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Phytochemical Analysis of *Clitoria ternatea* Leaves and Its Potential Antibacterial Activity against *Escherichia Coli*

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Abstract: *Clitoria ternatea* is traditionally used as medicine in Ayurveda and had been found to exhibit antibacterial activities due to its rich phytochemical contents. Due to the issue of resistant bacteria emergence and side effects of synthetic antibacterial agents, investigation of plant's antibacterial potential is important. In this study, the methanolic *C. ternatea* leaves extracts were investigated for phytochemical content and antibacterial activity. Phytochemical content was investigated quantitatively focusing on the total phenolic content (TPC) and total flavonoid content (TFC), determined by Folin-Ciocalteau method and Aluminum-chloride method, respectively. The antibacterial potential of the plant's extract was analyzed by disk-diffusion method of concentrations (12.5, 25, 50, 100 mg/mL), ampicillin and methanol act as positive control and negative control, respectively. The extraction yield of methanolic *C. ternatea* leaves extracts obtained by maceration method is 8.16%. The TPC and TFC of *C. ternatea* leaves extract are 0.66116 ± 0.43455 mg GAE /g and 0.31333 ± 0.057735 mg QE /g respectively. The disk-diffusion antibacterial assay showed no inhibitory activity of *C. ternatea* extracts against *Escherichia coli*. This might be attributed to the lack of potency of *C. ternatea* extracts at their current concentration, and the low content of TPC and TFC in the extracts. This had partially proved that concentration of the extracts used is crucial in antibacterial activities. Discrepancy of antibacterial results in *C. ternatea* observed between different studies might be attributed to the different methodologies. In conclusion, *C. ternatea* has been seen as a high potential plant in terms of antibacterial activity, but conditions during experiment poses high impact on the result of antibacterial assay. The findings from this study had provided valuable information to the field of phytochemistry and attempted to broaden the uses of medicinal plants, in which this can indirectly contribute to preservation of traditional knowledge and conservation of biodiversity.

Keywords: *Clitoria ternatea*, *Escherichia coli*, antibacterial activities, total phenolic content, total flavonoid content

1. Introduction

Bacteria like *Vibrio cholerae*, *Clostridium difficile*, *Escherichia coli, Bacillus coli* and *Shigella* species are capable of causing diarrhea, usually through gastrointestinal tract infection [1] [2]. There are an estimated 1.7 billion diarrhea cases annually, it is also one of the main reasons of premature death in children [3]. The invention of antibiotics temporarily fights the bacteria infections and increases the survival rate of patients. There is a 36% increment of antibiotics use by humans between the year 2000 to 2010, but there are 20% of deaths around the globe because of infectious diseases [4]. This is related to the emergence of antibiotic-resistant bacteria, capable of causing serious infections and increase the mortality rate [5]. In addition, synthetic antibiotics can cause undesirable side-effects, including skin rashes, allergy, gastrointestinal symptoms, nephritis and disturbance in the nervous systems [6] [7]. Facing the current scenario, novel antibacterial substances are required and plant-derived substances have shown

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effective antibacterial activity [8]. The antibacterial activities shown by the traditional medicinal plants are attributed to the bioactive compounds, mainly secondary metabolites including triterpenoids, alkaloids, phenols, and flavonoids [9] [10].

Malaysia is blessed with rich plant biodiversity, with approximately 15, 000 species of vascular plants documented [11]. Human activities like logging and introduction of invasive species lead to the current scenario of climate change, habitat degradation and fragmentation, threatening plant biodiversity [12]. Human had been making efforts like natural reserves, wild nurseries, botanical gardens and seed banks to ensure the future availability of commercially and medicinally important plants that are part of biodiversity [13]. Some traditional medicinal plants such as *Clitoria ternatea* are being underutilized, and they possess enormous medicinal value for the health care field. Therefore, by broadening their uses and values will drive the conservation efforts. Biodiversity can also benefit from the conservation efforts on medicinal plants, for instance the natural reserves of medicinal plant would also allow the survival of other biodiversity in the area. In addition, only the tip of an iceberg, particularly not over 10% of the rich plant species available on planet earth are being utilized, showing plant's potential yet to be discovered [14]. *Clitoria ternatea* Linn*,* commonly known as butterfly pea or *telang* in Malay is a pantropical species that is naturalized in tropical Asia, considered a medicinal plant [15]. Traditionally, seeds, roots and leaves of *Clitoria ternatea* are used for "Medhya Rasayana", a rejuvenating medicine in Indian Ayurveda [16] [17]. *Clitoria ternatea* is commercially cultivated in Thailand as an ornamental plant [18]. In Malaysia, the flower of *Clitoria ternatea* is utilized primarily as a natural food colorant for local dishes like nasi kerabu and kuih tekan [19]. The Temuan community in Gunung Ledang, Johor use the flower as a natural blue colorant [20]. *Clitoria ternatea* also exhibits various biological activities, as it is being viewed as a potential antidepressant, anticonvulsant, anti-inflammatory, anthelmintic, antipyretic, antimicrobial and anti-diarrhea [21] [22] [23]. Different parts of *Clitoria ternatea* had been identified to possess antibacterial activity against both gram-positive and gram-negative bacteria with the leaves exhibiting the foremost inhibitory activities [24]. The *in vitro* biological activity assays revealed that leaves of *Clitoria ternatea* possess untapped potential. There are relatively limited studies on the antibacterial activities and associated phytochemicals of *Clitoria ternatea* grown in Johor, especially in Bukit Gambir. One known previous study of the plant was in Pagoh [25].

Therefore, this study focuses on determining the total phenolic content (TPC) and total flavonoid content (TFC) of the *Clitoria ternatea* leaves. Then, the antibacterial activities of the leaves' crude extracts are analysed by disk-diffusion method against *Escherichia coli*. This study would therefore be able to contribute valuable information to the field of phytochemistry. In addition, correlating traditional medicinal plants such as *Clitoria ternatea* with its biological activity would broaden the application of traditional medicinal plants. Thus, indirectly contribute to the preservation of traditional knowledge and conservation of biodiversity.

2. Methodology

2.1 Plant Sample Collection, Identification and Authentication

The fresh plant sample of *Clitoria ternatea* was collected in November 2021, from a home garden in Bukit Gambir, Tangkak District, Johor, Malaysia (Latitude: 2°12'56.73" N, Longitude: 102º39'32.97" E, Elevation: 19m). The plant sample was made into an herbarium voucher specimen, and authenticated by Prof. Madya Dr. Alona Cuevas Linatoc from the Department of Technology and Natural Resources, Universiti Tun Hussein Onn Malaysia (UTHM). The herbarium voucher specimen was then deposited to the UTHM Repository Room for future reference.

2.2 Plant Sample Preparation and Extraction

Clitoria ternatea fresh leaves were washed under tap water and subsequently by distilled water for thorough cleaning. Then, the leaves were dried in the oven at 50°C for 48 hours [26] and the next step proceeded after the moisture content of the dried leaves were confirmed to be below 10% using a moisture analyzer. The dried leaves were pulverized using a blender into powdered form and stored in a zip lock bag, it is then stored in a desiccator with silica gel included before further steps [27]. The methanolic extraction of the plant sample was carried out according to [28] and [29] with modifications. The powdered plant sample was macerated with methanol in a 1:10 ratio with 56g of sample and 560 mL of methanol, and placed inside the incubator shaker at a constant temperature of 27ºC for 3 days. The extract was filtered through the Whatman filter paper after 3 days and the solvent was removed using a rotary evaporator at 45ºC bathwater until it yielded a sticky, viscous liquid. The sample was then poured into small steel plates and covered in loose aluminum foil in a fume hood for a week to obtain a semi-dried extract. The semi-dried extracts were placed in a universal bottle with a temperature of 4ºC before further use [30]. The yield percentage (%) [31] was calculated according to the equation below:

$$\text{Percentage of yield (\%)} = (\text{weight of extract (g)} / \text{weight of sample (g)}) \times 100\% \quad (1)$$

2.3 Total Flavonoid Content (TFC)

The TFC was estimated through the aluminum chloride method according to [32] and [33] with modifications. The 1 mL extract were incubated for 5 minutes after the addition of 4 mL deionized water and 0.3 mL 5% (*w/v*) sodium nitrite ($NaNO_2$). After 5 minutes, the 0.3 mL of 10% (*w/v*) aluminum chloride ($AlCl_3$) was mixed and incubated at room temperature for 6 minutes. Then, 2 mL of 1M sodium hydroxide (NaOH) was added and immediately followed by the addition of 2.4 mL of deionized water to obtain a final volume of 10 mL. The mixture was mixed thoroughly using a vortex mixer and incubated for 15 minutes. After 15 minutes, the solutions were poured in cuvettes and measured for absorbance at 510 nm using a uv-vis spectrophotometer. Quercetin was used to prepare standard stock solutions, and standard solutions ranged from 20 µg/mL to 100 µg/mL were prepared to plot the standard curve using a simple dilution method. TFC was calculated according to [34] by the following equation:

$$\text{Total Flavonoid Content} = c \times (v/m) \quad (2)$$

Where c is the quercetin concentration calculated from the standard curve in mg/ml, v is the volume of extract in milliliter, while m is the mass of the extract in gram. The total flavonoid content (TFC) was conveyed as mg quercetin equivalents (QE) per gram of dry weight (mg QE/g). The procedure was done in triplicates, and the results were presented in the form of Mean ± Standard Deviation.

2.4 Total Phenolic Content (TPC)

The TPC was estimated through the Folin-Ciocalteu reagent method based on the methods of [33] and [35] with modifications. The 0.5 mL of extract was incubated at room temperature for 8 minutes after the addition of 2.5 mL of 10% (*v/v*) Folin-Ciocalteu reagent. Then, 2 mL of 7.5% (*w/v*) sodium carbonate (Na_2CO_3) was added and the mixture was thoroughly vortexed with a vortex mixer and incubated for 1 hour at room temperature without exposing to light. After 1 hour, the solutions were poured in cuvettes and measured for absorbance at 765 nm utilizing a uv-vis spectrophotometer. Gallic acid was used to prepare standard stock solutions, and standard solutions ranged from 20 µg/mL to 100 µg/mL concentrations were prepared to plot the standard curve using a simple dilution method. TPC was calculated according to [36] by the below equation:

$$\text{Total Phenolic Content (TPC)} = c \times (v/m) \quad (3)$$

Where c is the gallic acid concentration calculated from the standard curve in mg/mL, v is the extract volume in milliliter, while m is the mass of the extract in gram. The total phenolic content (TPC) was conveyed as mg gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g). The procedure was done in triplicates, and the results were presented in the form of Mean ± Standard Deviation.

2.5 Preparation of Different Concentration of Crude Extracts and Standardization of Inoculum

The different concentrations of plant crude extracts were prepared, according to [37] and [38] with modifications. 100 mg/mL of the extract solution was prepared by dissolving 0.6 g of the extract in 6 mL of methanol in a sterile universal bottle. Two-fold serial dilution was carried out to obtain three concentrations of 50 mg/mL, 25 mg/mL, and 12.5 mg/mL. All of the extracts were stored in the refrigerator at a temperature 4ºC before further use. The *Escherichia coli* stock was inoculated on Mueller Hinton Agar (MHA) using streak plate method and was placed in the incubator for 24 hours at 37ºC of obtaining isolated bacterial colonies. The inoculum of *Escherichia coli* was standardized according to the 0.5 McFarland standard solution by suspending several colonies from the culture plate into the 0.85% saline solution in a universal bottle using an inoculation loop [39]. The inoculum was standardized to approximately 1.5×10^8 CFU/mL by comparing the turbidity of the suspension with the turbidity of 0.5 McFarland standard solution in front of a Wickerham turbidity card in the presence of adequate lighting.

2.6Antibacterial Activity by Disk-diffusion Assay

The antibacterial activities of the *Clitoria ternatea* crude extracts was determined using a standard disk-diffusion method according to [40] and [41] with modifications. 6 mm diameter autoclaved Whatman No. 1 filter paper discs were impregnated with 20 µL of plant extract of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL concentrations using a micropipette. Methanol-loaded discs were used as negative control, while ampicillin (500 µg/mL) was used as a positive control in each plate [42]. Forceps was used to place the impregnated discs over the Mueller Hinton Agar (MHA) plates that have been inoculated with bacteria *Escherichia coli*. The inoculum was standardized according to the 0.5 McFarland standard that possess a turbidity that is similar to the appearance of a 1.5×10^8 CFU/mL bacteria suspension prior to the procedure. The inoculation was done by using a sterile cotton swab to create a bacteria lawn on

the MHA. The inoculated MHA plates were left for 15 minutes to avoid excess surface moisture by allowing them to be absorbed into the MHA [32]. The MHA plates were kept at room temperature for 1 hour in advance of incubation for 24 hours at 37ºC inside an incubator to facilitate the proper diffusion of extracts into the MHA [41]. After 24 hours, the MHA plates were removed from the incubator, and the zone of inhibition, which is the area devoid of bacterial growth surrounding the filter paper discs was measured in mm using a ruler. The procedure will be done in triplicates, and the diameter of inhibition was expressed in Mean ± Standard Deviation.

3. Results and Discussion

3.1 Extraction Yield

The extraction yield is expressed as yield percentage (%), is a measurement of crude extracts obtained from plant materials [43]. The yield percentage (%) of the methanolic extract of *Clitoria ternatea* leaf using maceration method in this study as presented in Table 1 is 8.16%. However, it is a semi-dried sample being used for further analysis due to time constraints, as the extract did not completely dry out even after being placed in the fume-hood for a week.

Table 1 - The yield percentage (%) of the methanolic extract of *Clitoria ternatea* leaves

Extraction method	Yield percentage (%)
Methanolic maceration	8.16%

The yield (%) of 8.16% in this study is considered relatively high compared to the study of [44] that is 3.05% using successive hot continuous Soxhlet extraction. Methanolic extract of *Clitoria ternatea* leaves had an extraction yield of 20.29 ± 0.23% involving the utilization of 80 ± 1 °C water bath for 60 minutes as shown by another study [43]. A previous study [45] on the *Clitoria ternatea* leaves extracted by acetone, isopropyl alcohol and petroleum ether had extract yield of 16.2%, 12.2% and 9.7%, respectively. It can be said that the processing conditions can have an enormous influence on the extraction yield of bioactive compounds [46] as types of extraction solvent and extraction methods showed different extraction yields, not to forget the initial condition of the plant materials. In this study, the first and second batch of the *Clitoria ternatea* leaves collected were dried until the moisture content of the materials was less than 10%, that is 7.79% and 8.78%, respectively. A dried plant sample of moisture content below 10% is said to be advantageous and this can be observed that the crushed fresh leaves of *C. ternatea* macerated with 95.5% methanol for 72 hours only obtained an extraction yield of 1.6% [47]. Generally, it can be said that it is preferable to extract dried plant materials for higher extraction yield since drying can inhibit enzymes that can degrade the phytochemicals and the materials can be stored for a longer time [48] [49].

3.2 Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

Total phenolic content (TPC) and total flavonoid content (TFC) had been conducted in this study since phenolic compounds and flavonoids are associated closely with the antibacterial activity of plants [50] [51]. Phenolic compounds including flavonoids are found abundantly in *Clitoria ternatea*, especially in the leaf part [52]. Phenolic compounds such as gallic acid, quercetin, coumaric acid, catechol, and tannic acid possess inhibitory activities on the bacteria [50]. Flavonoids possess inhibitory activity against *Escherichia coli* through two mechanisms, which are the inhibition of synthesis of nucleic acid and cause damage to the cell membrane of the bacteria [53]. Moreover, in the study of [54] on hydro-ethanolic and hydro-methanolic *Clitoria ternatea* leaf extracts, the authors revealed a positive correlation between antibacterial activity and both total flavonoid content (TFC) and total phenolic content (TPC) against *Staphylococcus aureus* and *Escherichia coli*.

Table 2 - The Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) of the methanolic extract of *Clitoria ternatea* leaves

Total Flavonoid Content (QE mg/g)	Total Phenolic Content (GAE mg/g)
0.31333 ±0.057735	0.66116 ± 0.43455

In this study, Total phenolic content (TPC) was conducted on the methanolic extract of *Clitoria ternatea* leaves utilizing gallic acid in the Folin-Ciocalteu's method. The standard curve was plotted according to the absorbance (nm) of the different concentrations of gallic acid standards, where a regression equation $y = 0.0112x + 0.0569$ and a coefficient of determination (R^2) = 0.9877 was obtained as shown in Fig. 1. The total phenolic content (TPC) obtained of the methanolic *Clitoria ternatea* leaf extract in this study is 0.66116 ± 0.43455 mg GAE/g as shown in Table 2.

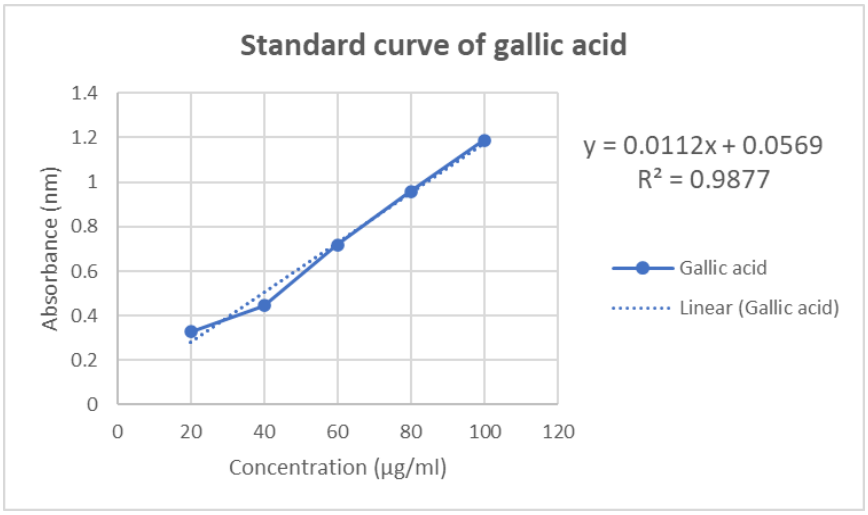


Fig. 1 - The standard curve of gallic acid for total phenolic content (TPC)

Total flavonoid content (TFC) was conducted utilizing the Aluminum-chloride method. Quercetin was used as standard to plot the standard curve, where a regression equation y = 0.001x + 0.0072 and a coefficient of determination (R^2) = 0.9909 was obtained as shown in Fig. 2. The total flavonoid content (TFC) obtained of the methanolic *Clitoria ternatea* leaf extract is 0.31333 ± 0.057735 mg QE/g as demonstrated in table 2.

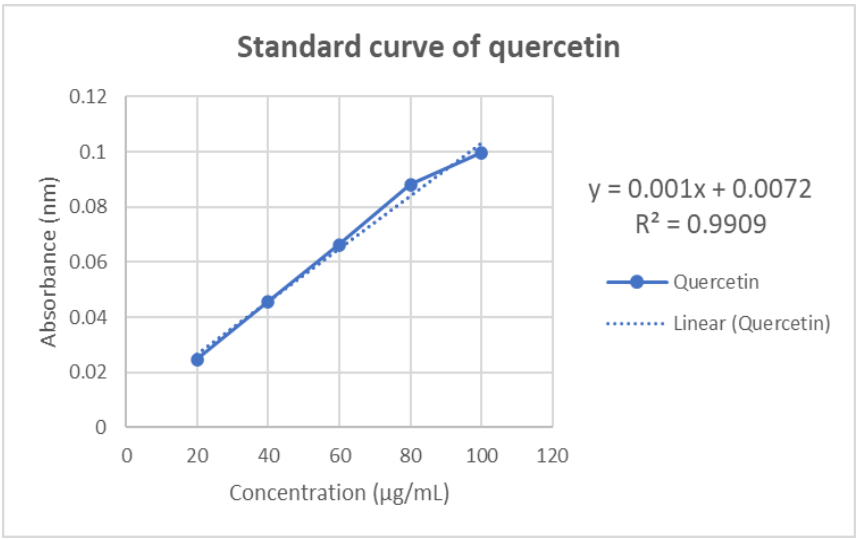


Fig. 2 - The standard curve of quercetin for total flavonoid content (TFC)

This study observed that the methanolic extract of *Clitoria ternatea* possesses higher total phenolic content (TPC) than total flavonoid content (TFC). This is consistent with precedent studies by [43], [55] and [56]. For instance, a previous study [43] found out that the total phenolic content (TPC) of *Clitoria ternatea* leaves methanolic extract is 73.03 ± 0.12 mg GAE/g extract, while the total flavonoid content (TFC) is 8.85 ± 0.13 mg QE/g extract. Besides, the hydro-methanolic extract of *Clitoria ternatea* leaves showed 25.92 ± 0.4 mg QE/g extract for total flavonoid content (TFC) and 75.21 mg GAE/g extract for total phenolic content (TPC). Another study [55] also showed that the methanolic extract of *Clitoria ternatea* leaves has higher total phenolic content (TPC) which is 358.99 ± 6.21 mg/g GAE than total flavonoid content (TFC) that is 123.75 ± 2.84 mg/g catechin equivalent (CE). The methanolic extract of blue-flowered *Clitoria ternatea* leaves possess higher content of total phenolic content (25mg GAE/ g dried weight) than total flavonoid content (10 mg QE/ g dried weight) [56]. This finding is theoretically normal where the total phenolic content (TPC) is higher than total flavonoid content (TFC) as flavonoids is considered a sub-class of the phenolic compounds, where there is other non-flavonoid phenolic compounds in the extract [57] [58].

If compared to precedent studies, it can be seen that the obtained value of total phenolic content (TPC) and total flavonoid content (TFC) in this study are different from others. There are a myriad of possibilities that can contribute to this scenario. For instance, the total phenolic content (TPC) in this study is 0.66116 ± 0.43455 mg GAE/g, it is comparative lower than value obtained by the similar study that extract *Clitoria ternatea* leaves using 70% methanol showed results of total phenolic content (TPC) of 64.8 ± 0.1 mg GAE/g plant material [59]. The utilization of hydro-

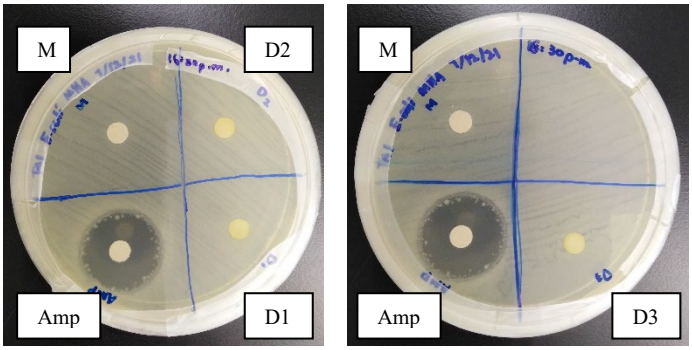
methanol has higher polarity than pure methanol, in which the polar phenolic compounds are more soluble in solvent with higher polarity [60]. During extraction, there is a tendency for the solvents to extract compounds according to their polarity as shown as a study on *Clitoria ternatea* petals [61]. The methanol as the polar solvent extracted hydrophilic substances including anthocyanins, kaempferol and quercetin, while the mixture of ethyl acetate and hexane as non-polar solvent had extracted hydrophobic tocopherols, phytosterols and fatty acids. *Clitoria ternatea* petals extraction also shows that the obtained total phenolic content (TPC) of petals extracted by the mixture of methanol and water in a 1:1 ratio is higher than petals that are extracted only by methanol [62]. While another previous study [63] showed that the total phenolic content (TPC) of methanolic *Clitoria ternatea* leaf extract obtained by Soxhlet apparatus is 22.63 ± 0.19 of gallic acid equivalent. There is a possibility that Soxhlet extraction method is more efficient in extracting phenolic compounds if compared to maceration. There has been precedent on the study of *Moringa oleifera* leaves, where Soxhlet extraction method showed higher total phenolic content (TPC) than the maceration method for an extraction time of 1 and 2 hours [64]. The heat applied during Soxhlet extraction might facilitate the breakdown of cell walls of plant materials and allow the release of bound phenolic compounds, thus increasing the efficiency of phenolic compounds extracted [65]. In addition, other content of phytochemicals such as sugars, carotenoids and ascorbic acid [66], or the method of extraction, the geographical variation and the duration of reaction can affect the phytochemical contents.

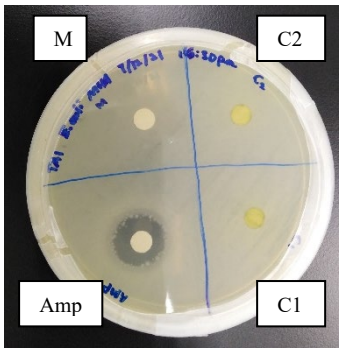
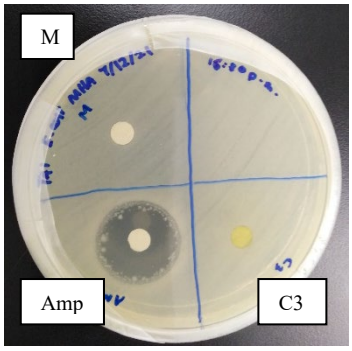
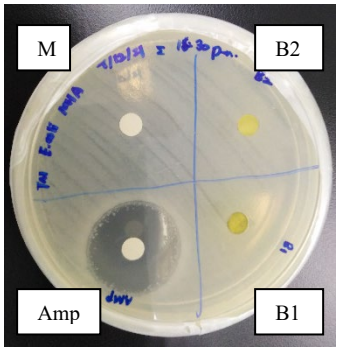
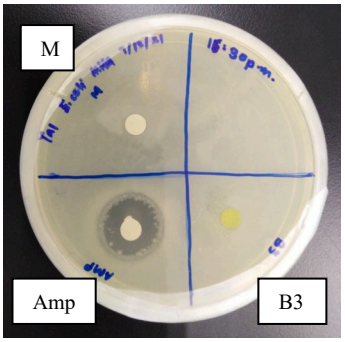
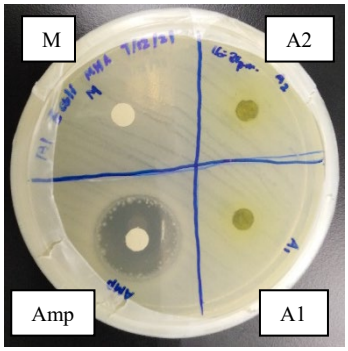
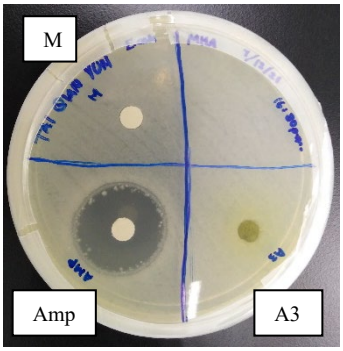
The biological factors such as DNA variation of plants in different geographical locations, and environmental factors can influence the phytochemical content in the plant, especially the leaves, which are more sensitive to the surrounding environmental conditions than other plant parts [67]. Even in the same country, various environmental factors such as climate, altitude, and amount of rainfall can influence the bioactive compounds available in the plant [68]. Thus, the variation of total flavonoid content (TFC) and total phenolic content (TPC) in this study and other precedents could be explained by the variation between plant materials, and extraction methods also play an important role. This can be supported by the study of [69] on the influence of methods of extraction toward total phenolic content (TPC) and total flavonoid content (TFC). The authors found out that manipulating parameters related to extraction such as properties of solvent, duration and temperature on the extraction of *Clitoria ternatea* flower affects the phenolic and flavonoid content. In order to increase the extraction efficiencies toward desirable phytochemicals, the nature of plant materials and the properties of targeted compounds should be taken into consideration when setting up optimal extraction parameters [70].

3.3 Antibacterial Activity

Table 3 and Table 4 show the result of antibacterial activity of *Clitoria ternatea* methanolic leaf extracts in different concentrations using the disk-diffusion method against *Escherichia coli,* a gram-negative bacterium that can cause gastrointestinal infections and diarrhea in humans. Positive control and negative control were placed on each plate, which are ampicillin and methanol solvent, respectively. As shown in Table 3, it can be clearly seen that all of the different concentrations of *Clitoria ternatea* methanolic leaf extracts showed no inhibition against *Escherichia coli*. The negative control shows no inhibitory activity, proving that the methanol solvent effect on the assay is negligible and does not interfere with the results.

Table 3 - Antibacterial activity of methanolic extract of *Clitoria ternatea* leaves against *Escherichia coli* in different concentrations

Concentration (mg/mL)	Samples
12.5	

25.0		
50.0		
100.0		

*Note: A1,A2,A3 = 100 mg/mL sample, B1,B2,B3 = 50 mg/mL sample, C1,C2,C3 = 25 mg/mL sample, D1,D2,D3 = 12.5 mg/mL sample, M = methanol, Amp = ampicillin

While only the positive control, which is the antibiotic ampicillin (0.5 mg/mL) showed inhibition against *Escherichia coli*. The edge of the inhibition zone exhibited by the ampicillin that possesses several sparse colonies of *Escherichia coli* might be due to the concentration of ampicillin (0.5 mg/mL) being not potent enough to completely inhibit the *Escherichia coli*. In addition, the nature of disk-diffusion that requires the diffusion of antibacterial substances to travel through the Mueller-Hinton Agar (MHA) will create a concentration gradient [71]. This will cause a decrease in the amount of antibiotics in a region further away from the discs. Variation in the inhibition zone created by ampicillin disks might be attributed to the improper pipetting technique when loading the ampicillin solution onto the paper disks. In addition, the condition of the Mueller-Hinton Agar (MHA) might also contribute to the variation in the inhibition zone. The variation in the amount of agar poured in the plate cause variation in agar thickness. Where agar thickness will affect the diffusion of antibiotic, with thicker agar showing smaller inhibition zone [72]. Based on Table 4, the inhibition zone of ampicillin is between 13 mm to 22 mm. The result of methanolic *Clitoria ternatea* leaf extract that shows no inhibition is different from a previous study, where the methanolic *C. ternatea* leaf extracts at 100 mg/mL concentration shown inhibitory activities against *Escherichia coli* when tested using disk-diffusion method [24].

Table 4 - The antibacterial activity measured in mm of methanolic *Clitoria ternatea* leaves extract against *Escherichia coli*, ampicillin and methanol solvent

Concentration (mg/mL)	Sample (Triplicate)	Diameter of inhibition zone (mm)		
		Sample	Ampicillin	Methanol
100	A1	-	19	-
	A2	-		
	A3	-	22	-
50	B1	-	22	-
	B2	-		
	B3	-	16	-
25	C1	-	13	-
	C2	-		
	C3	-	20	-
12.5	D1	-	20	-
	D2	-		
	D3	-	21	-

Another study showed that leaf extract of *C. ternatea* extracted using 60% methanol showed inhibitory activity towards *Escherichia coli* at every concentration using the disk-diffusion method [52]. Instead of using methanol as solvent, usage of solvents like water and acetone for *Clitoria ternatea* leaves extraction showed promising antibacterial effect on *Escherichia coli* [73]. *Clitoria ternatea* leaves extracted by ethanol, ethyl acetate and aqueous also showed inhibitory activities toward *Escherichia coli* at 100 mg/mL concentration, with ethanol exhibit maximum inhibitory activity [74]. *Clitoria ternatea* leaves extracted by petroleum ether, acetone and isopropyl alcohol also showed inhibitory activities against *Proteus mirabilis* [45]. These previous studies showed that different solvents used during the *Clitoria ternatea* leaves extraction could result in variation in terms of inhibitory zone. This might be attributed to the fact that distinct solvents can extract different antibacterial-related phytochemicals.

Then, all different concentrations (10, 20, and 30µl) of the methanolic *Clitoria ternatea* extracts obtained by Soxhlet extraction inhibit the growth of *Escherichia coli* and *Pseudomonas aeruginosa* when tested with agar well diffusion method [75]. Using well-diffusion methods, the methanolic extract of *Clitoria ternatea* leaf obtained by maceration showed inhibition against *Escherichia coli* [76]. The methanolic extract of *Clitoria ternatea* leaf using the maceration method showed inhibitory activity against *Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae* and *Salmonella typhi* when tested using agar well diffusion method [77]. However, when tested using the disk-diffusion method by different concentrations, only the first dilution (10^{-1}) showed inhibitory activity towards *Escherichia coli*, while the further diluted extracts did not inhibit the growth of the bacteria. Antibacterial assays such as the disk-diffusion method that relies on the diffusion of antibacterial substances like flavonoids might be inaccurate as some flavonoids with strong inhibitory activities against bacteria might possess a lower diffusion rate [78]. These four studies showed that the methanolic *Clitoria ternatea* extracts possess inhibitory activities against *Escherichia coli*, which are in contrast to this study. However, the use of different antibacterial activity assay might be contributing to the discrepancies of the result with previous studies.

There are also precedent studies that align with the findings of this study. For instance, the study by [56] showed that methanolic extraction of *C. ternatea* blue flowered leaf extracts obtained using Soxhlet extraction method showed no inhibitory activities against *Escherichia coli* and *S. aureus,* but can inhibit *Klebsiella pneumoniae* and *P. aeruginosa* at concentration of 14 mg and 10 mg respectively. A similar observation [25] shows that the methanolic extract of *Clitoria ternatea* leaves exhibit no antibacterial activities. The *C. ternatea* leaf with and without water stress treatments that are obtained by maceration method using methanol showed no inhibition against both gram-positive and gram-negative bacteria, specifically *Bacillus cereus* and *Pseudomonas aeruginosa.* The authors speculated that the concentration contributed to the non-inhibition zone observation, since only 20 mg/mL concentration was used in the study.

In addition, the acidified ethanolic extract of *Clitoria ternatea* also shows no inhibition activities toward *Escherichia coli* and *Yersinia enterocolitica* [46]. However, bacteria like *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* that are categorized as gram-positive bacteria had been inhibited by the extracts, the authors speculated that it is possible that negative bacteria possess an outer membrane in the cell wall that causes the extracts to be less permeable, thus less susceptible than gram-positive bacteria. *Bacillus cereus* and *Staphylococcus aureus* are more susceptible to the methanolic extracts of *Clitoria ternatea* leaves, indicated by larger zone of inhibition using disk diffusion assay if compared to gram-negative bacteria including *Klebsiella pneumonia*, *Proteus vulgaris*, and *Salmonella typhi* [79]. In addition, previous study [77] showed that generally gram-positive bacteria like *Staphylococcus aureus* are more susceptible to the methanolic *Clitoria ternatea* leaf extracts if compared to gram-negative bacteria. This might be a possible reason why the methanolic *Clitoria ternatea* leaf extract is not potent

enough to inhibit the gram-negative bacterium *Escherichia coli.* This is due to the inherent cell membrane structure of gram-negative bacteria that provide less permeability for plant extracts [80].

The discrepancy of results present in the different antibacterial studies of *Clitoria ternatea* leaves were observed through the literature reviewed in the previous paragraphs. A similar observation was made by the study of [81] where the authors found that discrepancies of results with previous literature might be caused by different methodologies, and possibility that active antibacterial compounds were not extracted. Similar conclusion had also been drawn by Biswas *et al.* [82] through the study on *Psidium guajava* L. leaf extracts, where the authors found out that their results aligned and at the same time contrasted to other similar studies on *P. guajava* L leaf extracts. Therefore, the authors concluded that different methodologies used in the studies would create different results, and the active substances contributing to antimicrobial activity of the plant crude extracts should be further investigated. In the case of *Clitoria ternatea*, there is a protein known as "finotin" that is isolated from the water extract of *Clitoria ternatea* seeds that has been identified to be responsible for antifungal, antibacterial and insecticidal properties [83]. While for the flowers of *Clitoria ternatea*, anthocyanin known as ternatin found in *C. ternatea* flower extract is reported to be the active substance contributing to antimicrobial activity [46] [84]. Cyclotides isolated from the whole plant of *Clitoria ternatea* inhibit the gram-negative bacteria including *Pseudomonas* aeruginosa, *Escherichia coli*, and *Klebsiella pneumonia* using radial diffusion assay [85]. However, the active substance that contributes to the antibacterial activity of *C. ternatea* leaf extracts has not yet been investigated in depth. Quercetin that is found in the leaves of *Clitoria ternatea* is only speculated to be responsible for the antibacterial activities of *Clitoria ternatea* [74]. Further investigation and isolation of active substances that are responsible for the antimicrobial activities of *Clitoria ternatea* crude extracts is also recommended [24].

In order to increase the sensitivity of biological assay, [86] stated that it is required to increase the concentration of targeted compounds in the sample. While it is being proven that the total phenolic content (TPC) and total flavonoid content (TFC) are related to the antibacterial activity [54], thus increasing the concentration of extracts used can naturally increase the possibility of exhibiting inhibitory activities against bacteria. The result of this study that showed no inhibitory activities of methanolic *Clitoria ternatea* leaves against *Escherichia coli* might be related to the lack of potency of the concentrations (12.5, 25, 50, 100 mg/mL) used. Besides, the methanolic crude extract of *Clitoria ternatea* leaves had been proven to possess low toxicity and require dosage more than 2g/kg mice body weight in oral administration to cause toxic effect [87]. Therefore, the lack of potency of the extracts used in this study against *Escherichia coli* is reasonable and partially proved that concentration of extracts in antibacterial assay is important. Through the review of related previous studies, the result of this study aligned with some of the precedents while in opposition to others. Undeniably, it is the differences between methodologies in terms of antibacterial assay and plant sample extraction that leads to the differences in obtained results. The disk-diffusion antibacterial assay is an inexpensive and convenient way to qualitatively determine the antibacterial activities of plant extracts. However, the method is not without its limitations, for instance the difficulty of potential antibacterial substances to diffuse through the Mueller-Hinton Agar (MHA) might contribute to the result of this study showing no inhibition for *Escherichia coli*. In terms of plant sample extraction, different extraction methodologies including solvent type, solvent concentration and extraction methods will result in different extraction results, and not to be forgotten is the origin of *Clitoria ternatea* plant materials used in the studies are from different regions in the world. This has proved that methodologies are crucial in plant extract antibacterial assay, where some methodologies are shown to be less effective in extracting antibacterial related phytochemicals. The different environmental factors and variation between *Clitoria ternatea* population could also contribute to the availability of different phytochemicals in the plant. There is also a possibility that the active antibacterial compounds are absent or in an amount too small that shows no potency in the plant extract due to a myriad of reasons. Several possible factors that could affect the antibacterial assay of this study in terms of methodologies have been identified, however the time constraint posed has been limiting this study. Further study to verify the effects of the possible factors on the antibacterial activities of *Clitoria ternatea* is recommended.

4. Conclusion

The study had quantitatively determined the content of phenolic compounds and flavonoids for methanolic *Clitoria terntea* leaves extract, where the TPC is 0.66116 ± 0.43455 GAE mg/g and TFC is 0.31333 ±0.057735 QE mg/g. It is being identified that extraction methods including polarity of extraction solvent and plant materials can affect the phytochemical content of plants. The study had also identified several possible factors that negatively affect the antibacterial assay of *Clitoria ternatea* extracts and provided valuable information in related fields for future researchers to be aware of and actions can be taken to avoid undesirable results. All of the four concentrations of *C. ternatea* leaves methanolic extract showed no inhibitory activities against *Escherichia coli.* Several possibilities had been identified, for instance the lack of potency of the extracts associated with the low content of flavonoid and phenolic compounds. Thus, it is partially proved that the concentration of plant extracts used in antibacterial assay is crucial, especially plant extract with low toxicity such as *C. ternatea*. Among the many different types of antibacterial assay, disk-diffusion is not without its limits, since the nature of antibacterial phytochemicals could affect their diffusion capacity, and thus influence the result of antibacterial assay. Diverse results have been observed from the different antibacterial studies on *C. ternatea* due to the differences in methodologies, there is also a possibility that the antibacterial substances in *C. ternatea* leaves are not extracted or in an amount too small to be effective. Since it is

found that a myriad of factors could affect the results of the antibacterial assay, it is still premature to claim that *C. ternatea* has a low potential as a medicinal plant that exhibits antibacterial activity. It should be emphasized that there is no definite result that proves *C. ternatea* leaves extracts pose no inhibitory activities against diarrhea-causing bacteria like *E. coli*. This study act as one that attempt to broaden the uses of one of the many traditional medicinal plants, so that more attention can be attracted toward the plants in Malaysia and their usefulness can be acknowledged. This can indirectly contribute to the conservation of biodiversity since actions will definitely be taken in order to sustainably utilizing the medicinal plants to ensure their future availability, and indirectly contribute to the conservation of traditional knowledge.

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