Combination of Ketepeng Cina (*Cassia alata* Linn) And Lidah Buaya Buaya (*Aloe vera*) As Antibacterial Agent Against *Staphylococcus aureus* And *Pseudomonas aeruginosa*

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Abstract

Infection is one of the biggest health problems in Indonesia. Infection can be caused by pathogenic microorganisms such as Staphylococcus aureus and Pseudomonas aeruginosa. Treatment with antibiotics is the primary choice to manage infections caused by bacteria. Previous research showed that many plants have been tested for their antibacterial activity such as ketepeng cina (Cassia alata) and lidah buaya (Aloe vera). Cassia alata and Aloe vera alkaloids, flavonoids, anthraquinones, saponins, tannins contain similar active compounds as antimicrobials. Based on this, the purpose of this study was to determine the antibacterial activity of the combination of methanol extracts of C. alata and A. vera against S. aureus and P. aeruginosa bacteria. The antibacterial activity test was carried out using the Kirby-Bauer method. The single extract of C. alata and A. vera had the greatest antibacterial activity against S. aureus at a concentration of 10 mg/disk with zones of inhibition of 29.5±0.7 mm and 21.5±1.4 mm, respectively. The single antibacterial activity test of both extracts against P. aeruginosa bacteria showed no inhibition zone. The test results showed that the combination of 10 mg/disk C. alata methanol extract and 10 mg/disk Aloe vera could inhibit the growth of S. aureus with an average inhibition zone of 30.75±1.7 mm. However, the lower ratio of both extracts resulted in a likely antagonistic effect against S. aureus. Phytochemical screening tests showed that these two extracts had the same compounds, namely phenolics, flavonoids, anthraquinones. Contact bioautography showed that mainly phenolics, flavonoids, and anthraquinones were responsible in the antibacterial properties.

Keywords: *Cassia alata, Aloe vera, Staphylococcus aureus, Pseudomonas aeruginosa*

INTRODUCTION

Infection disease caused by microbial pathogens is one of the major health problems in the world, especially in tropical countries such as Indonesia (Tauran Id *et al.*, 2022). *Staphylococcus aureus* and *Pseudomonas aeruginosa*, are bacterial pathogens notoriously known as the cause of many infectious diseases that attack the skin, respiratory, and even the urinary tract (Tong *et al.*, 2015; CDC, 2019). Due to the high resistant nature of both *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Exner *et al.*, 2017; Gupta and Datta, 2019; Breijyeh *et al.*, 2020), many researchers are now gearing towards the exploration of natural resources trying to discover a new agent to combat antibiotic resistance. Considering the above, the vast majority of natural resources that play a significant empirical role in the community are yet to be explored scientifically and this has made this type of research popular (Sánchez *et al.*, 2020; Nurhayati *et al.*, 2019; Fatmawati *et al.*, 2020).

Ketepeng cina or *Cassia alata* (CA) leaves were empirically used as a therapeutic for skin ulcers, itchiness, and fungus infection. It was previously reported that the ethyl acetate extract of CA could inhibit the growth of *S. aureus* resulting in 14.7 mm zone of inhibition due to its abundant secondary metabolite content (Toh *et al.*, 2023). (Toh *et al.*, 2023). On the other hand, *Aloe vera* (AV) has a long history in many cultures as part of traditional medicine. AV is reported to have over 75 different compounds, and hence has diverse pharmacological actions including anti inflammation, antibacterial and antioxidant (Sánchez *et al.*, 2020). AV contained anthraquinones which was claimed to be responsible for its antibacterial property

against *S. aureus* with the largest zone of inhibition of 12.81 mm (Dewi and Marniza, 2019).

Nurhayati *et al*, (2019), formulated a gel made of a combination of CA and AV leaves which was intended to treat fungus infection by *Malassezia furfur* since it was previously reported to establish similar activity as 2% ketoconazole in the inhibition of *M. furfur* growth. Consequently, based on the approach described by Nurhayati *et al*., (2019) and also supported by preceded discoveries of both plants' pharmacological activities (Sánchez *et al.*, 2020; Nurhayati *et al.*, 2019; Fatmawati *et al.*, 2020), we aimed to further investigate CA and AV compatibility especially regarding their antibacterial properties by combining the methanol extract of both CA and AV plants in the antibacterial activity assay against Gram-positive and negative bacteria namely *S. aureus* and *P. aeruginosa* respectively.

RESEARCH METHODOLOGY

Materials

Cassia alata and *Aloe vera* leaves were obtained from Tawangmangu, Central Java, and subsequently followed with plant identification by Plants Systematic Morphology Team at Universitas Setia Budi, Surakarta (reference letter: 01-02/Det.Lab/V/2022). The leaves were then washed, dried, and powdered. Leaves extraction was performed by maceration method using 1:5 ratio of methanol as the solvent. *Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from Pharmaceutical Biology Laboratory of Universitas Muhammadiyah Surakarta. Bacterial cultures were then streaked on Mannitol Salt Agar (MSA) (Sigma) and Mac Conkey Agar (MCA) (Sigma) for *Staphylococcus aureus* and *Pseudomonas aeruginosa* respectively before being transferred into liquid Broth Heart Infusion (BHI) (Sigma) media, meanwhile Mueller-Hinton Agar (MHA) (Sigma) was used in the antibacterial susceptibility test against four antibiotic discs (Merck) of Gentamicin 16 µg, Ampicillin 10 µg, Cotrimoxazole, Tetracycline 30 µg, Chloramphenicol 30 µg, and Ciprofloxacin 5 µg. Furthermore, dimethylsulfoxide (DMSO) was used as a solvent to dilute the extracts. Dragendorff's reagent, ammonia vapor, $FeCl_3$, SbCl, KOH 10%, and Bouchardat reagent were incorporated in thin layer chromatography-direct bioautography technique to qualitatively identify the group of active chemical compounds contained in the extracts (Wagner and Bladt, 1996). All glassware were sterilized using dry heat method in the oven at 170^{o}C for two hours while the media and sterile liquids in autoclave at 121^{o}C for 20 minutes.

Extracts preparation

500 grams of the separated samples of CA and AV powdered leaves were macerated with 3.5 litres of methanol for 3 days. The concoctions were stirred to homogenize twice daily followed by filtration which leaves the solid materials to be re-macerated twice in each with 2.5 litres of the solvent. All the filtrates were then collected and subsequently concentrated in the rotary evaporator and water-bath at 60^{o}C (Sesa *et al.*, 2014). The working solutions were formulated by diluting the extracts in DMSO in the following concentrations, 200; 400; 600; 1000 mg/mL with the loading doses of 4; 6; 8; and 10 mg/disc. The gradual loading doses were determined based on trials and errors.

Antibiotic susceptibility test

Staphylococcus aureus and *Pseudomonas aeruginosa* were grown in BHI media for 24 hours. The following day, 200 µl of the BHI culture of both bacteria were each separately transferred to a sterile. Consequently, 0.9% of sterile NaCl was added until the turbidity was equal to that of 0.5 Mc Farland standard ($1{,}5x10^8$ CFU/mL). 200 µl of the 0.5 Mc Farland suspension was then inoculated onto MH against Gentamicin 16 µg, Ampicillin 10 µg, Cotrimoxazole, Tetracycline 30 µg, Chloramphenicol 30 µg, and Ciprofloxacin 5 µg antibiotics. DMSO was treated as a negative control, while each

Table 1. Single extract antibacterial activity of *Cassia alata* and *Aloe vera*

Extract	Zone of inhibition (mm)						
	Concentrations/Loading doses (mg/disc)					K+ Ciprofloxacin	K- DMSO
	2	4	6	8	10	5 µg/disc	10 µL/disc
Staphylococcus aureus							
Cassia alata	15±1,7	22,5±0,7	24,3±1,06	28±2,82	29,5±0,7	37,5 ± 2,1	6,0 ± 0,0
Aloe vera	12,3±1,4	13,7±1,8	15,6±1,5	18,5±0,7	21,5±1,4	37,5 ± 2,1	6,0 ± 0,0
Pseudomonas aeruginosa							
Cassia alata	6,0 ±0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0
Aloe vera	6,0 ±0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0	6,0 ± 0,0

*Disc diameter: 6 mm, ** Data presented by means ±SD, n=2

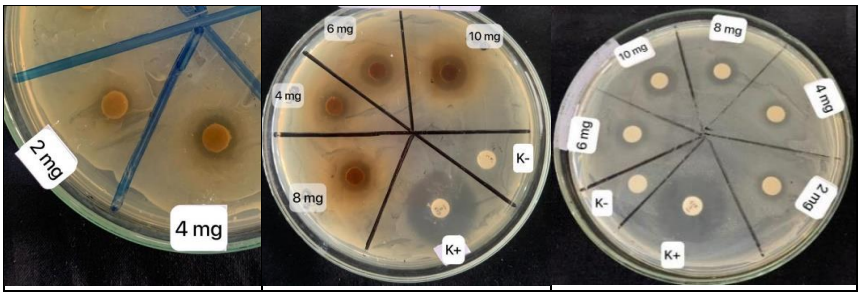
experiment was performed in two replicates (Hudzicki, 2012).

Antibacterial activity test

The antibacterial potency of the extracts was determined using disc diffusion method for both single extract and combination. 10 µl of each working solutions of the extracts were loaded into the blank discs with final concentration for single extract assay of 2, 4, 6, 8, 10 mg/disc for both extracts and left for approximately 30 minutes in an enclosed sterile space to dry out the solvent. Meanwhile, 200 µl of 0.5 Mc Farland standard bacterial suspension was spread onto MH media and was kept at room temperature for 10 minutes. Afterwards, the loaded discs were placed on the media which also included negative and positive control discs, and were incubated for 24 hours at 37ºC. Following 24 hours of incubation, the zone of inhibition for each disc was measured (Rahardjo *et al.*, 2017; Hudzicki, 2012). The antibacterial experiment for the combination of both extracts were performed similarly with the following loading doses respectively for *Cassia alata*+*Aloe vera*, 8+2; 6+4; 4+6; 2+8 mg/disc. Combination of the loading doses were determined based on trials and errors. Each experiment was done in two replicates.

Plants phytochemical screening

The phytochemical content of both extracts was investigated using thin layer chromatography (TLC) methods. Silica gel F254 as stationary phase and 8:2 ethyl actetate:n-hexane as a mobile phase was used in the TLC system. The development of the plate was done twice using similar system, prior to the development three samples (0.1%

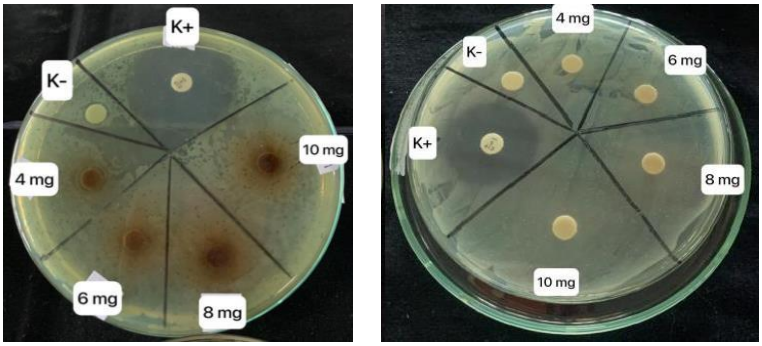


A **B**

Figure 1. Antibacterial activity assay of *Cassia alata* (A) and *Aloe vera* (B) against *Staphylococcus aureus*. K+ and K- represent positive control ciprofloxacin 5 µg/disc and negative control DMSO respectively. Zone of inhibition was observed in all concentrations of both extracts. Disc diameter: 6 mm.

Cassia alata extract, 5% *Aloe vera* extract, and the combined extract) were spotted at the lower line of the silica plate. Subsequently, the RFs of the spots were determined and visualized using ammonia vapor, $FeCl_3$, $SbCl_3$, KOH 10%, and Bouchardat reagents (Wagner and Bladt, 1996; Jangnga *et al.*, 2018; Muflihah *et al.*, 2020).

vera (AV) extracts. Antibiotic susceptibility test was performed to mainly determine positive control for the experiment as well as to evaluate the susceptibility of the bacteria used in the research. The less susceptible bacteria against antibiotics are the more unlikely the plant extracts would be active considering the unresponsive behaviour



A **B**

Figure 2. Antibacterial activity assay of *Cassia alata* (A) and *Aloe vera* (B) against *Pseudomonas aeruginosa*. K+ and K- represent positive control ciprofloxacin 5 µg/disc and negative control DMSO respectively. No inhibition zone was observed in the culture against both extracts. Disc diameter: 6 mm.

Contact bioautography

Contact bioautography was performed in order to identify the active compound groups responsible for the antibacterial activity by inhibiting bacterial growth. The developed silica plate was vapored out at room temperature to remove the residual mobile phase. The plate was then placed onto the 200 µl of 0.5 Mc Farland bacterial suspensions inoculated MH media and was left up to 30 minutes at room temperature to let the eluted phytochemicals diffuse into the agar. Thereafter, the plates were incubated for 24 hours at 37ºC. Following 24 hours of incubation, the zone of inhibition presence identical to the previously identified RFs of the spots was observed (Ramadhani *et al.*, 2017). Each experiment was done in two replicates.

RESULT AND DISCUSSION

The yield obtained from the extraction process was relatively low at 16% and 3% respectively for *Cassia alata* (CA) and *Aloe*

against standard antibiotics (Suganya *et al.*, 2022). The antibiotic susceptibility test on *S. aureus* and *P. aeruginosa* showed that the largest zone of inhibition was found to be against Ciprofloxacin 5 µg for both of the bacterial species at 37 and 36 mm respectively. Ciprofloxacin is a broad-spectrum and a bactericidal aminoglycosides antibiotic that targets the 30S ribosome subunit resulting in an inhibition of protein synthesis (Hodges, 2013).

Prior to the antibacterial assay of the combined two extracts, the potency of each single extract was evaluated against the two bacterial species**. Table** 1 provides the zone of inhibition data for single extract experiments. It is noteworthy that while both extracts possessed antibacterial activity against *S. aureus* (**Figure** 1), they did not inhibit the growth of *P. aeruginosa* showing selectivity against Gram positive bacteria. However, a significant increase in the diameter of the inhibition zone exponentially in correlation with the escalated dose of both

Table 2. Qualitative identification of compounds content by TLC in *Cassia alata* and *Aloe vera* extracts

Sample	Rf	Amonia vapor		$FeCl_3$	Bouchardat	Dragendorff	KOH 10%		$SbCl_3$	Compound content
		Vis	UV 366	Vis	UV 366	Vis	UV 366	UV 366+oven	UV 366	
Cassia alata	0	-	-	-	-	GY	-	-	-	Alkaloids
	0,76	-	-	-	-	-	BR	R	-	Anthraquinones
	0,9	Y	B	-	RO	-	-	-	-	Flavonoids, Alkaloids
	0,98	-	-	D	-	-	-	-	-	Phenolics
Aloe vera	0,7	-	-	-	-	-	-	R	-	Anthraquinones
	0,88	Y	B	-	-	-	-	-	-	Flavonoids
	0,9	-	-	D	-	-	-	-	-	Phenolics
Combination	0,75	-	-	-	-	-	-	R	-	Anthraquinones
	0,8	-	-	-	RO	-	-	-	-	Alkaloids
	0,82	Y	B	-	-	-	-	-	-	Flavonoids
	0,96	-	-	D	-	-	-	-	-	Phenolics

Vis= visible light
Y= yellow fluorescent
B = blue fluorescent
R = red
D = dark
RO= reddish orange
GY= greenish yellow
BR= brownish red

extracts (p=0.034 and p=0.02 for CA and AV respectively) was observed in antibacterial activity assay against *S. aureus* (**Figure** 2). This result supports previous reports which stated that the difference in cell wall components between Gram-positive and negative bacteria plays role in the survival of the bacterial cells (Exner *et al.*, 2017; Gupta and Datta, 2019; Miller, 2016). The outer layer of Gram-negative bacteria's cell is hydrophobic and therefore acts as an additional protection leading to a more resistant nature against foreign substances (Exner *et al.*, 2017; Gupta and Datta, 2019; Miller, 2016; Breijyeh *et al.*, 2020). The antibacterial activity of the combined extracts was performed to determine the correlation between two different extracts. It is expected

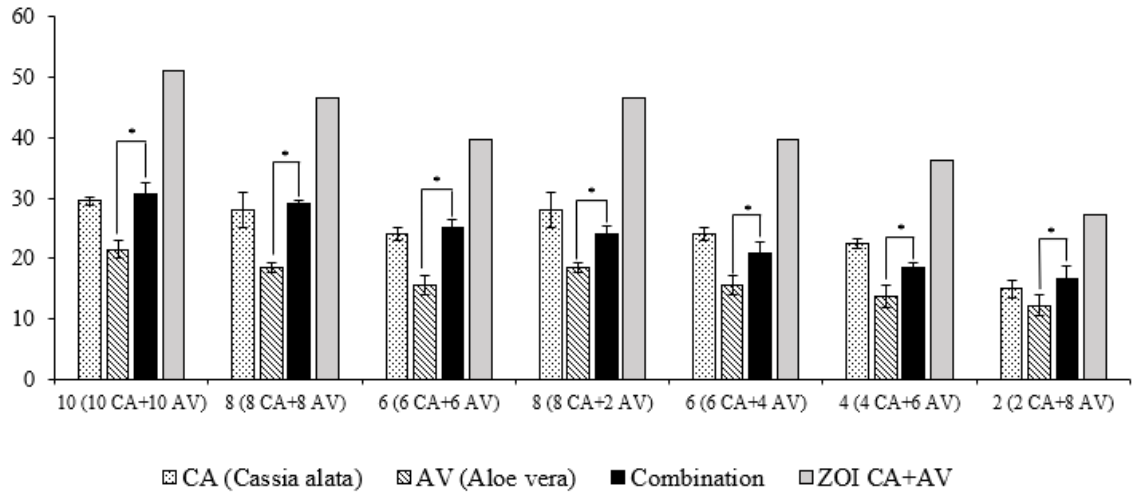


Figure 3. Antibacterial assay results from the combination of *Cassia alata* (CA) and *Aloe vera* (AV) extracts in various concentrations against *Staphylococcus aureus* (black bars). The concentrations of combined extracts are presented below X-axis inside the parentheses for CA and AV respectively in mg/disc. Solid grey bar denotes the total amount of zone of inhibition (ZOI) generated in a single treatment of CA and AV extracts. No significant differences between CA vs combined extracts whereas on the contrary for AV vs combined extracts ($p<0.05$).

that the combination between the two extracts was to be synergistic as both were previously reported as potent antibacterial agents against many bacterial species. Considering the abovementioned results, CA was claimed to be a potent antibacterial agent against various ranges of Gram-negative and positive bacteria, including *S. aureus* and *P. aeruginosa* (Khan *et al.*, 2001). Similarly, AV was also found to be active against oral pathogens such as *Actinobacillus actinomycetemcomitans Clostridium bacilli*, *Streptococcus mutans and Staphylococcus aureus* among other type of bacteria (Jain *et al.*, 2016; Sánchez *et al.*, 2020).

the combination assay, the total amount of ZOI between the single treatment of CA and AV extracts in all groups was a lot higher than the combined extracts (**Figure** 3). Therefore, it implies that rather than a synergistic correlation between methanol extracts of CA and AV combination (Bliss, 1939), they seem to be antagonistic when the ratio of both extracts is not equal

TLC chemical identification assay revealed positive results in various compound groups namely anthraquinones, flavonoids, and phenolics in both CA and AV, while the alkaloids group was only detected in CA extract (**Table** 2) (Wagner and Bladt, 1996;

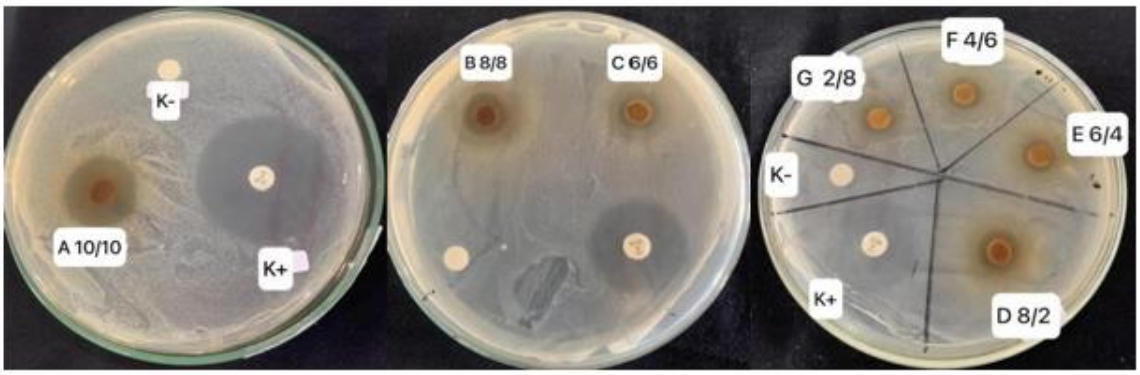


Figure 4. Results of antibacterial activity assay from combined extracts *Cassia alata* (CA) and *Aloe vera* (AV) against *Staphylococcus aureus*. Assay was performed in two replicates. Various concentrations are represented by following labels, A= 10 CA +10 AV; B= 8 CA+8 AV; C= 6 CA+6 AV; D= 8 CA+2 AV; E= 6 CA+4 AV; F= 4 CA+6 AV; G= 2 CA+8 AV; K+= Ciprofloxacin 5 µg; and K- = DMSO. All concentrations are in mg/disc.

However, based on our result, there is no significant difference between CA and combined extract even though the trend shows a slight increase in the zone of inhibition where similar concentrations of CA and AV were applied. On the other hand, the trend seems to disappear when CA and AV extracts were in different proportions (**Figure** 3 and 4). Furthermore, we observed a significant difference between AV and combined extracts, where the ZOI of combined extracts is higher for single AV extract treatments, in all the experimental groups. Therefore, our results indicate that CA acted as a stronger antibacterial agent than AV against *S. aureus.* Nevertheless, regarding

Muflihah *et al.*, 2020). However, it is noteworthy that the development system for the TLC method needs to be optimized since not all the extracts at the bottom were eluted, which likely caused many other compound groups left undetected. Subsequently, contact bioautography was performed on the extracts against *S. aureus.* **Figure** 5 shows that the clear inhibition zones were observed at Rf of 0.94; 0,81; and 0.72 which were previously identified as phenolics, flavonoids, and anthraquinones groups respectively. Besides, it was earlier reported that flavonoids act as antibacterial agents by inhibiting the synthesis of nucleic acids, distorting cell membrane's functions, and energy metabolism (Xie *et al*.,

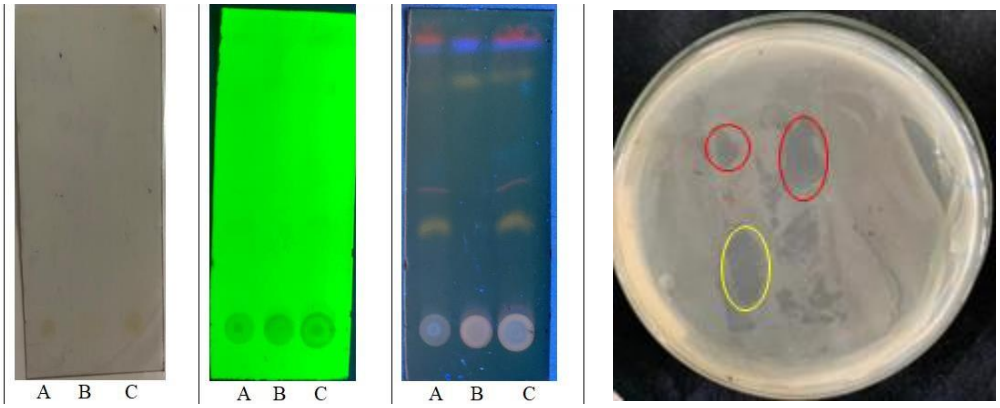


Figure 5. Results of contact bioautography method of (A) Cassia alata, (B) Aloe vera, and (C) combination of extracts (far right) against Staphylococcus aureus preceded by TLC identification on silica gel F254 and visualisation in visible light, UV 254, and UV 366 respectively (from far left). Zone of inhibition was observed at Rfs of 0.94; 0.81; and 0.72 (red circles). Assay was performed in two replicates. Yellow circle indicates irradical ZOI where bacterial growth is not completely inhibited.

2015). On the other hand, phenolic compounds were also claimed as a potent antibacterial agent that obstructs the new cell wall synthesis and disrupts the membrane cell resulting in cell death (Miller, 2016).

In addition, some of the phenolic compounds have lipophilic nature which would attach to the bacteria's cell wall causing damage (Bouarab-Chibane *et al*., 2019). Furthermore, anthraquinones were also reported to have an antibacterial property by binding to the DNA templates, thereby preventing DNA replication and protein synthesis, and consequently, inhibiting bacterial growth (A'lana *et al*., 2017). In addition, slight bacterial growth inhibition was observed at the lower Rf (yellow circle, **Figure** 5) which was also positive by KOH 10% reagent indicating for other anthraquinones group compounds (Marliana *et al*., 2005). However, based on our findings they were likely less active than the other spots observed at the higher spots.

CONCLUSIONS

In conclusion, the methanol extracts of CA and AV are potent antibacterial agents by themselves against *S. aureus* but not to *P. aeruginosa* due to their flavonoids, phenolics, and anthraquinones content, however the combination of both did not result into synergistic antibacterial effect. Further research is necessary to determine the metabolite profiles of the extracts as well as to evaluate the antibacterial activity against other microorganisms.

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