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Extraction of anthocyanins from *Clitoria ternatea L.* petals in Vietnam and determination of its antioxidant and antimicrobial activities

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ABSTRACT

In this study, the effects of various factors on anthocyanins extracted from *ClitoriaTernatea L.* petals were determined. In addition, phytochemical properties, antioxidant ability, antimicrobial capacity, and application of anthocyanins extractions were also investigated. As a result, the highest proportion of anthocyanin, 1.21 mg/g fresh weight (FW), was obtained when extracting *ClitoriaTernatea L.* petals with 100% methanol, with the solvent/sampleratio of 10ml/g at 37°C for an hour. The highest total phenolic content (TPC) was $24.7x10^3 \pm 8.55x10^2$ µg gallic acid equivalent (GAE)/g FW, while the figures for total flavonoid content was 18.8±0.149 mg quercetin equivalent (QE)/g FW. The values for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity and the highest total antioxidant activity were EC_{50}=904.1µg/ml and $2.86x10^3 \pm 1.01x10^1$ µg ascorbic acid equivalent (AAE)/g FW, respectively. The inhibitions to *Staphylococcus aureus*, *Streptococcus mutans,* and *Pseudomonas aeruginosa* were shown with the minimum inhibitory concentration (MIC) of 90 mg/ml and 180 mg/ml. It also showed that the *ClitoriaTernatea L.* petal extract also had the potential to be developed into a pH indicator, however, further study should be carried out to obtain a highly qualified one.

Keywords: *Clitoria Ternatea L.* petal, anthocyanins, optimization, pH indicator, phytochemical properties.

INTRODUCTION

Clitoria Ternatea Linn – first called by Breyne, common named butterfly pea, is a plant belonging to a vine family, *Fabaceae*. The flower of *Clitoria Ternatea L.*, which can be blue or white color, is reported to contain numerous substances. Soluble minerals and soluble carbohydrates with high concentration, phenolics and flavonoids were found this kind of flower. Anthocyanins, the substances that contributed to the blue color of the petals, have been found. They are the acylated form, which has potentially high stability,

In addition to being used as food colorant and dyes, the flower has been used for the ornamental purpose all over the world. It is also proven for pharmacological activities and used as an Ayurvedic medicine for thousands of years. Its extract showed significant results in anticancer to different cell lines using the trypan blue dye exclusion method. The blue line flower also showed remarkable antioxidant activity to 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) [1-5].

Anthocyanins, which are one of the flavonoids' sub-groups, are water-soluble natural colorants. They contribute to the majority of pigments discovered in plants from orange, red to blue. They can be predominantly found in fruits of berries, apples, grapes, and others, flower petals, and partly found in other parts of the plants namely root or leaves [6]. The anthocyanins obtained from different sources are also of great variance. In addition to increasing

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animal attractions, anthocyanins also benefit human's health. They have been claimed for the potential of being food colorants and food additives due to their safety and high stability, while other articles have pointed out that they could show inhibition to cancer and chronic symptoms. Anthocyanins are easily affected by pH, light, temperature, metals, copigmentation and other substances [3,6]. Based on the effect of pH on anthocyanins color, pH indicators developed from its extract was considered [7].

Clitoria Ternatea L. flower is usually used as food colorant in addition to the use as tea ingredient. There is no official document for the Vietnamese traditional used of this flower published internationally.

The essence of this study was to investigate in the total anthocyanin content at the optimal condition of *Clitoria Ternatea L.* flower growing in Vietnam. Several characteristics of the extract at this condition included total phenolic content, total flavonoid content, antioxidant and antimicrobial activities were also determined. The pH sensitivity of this petal was also evaluated in order to develop a pH indicator.

Materials and methods

Materials

After pretreatment, only the *Clitoria Ternatea L.* petals collected in Phuong Lam, Dong Nai province, Vietnam were kept and stored at -20ºC until performing the experiment.

Screening for anthocyanins sources

Fresh and dried samples were screened for their anthocyanin content [8,9]. The samples were macerated in 100% methanol containing 0.1% (v/v) concentrated hydrochloric acid with a ratio of solvent/sample of 5ml/g at 37ºC for 1 hour. The extracts were then centrifuged and diluted with the mixture of ethanol: 1.5N hydrochloric acid (85:15 v/v). The absorbance at 535 nm was then measured by the followed formula and expressed as mg total anthocyanin content (TAC)/g fresh weight (FW):

Total anthocyanin content (TAC) (mg/g FW) =

$$\frac{A_{535nm}\ x\ dilution\ factor}{98.2\ x\ weight\ of\ fresh\ sample}$$

In which: A_{535nm} was the absorbance of the solution, 98.2 was the extinction coefficient value of cranberry anthocyanin in the mixture of ethanol and hydrochloric acid (mg/ml).

Components of the extracts were then analyzed using thin-layer chromatography (TLC) technique on cellulose plate and Forestal, glacial acetic acid : hydrochlori c acid : water (30:3:10), as the mobile phase [19].

Optimization of anthocyanins extraction

In this part, the effects of several parameters, including time of extraction, temperature, solvent/sample ratio, types of solvent and solvent concentration, on TAC would be studied**.** Sample containing a higher total anthocyanin proportion in the previous part was used to optimize the anthocyanins extraction. The extraction would follow the modified methods [8,9]. The sample was initially extracted with 100% methanol containing 0.1% (v/v) concentrated hydrochloric acid with a ratio of solvent/sample of 5ml/g at 37ºC for various durations of extraction. The optimal condition of one factor would be subsequently used to study the following factors. Its TAC was determined as mentioned in the previous part to determine the optimal conditions for each factor.

Characterization of the extract of Clitoria Ternatea L. petals

TPC, TFC, DPPH scavenging capacity and total antioxidant activity test procedures were adopted from Yadav and colleagues' work in 2017 with some modifications [10].

Determination of total phenolic content (TPC)

A 250µL aliquot of Folin-Ciocalteau's reagent and 250µL of 7.5% sodium carbonate solution were added to 100µL Gallic acid solutions (6.25-100 µg/mL) and incubated for 30 minutes at room temperature (RT) before measuring the absorbance at 765 nm. The process was applied to the extracts. Their TPC was expressed as µg GAE/ g FW.

Determination of total flavonoid content (TFC)

A mixture of 100µL of the Quercetin solution (0.75-10. mg/ml), 300µL methanol, 20µL of 10% Aluminum Chloride, 20µL ml of potassium acetate and 560µL of distilled water

was incubated at RT for 30 minutes, and then measured at 420nm. The procedure was applied to the extracts to evaluate the TFC of the extract expressed as mg QE/g FW.

Determination of antioxidant capacity

DPPH scavenging capacity: An amount of 0.75ml of 0.0067% DPPH solution was allowed to react with 0.25ml of extract or L-ascorbic acid at various concentrations for 20 minutes. The absorbance was measured at 517nm. The results were displayed as EC_{50}.

Total antioxidant activity: An amount of 0.1 ml of ascorbic acid (6.25-100 µg/mL) was allowed to react with 1ml of reagent solution at 95ºC for 90 minutes followed by absorbance measurement at 695nm. The same process was applied to the extracts. Its total antioxidant capacity was determined and then represented as µg AAE/g FW.

Determination of antimicrobial activity

Antimicrobial activity of the extracts was evaluated by employed procedure with modifications[11,12]. Discs containing the extracts with the concentration of 180 mg/ml were utilized to determine antimicrobial activity on plates spreading bacteria, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus*, *Streptococcus mutans*, *Pseudomonas aeruginosa*, while appropriate antibiotics were used as positive control. MIC was determined using discs containing extracts with various concentrations. The diameters of the clear zones were used in order to determine the antimicrobial power as well as the MIC of the extracts after overnight incubation at 37ºC.

Determination of pH sensitivity

pH indicators was developed as described by Syahirah L et al. (2018) [13]**.** For liquid pH indicator, color changes in pH 1 to 13 solutions after adding water extract of *Clitoria Ternatea L.* were recorded. While colors of pH paper containing the extract with pH 1 to 13 solutions was compared with those developed on the universal pH test paper strips.

Statistical analysis

All the experiments were triplicated. The results were then analyzed with t-test or ANOVA one-way and Turkey's Test using SPSS.

Results and Discussion

Screening for anthocyanins sources

The chart displayed the fresh sample had a statistically higher potential of TAC (1.056±0.0361 mg/g sample) than that of dried sample ($0.319\pm9.07\times10^{-3}$ mg/g sample) with an equivalent proportion, **Figure 1** ($p<0.05$). When analyzed with TLC, the fresh sample was confirmed to contain more compounds with higher concentration compared with the dried one. Their R_f was approximately similar, which was 0.813, **figure 2**.

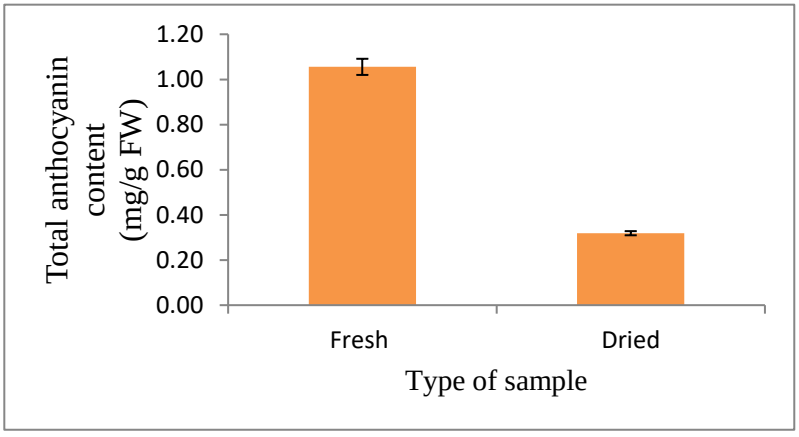


Figure 1. Total anthocyanin content of the fresh and dried samples

Figure 2. TLC result of (F) fresh sample and (D) dried sample

The difference could be caused by the pretreatment of samples. While the fresh petals were collected and stored at -20°C, the petals were dried at 40°C and ground. This treatment process might have significantly destroyed the TAC in the sample, which sensitive to temperature [3]. The brighter and broader band for fresh sample and the narrow and pale band for the dried sample in TLC, even though both bands had the same R_f value, indicating that the fresh sample had more anthocyanins with higher concentrations. Thus, fresh petals of *Clitoria Ternatea L.* were selected.

Optimization of anthocyanins extraction

As shown in **figure 3**, the sample extracted for 1 hour showed the highest yield of anthocyanins ($p<0.05$), followed by that of extraction for 1.5 hours. The optimal time extraction was similar to the one mentioned in a study in 2008 [14], however, was different from other research. The optimal duration for anthocyanins extraction was 1.5 hour [8,15], whereas it was reported to be up to 2.5 hours [16]. The extraction time of one hour was considered as the optimized time in order to evaluate other factor effects.

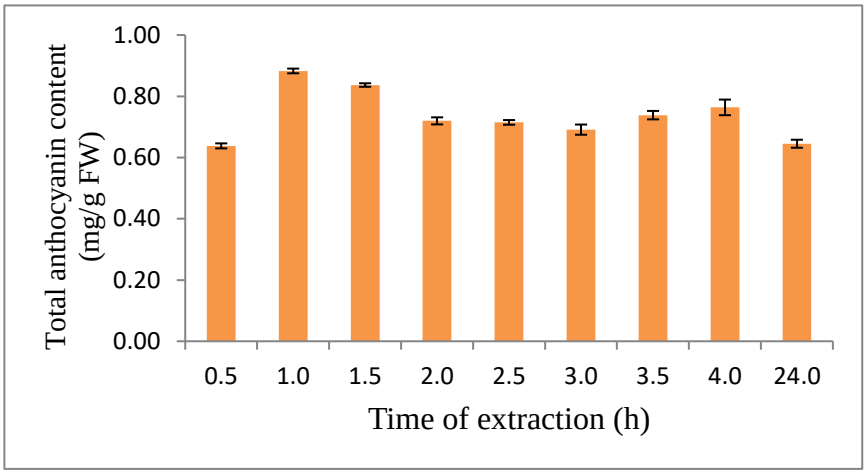


Figure 3. Total anthocyanin content of the fresh sample under the effect of time extraction

The optimal time extraction was applied with other initial conditions to investigate the effect of temperature on TAC. An increase in temperature from 4ºC to 37ºC resulted in an increase in TAC, the TAC extracted at 37ºC was the highest one (0.883 ± 7.64 x 10^{-3} mg/g FW) (P<0.05), **figure 4**. However, this acceleration was reversed when the temperature continued to reach higher, there was no significant difference among total anthocyanin content extract at other temperatures (p<0.05). This could be explained by the hydrolyzation of 3-glucoside structure and pyllirium ring [17]. The effective temperature for anthocyanins extraction about 35ºC was reported [15], meanwhile the study in 2008 concluded that extraction at 80ºC would bring the highest yield [14].

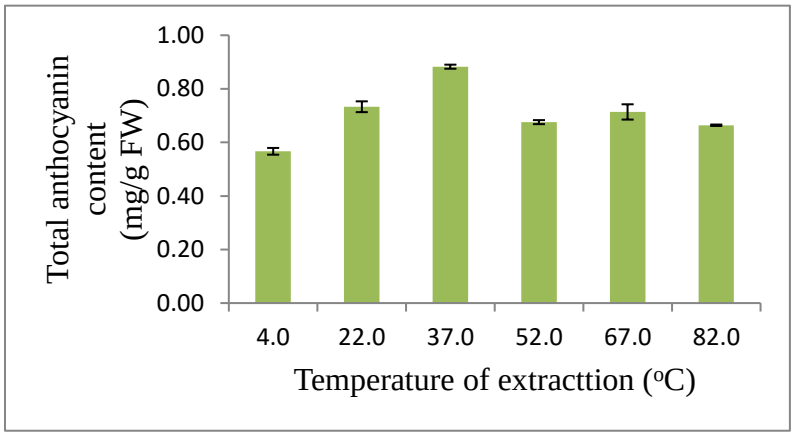


Figure 4. Total anthocyanin content of the fresh sample at different temperature

The effect of solvent/sample ratio, which was reported to affect the efficiency of anthocyanins extraction according to Cacace & Mazza (2003) [15], was identified under time extraction of 1 hour at 37ºC. The indifferences between extracts with different ratio of solvent/sample, except for the one extracted with the ratio of 10 ml/g, the highest one (1.21±4.61x10^{-2} mg/g FW) (p<0.05), **figure 5**, led to a conclusion that a reduction in TAC might be because high solvent/sample ratio could have caused further dilution, leading to a drop in the reading. This ratio was similar to the one reported in the study of Blackhall et al. (2018) [8], whereas this ratio was 15ml/g in another study [16].

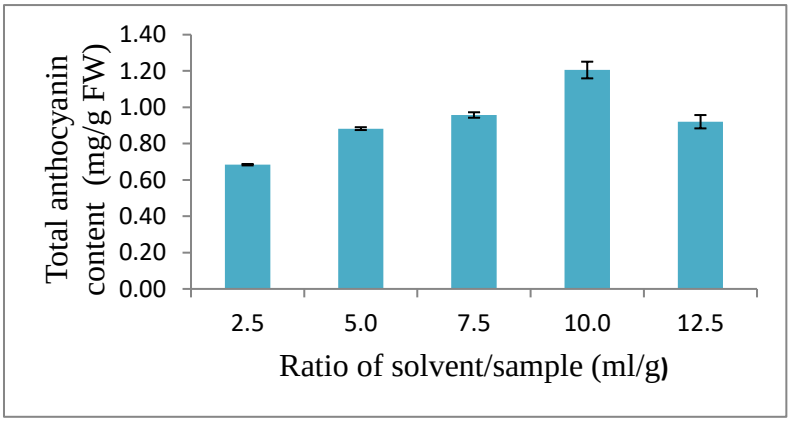


Figure 5 Total anthocyanin content of the fresh sample with variety ratio of solvent/sample

The suitable solvent would give potential result in anthocyanins extraction. Alcohols, especially methanol and ethanol containing low concentration of acid, were advised to be used due to their cell membrane denaturation property and provision a suitable medium for flavylium ions, a form of anthocyanin. Ethanol was preferred due to its safety for human consumption, especially in food industry [18,19]. In **figure 6** methanol containing 0.1% v/v concentrated HCl resulted in high and significant different TAC yield, $1.21\pm4.61x10^{-2}$ mg/g FW in compared to ethanol containing 0.1% v/v concentrated HCl, $0.967\pm1.05x10^{-2}$ mg/g FW ($p<0.05$). The ethanol solution, however, was still being used to identify the optimal conditions for future application in the food industry.

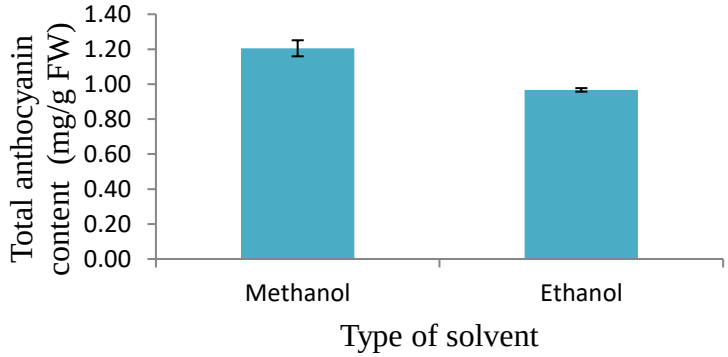


Figure 6. Total anthocyanin content of the fresh sample extracted by methanol and ethanol

The effect of concentration of solvent was carried out, subsequently. Water with various proportions were advised to be added to extract anthocyanins completely [6,18]. **Figure 7** showed that the extract with solution of 100% methanol yielded the highest TAC, followed by the extract with 100% ethanol mixture. There was no significant difference between total anthocyanin content extracting with other concentrations ($p<0.05$). The results were different from previous studies which could be due to different materials used in anthocyanins extraction. The exact explanation for this was still unknown.

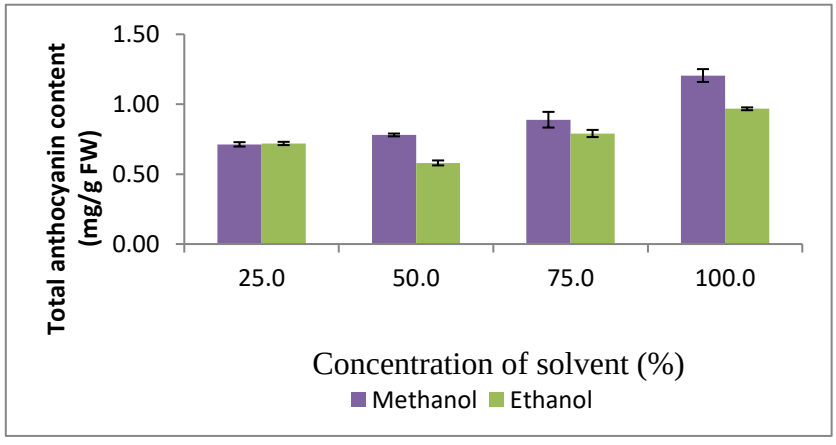
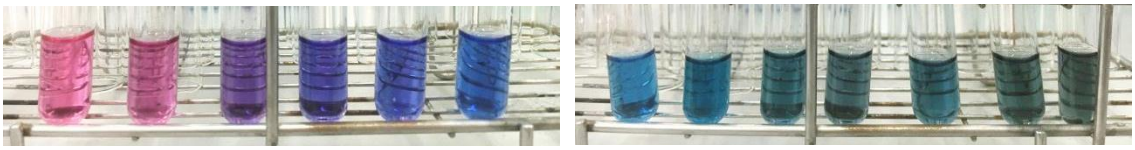


Figure 7. Total anthocyanin content of fresh sample extracted by various concentrations of methanol and ethanol



(a) (b)

Figure 8. Result of liquid pH indicator. (a) Color developed in pH 1 to 6. (b) Color developed in pH 7 to 13

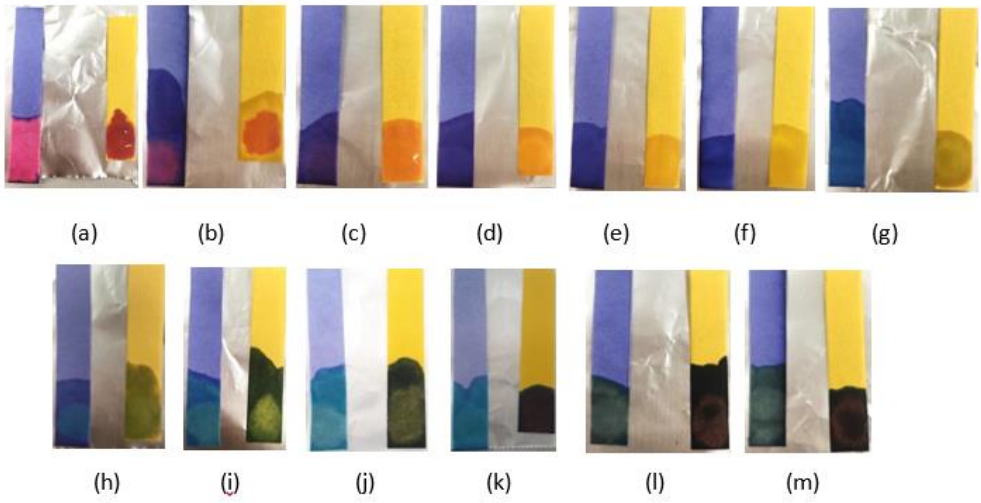


Figure 9 .Color changing indicated by pH paper and universal pH indicator respectively. From (a) to (m) color developed in pH 1 to 13

(a) (b) (c)

Figure 10 Results of antimicrobial activity of methanol extract against *Staphylococcus Aureus*. (a) methanol extract. (b) positive control. (c) negative control. The determination of antimicrobial activities of methanol extract and ethanol extract against other bacterial strains were performed similarly

Consequently, *Clitoria Ternatea L.* petal should be extracted for one hour at 37ºC with the solvent/sample ratio of 10ml/g of 100% methanol with 0.1% concentrated HCl to obtain an optimal amount of anthocyanins. Extract under these conditions using ethanol would yield the most efficient anthocyanins in food industry. Both extracts would then be evaluated for other characteristics in this study.

Characterization of the extract of Clitoria Ternatea L. petal

Determination of total phenolic content (TPC)

Phenolic compounds are compounds that are widely distributed in plants with several properties such as antioxidant, antimicrobial and health benefits [20-21].The means of TPC of two extracts were mentioned in **table 1**. TPC of methanol extract and ethanol extract was calculated from the linear regression equation derived from the standard curve of Gallic acid, $R^2 = 0.9986$. There was significantly higher TPC in methanol extract ($24.7x10^3±8.55x10^2$ µg GAE/g FW)and compare to that of ethanol extract ($18.8x10^3±2.46x10^2$ µg GAE/g FW) ($p<0.05$). This suggested that various types of solvent also affect the phenolic content. In this case, since methanol was more polar than ethanol, solvent polarity could have resulted in different amounts of TPC. These figures were slightly lower than the reported 26.72±2.17 mg GAE/g of dry flower[22] andmuch lower than the reported figure of 76.90 mg GAE/g FEW [20]. However, these data were higher than TPC of other anthocyanins sources, 2087.43±17.37 mg GAE/100 g FW[23] and 294mg GAE/100 g FW[24].

Table 1. Total phenolic content, total flavonoid content and total antioxidant activities of the extracts.

	Methanol extract	Ethanol extract
Total phenolic content (µg GAE/g FW)	$24.7x10^3±8.55x10^2$	$18.8x10^3±2.46x10^2$
Total flavonoid content (mg QE/ g FW)	18.8±0.149	15.5±0.091
Total antioxidant capacity (µg AAE/g FW)	$2.86x10^3±1.01x10^1$	$2.57x10^3±2.50x10^1$
DPPH scavenging capacity (EC_{50}) µg/ml	904.2±0.327	904.0±0.581
Total antioxidant activity (µg AAE/g FW)	$2.86x10^3±1.01x10^1$	$2.57x10^3±2.50x10^1$

Determination of total flavonoid content (TFC)

The proportion of flavonoids, the largest group of plant phenolic compounds [19], was observed in these extracts. The reliable data for TFC of the extracts, which were quantified by the standard curve of Quercetin with $R^2 = 0.9995$ were listed in **table 1**. It revealed that means of TFC of methanol extract was 18.8±0.149 mg QE/g FW, significant different compared to that of ethanol extract, 15.5±0.091 mg QE/g FW. Similar to figure for TPC, the TFC figure of methanol extract was also significantly higher than that of ethanol extract in spite of the same sources which suggested a further study on the effect of solvents on proportion of phenolic and flavonoid in *Clitoria Ternatea L.* The figure for TFC of methanol extract was higher than the reported one, 16.44 ± 0.02 mg QE/g FW [24], while TFC of ethanol extract was slightly lower.

Determination of antioxidant capacity

DPPH scavenging capacity: The DPPH radical was neutralized by the antioxidants, which caused the discoloration measuring at 517 nm. The efficacy of antioxidants was then expressed as EC_{50}, which was the antioxidant concentration that could reduce the concentration of DPPH by 50% [25]. The efficient concentration for scavenging of 50% DPPH (EC_{50}) of both extracts was shown in **table 1.** There was no significant difference between the EC_{50} of methanol extract and that of ethanol extract ($p<0.05$). The results could be affected by technical skills. The EC_{50} was higher than 0.76±0.03 mg/ml [24], and 84.15±1.50 µg/ml[26] which could be blamed on the differences in anthocyanin content and phenolic content. This meant that the Clitoria

Ternatea L. petals collected in Phuong Lam, Dong Nai province had less antioxidant ability than the petals harvested in Malaysia and Thailand, respectively. .

Total antioxidant activity: By measuring the absorbance of phosphomolybdenum complex produced after reducing Mo(VI) to Mo(IV), the ability of antioxidant would be determined [27]. The antioxidant activity of methanol extract ($2.86x10^3±1.01x10^1$ µg AAE/g FW) was presented in **table 1**, while that figure for ethanol extract was $2.57x10^3±2.50x10^1$ µg AAE/g FW ($p<0.05$). Methanol extract had a significantly higher antioxidant ability than ethanol extract. This difference could be from the difference in the proportion of anthocyanins, and phenolic compounds content of these extracts. This was unsurprising and data was consistent with prior findings.

Determination of antimicrobial activity

Methanol extract presented more effective antimicrobial inhibition to *Staphylococcus aureus,* and *Pseudomonas aeruginosa* compared to ethanol extract, except for *Streptococcus mutans* which they showed the same antimicrobial ability ($p<0.05$), **table 2**. The inhibition zones of these extracts were much smaller than those of the appropriate antibiotics. Leong et al. (2017) also reported that *Clitoria Ternatea L.* extract did not show inhibition to Escherichia coli [28]. However, an opposite result was found [2,12]. While MICs against *Staphylococcus aureus*, *Streptococcus mutans* were the same for both extracts, 90 mg/ml, the MIC of ethanol extract against *Pseudomonas aeruginosa*, 180 mg/ml, doubled that of the methanol extract. It could be claimed that both methanol extract and ethanol extract had the same inhibitory strength to *Staphylococcus Aureus,* and *Streptococcus mutans*. Methanol extract presented the greater inhibitory strength to *Pseudomonas aeruginosa* than ethanol extract. The MIC for *Staphylococcus aureus* was lower, the MIC for *Pseudomonas aeruginosa* was higher than the reported one [12]. This could be caused by the use of different strains of the bacterial species and their unknowingly developed resistance.

Table 2.Antimicrobial activities of Clitoria Ternatea L. extracts

	Zone of growth inhibition (mm)		
	Positive control	**Methanol extract**	**Ethanol extract**
Escherichia coli	17.9±0.404	-	-
Salmonella typhi	27.4±0.306	-	-
Staphylococcus aureus	32.2±0.152	15.5±0.351	10.5±0.153
Streptococcus mutans	35.2±0.451	11.4±0.100	11.3±0.208
Pseudomonas aeruginosa	36.0±0.208	11.4±0.322	8.40±0.173

'-' inhibition zone was not recorded

Determination of pH sensitivity

In acidic media, flavyliumcation was predominant, causing the solution to turn red. By increasing the pH, the form of carbinolpseudobase (colorless) of anthocyanins increased while there was a decrease in flavylium form that led to the fade in color at pH 4 to 5. Above pH 5, quinonoidal structure was the dominant responding of the color. This structure would turn to be chalcone structure when pH was over 7. Chalcone, colorless, could contribute to pale yellow color development. However, it was unstable [7,29-32] . The result in color changes for pH over 7 was found to be inconsistent, the reason remained unknown.The petals were extracted with water with pH adjusted to 7. Colors developed in liquid pH indicator showed different shades which could be distinguished from one another, changing from light pink to purple, then blue and turquoise accompanied pH changed from 1 to 11, except for the colors of buffer pH 12 and pH 13, turquoise

hue with the yellowish shade, that could not be distinguished instantly. The yellow shade disappeared immediately after few seconds adding the extract. This observation was quite different from the reported ones by TawadchaiSuppadit (2011) [33], who found the yellowish color at pH 12 and a light yellow color at pH 13, and yellow color at pH 12 by Abdullah and colleagues[34]. On the other hand, the pH paper showed longer lasting and more distinguishable colors for pH 3, 4 and 5 in compared to that of universal pH paper. However, it was better to use universal pH paper to differentiate pH 7 and 8. The pH paper showed clear different colors for pH 12 and 13, which was yellow with dissimilar shades. These colors faded after a few seconds exposed to the atmosphere, which can be caused by the instability of chalcone structure. Further study should be carried out in order to develop a stable pH indicator from natural sources.

Conclusion

In conclusion, the optimal extraction condition of *Clitoria Ternatea L.* petals was determined. The utilization of methanol as solvent yielded more effective result. However, ethanol could be applied to extract anthocyanins in food industry with comparable characteristics. It showed that the extracts also containing other phenolics and flavonoids, had noticeable antioxidant activity and antimicrobial ability. Slight differences between liquid pH indicator and pH paper showed, further study should be performed to develop a more stable and reliable pH indicator from natural sources.

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استخراج الأنثوسيانين من بتلات *Clitoria ternatea* L. في فيتنام وتحديد أنشطتها المضادة للأكسدة والميكروبات

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ملخص

في هذه الدراسة تم تحديد تأثير العوامل المختلفة على الانثوسيانين المستخرج من بتلات *ClitoriaTernatea* L. بالإضافة إلى ذلك، تم فحص الخصائص الكيميائية النباتية، والقدرةالمضادة للأكسدة، والقدرة المضادة للميكروبات، وتطبيق مستخلصات الأنثوسيانين .نتيجة لذلك، تم الحصول على أعلى نسبة من الأنثوسيانين، 1.21 مجم / جرام وزن طازج(FW) ، عند استخراج بتلات *ClitoriaTernatea* L. مع100 ٪ ميثانول، مع مذيب / عينة 10 مل / جمعند 37 درجة مئوية لمدة ساعة .أعلى محتوى فينولي إجمالي (TPC) كان 8.55x102± 24.7x103 ميكروغرام مكافئ حمض الغاليك / (GAE) جمFW ، بينما كانت أرقام إجماليمحتوى الفلافونويد 0.149 ± 18.8 مجم مكافئ كيرسيتين / (QE) جم .FW كانت قيم قدرة الكسح2 ،2- diphenyl-1-picrylhydrazyl (DPPH) وأعلى نشاط إجمالي لمضادات الأكسدة هي EC50 = 904.1 ميكروغرام / مل و 1.01x101 ± 2.86x103 ميكروغرام مكافئ حمض الأسكوربيك / (AAE) جمFW ، على التوالي. تم عرض مثبطات Staphylococcus aureusو Streptococcus mutans و Pseudomonas aeruginosa بأقل تركيز مثبط (MIC) يبلغ 90 مجم / مل و 180 مجم / مل .وأظهرت أيضًا أن مستخلص بتلة *ClitoriaTernatea* L. لديه أيضًا إمكانية تطويره إلى مؤشر الأسالهيدروجيني، ومع ذلك، يجب إجراء مزيد من الدراسة للحصول على واحد مؤهل تأهيلا عاليا.

الكلمات الدالة: *ClitoriaTernatea* L. :البتلة، الأنثوسيانين، التحسن، مؤشر الأس الهيدروجيني، الخواص الكيميائية النباتية.

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