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Inhibitory Activity of Distilled water and Ethanol Extracts of *Moringa oleifera* Leaves Against *Enterobacter aerogenes*

Andi Sayida Nurul Shafira[1], Metusalach[2], Nursinah Amir[3]

[1]Fisheries Science Study Program, Faculty of Marine and Fisheries Sciences, University of Hasanuddin, Makassar, Indonesia
[2]Department of Fisheries, Faculty of Marine and Fisheries Sciences, University of Hasanuddin, Makassar, Indonesia
[3]Department of Fisheries, Faculty of Marine and Fisheries Sciences, University of Hasanuddin, Makassar, Indonesia
*Corresponding Author

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Abstract**—** *Enterobacter aerogenes is one of the bacteria that has an important role in converting histidine to histamine in fish. When consumed, histamine in fish can cause poisoning and even death. The growth of bacteria that cause damage to fish can be inhibited in several ways, including the addition of compounds that have the potential to act as bacteriostatic or even bactericide. One of the plants that has the potential as a source of bactericide compounds is drumstic tree (Moringa oleifera* Lamk.*). This study aimed to determine the effectiveness of several concentrations of Moringa leaves extract using distilled water and ethanol in the inhibition of Enterobacter aerogenes bacteria as a histamine producer. The results showed that distilled water and ethanol extracts of Moringa leaves had an effective inhibition against Enterobacter aerogenes. The distilled water extract at a 40% concentration produced the largest inhibition zone (14.73 cm) while that of the ethanol extract at a 40% concentration was 10.57 cm. The distilled water extract of Moringa leaves is suitable for use because it is cheap, practical, safe for consumption and does not leave any unsafe residue on food.*

Keywords**— *Distilled watert extract, Enterobacter aerogenes, Ethanol extract, Inhibitory activities, Moringa oleifera Leaves.***

I. INTRODUCTION

Enterobacter aerogenes is one of the bacteria present in the guts and gills of fish this bacterium has enzyme *histidine decarboxylase* capable of converting the amino acid histidine in fish into histamine in warm conditions [1]**.** Poisoning due to consuming foods containing high histamine are characterized by symptoms of a rash, nausea, vomiting and diarrhea and may even lead to death [2]. Histamine can also cause allergies in some people [3].

Histamine is a poisonous compound produced by several types of red meat fish, especially in the scromobidae family, due to the activity of bacteria and enzymes [4].

Among the histamine-forming bacteria such as using *Raoultella terrigena*, *Enterobacter* spp., *Microbacterium testaceum*, *Brevibacterium mcbrellneri; Micrococcus diversus; Staphylococcus* spp., the *Enterobacter* spp., have the greatest ability in forming histamine. Several types of the *Enterobacter* bacteria known to form histamine are *Enterobacter aerogenes*, *Enterobacter agglomerans*, *Enterobacter amnigenus*, *Enterobacter cloacae*, and *Enterobacter intermedium* [5].

The growth of bacteria that cause damage to fish can be inhibited in several ways, including the use of bactericide compounds. One of the plants that have the potential as a bactericide is Moringa [6]. *Moringa oleifera* or Moringa leaves contain bioactive compounds *saponins, alkaloids, phytosterols, tannins, phenolics* and *flavonoids* [7]. Moringa leaves have active compounds that act as antibacteria [8]. Moringa leaves extract can inhibit activities of several types of bacteria, such as *Streptococcus* sp., *Pseudomonas fluoroscens*, *Proteus mirabilis* and fungi *Aspergillus flavus* [9].

Moringa grows well in tropical and subtropical areas on all types of soil and its resists dry season with drought tolerance for up to 6 months [10]. Moringa leaves are easily be found throughout Indonesia. However, most Indonesians recognize Moringa leaves only as a vegetable dish that can be mixed with other types of vegetables [11]. So, it will be very useful if Moringa leaves can be used to inhibit the growth of bacteria, especially *Enterobacter aerogenes* as histamine-forming bacterium in fish.

This study, therefore aimed to determine the inhibitory activities of distilled water and ethanol extracts of *Moringa oleifera* leaves against bacteria *Enterobacter aerogenes*.

II. MATERIALS AND METHODS

2.1 Collection of moringa leaves samples

Moringa oleifera leaves (± 1 kg wet weight) were collected from Pinrang & Selayar, South Sulawesi Province, Indonesia. The collected leaves were then cleaned and washed under running water. Upon drained, the leaves were air-dried under shade (Fig. 1a) to avoid direct sunlight exposure. The dried samples were then mashed into a powder form (Fig. 1b), sieved with a commercial sieve and stored in a closed glass container until used.

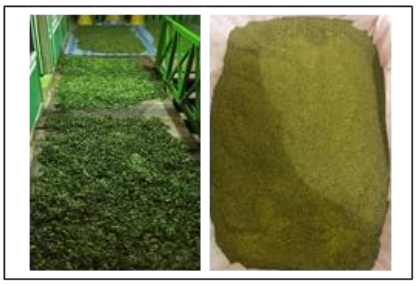

Fig 1. Air-drying process (a) Moringa leaves powder (b)

2.2 Preparation of moringa leaves estract

Extract of the Moringa leaves was prepared using two type of solvents, distilled water & ethanol. Extraction was performed by macerating (Fig. 2) 100 g of Moringa leaves powder in 500 mL of either distilled water or ethanol (1:5, w/v) for 48 h. The filtrate was then separated using Whatmann No. 1 filter paper. To recover solid extract, the water extract was lyophilized while the ethanol extract was dried using a vacuum rotary evaporator. The solid extract was stored in a capped vial until used.

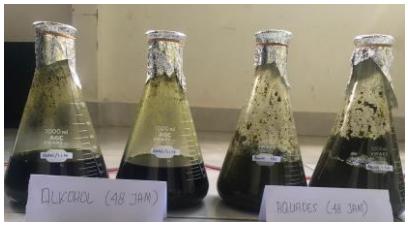

Fig.2: Maceration process for 48 hours

2.3 Nutrient agar media preparation

Nutrient agar (NA) media was aseptically prepared by dissolving 4 g nutrient agar powder in 200 mL of distilled water, then heating it to a boil with regular stirring. The prepared NA media was then sterilized in an autoclave at a temperature of 121ºC for 15 min at a pressure of 1 atm [12].

While in warm conditions (40 - 45 ºC) the sterile media was poured into 15 mL sterile petri dishes and allowed to stand until the media solidified.

2.4 Bacterial rejuvenation

Enterobacter aerogenes was obtained from the Indonesian Culture Collection LIPI, Indonesia, code InaCC B865. The bacteria were grown on NA media, inoculated using a loop needle etched on NA media then incubated at 30ºC for 24 h.

2.5 Preparation of bacterial suspension

Preparation of the suspension test was carried out by taking a loopful of the isolate, suspend it in 2 mL 0.9% NaCl solution in a sterile test tube and then homogenizing it with a vortex. The turbidity of the suspension was then compared with that of the standard 0.5 McFarland (1.5 x 10^8 CFU/mL).

2.6 Preparation of test solution extract

The concentrations of the extract solution used in this study were 10, 20, 30, and 40%. These concentrations were made by thoroughly dissolving 25, 50, 75 and 100 mg, respectively, of the dry extract in 250 µL of DMSO. The negative control was 50 µL DMSO and the positive control was 10 µg /mL of Amoxicillin.

2.7 Inhibition test against *E. aerogenes*

The inhibition test was performed following the *Kirby-Bauer* disk diffusion method. Warm NA media was poured into the sterile petri disk and let to cool. Further, the bacterial suspension of 100 µL which was comparable to a standard suspension of 0.5 McFarland (1.5 x 10^8 CFU/mL) was added to the petri dishes. The bacterial suspension was spread evenly on the surface of the NA and let to stand for 5 min. Then, the paper discs pre-impregnated with test solution were placed on top of the NA containing bacteria

and incubated for 24 h at 37˚C. Upon completion of incubation, the inhibitory activity was determined by measuring diameter of the inhibitory zone.

2.8 Data analysis

Data were analyzed by one-way ANOVA using the statistical package SPSS 26 for windows to determine the antibacterial effect of *M. oleifera* extract against *E. aerogenes*. The difference in antibacterial activity among the test treatments was assessed using Duncan's Multiple Range Test with an adjusted p-value at 0.05.

III. RESULTS

The antibacterial activity of the distilled water and ethanol extract of Moringa leaves was determined by measuring the diameter of the formed inhibition zone.. The anti-bacterial activity is present if there is a clear zone around the disc paper [13]. Figure 3 shows clear areas around the discs indicating the anti-bacterial activity of the Moringa leaves extracts.

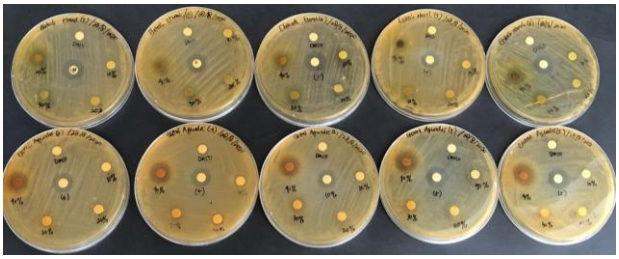

Fig 3: Inhibition zone of ethanol extract (upper row) & distilled water extract (bottom row)

Both the ethanol and water extracts of Moringa leaves showed positive results. The Inhibition zone diameter of Moringa leaves extracts is presented in Table 1. The 40% distilled water extract showed the largest diameter of inhibition, which was 14.73 mm, while the inhibition diameter of the positive control (Amoxicillin 10 µg/ml) was 13.98 mm and the 40% ethanol extract was 10.57 mm. This shows that the water extract of the Moringa leaves has an antibacterial ability comparable to the 10 µg/ml Amoxicillin antibiotic. The potential of an antimicrobial is estimated by comparing the growth inhibition zone to sensitive microorganisms from the inhibition of a concentration of a test solution to antibiotics [14].

Table 1. Diameter of Inhibitory Activities

Solvent Extract	Concentration (%)	Diameter of Inhibition (mm)
Etahonlic	10	7.01[a]
	20	7.17[a]
	30	8.17[a]
	40	10.57[b]
Distilled watert	10	7.90[ab]
	20	8.13[ab]
	30	9.56[ab]
	40	14.73[c]
Positive Control	Amoxicilin 10 µg/ml	13.98[c]
Negative Control	DMSO	0

The diameter of the zone of inhibition showed that the two extract at a 40% concentration had strong anti-bacterial activities in treating the *E. aerogenes*. The ability of the test material to inhibit the test bacteria is classified as very strong (>20 mm), strong (11-19 mm), moderate (5-10 mm) and weak (<5 mm) [15].

The results of ANOVA showed that the type of solvent and the concentration of the extract had significant effects on the activity of the *E. aerogenes*. The Duncan test showed that the distilled water extract of Moringa leaves at 40% showed a higher ($p<0.05$) inhibition activitiey against the *E. Aerogenes* compared to other concentrations of the water extract as well as to all concentrations of the ethanol extract tested. Previous study where three concentrations of [13] distilled water extract from *M. oleifera* had inhibitory diameters of 18, 19, 21 mm against *P. vulgaris* strain NCTC8196, while methanol and petroleum ether extracts did not show any effect on *P. vulgaris* bacteria [16]. *P. vulgaris* and *E. aerogenes* are of the same family.

The difference in the diameter of the inhibition zone between the distilled water and the ethanol extracts of Moringa leaves is caused by differences in the degree of polarity of each solvent. The polarity of distilled water is 9.0, while that of 96% ethanol is about 5.2. The principle of extraction is that polar substances only in polar solvents, and non-polar substances only dissolve in non-polar solvents [16]. The polarity of the solvent used determines the amount of the active substances because, in the extraction process, the principle of "like dissolves like" applies where the substance will only dissolve properly and get static if the solvent used has the same polarity level [17].

The phytochemical test results showed that the distilled water extract of Moringa leaves contained flavonoids and alkaloids. The ethanol, methanol, and ethyl acetate extracts of Moringa leaves contained alkaloid but they did not

contain flavonoids, whereas the n-hexane extract did not contain both alkaloids and flavonoids [18]. However, other study showed that the extract of Moringa leaves macerated in ethanol contained flavonoids and polyphenols [19]. The distilled water extract also contained steroids, triterpenoids, flavonoids, saponins, phenols, and tannins [12]. Nonetheless, both the distilled water and the ethanol extracts of Moringa leaves have the antibacterial ability. Still, the level of inhibition is different based on the solvent's degree of polarity.

The flavonoids, polyphenols, saponins, and tannins are active compounds in Moringa leaves with antibacterial properties [20]. According to [21], the flavonoid compounds in Moringa leaves extract have the same polarity as distilled water. Flavonoids are polyphenol group compounds widely distributed in plants in the form of glycosides that bind to sugar. Flavonoids are divided into several types, and each type has a different polarity depending on the number and position of the hydroxyl groups [21]. Polar solvents commonly used for flavonoid extraction are methanol, acetone, ethanol, water, and isopropanol.

Water as a solvent is polar, cheap, easy to obtain, stable, non-toxic, non-volatile, and flammable [22]. Water is also a recommended solvent for the food industry because it's leaves no residue in the extraction results so that the resulting product is safe for consumption [23].

According to [24], water is often referred to as the universal solvent because it dissolves many chemicals. Water is in a dynamic balance between the liquid and solid phases under standard pressure and temperature. In ionic form, water can be described as a hydrogen ion (H +) in association (bonding) with a hydroxide ion (OH-).

Ethanol is a volatile liquid commonly used as a solvent for most organic compounds. Ethanol, which is a semi-polar solvent, can dissolve both polar and non-polar compounds. Semi-polar solvents can induce polarity of non-polar solvent molecules. It acts as an intermediate solvent to mix non-polar and non-polar solvents. Ethanol has a high selectivity for reactions [25]. Therefore, selection of solvent must consider several factors, including selectivity, ability to extract, toxicity, ease of evaporation, and solvent price [25].

IV. CONCLUSION

The present study demonstrated that both the distilled water and the ethanol extracts of the Moringa leaves exhibited an antibacterial activity against *E. aerogenes*. At high concentration (40%), the antibacterial activity of the distilled water extract was more powerful than that of the ethanol extract. Nevertheless, both extracts were similar in their anti *E. aerogenes* activity at the concentrations of up to 30%. Since distilled water is considered to be safer than ethanol, it is recommended to use water for extraction of anti *E. aerogenes* from the Moringa leaves, especially if the extract is intended to be used to preserve food products. However, further study using different types of pathogenic and/or spoilage bacteria is warranted.

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