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SYNERGISTIC ANTIBACTERIAL AND ANTIOXIDANT EFFECTS OF *ALTERNANTHERA SISSOO* AND *COSMOS CAUDATUS* METHANOL EXTRACTS

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Abstract

Antibacterial and antioxidant properties are important in medical applications, nutrition and food preservation. A combination of these properties plays a significant role in promoting health, preventing disease, and maintaining overall well-being. As current drugs have become less effective and have come with bad side effects, this has increased the need for safer alternatives like plant-based remedies that are more effective. Thus, this study aims to evaluate the antibacterial and antioxidant activities of a crude methanolic extract from a mixture of *Alternanthera sissoo* (Brazilian spinach) and *Cosmos caudatus* (Ulam raja). The concentrations of methanolic crude extract used were 500 mg/mL, 250 mg/mL, 125 mg/mL and 62.5 mg/mL. Antibacterial activity was first tested using the disk diffusion method. However, minimal and no visible inhibition zones were observed at most of the evaluated concentrations used. Therefore, a Minimum Inhibitory Concentration assay using a 96-well plate was applied. The results showed that wells with concentrations between 500 mg/mL down to 7.81 mg/mL were clear, indicating complete inhibition of bacterial growth. Slight turbidity was observed at 3.91 mg/mL and 1.95 mg/mL, particularly in wells with *Bacillus cereus*, suggesting low effectiveness at lower concentrations. For antioxidant evaluation, the DPPH radical scavenging assay was conducted. The results demonstrated that antioxidant activity increased with higher extract concentrations. The highest concentration (500 mg/mL) recorded the lowest absorbance value at 517 nm (0.125), corresponding to the highest scavenging activity of 78.55%. As the concentration decreased, a gradual decline in antioxidant activity was observed, with the lowest still maintaining a strong activity at 68.81%. In conclusion, the methanolic extract of *Alternanthera sissoo* and *Cosmos caudatus* mixture has been proven to possess both antibacterial and antioxidant properties, highlighting its potential for future development.

Keywords: *Alternanthera sissoo*, *Cosmos caudatus*, Methanolic extract, Antibacterial activity, Antioxidant activity

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Introduction

Antibacterial and antioxidant properties are essential for creating natural health products as they help prevent infections, combating oxidative stress and improve overall health. While antibacterial agents have reduced mortality from bacterial infections, Antimicrobial Resistance (AMR) makes treatments less effective. According to Di Martino (2022), humans are exposed to free radicals and oxidants through internal processes such as normal cellular metabolism, and external sources like pollution, cigarette smoke, radiation, and certain medications. When the body is unable to effectively neutralize an excess of free radicals, this will lead to a condition known as oxidative stress. Consequently, this results in progression of chronic disease such as cancer, cataracts, autoimmune disorders and neurodegenerative conditions. Since free radicals are toxic to our body, the body has developed and antioxidant defense system involving free radical scavenging, metal ion binding, and enzymatic

mechanisms that neutralize reactive species as soon as they are formed (Chaudhary et al., 2023).

This study evaluated the antibacterial and antioxidant activities of crude extract mixture of *Alternanthera sissoo* (Brazilian spinach) and *Cosmos Caudatus* (Ulam raja). Since *Alternanthera sissoo* (*A. sissoo*) is packed with phytochemicals such as flavonoids, phenolic compounds and vitamins, it has shown that this plant has high antioxidant and antibacterial capacity which help to eliminate free radicals in the human body and reduce oxidative stress (Ikram et al., 2022). Similarly, *Cosmos caudatus* (*C. caudatus*) is also known for its high anti-oxidant capacity and other medicinal benefits such as anti-diabetic and anti -hypertensive, anti-inflammatory effects and antimicrobial activity. This plant is rich in ascorbic acid, quercetin and chlorogenic acid which are responsible for medicine properties of the plant (Chaudhary et al., 2023).

Therefore, it is important to analyze the antibacterial and antioxidant properties of the mixture between these two plants due to their potential to provide health benefits when used together. As people become interested in traditional medicinal plants and herbs, a thorough research of these plants need to be done since it could lead to the invention of new products related to infections, oxidative stress and chronic diseases. To investigate the antibacterial and antioxidant activity, the methanol extract of *A. sissoo* and *C. caudatus* will be tested against bacteria strains to determine the efficacy of plant mixture against bacteria while also measuring their antioxidant activity.

Methods

Chemicals

Methanol 80%, Mueller-Hinton Agar (MHA), Nutrient broth, Ampicillin powder, Ampicillin disc, Ascorbic acid, DPPH solution, Gram-positive bacteria, *Bacillus cereus* (*B. cereus*) and *Staphylococcus aureus* (*S. aureus*), and Gram-negative bacteria, *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*)

Apparatus

Mortar and pestle, Analytical balance, Whatman No. 1 filter paper, Rotary evaporator, Universal bottles, Petri dishes, Blank discs, Sterile cotton swab, Sterile forceps, Incubator, Spectrophotometer, Beakers, Flasks, Sterile gloves, Statistical software (SPSS)

Preparation of Bacteria

Bacteria cultures (*B. cereus*, *S. aureus*, *E. coli* and *K. pneumoniae*) were obtained from the Unit Bank Kultur, School of Biology, UiTM Negeri Sembilan Branch. Each bacterial strain was grown in Mueller Hinton broth and was then incubated at 37°C for 24 hours (Astutiningrum & Feroniasanti, 2017).

Preparation of Extracts

The dried leaves were grounded into a fine powder using mortar and pestle. Methanol was used as the solvent to extract bioactive compounds. 20g of each plant sample were weighed and soaked in 250 mL of methanol for 48 hours. The mixture was filtered using Whatman No. 1 filter paper. The filtered extract was concentrated using a rotary evaporator, whose water boiling point was set at 40°C and vacuum at 337 mbar, leaving behind a crude extract. The crude extract produced after being processed in a rotary evaporator was 2.84g. The crude extract then was transferred to a universal bottle and stored at 4°C until further analysis (Sia et al., 2020).

Antibacterial activity by Disk Diffusion Assay

Bacterial cultures (*B. cereus, S. aureus*, *E. coli*, and *K. pneumoniae*) were spreads onto MHA agar plates using sterile cotton swabs on four separate petri dishes. 1000 mg/mL stock solutions of the leaf mixture extract were diluted to 500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL concentrations using serial dilution method. Sterile blank discs were soaked in each dilution and were placed on the inoculated agar surface using sterile forceps with around 30 mm gap between each disk. Ampicillin and sterile blank disc soaked with methanol were also placed on agar surface as positive control and negative control. The sterile blank discs were being lightly pressed down to ensure the contact with agar. This

process was repeated with other plates 3 times. All plates then were incubated at 37°C for 24 hours, and the inhibition zone (mm) was measured and recorded (Astutiningrum & Feroniasanti, 2017).

Antibacterial activity by Minimum Inhibitory Concentration

The antibacterial activity was further tested using Minimum Inhibitory Concentration method in a 96-well microplate. A stock solution was prepared by preparing the highest concentration of 500 mg/mL. In the first well, 100µL of stock solution was added. Then, 50µL of Nutrient broth was added from well 2 to well 9. A two-fold serial dilution was carried out by transferring 50µL from well 1 to well 2, mixing thoroughly and repeating the process sequentially up to well 9. After dilution, 50µL of bacterial suspension was added to wells 1 to 9, also to well 11 and 12. Well 11 served as positive control, containing 50µL of ampicillin and 50µL of bacteria suspension, while well 12 served as negative control with 50µL of Mueller Hinton broth and 50µL of bacteria suspension. Each bacteria was assigned to a specific row on the microplate, with Row A for *B. cereus,* Row B for *S. aureus*, Row C for *E. coli*, and Row D for *K. pneumoniae*.

Antioxidant Activity by DPPH Radical Scavenging Assay

About 4 ml mixture of ascorbic acid and methanol was used as control, while 4 ml methanol was used as blank. The DPPH solution was prepared by adding 2.5 mg of DPPH with 100 mL methanol. Then, 10µL of each different concentration (500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL) were mixed with 4 mL of DPPH solution. The mixture was gently homogenized then stored in a dark environment for 30 minutes (Ikram et al., 2022).

The absorbance of the samples at 517 nm were measured using spectrophotometer and the DPPH scavenging activity were calculated by determining the percentage of DPPH scavenged using formula:

$$\% \text{ Scavenging activity} = \frac{(AC - AS)}{AC} \times 100$$

Where AC is Absorbance of the control while AS is Absorbance of sample.
This process was repeated 3 times and graph calibration of the curve was constructed.

Data analysis

The data for antibacterial and antioxidant activity were repeated three times for each experiment. Statistical analysis was carried out using SPSS software version 20.0, with results presented as mean ± standard deviation. Analysis of Variance (ANOVA) was used to compare the mean differences in concentrations of the plant mixture. Statistical significance was determined at $p < 0.05$, showing the differences between groups if the p-value was less than 0.05.

Result and Discussion

Antibacterial activity by Disk Diffusion Assay

The methanolic extract of *A. sissoo and C. caudatus* mixture was evaluated for its antibacterial activity against all selected bacteria using disk diffusion assay. The extract was tested at four concentrations: 500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL with ampicillin served as positive control and methanol served as negative control. The result is presented in Figure 1.

The result showed an unexpected outcome where little to no visible zones were observed for most of the tested concentrations. Typically, an increase in concentration would correspond to a larger inhibition zone but the result showed otherwise. Only the positive control, ampicillin produced a clear inhibition zone which validated the reliability of the experiment procedure and susceptibility of bacteria. The negative control, methanol, shows no antibacterial activity as expected.

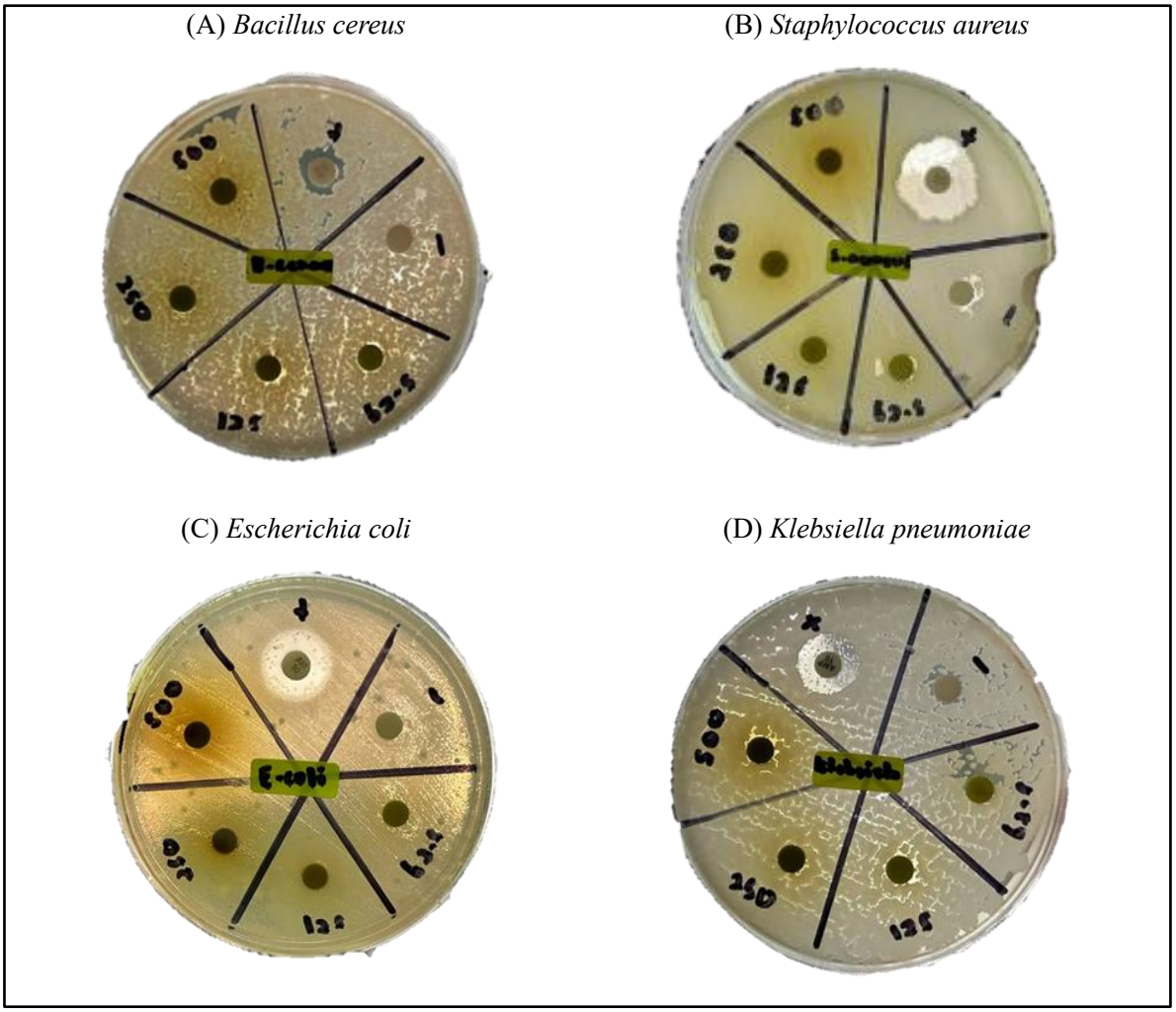

Figure 1. Inhibition zones of *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumonia* after soaked with different concentrations of the extract (500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL), positive control and negative control.

The absence of antibacterial activity especially at higher concentration may be attributed to several possible factors. One possible explanation is the combination of these two plant species might have interacted negatively, thereby reducing their individual antibacterial effectiveness. This phenomenon is called antagonistic effect which occurs when the antimicrobial activity of two compounds in combined plants is less than the sum of their individual effects (Vaou et al., 2022). However, this explanation may not be applicable in this case, as there is still a slight presence of inhibition zone, which suggests that the antagonistic interactions are not likely to be the main factor.

Slight inhibition zone was observed at lower concentrations, while higher concentration showed no inhibition. This can be observed at *B. cereus* plate, where concentrations of 62.5 mg/mL and 125 mg/mL produced the largest inhibition zones, measuring 8 mm and 7 mm respectively, while the 500 mg/mL concentration showed no inhibition. This result could be attributed due to the extract may have been too viscous or may have precipitated, preventing the active compounds from diffusing effectively. Some bioactive compounds might degrade or interact negatively with other compounds when too concentrated (Bhadange et al., 2024).

Antibacterial activity by Minimum Inhibitory Concentration Test

Since the disk diffusion assay did not show clear positive results, a Minimum Inhibitory Concentration (MIC) test was conducted to further investigate the antibacterial potential. The results, as presented in

Figure 2 show positive results of antibacterial activity against selected bacteria. Both positive and negative control confirms the validity of this experiment as the positive control in well 11 appeared clear, indicating inhibition of bacterial growth. In contrast, negative control in well 12 appeared cloudy, confirming bacterial growth.

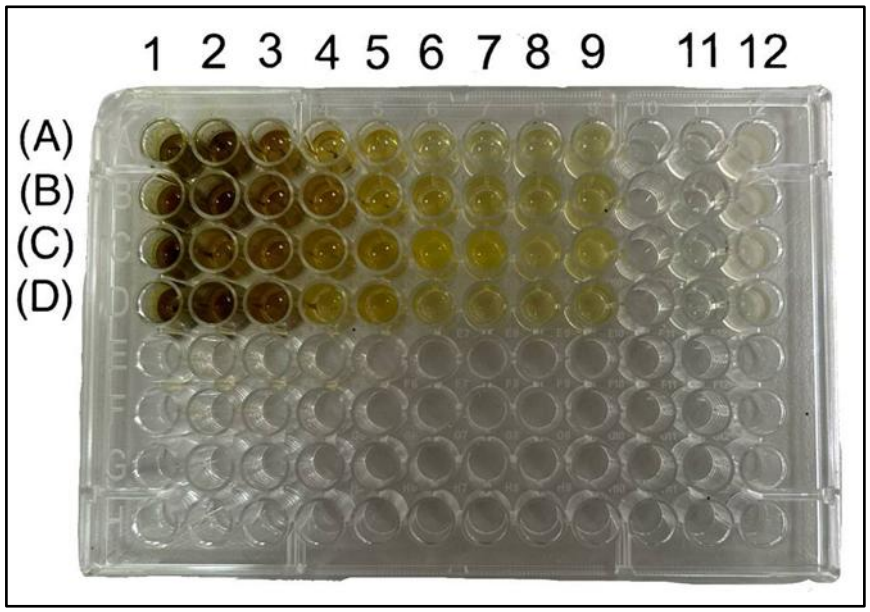

Figure 2. The results of Minimum Inhibitory Concentration (MIC) of *Alternanthera sissoo* and *Cosmos caudatus* mixture against; (A) *Bacillus cereus*, (B) *Staphylococcus aureus*, (C) *Escherichia coli* and (D) *Klebsiella pneumonia* with positive and negative control.

Wells 1 to 7, which represent concentrations ranging from 500 mg/mL to 7.81 mg/mL remained clear for all tested bacteria. This indicates that the extract was able to effectively inhibit bacterial growth at these concentrations, supporting the presence of bioactive compounds such as flavonoids, tannins, saponins and other phytochemicals known for their antimicrobial properties. Saponin, for example, can disrupt bacterial membranes which allow easier penetration of antibacterial agents to kill bacteria (Cankaya & Somuncuonglu, 2021). However, wells 8 (3.91 mg/mL) and 9 (1.95 mg/mL) showed slight cloudiness for *B. cereus,* indicating that lower concentrations may not be sufficient to completely inhibit bacterial growth. The successful inhibition in MIC test compared to previous disk diffusion assay is probably due to higher sensitivity of the broth dilution method. In the MIC test, the extract is fully dissolved, allowing better interaction with bacteria and enabling even low concentrations to show antibacterial effects. In contrast, agar diffusion is less sensitive, meaning that weak antibacterial effects might not show up and a larger amount of test substance may be required to detect antibacterial activity (Hossain, 2024). This limitation may cause false negatives, where antibacterial activity is present but not visually detected. Thus, the MIC method effectively validates the antibacterial properties of the extract despite the lack of inhibition in the disk diffusion assay.

Antioxidant activity by DPPH Radical Scavenging Assay

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay method is a widely used method to evaluate the antioxidant activity of leaf extracts in methanol. In this assay, DPPH interacts with antioxidant compounds by donating hydrogen atom or electron to DPPH radical, reducing it to the non-radical form, 1,1-diphenyl-2-picrylhydrazine. This reduction results in a color change from deep purple to a pale yellow, which reflects the scavenging ability of the extract (Hussen & Endalew, 2023). The antioxidant activity was measured at a wavelength of 517 nm, with the mixture of extracts tested at concentrations of 500 mg/mL, 250 mg/mL, 125 mg/mL, and 62.5 mg/mL, as presented in Table 1. The superscript letters a, b, c and d assigned to each absorbance value indicate the differences in antioxidant activity among different concentrations are statistically significant ($p < 0.05$).

Table 1. Antioxidant activity of methanol extract of *Alternanthera sissoo* and *Cosmos caudatus mixture*

Concentration (mg/ml)	Absorbance at 517 nm	% Of Antioxidant Activity
500	0.118 ± 0.0075^{a}	78.12
250	0.134 ± 0.0141^{b}	74.62
125	0.136 ± 0.1363^{c}	74.53
62.5	0.171 ± 0.1710^{d}	68.18
Control	0.539	0

Note: Superscript letters (a, b, c, d) differ to show significant differences

Based on Table 1, the extracts displayed significant antioxidant activity, with the percentage of antioxidant activity increasing as the concentration increases. At 517 nm, the highest concentration, 500 mg/ml produced the lowest absorbance mean of 0.118, giving the highest percentage at 78.12%. As concentration decreases, a gradual decline of antioxidant percentages can be seen with the lowest value being at 68.81%, which still gives strong antioxidant activity. These findings support the study by Gulcin and Alwasel (2023), who reported that DPPH activity is directly proportional to increasing extract concentrations due to higher presence of antioxidant compounds at greater concentrations.

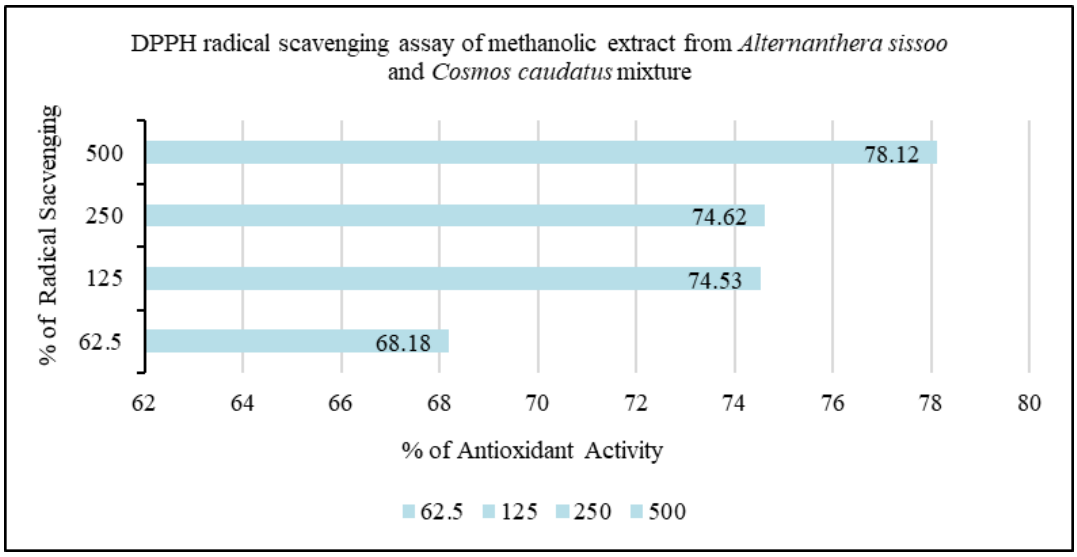

Figure 3. DPPH radical scavenging assay of methanolic extract from *Alternanthera sissoo* and *Cosmos caudatus* mixture

Based on Figure 3, the methanolic extract of *A. sissoo* and *C. caudatus* exhibited a concentration-dependent increase in antioxidant activity as measured by DPPH radical scavenging assay. The x-axis of the graph represents the different concentrations of the methanolic plant extract mixture, while the y-axis shows the percentage of DPPH radical scavenging activity. As the concentration increases, the percentage also increases showing higher antioxidant activity. This graph indicates that higher concentrations of the extract are more effective in neutralizing free radicals.

According to Seyedreihani et al. (2016), *C. caudatus* contains several bioactive compounds such as quercitrin, catechin and rutin which are known to neutralize free radicals for antioxidant activity. Similarly, a study by Saengha et al. (2022) reported that *A. sissoo* is rich in flavonoids, myricetin, rutin and ferulic acid. Therefore, it is proven that the results obtained in this study is aligned with previous study, and can be concluded that the observed antioxidant activity is largely attributed to the presence of these phytochemicals.

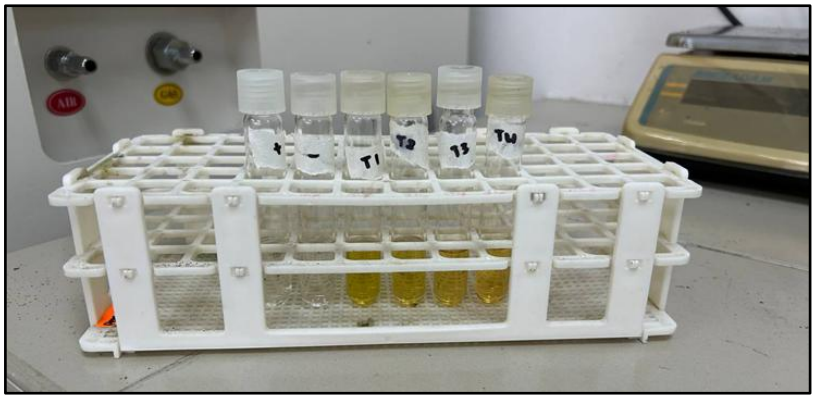

Figure 4. Mixture of different concentrations of methanolic *Alternanthera sissoo* and *Cosmos caudatus* with DPPH. (T1= 500 mg/mL, T2 = 250 mg/mL, T3 = 125 mg/mL, T4 = 62.5 mg/mL, positive and negative control)

This conclusion is further supported by the visual changes observed in DPPH assay, as shown in Figure 4. As antioxidants reduce DPPH, the solution changes from purple to yellow. The more intense the yellow, the higher antioxidant activity. Tubes T1 to T4 display a gradient of yellow intensity, with T1 showing the darkest yellow, indicating the highest antioxidant activity. This color change confirms that antioxidant activity increases with extract concentration.

Conclusion

In conclusion, this study successfully met its objectives, which the methanolic extract of *A. sissoo* and *C. caudatus* mixture has been proven to possess both antibacterial and antioxidant properties. Although disk diffusion assay showed minimal inhibition zone, the MIC results supported the presence of antibacterial activity against Gram-positive and Gram-negative bacteria at specific concentrations. Additionally, the DPPH assay demonstrated a concentration-dependent increase in antioxidant activity, with higher concentrations showing stronger radical-scavenging effects. These results not only support previous studies reporting the benefits of these two plants, but also highlight their potential for functional applications in food, healthcare, and pharmaceutical industries.

To enhance the scope and reliability of future studies, it is recommended to explore different extraction methods such as ethanol to yield a higher concentration of bioactive compounds. Besides, testing a wider range of bacterial strains, especially those that are resistant to antibiotics to provide a broader understanding of the extract's antibacterial potential. Furthermore, investigating synergistic effects with other plant extracts could also reveal enhanced efficacy and provide insights into possible therapeutic applications. These steps will help validate the extract's full potential and support its development for pharmaceutical or nutraceutical use.

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Author Contribution

Nur Aina Amirah Zakaria – sample collection, data processing, analysis manuscript writing; Ida Muryany Md Yasin – supervision, review, editing

Conflict of Interest

Authors declare no conflict of interest

Declaration on the Use of Generative AI

Authors declared ChatGPT is generative AI used in the manuscript for language enhancement, grammar correction and idea generation.

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