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Determination of the antioxidant property from the flavonoid rich subextract of *Clitoria ternatea*

Rowel Paningbatan Catchillar [1, 2, 3, 4], Jan Karlo Tiongson Ecalne [1, 5, 6], Juliann Marie Abaday Cayaba [5], Claire Noelle Piit Aguipo [5], Rhisand Marie Rosallo Gadian [5], Anne Kathleen Villaseran Osia [5], Bridget Orillaneda Saludes [5], Danica Jen Tagapan Soria [5], Renil Phi Venice Saligumba Torion [5], Sandra Montemayor Valentin [3, 7], Sandra Enriquez Laguimun [1, 6], Jan Ken Emmanuel Tiongson Ecalne [1], Mylene Sevilla Andal [1] and Saffia Anzures Abu-Shendi [1]

[1] *School of Pharmacy, Centro Escolar University, 9 Mendiola St., San Miguel, Manila, Philippines 1005.*
[2] *School of Pharmacy, Far Eastern University, Regalado Ave., West Fairview Quezon City, Philippines 1118.*
[3] *College of Pharmacy, Virgen Milagrosa University, Dr. Martin P. Posadas Avenue, San Carlos City, Pangasinan, Philippines 2420.*
[4] *School of Pharmacy, Philippine Women's University, 1743 Taft Avenue, Manila Philippines 1004.*
[5] *Allied Health Program, Lourdes College, Cagayan De Oro City, Philippines 9000.*
[6] *College of Pharmacy, Adamson University, Ermita, Manila, Philippines 1000.*
[7] *School of Advanced Studies, Saint Louis University, Baguio City, Philippines 2600*

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Abstract

This study evaluated the phytochemical, total phenolic and flavonoid content, and antioxidant property of the *Clitoria ternatea* flower extract using, 2-diphenyl-1picrylhydrazyl (DPPH) free radical scavenging activity. *Clitoria ternatea* flower sample were collected from Herbanext Laboratories Inc., was oven dried at 60°C for 6hrs, then macerated using 70% ethanol and concentrated using a rotary evaporator at 60°C to obtain a syrupy crude extract, the sample was subjected to phytochemical screening to check for the presence of flavonoids and phenols, and was further used for sub-extraction using water, n-hexane and ethyl acetate as partitioning solvents and analyzed for its total phenolic and flavonoid content. The sub-extract with the highest concentration of phenols and flavonoids were then subjected to DPPH free radical scavenging activity. The results revealed that among the 3 sub-extracts subjected to total phenolic and total flavonoid content, the ethyl acetate (174.60 ± 5.32 mg GAE/g & 347.27 ± 8.79 mg QE/g) sub-extract has the highest concentration, compared to the aqueous (42.34 ± 1.84 mg GAE/g & 29.49 ± 5.28 mg QE/g) and n-hexane (15.14 ± 0.99 mg GAE/g & 272.42 ± 2.29 mg QE/g) sub-extracts, the flavonoid rich sub-extract was then subjected to antioxidant assay using the DPPH free radical scavenging activity, the result showed that the extract exhibits concentration dependent activity with an IC50 value of 75.13 ± 3.14 ppm compared to the standard gallic acid (1.74 ppm). Further investigation using different parts of the plant and various purification methods are recommended to confirm the potential use of *Clitoria ternatea* as potential source of antioxidant compound and maximize its use.

Keywords: *Clitoria ternatea*; Phenol; Flavonoid; Antioxidant property; DPPH

1. Introduction

Oxidative stress is a damaging phenomenon caused by free radicals and oxidants that harms cell membranes and other biomolecules such as proteins, lipids, and deoxyribonucleic acid [1]. Overproduction of free radicals can cause oxidative damage to biomolecules, which can result to chronic diseases in people, including aging and other metabolic and

* Corresponding author: Rowel P. Catchillar

degenerative conditions. [2]. To counter these conditions, antioxidants were widely used since antioxidants can decrease the impact of oxidation by scavenging free radicals from the body cells, gaining popularity among consumers. There are two types of antioxidants, natural and synthetic antioxidants. Natural antioxidants are essentially phenolic and may be found in plants, such as fruits, vegetables, seeds, leaves, barks and roots [3]. Despite the abundance of synthetic antioxidants in the market, the majority of consumers still prefer to use natural antioxidants than synthetic compounds, as synthetic antioxidants were proven to have potential adverse effects [4] [5]. In the study of Kornienko et al., in 2019 [6], the use of synthetic antioxidants in excess concentrations can harm DNA and hasten senescence. In addition, butylated hydoxyanisole(BHA) and butylatedhydroxy toluene (BHT) which are popular synthetic antioxidants are known to cause significant adverse events in animal models particularly its association to carcinogenesis [7]. Amongst the phenolics, flavonoids is one of the compounds that stood popular as an antioxidant agent. The majority of flavonoids are proven to be effective radical scavengers and capable of neutralizing variety of free radicals and oxidants. [8]. The following potential health benefits of flavonoids have encouraged researchers globally to find a new potential natural source of an effective antioxidant [9]. *Clitoria ternatea,* commonly known as Pukingan, Asian pigeonwings, or Blue Ternate which belongs to the family Fabaceae, is widely gaining popularity among Southeast Asian countries particularly the Philippines, because of its folkloric and traditional use in rural communities, in particular, the flower is consumed to improve cognitive functions, and treat illness such as fever, inflammation, pain and diabetes[10]. Recent studies have shown that the flowers from *Clitoria ternatea* is abundant in anthocynanins, a water-soluble flavonoids that can be utilized as a potent antioxidant compound [11] [12]. The current study seeks to investigate the antioxidant property of the flavonoid rich sub-extract of *Clitoria ternatea* using 2-diphenyl-1picrylhydrazyl (DPPH) free radical scavenging activity.

2. Material and methods

The materials used in the study are of analytical grade and were provided by Herbanext Laboratories Inc. All other excipients and solvent are also analytical grade, except when otherwise specified.

2.1. Plant Collection and Authentication

Clitoria ternatea flowers were collected between the months January-February 2022 and was authenticated by Herbanext Laboratories Inc.

2.2. Preparation of the Crude Ethanolic Flower Extract of *Clitoria ternatea*

Flower sample of *Clitoria ternatea* were washed thoroughly with tap water to remove adhering dirt and other extraneous articles. The flower sample was then oven dried at 60°C for 6hrs and pulverized using a Wiley mill. The ground dried flowers were macerated with 3 x 1L portions of 70% ethanol in a stainless-steel percolator. Combined ethanol extracts were concentrated using a rotary evaporator at 60^0C for 5 hours to obtain a syrupy crude extract. The % yield was of the crude ethanolic flower extract was calculated using the formula:

$$\% \text{ Yield } = \frac{\text{Weight of crude ethanolic flower extract obtained}}{\text{Weight of ground dried flowers}} \times 100$$

2.3. Qualitative Phytochemical Screening

The crude ethanolic flower extract of *Clitoria ternatea* was initially screened for phytoconstituents including flavonoids, phenols, saponins, tannins, terpenoids and proteins [13,14,15].

2.3.1. Flavonoids

One (1) gram of crude ethanolic flower extract was mixed with pieces of magnesium ribbon and concentrated HCl. Pink color will indicate the presence of flavonoids.

2.3.2. Phenols

One (1) mL of 2% ferric chloride solution was added to 1 gram of crude ethanolic flower extract. The appearance of black coloration would indicate the presence of phenols.

2.3.3. Saponins

One (1) mL of water was added to 1 gram of crude ethanolic flower extract. The appearance of stable foam indicates the presence of saponins.

2.3.4. Tannins

The crude ethanolic flower extract is mixed with 2 mL of water and 1 or 2 drops of ferric chloride solution. The appearance of blue color / green-black color indicates the presence of tannins.

2.3.5. Terpenoids

One (1) mL of chloroform and 1 mL of sulfuric acid to the crude ethanolic flower extract. The appearance of reddish-brown color indicates the presence of terpenoids.

2.3.6. Proteins

The crude ethanolic flower extract was added drop wise of a million reagents. The color formation of red upon heating indicates the presence of proteins.

2.4. Solvent partitioning of *Clitoria ternatea* crude ethanolic flower extract

In a separatory funnel, about 100 grams of the crude ethanolic flower extract was suspended in 50 ml of distilled water and allowed to partition with 3 x 100 ml portions of n-hexane. The aqueous layer was subsequently allowed to partition with 3 x 100 ml portions of ethyl acetate. The aqueous, n-hexane and ethyl acetate extracts were concentrated using a rotary evaporator at not more than 50^0C to achieve dried consistency of the sub-extracts.

2.5. Choice of the Flavonoid Rich Sub-extract

All sub-extracts (aqueous, n-hexane and ethyl acetate) were subjected to the following tests: total phenolic content and total flavonoid content to determine the sub-extract with the highest phenol and flavonoid content. The flavonoid rich sub-extract will be chosen for the DPPH antioxidant assay.

2.5.1. Total Phenol Content

The total phenol content of *Clitoria ternatea* sub-extracts were determined using the Folin - Ciocalteau colorimetric method with modifications. One-half milliliter (0.5) mL of diluted sample extract solution was mixed with the 2.5 mL of 10% Folin–Ciocalteau reagent and allow to stand for 3-8 minutes, 7.5% calcium carbonate was then added which will then be covered with aluminum foil, vortexed, and incubated for 1 hour at room temperature in a dark room. The absorbance was measured at 765 nm (UV- Spectrophotometer). The same procedure was done using Gallic acid as the standard and was used for establishing the standard curve. The results were expressed as mg of gallic acid equivalents/g of extract [16,17].

2.5.2. Total Flavonoid Content

Total flavonoid content of each sub-extracts *of Clitoria ternatea* sub-extracts were estimated using aluminum chloride method. One-half milliliter (0.5) mL of diluted sample extracts were added with 0.15 mL of 10% aluminum chloride ($AlCl_3$) and 2.0mL distilled water, the mixture was allowed to stand for 6 minutes, then added with 0.5mL of 1M hydrochloric acid and 0.5mL sodium acetate, afterwards, the solution was vortexed and incubated for 10 minutes at room temperature. The absorbance will be measured at 425 nm. The same procedure was done using Quercetin as a standard to establish a calibration curve. The concentrations of total flavonoids content were calculated as quercetin mg/g [16,17].

2.6. DPPH Free Radical Scavenging Activity

The flavonoid rich sub-extract of *Clitoria ternatea* was diluted to the following concentrations parts per million (ppm): 33 ppm, 50 ppm and 67 ppm. Each sample dilutions were portioned into 190 µl in a 96 well microtiter plate. Ten (10) µl of 2mM DPPH solution was added to the samples and automatically shake for 30 seconds at 6rpm. The samples were then covered with aluminum foil and incubated at room temperature in a dark area for 30 minutes, afterwards the solution was subjected to absorbance reading using microplate reader at 490 nm. The same procedure was done using Gallic acid as standard with a concentration of 0, 0.25, 0.75, 1.0, 2.50, and 3.0 ppm [18]. The scavenging activity was calculated using the following formula:

$$\% \text{ scavenging activity} = \frac{\text{A control} - \text{AS control}}{A\ control} \times 100$$

Where;
$A_{control}$ = Absorbance of the control (solution in which no antioxidant was added)
$AS_{control}$ = Absorbance of the extract solution

3. Results and discussion

3.1. Percentage yield of *Clitoria ternatea* crude ethanolic flower extract

The percentage yield of *Clitoria ternatea* ethanolic flower extract was computed and resulted in 58.26%, which is significantly high in terms of quantity and therefore can be utilized further in research.

3.2. Phytochemical Screening *Clitoria ternatea* crude ethanolic flower extract

The qualitative phytochemical screening of the ethanolic flower extract of *Clitoria ternatea* is presented in Table 1.

Table 1 Phytochemical Result of the *Clitoria ternatea* Crude Ethanolic Flower Extract

Phytoconstituent	Result
Flavonoid	-
Glycoside	-
Phenol	+++
Saponin	+++
Tannin	++
Phytosterol	-
Terpenoid	+
Protein	-

(-) absent, (+) low, (++) moderately present, (+++) highly present.

The results of the qualitative phytochemical screening of *Clitoria ternatea* crude ethanolic flower extract is presented in Table 1. The crude ethanolic flower extract have shown presence of phenol, saponin, tannins and terpenoid compounds, from which phenol and saponins reflects abundant concentration based on qualitative results, in addition, the results shows that other metabolites particularly flavonoids is absent, this result is possible due to the masking of secondary metabolites during the phytochemical screening, to confirm this data, quantitative analysis using the total phenol and total flavonoid content is employed to validate and reconcile results.

3.3. Determination of the Total Phenol & Total Flavonoids Content

Table 2 summarizes the total phenolic and total flavonoid content of the 3 sub-extracts of *Clitoria ternatea* crude ethanolic flower sub-extract.

Table 2 Total Phenolic and Total Flavonoid Content *of Clitoria ternatea* flower sub-extracts

SUBEXTRACT	TOTAL PHENOLIC CONTENT (mg GAE/g)	TOTAL FLAVONOID CONTENT(mg QE/g)
n-Hexane	15.14 ± 0.99	272.42 ± 2.29
Ethyl acetate	174.60 ± 5.32	347.27 ± 8.79
Aqueous	42.34 ± 1.84	29.49 ± 5.28

GAE: Gallic acid equivalent; QE: Quercetin equivalent

Table 2 shows the total phenolic content and total flavonoid content of the three sub-extracts of *Clitoria ternatea* crude ethanolic flower extract. The ethyl acetate sub-extract has the highest phenolic and flavonoid content with 176.60 ± 5.32 mg GAE/g and 347.27 ± 8.79 mg QE/g respectively. The quantitative assay of TPC and TFC confirmed that *Clitoria ternatea* can be a potential abundant source of phenolic and flavonoid compounds which can be further extracted and purify as new source of antioxidant compounds. The abundance of the flavonoid content of the ethyl acetate extract is unusually high, which implies that other polyphenolic substances, may have been present in the sample, the test also involves the oxidative coupling between phenolic groups of any flavonoids and does not represent quercetin alone but all polyphenolic substances. At this point, the Ethyl acetate sub-extract is therefore designated as the flavonoid-rich sub-extract and will be used in the antioxidant assay using the DPPH free radical scavenging activity.

3.4. DPPH Radical Scavenging Assay

Table 3 and 4 shows the % DPPH scavenging effect of the flavonoid rich sub-extract of *Clitoria ternatea* at different concentrations and the IC_{50} of the flavonoid rich sub-extract and gallic acid respectively.

Table 3 DPPH Free Radical Scavenging Activity of the Flavonoid rich sub-extract

Flavonoid Rich Subextract (ppm)	% Inhibition
33 ppm	25.130 %
50 ppm	35.101 %
67 ppm	42.767 %

Table 4 IC50 value of Flavonoid Rich Subextract of *Clitoria ternatea* and Gallic acid

Sample	IC50 (ppm)
Flavonoid Rich Sub-extract	75.13 ± 3.14 ppm
Gallic acid	1.74 ppm

The presence of the Flavonoid Rich sub-extract of *Clitoria ternatea* on the DPPH free radical has caused low to moderate scavenging activity with concentration dependent activity as shown on Table 3 in which extracts with higher concentrations exhibits higher scavenging activity of the radical, however, in terms of the IC_{50}, the flavonoid rich sub-extract has significant difference to the standard control (gallic acid) with an IC_{50} of 75.13 ± 3.14 ppm and 1.74 ppm respectively. The inferior radical scavenging activity of the flavonoid rich sub-extract of *Clitoria ternatea* compared to gallic acid is possible due to an insufficient purification method to extract flavonoids and other phenolic compounds, despite undergoing solvent partitioning method, however, it is still noteworthy that the plant sample *Clitoria ternate*a has still the potential to be further extracted & purified to obtain compounds with effective antioxidant properties.

4. Conclusion

The result of this study showed that the crude ethanolic flower extract of *Clitoria ternatea* contain a significant amount of phenol and flavonoid compounds based on qualitative phytochemical screening which reflects on the total phenolic and total flavonoid content of the three sub-extracts used in the study, furthermore the ethyl acetate sub-extract of *Clitoria ternatea* crude ethanolic flower extract has proven to contain the highest amount of phenolic and flavonoid compound with a value of 174.60 ± 5.32 mg GAE/g and 347.27 ± 8.79 mg QE/g respectively. In the DPPH radical scavenging activity assay, the ethyl acetate sub-extract of *Clitoria ternatea* crude ethanolic flower extract has found to have low to moderate antioxidant activity in a concentration dependent mechanism, with an IC_{50} of 75.13 ± 3.14 ppm compared to gallic acid of 1.74 ppm, although not comparable to the standard, the research has proven that *Clitoria ternatea* still exhibits scavenging activity on free radicals and can be a potential new source for flavonoids and phenolic compounds with promising antioxidant activity. Further investigation using different parts of the sample and various purification methods are recommended to maximize its use.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors have no conflicts of interest to declare.

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