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Antibacterial activity of ethanol extracts of *Hibiscus tiliaceus* L. leaves from different extraction methods against *Escherichia coli* and *Staphylococcus aureus*

Devi Nisa Hidayati*, Hanik Rohmatul Maghfiroh, Aysah Safitri
Faculty of Pharmacy, Universitas Wahid Hasyim, Semarang
Jl. Menoreh Tengah X/22, Sampangan, Semarang, Indonesia

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ABSTRACT

The leaves of sea hibiscus (*Hibiscus tiliaceus* L.) are known to have good antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, which is associated with the saponin, flavonoid, polyphenol, and tannin contents. Chemical compounds in plant extracts are, however, influenced by the extraction method used. This study aimed to determine the antibacterial activity of the ethanol extracts of *Hibiscus tiliaceus* L. leaves obtained using four different extraction methods against *E. coli* and *S. aureus.* In the maceration, percolation, reflux, or Soxhlet method, 200 g of the crude drug (i.e., dried sea hibiscus leaf powder) was extracted with 96% ethanol, and the derived extract was tested at 1%, 2%, 3%, 4%, and 5% concentrations. The disk diffusion method was used to test the antibacterial activity by observing and statistically analyzing the diameter of the zones of inhibition. Results showed that all the ethanol extracts have antibacterial properties against *E. coli* and *S. aureus*, with the maceration-produced extract forming significantly different zones of inhibition from the other methods (percolation, reflux, and the Soxhlet) at all concentrations.

Keywords: *Hibiscus tiliaceus* L., Several extraction methods, *Escherichia coli, Staphylococcus aureus*

Corresponding author:
Devi Nisa Hidayati
Faculty of Pharmacy, Universitas Wahid Hasyim, Semarang
Jl. Menoreh Tengah X/22, Sampangan, Semarang
Email: devinisahidayati@unwahas.ac.id

INTRODUCTION

Infectious diseases are one of the leading causes of death in children (WHO, 2015). They are caused by the growth of various pathogenic microorganisms, such as bacteria, fungi, viruses, and parasites (Mandel et al., 2010). Among the bacterial infectious agents are *Escherichia coli* and *Staphylococcus aureus*. *E. coli* can cause diarrhea, which is a major cause of child morbidity and mortality in many countries, including Indonesia. Approximately 1.8 billion people die each year due to diarrhea (Kementrian Kesehatan Republik Indonesia, 2012). *S. aureus* causes skin disease, namely impetigo, with an incidence rate of 1.72% (Rizani et al., 2015).

The high incidence of infections encourages the development of numerous treatments to deal with this problem. One of the natural ingredients with scientifically proven antibacterial properties is sea hibiscus leaves (*Hibiscus tiliaceus* L.). The leaf ethanol extract derived using maceration proves effective in destroying or inhibiting the growth of *E. coli* and *S. aureus* (Ramproshad et al., 2012). Moreover, the leaves contain various antimicrobial compounds, such as tannins, flavonoids, and saponins (Surahmaida et al., 2020).

These antibacterial activities may, however, vary depending on the extraction technique used. For instance, temperature (i.e., hot or cold extraction) greatly affects the compounds or secondary metabolites drawn out of plant materials and the extract's antibacterial activity (Suhardiman et al., 2018). The Soxhlet and reflux are examples of hot extraction, while maceration and percolation are cold extractions (Patel et al., 2019). Maceration and reflux have the same principle of immersing powdered crude drug or specimen in solvents, although there is a difference in tha reflux requires heat (Sahne et al., 2016).

Reflecting on previous studies, this research aimed to investigate the difference in the antibacterial activity of the sea hibiscus leaf extracts obtained using four methods (maceration, percolation, reflux, and the Soxhlet) against *E. coli* and *S. aureus*. In doing so, the method that generates the greatest antibacterial activity can be determined and selected as the most favorable extraction for sea hibiscus leaves.

MATERIALS AND METHODS

Materials

The research materials were green sea hibiscus leaves (harvested in Pati, Central Java, Indonesia), 96% ethanol, *Escherichia coli* and *Staphylococcus aureus* obtained from the Microbiology Laboratory of the Faculty of Medicine, Universitas Muhammadiyah Semarang, 0.9% NaCl (Brataco), and chloramphenicol 30 µg/disk (OXOID Limited). The research equipment included a laminar air flow (AIRTECH), incubator (BINDER), and micropipette (SOCOREX).

Methods

Preparation of crude drug (sea hibiscus leaf powder)

Mature green sea hibiscus leaves were picked and then authenticated to ensure the right ingredients were used. Nine kg of the leaves were wet-sorted, washed with running water, and then dried in a drying cabinet at a temperature of 40–50℃. Afterward, the dried leaves were sifted using a 40 mesh sieve to obtain crude drug with <10% water content.

Ethanol extractions of sea hibiscus leaves

Maceration

Two-hundred grams of the crude drug were put in a maceration vessel containing 2,000 mL of 96% ethanol as the solvent. The powder-to-solvent was 1:10. In this vessel, maceration was conducted for 5 days, comprising 3 days of maceration and 2 days of re-maceration. The resulting maceration and re-maceration filtrates were combined and then thickened in a rotary vacuum evaporator at a temperature of 50℃ to obtain a thick extract (Mahasuari et al., 2020).

Reflux extraction

Two-hundred grams of the crude drug were put in a round-bottom flask and soaked in 500 mL of 96% ethanol (solvent) for 2 h; then, the filtrate was separated from the dregs. Afterward, the dregs were added back into the solvent. The process was carried out 3 times for 2 h each. The resultant filtrates were combined and thickened in a rotary vacuum evaporator at 50℃ to make a thick (Laksmiani et al., 2015).

Soxhlet extraction

Two-hundred grams of the crude drug were placed in a filter paper and then inserted into the Soxhlet apparatus. Then, 700 mL of 96% ethanol (solvent) was added and circulated through the extractor 25 times. The process continued until a clear solution formed in the tube. Finally, the results were condensed using a rotary vacuum evaporator at 50℃ to obtain a thick extract.

Percolation

Two-hundred grams of the crude drug were soaked in 3000 mL of 96% ethanol (solvent) and placed in the percolator. Soaking was carried out for 24 h, then the filtrate was removed slowly. The top part was added with solvent continuously until clear filtrate droplets were obtained. The results were condensed using a rotary vacuum evaporator at 50℃ to produce a thick extract.

Antibacterial activity test

Preparation of the testing media

The testing media were Mueller-Hinton agar (MHA), nutrient agar (NA), and nutrient broth (NB). They were prepared differently: 3.8 g of MHA was dissolved in 100 mL of distilled water, 1.4 g of NA in 50 mL, and 8 g of NB in 20 mL. Each was heated until homogeneous and then sterilized by autoclaving.

Bacteria rejuvenation and preparation of bacterial suspension

One colony of a pure culture of *E. coli* was seeded on the surface of NA, suspended in NB, and then incubated for 24 h at 37℃. The suspension was diluted until the turbidity was equivalent to the 0.5 McFarland standard. This process was repeated for *S. aureus*.

Disk diffusion method

The ethanol extract was dissolved in DMSO to create test solutions with 1%, 2%, 3%, 4%, and 5% concentrations. Ten µL of each test solution, negative control (DMSO), and positive control (the antibiotic chloramphenicol 30 µg/disk) was dripped onto a sterile disk paper. Next, the disk paper was applied to MHA inoculated with the test bacteria; then, the plate was incubated at 37℃ for 24 h. Finally, the antibacterial activity was determined by observing the diameter of the zone of inhibition formed around the paper disk. This assay was conducted in triplicates (Hidayati et al., 2019).

Compound detection tests

Each extract produced by maceration, percolation, reflux, and the Soxhlet method was screened for saponins, alkaloids, tannins, phenolics, and flavonoids, as explained below.

Saponins

The ethanol extract was dissolved in water and then shaken for a few minutes. It contains saponins if the foam is stable for 5 minutes (Sarker et al., 2006).

Alkaloids

The extract was dissolved in 10 mL of 2 N HCl. Then, the solution was divided and poured into four tubes. Tube 1 acted as a control (without treatment), whereas tubes 2, 3, and 4 were added with Dragendorff's reagent, Wagner's reagent, and Mayer's reagent, respectively. Precipitate appearing in two to three tubes indicates the presence of alkaloids (Sarker et al., 2006).

Tannins

The extract was poured with hot distilled water and then divided into two tubes. The first tube was added with 1% NaCl, and the second one was added with 1% NaCl and 5% gelatin. Precipitate appearing in tube indicates the presence of tannins (Sarker et al., 2006).

Phenolics

One mL of the extract was added with ethanol, followed by 2–3 drops of 5% $FeCl_3$ (Sarker et al., 2006).

Flavonoids

The extract was added with ethanol, followed by Mg powder, amyl alcohol, and concentrated HCl. Flavonoids will dissolve in the upper phase (amyl alcohol layer) and develop a color Greenish-yellow or yellowish-pink (Sarker et al., 2006).

Data Analysis

The ethanol extract of sea hibiscus leaves is said to have antibacterial activity if a clear region is formed around the paper disk, which is expressed as the diameter of the zone of inhibition. The diameters were then analyzed statistically to determine differences between the tested extraction methods (i.e., maceration, percolation, the Soxhlet, and reflux) in killing or inhibiting the growth of *E. coli* and *S. aureus*.

RESULTS AND DISCUSSION

Percent extraction yields

The drying process of 9 kg of fresh sea hibiscus leaves produced 3.1 kg of powder (crude drug) or 34.4% yield with 7% water content. Each extraction method used 200 g of the crude drug. The ethanol extracts derived from several extraction methods are presented in Table 1.

Table 1. Yields of ethanol extraction of sea hibiscus (*Hibiscus tiliaceus* L.) leaves using four different methods

Method	Derived ethanol extract (in % yield)
Maceration	10.9
Percolation	17.5
Reflux extraction	19.6
The Soxhlet method	8.3

As seen in Table 1, reflux extraction produced the highest yield among the tested methods, presumably because it uses heat. High temperature is one of the factors affecting the speed of extraction by increasing the desorption of active compounds from plants and, thus, damaging plant cells. Aside from reflux, the Soxhlet method is also heat-assisted. However, it produced a lower yield, which might be caused by the smaller amount of solvent it used. The % yields, from the highest to the lowest, were provided by reflux, percolation, maceration, and the Soxhlet. In addition, the viscous extracts derived from the four methods had similar organoleptic characteristics: thick texture, blackish-green color, and distinctive odor of sea hibiscus leaves.

Antibacterial activities of the ethanol extracts

The ethanol extracts from the four extraction methods were tested against two bacteria, *Escherichia coli* and *Staphylococcus aureus*, to determine their antibacterial activities based on the diameter of the zone of inhibition around the paper disk. In the assay, DMSO was used as a negative control to see, if any, the solvent's effect on bacterial growth and to ensure that the antibacterial effect came solely from

the ethanol extract. The agar diffusion method was chosen because of its fast, effortless, and simple operation (Jawetz et al., 2005).

Results showed zones of radical inhibition in all concentrations of the ethanol extracts tested against *E. coli* (Figure 1) and *S. aureus* (Figure 2). A radical inhibition zone is characterized by a clear area around the paper disk that is not overgrown with bacteria. In the assay against *E. coli*, the average zone diameter ranged from 9.82–12.72 mm for the maceration-derived ethanol extract, 7.33–11.37 mm for reflux, 6.24–9.35 mm for percolation, and 7.65–10.81 mm for the Soxhlet method. In the assay against *S. aureus*, it varied from 15.50–17.44 mm for maceration, 10.30–13.30 mm for reflux, 8.20–12.60 mm for percolation, and 8.80–14.70 mm for the Soxhlet method (Table 2). At the highest concentration, 5%, the ethanol extracts from maceration, reflux, percolation, and the Soxhlet method created a zone diameter of 12.72 mm, 11.37 mm, 9.35 mm, and 10.81 mm, respectively. These numbers showed that maceration produced the largest diameter.

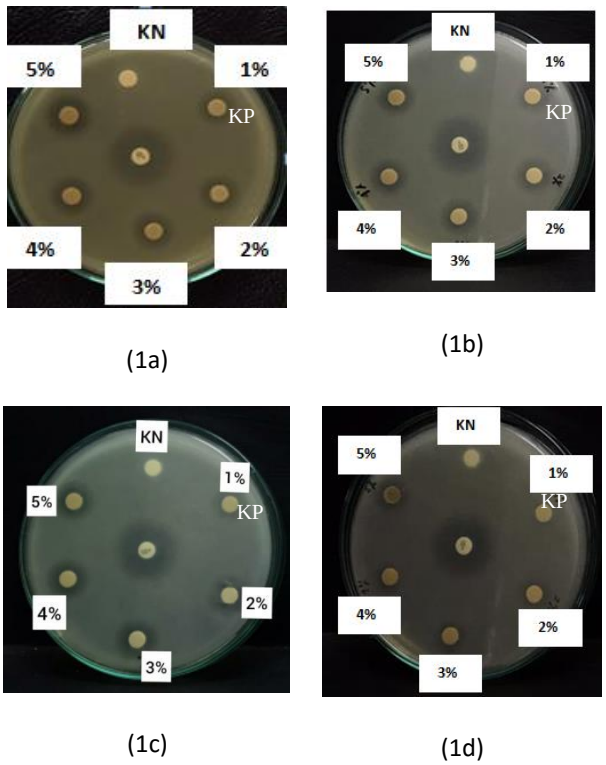

Figure 1. Antibacterial activity of the ethanol extracts of sea hibiscus (*Hibiscus tiliaceus* L.) leaves derived from four extraction methods against *Escherichia coli*. Maceration (1a), reflux (2b), the Soxhlet method (1c), and percolation (1d). KN (negative control), KP (positive control)

In a previous study, the ethanol extract of sea hibiscus leaves obtained by maceration formed a zone diameter of 12 mm for *E. coli* and 9 mm and 15 mm for *S. aureus* at 500 µg/disk (Ramproshad et al., 2012). In this research, the 5% concentration is equivalent to 500 µg/disk, and the same ethanol extract inhibited the growth of *E. coli* by forming a zone diameter of 12.72 mm, which is close to the previous study result. As for the assay against *S. aureus*, the zone diameter was 17.44 mm. From these diameters, it can be inferred that the ethanol extract has an antibacterial effect on *E. coli* and *S. aureus* but is more potent on the latter, all of which correspond to the previous study results (Ramproshad et al., 2012). Among the factors responsible for the different zone diameters between the current and prior studies is the area where the plant material was harvested, which can vary the associated compounds (Andriani et al., 2017).

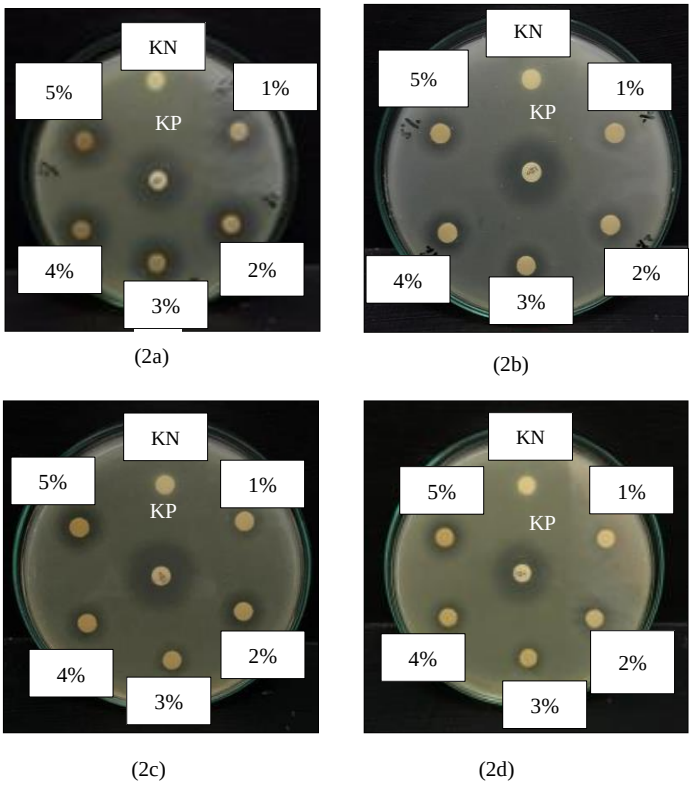

Figure 2. Antibacterial activity of the ethanol extracts of sea hibiscus (*Hibiscus tiliaceus* L.) leaves derived from four extraction methods against *Staphylococcus aureus*. Maceration (2a), reflux (2b), the Soxhlet method (2c), and percolation (2d). KN (negative control), KP (positive control)

Subsequent statistical analyses revealed that the heat-assisted methods (reflux and the Soxhlet) produced ethanol extracts with the same activity. On the contrary, maceration gave an antibacterial effect on *E. coli* and *S. aureus* that was significantly different from the other three methods (reflux, percolation, and the Soxhlet). In addition, the antibacterial effect of the Soxhlet-derived extract on *S. aureus* was significantly different from reflux and percolation. Statistical results indicated that using different extraction methods could affect the extract's antibacterial activity. Maceration produced zones of inhibition with the widest diameter average and antibacterial effects on both bacteria that were significantly different from reflux, percolation, and the Soxhlet. Thus, it can be concluded that maceration is preferred for extracting sea hibiscus leaves to inhibit the growth of *E. coli* and *S. aureus*.

Phytochemical screening found that the ethanol extracts of sea hibiscus leaves derived from these four extraction methods contained saponins, phenolics, and flavonoids (Table 3). These results are in line with previous research (Kumar et al., 2008).

Table 2. Antibacterial activity of the ethanol extracts of sea hibiscus (*Hibiscus tiliaceus* L.) leaves obtained with several extraction methods against *Escherichia coli* (EC) and *Staphylococcus aureus* (SA) (triplicates)

Extraction method	Concentration	Average diameter of the inhibition zone (mm)	
		EC (mean±SD)	SA (mean±SD)
Maceration	1%	9.82±0.70	15.50±0.08
	2%	10.71±0.75	16.00±0.38
	3%	11.63±1.19	16.30±0.10
	4%	11.99±1.01	17.10±0.49
	5%	12.72±1.60	17.44±0.18
Reflux	1%	7.33±1.01	10.30±0.18
	2%	8.50±0.72	11.30±0.29
	3%	9.99±0.35	11.80±0.53
	4%	10.72±0.29	12.70±0.60
	5%	11.37±0.16	13.30±0.13
Percolation	1%	6.24±0.14	8.20±0.24
	2%	7.47±0.35	9.30±0.20
	3%	7.92±0.66	11.00±0.67
	4%	8.74±0.65	11.60±0.39
	5%	9.35±0.20	12.60±0.30
The Soxhlet	1%	7.65±0.99	8.80±0.65
	2%	8.29±0.27	11.20±0.88
	3%	9.51±0.27	12.10±1.17
	4%	10.12±0.72	12.60±0.26
	5%	10.81±0.59	14.70±1.48
Positive control (Chloramphenicol)	30 µg/disk	17.86±0.64	20.20±0.09
Negative control		-	-

Table 3. Phytochemical screening results of the ethanol extract of sea hibiscus (*Hibiscus tiliaceus* L.) leaves obtained from four extraction methods

Phytochemicals	Results	
Saponins	All extraction methods: Stable foam for 5 minutes	+
Alkaloids	All extraction methods: No precipitate	-
Tannins	All extraction methods: No precipitate	-
Phenolics	All extraction methods: Color change to blackish-green	+
Flavonoids	Maceration, percolation, the Soxhlet: Color change to greenish-yellow; Reflux: Color change to yellowish-pink	+

Based on Table 3, it is suspected that three compounds are responsible for the extract's antibacterial activity against *E. coli* and *S. aureus*. Saponins are secondary metabolites that taste bitter and will foam when in contact with water. Moreover, saponins are good antimicrobial agents that suppress bacterial growth by reducing the surface tension of the cell walls (Widodo, 2005). As an antibacterial, flavonoids work by the mechanism of denaturing protein to stop the metabolic activity of bacterial cells (Estri & Anggarbeni, 2015). Phenolics act as a toxin in the protoplasm, damaging and penetrating the cell wall. To damage the cell wall, the interaction of cations and anions on it can be separated by affinity, which

is followed by increased membrane permeability that can lead to cell leakage (Rosidah et al., 2014). The active compounds in hibiscus leaves are polyphenols, flavonoids, terpenoids, steroids and glycosides (Samsudin et al., 2019).

CONCLUSION

Different extraction methods can affect the antibacterial activity of the ethanol extract of sea hibiscus (*Hibiscus tiliaceus* L.) leaves against *Escherichia coli* and *Staphylococcus aureus*. Maceration produces ethanol extracts with the most potent antibacterial effects at 1%, 2%, 3%, 4%, and 5% concentrations. It is evident in the formation of zones of inhibition with an average diameter that is significantly different from the other methods (percolation, reflux, and the Soxhlet).

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