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Research Article

Bioactivity of *Clitoria ternatea* Crude Extracts Against Pathogenic Bacteria

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ABSTRACT

Clitoria ternatea, sometimes referred to as the Asian pigeon wings blue pea, the butterfly pea, or the Darwin pea, is a Fabaceae plant species that has been shown to possess antibacterial effects against several pathogenic microbes. Hence, the present study has been carried out to access the antibacterial activity of *C. ternatea* flower extracted with water and methanol against pathogenic bacteria. The well and disk diffusion assays were performed to determine the antibacterial activity of *C. ternatea* flower extracts. The efficacy of the extracts was then evaluated via broth microdilution assay to obtain MIC and MBC values and the growth reduction assay. Meanwhile, the DPPH scavenging test was used to assess the antioxidant activity of the crude extracts. The results of the well and disc diffusion assays showed that Gram-positive bacteria were more sensitive to both extracts compared to Gram-negative bacteria. Meanwhile, the methanolic extract showed higher antibacterial activity on both Gram-positive and Gram-negative bacteria compared to the aqueous extract. The results of the MIC and MBC tests showed that the methanolic extract was bactericidal to both Gram-positive and Gram-negative bacteria. The aqueous extract, however, demonstrated bacteriostatic activity against Gram-negative bacteria and bactericidal activity solely against Gram-positive bacteria. After a 24-h exposure period, a growth reduction assay showed that the methanolic extract could suppress both Gram-positive and Gram-negative bacteria by up to 99%. Meanwhile, the aqueous extract showed an inhibition percentage value ranging from 75% to 96% after an incubation period. The aqueous extract had the lowest antioxidant activity, with an EC_{50} value of 87.78 µg/mL, whereas the methanolic extract had a fair amount of antioxidant activity when compared to the control (quercetin), according to the DPPH scavenging assay. The present study suggests that *C. ternatea* extracts as a potential antibacterial agent against pathogenic bacteria with significant antioxidant activity and this activity may be due to the presence of anthocyanin and its derivatives.

Key words: Antibacterial activity, antioxidant activity, *Clitoria ternatea*, DPPH scavenging test, well diffusion and disc diffusion assays

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INTRODUCTION

Malaysia is blessed with vast biodiversity and is recognized as one of the mega-diverse countries worldwide. As a tropical country, Malaysia is home to a variety of beneficial plants, especially in its rainforest. Since it was planted for its bloom and as an attractive plant, the butterfly pea, or *Clitoria ternatea*, is one of the useful plants that is abundantly found in Malaysia. On the east coast of Peninsular Malaysia, local people use the butterfly pea flower as one of the ingredients in the making of "*Nasi Kerabu*" or Salad Rice whereby this flower gives the blue color to the rice. On the other hand, it is also used in traditional cuisine by Straits-born Chinese or Baba Nyonya to produce "*pulut tai-tai*" or blue glutinous rice cakes. The woody genus *C. ternatea* is distinguished by its colorful, papilionaceous blooms, and infundibular calyx with persistent bracteoles, stipules, and stalked ovaries (Gupta *et al*., 2010).

Numerous bioactivity features, such as antibacterial, antibiofilm, antioxidant, and anticancer activities, have been linked to the butterfly pea plant. For instance, *Clitoria ternatea* L. corolla extract was found to have antibiofilm efficacy against *Streptococcus mutans* ATCC 25175 and *Staphylococcus aureus* ATCC 6538 (Satria *et al*., 2022). Their research demonstrated that the extract significantly inhibited the growth of the test bacteria, *S. aureus*, and *S. mutans* by causing their cells to leak intramembrane fluid. Additionally, Senarathna *et al*. (2021) demonstrated that the leaves and flowers of *C. ternatea*

possess antibacterial activity against *S. aureus* and *Escherichia coli*. They discovered that ethanolic leaf and floral extracts showed greater inhibitory zones against *S. aureus* than methanolic sample extracts. Methanolic leaf and flower extracts showed greater zones of inhibition against *E. coli* than did ethanolic sample extracts. On the other hand, Jamil and Pa'ee (2002) reported that *C. ternatea* with vibrant blue flowers possesses antimicrobial properties that are beneficial to human health. All parts of *C. ternatea* including the flower, have potential antimicrobial activity against *Aeromonas hydrophila*, *Bacillus cereus*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *S. agalactiae*, and *S. aureus*. *C. ternatea* flowers have been reported to show antibiofilm activity against *P. aeruginosa*. Anthocyanin-rich fractions of *C. ternatea* flowers were effective against *P. aeruginosa* with significant biofilm inhibitory activity of 64.0 ± 1.1% (Jeyaraj *et al*., 2022b). For their significant antioxidant activity, Lakshan *et al*. (2020) measured the total phenolic content (TPC), total flavonoid content (TFC), ferric-reducing antioxidant power (FRAP), and DPPH radical scavenging activity of three varieties of Sri Lankan *C. ternatea*, including white flower with normal keel petals (WSPF), blue flower with normal keel petals (BSPF), and blue flower with enlarged keel petals (BMPF). They revealed that these three flower types had considerable concentrations of biologically active compounds, which suggests that the flower samples had significantly greater concentrations of TPC, TFC, and free radical scavenging activity. Additionally, they hypothesized that these three types have the potential to be anti-cancerous natural herbal medications due to their relatively greater levels of TPC, TFC, and antioxidant capacity.

Millions of bacteria, fungi, and viruses that make up the skin microbiota, such as *S. aureus*, and *S. epidermidis* reside on our skin. *S. aureus* is a significant human pathogen that causes several clinical diseases and is the most common bacteria involved in skin infections worldwide, regardless of the patient's age, the weather, or the location of the infection. It is a major contributor to bacteremia, infectious endocarditis, skin, and soft tissue infections, pleuropulmonary infections, and infections brought on by medical devices (Tong *et al*., 2015). The main clinical skin manifestations are associated with a small number of the bacteria's toxins, which cause a wide variety of clinical manifestations. Exfoliatins, enterotoxins, and toxin shock syndrome toxin 1 are the main toxins connected to most dermatological complaints caused by *S. aureus*. There may be other, less common cutaneous symptoms in endocarditis and bacteremia (Del Giudice, 2020).

In the meantime, *S. epidermidis*, a skin pathogenic bacterium, frequently results in infections of medical devices due to its ability to form biofilms, which offer protection against drugs and host immune response. This strain possesses a much greater number of virulence factors mostly depending on its capacity to build biofilms to spread infection. When *S. epidermidis* adheres to a surface, it changes its phenotype, and an extracellular polymeric substance (EPS) is produced as a protective coating, forming a bacterial community known as a biofilm (Vitale *et al*., 2021). Despite being a Gram-positive coccus that is prevalent on human skin and mucous membranes, *S. epidermidis* also has the potential to be detrimental when it comes to illnesses associated with healthcare (HAI). It is one of the main pathogens that cause infections associated with medical equipment like catheters and prostheses, because of the expression of virulence factors, particularly the creation of biofilms (Dodou *et al*., 2017).

Klebsiella pneumoniae and *P. aeruginosa* are opportunistic pathogens that may cause skin infections such as cutaneous infections. An opportunistic bacterium called *K. pneumoniae* can be found in a variety of microbiological environments, including soil, food, and the skin, intestines, and feces of animals. A urinary tract infection, pneumonia, and bacteremia have all been linked to *K. pneumoniae* (Hartantyo *et al*., 2020). Some potentially dangerous dermatoses, such as ecthyma gangrenosum, which suggests immunosuppression or blood poisoning, especially in children, are caused by *P. aeruginosa*, a common Gram-negative bacillus recognized by its greenish color and sweet-smelling scent. When severe or localized in skinfolds, it frequently colonizes long-term wounds, severe burns, and spongiotic or acantholytic dermatoses. Additionally, a nail condition or folliculitis associated with aquatic sports (chloronychia) may be involved (Morand & Morand, 2017).

Particularly in immunocompromised patients with underlying diseases like chronic pulmonary obstruction, diabetes mellitus, or cancer, *K. pneumoniae*, one of the Gram-negative opportunistic pathogens, frequently causes bloodstream infections, respiratory infections, and urinary tract infections that emerge in hospitals. According to reports, immunocompromised patients infected with isolates of the multidrug-resistant *K. pneumoniae* had a high mortality rate of 18 to 49%. Extended-spectrum-lactamases (ESBLs) produced by *K. pneumoniae* have proliferated globally throughout time and pose a serious hazard to human health (Moo *et al*., 2021).

Over the past years, antibiotic resistance in bacteria has become a major problem around the world, jeopardizing our ability to successfully treat illnesses. Antibiotic resistance-associated factors have significantly expanded in bacterial populations due to their mobility and contagiousness. The issue is mostly caused by antibiotic-driven selection and bacterial genomic flexibility. Due to genome evolution and the emergence of highly effective multidrug-resistant clades in many diseases, this has grown to be a significant issue (Klemm *et al*., 2018).

This study aims to evaluate the antibacterial efficacy of butterfly pea flower extract against various skin pathogenic and foodborne bacteria. This objective is based on earlier research that highlighted the advantages of the butterfly pea flower as an antioxidant, cytotoxic, and antimicrobial agent (Jeyaraj *et al*., 2022b). By using the blue hue of *C. ternatea* as a natural colorant, the knowledge and information from the present study could serve as a benchmark in the development of handwash formulation.

MATERIALS AND METHODS

Collection of samples

The healthy flower samples were collected from Kota Warisan Sepang (2° 49' 22'' North, 101° 42' 4'' East). The samples were delivered to the UiTM Shah Alam Microbiology Laboratory within two hours after being put in Ziplock plastic bags and kept in an ice box. The flower samples were cleaned under running water to get rid of any dirt, rinsed, and dried for 24 h in a 60 °C oven.

Plant extraction

A hundred grams of dried powdered *Clitoria ternatea* was soaked in 200 mL of methanol and distilled water for 24 h at room temperature (30 ± 2 °C). The soaked material was filtered using Whatman filter paper (No.

1) and concentrated using a Rotary evaporator at 55 °C to obtain the methanolic extract. The concentrated crude extract was placed in a fume hood to remove the excessive solvent. For aqueous extract, the soaked sample was filtered using Whatman filter paper (No. 1) and the filtrate was then freeze-dried for a week using a freeze dryer. Both crude extracts were kept in a desiccator until further use (Jeyaraj *et al*., 2022a).

Microorganisms and cultural maintenance

The skin pathogenic bacteria *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumonia,* and *Pseudomonas aeruginosa* (all are clinical isolates) were provided by the Microbiology Laboratory, School of Medical Sciences, Health Campus, Universiti Sains Malaysia. On Mueller-Hinton agar (MHA), the bacterial strains were grown and incubated for 24 h at 37 °C. The glycerol stock was prepared to preserve the cultures and stored in a freezer (-18 °C). The cultures were revived every three months to ensure their viability and purity.

Inoculum preparation

Two or three colonies of pathogenic bacteria were taken from the agar plate and introduced into a Universal bottle containing 5.0 mL of 0.85% (w/v) physiological saline solution. The mixture was vortexed and compared with 0.5 McFarland to obtain a bacterial suspension of 1 × 10^8 CFU/mL (Taufiq & Darah, 2019).

Well diffusion assay

The method described by Hamza *et al*. (2021) was employed to determine the antibacterial activity of crude extracts of *C. ternatea.* A 3 mm diameter hole was punched through the solidified MHA using a cork borer, and test bacteria were then added to the plate. The plates were then incubated at 37 °C for 24 h after the crude extracts (1 mg/mL) were added to each well. The chloramphenicol (30 µg/mL) and distilled water were set as positive and negative controls, respectively. After an incubation period (24 h), the inhibition zone formed around the wells was measured and recorded.

Disc diffusion assay

The antibacterial activity of *C. ternatea* crude extracts against several pathogenic bacteria was evaluated using the Kirby Bauer Disc diffusion assay according to the CLSI procedures (CLSI, 2022). Bacterial inoculum was prepared as the previous method (subtopic: inoculum preparation). A concentration of 1 mg/mL extract was prepared by diluting 100 mg of extract into 100 mL 5% (v/v) dimethyl sulfoxide (DMSO). A volume of 20 µL of the extract was impregnated into a 6 mm blank sterile antibiotic disc. On Mueller Hinton agar (MHA), which had previously been colonized by test bacteria, the air-dried disc was then put. For 24 h, the plate was incubated at 37 °C. The negative and positive controls used were DMSO and chloramphenicol (30 µg/mL), respectively. The inhibition zone that formed around the wells after an incubation period (24 h) was measured and recorded.

Evaluation of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

According to the procedure outlined by Taufiq and Darah (2019), the MIC values of the crude extracts were assessed using a sterile U-shaped microtiter plate (96 wells). Chloramphenicol was used as a standard drug, while the negative control was DMSO. MIC values were established as the lowest concentration of the *C. ternatea* extract that could prevent the test bacteria's ability to grow visibly. The MBC values of the extracts were determined upon a reading of MIC values. A sterile cotton stick was dipped in the wells showing no visible bacterial growth (from lowest to highest concentration) and swabbed on the MHA plate. The plate was then incubated at 37 °C for 24 h. The MBC value was recorded as the lowest extract concentration which resulted in 99.9% of test bacterial reduction.

Bacterial growth reduction

The efficacy of the crude extracts was measured according to the method described by Jalil *et al*., (2022b) with slight modifications. Each crude extract was prepared in this study at a 2 × MIC level or MBC value. For treatment, a volume of 8.9 mL Mueller Hinton broth (MHB) was introduced into a 50 mL Erlenmeyer flask and subsequently added with 1.0 mL of crude extracts at a final concentration of 2 × MIC. The flask was then filled with 0.1 mL of bacterial suspension, which had a final bacterial concentration of 5 × 10^8 CFU/mL, and it was shaken at 150 rpm for 24 h at 37 °C. After 24 h of the incubation period, a volume of 1.0 mL mixture was withdrawn. The spread plate count was performed, and the bacterial colony was calculated as a colony-forming unit (CFU).

DPPH scavenging activity

Clitoria ternatea crude extracts were tested for antioxidant activity using the method published by Shekhar and Anju (2014). A volume of 0.1 mM 1, 1-diphenyl-2-picrylhydrazyl (DPPH) solution in ethanol was prepared. Methanolic and aqueous extracts were diluted in the ethanol at different concentrations (5,10, 15, 20, & 25 µg/mL). By performing in the 96-well microtiter plate, 100 µL of the ethanolic DPPH solution was added into the well containing different concentrations of crude extracts and shaken vigorously. The mixture was allowed at room temperature (30 ± 2 °C) for 30 min and the absorbance was then measured by using a spectrophotometer at 517 nm. Quercetin was used as a standard reference. The crude extracts' IC_{50} value [sample concentration required to inhibit 50% of the DPPH free radicals] was calculated by plotting a Log dose inhibition curve. The percentage of the scavenging effect was determined by using the following equation:

$$\text{DPPH scavenging activity (\%)} = \frac{A_c - A_s}{A_c} \times 100$$

Where A_c and A_s were the absorbance of the control and the sample, respectively.

Statistical analysis

All tests were run in triplicate (n=3), and the data were presented as mean standard deviation (SD). Using SPSS 15.0, one-way ANOVA was used to analyze the data, and the Duncan test was used to determine whether there were any differences in the means. The results were considered statistically significant if p<0.05.

RESULTS & DISCUSSION

Extraction of methanolic and aqueous crude paste of *C. ternatea*

Extraction is the first and most crucial stage in the evaluation of medicinal plants and define the necessary chemical components from the plant materials. The primary steps were pre-washing, freeze-drying, or drying of plant materials, grinding to provide a homogenous sample, and frequently enhancing the kinetics of analytical extraction as well as increasing the contact of the sample surface with the solvent system. The right procedures must be used when creating the extract from plant samples to make sure that any potential active components are not lost, changed, or destroyed. The particular characteristics of the bioactive compounds being targeted determine the solvent system that should be used in the major part. It is possible to extract the bioactive compounds from natural products using a variety of solvent systems (Sasidharan *et al*., 2011). Table 1 shows the yield of methanolic, and aqueous crude extracts obtained after solvent and free-dried extractions, respectively. The outcome showed that the methanolic extract's yield (9.1%) was much higher than the aqueous extract's (8.1%). Similar findings were made by Sharmin *et al*. (2016), who found that the methanol extract from pomegranate waste had a higher yield of bioactive compounds than the water extract (14.1%), including total phenolic content (18.3%). Tan *et al*., (2013) revealed that solvent extraction could give a fair recovery, however, it also has some disadvantages such as loss of quality and increasing cost during the evaporation process.

Table 1. The yield of methanolic and aqueous crude extracts paste of *Clitoria ternatea*

Extracts	Weight of *Clitoria ternatea* powder (g)	Weight of crude extracts paste (g)	Yield (%)
Methanolic	100	9.10	9.1
Aqueous	100	8.06	8.1

Agar well diffusion assay

Over the past 50 years, the agar diffusion assay has become a vital tool for determining bacteria sensitivity to antibiotics. There are numerous varieties for it, including the cup method, the paper disc method, the standardized single disc approach, well diffusion, and others. The agar diffusion method's precision and repeatability are impacted by several factors, including the selection of the cut-off size for the inhibitory zones and breakpoints, temperature, and the thickness and consistency of the gel (Bonev *et al*., 2008). Therefore, agar well diffusion was used to test the antibacterial efficacy of *C. ternatea* crude extracts. Table 2 shows the antibacterial activity of the aqueous and methanolic extracts of *C. ternatea* against pathogenic bacteria which are two Gram-positive and two Gram-negative. The aqueous extract was observed to inhibit *S. aureus* and *S. epidermidis* with the diameter of the inhibition zone values of 12.6 ± 0.6 mm and 11.7 ± 0.6 mm, respectively. Meanwhile, the methanolic extract exerted antibacterial activity against *S. aureus* and *S. epidermidis* with the diameter of the inhibition zone values of 13.7 ± 0.6 mm and 14.3 ± 1.2 mm, respectively. For Gram-negative bacteria, the antibacterial activity of aqueous and methanolic extracts towards *K. pneumoniae* was 10.7 ± 0.6 mm and 12.3 ± 2.5 mm, respectively. Meanwhile, the aqueous and methanolic extracts exerted antibacterial activity against *P. aeruginosa* with the diameter of inhibition zone value of 10.3 ± 6.6 mm and 11.3 ± 0.5 mm, respectively. The present study revealed that the *C. ternatea* methanolic extract showed higher antibacterial activity against test bacteria compared to the aqueous extract based on statistical analysis (p<0.05). This result was in line with that of Parekh *et al*. (2006), who found that plant extracts in an organic solvent (methanol) exhibited a more antibacterial effect than extracts in water. These results are due to the polarity of the compounds and, their capacity to dissolve or diffuse in different mediums.

Table 2. Antibacterial activity of crude extracts of *Clitoria ternatea* against pathogenic bacteria on agar well diffusion assay.

Test bacteria	Diameter of inhibition zone (mm)		
	Aqueous extract	Methanolic extract	Chloramphenicol
Gram-positive bacteria			
Staphylococcus aureus	12.6 ± 0.6^{a}	13.7 ± 0.6^{b}	21.3 ± 0.6^{c}
Staphylococcus epidermidis	11.7 ± 0.6^{b}	14.3 ± 1.2^{a}	20.7 ± 0.6^{c}
Gram-negative bacteria			
Klebsiella pneumoniae	10.7 ± 0.6^{c}	12.3 ± 2.5^{c}	22.7 ± 1.5^{b}
Pseudomonas aeruginosa	10.3 ± 6.6^{c}	11.3 ± 0.5^{d}	24.7 ± 0.6^{a}

*Different superscript shows significant differences in the means

Disc diffusion assay

The Kirby-Bauer disc diffusion susceptibility test was used to evaluate the susceptibility or resistance of pathogenic aerobic and facultative anaerobic bacteria to various antibiotic agents. Table 3 displays the disc diffusion assay results for *C. ternatea* extracts' antibacterial efficacy against harmful microorganisms. The aqueous and methanolic extracts inhibited the Gram-positive bacteria, *S. aureus* and *S. epidermidis* with the diameter of the inhibition zone of 10.0 ± 2.7 mm, 12.3 ± 2.5 mm, and 10.3 ± 1.5 mm, 11.8 ± 1.5 mm, respectively. Meanwhile, Gram-negative bacteria, *K. pneumoniae,* and *P. aeruginosa* were inhibited by aqueous and methanolic extracts with the diameter of inhibition zone of 9.7 ± 1.56 mm, 10.7 ± 0.6 mm, and 9.7 ± 3.2 mm, 10.3 ± 0.6 mm, respectively. The present study showed almost similar finding reported by Kamilia *et al*. (2009) who revealed the methanolic extract of *C. ternatea* flower inhibited the growth of *P. aeruginosa* and *S. aureus* with the inhibition zone of 11.3 ± 1.5 mm and 13.0 ± 1.0 mm, respectively. The findings also revealed that Gram-negative bacteria were least susceptible to both aqueous and methanolic extracts compared to Gram-negative bacteria (p<0.05). Jalil and Ibrahim (2022) suggest that structural and morphological differences between Gram-positive and Gram-negative microorganisms could explain why the two types of bacteria have different sensitivity levels to the extract. Gram-negative bacteria often have more complex structures than Gram-positive bacteria, such as the outer membrane layer, thin peptidoglycan layer, and periplasm. The outer membrane of gram-negative bacteria inhibits flow and functions as a selective barrier, obstructing antibiotics while allowing the supply of vital nutrients to the cell. Additionally, porins, membrane proteins that serve as selective channels for the transit of hydrophilic molecules of a specific size into the periplasm, are joined to the outer membrane (Miller & Salama 2018). The current investigation also showed that the diameter of the inhibition zone was considerably larger in the good diffusion assay (Table 2) than in the disc diffusion assay. This finding was consistent with Jalil *et al*. (2021) who claimed that agar plug diffusion and disc diffusion assays produced differing results for various test microorganisms' susceptibility to an extract. This phenomenon may be influenced by the concentration of bioactive substances, the depth of the agar medium, and the diffusion rate. Additionally, the creation of the inhibitory zone was influenced by dissipative diffusion behavior, agar's diffusion rate, and depth, substrate loss during diffusion, antibiotic degradation, and antibiotic removal by the microbial film (Bonev *et al*., 2008).

Table 3. Antibacterial activity of crude extracts of *Clitoria ternatea* against pathogenic bacteria on disc diffusion assay.

Test bacteria	Diameter of inhibition zone (mm)		
	Aqueous extract	Methanolic extract	Chloramphenicol
Gram-positive bacteria			
Staphylococcus aureus	10.0 ± 2.7[a]	12.3 ± 2.5[a]	22.3 ± 2.1[c]
Staphylococcus epidermidis	10.3 ± 1.5[a]	11.8 ± 1.5[b]	21.3 ± 0.6[d]
Gram-negative bacteria			
Klebsiella pneumoniae	9.7 ± 1.5[a]	10.7 ± 0.6[c]	24.3 ± 0.6[b]
Pseudomonas aeruginosa	9.7 ± 3.2[a]	10.3 ± 0.6[c]	25.3 ± 0.6[a]

*Different superscript shows significant differences in the means

Determination of MIC and MBC values

The MIC and MBC values for both crude extracts against the test pathogenic bacteria are shown in Table 4. According to the current study, the aqueous extract's MIC and MBC values against Gram-positive bacteria were 250 µg/mL and 1000 µg/mL, respectively. As for methanolic extract, the MIC and MBC values were 250 µg/mL and 500 µg/mL, respectively. Meanwhile, the MIC and MBC values of the aqueous and methanolic extracts against Gram-negative bacteria were 250 µg/mL, 2000 µg/mL, and 250 µg/mL,1000 µg/mL, respectively. Gram-positive bacteria were found to be more sensitive to both extracts than Gram-negative bacteria (p<0.05). It is also interesting that the methanolic extract was more effective against both Gram-positive and Gram-negative bacteria than the aqueous extract. It was hypothesized that the extraction of bioactive substances from the flower of *C. ternatea* was impacted by the solvent's polarity. Nawaz *et al*. (2020) revealed that the phytochemical content and extraction yield of plants were significantly influenced by the polarity of extracting solvents. The results were in line with those reported by Karaman *et al*. (2002), who found that the methanol extract of *Juniperus oxycedrus* inhibited the growth of 57 strains of 24 bacterial species, including those belonging to the genera of *Acinetobacter*, *Bacillus*, *Brevundimonas*, *Brucella*, *Enterobacter*, *Escherichia*, *Micrococcus*, *Pseudomonas*, *Staphylococcus*, and *Xanthomonas*, while the aqueous extract had no antibacterial effect. Since the MBC/MIC ratio was less than or equal to 4, the current investigation further showed that the aqueous and methanolic extracts have bactericidal action against all test microorganisms other than *K. pneumoniae* and *P. aeruginosa*. According to Jalil *et al*. (2022), antimicrobial compounds are considered bacteriostatic agents when the MBC/MIC ratio is greater than 4, and bactericidal agents when it is 4 or below. However, both bacteriostatic and bactericidal agents have advantages as an antibiotic against pathogenic bacteria in which bacteriostatic antibiotics do not reduce the number of bacteria; instead, they stop their growth, whereas bactericidal agents reduce their numbers. Bacteriostatic antibiotics allow the immune system to combat infections even when the bacteria are still alive, in contrast to bactericidal antibiotics. For instance, some bacteriostatic drugs work well in cases of streptococcal and clostridial gangrene. This is because they stop the toxins from being produced, which are the main contributors to morbidity.

Table 4. Determination of MIC and MBC values of the crude extracts via broth microdilution assay

Test bacteria	Aqueous extract			Methanolic extract		
	MIC (µg/mL)	MBC (µg/mL)	Ratio (MBC/MIC)	MIC (µg/mL)	MBC (µg/mL)	Ratio (MBC/MIC)
Gram-positive bacteria						
Staphylococcus aureus	250	1000	4 (bactericidal)	250	500	2 (bactericidal)
Staphylococcus epidermidis	250	1000	4 (bactericidal)	250	500	2 (bactericidal)
Gram-negative bacteria						
Klebsiella pneumoniae	250	2000	8 (bacteriostatic)	250	1000	4 (bactericidal)
Pseudomonas aeruginosa	250	2000	8 (bacteriostatic)	250	1000	4 (bactericidal)

Bacterial growth reduction

The efficacy of the crude extracts was assessed against pathogenic bacteria for 24 h. Table 5 shows the reduction in bacterial growth after being treated for 24 h with aqueous and methanolic extracts. *S. aureus* growth was reduced up to 96.52 and 99.93% after 24 h of treatment with aqueous and methanolic extracts, respectively. Meanwhile, the growth of *S. epidermidis* was reduced to 95.87% and 99.89% after 24 h of treatment with aqueous and methanolic extracts, respectively. For Gram-negative bacteria, after being exposed to aqueous and methanolic extracts, *K. pneumoniae*'s growth was inhibited up to 82% and 99%, respectively. Whereas the growth of *Pseudomonas aeruginosa* was reduced to 79.75% and 99.66%, after being exposed to both aqueous and methanolic extracts, respectively. The growth reduction result was in line with MIC and MBC values (Table 4) showing the efficacy of the extracts against test bacteria. Besides that, the aqueous extract may exert a bacteriostatic effect against *P. aeruginosa* and *K. pneumoniae* since the percentage of bacterial reduction was in the range of 79.75% and 82.21%, respectively. A previous study by Jalil *et al*. (2021) on time-killing revealed this pattern and they concluded that the efficacy of the extract is dependent on dose and exposure time. Similar findings were made by Saeloh and Visutthi (2021), who found that the reference strains of *S. aureus* ATCC29213, Methicillin-resistant *S. aureus* NPRC001R, and *E. coli* ATCC25922 were all susceptible to the antimicrobial effects of *Piper betle* extract. Time-Kill study is an analytical microbiological technique used to evaluate microorganism susceptibility and it is employed to study a bacterial strain's resistance to an antimicrobial agent.

Table 5. Bacterial growth reduction after 24 hours of treatment with the crude extracts

Test bacteria	Aqueous extract			Methanolic extract		
	Without extract	Extract	Bacterial reduction (R% ± SD)	Without extract	Extract	Bacterial reduction (R% ± SD)
Gram-positive bacteria						
Staphylococcus aureus	2.33×10^8	8.10×10^6	**96.52**[a]	2.33×10^8	1.60×10^5	**99.93**[a]
Staphylococcus epidermidis	2.16×10^8	8.90×10^6	**95.87**[b]	2.16×10^8	2.45×10^5	**99.89**[a]
Gram-negative bacteria						
Klebsiella pneumoniae	1.63×10^8	2.90×10^7	**82.21**[c]	1.63×10^8	7.80×10^5	**99.52**[a]
Pseudomonas aeruginosa	1.21×10^8	2.45×10^7	**79.75**[d]	1.21×10^8	4.10×10^5	**99.66**[a]

*Different superscript shows significant differences in the means

Determination of antioxidant activity

The DPPH scavenging activity of quercetin and *C. ternatea* crude extracts is shown in Figure 1. The results showed that crude extracts' ability to scavenge DPPH was dose-dependent, with an increase in extract concentration having the potential to do so. Table 6 shows the EC_{50} values of quercetin, aqueous and methanolic extracts. The finding exhibited that the methanolic extract possesses a higher scavenging ability compared to the aqueous extract ($p<0.05$). This finding supported the previous result (Table 2, Table 3, Table 4) in which the methanolic extract showed significantly higher antibacterial activity compared to the aqueous extract. This might be because the methanolic extract has more antioxidant components than the aqueous extract does. Antibacterial action has been linked to antioxidant substances like phenolic compounds, flavonoids, alkaloids, and tannins. For instance, Mandal *et al*. (2017) revealed that phenolic compounds such as rutin, tannic acid, epigallocatechin gallate, and eugenol exhibited significant antibacterial activity against *P. aeruginosa* and *S. epidermidis*. Additionally, flavonoids have been found to have antibacterial activity against a variety of pathogenic microorganisms. These compounds are thought to work by inhibiting several processes, including nucleic acid synthesis, cytoplasmic

membrane function, membrane permeability, porin on cell membranes, inhibition of energy metabolism, and attenuation of pathogenicity (Xie *et al*., 2015). In addition to that, Raji *et al*. (2019) reported the capability of alkaloids to exhibit antibacterial activity with inhibition of toxin production and protein transcription as their mode of action. Meanwhile, tannins showed potential antibacterial activity by interrupting bacterial metabolism, depriving the required compounds for microbial growth, and inhibiting enzyme activity. On the other hand, Jeyaraj *et al*. (2022a) reported *C. ternatea* flower extract possesses anthocyanin that has the potential to be used and developed as a functional food ingredient or nutraceutical agent. They also revealed that the higher antimicrobial activity is due to the higher anthocyanin content compared to the crude extracts which had a higher content of flavanols. According to Cerezo *et al*. (2020), anthocyanin derivatives including cyanidin, delphinidin, petunidin, and malvidin may have acted synergistically for the antibacterial activity of anthocyanin-rich fractions against bacterial strains such as *E. coli*, *B. cereus*, and *P. aeruginosa*. These compounds were found to affect bacterial cell membrane structure leading to the inactivation of crucial enzymes, affecting gene expression, and impairment of the metabolism of bacteria which may affect their growth and reproduction. Moreover, anthocyanins were found to have disrupted the tricarboxylic acid cycle (TCA) cycle and this leads to weakened cellular respiration and inadequate energy supply leading to death (Sun *et al*., 2018).

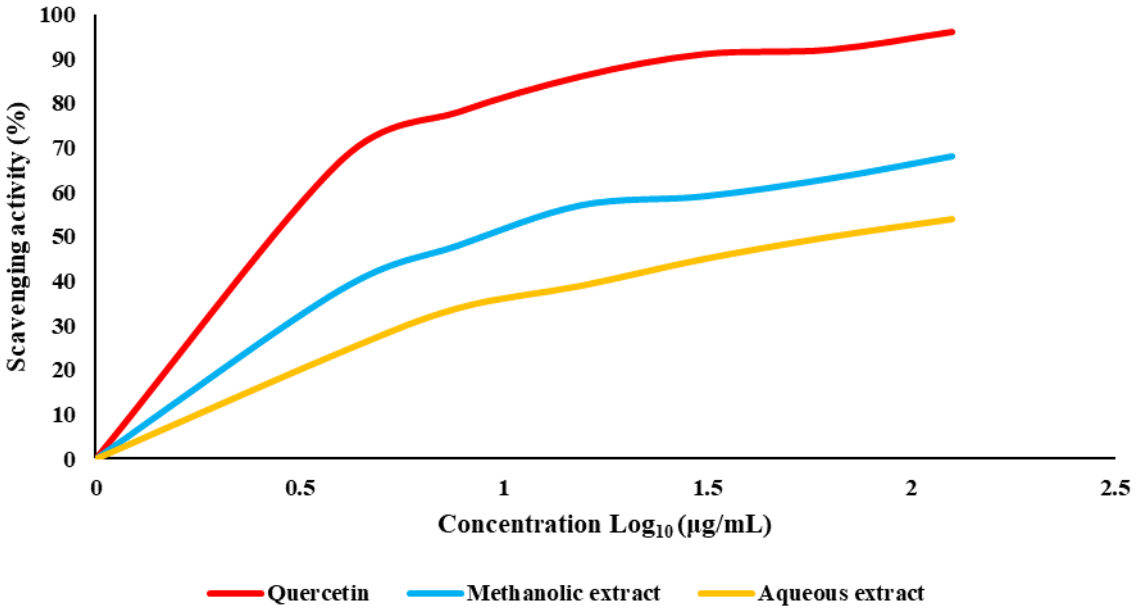

Fig.1. Scavenging effect of DPPH radicals on quercetin, methanolic and aqueous extract at different concentrations.

Table 6. EC_{50} values of quercetin, aqueous and methanolic extracts

Sample	EC_{50} (µg/mL)
Quercetin	18.10[a]
Methanolic extract	44.43[b]
Aqueous extract	87.78[c]

*Different superscript shows significant differences in the means

CONCLUSION

The present study successfully revealed the potential of *Clitoria ternatea* extracts as antibacterial agents against several pathogenic bacteria, which possess the ability to inhibit Gram-positive and Gram-negative with a diameter of inhibition zone ranging from 10.3 ± 6.6 to 14.3 ± 1.2 mm (well diffusion assay) and 9.7 ± 1.56 mm to 12.3 ± 2.5 mm (disk diffusion assay), respectively. The MIC and MBC values revealed that both aqueous and methanolic extracts exerted bactericidal effects toward Gram-positive bacteria. However, for Gram-negative bacteria, aqueous and methanolic extracts showed bacteriostatic and bactericidal effects, respectively. Furthermore, bacterial growth reduction analysis exhibited that methanolic extract could eliminate the bacterial cells of Gram-positive bacteria up to 99%. After 24 h of exposure, the aqueous extract can only inhibit 79% of Gram-negative bacteria and 95% of Gram-positive bacteria. As for the DPPH scavenging activity, the methanolic extract had better free radical scavenging activity when compared to the aqueous extract with the value of EC_{50} being 44.43 µg/mL. This shows that *C. ternatea's* methanolic extract can be a rich source of natural antioxidants and has a lot of promise for application in the creation of cosmeceutical products like handwash. It is noteworthy that the antibacterial and antioxidant activity of *C. ternatea* flower extract may be due to the presence of anthocyanin and its derivatives. Further studies are needed to purify the compounds involved in the antibacterial activity of these extracts and determine the mode of action of these extracts against test bacteria. The toxicity analysis of the extracts also needs to be performed to ensure that they are safe to be used in the formulation of cosmeceutical products.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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