

# Potential Antagonistic Interactions of *Cassia alata* L. Leaf Extract and Commercial Antibiotics against *Staphylococcus aureus*: An In Vitro Evaluation

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**Abstract:** Several in vitro studies have demonstrated that the combination of plant extracts with antibiotics may reduce the minimum inhibitory concentration (MIC) of antibiotics against resistant microorganisms. Interactions between plant extracts and antibiotics can be synergistic, additive, or antagonistic. This study aimed to evaluate the potential interaction between *Cassia alata* leaves methanol extract and five selected conventional antibiotics in inhibiting the growth of *Staphylococcus aureus* ATCC 29213. Antibacterial activity was assessed using the well diffusion and paper strip methods. The methanol extract of *C. alata* leaves, when tested singly at a concentration of 100 mg/mL, produced an average inhibition zone of 11.74 mm. Four antibiotics, namely ciprofloxacin, clindamycin, chloramphenicol, and tetracycline, exhibited stronger antibacterial activity with inhibition zones ranging from 19.56–27.26 mm, whereas co-trimoxazole showed no inhibitory effect. The combination of *C. alata* leaves methanol extract with ciprofloxacin, clindamycin, chloramphenicol, tetracycline, and cotrimoxazole resulted in inhibition zones of  $22.26 \pm 0.703$  mm,  $22.54 \pm 1.222$  mm,  $17.91 \pm 1.094$  mm,  $17.57 \pm 0.652$  mm, and  $9.30 \pm 1.180$  mm, respectively. The combined treatments have potential antagonistic effects, as the inhibition zones were smaller than the sum of the individual activities of the extract and antibiotics. Therefore, the combination was considered less effective in suppressing growth of *S. aureus* ATCC 29213

**Keywords:** Antibiotics; *Cassia alata* L.; Combination effect; *Staphylococcus aureus*

## Introduction

Skin and soft tissue infections (SSTIs) are infections caused by bacteria, such as *Staphylococcus aureus*, that infect areas where the skin's protective barrier has been compromised, such as wounds or post-operative infections (Lacey *et al.*, 2016). The spread of *S. aureus* on the skin and soft tissues causes several diseases, such as bacteremia, endocarditis, skin infections, and pleuropulmonary infections (Gnanamani *et al.*, 2018). This makes *S. aureus* the most common cause of skin and soft tissue infection worldwide, with high infection rates in many

regions (Esposito *et al.*, 2017).

*S. aureus* bacterial infections are becoming an increasingly serious problem due to the emergence of conditions where some *S. aureus* become resistant to various antibiotics. This case emerged alongside the increasing use of antibiotics, which caused *S. aureus* to undergo various genetic variations and mutations in their genome (Alghamdi *et al.*, 2023). *S. aureus* resistance has worsened since it was discovered that *S. aureus* has become resistant to several antibiotics such as methicillin, penicillin, and other common antibiotics (Nandhini *et al.*, 2022). *S. aureus* resistance is a danger

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in the world of health because it causes therapy failure and increases the mortality rate by up to 64% compared to non-resistant bacterial infections (Yuliana *et al.*, 2025). A study also reported that of 567 patients with SSTI in three cities in Indonesia, 257 (45.3%) were infected with *S. aureus*, and 8 (3.1%) were infected with Methicillin-Resistant *S. aureus*. Due to the high level of danger and spread, it is necessary to implement effective alternative treatments to prevent *S. aureus* infections (Santosaningih *et al.*, 2018).

One of the plants commonly used as herbal medicine in Asia is *C. alata*, also known as ketepeng cina in Indonesia. *C. alata* is widely used as an alternative medicine because it has been proven have minimal side effects in clinical treatment and has various biological activities (Fatmawati *et al.*, 2020). Based on research, showed that ethanol extracts of *C. alata* leaves have antibacterial effects inhibiting the growth of several bacteria that cause skin infections, such as *S. aureus*, *S. epidermidis*, *Pseudomonas aeruginosa*, and *Propionibacterium acnes* with an inhibition zone diameter more than 15 mm (Fitriani *et al.*, 2023).

The primary goal of antibiotic-plants extract interaction research has been to identify and isolate putative resistance modulators from medicinal plants. However, it is necessary to clarify how plant extracts and antimicrobial medications can have both synergistic and antagonistic effects. Synergism experiments between terpenes and penicillin against MRSA and *Escherichia coli* revealed a synergistic impact due to the interaction between carvone and penicillin, as opposed to the antagonistic effect of thymol and penicillin against MRSA strains (Vaou *et al.*, 2021). Another study reported that a combination of *Daphne genkwa*, with oxacillin revealed synergism against MRSA (Kuok *et al.*, 2017).

To the best of our knowledge, research on the combination of *C. alata* leaves methanol extract specifically with commercial antibiotics ciprofloxacin, cotrimoxazole, chloramphenicol, tetracycline, and clindamycin against *S. aureus* ATCC 29213 is still limited. Therefore, this study aimed to evaluate the combined antibacterial effect of methanolic extract of *C. alata* leaves with five commercial antibiotics in treating *S. aureus* ATCC 29213. This is particularly relevant considering the increasing prevalence of antibiotic-resistant *S. aureus* infections, which pose a significant global public health burden. The finding of this research are anticipated to support local communities develop treatments using this plant and contribute to the development of studies on the management of an infection that attacks the skin and underlying soft tissue.

## Method

This study was conducted from July 2024 to

April 2025 at Bengkulu University. In this study, *C. alata* leaves were collected from the Bengkulu City area in Bengkulu Province, Indonesia. The antibiotic samples used were five types of commercial antibiotics: ciprofloxacin, cotrimoxazole, chloramphenicol, tetracycline, and clindamycin. The bacterial strain used was *Staphylococcus aureus* ATCC 29213.

### Sample Preparation and Extraction

Fresh *C. alata* leaves were chopped into small pieces after being cleared of twigs, broken leaves, and stems. The samples were then air-dried indoors for six days. The dried leaf samples were then weighed at 300 g and macerated with 3 L of methanol for 7 days. Following maceration, the resulting mixture was passed through filter paper. The solvent contained in the macerate was then removed using a rotary evaporator maintained at 45°C with low pressure to yield a concentrated extract of *C. alata* leaves. The re-maceration process continued until the maceration solvent was clear (Adfa *et al.*, 2021).

### Preparation of Media

In the present experiment, nutrient agar (NA) and nutrient broth (NB) were used as Media. Preparation of the NA medium involved dissolving 9.8 g of nutrient agar with 350 mL of sterile water in an Erlenmeyer flask. The medium was then heated using a hot plate and stirred using magnetic stirrer until completely dissolved. Following sterilization, approximately 15 mL of NA medium was dispensed into petri dishes and left to solidify. The prepared NA plates were subsequently incubated for 24 h in an incubator. For the preparation of NB medium, 0.4 g of nutrient broth was accurately weighed and transferred into an Erlenmeyer flask, after which 50 mL of sterile water was added. The medium was then heated using hot plate, stirred using a magnetic stirrer, and sterilized (Bahri *et al.*, 2023).

### Preparation of Bacteria Culture

The bacteria *Staphylococcus aureus* ATCC 29213 were revived by inoculating 1 loop of pure culture on nutrient agar medium. The inoculated medium was subsequently incubated at 37°C. *S. aureus* Colonies from the nutrient agar medium were suspended one loop in 15 mL of nutrient broth medium in an Erlenmeyer flask and shaken until homogeneous using a magnetic stirrer. The medium was then incubated for 24 h (Nurwahida *et al.*, 2025).

The optical density of the bacterial suspension was determined using a UV-Vis spectrophotometer at wavelength of 625 nm, with the absorbance value adjusted to be in the range of 0.08–0.10. If the measured absorbance value exceeded or fell below the set range,

the suspension was diluted using a sterile nutrient broth solution until the correct value was obtained (Shari *et al.*, 2025).

#### *Antibacterial Activity Combination Test Using Well Diffusion Method*

The prepared NA medium was then inoculated with a standard suspension of *S. aureus* ( $1.5 \times 10^8$  CFU/mL). The NA medium inoculated with the bacteria suspension was then poured into sterile petri dishes, 15 mL each, using a syringe and left to solidify. Each solidified medium was then made into 4 holes with a diameter of 6 mm, which were then given antibiotic solution (0.25 mg/mL), extract solution (100 mg/mL), negative control (DMSO 100%), and combination solution using a micropipette, each amounting to 20  $\mu$ L. After that, the medium was incubated at 37°C for 24 h, after which the diameter of the inhibition zone was measured using a caliper in four replicates (Nurhasana *et al.*, 2023).

#### *Combination Effect Test Using the Paper Strip Method*

Methanol extracts of *C. alata* and five selected antibiotics were each dropped 30  $\mu$ L onto different Whatman (No.3) paper strips measuring 3 x 0.5 cm. The paper strips were then placed on NA agar media that had been previously inoculated with the test bacteria. The paper strips each containing the extract and the tested antibiotics were positioned to intersect at a right angle (90°) on a Petri plate. The petri dish was subsequently incubated at 37°C for 24 h. Measurement of the inhibition zone was performed using a caliper, after which the diameter of the clear zone was deducted with the paper strip diameter. The test was conducted in duplicate (Hamni *et al.*, 2022).

#### *Data Analysis*

Data analysis of the antibacterial test using well diffusion and paper strip was performed by determining the mean inhibition zone diameter of *S. aureus* growth and standard deviation using Microsoft Excel (Arisurya *et al.*, 2026). The inhibition effect of extracts and antibiotics was classified into four type's according to the diameter of the inhibition zone formed, namely: > 20 mm (highly sensitive), 15-19 mm (very sensitive), 9-14 mm (sensitive), and < 8 mm (not sensitive) (Ponce *et al.*, 2003).

The combination effect was determined by observing the pattern of the clear zone and was described as synergistic, additive/indifferent, or

antagonistic (Hanifa *et al.*, 2022). Referring to Berenbaum (1977), the combination is categorized as synergistic if the combination result exceeds the total sum of the individual inhibition zones, antagonistic if the combination result is below the total sum of the individual inhibition zones, and additive if the combination result is equal to the total sum of the individual inhibition zones.

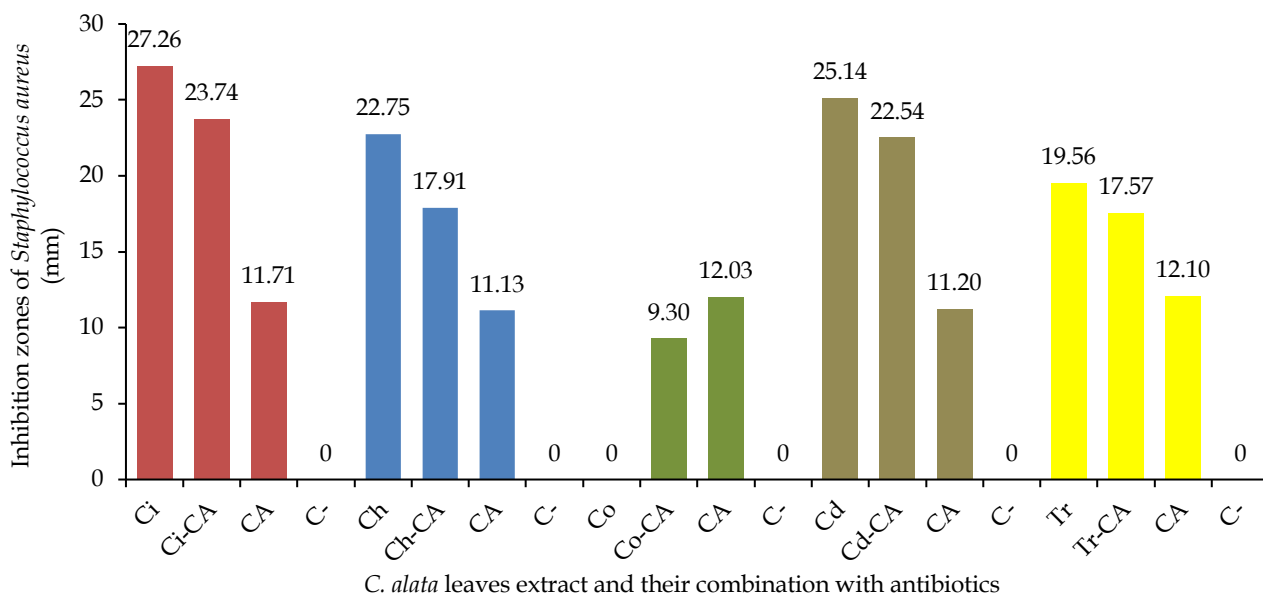
## **Result and Discussion**

#### *Extraction*

The extract was prepared by macerating pieces of *C. alata* leaves with methanol. The use of methanol in the maceration process aims to dissolve polar and nonpolar compounds, making it suitable for extracting secondary metabolite content in plants (Saputra *et al.*, 2018). The polar solvent, making it dominant in extracting compounds such as phenolic compounds, flavonoids, saponins, and alkaloids, which can act as antibacterial agents (Setiawan *et al.*, 2025). The maceration process was also chosen because, without heating, the bioactive compounds in the extract would not be damaged, allowing more secondary metabolites to be extracted (Handarni *et al.*, 2020). Through a 7-day maceration process with re-maceration, a concentrated extract of 98.67 g was produced with a yield of 32.89%.

#### *Antibacterial Activity of Cassia alata Leaves Methanol Extract with Selected Antibiotics Using the Well Diffusion Method*

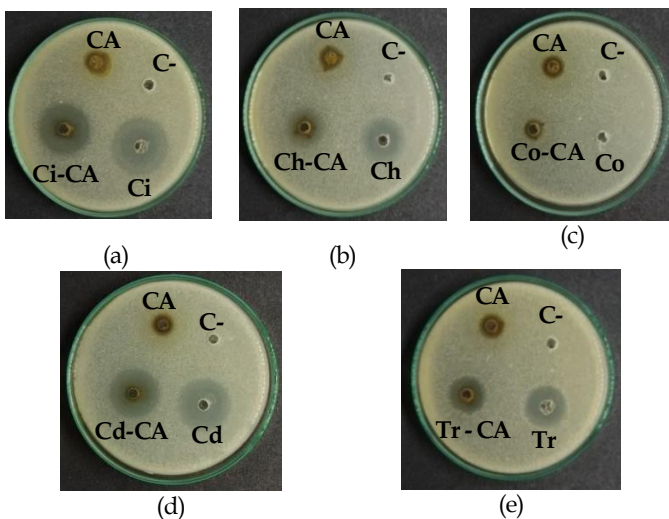
The antibacterial activity test of methanol extract of *C. alata* leaves, five selected antibiotics, and a combination of both was tested using the well diffusion method with four replications. Based on the visualization of the clear zone from the well diffusion test, it was found that the combination of 10% methanol extract of *C. alata* leaves with the antibiotics tetracycline, ciprofloxacin, clindamycin, chloramphenicol, and cotrimoxazole, each at 250 ppm, produced the same pattern, where the inhibition zone of the combined *C. alata* leaves extract and the test antibiotics was smaller than the sum their individual action of antibiotics and *C. alata* leaves extract. The visualization in Figure 2 shows that the methanol extract of *C. alata* leaves has the potential to have an antagonistic or attenuating effect when combined with the five selected antibiotics (Burt, 2004).



**Figure 1.** Inhibition zone diagram of *S. aureus* growth after treatment with *C. alata* leaves methanol extract, five selected antibiotics, and their combination

Note: CA = *C. alata* leaves extract, Ci = Ciprofloxacin, Ch = Chloramphenicol, Co = Cotrimoxazole, Cd = Clindamycin, Tr = Tetracycline, and C- = 100% DMSO

Figure 1 shows the inhibition zone of *S. aureus* against *C. alata* leaves extract (CA) were 11.71, 11.13, 12.03, 11.20, and 12.10 mm. The overall average was 11.74 mm. This data is almost similar to that of a study, found that 10% *C. alata* extract had an inhibition zone of  $11.63 \pm 0.405$  mm (Fitriani *et al.*, 2023). Since the inhibition zone was less than 15 mm, the *C. alata* extract was considered sensitive to *S. aureus* (Ponce *et al.*, 2003).



**Figure 2.** The inhibition zone of *S. aureus* growth after treatment with *C. alata* leaves methanol extract, five selected antibiotics, and their combination, (a) Ciprofloxacin; (b) Chloramphenicol; (c) Cotrimoxazole; (d) Clindamycin; (e) Tetracycline

Note: CA = *C. alata* leaves methanol extract, Ci = Ciprofloxacin, Ch = Chloramphenicol, Co = Cotrimoxazole, Cd = Clindamycin, Tr = Tetracycline, and C- = 100% DMSO

Among the five individual antibiotics tested, four exhibited a greater inhibition zone diameter of *S. aureus* than the combination of the methanol extract from *C. alata* leaves with these antibiotics. The inhibition zone diameters indicated that ciprofloxacin (27.26 mm), clindamycin (25.14 mm), and chloramphenicol (22.75 mm) exhibited highly sensitive antibacterial activity. Tetracycline (19.56 mm) showed a sensitive inhibition zone diameter, whereas cotrimoxazole displayed no antibacterial effect. This finding aligns with a research who noted that *S. aureus* is resistant to cotrimoxazole (Taura *et al.*, 2013). As of 2012, the resistance level of Gram-negative bacterial to cotrimoxazole reached 79.8% (Batra *et al.*, 2017).

Plants of the *Cassia* genus, such as *Cassia alata*, have been identified as containing several groups of secondary metabolites, including alkaloids, anthraquinones, flavonoids, triterpenoids, sterols, phenylpropanoids, and  $\gamma$ -naphthopyronones. However, anthraquinones and flavonoids are the main groups that are quite abundant in plants of the *Cassia* genus (Khurm *et al.*, 2021). Based on previous studies, compounds such as flavonoids and anthraquinones are natural antibacterial sources found in *C. alata* leaves (Lathifah *et al.*, 2021). Compounds such as aloe emodin, emodin and kaempferol exhibit excellent antimicrobial activity against *Staphylococcus aureus*, including MRSA (Yon *et al.*, 2023).

Phenolic compounds, such as kaempferol and its derivatives, in *C. alata* leaves exhibit antibacterial activity against pathogenic bacteria. These phenolic

compounds, produced by the plant's secondary metabolism, can directly destroy cell membrane, resulting in bacterial cell death. In addition to cell membrane disruption, kaempferol and its derivatives can induce apoptosis, DNA fragmentation, DNA gyrase inhibition, and DNA helix inhibition in bacteria (Lin *et al.*, 2020; Periferakis *et al.*, 2022). Several anthraquinone derivatives such as rhein, aloe-emodin, and aloe-emodin-8-O- $\beta$ -D glucoside in *C. alata* also active against Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Helicobacter pylori*, *Neisseria gonorrhoeae*, as well as Gram-positive bacteria such as *Staphylococcus* species. The antibacterial mechanisms of anthraquinone are diverse and include weakening of cell walls, changes in metabolic pathways, and DNA inclusion (Malmir *et al.*, 2017). Research also reported that emodin is a strong antibacterial compound against MRSA with MIC 4  $\mu$ g/mL (Li *et al.*, 2021).

The combination of *C. alata* leaves methanol extract with five selected antibiotics resulted in an inhibition zone diameter that was less than four antibiotics singly (ciprofloxacin, clindamycin, chloramphenicol, tetracycline), and the sum their individual action of antibiotics and *C. alata* leaves extract. The combined treatment of *C. alata*-ciprofloxacin (CA-Ci), *C. alata*-clindamycin (CA-Cd), *C. alata*-chloramphenicol (CA-Ch), *C. alata*-tetracycline (CA-Tr), *C. alata*-cotrimoxazole (CA-Co) exhibited average inhibition zone diameters of 22.26 $\pm$ 0.703 mm, 22.54  $\pm$  1.222 mm, 17.91  $\pm$  1.094 mm, 17.57  $\pm$  0.652 mm, and 9.30  $\pm$  1.180 mm, respectively. An overall decline in inhibition zone diameter was observed when *C. alata* extract was applied in combination with five antibiotics, indicating an antagonistic or weakening effect. These findings suggest that the combination effect not caused by single compound alone. but rather each identified constituent plays a role in contributing to the effect to varying degrees (Sanhueza *et al.*, 2017). The antagonistic effect of combined extracts and antibiotics also involves complex interactions between the agents. One of the key mechanisms involves modulation of gene expression related to antibiotic resistance, biofilm formation, and virulence factors. For example, cinnamaldehyde acts synergistically with  $\beta$ -lactam antibiotics by targeting the staphylococcal accessory regulator SarA, which in turn decreases the expression of the *mecA* gene (Li *et al.*, 2024).

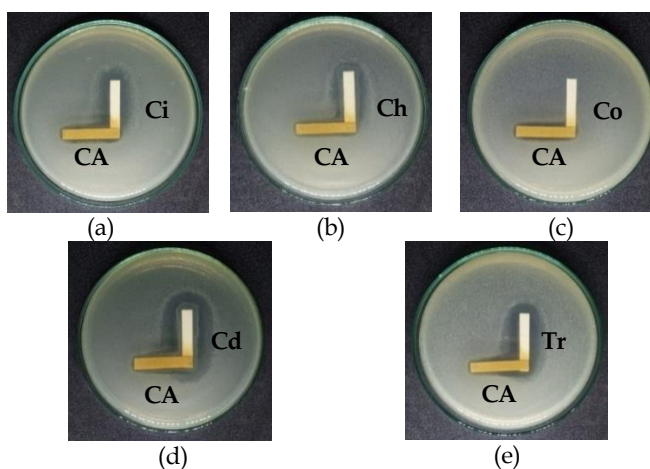
The indication of antagonistic effect of the combinations also occurred because of the bacteriostatic and bactericidal properties of the bioactive compounds in the *C. alata* methanol extract and the antibiotics, which weakened each other (Ishak

*et al.*, 2025). Secondary metabolites from *C. alata* leaves may inhibit the binding of antibiotic compounds to receptors by competing at the active site of bacteria and altering receptor function, thereby reducing the effectiveness of antibiotics in inhibiting the growth of *S. aureus* (Susanti & Asri, 2024). This is because, antagonistic behavior is believed to happen in three ways: first, when there is a mix of bacteriostatic and bactericidal antimicrobials; second, when the antimicrobials work at the same site or use the same mechanism of action; and third, when the antimicrobials affect each other (Aelenei *et al.*, 2016; El-Sakhawy, 2023).

The possibility of incompatibility between the antibacterial effects of compounds in the extract and antibiotics is one of the causes of the potential antagonistic effects. This effect means that the methanol extract of *Cassia alata* L. leaves is not recommended for combination with the five commercial antibiotics chloramphenicol, ciprofloxacin, tetracycline, clindamycin, and cotrimoxazole, as it reduce the effectiveness of these antibiotics in controlling the growth of *S. aureus*.

#### *The Combined Effect of Cassia alata Leaves Methanol Extract with Selected Antibiotics Using Paper Strip Method*

As shown in Figure 3, the antibiotics combination with *C. alata* leaves methanol extract show smaller inhibition zone compared to the combined inhibition zone of single antibiotics and single extracts. A similar pattern was observed for the five combinations of extracts with antibiotics tested.



**Figure 3.** The combined effect of methanol extract of *C. alata* leaves 10% with selected antibiotic 250 ppm using the paper strip method on *S. aureus* bacteria, (a) Ciprofloxacin; (b) Chloramphenicol; (c) Co-trimoxazole; (d) Clindamycin; (e) Tetracycline with CA= *C. alata* leaves methanol extract

Note: Ci = Ciprofloxacin, Ch = Chloramphenicol, Co = Cotrimoxazole, Cd = Clindamycin, Tr = Tetracycline and C- = 100% DMSO

During testing, the diameter of the inhibition zone at a 90° angle from the paper strip changed to become smaller, which indicates that the combination of *C. alata* leaf methanol extract with the five antibiotics had a potential antagonistic effect. The similarity of the potential antagonistic combination effect produced between the well method and the paper strip method proves that the methanol extract of *C. alata* leaves is not suitable when combined with several types of commercial antibiotics such as chloramphenicol, ciprofloxacin, tetracycline, clindamycin, and cotrimoxazole in the treatment of *S. aureus* bacterial infections.

### Conclusion

The antibacterial activity of the combination of *C. alata* leaves methanol extract with the five selected antibiotics showed a decrease in the diameter of the inhibition zone compared to the extract and antibiotics alone. The single methanol extract of *C. alata* showed antibacterial activity that was classified as sensitive with an average inhibition zone diameter of 11.74 mm. Meanwhile, four single antibiotics (except cotrimoxazole) showed a higher inhibition zone diameter, and the antibacterial activity was classified as very sensitive to highly sensitive. The combination effect of the methanol extract of *C. alata* leaves with chloramphenicol, ciprofloxacin, tetracycline, clindamycin, and cotrimoxazole showed a potential antagonistic effect, characterized by a decrease in the inhibition zone. Based on the result, the combination of methanol extract of *C. alata* leaves with 5 commercial antibiotics was considered less effective in suppressing the growth of *S. aureus* ATCC 29213. In the future, further calculations are needed to determine the quantitative effects of the combination of *C. alata* leaves extract and antibiotics by calculating the FIC and FBC indices.

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### Author Contributions

Conceptualization, project administration, M.A.; resources, M.A., A.A., and K.W.; methodology, O.R.M., P.R., S.B.T.S., and W.S.; data collection and processing, O.R.M., W.S., A.A., and K.W.; analysis and interpretation, O.R.M., A.A., K.W., and M.A.; writing—original draft preparation, O.R.M.; writing—review and editing, supervision, M.A., and S.Y.S.; final approval, O.R.M., P.R., S.B.T.S., W.S., A.A., K.W., S.Y.S., and M.A.

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### Conflicts of Interest

The authors declare no conflict of interest.

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