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RESEARCH ARTICLE

In Vitro Evaluation of Antibacterial Activity from *Nephelium lappaceum L.* Leaf Ethanolic Extract and Fraction against Some Foodborne Pathogens

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Abstract

Food borne pathogens are causing foodborne illness with significant effects on human health. Some of the most common foodborne bacteria that cause foodborne illness are *Bacillus cereus* and *Shigella dysenteriae. Nephelium lappaceum* L. is known to have antibacterial potential because of these plants have secondary metabolites such as flavonoid and polyphenols. The ethanolic extract and fractions of *Nephelium lappaceum* L. was investigated for antibacterial activities including determine of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against foodborne pathogens. The extraction of *N. lappaceum* L. was done by maceration method with 70% ethanol and fractionationed by liquid-liquid extraction to obtain ethyl acetate, water, and n-hexane fractions. Secondary metabolite content and profile determination of *N. lappaceum* L. was done by phytochemical screening and thin layer chromatography. The antibacterial activities were determined using the agar diffusion method, MIC and MBC value determined by dilution method using the test tube. The results showed that the ethanolic extract, ethyl acetate fraction and water fraction of *N. lappaceum* L. had antibacterial activity against *Bacillus cereus* isolate and *Shigella dysenteriae* bacteria. The value of MIC and MBC of the extract in the concentration range between 0.08%(w/v)-0.15%(w/v) against *B. cereus* isolate and 2.5%(w/v) – 5%(w/v) against *S. dysenteriae,* which the ethyl acetate fraction in concentration range between 0.04%(w/v) - 0.08%(w/v) against *B. cereus* isolate and 1.25%(w/v)-2.50%(w/v) against *S. dysenteriae* bacteria. The ethyl acetate fraction is the most active fraction because it had the best activity, MIC and MBC values when compared to the results against both of test bacteria.

Keywords: *Antibacterial, Bacillus cereus, Nephelium lappaceum L., Shigella dysenteriae, Food borne pathogens.*

Introduction

Foodborne disease is an important global concern because can cause significant morbidity, mortality, and economic costs [1, 2]. Foodborne pathogens can be caused by viruses, bacteria, parasite, or biological agents that can cause foodborne illness or foodborne disease may be transmitted to humans by contaminated food [3, 4]. Foodborne illness occurs when consuming food contaminated with pathogen and it is ingested in the human host, or when a toxigenic pathogen that produce toxins in the food product and then ingested by human host. Incubations period of foodborne infections since the time of ingestion to symptoms occurs is much longer than food borne intoxication [5].The most food borne outbreaks reported in 2015 at European Union were caused by bacterial agents [6]. The most common symptoms of foodborne illnesses include vomiting or diarrhea (more than three loose stools in 24 hours, fever, abdominal cramping, headache, and dehydration [7].

Some of the most common foodborne bacteria that cause foodborne illness are *Bacillus cereus* and *Shigella dysenteriae*. *B. cereus* is a Gram-positive, facultative anaerobic bacteria and produces spores that heat-resistant and survive when freezing and drying [8, 9]. *B. cereus* can induce gastroenteritis, bloody diarrhea and emetic poisoning caused by its ability two types of toxins, enterotoxins can cause diarrhea syndrome and cereulide can cause emetic syndrome [10-12].

Time from ingestion to symptom onset for diarrhea syndrome (enterotoxins) from 10-16 hours [13] and 1-5 hours for emetic syndrome (cereulide or emetic toxin) with duration of illness from 24-48 hours [5, 9]. *S. dysenteriae* is one of species from *Shigella* spp can cause an acute enteric infection which is commonly shigellosis or bacillary dysentry [13, 14]. *S. dysenteriae* is a Gram-negative, non-motile forming, and facultatively anaerobic rods bacteria [9].

S. dysenteriae is one of the most important causes of acute diarrhea and dysentery infection. The 50% cases of bloody diarrhea caused by *Shigella*-associated diarrhea [15].All species of *Shigella* produces a potent cytotoxin called Shiga toxin that causes illness is more severe, more prolonged, and more frequently fatal [16].Time from ingestion to symptom onset from 4-7 days with duration of illness from 24-48 hours [13].

Most cases of acute infectious diarrhea caused by foodborne pathogen usually use of broad-spectrum antibiotics for treatment of infection. The used of broad-spectrum antibiotics for the infections therapy or treatment can cause some side effects include kidney problems, abnormal blood clotting, blood disorders, defenses system and resistance. Antibiotics resistance can cause an increase in mortality and morbidity rate in the world [15].

Therefore, it is required for development and to find for natural antibacterial effects from secondary metabolites contained in the medicinal plants to avoid from antibiotics side effects and to treat infection caused by foodborne pathogen. The secondary metabolites include polyphenols, flavonoids, terpenoids and essential oils that have antibacterial activity effects [17, 18]. One of the potential plants used for infections treatment and various diseases is *Nephelium lappaceum* L from Sapindaceae Family, popularly known as "Rambutan". Traditionally the *N. lappaceum* has been used as a remedy for diabetes and high blood pressure [19].In another study, antibacterial activities of various extract the N. lappaceum have been reported. The Rambutan peel methanol extract has antibacterial activity against Gram-negative bacteria (*Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, and Enterococcus faecalis*) [20].

The Rambutan seed methanol extract has antibacterial activity with MIC value is 40 g/mL against *S. epidemidis* [21]. The rambutan peel crude extract has antibacterial activity against *E. coli* and *S. aureus* [22]. Therefore, the objective of this research was to determine antibacterial activity from the *N. lappaceum* L. leaf ethanolic extract and fractions against *B. cereus* isolate and *S. dysenteriae* ATCC 13313.

Materials and Methods

Chemical and Reagents

The chemical materials for solvents and reagents used in this research are chemical materials with analytical grade. The solvents and reagents used are ethanol (Merck, Germany), ammonia (Merck, Germany), chloroform (Merck, Germany), hydrochloric acid (Merck, Germany), iodide mercury (Merck, Germany), potassium iodide (Merck, Germany), magnesium powder (Merck, Germany), amyl alcohol (Merck, Germany), ferric chloride (Merck, Germany), gelatin solution (Merck, Germany), diethyl ether (Merck, Germany), sodium hydroxide (Merck, Germany), dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany), Mueller-Hinton Agar (Oxoid, Basingstoke, UK), Mueller-Hinton Broth (Oxoid, Basingstoke, UK), and distillated water.

Bacteria Culture Preparation

Bacillus cereus isolate used in this research obtained by isolation from expired and rotten food, while *Shigella dysenteriae* bacteria used in this research are *S. dysenteriae* ATCC 13313 pure strain. Both test bacteria are collections and maintained at

Pharmaceutical Microbiology and Biotechnology Laboratory, Department of Biology Pharmacy, Universitas Padjadjaran.

Plant Preparation and Determination

The leaf of *N. lappaceum* L. was collected from Subang and processed into simplicia in Lembang, West Java – Indonesia. Whole parts of *N. lappaceum* L. were determined at Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Sciences, Universitas Padjadjaran.

Extraction of *N. lappaceum L.*

The dried *N. lappaceum* L. leafs was extracted with 70% ethanol with sample solvent ratio of 1:5 by maceration method for 24 hours in triplicate at room temperature and the solvent is replaced every 24 hours. The macerate was concentrated with rotary evaporatory at 68 °C and then further concentrated with waterbath at 50 °C to obtained thick extract.

Fractionation

N. lappaceum L. leaf extract fractionation method was performed using Liquid-liquid Extraction. A total of 20 g thick extract was dissolved in 100 mL of water and put into a separating funnel. The n-hexane solvent was added with a volume ratio 1:1 with the water into the funnel and then shaken while the existing air was occasionally released and allowed to remain until the two phases separate. The n-hexane and water fraction were separated, this process was repeated several times until the n-hexane phase was no longer colored.

At the same separating funnel, the fractionation was continued by the addition of ethyl acetate solvent with the same amount of water, then shaken and separated using the same procedure was performed as the n-hexane fraction. The result obtained three fractions consisting of water fraction, n-hexane fraction, and ethyl acetate fraction. All fractions were evaporated and concentrated using a rotary evaporator. The yield of the fraction obtained is calculated.

Phytochemical Screening

The method of Farnsworth was used to determine the presence of secondary metabolites content such as alkaloid, polyphenolic, tannin, flavonoid, quinone, saponin, monoterpene & sesquiterpene, steroid & triterpenoid compound in *N. lappaceum* L [23].

Antibacterial Activity Extract and Fraction Test

Agar diffusion with a perforation technique was performed for antibacterial activity method using Clinical and Laboratory Standards Institute (CLSI) has suggested the guideline with some modification. Preparation of sample solution for extract and fractions were made with some variation of concentration. The extract was dissolved with dimethyl sulfoxide (DMSO) and water with a volume ratio 1:9.

The turbidity of each bacterial suspension (*B. cereus* or *S. dysentryae*) was adjusted to 1.5 x 10 CFU/mL and a total of 20 µL of bacterial suspension overnight cultured was spread with sterile spreader or cotton swab into the surface of the 20 mL of molten agar which contain Mueller-Hinton Agar (MHA) medium were poured into Petri dishes (diameter: 90 mm) and left for a few minutes to allow the bacteria to adapt with MHA medium.

The agar plates have been perforated using perforator (diameter: 9 mm) aseptically, when the plates were aseptically dried. A total of 100 µL soluble extract or fractions solutions with various concentrations were placed into each well and allowed to set. After that, all the plates were incubated at 37°C for 16-18 hours and measured the diameter of bacterial inhibition using calipers [24, 25].

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC and MBC test were analyzed using the microdilution method through broth dilution. Preparation of sample solution made with some variation of the smallest concentration for extract and the most active fraction still have activity based on antibacterial activity.

After dilution of the extract or the most active fraction, added an overnight culture of bacterial suspension (*B. cereus* or *S. dysenteriae*) into microplate and then incubate at 37 °C for 16-18 hours. The MIC and MBC values were determined by a subculture first from each well of microplate was inserted with micropipette into the MHA agar plates and spreaded using a spreader

aseptically. The plates were incubated at 37 °C for 16-18 hours [24, 25].

Results

Plant Determination

The *N. lappaceum* L. determination was done at Plant Taxonomy Laboratory, Department of Biology, Faculty of Mathematics and Sciences, Universitas Padjadjaran. The determination results showed that the plants used in this research included in Kingdom Plantae, Sapindaceae Family, Genus of *Nephelium*, and *Nephelium lappaceum* L. Species.

Extraction of *N. lappaceum* L.

The *N. lappaceum* L. leaf used for the extraction process using maceration method was 500 g which was soaked in 70% ethanol for 24 hours in triplicate at room temperature and the solvent is replaced every 24 hours. The extraction process was obtained 116.97 g crude extract with the yield of crude extract was 23.394%. The crude extract characteristic has rubbery and hard consistency, brownish black color, bitter taste, and specific odor.

Extract Fractionation

The extract fractionation process from 20 g of extract was obtained three fractions, namely 8.98 g of water fraction with the yield 44.9% (w/w), 0.18 g of n-hexane fraction with the yield 0.9% (w/w), and 5.53 g of ethyl acetate fraction with the yield 27.65% (w/w).

The organoleptic observation showed that the water fraction form was sticky, viscous liquid and brown color; the n-hexane fraction form was sticky, viscous liquid and green color; the ethyl acetate fraction form was viscous liquid, rather hard because too sticky, shiny, and blackish green color. All the fractions had a specific odor and bitter taste.

Phytochemical Screening

Table 1: The phytochemical screening of *N. lappaceum* L. results

Secondary Metabolites	Result
Alkaloids	-
Polyphenols	+
Tannins	+
Flavonoids	+
Monoterpens dan Sesquiterpens	+
Quinone	-
Saponins	+
Steroid dan Triterpenoid	-

*(+): detected; (-): absence / not detected

The phytochemical screening of *N. lappaceum* L. results showed in Table 1. It can be concluded that the *N. lappaceum* L. leaf contained some secondary metabolites such as flavonoid, polyphenol, tannins, saponins, monoterpens, and sesquiterpens. In another study, the *N. lappaceum* L. leaf positive for alkaloids, saponins and tannins, thus the results were quite similar [26].

Antibacterial Activity of Extract and Fractions Results

The extract and three fractions of *N. lappaceum* L. were tested for in vitro activity of antibacterial effect against *B. cereus* isolate and *S. dysenteriae*. The results showed that the extract, water fraction and ethyl acetate fraction had antibacterial effect against both of test bacteria with various concentrations are shown in Table 2 and Table 3.

Table 2: The antibacterial activity of extract and fractions against *B. cereus* isolate results

Sample	Diameter Zone of Inhibition (mm) from each Concentration*			
	5% w/v	10% w/v	20% w/v	40% w/v
Ethanolic extract	4.20 ± 0.02	6.76 ± 0.02	9.82 ± 0.02	11.42 ± 0.02
N-hexane fraction	-	-	-	-
Ethyl acetate fraction	7.52 ± 0.02	8.52 ± 0.02	11.53 ± 0.02	13.56 ± 0.02
Water fraction	4.34 ± 0.02	6.70 ± 0.02	9.54 ± 0.02	11.93 ± 0.02

*data are the average of three replicates.

Table 3: The antibacterial activity of extract and fractions against *S. dysenteriae* results

Sample	Diameter Zone of Inhibition (mm) from each Concentration*			
	5% w/v	10% w/v	20% w/v	40% w/v
Ethanolic extract	6.70 ± 0.02	9.45 ± 0.02	10.93 ± 0.02	13.80 ± 0.02
N-hexane fraction	-	-	-	-
Ethyl acetate fraction	7.70 ± 0.02	10.76 ± 0.02	12.18 ± 0.02	14.43 ± 0.02
Water fraction	1.43 ± 0.02	1.52 ± 0.02	2.31 ± 0.02	5.64 ± 0.02

*data are the average of three replicates.

It can be concluded if the ethyl acetate is the most active fraction, because this fraction in the lowest concentration (5% w/v) gave better activity compared with the extract and another fractions. The size of bacterial inhibition zone was directly proporsional to increase as indicated by increase of the extract concentration.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Extract and the Most Active Fraction Results

The determination of MIC and MBC results (Table 4 and Table 5) showed that the ethyl acetate fraction gave better activity to inhibit the growth of bacterias compared to the extract. The MIC and MBC value of extract (Table 5) in concentration range between 0.08% (w/v) -0.16% (w/v) against *B. cereus* isolate, and 2.5% (w/v) -5% (w/v) against *S. dysenteriae*. While the MIC and MBC value of ethyl acetate fraction in concentration range between 0.04% (w/v) -0.08% (w/v) against *B. cereus* isolate and 1.25% (w/v) - 2.5% (w/v) against *S. dysenteriae*.

Table 4: Determination of MIC and MBC of extract against both of test bacteria results

Extract Concentration (% w/v)	Bacterial Growth of *S. dysenteriae*	Bacterial Growth of *B. cereus* isolate
5.00	-	-
2.50	+	-
1.25	+	-
0.62	+	-
0.31	+	-
0.16	+	-
0.08	+	+
0.04	+	+

*(+): presence; (-): absence

Table 5: Determination of MIC and MBC of ethyl acetate fraction against both of test Bacteria results

Extract Concentration (% w/v)	Bacterial Growth of *S. dysenteriae*	Bacterial Growth of *B. cereus* isolate
5.00	-	-
2.50	-	-
1.25	+	-
0.62	+	-
0.31	+	-
0.16	+	-
0.08	+	-
0.04	+	+

*(+): presence; (-): absence

Discussion

The extraction process was performed using maceration method with 70% ethanol as the solvent to obtain secondary metabolites contained in *N. lappaceum* L. leaf. This method is used to prevent loss of thermolabile compounds that usually being lost due to high temperatures during the extraction process because of this method does not use heating during extraction. Ethanol was selected as the solvent because it is a universal solvent and can attract secondary metabolites compounds which are polar and non-polar compounds. Liquid-liquid extraction (LLE) was used to extracting a solute from a solution in a certain solvent with other solvents [27].

In this research, LLE is a separation process using three different solvents based on their polarity degree.

The solvents used are n-hexane (non-polar), ethyl acetate (semi-polar), and water (polar). The compounds contained in the extract will be separated based on polarity degree. Antibacterial activity test was qualitatively evaluated by agar diffusion method with perforation technique that only can identify presence or absence of the antibacterial activity from extract or fractions against test bacteria.

Antibacterial effect was observed from the bacterial inhibition zone formed at around the well perforation in MHA medium plates that zone indicated the extract can inhibit growth of bacteria and the diameter of bacterial inhibition zone measured using calipers. The inhibition zone formed indicates that the secondary metabolite compounds are contained in the extract or fractions can inhibit the bacterial growth. The MIC and MBC determination were aimed to evaluate the effectiveness of extract and the most active fractions with variation concentration in inhibiting both of test bacterial growth.

The MIC value determination aims to find the lowest concentration of extract and the most active fractions that can inhibit bacterial growth that suggesting, while MBC does not have bacterial growth. The MIC and MBC value in this research indicates that the extract and the most active fraction are bacteriostatic at lower concentrations and bactericidal at higher concentration.

The MIC and MBC determination results was carried out from the turbidity level of solution in microplate well that indicating the presence or absence of bacterial growth. In this research, antibacterial activity effect was probably derived from secondary metabolites such as flavonoid, polyphenol, tannins, saponins, monoterpens, and sesquiterpens.

Relate studies of antibacterial activity from extract containing flavonoids [17], saponins [17], polyphenols [17], tannins [17], terpenoids and essential oils [18] had antibacterial activity effect against various bacteria [16-18]. Flavonoids and polyphenols are phenolics compounds that have antibacterial activity by the sites and number of hydroxyl groups (-OH) on phenolic groups that increased hydroxylation results in increased toxicity [18, 28]. Flavonoids probably can cause damage to the permeability of bacterial cell walls because the form complex compounds with proteins and inhibit bacterial metabolic processes by inhibiting the use of oxygen and causing cell death [29].

Polyphenols or phenols can denaturate the components of peptidoglycans due to the hydrogen bonding between phenols and protein, causing the protein structure in the peptidoglycan to be damaged and the structure of the bacterial cell wall not to form which results on cell death [30].

Saponins have the ability to reduce surface tension in bacterial cell walls and are capable of damaging bacterial permeability that can cause enzyme leakage, cell leakage and release of genetic material from intracellular, then lead to cell death [17]. Tannins can cause cell wall damage by disrupting the permeability of the cell membranes and the cells cannot maintain their life activities [17, 31]. Terpenoids and essential oils have mechanism of action to involve cell membrane disruption by lipophilic compounds [18].

Conclusion

The results of this research can be concluded that the *Nephelium lappaceum* L. leaf ethanolic extract, ethyl acetate fraction and water fraction had antibacterial activity against *Bacillus cereus* isolate and *Shigella dysenteriae* ATCC 13313 was probably derived from flavonoid, polyphenol, tannins, saponins, monoterpens, and sesquiterpens contained in the *N. lappaceum* L. leaf. The ethyl acetate fraction is the most active fraction because it had the best activity, MIC and MBC values when compared to the results against both of test bacteria.

The ethyl acetate is the most active fraction, because this fraction in the lowest concentration (5% w/v) gave better activity compared with the extract and another fraction. The MIC and MBC value of ethyl acetate fraction in concentration range between 0.04% (w/v) -0.08% (w/v) against *B. cereus* isolate and 1.25% (w/v) -2.5% (w/v) against *S. dysenteriae*.

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