 1, 2, and 3.

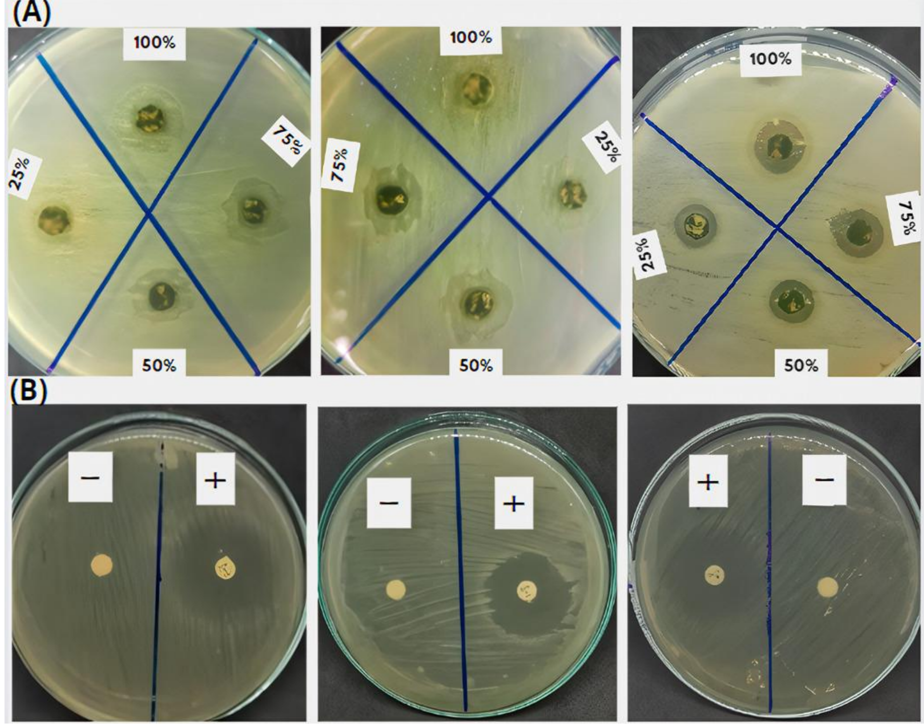

Figure 4. Antibacterial activity results test of *D. regia* leaves ethanolic extract against *S. thypimurium* (A) Concentrations 100%, 75%, 50%, and 25%, replication 1, 2 and 3; (B) Control + (ciprofloxacin) and control – (10% ethanol), replication 1, 2 and 3.

Table 4. The diameter of inhibition zones of *D. regia* leaves ethanolic extract

Sample Concentration (%)	Mean Inhibition Zone Diameter (mm) ± SD		Category
	Escherichia coli	*Salmonella thypimurium*	
25%	5.26 ± 0.27	5.05 ± 0.07	Medium
50%	6.37 ± 0.15	7.01 ± 0.13	Medium
75%	7.27 ± 0.08	8.77 ± 0.10	Medium
100%	10.18 ± 0.17	10.04 ± 0.18	Strong
Control +	30.02 ± 2.07	28 ± 1.53	Very strong
Control –	0 ± 0	0 ± 0	-

According to the categorization in [39], the antibacterial inhibition zones are grouped into four categories: diameters of inhibition zones less than 5 mm are categorized as weak, 5–10 mm as medium, greater than 10–20 mm as strong, and over 20 mm as very strong. Based on these criteria, the antibacterial activity of *D. regia* leaf extract against *E. coli* and *Salmonella typhimurium* at 25%, 50%, and 75% is classified as medium. In comparison, it is classified as strong at a concentration of 100%. This indicates that higher concentrations of the antibacterial material result in larger inhibition zones, aligning with prior findings that suggest the greater the concentration of an extract, the more bioactive components it contains, thus enhancing the inhibition zone diameter [40].

Research supports the notion that flavonoid compounds possess antibacterial properties. Specifically, kaempferol has been identified as an effective antibacterial flavonoid, particularly against *E. coli*, where it inhibits the enzyme DNA gyrase, a critical agent in bacterial replication and DNA strand tension relief during DNA replication [41]. The antibacterial efficacy of flavonoids is often attributed to their chemical structure, notably the presence and position of methoxyl groups at C-8 in ring A and hydroxyl groups at position 3 in ring C. Furthermore, flavonoid B rings can intercalate or form hydrogen bonds with the nucleic acids, inhibiting bacterial nucleic acid production [41, 43].

In addition to flavonoids, phenolic compounds in *D. regia* leaf extract, such as coumarin, have demonstrated antibacterial activity. Coumarin targets the B subunit of DNA gyrase in bacteria, preventing ATPase activity and hindering DNA supercoiling, contributing to its antibacterial properties [44]. Additionally, eugenol, belonging to the phenol chemical class and also known as phenylpropanoid, has been documented to disrupt the cytoplasmic membrane of gram-negative bacteria, leading to structural cell changes and leakage of cellular contents, underscoring its antibacterial mechanism [45].

In the negative control, which utilized 10% ethanol, no inhibition zones were observed around the paper discs for the two test bacteria. This confirms that the 10% ethanol used as a solvent does not possess antibacterial activity. Conversely, the positive control, ciprofloxacin, demonstrated significant antibacterial efficacy, producing inhibition zones measuring 30.02 mm against *E. coli* and 28 mm against *Salmonella typhimurium*, categorizing it within the "very strong" inhibition range. Ciprofloxacin, a fluoroquinolone class antibiotic, is known for

its bactericidal properties and its ability to inhibit both gram-positive and gram-negative bacteria, particularly those from the Enterobacteriaceae family such as *E. coli*, *Salmonella spp.*, *Shigella spp.*, and *Neisseria* [46]. It acts by inhibiting bacterial DNA gyrases—topoisomerase II and IV—disrupting DNA replication, causing DNA damage, and ultimately leading to cell death [47].

The average diameter of the inhibition zones reported in Table 4 is lower than those found in previous studies. This discrepancy could be attributed to several factors, primarily the nature of the test bacteria. *E. coli* and *Salmonella typhimurium* are gram-negative bacteria characterized by a protective three-layered barrier. The critical outer membrane, composed of phospholipids, lipopolysaccharides (LPS), and proteins, notably contributes to bacterial resistance against antibacterial substances. The hydrophilic nature of the outer membrane and the enzymatic activity in the periplasmic space can degrade external molecules. At the same time, the negatively charged LPS protects the bacterial cells against antibacterial agents [43, 48]. Other influencing factors include the metabolic activity and inherent sensitivity of the bacteria, as well as the concentration of the treatment and the type of solvent used, which affect the solubility and efficacy of the active compounds [40].

The statistical analysis indicated that the inhibition zones observed are normal as $p > 0.05$. Homogeneity tests revealed consistent data for *E. coli* with a significance value of 0.480 ($p > 0.05$) and for *Salmonella typhimurium* at 0.592 ($p > 0.05$), confirming the uniformity of data distribution. Subsequent analysis using one-way ANOVA showed significant results with a 0.000 ($p < 0.05$) value for both *E. coli* and *S. typhimurium*. These findings corroborate that both the ethanol extracts of *D. regia* leaves at various concentrations and the positive controls are effectively inhibitory against the growth of *E. coli* and *S. typhimurium*

CONCLUSION

The antibacterial activity tests conducted in this study demonstrate that *Delonix regia* leaf extract can effectively inhibit the growth of *E. coli* and *Salmonella typhimurium*. It was observed that the higher the concentration of the *D. regia* leaves extract, the larger the inhibition zone formed against these bacteria. This effect is attributable to the rich content of bioactive components within the extract. The antibacterial properties of the *D. regia* leaf extract are significantly influenced by the secondary metabolites present in the leaves. Characterization studies identified polyphenolic compounds, specifically flavonoids and phenolics, as prevalent in the D. regia leaves extract and crucial in inhibiting bacterial growth. Phytochemical screenings further confirmed the presence of flavonoids, alkaloids, saponins, tannins, and phenolic compounds in the extract, reinforcing the therapeutic potential of *D. regia* leaves.

ACKNOWLEDGMENT

This research was supported by an

internal research grant from the Faculty of Mathematics and Natural Sciences (FMIPA), Brawijaya University, contract number: 2612.2/UN01.F09/PN/2024

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