Original Research Article

Antioxidant activity and phytochemical constituents of Philippine Clitoria ternatea flowers as a potential therapeutic agent against infectious diseases

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Infectious diseases have always been present throughout human history, in which numerous emerging and re-emerging pathogens have been documented. Various strategies have been established to combat these pathogens: from vaccinations, to optimized drug delivery routes, to natural products such as antioxidants and flavonoids. This study evaluated the antioxidant activity of the ethanolic extract of the anthocyanin-rich Clitoria ternatea flowers, from the Philippines, by analyzing its total phenolic content and DPPH radical scavenging activity. The raw materials used in this study were collected from Victoria, Tarlac, Philippines, which are then macerated and soaked in 95% ethanol for 24 hours before extraction. The extract was then filtered using coarse filter paper and concentrated using a rotary evaporator. The samples were subjected to heavy metals analysis, Total phenolic content and IC50 from DPPH radical scavenging activity of the C. ternatea extract were found to be 3.9519 ± 0.1 mg GAE / 100 g and 53.6913 mg/kg, respectively. Phytochemical screening of the ethanolic extract of C. ternatea revealed the presence of triterpenes, saponins, reducing sugars, tannins, sterols, flavonoids, and alkaloids. Results show the potent antioxidant properties and the abundance of flavonoids of C. ternatea extract which may encourage further studies to evaluate its possible applications as plant-derived antioxidants for the therapeutic management of various infectious diseases.

Keywords: Antioxidant activity, DPPH radical scavenging activity, phytochemical constituents, Clitoria ternatea

INTRODUCTION

Infectious diseases have been plaguing humanity throughout history (Morse, 1995). From the bubonic plague in 1346, smallpox in 1817, cholera in 1854, the recurrences of influenza during the years of 1847, 1918, and 1976 (Lederberg, 2000 and Simonsen et al., 2016) to the emerging and re-emerging infectious diseases such as influenza, Lyme disease, HIV infection, West Nile, Zika, and Dengue viruses, SARS, Ebola, and COVID-19 (Bryukhanova et al., 2020 and Fauci, 2001). There are numerous factors involved on why there is emergence and re-emergence of infectious diseases, which include (but are not limited to) travel, demographic, environment, lifestyle, climate, and even diet (Wilson, 1995 and Morse, 2001).

Several measures have been developed against infectious diseases, from DNA immunization and immunotherapeutic developments (Watts and Kennedy, 1999), antiviral and antibacterial therapy (Chen et al., 2021) through properly targeted delivery strategies (Loretz et al., 2021), to modern and more sophisticated strategies such as antimicrobial photoinactivation (St. Denis et al., 2011), the use of adenoviral vectors (Zhang and Zhou, 2015), and the application of nanotechnology through the use of
nanoparticles as drug carriers (Kirtane et al., 2021; Lin et al., 2021). Aside from those, the existence of natural products-based therapeutics is also widely known, which can synergize with the already established therapeutic strategies (Hemaiswarya et al., 2008).

There has been a growing interest in using natural antioxidants as a promising alternative to the current synthetic ones (Papas, 1999). An antioxidant is a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. It may delay or inhibit cellular damage by acting as a radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agent (Frie et al., 1988). Artificial and natural antioxidants are used in the food, cosmetic, and therapeutic industries. However, issues of the physical properties of some synthetic antioxidants, strict legislation on the use of artificial food additives, and the health and ecological concerns attributed to their use have impacted consumer preferences.

Additionally, the potential therapeutic properties of flavonoids have been expounded. Flavonoids are known as the secondary metabolites of phytochemical synthesis and is present in plants in the form of aglycones or glycosides. Being part of the human diet, these secondary metabolites are associated with health promoting effects in a broad spectra, through improvement of cell signaling and nutrient metabolism (Biharee et al., 2020). Flavonoids are also known to possess antioxidant and antibacterial properties. These molecules tend to interfere with the pathogens’ ability to infect a host cell (i.e. high affinity binding with SARS-CoV-2 spike protein (Rupasinghe, 2020)), disruption of pathogen cellular membrane (i.e. inhibition of FAS-II pathway in gram-negative bacteria (Biharee et. al., 2020)), and overall disruption of a pathogen’s ability to reproduce (i.e. inhibition of 3CL protease, an enzyme vital for viral replication of COVID-19 virus (Russo, et al., 2020)).

*Clitoria ternatea* L. (family: Fabaceae), commonly known as blue ternaite and butterfly pea, is a perennial herbaceous plant widely distributed in tropical zones, such as Asia, and Southern and Central America. The flowers, whose bright blue color is attributed to the high anthocyanin content, are used as decoration and natural coloring agents. Moreover, the plant has also been traditionally used as a treatment for snakebites, scorpion stings, fever, and skin diseases, among others (Mukherjee et al., 2008). Several studies have reported the various pharmacological effects of the plant, such as antioxidant, antimicrobial, anti-inflammatory, antipyretic, antipilidemic, and analgesic (Gupta et al., 2010). Characterization of *C. ternatea* L. flowers revealed the presence of ternatin anthocyanins, namely, A1-A3, B1-B4, C1-C4, and D1-D3, as well as phenolic flavonoids such as quercetin, robinin, clitorin, and kaempferol derivatives (Nair et al., 2015; Turnos, 2021).

Last December 2019, the world witnessed the unfolding of a new pandemic caused by SARS-CoV-2, the causative agent of COVID-19. Since then, the importance of discovering vaccine types and other therapeutic agents that can be used against the virus is of paramount significance (Yuki et al., 2020). Coronaviruses (CoVs) belong to a family of enveloped viruses with a positive sense, single-stranded RNA genome. These viruses cause several illnesses ranging from upper respiratory tract infections to lower respiratory tract infections including bronchitis, pneumonia, and severe acute respiratory syndrome (SARS). Reports mentioned that the most dangerous disease outcomes are in the elderly, immunocompromised patients, and infants (Schoeman and Fielding, 2019; Gralinski and Baric, 2015). Viral pneumonia caused by SARS-CoV-2 induces an inappropriate immune response in the lung tissues characterized by the excessive production of proinflammatory cytokines, which contributes to endogenous oxidative stress (Zhao, 2020). The production of reactive oxygen species (ROS) increases due to oxidative stress, which promotes viral multiplication (Reshi et al., 2014). Many of the tested conventional drugs and antiviral therapies proved ineffective in treating SARS-CoV infections (Diniz et al., 2020). Several studies have been conducted to assess the antioxidant activity of plant compounds against viral infections. It was reported that antioxidant flavonoids, including (+) catechin, luteolin, apigenin, quercetin, and quercetin-7-rhamnoside were successful in inhibiting ROS accumulation and apoptosis of cells infected with different coronavirus (Song et al., 2011; Liang et al., 2015; Hashim, 2021).

Critical therapeutic targets of SARS-CoV 2 by flavonoids have been established to inhibit the binding through the cell membrane and translation of the viral RNA (Alzaabi et al., 2021). Spike proteins, ACE-2 receptors, and human type-2 transmembrane serine (TMPRSS2) possess potential binding sites for flavonoids to inhibit the binding of the virus on the cell membrane. (Astuti and Ysrafil, 2020; Russo et al., 2020). Viral RNA translation mechanisms can also be inhibited with the use of flavonoids, by targeting specific enzymes responsible for the translation and proteolysis of the viral genomic (+) RNA such as the Main protease, 3CL protease, and the RNA-dependent RNA polymerase (Alzaabi et al., 2021; Oostra et al., 2007).

Some notable flavonoids that are known to inhibit the critical therapeutic targets of SARS-CoV-2 are baicalin, which has the highest binding affinity for Spike proteins (Pandey et al., 2021), quercetin and its derivatives as a 3CL protease inhibitor (Santana et al., 2021), and kaempferol’s mechanisms of action that include inhibition of 3a viral protein that is being expressed in infected cells (Ahmadian et. al., 2020; Schwarz et al., 2014) and its immunomodulatory effects such as caspase-1 activation along with its stimulatory effect on NLRP3 inflammasome that is necessary for IL-1β secretion and pyroptotic cell death (Ahmadian et al., 2020). *C. ternatea* L contains abundant concentrations of both quercetin and kaempferol and their respective derivatives (Santana et al., 2021).

This study, therefore, aims to evaluate the antioxidant activity of *Clitoria ternatea* (blue pea) flowers by determining its total phenolic content and free radical scavenging activity. Moreover, this study also aims to
determine the secondary metabolites present in the *C. ternatea* extract.

**METHODOLOGY**

**Collection of plant material and ethanolic extraction**

*C. ternatea* flowers were obtained from Victoria, Tarlac, Philippines. The fresh plant materials (200 g) were macerated in 300 mL of 95% ethanol for 24 hours. The extract was filtered through a coarse filter paper and then subjected to *in vacuo* concentration using a rotary evaporator at 60°C (Buchi R300 Rotary Evaporator). The crude extract was stored in a desiccator before use for analyses (Saejung et al., 2021).

**Heavy metals analysis**

Heavy metals analysis of fresh plant materials was conducted at the Standards and Testing Division, Industrial Technology Development Institute - Department of Science and Technology in Bicutan, Taguig City. Cadmium and lead were determined through atomic absorption spectroscopy (Shimadzu AA-6880), and the presence of arsenic was determined through atomic absorption spectroscopy coupled with Hydride Vapor Generator (Shimadzu AA-6880 with Shimadzu HVG-1), both following the modified AOAC Method 986.15 (AOAC INTERNATIONAL, 2012). Mercury was determined using a direct mercury analyzer (Milestone DMA-80) according to the US EPA 7473 method (US EPA, 1998).

**Evaluation of Antioxidant activity**

**Total Phenolic Content (TPC)**

The total phenolic content (TPC) of the *C. ternatea* extract was measured using the Folin-Ciocalteu method with some modification. The Folin-Denis reagent and 35% w/v Na2CO3 (0.5 mL) were added to the sample extract (1.0 mL). The mixture was incubated in the dark for 30 minutes at room temperature for the reaction to occur. The absorbance was measured at 765 nm wavelength using a UV-VIS spectrophotometer (Hitachi U-2900 Spectrophotometer), Gallic acid in the concentration of 25 to 1000 ppm was used as standard and in calibration. Analysis was done in triplicate and one for recovery. In the Total Phenolic Content (TPC), value was expressed in mg Gallic Acid Equivalents (GAE) / 100 g (Evans 2009; Stankovic, 2011).

**Total Phenolic Content**

\[
T = \frac{(Corrected \ Balance - Intercept)}{Slope \times \text{ Dilution Factor}} \times \frac{1}{\text{Weight of sample}}
\]

**2,2-diphenyl-1-picrylhydrazyl (DPPH) Free radical scavenging assay**

Antioxidant capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined based on the DPPH free radical scavenging assay method with some modifications. A 1.0 mL of extract or ethanol (as control) was added to the test tubes. Then, 3.0 mL of DPPH reagent (0.1 mM) was added to the sample extract or control. The mixture was then incubated in the dark for 30 minutes at room temperature. The absorbance of the mixture was measured by UV-VIS spectrophotometer (Hitachi U-2900 Spectrophotometer) at 517 nm wavelength (Stankovic, 2011). All samples and control were done in triplicate. The percentage scavenging activity was determined by using the following formula:

\[
\% \text{ scavenging activity} = \left(1 - \frac{A_{\text{Sample}}}{A_{\text{Control}}}\right) \times 100
\]

where \(A_{\text{control}}\) is the absorbance of the control alone, while \(A_{\text{sample}}\) is that of radical with sample (Evans, 2009; Stankovic, 2011). The concentration-response curve was constructed using the Hill equation:

\[
Y = \frac{Y_{\text{max}}}{1 + \left(\frac{X}{IC_{50}}\right)^{Hill \ coefficient}}
\]

where \(Y\) is the response value at concentration \(X\), \(Y_{\text{max}}\) is the maximum response, \(X\) is the given concentration, and \(IC_{50}\) is the concentration that produces a 50% inhibitory response.

**Phytochemical screening**

The crude ethanol extract was analyzed for the presence of sterols, triterpenes, flavonoids, alkaloids, saponins, reducing sugar, and tannins. Results were reported as presence (+) or absence (-) of the phytochemicals tested. (Evans, 2009)

**Liebermann-Burch test for sterols and triterpenes**

A small amount of the dried extract was dissolved in acetic anhydride. The soluble portion was decanted afterward. To this, 1-2 drops of concentrated sulfuric acid were added. A pink to red color is indicative of triterpenoids while blue color indicates the presence of steroids.

**Magnesium turning test for flavonoids**

One milliliter or a small amount of the dried alcoholic extract was treated with 1.0 mL of 10% HCl and magnesium turnings. The red coloration is observed for positive results.

**Mayer’s test for alkaloids**

The dried alcoholic extract was extracted with 1% HCl and filtered. Then, 2 drops of Mayer’s reagent were added to the filtrate. A cream-colored precipitate is indicative of the presence of alkaloids.
Heavy metals content of *C. ternatea* flowers

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium (Cd), mg/kg</td>
<td>Not detected</td>
</tr>
<tr>
<td>Lead (Pb), mg/kg</td>
<td>Not detected</td>
</tr>
<tr>
<td>Arsenic (As), µg/kg</td>
<td>Not detected</td>
</tr>
<tr>
<td>Mercury (Hg), µg/kg</td>
<td>0.751</td>
</tr>
</tbody>
</table>

*Method: AOAC Official Method 986.15*

**Method: US EPA 7473**

Detection limit: 0.987 mg/kg, 1.35 mg/kg, 3.65 µg/kg, 7473 µg/kg

Froth test for saponins

The alcoholic extract was dissolved in hot water and then filtered. The filtrate/aqueous extract was shaken vigorously. The presence of froth, honeycomb in nature, which should persist for at least 30 minutes is indicative of saponins.

Fehling’s test for reducing sugar

The alcoholic extract was dissolved in hot water and then filtered. 2 mL of the filtrate was placed in two (2) test tubes. To the first tube, 1.0 mL of diluted HCl was added while the second tube served as the control. Both tubes were placed in a boiling water bath for 5 minutes and then allowed to cool. The samples were neutralized with anhydrous sodium carbonate until no effervescence was produced. Then, 1.0 mL of Fehling’s solution was added. The tubes were heated in the water bath for 2 minutes. An increase in the amount of brick red ppt in the hydrolyzed sample is indicative of the presence of reducing sugar.

Ferric chloride test for tannins

The dried alcoholic extract was extracted with hot water and the aqueous extract was filtered. 1-2 drops of the Ferric Chloride T.S. were added to the filtrate. Greenish or bluish-black coloration indicates the presence of tannins.

Statistical Analysis

Determination of total phenolic content and radical scavenging activity was done in triplicate trials and data were expressed as means ± SD.

RESULTS AND DISCUSSION

Heavy metals analysis

The results of the heavy metals analysis of the fresh *C. ternatea* flowers are presented in Table 1. Based on these results, cadmium, lead, and arsenic were not detected in *C. ternatea* flowers. However, 0.751 µg/kg of mercury, which is significantly lower compared to the allowable concentration of 7473 µg/kg, was present which could have been absorbed from its soil via its root.

Antioxidant Activity

The in vitro assessment of antioxidant activity of plant extracts has been widely used for preliminary assessment of their potential beneficial properties (Shahidi and Zhong, 2015). In this particular study, the antioxidant activity of the ethanolic extract of *C. ternatea* flowers was evaluated through the determination of total phenolic content and DPPH radical scavenging activity.

Total Phenolic Content

Polyphenols are secondary metabolites of plants, largely found in fruits and vegetables. They are most noted for their antioxidant activities, which can be attributed to their structure. The highly conjugated system and certain hydroxylation patterns allow the polyphenols to suppress the generation of free radicals by donating an electron or hydrogen atom. They also utilize chain-breaking mechanisms by neutralizing the radicals to become less reactive, thus stopping the chain reactions (Rice-Evans et al., 1996). In this study, the total phenolic content of the *C. ternatea* ethanolic extract obtained from the linear regression equation of the gallic acid standard curve (y = 0.0091719x – 0.0323654, R² = 0.9999) was 3.9519 ± 0.1 mg GAE / 100 g.

DPPH Radical Scavenging Activity

DPPH assay is a widely used method for measuring antioxidant activity of plant extracts. DPPH is a stable free radical that acts as a hydrogen radical scavenger. Antioxidants react to the stable free radicals from DPPH by donating hydrogen, resulting in the formation of non-radical DPPH-H. This reaction can be observed through a color change in the test solution from violet to yellow (Khatoon et al., 2013).

Figure 1 shows the graph generated from plotting the different concentrations of *C. ternatea* extracts against the resulting percentage radical scavenging activity. Following the equation for concentration-response curve, the IC₅₀ was determined to be 53.6901 mg/kg.

Flavonoids are a large group of polyphenolic compounds with benzo-γ-pyridine structure. These are ubiquitously present in plants, from fruits to leaves and other parts. As a dietary component, flavonoids exhibit health-promoting properties due to their high antioxidant capacity in vivo and in vitro (Cook and Samman, 1996). The configuration, substitution, and the total number of hydroxyl groups on the structures substantially influence several mechanisms of antioxidant activity such as radical scavenging and metal ion chelation ability. The β-ring hydroxyl configuration is the most significant determinant of scavenging of ROS and RNS because it donates hydrogen and an electron to hydroxyl, peroxy, and peroxy nitrite radicals, stabilizing them and giving rise to a relatively stable flavonoid.
radical (Hashim and Fry, 2020).

Anthocyanin, found abundant in *C. ternatea* flowers, is one of the subclasses of flavonoids. There are six known major anthocyanidins, namely, pelargonidins, cyanidins, delphinidins, peonidins, petunidins, and malvidins. Their structures reversibly undergo pH-dependent transformation in aqueous solution, which can be observed as a color change in the solution (Brouillard and Delaporte, 1977). Anthocyanins are potent antioxidants in vitro. They have the ability to quench free radicals and terminate the chain reaction effectively. In a study conducted to assess the antioxidant activity of anthocyanins at neutral pH using oxygen radical absorbance capacity (ORAC) assay, cyanidin-3-glucoside had the highest ORAC value, 3.5 times as potent as Trolox, a water-soluble vitamin E analog (Wang et al., 1997).

**Phytochemical screening**

Crude ethanolic extract of the fresh *C. ternatea* flowers were prepared and used for phytochemical screening (Table 2). The presence of various phytochemical constituents contributes to the biological activities of this plant as previously mentioned.

**CONCLUSION**

The antioxidant activity of *C. ternatea* extract as determined by its total phenolic content expressed as gallic acid equivalent was 3.95 mg GAE / 100 g total phenolic count and by DPPH radical scavenging activity was found to have an IC$_{50}$ value of 53.69 mg/kg. On the other hand, phytochemical screening of the ethanolic extract revealed the presence of triterpenes, saponins, reducing sugar, tannins, sterols, flavonoids, and alkaloids. The presence of flavonoids in the extract is the main contributor of the antioxidant activity of *C. ternatea*. Thus, *C. ternatea* is a good source of antioxidants, which might be useful in preventing the progress of various oxidative conditions. Furthermore, this study may encourage further research to evaluate and characterize plant-derived antioxidants and...
flavonoids for the therapeutic management of infectious diseases.

Conflicts of Interest
The authors declare that there are no conflicts of interest upon writing of this manuscript.

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