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Clitoria Ternatea: A Comprehensive Study on Extraction, Phytochemical Characterization, Antioxidant & Antimicrobial Properties, and Inorganic Elemental Analysis

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Abstract

North-eastern India is having rich biodiversity. Phytochemical are non-nutritive, chemical compounds occurs naturally on plants during metabolic processes and they have diverse proactive properties or disease preventive properties. Plants are known to produce these chemicals to protect them. While recent research demonstrates that they can also play an important role in protecting humans against diseases. Even some of these plants are in use as traditional medicine for centuries. Methanol extract showed the presence of most phytochemicals. Distilled water and acidified water extracts showed moderate phytochemical diversity. Ether extract showed presence of lipophilic phytochemicals like lipids, steroids, terpenoids, and triterpenoids. The TPC values for *Clitoria ternatea* range from 50-350 mg GAE/g. The methanolic extract of *Clitoria ternatea* showed higher total phenolic content of 209.2 mg GAE/g compared to the aqueous extract, which shows 122.1 mg GAE/g. The results indicate that methanol is a more efficient solvent for extracting phenolic compounds from *Clitoria ternatea*. The inorganic composition of plants, in a solid state, was determined using ED-XRF. The amount of silver, barium, cadmium, chromium, iron, nickel, lead, zinc, calcium, potassium, sodium, magnesium in plants were determined using ICP-OES. The most significant findings in this section include high concentrations of Potassium (K) in Asian pigeonwings followed by Calcium (Ca). According to the toxicity level, Cadmium (Cd) and Lead (Pb) levels are within safe limits. Hence, further research should be carried out to identify the active biomolecules or Phyto-molecules from the plants and determine the effects of them *in vitro* as well as *in vivo*.

Keywords: *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Streptococcus*, *Enterobacter aerogenes*, *Bacillus subtilis*, phytochemical, antioxidant activity, antibacterial activity, Total phenolic compound, *Clitoria ternatea*.

Introduction:

Herbal plants such as the Asian Pigeonwings flower (*Clitoria ternatea*) are widely used as traditional medicine. Some of the active substances make the plant to have the ability as an antibacterial compound.

The active substances are alkaloids, tannins, phenols, saponins, and flavonoids **[1, 2]**.

The plant has numerous pharmacological actions, such as antibacterial, antioxidant, anticancer, hypolipidemic, cardiovascular, central nervous, respiratory, immunological, anti-inflammatory, analgesic, and antipyretic. The antioxidant activities of the flower are rarely used in cosmetic industries. It uses as an antiaging product with its antioxidant agents, such as phenolic and flavonoid **[3, 4, 5]**.

The taxonomic classification of Asian Pigeonwings flower is like- **Kingdom**: Plantae; **Division**: Tracheophyta; **Class**: Magnoliopsida; **Superorder**: Rosanae; **Order**: Fabales; **Family**: Leguminosae; **Genus**: *Clitoria*; **Species**: *ternatea*. The Common name are: Butterfly pea, Asian Pigeon wings flower.

The leaf extract of *Clitoria ternatea* being colourful, so after purification it is used as a natural colouring agent or natural dye in various food industries. Being a traditional medicinal herb it is the only species among the *Fabaceae* family that is bestowed with the presence of cyclotides. Highest concentrations of cyclotides are found to be present in the roots of *Clitoria ternatea*. This compound is greatly possessing antibacterial and immune stimulating activities **[6, 23]**.

The seeds also having lectin that agglutinates trypsin-treated human B erythrocytes. The flower extract of the plant act as a good anti-inflammatory, analgesics and also as antidiabetic agent. The bark is used as a diuretic and laxative. For centuries the extract of the plant has been used as a memory enhancer, antidepressant, stress reducer, anxiolytic, sedative and tranquillizing agent **[5]**. The extract of the whole plant is used in treating sexual ailments such as gonorrhoea and infertility. Among the various parts of the plant extracts, the extract of the seed has an effective larvicidal activity **[24]**.

In immunocompromised patients with underlying diseases like chronic pulmonary obstruction, diabetes mellitus, or cancer, *K. pneumoniae*, one of the Gram-negative opportunistic pathogens, frequently causes bloodstream infections, respiratory infections, and urinary tract infections that emerge in hospitals. According to reports, immunocompromised patients infected with isolates of the multidrug-resistant *K. pneumoniae* had a high mortality rate of 18 to 49% **[7, 22]**.

Over the past years, antibiotic resistance in bacteria has become a major problem around the world. Antibiotic resistance-associated factors have significantly expanded in bacterial populations due to their mobility and contagiousness **[8, 25]**. The issue is mostly caused by antibiotic-driven selection and bacterial genomic flexibility. Due to genome evolution and the emergence of highly effective multidrug-resistant clades in many diseases, this has grown to be a significant issue **[9]**.

Materials and Methods:

Sample collection

A 5 kg of healthy and fresh butterfly pea flowers were collected from a local plantation in Bhubaneswar, Odisha market. After collection, the flowers were washed, then air-dried overnight and continued in an oven at 40ºC. The dried pigeonwing flower samples were blended and sieved.

Extraction

The fine powder of the pigeonwings flower was extracted using the maceration method. The first maceration extraction was done using 150 grams of fine powder of pigeonwings flower with different solvents for seven days. The extraction process was continued for two more days. The sample was filtered a second time to obtain the second filtrate. The two filtrates obtained by using Soxhlet apparatus and concentrated using a rotary evaporator at a temperature of 60ºC **[6]**.

Phytochemical screening

***Alkaloid*:** Few ml of extract and few drops of Mayer's reagent added by side of test tube. A white and

creamy precipitate indicates presence of alkaloids.

***Amino Acid*:** 1 ml of extract was treated with few drops of Ninhydrin reagent. Appearance of purple colour shows the presence of amino acid.

***Anthraquinone Glycoside*:** To 2.0 mL of each extract of the plant, concentrated ammonia (1.0 mL) was added. The formation of a red-rose colour indicates a positive result, suggesting the presence of anthraquinone glycosides.

***Cardiac Glycosides*:** To the solution of extract, add glacial acetic acid, few drops $Fecl_3$ and conc. Sulphuric acid. Then observe reddish brown colour at the junction of two-layer shows presence of CO_2.

***Flavonoids*:** On 1.0 mL of each acidic extract, sodium hydroxide (4.0 M) solution was added until the pH reached 10. The formation of yellow colour indicates a positive result, suggesting the presence of flavonoids.

***Lipids*:** 0.5 N alcoholic potassium hydroxide was added along with a drop of phenolphthalein to 10 ml of the extract. The mixtures were incubated on water bath for 1 hour. The presence of lipids was indicated by the formation of foam or soapy layer.

***Phenol*:** 2 ml of extract and 2 ml of $FeCl_3$ will give the appearance of deep bluish green colour solution which shows the presence of phenol.

***Phlobatannin*:** 10 ml of aqueous extract of flower was boiled with 1% HCl. Presence of phlobatannin was indicated by the thick red precipitate deposition in the bottom.

***Proteins*:** 1 ml of methanolic extract was taken and added a few drops of nitric acid to the sides of the test tube very gently. Within few seconds the formation of yellow colour indicates the presence of proteins in the sample (Xanthoprotein test).

***Reducing sugar*:** Mix 1 ml of extract and 5-8 drop of Fehling's reagent and boiled it. A brick red colour precipitated is observed.

***Saponin*:** 5 ml of extract boiled in 10 ml of D.W. in test tube, shake vigorously for 30 sec. and allowed to stand for half an hour formation of forth indicates the presence of saponins.

***Steroids*:** 2 ml of chloroform was added to extract and few drops of concentrated H_2SO_4. The presence of steroids was indicated by the appearance of red colour in the upper layer while yellow with greenish fluorescence appears in the H_2SO_4 layer.

***Tannins*:** a few drops of ferric chloride (5%) were added to the aqueous extract (2.0 mL). The formation of a blue or green colour indicates a positive result, suggesting the presence of tannins. The formation of a blue colour suggests the presence of hydrolysable tannins, while the green colour suggests the presence of condensed tannins.

***Terpenoids*:** To 1 ml of the aqueous extract 1 ml of chloroform was added, mixed well and left for 5 minutes, 1 ml concentrated H_2SO_4 was added after 5 minutes. The presence of terpenoids was indicated by the appearance of greyish layer.

***Triterpenoids*:** 2 ml of extract was added with 5 drops of concentrated Sulphuric acid and kept undisturbed. Presence of triterpenoids was indicated by the appearance of greenish blue colour.

Estimation of total Phenolic content

The phenolic content of the methanolic and aqueous extracts of each sample prepared in the "Phytochemical screening" section was estimated. A volume of 0.5 mL of each extract was mixed with a diluted Folin-Ciocalteu reagent (2.5 mL) (prepared by diluting the concentrated reagent with a dilution factor of 10) and shaken. After standing for 5 minutes at room temperature, sodium carbonate solution (2.0 mL, 75 g/L) was added to each test sample and vortexed [8,9]. After standing for an hour at room

temperature, the absorbance of the solutions was measured at 760 nm using an UV/VIS spectrophotometer. Three replicates were conducted for each extract. The concentration of phenolic content was calculated by preparing a standard curve of gallic acid [a solution of gallic acid (10 mg) in 100 mL of distilled water was prepared (as a 100 µg/mL standard], which was used to prepare standard solutions of 5, 10, 20, 40 and 80 µg/mL by serial dilution]. The phenolic content of each extract was reported as a Gallic Acid Equivalent (GAE) **[21]**.

Inorganic composition

The inorganic composition of each plant sample was determined by using Epsilon-1 Energy Dispersive X-Ray Fluorescence (ED-XRF). Non-destructive measurement of the inorganic composition was accomplished with 15 W, 50 kV, and 1500 µA. The instrument contained a silver X-ray tube and was equipped with a small spot camera with a 1 mm^2 spot size to select the best spot for targeting. The analysis was performed with the help of Epsilon software for analyzing the X-ray spectra. Prior to analysis, the dry samples were grinded and then pressed onto a tablet by a compressor, and the analysis was repeated three times for each plant sample **[19, 20]**.

Mineral profile

The amounts of minerals, such as: silver, barium, cadmium, chromium, iron, nickel, lead, zinc, calcium, potassium, sodium, magnesium in each sample were determined by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). First, using a 1000 ppm multi-element stock solution containing all the minerals, a 20 ppm elemental mother solution was prepared, from which a series of standard solutions with concentrations of 400, 200, 100 and 50 ppb were prepared for calibration. Then, a 500 mg amount of each plant sample was boiled in distilled water (25 mL) for 20 minutes. The samples were filtered and acidified with concentrated nitric acid (1.0 mL), and then diluted with a dilution factor of 100 using distilled water before analysis. The mineral profile of the plant samples of interest was determined with the aid of ICP-OES. Three replicates were conducted for each extract **[17, 18]**.

Antioxidant Activity using DPPH Radical Scavenging Method

The antioxidant activity of the plant extracts was determined against DPPH. A methanolic dilution of DPPH 1×10^{-4} M was prepared. Aliquots of 1 mL of each sample in the methanolic extract were collected (at 4 different concentrations: 0.1, 0.5, 1, and 2 mg/mL; two replicates per sample and concentration) and had 2 mL of methanolic dilution of DPPH added. The mixture was kept in the dark at room temperature for 16 mins, and absorbance was measured at 517 nm in a UV spectrophotometer. The blank was prepared with the methanolic dilution of DPPH. The results were expressed in milligram equivalents of Quercetin per milligram of dry weight. The calibration line was established using the following concentrations of Quercetin: 0.001, 0.002, 0.005, 0.01, 0.02, and 0.04 mg/mL **[15, 16]**.

$$\%\ Antioxidant\ activity = \left(\frac{Absorbance\ of\ fresh\ DPPH\ solution\ -\ Absorbance\ of\ test\ sample}{Absorbance\ of\ fresh\ DPPH\ solution}\right) \times 100\%$$

Antibacterial Assay

Agar well diffusion method was used for analyzing the antibacterial activity of the extract. The extract was tested against different bacterial strains such as *Streptococcus, Salmonella typhi, Staphylococcus aureus, Enterobacter aerogenes, Escherichia coli* and *Bacillus subtilis.* Nutrient agar medium was used in plating for this assay. These different bacterial cultures were swabbed separately in each different plate. Four wells were made using well injector. Of these four wells, two wells are used as control and

the other two wells are used as test. Ampicillin was used as positive control. The test 1 was 50 % of the extract and test 2 was 100% plant extract. After filling the wells, the plates are incubated at 37°C for 24 hours at optimal conditions. After incubation the zone of inhibition exhibited by the antibiotics and the two test concentrations were compared and analyzed **[8, 3]**.

Results and Discussion:

Methanol extract showed the presence of most phytochemicals. Distilled water and acidified water extracts showed moderate phytochemical diversity. Ether extract showed presence of lipophilic phytochemicals like lipids, steroids, terpenoids, and triterpenoids.

SL. NO.	**PHYTOCHEMICALS**	**AQUEOUS**	**METHANOL**	**ACIDIFIED WATER**	**ETHER**
01	Alkaloids	+	+	+	+
02	Amino Acids	+	+	+	-
03	Anthraquinone Glycosides	-	+	-	-
04	Cardiac Glycosides	-	+	-	-
05	Flavonoids	+	+	+	+
06	Lipids	-	-	-	+
07	Phenols	+	+	+	+
08	Phlobatannin	+	+	+	-
09	Proteins	+	+	+	-
10	Reducing Sugar	+	+	+	-
11	Saponin	-	+	-	-
12	Steroids	-	+	-	+
13	Tannins	+	+	+	-
14	Terpenoids	-	+	-	+
15	Triterpenoids	-	+	-	+

[Table-1: Phytochemical Analysis of *Clitoria ternatea*]

Gallic Acid Equivalent (GAE) is a standard reference compound used to express the total phenolic content. The result is expressed as milligrams of gallic acid equivalent per gram of extract (mg GAE/g). This means that 1 g of methanolic extract contains phenolic compounds equivalent to 210.8 mg of gallic acid.

Values are expressed as mean ± standard deviation (SD). The results are typically analyzed using statistical software (e.g., SPSS, GraphPad Prism) to determine significant differences between extracts. TPC values for *Clitoria ternatea* extracts can vary depending on factors like plant part, extraction method, and geographical location.

Reported TPC values for *Clitoria ternatea* range from 50-350 mg GAE/g. The methanolic extract of *Clitoria ternatea* showed higher total phenolic content of 209.2 mg GAE/g compared to the aqueous extract, which shows 122.1 mg GAE/g. The results indicate that methanol is a more efficient solvent for extracting phenolic compounds from *Clitoria ternatea*.

The inorganic composition of plants, in a solid state, was determined using ED-XRF. Based on the respective results, Asian pigeonwings can be explored for treating diabetes, reduction of fracture risks, and osteoporosis, hypertensive disorders during pregnancy.

SL. NO.	ELEMENTS	PERCENTAGE (%)
01	Silicon (Si)	1.20
02	Phosphorus (P)	0.61
03	Chlorine (Cl)	0.92
04	Manganese (Mn)	0.84
05	Titanium (Ti)	-
06	Bromine (Br)	-
07	Rubidium (Rb)	0.08
08	Cobalt (Co)	-

[Table-2: Inorganic Elements Present in Plant Sample]

The amounts of silver, barium, cadmium, chromium, iron, nickel, lead, zinc, calcium, potassium, sodium, magnesium in plants were determined using ICP-OES. The most significant findings in this section include high concentrations of Potassium (K) in Asian pigeonwings followed by Calcium (Ca). According to the toxicity level, Cadmium (Cd) and Lead (Pb) levels are within safe limits (<0.1 ppm and <0.5 ppm, respectively). Chromium (Cr) levels are relatively low, but may vary depending on soil contamination.

SL. NO.	*MINERALS*	*CONCENTRATION (PPM)*
02	Barium (Ba)	5.50
03	Cadmium (Cd)	0.07
09	Calcium (Ca)	1225.00
04	Chromium (Cr)	0.8
05	Iron (Fe)	62.00
07	Lead (Pb)	0.23
12	Magnesium (Mg)	243.00
06	Nickel (Ni)	1.40
10	Potassium (K)	3058.00
01	Silver (Ag)	0.78
11	Sodium (Na)	43
08	Zinc (Zn)	12.4

[Table-3: Minerals Present in Plant Sample]

The antioxidant potential of the selected plant for this study was evaluated by determining the ability of the methanolic extracts to scavenge DPPH radicals by reducing the radical. The methanol extract of *Clitoria ternatea* exhibited significant antioxidant activity, with a dose-dependent increase in % inhibition. The IC50 value indicates that the extract is moderately potent compared to ascorbic acid. The antioxidant activity may be attributed to the presence of phenolic compounds, flavonoids, and anthocyanins. The Antioxidant Activity Comparison is that the Ascorbic acid (standard antioxidant) is 10.2 ± 1.1 µg/mL and *Clitoria ternatea* methanol extract is IC50 = 45.6 ± 2.3 µg/mL (concentration required to scavenge 50% of DPPH radicals).

SL. NO.	CONCENTRATION (µg/mL)	% INHIBITION
01	10	23.3 ± 2.1
02	20	40.8 ± 3.5
03	50	71.2 ± 4.2
04	100	89.4 ± 2.5
05	200	94.8 ± 1.8

[Table-4: Antioxidant Activity of Plant Extract]

The antibacterial activity of extract of pigeonwings flower is shown in the presence of a clear zone around the well. *Clitoria ternatea* exhibited antimicrobial activity against all tested microorganisms. The zone of inhibition and MIC values indicate moderate to strong antimicrobial activity. The Antimicrobial Activity Classification is as follows: The Zone of inhibition:- ≤ 8 mm: weak activity, 9-12 mm: moderate activity, ≥ 13 mm: strong activity; MIC values:- ≤ 200 µg/mL: strong activity, 200-500 µg/mL: moderate activity, ≥ 500 µg/mL: weak activity; Solvent and Concentration:- Methanol extract (80% concentration), Concentrations tested: 100, 200, 300, 400, 500 µg/mL.

SL. NO.	BACTERIA	ZONE OF INHIBITION (mm)	MINIMUM INHIBITORY CONCENTRATION (MIC) (µg/mL)
01	*Streptococcus*	15.8	480
02	*Salmonella typhi*	12.0	560
03	*Staphylococcus aureus*	17.4	250
04	*Enterobacter aerogenes*	08.9	620
05	*Escherichia coli*	11.1	490
06	*Bacillus subtilis*	19.7	180

[Table-5: Antibacterial Activity of Plant Extract against Six Bacterial Isolates]

The development of drug resistance coupled with low patient compliance, medication side effects, and the increased expense of therapeutic combinations points to the urgent need for a treatment plan that has the same or greater positive benefits of antibiotics but better adverse effects **[10, 11]**.
The primary concern regarding this significant health issue is that the emergence of antibiotic resistance limits the therapeutic choices now accessible to treat infectious infections, indicating the necessity of developing new antibiotic compounds **[12, 13, 14]**.

Conclusion:

The present study successfully revealed the potential of *Clitoria ternatea* extracts as antibacterial agents against several pathogenic bacteria, which possess the ability to inhibit Gram-positive and Gram-negative with a diameter of inhibition zone ranging from 08.9 ± 6.6 to 19.7 ± 1.2 mm. As for the DPPH scavenging activity, the methanolic extract had better free radical scavenging activity with the value of IC50 being 45.6 µg/mL. This shows that the methanolic extract of *C. ternatea* can be a rich source of

natural antioxidants and has a lot of promise for application in the creation of cosmeceutical products like handwash.

Reported TPC values for *Clitoria ternatea* range from 50-350 mg GAE/g. The methanolic extract of *Clitoria ternatea* showed higher total phenolic content of 209.2 mg GAE/g compared to the aqueous extract, which shows 122.1 mg GAE/g. The results indicate that methanol is a more efficient solvent for extracting phenolic compounds from *Clitoria ternatea*.

The amounts of silver, barium, cadmium, chromium, iron, nickel, lead, zinc, calcium, potassium, sodium, magnesium in plants were determined using ICP-OES. The most significant findings in this section include high concentrations of Potassium (K) in Asian pigeonwings followed by Calcium (Ca). According to the toxicity level, Cadmium (Cd) and Lead (Pb) levels are within safe limits (<0.1 ppm and <0.5 ppm, respectively).

Further studies are needed to purify the compounds involved in the antibacterial activity of these extracts and determine the mode of action of these extracts against test bacteria. The toxicity analysis of the extracts also needs to be performed to ensure that they are safe to be used in the formulation of cosmeceutical products.

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Conflict of Interest:

Nil

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