Identification of Food Natural Antimicrobe Compound from Waru Leaves (*Hisbicus tillacaeus L.*) Extract by GC-MC

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Abstract: Chicken is the most common source of animal protein in Indonesia due to its high protein content and low price. However, because of was not a standardized process, chicken meats sold in the traditional market is mostly contaminated by phatogen bacteria such as Escherichia coli. Previous research indicated of waru (Hibiscus tillaceus L.) inhibited Escherichia coli growth. Therefore in this research, had aim 1) to study whether the antimicrobial activity of extracts of waru/hisbiscus by GCMS, 2) to find out the activity of waru that have antimicrobial activity against Escherichia coli contaminant. The results showed that the antimicrobial activity of waru leave extracts depends on the respective proportion in the blend. Increasing antimicrobial activity was observed when waru leaves extract concentration was an increase in the mixture. The results of the research showed that content of the leaves of waru was dominated by Phytol (38%); methyl ester (22,1%); Pentadecanoic acid,14-methyl-Squalene (6,58%); Bis (2-Ethylhexyl) phthalate (5,94%); and ester compound, that had as an antimicrobe potency.

1 INTRODUCTION

The microbes that often contaminate meat or high protein food are pathogenic microbes, such as, Escherichia coli and Salmonella sp. (Sartika et al., 2019). Contamination of pathogenic microbes can cause various diseases, such as fever, typhoid, diarrhea, etc. or often referred to as a foodborne disease. Djafaar and Rahayu (2007) explained that Escherichia coli contamination can cause denatured protein on meat and produce toxin compounds. These toxin compounds can cause several cases of foodborne disease, one of them as diarrhea (Jawetz et al., 1995). Escherichia coli or pathogenic microbes contamination needs to be inhibited to reduce the number of pathogenic microbes contamination and prevent damage to chicken meat.

Contamination of pathogenic microbes on high protein foodstuffs, meat, generally, can be inhibited by cooling treatment. The other inhibition microbe treatments, such as the addition of salt, sugar, acid, and preservatives with synthetic or chemical preservatives (Usmiati, 2010). However, the methods that use preservatives with synthetic or chemical preservatives can have a serious impact on consumers. So, need a natural preservative method that has a low risk of health. Therefore, it is necessary to develop more effective antimicrobials such as natural antimicrobials in inhibiting pathogenic microbes contamination on meat. Natural antimicrobials are recommended because not cause side effects or negative effects. The natural preservative method can use by a natural antimicrobe from natural resources.

Indonesia is a country that has various natural resources, such as waru (Hisbicus Tillacaeus L.). Waru is a popular plant in Indonesia. The leaves of waru were presumed can act as a natural antimicrobial because contain antimicrobial compounds. The waru leaves contain saponins, flavonoids, tannins, and polyphenols. Meanwhile, teak leaves contain flavonoids, saponins, tannins, katekat tannins, quinones, steroids/triterpenoids (Hartati et al., 2007). The antimicrobial can be explored from environmental such as dragon fruit leather (Sartika *et al.,* 2019); plant extract (katalinic *et al*., (2006); kapok bananas (Ningsih and Nurmiati (2013); gambir (Pambayun *et al*., (2007); red rosella (Putri *et al*., (2006) and bacteriophage (Sartika *et al.*, 2002).

Antimicrobial compounds from waru leaves can be isolated through extraction processes such as maceration, soxhlet process, percolation, reflux, and

so on. Maceration is an extraction method that is simple, easy to perform, and does not require high costs. The macerated extract of hibiscus leaves was effective in inhibiting the growth of Bacillus subtilis and Escherichia coli. In the fact, the content of waru leaves does not found exactly. So, this research aimed was to find the waru content with use by GCMS.

2 MATERIAL AND METHOD

2.1 Material

Materials used in this research were waru leaves that were collected from Lampung Timur of Lampung province, ethanol 70%, polar dissolvent, aqua distillate, and alcohol 70%. type of Equipment used included knife, basin, blender, filter paper, macerator, beaker tube, Erlenmeyer tube, vacuum rotary evaporator, scale tube, stirrer, Gas Chromatography-Mass Spectrometry (GC-MS).

2.2 Study Area

Waru leaves were obtained from Sribahwono Village, Bandar Sribhawono District, East Lampung Regency, Lampung Province. Selected samples of waru/hisbiscus that were not too old, not too young, healthy, and not moldy were included (Figure 1).
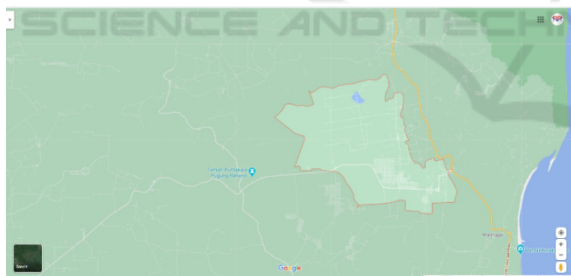

Figure 1: Location of Desa Sribahwono indicating the sampling sites of Hibiscus tiliaceus L.; 5°18' 40.76" S | 105° 44' 31.24" E.

2.3 Method

This research was conducted in two stages. The first stage was the preparation and extraction process of waru leaves sample at The Laboratory of Chemistry, Faculty of Sciences at Lampung University. The second stage of the research was to test the chemical compounds that contained the waru leaves by using Gas Chromatography-Mass Spectrometry (GC-MS) which was conducted in the Integrated Laboratory of Lampung University.

2.3.1 Powder Making and Leaf Extract (Ningsih et al., 2013)

The leaves are cleaned first using clean water to remove dirt, then cut into small pieces. Furthermore, it is oven using 500C temperature for 24 hours. The use of temperature and time of the oven is intended to prevent the active compound in the leaves from being damaged (Putri et al., 2014). After that, the dry leaves are ground using a blender to produce leaf powder. Then the leaf powder is sieved using a 40 mesh sieve to obtain a uniform leaf powder. Sembiring et al. (2006) reported that 40 mesh powders can produce a high yield of active substances after the extraction process. The powdered leaves of waru and teak leaves that have been obtained are then mixed according to the proportions for each treatment. After that, the powder was immersed in boiling distilled water (1000 C) for 10 minutes. The use of boiling distilled water (1000 C) as a solvent aims to improve the solubility of the extract (Pambayun et al., 2007). Furthermore, it is filtered using filter paper to obtain leaf extract.

2.3.2 Total Microbial Decrease Test

The total microbial reduction test was carried out in several stages. First, the Escherichia coli bacteria were rejuvenated (Suwandi, 2012) and made a turbidity standard of 0.5 Mc Farland (Sutton, 2011). Then the bacterial suspension was made, where the bacterial suspension was compared to the standard 0.5 Mc Farland using a spectrophotometer with a wavelength of 600 nm. After that, total Escherichia coli testing was carried out on fresh fillet chicken meat, tested the antimicrobial inhibition of each treatment using disc paper (Suwandi, 2012), and decreased total Escherichia coli in chicken meat.

2.3.3 Data Analysis

Antimicrobial inhibition test data, total reduction test for Escherichia coli in chicken meat, test for phenol content of chicken meat, and test for the degree of acidity (pH) were further tested with the descriptive method. Then the test data for the application of antimicrobial activity and sensory tests were further tested using the Microsoft Excel application (Anova and LSD).

3 RESULTS AND DISCUSSION

3.1 Waru Characteristic by GC-MS Method

The preparation process of this research was the waru leaves were taken in the morning, washed, and dried to remove dust on the material (figure 2). It was shredded into smaller sizes. Then, the small slice of waru was dried to remove water content using an oven. The waru leaves dry powder, 500 grams of dry weight, was taken to make extraction by using ethanol 70% solvent.

Figure 2: Extraction of Waru leaves.

The step of Chemical Compounds research was 3 µl of the sample (waru leaves extract) which had been washed with an ethanol solvent that was taken by using a gas chromatograph inlet. The sample was injected into a gas chromatograph by the following conditions: Gas Chromatograph with AutoSampler (Agilent Technologies 5973 N) and Mass Selective Detector 5873 I; Capillary Column (Innowax) with dimension of 60 m length, 0.2mm wide, 0.25 mm film thickness; 290°C injector temperature; 290°C temperature detector; temperature program of 90°C(150 minutes) - 290°C (20 minutes); carriage gas – Helium 1 ml/min with the constant flow; 1uL Split (ratio 50:1) injection volume; ethanol solvent. The analysis of chemical compounds contained in waru leaves extract was conducted by using Gas Chromatography-Mass Spectrometry (GC-MS). Chromatograph results are presented in Figure 3.
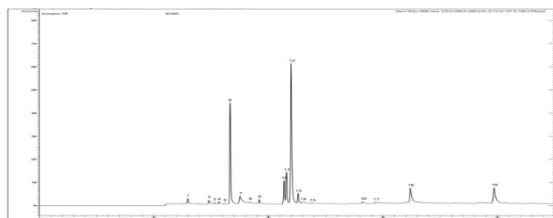

Figure 3: Chromatograph graphic of the waru leaves extract.

The result of chromatograph graphic showed that the waru had characteristic content from high to low rating, as follows, Phytol (38,70%); Pentadecanoic acid,14-methyl-,methyl ester (21,11%); 9,12,15-Octadecatrienoic acid,methyl-ester, (Z,Z,Z)- (8,24%); 8,11-Octadecadienoic acid, methyl ester (5,59%). The other compound of waru leaves content by GCMS was summarized completely in Table 1.

Table 1: Waru Chemical compounds.

The Chemical Compound	Area	% Area
1. 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	16.978	1,39
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	9.722	0,80
2H-Pyran,2-(7-heptadecynyloxy)tetrahydro-	7.685	0,63
3,7,11,15-Tetramethyl-2-hexadecen-1-ol	6.447	0,53
11-Hexadecenoica cid, methyl ester	2.510	0,21
Pentadecanoic acid,14-methyl-,methyl ester	258.045	21,11
n-Hexadecanoic acid	58.626	4,80
n-Hexadecanoic acid	8.092	0,66
Heptadecanoic acid,methyl ester	12.992	1,06
8,11-Octadecadienoic acid,methyl ester	68.372	5,59
9,12,15-Octadecatrienoic acid,methyl ester,(Z,Z,Z)-	100.746	8,24
Phytol	472.954	38,70
Octadecanoic acid,methyl ester	29.407	2,41
9,12,15-Octadecatrienoic acid,2,3-dihydroxypropyl ester, (Z,Z,Z)-	5.547	0,45
9,12-Octadecadienoic acid,methyl ester, (E,E)-	2.505	0,20
Pentatriacontane	5.673	0,46
Cedran-diol,8S,14-	2.878	0,24
Bis(2-ethylhexyl) phthalate	72.592	5,94
Squalene	80.480	6,58

3.2 Antimicrobial Inhibition

The results of the research showed that the proportion of hibiscus leaves gave an effect on the inhibition zone width. The increase of concentration would increase the zone inhibition. The highest of inhibition

zone was reached at the 25% (4,567 ± 0,320) and the lowest was at the 0% (1,278 ± 0,298) concentration level. The following figure describes that the effect of hibiscus extract concentration (Figure 4).
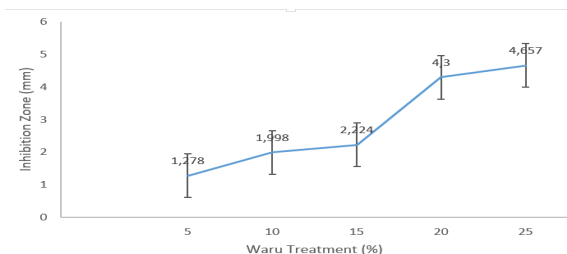

Figure 4: The effect of the hibiscus extract on *Escherichia coli* inhibition.

The research result showed that the concentration of waru/hisbiscus leaves affected inhibition zone width, which described that waru was effective against Escherichia coli bacteria. The best treatment was reached out at a 25% concentration level This happened probably because the highest of waru concentration had the highest antimicrobial compounds. According to Oktavia (2018), hibiscus leaf extract at a concentration of 25% has a flavonoid level of 21.7398%. Meanwhile. Reducing Escherichia coli by Waru or hisbiscus on chicken meat meat.

The total yield of Escherichia coli in chicken fillets from Pasar Tempel, Rajabasa Raya, Bandar Lampung was 2,775 ± 0.775 x 104 colonies/gram. This case indicates that Escherichia coli contamination on the fillet chicken meat upper than the maximum contamination limit set by SNI-7388-2009 (1x101 colony/gram). Escherichia coli contamination can be caused by the unhygienic handling process. Therefore, Escherichia coli contamination in chicken meat can be prevented by performing hygienic handling with the best possible process or added preservative.

The results of the analysis of variance showed that the proportion of the mixture of hibiscus leaves and teak leaves had a significant effect on the total reduction of Escherichia coli bacteria. The results of further testing with the least significant difference (LSD) at the 5% level showed that the control treatment was not significantly different from the W6J6 treatment (0% of waru leaves and 25% of teak leaves), but was significantly different from other treatments. The treatments of W3J3 (15% waru leaves and 10% teak leaves), W4J4 (10% waru leaves and 15% teak leaves), and W5J5 (5% waru leaves and 20%) were not significantly different. Then the W2J2 treatment (20% waru leaves and 5% teak leaves) was significantly different from the W1J1 treatment (25%

waru leaves and 0% teak leaves) and the two treatments were significantly different from other treatments. The graph of the effect of mixing the extract of hibiscus leaves and teak leaves on the decrease in total *Escherichia coli*.
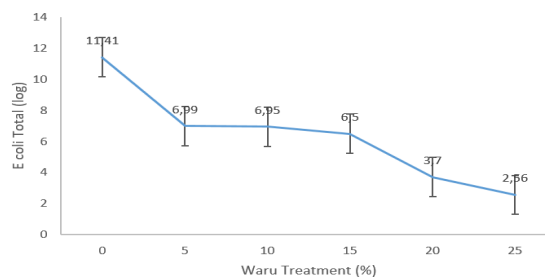

Figure 5: The effect of the extract of hibiscus/waru leaves on the *Escherichia* coli total.

Figure 5 described that the highest proportion of hibiscus/waru leaves gave an effect to the *Escherichia* coli total was highest too. Thus, the results of the total microbial reduction test were in line with the antimicrobial inhibition zone test. The concentration level at 25% hibiscus leaves treatment showed the highest total reduction of *Escherichia* coli on chicken meat with a decreasing percentage of 76.12%. Meanwhile, the resulting research showed that the lowest treatment (0%) tend to decrease of *Escherichia* coli on chicken meat was lowest too (log *E*. coli total was 6.59%). So, Extract waru leaves can be categorized as an antimicrobial or a bacteriostatic compound. According to Pelezar and Chan (1988), antimicrobials can work bacteriostatically (inhibit microbial growth) and bactericidal (kill microbes). Characteristics of *Escherichia* coli colonies that grow on Eosin Methylene Blue (EMB) media are described in Table 1.

Table 2: Characteristics of Escherichia coli colonies growing on Eosin Methylene Blue (EMB) media.

Treatment	Colony Characteristics of *Escherichia coli*
Control	Darkcore purple with metallic green color. Grows on the base, middle, and surface of the media
25%	Dark purple core. Grows on the base of the medium
20%	Dark purple core. Grows in the middle, and base of the medium
15%	Dark purple core. Grows in the middle, and base of the medium
10%	Darkcore purple with metallic green on the base, middle, and surface
5%	Darkcore purple with metallic green on the base, middle, and surface

In Table 2, it can be seen that the characteristics of Escherichia coli colonies that grow on Eosin Methylene Blue (EMB) media are different. The results showed that the addition of hibiscus leaf extract with a greater proportion could prevent the formation of the metallic green color of Escherichia coli colonies on Eosin Methylene Blue (EMB) media. This is evidenced by the growth of the Escherichia coli bacteria forming colonies with dark purple cores without metallic green on EMB media treated by a 25% waru leaves, 20% waru leaves, and 15% hibiscus leaves. While the EMB medium was treated with 0% waru leaves, 5% waru leaves, 0% waru leaves formed Escherichia coli bacteria colonies which are dark purple with a metallic green color. According to Connie et al. (2015) on EMB media, *Escherichia* coli bacteria can ferment lactose quickly and produce acid. As a result of acidic conditions, eosin will change color from clear to dark purple which is usually accompanied by metallic green. This is supported by the statement by Molita (2017) which states that bacteria that ferment lactose from EMB media produce colonies with dark cores with black dots and a metallic green sheen. *Escherichia* coli colonies that did not form a metallic green color on EMB media were treated with the extract with more mixture of leaves indicating that the lactose fermentation process was not running optimally.

4 CONCLUSIONS

The Extract waru leaves had an antimicrobe potency and can be categorized as an antimicrobial or a bacteriostatic compound, it can be seen on the resulting test using by GC-MS and inhibition zone method. The GC-MS test result showed that the waru had characteristic content from high to low rating, as follows, Phytol (38,70%); Pentadecanoic acid,14-methyl-,methyl ester (21,11%); 9,12,15-Octadecatrienoic acid,methyl ester,(Z,Z,Z)- (8,24%); 8,11-Octadecadienoic acid,methyl ester (5,59%). The highest proportion of hibiscus/waru leaves gave an effect to the *Escherichia* coli total was highest too. Thus, the results of the total microbial reduction test were in line with the antimicrobial inhibition zone test. The concentration level at 25% hibiscus leaves treatment showed the highest total reduction of *Escherichia* coli on chicken meat with a decreasing percentage of 76.12% (inhibition zone method).

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