

Biological activity of the *Clitoria ternatea* flower aqueous extract planted in Binh Duong province, Vietnam

Hung, T.N. and *Manh, T.D.

Institute of Applied Technology, Thu Dau Mot University, Thu Dau Mot City, Binh Duong, 820000, Vietnam

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Abstract

Clitoria ternatea L. is commonly called the blue pea flower, an herbaceous plant that is used for traditional medicine, natural food colorants and beverages thanks to its antioxidant properties and the consequent health benefits. This study aimed to evaluate the bioactivity and safety of *Clitoria ternatea* flower (CTF) aqueous extracts. In this study, we evaluated the biological activity of CTF aqueous extracts at concentrations from 20 to 120 µg/mL through the test of 1,1-diphenyl-2-picrylhydrazyl (DPPH), ACE inhibition, antibacterial activity and cell viability assay to test for HEK.293 nephrotoxicity. The results indicated that CTF aqueous extract exhibited the highest antioxidant activity and ACE inhibitor activity at the concentration of 120 µg/mL. Besides, the antibacterial activity of the aqueous extract against *Staphylococcus aureus* and *Bacillus* sp. at 80 µg/mL concentration was similar to that of 120 µg/mL. The aqueous extract at 120 µg/mL did not exhibit cytotoxic effects on HEK.293 cells. The results of this study suggested that CTF can be used as a natural beverage, a kind of tea to improve human health.

1. Introduction

Clitoria ternatea L. (butterfly pea or blue pea flower) is a perennial herbaceous plant from the Fabaceae family (Mukherjee *et al.*, 2008). This plant is popularly distributed in tropical regions such as India, the Philippines, and Vietnam. In Vietnam, it is mainly grown as ornamental plants around the house in Southern Vietnam, including Binh Duong. Different parts of this plant have been used in traditional medicine and Ayurvedic medicine in some Asian countries such as China and Sri Lanka to treat a variety of disorders such as anasarca, ascites, hemicrania, liver problems, fever, inflammation, pain and enlargement of abdominal viscera (Mukherjee *et al.*, 2008; Jeyaraj *et al.*, 2021). In addition, it was also used as a natural food colorant (Havananda and Luengwilai, 2019; Oguis *et al.*, 2019), a functional natural beverage to manage oxidative stress associated with chronic diseases (Lakshan *et al.*, 2019), drunk daily as a kind of tea for preventing aging and hazards of heart disease and used in husbandry, a forage. The crude flower extract has been shown to be a natural antioxidant source, specifically with antimicrobial, anticancer, anti-inflammatory, cytotoxic and antidiabetic activities (Chayaratanasin *et al.*, 2015; Shen *et al.*, 2016; Leong *et al.*, 2017; Borikar *et al.*, 2018; Mehmood *et al.*, 2019; Jeyaraj *et al.*, 2021). These benefits to human health are due to compositions found in the extract as

phenolic compounds, mainly the ternatins anthocyanins, flavonols, kaempferol, quercetin and mirecithin (Kazuma *et al.*, 2003; Mukherjee *et al.*, 2008; Zakaria *et al.*, 2018), lipophilic compounds (Shen *et al.*, 2016). However, the scientific proof of these pharmaceutical values in the aqueous extract of *Clitoria ternatea* flower (CTF) has not been demonstrated sufficiently, especially in its dosage. This study was carried out to assess the healthcare potential of aqueous extracts of CTF such as antibacterial activity, free radicals scavenging activity, angiotensin-converting enzyme (ACE) inhibitory activity, and cell viability level. The obtained results will help clarify CTF as a natural drink, a kind of tea to improve health.

2. Materials and methods

2.1 Preparation of *Clitoria ternatea* flower aqueous extract

The fresh flower samples were collected between 6 am to 8 am in Binh Nham ward, Thuan An City, Binh Duong Province, Vietnam (11° 09' 103''N and 106° 62' 149''E). A total of 1 kg sample was then washed and extraction was carried out with 5 L of distilled water in a vacuum concentrator (ECO DRY HP, Eco-Techno SRL., Italy) at 60°C for 3 hr at 50 psi vapor pressure and -0.735 to -0.27 atm vacuum conditions, to obtain the

*Corresponding author.

Email: manhtd@tdmu.edu.vn

aqueous extract. The extraction process was repeated 2 more times until finally all the components in the flower were exhausted. The entire aqueous extract from the CTF obtained was vacuum-concentrated until completely dried up to obtain the final aqueous extract from the CTF. This sample was stored at 4°C until use.

2.2 1,1-diphenyl-2-picryl-hydrazyl assay

The antioxidant activity of the extract was determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay, as described by Sang *et al.* (2018) by mixing carefully 100 µL of the aqueous extract of CTF at 20, 40, 60, 80, 100, 120 µg/mL concentrations with 100 µL of DPPH solution then incubating in the dark at room temperature for 30 mins. Measuring the absorbance of the mixture at 490 nm was conducted with a UV-spectrophotometer (Biowave DNA, Cambridge, England). Ascorbic acid was used as the antioxidant standard. Each concentration was performed in triplicate. DPPH scavenging ability was determined by the following formula:

$$\text{DPPH scavenging effect (\%)} = \left[\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right] \times 100.$$

2.3 ACE inhibition assay

ACE inhibitory activity was determined according to the method described by Chaudhary *et al.* (2013) with some modifications as follows: Preparing the aqueous extract of CTF at 20, 40, 60, 80, 100, 120 µg/mL concentration, then 50 µL of this sample was incubated with 50 µL of ACE solution (25 mU/mL) in sodium borate buffer at 37°C for 10 mins; adding 150 µL solution of 8.3 mM hippuryl-L-histidyl-L-leucine (Hip-His-Leu, HHL), with 50 mM sodium borate buffer and 0.5 M NaCl at pH 8 and incubated at 37°C for 30 mins; adding 250 µL of 1.0 M HCl to stop the reaction; adding 0.5 mL of ethyl acetate to the mixture and centrifuged at 800 rpm for 15 mins at 5°C; taking out 0.2 mL from the upper layer by a micropipette and transferring it into a test tube then letting it evaporate at room temperature for 2 hrs under vacuum; dissolving the hippuric acid in 1.0 mL of distilled water and measuring the absorbance of the solution at 228 nm by the UV-spectrophotometer (Biowave DNA, Cambridge, England). All the experiments were performed in triplicate for each concentration of the sample. Captopril was used as the standard at a concentration of 3.6 ng/mL (Park and Jhon, 2010). Distilled water was used as a blank for each sample. The percentage inhibition of ACE was calculated following the formula:

$$\text{Percentage inhibition} = \frac{(A - B)}{(A - C)} \times 100$$

Where A is the optical density with ACE but without an inhibitor. B is the optical density in the presence of ACE and inhibitor. C is the optical density without ACE and inhibitor.

2.4 Antibacterial activity of *Clitoria ternatea* flower aqueous extract

The antibacterial activity was determined by the agar diffusion method according to Nguyen *et al.* (2011). The test bacteria including *Bacillus* sp. (IAL 55), *Vibrio parahaemolyticus* (ATCC 17802), *Staphylococcus aureus* (ATCC 13565), and *Streptococcus mutans* (ATCC 7644) were proliferated in Tryptone soya broth (TSB) for 24 hrs at 35°C. The bacterial suspensions were spread carefully (0.1 mL of 10⁸ CFU/mL) on the Tryptone soya agar (TSA) petri dish, dried for 30 minutes and proceeded to create 5 wells with a sterile cork borer 8 mm in diameter. The CTF aqueous extract at 0, 40, 80, and 120 µg/mL concentration was previously prepared and then added to the wells. The plates were incubated at 30°C for 24 hrs. Tetracycline at 40 µg/mL concentration was used as the positive control (Ekprasert *et al.*, 2020). Each plate included 5 wells containing CTF aqueous extract and tetracycline and 10 repetitions were done for the above bacteria. The results were expressed as the diameter of the inhibition halo (mm).

2.5 Cell viability assay

Cell viability level was determined by MTT assay. In brief, the renal HEK.293 cells (10⁵ cells/mL) were incubated with the aqueous extract of CTF at 0, 20, 40, 60, 80, 100, 120, and 140 µg/mL concentrations for 24 hrs. The medium was removed, and the cells were incubated with an MTT solution of 1 mg/mL for 4 hrs. Removing the supernatant and adding DMSO solution was to solubilize formazan salt. The amount of formazan salt was determined by measuring absorbance at 540 nm using a microplate reader (Tecan Austria GmbH, Grodig/Salzburg, Austria). The cell viability was calculated as a percentage compared to that of the blank (Ngo *et al.*, 2019). All concentration tests were done in triplicate.

2.6 Statistical analysis

All data were analyzed and expressed as the mean ± standard deviations (SD) of replicates by using Statgraphics Centurion XV software (Statpoint Technologies, Inc., Virginia, United States) with a significant difference of 5%.

3. Results and discussion

3.1 Assessing the free radical scavenging activities of *Clitoria ternatea* flower aqueous extract

Plants have long been considered natural antioxidants against many diseases associated with oxidative stress (Admassu *et al.*, 2018). Therefore, free radical scavenging activities of CTF aqueous extract were used to assess the antioxidant ability of the plant extract compared with an ascorbic acid standard, the results are shown in Figure 1. Increasing the concentration of CTF aqueous extract and ascorbic acid from 20 to 120 $\mu\text{g/mL}$ showed a proportional relationship with DPPH activity, reaching its highest of 73% with 120 $\mu\text{g/mL}$ CTF aqueous extract. The results indicated that the DPPH activity of CTF aqueous extract at 120 $\mu\text{g/mL}$ concentration was higher than that of ascorbic acid at the same concentration. This is explained by CTF aqueous extract having high anthocyanin contents, which plays a role as an antioxidant factor (Mattioli *et al.*, 2020). According to Zhao *et al.* (1989), polyphenols or flavonoids are more potent antioxidants than ascorbic acid and vitamin E. The results of this study are similar to a previous study (Escher *et al.*, 2020) where anthocyanin extracted from CTF aqueous extracts showed 65% DPPH radical inhibition. This extract has higher antioxidant activity compared with the aqueous extract of elderberry fruit studied by Duymuş *et al.* (2014). Now, natural antioxidant compounds like anthocyanin are often used as a potential antioxidative and antiaging source (Boonsong *et al.*, 2016). They are also added to food to delay the oxidation of food during storage and processing (Zhang *et al.*, 2010).

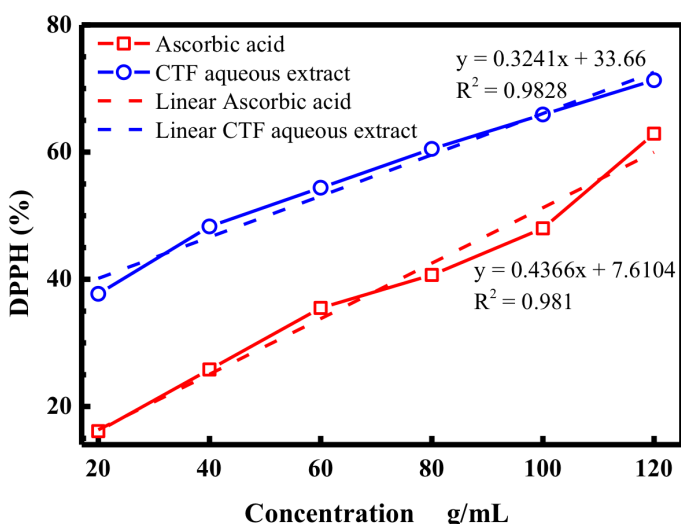


Figure 1. Antioxidants activity of CTF aqueous extract by DPPH.

3.2 Assessing the ACE inhibition activity of the aqueous extract of *Clitoria ternatea* flower

In addition to the antioxidant capacity of the CTF

aqueous extracts, they also have the ability to inhibit ACE, which is shown in Figure 2. The results showed that when increasing the concentration of the CTF aqueous extract from 20 to 120 $\mu\text{g/mL}$, ACE inhibition activity was also increased, achieving 75.1% inhibitory efficacy at the concentration of 120 $\mu\text{g/mL}$. Meanwhile, the ACE inhibitory efficiency of captopril was 87% at a concentration of 3.6 ng/mL . Hence, the CTF aqueous extract exhibited relatively high ACE inhibitory performance at a concentration of 120 $\mu\text{g/mL}$ and would be applicable in the treatment of hypertension. Captopril is known to be a synthetic inhibitor of ACE activity, which has been reported to cause some potential side effects in susceptible patients such as cough and skin rash (Marczak *et al.*, 2003).

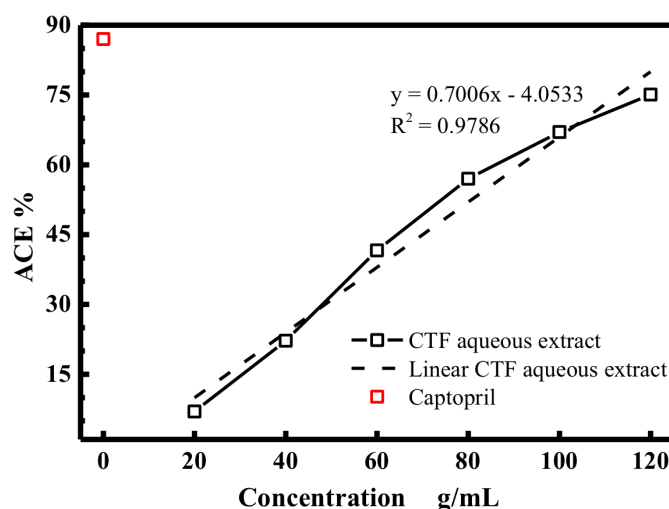


Figure 2. ACE inhibition activity of CTF aqueous extract.

The ability to inhibit ACE inherent in the CTF aqueous extract has been previously studied (Jung *et al.*, 2006; Chaudhary *et al.*, 2013; Mattioli *et al.*, 2020) because it contains high amounts of anthocyanins, flavonoids, hydrolysable tannins, phenylpropanes, proanthocyanidins, xanthenes, fatty acids, terpenoids alkaloids, oligosaccharides, and peptide amino acids. According to Guerrero *et al.* (2012), there are 17 flavonoids, quercetin-3-rutinoside, quercetin, kaempferol, and epicatechin which present above 42% inhibitory activity of ACE-I. The difference in ACE inhibition activity could be due to the position and the number of hydroxyl groups, combined with the presence of double bonds in the rings forming stable complexes chelating with the zinc in the active site of ACE-I (Loizzo *et al.*, 2007). Therefore, the ACE inhibitory activity present in the CTF aqueous extract could substitute for captopril.

3.3 Assessing the antibacterial activity of the aqueous extract of *Clitoria ternatea* flower

To limit the phenomenon of antibiotic-resistant bacteria, CTF aqueous extract was used to evaluate its

Table 1. Antibacterial activity of CTF aqueous extract.

Bacteria	Tetracycline (40 µg/mL)	CTF aqueous extract (µg/mL)			
		0	40	80	120
<i>Streptococcus mutans</i>	14.6±0.3 ^a	0	0	0	4.3±0.3 ^b
<i>Vibrio parahaemolyticus</i>	17.4±0.4 ^a	0	0	0	3.3±0.3 ^b
<i>Staphylococcus aureus</i>	13.4±0.7 ^a	0	9.3±0.5 ^c	11.7±0.5 ^b	12.3±0.5 ^b
<i>Bacillus</i> sp.	10.0±0 ^b	0	9.3±0.7 ^c	13.3±0.5 ^a	13.7±0.6 ^a

Values are presented as mean±SD, n = 10. Values with different superscripts within the same row are statistically significantly different at a confidence interval of 95%.

antibacterial ability against common harmful bacteria such as *S. mutans*, *V. parahaemolyticus*, *Bacillus* sp. and *S. aureus*. The results showed that the CTF aqueous extract exhibited antibacterial activity against all bacterial strains tested (Table 1), and the antibacterial activity increased with increasing concentration of CTF aqueous extract from 0 to 120 µg/mL. The highest inhibitory ability for *S. aureus* and *Bacillus* sp. were recorded at 80 µg/mL CTF aqueous extract with diameters of the inhibitory halo of 11.7±0.5 and 13.3±0.5 mm, respectively, which was not different from those of the 120 µg/mL concentration (Figure 3). However, the CTF aqueous extract slightly inhibited the growth of *S. mutans* and *V. parahaemolyticus* at a concentration of 120 µg/mL in this study. Meanwhile, tetracycline was widely used as a broad-spectrum antibiotic (positive control) against pathogenic bacteria (Ekprasert *et al.*, 2020). Compared with tetracycline, the CTF aqueous extract more effectively inhibited *Bacillus* sp. The results of this study are also consistent with the study of Leong *et al.* (2017) and Mahmud *et al.* (2018), in which *C. ternatea* extract was reported to be able to efficiently inhibit *B. subtilis* and *S. aureus*. The potent antibacterial activity of the CTF aqueous extract in this study could be attributed to the presence of flavonoid compounds, especially anthocyanins (Leong *et al.*, 2017; Jeyaraj *et al.*, 2021), and their synergistic effects in the crude extract (Dhiman *et al.*, 2011). The findings from this study suggest the potential of CTF being taken daily as tea to limit the growth of harmful bacteria in the oral cavity and digestive tract due to their antibacterial activity.

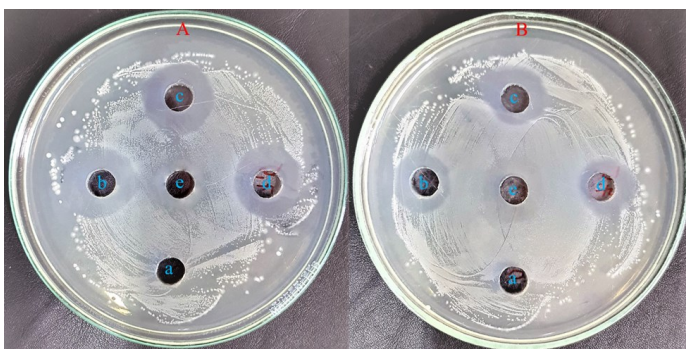


Figure 3. Antibacterial activity of CTF aqueous extract with (A) *Bacillus* sp. and (B) *Staphylococcus aureus* at (a) 0, (b) 40, (c) 80, (d) 120, (e) 40 µg/mL tetracycline.

3.4 Effect of extract on cell viability

In order to screen safe and effective pharmaceutical plants for the purpose of protecting human health, studies on nephrotoxicity by different dosages are very important and necessary. In this study, the *in vitro* cytotoxic effect of CTF aqueous extract was examined on renal HEK.293 cells by MTT colorimetric assay, the results are shown in Figure 4. The results showed that the survival of HEK.293 cells in the kidney at 20-120 µg/mL concentration of CTF aqueous extract was evaluated in the range of 82.9-91.1% when compared with the blank sample (no addition of extract). This result indicated that the CTF aqueous extract had no effect on renal cells at the concentration range from 20 to 120 µg/mL. Increasing concentrations up to 140 µg/mL are shown to start initiating cytotoxicity. There have been many studies on CTF aqueous extracts against Hs27, A549, HCT8, and IMR90 cell lines (Neda *et al.*, 2013; Escher *et al.*, 2020), all showing no cytotoxicity.

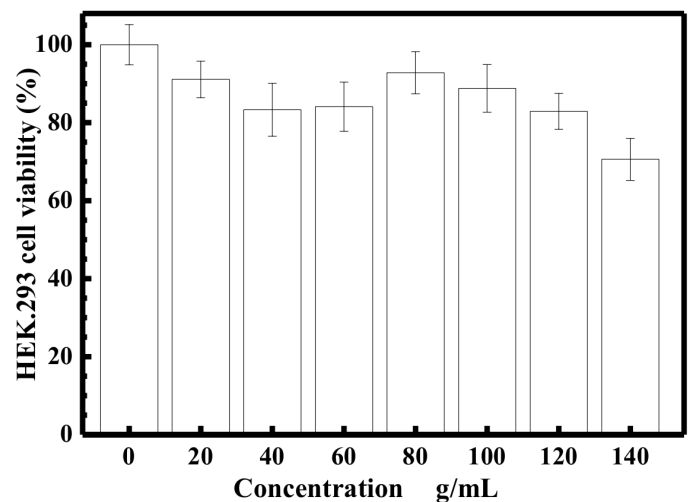


Figure 4. Effect of CTF aqueous extract on HEK.293 cell viability.

From the data obtained from CTF aqueous extracts, it was shown that they have antioxidant activity, ACE inhibitory activity, antibacterial activity, and HEK.293 cell viability. These abilities have also been demonstrated by many previous studies (Kazuma *et al.*, 2003; Mukherjee *et al.*, 2008; Guerrero *et al.*, 2012; Chayaratanasin *et al.*, 2015; Leong *et al.*, 2017; Zakaria *et al.*, 2018) because the CTF aqueous extract contains

many anthocyanin and flavonoid compounds. Thus, the aqueous extract can be used daily as a tea to limit the growth of harmful bacteria in the oral cavity and gastrointestinal tract, hypoglycemia, and enhance human health.

4. Conclusion

It was concluded that CTF aqueous extracts exhibited biological activity as well as were safe for renal cells. The results obtained showed that the CTF aqueous extract exhibited 73% DPPH activity, 75.1% inhibitory efficacy of ACE, and no cytotoxic effect on renal cells at 120 µg/mL concentration. In addition, they exhibited inhibition zone diameters of 11.7±0.5 and 13.3±0.5 mm against *S. aureus* and *Bacillus* sp. at 80 µg/mL CTF aqueous extract, respectively. The resulting data also confirm that the aqueous extract is safe and can be taken daily as a health-promoting tea for lowering blood pressure.

Conflict of interest

The authors declare no conflict of interest.

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