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A beneficial combination between noni seed (*Morinda citrifolia*) and Temulawak (*Curcuma xanthorriza*) rhizome extracts as antibacterial against *Escherichia coli* and *Staphylococcus aureus*

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A Beneficial Combination Between Noni Seed (*Morinda citrifolia*) and Temulawak (*Curcuma xanthorriza*) Rhizome Extracts as Antibacterial Against *Escherichia coli* and *Staphylococcus aureus*

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Abstract. Noni (*Morinda citrifolia*) and temulawak (*Curcuma xanthorrhiza*) are medicinal plants that contain flavonoids and phenolic compounds which are known to have antibacterial activity. The use of a combination of medicinal ingredients has the potential to produce synergistic antibacterial activity. This study aims to determine the antibacterial effect of the combination of noni seed and temulawak extract against *Escherichia coli* and *Staphylococcus aureus* bacteria. Extraction of the herbal materials was carried out by maceration for 5 days. Analysis of total flavonoids and total phenol levels was conducted using the UV-Vis spectroscopy. Antibacterial activity was performed out by liquid microdilution method using a checkerboard to obtain MIC (Minimum Inhibitory Concentration) values of single or combined extracts. The synergistic antibacterial effect of the extract combination was measured based on the FICI (Fractional Inhibitory Concentration Index) value. The results showed that noni seed extract and temulawak rhizome contained total flavonoids, respectively 12.46 ± 0.73 and 5.26 ± 0.74 mgQE/g extract, while total phenols were 10.95 ± 0.14 and 6.18 ± 0.12 mgGAE/g extract, respectively. The combination of the two extracts produces an additive effect in inhibiting the growth of *Escherichia coli* and a synergistic effect on *Staphylococcus aureus*. Single extracts of noni seeds and temulawak have MIC values against *E. coli* of 12% and 40% respectively. When combined, the MIC was obtained from a mixture of 6% noni seed extract and 20% temulawak. For *S. aureus*, the MIC values of single extracts of noni seeds and temulawak were 6% and 10%, respectively. However, MIC of the mixture decreased significantly to 0.75% for noni seed and 2.50% for temulawak extracts. Write the MIC value of single and combination here

INTRODUCTION

Curcuma is a genus of medicinal plants that grows widely in Indonesia and is used for cooking and medicinal seasoning. Curcuma is often used as a traditional medicine to treat infections caused by pathogenic bacteria such as *E. coli* and *S. aureus*. Previous study showed that extract of Curcuma xanthorriza (temulawak) has antibacterial activity against *E. coli* with MIC and MBC (Minimum Bactericidal) values of 12.5% and 25% respectively. [1]. Temulawak (*C. xanthorrhiza*) has been reported to exhibit antidiuretic, anti-inflammatory, antioxidant, antihypertensive, antihepatotoxic, antibacterial, and antifungal activities. Temulawak rhizome ethanol extract contains a group of flavonoids and terpenoid/steroid compounds [2,3]. Several studies have stated that temulawak rhizome contains bioactive compounds consisting of starch, protein, curcuminoite (a yellow dye consisting of two components, namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin), and essential oils [4,5].

Besides Curcuma, the noni plant (*Morinda citrifolia*) also has various benefits ranging from its fruit, leaves, seeds and roots. *M. citrifolia* contains alkaloids, flavonoids, glycosides and glucose. Indian noni fruit contains steroids, cardiac glycosides, phenols, tannins, terpenoids, alkaloids, carbohydrates, flavonoids, lipids and fats, saponins and acid compounds [6,7]. Noni seeds are blackish brown, small and flat. Noni seeds contain phenolic compounds, flavonoids, carotenoids, vitamin C, tannins, polyunsaturated fatty acids, phytosterols and tocopherols [8]. Among the noni plant parts, noni seeds are reported to have the greatest antibacterial activity compared to the leaves and fruits against the growth of E. coli and S. aureus [9]. However, noni seeds have not been utilized optimally in the development of traditional medicine.

Herbal treatment by administering a combination of plants has the potential to cause a more effective therapeutic effect than using only one plant. The active components contained in a combination of plant extracts can provide therapeutic effects that help each other in increasing their efficacy, namely synergistic effects [10]. The combination of antibacterial compounds that have synergistic effects can increase antibacterial sensitivity thereby preventing resistance to antibacterial compounds [11].

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Based on the description above, it is necessary to explore the use of combinations of plant extracts that produce synergistic effects thereby increasing the success of treatment. Because in previous studies, extracts of noni seeds and temulawak rhizome were only tested as single extracts, this study was conducted to determine the synergism effect of the combination of the two plants against E. coli and S. aureus bacteria. Information on the existence of a synergistic effect provides benefits in the use of treatment using a combination of active ingredients compared to the use of a single ingredient.

MATERIAL AND METHOD

Chemicals

All chemicals used for extract preparation and chemical analyses were purchased from Merck (USA) with pro analyte grade, while for microbial test were from Oxoid (UK).

Bacterial Strain and Growth

The bacterial used in this present study was *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli (ATCC 25922)*. These were subcultured in Mueller-Hinton Agar (MHA) at 37°C, 24 h before use.

Extract Preparation

Noni seed extract (NSE) was produced from the extraction by maceration where as much as 500 g of dry simplicia powder was soaked overnight in 96% ethanol solvent as much as 1000 mL. The obtained macerate was evaporated using a rotary evaporator until a thick extract is formed. Temulawak rhizome extract (TRE) was made in the same way as NSE.

Determination of Total Flavonoid Content

A total of 0.5 mL of the extract solution and a series of reference solutions (quercetine) were pipetted separately into each tube. Then each was added 1.5 mL of ethanol P, 0.1 mL of 10% aluminum chloride P, 0.1 mL of 1M sodium acetate and 2.8 mL of water. The mixture was then shaken and left for 30 min at room temperature. After that, the absorption is measured at a maximum absorption wavelength of 435 nm [12].

Determination of Total Phenolics Content

To 1 mL of the extract solution and a series of reference solutions (gallic acid) in each tube, 5 mL of Folin-ciocalteu LP dilution (7.5% in water) was added. The mixture was allowed to stand for 8 min, 4 mL of 1% NaOH was added, incubated for 1 hour. The absorption of each solution was measured at a maximum wavelength of approximately 730 nm [12].

Antibacterial Activity Test

The test solutions used were noni seed extract with a concentration series of 0.37; 0.75; 1.50; 3; 6; and 12%, as well as temulawak rhizome extract with a concentration series of 1.25; 2.50; 5; 10; 20; and 40%. The antibacterial activity test of the combination of extracts in this study was carried out using the microdilution checkerboard assay. In this assay, two antimicrobial agents (extracts) are examined through double serial dilutions, and the concentration of each extract is evaluated individually as well as in combination. In the concentration gradient series, 100 µL of extract solution was combined with 100 µL of S. aureus bacterial suspension, followed by an incubation period of 18-24 hours at 37°C to assess the minimum inhibitory concentrations of individual NSE and TRE extracts. For the determination of the FICI (Fractional Inhibitory Concentration Index), 50 µL of each concentration of NSE solution was mixed with 50 µL of TRE solution from the prepared concentration series, and then 100 µL of bacterial suspension was added. This mixture was incubated for 18-24 hours at 37°C [11,12].

Data Analysis

MIC (Minimum Inhibitory Concentration) data was obtained from the smallest concentration of the test solution that appears clear in liquid BHI broth media. The MBC (minimum bactericidal concentration) data was obtained from the smallest concentration which was not overgrown with bacteria after being streaked on MHA media. The MIC data obtained was used to calculate the FICI value. FICI was determined through the application of the following equation:

$$FICI = \frac{MIC\ (EDLB) in\ combination}{MIC\ (EDLB)} + \frac{MIC\ (EDK) in\ combination}{MIC\ (EDK)}$$

RESULTS AND DISCUSSION

Extract characteristics

Characterization of the extracts is conducted to assess the quality of the extracts used as test samples. The results of organoleptic tests, which encompass attributes like taste, smell, and color, along with the determination of water content in both noni seed and temulawak rhizome extracts, are presented in Table 1. These findings conform to the quality standards prescribed by the Indonesian Herbal Pharmacopeia. Analyzing Table 1, it can be established that the yield of noni seed extract meets the specified requirements, as it yields 14.36%, surpassing the Indonesian Herbal Pharmacopeia's minimum requirement of 10.10%. In contrast, the yield of temulawak rhizome extract stands at 10.69%, falling short of the stipulated requirement of at least 18.00%. This variance in yield results may be attributed to factors such as the quality of the raw materials, suboptimal extraction conditions, or even potential material losses during the extraction process, such as evaporation or precipitation, leading to a reduced overall yield [13,14].

TABLE 1. Organoleptic, water content and yield of extracts

Samples	Organoleptic			Water content (%)	Yield (%)
	Color	Smell	Taste		
Noni seed extract (NSE)	Dark brown	Special smell	Bitter	5.41%	14.36
Temulawak rhizome extract (TRE)	Brownish yellow	Special smell	Bitter	2.47%	10.69

Phytochemical screening of extracts

TABLE 2. Extract phytochemical screening

No	Compound groups	Reagents	Results	
			NSE	TRE
1	Flavonoids	Mg powder + HCL 2N	+	+
2	Saponins	HCL 2N	+	+
3	Tannins	FeCl3 1%	+	+
4	Alkaloids	HCL 2N	-	-
5	Terpenoids	H2SO4	+	+
6	Phenolics	FeCl3	+	+

According to research by Qulub (2018) and Oktaviana (2019), the compounds contained in noni seeds include flavonoids, alkaloids, tannins, saponins, steroids and phenols [15][16]. In the noni seed extract samples tested, the results showed that the noni seed extract samples contained secondary metabolites including flavonoids, saponins, tannins, terpenoids and phenolics (Table 2). There are differences in secondary metabolites between the previous studies and this research conducted, that is, no alkaloid content was found in the research conducted. This can occur due to differences in the types or varieties of plants used, the location of the plants growing and the differences in the processing of the plants. In addition, the alkaloid levels that is too small can also affect the reaction formed in the test tube.

According to research by Farida (2018) and Putri (2017) the compounds contained in temulawak rhizomes are flavonoids, saponins, tannins, terpenoids, and essential oils [17,18]. In the curcuma rhizome extract samples

tested, the results showed that the temulawak rhizome extract samples contained flavonoids, saponins, tannins, terpenoids and phenolics. Based on the test results obtained, there are similarities in the content of secondary metabolites in temulawak rhizome extract with research that has been done by other researchers before.

Total Flavonoid and Phenolic Contents

The calculation of total flavonoid content in noni seed extract has yielded varying results. According to Table 3, the average total flavonoid content is 12.46 mg of quercetin equivalents (QE) per gram of extract, indicating that 1 gram of noni seed extract contains 12.46 mg of flavonoids. In contrast, a different study reported a total flavonoid content of 6.18 mg of QE per gram of extract, implying that 1 gram of noni seed extract contains 6.18 mg of flavonoids. These findings highlight the variability of flavonoid levels in noni seed extract [15].

TABLE 3. Total flavonoid and phenolic contents of the extracts

Extract	Total flavonoid (mgQE/g extract)	Total phenolic (mgGAE/g extract)
NSE	12.46 ± 0.73	10.95 ± 0.14
TRE	5.26 ± 0.74	6.18 ± 0.12

The total flavonoid content in temulawak rhizome extract was found to be 5.26 mg of quercetin equivalents (QE) per gram of extract, indicating that 1 gram of the extract contains 5.26 mg of flavonoids. These results differ from previous studies, where the total flavonoid content was reported as 346.20 mg of QE per gram of extract, or 34.62% QE, signifying that 1 gram of the extract contains 346.20 mg of flavonoids. [19].

In noni seed extract, the total flavonoid content of the samples tested was greater than the results of previous studies, and in temulawak rhizome extract the total flavonoid content of the samples tested was lower than the results of previous studies. This difference can be caused by genetic variation and the age of each plant, the older the plant, the greater the total flavonoid levels, the existence of environmental factors that can affect the production of flavonoids in plants, where environmental conditions that are not optimal can cause low total flavonoid levels. . In addition, differences in the methods used or interactions with other factors can also affect the total flavonoid levels produced [20].

Based on Table 3, the total phenolic content of noni seed extract averaged 10.95 mgGAE/g extract, which means that 1 gram of noni seed extract contains 10.95 mg of phenol. Meanwhile, based on a previous study, the total phenolic content of noni seed extract was 108.43 mgGAE/g extract, which means that 1 gram of noni seed extract contains 108.43 mg of phenol [15]. In temulawak rhizome extract, the average total phenol content was 6.18 mgGAE/g extract, which means that 1 gram of temulawak rhizome extract contains 6.18 mg of phenol. Meanwhile, according to the different study, the total phenol content of temulawak rhizome extract is 7.80% GAE or equal to 78.00 mgGAE/g extract, which means that 1 gram of temulawak rhizome extract contains 78.00 mg of phenol [19].

In noni seed extract and temulawak rhizome extract, the total phenol content of the samples tested was lower than the results of previous studies. This difference can be caused by genetic variation and the age of each plant, the older the plant, the greater the total phenol content, the presence of environmental factors that can affect phenol production in plants, where environmental conditions that are not optimal can cause low total phenol levels. In addition, differences in the methods used or interactions with other factors can also affect the total phenol content produced [20].

Antibacterial activity assay

Antibacterial activity was determined by the microdilution method on a checkerboard (Fig. 1 and Fig. 2). The results showed that the MIC of the noni seed extract was in the first row well (well M) of 12% against *E. coli*, while the MIC of temulawak rhizome extract was in the first column well (well T) which was 40%. The MIC of the extract combination was found in the MT wells which contained 6% NSE and 20% TRE. Furthermore, testing for MBC (minimum bactericidal concentration) was carried out by scraping the concentration that was suspected of inhibiting bacteria [21]. The results of the MBC of noni seed extract were 12%, while the MBC of temulawak rhizome extract was 40%, and the combined MBC was 6% NSE + 20% TRE. Thus, the MBC value is the same as the MIC value in both single and combined extracts.

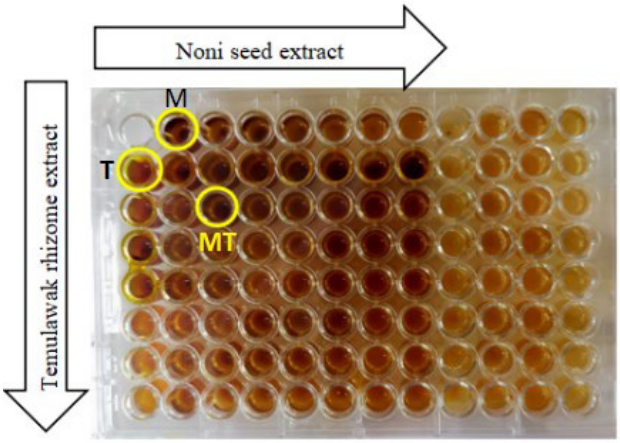

FIGURE 1. Antibacterial activity against *E. coli*. M, T and MT are wells which showed inhibition of bacterial growth after treatment with NSE (well M), TRE (well T) and NSE-TRE (well MT) combination

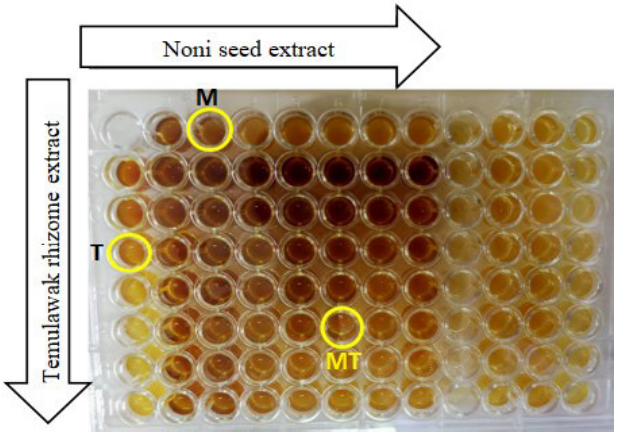

FIGURE 2. Antibacterial activity against *S. aureus*. M, T and MT are wells which showed inhibition of bacterial growth after treatment with NSE (M), TRE (T) and NSE-TRE (MT) combination

In the antibacterial activity test against *S. aureus*, the MIC yield of individual noni seed extract was 6% (well M), while temulawak rhizome extract was 10% (well T). Meanwhile, combined MIC was found in the MT wells which contained a mixture of 0.75% NSE and 2.50% TRE. After being etched on solid media, the MBC value of noni seed extract was 6%, while temulawak rhizome extract was 10%. The combined MBC value was obtained from a mixture of 0.75% NSE and 2.50% TRE, the same value as the MIC.

The MIC and MBC values of both single and combined extracts in the activity test against E. coli were different from those of S. aureus. This was influenced by the difference in total flavonoid and total phenolic content in the two extracts. In addition, noni seeds and temulawak rhizomes contain secondary metabolites which also act as antibacterial, namely saponins and tannins [22]. Flavonoids and saponins have the ability to deactivate proteins or enzymes in the bacterial cell wall which causes the protein structure to be damaged and the bacterial cell to lyse. Meanwhile, tannins, which are polyphenolic compounds, work by damaging cell walls and deactivating enzymes and the function of the genetic material of bacteria [18].

FICI (Fractional Inhibitory Concentration Index)

The antibacterial effect of a combination of extracts is typically interpreted based on the Fractional Inhibitory Concentration Index (FICI) values. If the FICI value is ≤ 0.5, it indicates a synergistic effect, meaning that the combination is more effective against the bacteria than individual extracts used alone. If the FICI value is > 0.5 and ≤ 1.0, it suggests an additive effect, where the combination is effective but not significantly more so than using the extracts separately. If the FICI value is > 1.0 and ≤ 4.0, it is considered indifferent, implying that the combination doesn't have a remarkable impact. If the FICI value is > 4.0, it is categorized as antagonistic, meaning the combination is less effective than using the extracts on their own [23]. Based on the FICI value calculations

presented in Table 4, the data indicates that the combined effect of noni seed extract and temulawak rhizome extract is additive when tested against E. coli bacteria and synergistic when tested against S. aureus bacteria.

TABLE 4. Fractional Inhibitory Concentration Index

Data	MIC NSE single	MIC TRE single	MIC combination (NSE:TRE)	FICI	Interpretation
E. coli	12	40	6 : 20	1	Additive
S. aureus	6	10	0.75 : 2.50	0.375	Synergistic

The term "additive effect" in this context signifies that the activity of a combination of extracts is essentially equivalent to that of a single extract [24]. In the context of this study, an additive effect means that the combined impact of noni seed extract and temulawak rhizome extract is comparable in terms of inhibitory effects to the use of each individual plant extract. While a "synergistic effect" indicates that the combined impact of two plants can provide therapeutic benefits that enhance each other's effectiveness when compared to their individual use [25]. In this study, a synergistic effect implies that the combined effect of noni seed extract and temulawak rhizome extract results in a greater inhibitory effect than the use of each plant extract separately.

The variation in the combination effect observed against *E. coli* and *S. aureus* at the same concentration levels may be attributed to the distinct properties and characteristics of these two bacteria. *Escherichia coli* is a gram-negative bacterium with a more complex cell wall structure, which can pose challenges for antibacterial compounds in terms of penetration. The cell wall of gram-negative bacteria is composed of peptidoglycan, lipopolysaccharide, and lipoprotein. In contrast, *Staphylococcus aureus* is a gram-positive bacterium with a simpler cell wall structure, allowing antibacterial compounds to more easily enter the cells [26,27].

CONCLUSION

The noni seed extract (NSE) exhibited a higher total flavonoid content (12.46 ± 0.73 mgQE/g extract) and total phenolic content (10.95 ± 0.14 mgGAE/g extract) compared to the temulawak rhizome extract (TRE) with values of 5.26 ± 0.74 mgQE/g extract and 6.18 ± 0.12 mgGAE/g extract, respectively. When combined, the NSE and TRE showed inhibitory effects on bacterial growth with minimum inhibition concentration (MIC) values of 6% NSE & 20% TRE against *Escherichia coli* and 0.75% NSE & 2.50% TRE against *Staphylococcus aureus*. It is important to note that the combination of NSE and TRE exhibited an additive antibacterial effect against *E. coli* with an FICI value of 1. On the other hand, their combination showed a synergistic effect against *S. aureus*, with an FICI value of 0.375, signifying an enhanced inhibitory effect when used together. These results suggest the potential of this combination as an effective antimicrobial strategy, especially against *S. aureus*.

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