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Original Article

INTERACTION EFFECT OF *APIS TRIGONA* HONEY, ETHANOLIC EXTRACTS KEMUNING (*MURRAYA PANICULATE*), YAKON (*SMALLANTHUS SONCHIFOLIUS*) AND THEIR COMBINATION AGAINST STAPHYLOCOCCUS AUREUS INFECTIONS

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ABSTRACT

Objective: This study was investigated the antibacterial interaction of *Murraya paniculate* extract, *Smallanthus sonchifolius* extract**,** *Apis trigona* honey and their combinations for their interaction effect against *Staphylococcus aureus* ATCC 29213.

Methods: All extracts and honey were evaluated for antibacterial interaction effects both alone and in combination. The disk diffusion method was employed with clindamycin phosphate as the standard antibiotic. The minimum inhibitory concentration (MIC) of the most potent extract was determined using microdilution assays and performed in line with CLSI guidelines.

Results: Among all, *S. sonchifolius* extract provided the most effective inhibitory activity in higher inhibition than clindamycin phosphate with the range MIC value of 12.5-25% w/v. However, significant different interactions (synergistic, additive and antagonistic) were observed between honey and plant crude extracts. The *S. sonchifolius* extract displayed additive interaction with *M. paniculate* extract but antagonistic with *A. trigona* honey. The antagonistic interaction also produced when *M. paniculate* extract combined with *A. trigona* honey. Consequently, their total combination of all tested sample produced an additive interaction.

Conclusion: Thus, we concluded that their combination was ineffective to be used as the antibacterial cocktails against *S. aureus* infections.

Keywords: *Murraya paniculate, Smallanthus sonchifolius, Apis trigona*, Interaction, Additive, Antagonistic, *Staphylococcus aureus*

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INTRODUCTION

Staphylococcus aureus colonizes enormous parts of human populations as well as to being one of the most important human infections. Several reports claimed that nasal cavities are the predominant colonization site [1, 2], and that 20% of the population is a permanent carrier, whereas 60% is an intermittent carrier. Although the human throat has received less attention as a carriage site, several studies have concluded that it is the most prevalent [3, 4]. The risk and result of nosocomial staphylococcal bacteraemia caused by *S. aureus* nasal carriers and noncarriers varies dramatically, and bacteraemia is three times more common in carriers than in noncarriers [5]. As a result, potential antibiotics are critical in avoiding infections.

Antibiotic resistance in bacteria is a critical worldwide issue. Over the last few decades, extensive over-prescription and self-medication of therapeutically accessible antibiotics has resulted in the long-term exposure of pathogenic microbes to these antibiotics [6]. Currently, more than 70% of harmful microorganisms have gained antibiotic resistance [7]. As a result, newer antimicrobials and/or ways to combating the issue are urgently required. Natural materials and traditional treatments may hold the key to discovering novel antibacterial agents. Pharmaceutical businesses have developed new antibiotics during the previous three decades [8]. Thus, it is critical to discover novel antimicrobial medicines or methods for treating infectious disorders caused by drug-resistant bacteria [9].

Few antibiotics are authorized by regulators, reflecting both the difficulties of producing such drugs and the fact that antibiotic discovery initiatives at numerous large pharmaceutical corporations have been abandoned in the last decade [10]. As a result, the output of drug pipelines is simply not well positioned to control resistant infections, despite efforts by academic institutions and smaller enterprises to fill the gap. Combination treatment is an emerging method for combating such diseases. Combining two antibiotics is emerging as a viable treatment strategy [11]. Broad-spectrum coverage for the initial therapy of severely infected patients, polymicrobial infections, and prevention of selection of resistant microorganisms when the causal organism has a high mutation rate to the antibiotic indicated, reduction of dose-related toxicity, and antimicrobial synergistic activity are reasons that justify the use of antimicrobial combinations [12]. Few studies have indicated that combining plant extracts can boost their efficiency against certain bacterial infections [13-15]. With this in mind, our research team investigated and confirmed that kemuning leaves (Murraya paniculata L. Jack), yacon leaves (Smallanthus sonchifolius) and Apis trigona bee honey contain several phytochemical compounds which exhibit bactericidal effect against diverse Gram-positive bacteria [16-18]. In this study, we further investigated the antibacterial efficacy of those plants extract and honey in combination against S. aureus to answer whether any different antibacterial effects occur between the single extract and their combination.

MATERIALS AND METHODS

Extraction of kemuning and yacon

Plant parts were locally collected from Manoko Garden, West Java, Indonesia and *Apis trigona* was obtained from Ciburial Honey Bee Cultivation. All samples were authenticated by the experts from Biology Department, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Indonesia. As the results, our samples were confirmed as kemuning (*Murraya paniculata* L. Jack) and yacon (*Smallanthus sonchifolius* Poepp. H. Rob) (No. 531/HB/02/2017) and *Apis trigona* (557/HB/02/2018). Each powdered of the leave (500 g) was macerated in 1 L of 70% alcohol for 3 d. This filtrate was extracted with a rotatory evaporator after filtering through a Whatman number 1 filter paper and maintained at 55 °C until fully dry. Those concentrated extracts and honey were kept in a sterile screw-capped vial at 20 °C and dissolved in dimethylsulfoxide (DMSO; Merck, Germany) before use.

Preliminary phytochemical screening

Kemuning, yacon leaves extracts, and *A. trigona* honey were subjected to preliminary qualitative phytochemical screening for the identification of alkaloids, flavonoids, saponins, steroids, and tannins using previously reported methods [19, 20]. To test the alkaloids, 2 ml of each extract was mixed with 2 ml of 10% aqueous hydrochloride acid. 1 ml of the filtrate was treated with a few drops of Mayer's reagent. The presence of alkaloids in the extract and honey were shown by the appearance of creamy precipitate, and for Wagner's reagent, 1 ml of the extract and honey were treated with a few drops of the reagent. The presence of alkaloids in the extract and honey were also confirmed by a reddish-brown precipitate. To test the flavonoids, 3 ml aliquot of the filtrate was made alkaline with sodium hydroxide (NaOH). A yellow color developed which indicated the possible presence of flavonoids compounds. To detect the saponins, 2 g of the powdered extract and honey were placed into a test tube, 5 ml of water was added and it was shaken strongly. The whole tube was added and it was filled, which last for some minute. The presence of bubbles indicated presence of saponin. To detect the steroids, 5g of sample powder were dissolved in 5 ml of chloroform. After then, it was filtered. To make the bottom layer, 2 ml of concentrated sulfuric acid were carefully added. The existence of a steroidal ring is indicated by a reddish-brown tint at the interface. To detect the tannins, 5% ferric chloride solution was added to 2-3 ml of extract and honey, drop by drop. The presence of tannins is indicated by a dark green precipitate.

Determination of antistaphylococcal activities

Overnight suspensions of *Staphylococcus aureus* were prepared following inoculation of Muller Hinton broth (MHB; Oxoid) with three to five well-isolated colonies from Tryptic Soy Agar (TSA, Oxoid). The suspension was adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/ml). A stock solution of the herbal recipe (100 mg/ml in 10% DMSO) was diluted in DMSO to produce several working solutions of 250, 125, 62.5, and 31.25 mg/ml. A 250 mg/ml clindamycin phosphate was used as the positive control. The antistaphylococcal potential of each sample was determined using the agar well diffusion technique. The standardized inocula (20 µl) were put into individual plates with MHA growth medium. A sterile copper borer with a 6 mm diameter was used to form wells in the solidified growth media in the plates. Each well was labelled appropriately and individually filled with 100 µl of testing materials. Before starting the bacterial growth, the inoculation petri plates were kept at room temperature for an hour to allow for treatment diffusion. The plates were then incubated at 37 °C for 24 h before measuring the zones of inhibition (ZOI) surrounding the wells. The results were then statistically analysis using ANOVA to determine the antistaphylococcal effect of those tested sample.

Synergistic antibacterial assay

The synergistic antibacterial interaction of kemuning extract, yakon extract**,** *A. trigona* honey, and their combinations for their interaction effect against *S. aureus* ATCC 29213 were investigated in this study using the agar well diffusion assay. The tested media was prepared with the same procedure as previous antibacterial assay. The two extracts were combined by adding 50 µl of each extract (500 mg/ml) to the same well. Kemuning and yakon leaf extract (KY), yakon leaf extract and *A. trigona* (YA) bee honey, and kemuning leaf extract and *A. trigona* bee honey (KA) were the pattern combinations investigated, presented in table 1. Each test extract concentration (KYA) in the combination of three extracts was 75 mg/ml (0.75g extract in 1 ml DMSO solvent) with each volume of 33.3 µl in the same well. All test medium were incubated at 37 °C for 24 h. A calliper is used to measure the inhibition zones created around the test hole.

Table 1: Extracts and honey combination

Extracts and honey (500 mg/ml)	Combination		
	Kemuning	Yakon	Honey
Kemuning	-	1:1	1:1
Yakon	1:1	-	1:1
Honey	1:1	1:1	-

Minimum inhibitory concentration determination

To determine the MICs of the most potential sample, broth microdilution assays were performed in line with CLSI guidelines [21]. Each well contained 100 μl of tested antistaphylococcal agent (250 mg/ml diluted to 0.4875 mg/ml) and 100 μl of the bacterial suspension and incubated for 24 h at 37 °C. The MIC was then determined as the lowest concentration, showing no growth using optical density (OD) at 595 nm (OD595 nm) on a microplate reader. The MIC result then subcultured by dropping 10 µl of the subculture result on to the surface of agar media. The assay was repeated in triplicate.

RESULTS

Extraction yield and phytochemical contents

The ethanol extract yield, measured as the weight of the extract divided by the weight of the crude herb powder, was 24.9 and 2.3% (w/w) for kemuning and yacon, respectively. The results of the phytochemical analysis provided evidence of the presence of alkaloids, flavonoids, saponins, tannins and steroids in the leaf extracts of kemuning and *A. trigona* honey, except that steroids was not found in yakon extract, presented in table 2.

Table 2: Phytochemical contents

Samples	Phytochemical contents				
	Alkaloids	Flavonoids	Saponins	Tannins	Steroids
Kemuning	+	+	+	+	+
Yacon	-	+	+	+	+
Honey	+	+	+	+	+

Notes: (+) presence; (-) absence

Table 3: Antistaphylococcal activity

Sample	Diameter of Inhibition (mm) in certain concentration (mg/ml)			
	250	125	62.5	31.25
Kemuning	19.00±0.25	17.10±0.55	13.88±0.48	7.73±1.08
Yakon	20.05±1.00	18.43±0.72	17.64±1.39	13.8±2.80
Honey	11.25±0.50	10.58±0.08	9.38±0.13	7.88±0.38
Clindamycin phosphate (250 mg/ml)	20.88±0.13			

Note: perforator diameter= 6 mm, data showed as mean and deviation standard, n=3

Antistaphylococcal activity

The antistaphylococcal activities of both leaf extracts and the *Apis trigona* honey are demonstrated in table 3. As the results, all tested samples exerted potent inhibition against *S. aureus*. Of the plant extracts, Yacon extract showed the most active antibacterial while *Apis trigona* honey had the lowest mean total inhibition.

S. aureus gave different sensitivity responses to the extracts and honey at the same concentration. To observe the extent of the difference in the inhibitory potential of each sample, a statistical analysis was carried out as shown in table 4. Effect of the extract on the difference in the inhibition diameter was statistically analysed using the ANOVA test. The results of the ANOVA test above obtained the value of Sig. Sig. (0.000)<0.05 means that there is a difference in the inhibitory potential of the all samples at a concentration of 31.25, 62.5, 125, and 250 mg/ml.

Synergistic antibacterial assay

Significant different interactions (synergistic, additive and antagonistic) were observed between honey and plant crude extracts, shown in table 5. The effect of interaction was determined by observing the diameter of inhibition both single and combination. Interactions are additive when their combined effect equals the total of their individual effects, synergistic when the combined impact exceeds the sum of their individual effects, and antagonistic when the combined effect is less than the sum of their individual effects [22]. The *S. sonchifolius* displayed additive interaction with *M. paniculate* extract but antagonistic with *A. trigona* honey. The antagonistic interaction also produced when *M. paniculate* extract combined with *A. trigona* honey. Consequently, their total combination of all tested sample produced an additive interaction.

Table 4: Statistical analysis result

	Sum of squares	Df	Mean square	F	Sig.
Between Groups	678.425	14	48.459	13.629	0.001
Within Groups	53.333	15	3.556		
Total	731.757	29			

Table 5: Synergistic antibacterial results

Tested sample (500 µg/ml)	Diameter of inhibition (mm)
Yacon and kemuning	19.20±0.05
Yacon and *A. trigona* honey	16.15±0.50
Kemuning and *A. trigona* honey	16.13±1.03
Yacon, kemuning and *A. trigona* honey	18.72±0.51

Data showed as mean and deviation standard, n=3

Table 6: MIC and MBC values of Yacon leaf extract

Extract concentration (mg/ml)	Bacterial growth	
	MIC	MBC
0.48	+	+
0.97	+	+
1.95	+	+
3.96	+	+
7.81	+	+
15.6	+	+
31.2	+	+
62.5	+	+
125	-	-
250	-	-
Control (+)	+	+
Control (-)	-	-

MIC and MBC values

MIC and MBC of yacon leaf extracts in a single extract was determined. The MIC values for the extracts ranged between 0.4875 mg/ml and 250 mg/ml, presented in table 6. Yacon leaf extract with a MIC of 125 mg/ml and MBC of 250 mg/ml was the most potential plant extract against *S. aureus*.

DISCUSSION

Antimicrobial resistance is now regarded as a major barrier to combating infectious diseases, so the goal of this study is to shed light on some antimicrobial interactions of some plant extracts against *S. aureus* in order to provide practitioners and infectious disease specialists with ideas for laying strategic control of Staphylococcal infections by using those antimicrobials. The antibacterial interaction of *M. paniculate* extract, *S. sonchifolius* extract**,** *A. trigona* Honey, and their combinations for their interaction effect against *S. aureus* ATCC 29213 was investigated in this study.

M. paniculata leaves exhibit antibacterial capabilities against human infections due to their high phenolic and flavonoid content, which supports antibacterial activity [23]. *S. sonchifolius* has been implicated in antibacterial activities [24]. *S. sonchifolius* tubers contain fructooligosaccharide and phenolic compounds [24, 25], whereas the leaves include numerous kaurene diterpenoids, acetophenone-type phytoalexins, and melampolide-type sesquiterpene lactones [26]. According to the findings, the antibacterial activity of *S. sonchifolius* may be safely attributed to enhydrin because polymatin B and allo-schkuhriolide had no action against *S. aureus* strains. Meanwhile, the *Trigona sp.* honey demonstrated inhibitory activity against *E. coli* and *S. aureus* at least in the concentration of 12.5%. These results showed that *S. aureus* was more liable than *Salmonella typhi* and *E. coli* [27]. Those studies strengthened the reason to investigate the efficacy the combination of those strong antibacterial agent.

During the last few decades, numerous biologically and medicinally important phytochemicals, which are also found in both leaf extracts and *Apis trigona* honey, including alkaloids, flavonoids, tannins, steroids, saponins, have been reported in increasing its medicinal importance [28]. The phytochemicals play vital roles in plant defence mechanism against different microbial infections [29].

Alkaloids might be the main antimicrobial components as their antimicrobial activity has been reported earlier [30, 31]. Flavonoids are phytochemical substances that have been demonstrated to have a broad antibacterial range via several mechanisms [32-35]. Several studies have reported various antibacterial mechanisms of flavonoid, including the inhibition of nucleic acid synthesis, the interference of cytoplasmic membrane function and energy metabolism, the reduction of bacterial adhesion to form biofilm, the interruption of porin, and the reduction of membrane permeability [36-40]. Many plants which biosynthesize saponins was also found to inhibit the growth of *S. aureus* isolate by disturbing the permeability of bacterial membrane cells [41-44]. The integrated of other phytochemical substance in all leaf extracts had strengthened their antibacterial potency. Alkaloids also have an antibacterial mechanism that is almost the same as other phytochemical compounds found in all extracts of this plant, such as inhibition of bacterial cell wall synthesis, bacterial metabolism, nucleic acid and protein synthesis, also disturbing the permeability of bacterial cell membrane [45, 46].

In this study, antimicrobial activity for yakon leaf extract was detected as the most active antibacterial af all tested samples and *Apis trigona* honey had the lowest mean total inhibition. For almost a century, the concept of synergistic interactions between medications and substances has been a major concern in the biomedical world. The synergistic determined when the combined impact exceeds the sum of their individual effects [22]. Understanding the interactions between medications is becoming increasingly important as complicated illnesses are treated with numerous therapeutic combinations. The concept of 1+1 = 2 is not intriguing, and it is widely accepted and understood across many academic areas and even civilizations throughout the world. As a result, saying 1+0 = 2, 0+0 = 1, or even 1+1 = 0 is extremely paradoxical. That paradigm, however, is a very simple model for comprehending synergist interactions, or synergy. Synergy is typically described as the combined impact of two or more agents that is larger than the predicted additive effect of said agents. Returning to the 1+0 = 2 example [47,48], it may be stated that a synergistic interaction is taking place. Unfortunately, quantifying such interactions is far from straightforward in reality. This synergistic interaction has the ability to maximize therapeutic impact while decreasing detrimental effects or side effects when using a certain pharmacological regimen [49,50]. If two medications work synergistically, smaller dosages of each treatment might be utilized, resulting in fewer side effects while still achieving the intended aim. Our study found that significant different interactions (synergistic, additive and antagonistic) were observed between honey and plant crude extracts. The *S. sonchifolius* displayed additive interaction with *M. paniculate* extract but antagonistic with *A. trigona* Honey. The antagonistic interaction also produced when *M. paniculate* extract combined with *A. trigona* honey. Antagonism is the contrary of synergy; it develops when the combined impact of two or more substances is less than predicted. Consequently, their total combination of all tested sample produced an additive interaction. In most cases, the baseline impact for synergy detection methods is an additive effect. When there is no synergy, it is the impact that is theoretically predicted from the combination of many medications [51]. Thus, we concluded that their combination was ineffective to be used as the antibacterial cocktails against *S. aureus* infections. Yakon leaf extract solely enough to inhibit *S. aureus* with the with the range minimum inhibitory concentration (MIC) value of 125-250 mg/ml.

CONCLUSION

From the results of this study, we conclude that the antistaphylococcal activities of *S. sonchifolius*, *M. paniculate* and *Apis trigona* honey were provide interaction in combination. However, the interaction did not produce synergistic effect against *S. aureus*. The findings of this study point to the need of deliberately selecting extracts to maximize synergisms while minimizing antagonisms in antistaphylococcal activity.

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AUTHORS CONTRIBUTION

All the authors contributed equally.

CONFLICT OF INTERESTS

Declared none

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