

In Vitro Establishment, Induction and Propagation of Callus Cultures in *Clitoria ternatea*

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Abstract: *Clitoria ternatea*, commonly known as butterfly pea, is a medicinal and economically important plant known for its diverse bioactive compounds, including ternatins, flavonoids, triterpenoids and cyclotides. Ternatins, a group of anthocyanin pigments, contribute to the characteristic blue colour of its flowers and are associated with antioxidant activity, while flavonoids such as quercetin and kaempferol are reported for their antioxidant and anti-inflammatory properties. Cyclotides are cyclic peptides with reported antimicrobial and insecticidal activities. The plant has applications as a natural blue colourant, pH-responsive indicator, natural dye and potential source of bioactive compounds. However, the production and accumulation of these metabolites in naturally grown plants may vary with seasonal, environmental and geographical conditions. Plant tissue culture provides an alternative approach for establishing plant material under controlled conditions. In the present study, in-vitro cultures of *C. ternatea* were established using embryo culture and callus induction on Murashige and Skoog (MS) medium supplemented with suitable plant growth regulators. The cultures were incubated at $25 \pm 2^\circ\text{C}$ under a 16-hour light and 8-hour dark photoperiod. Preliminary phytochemical analysis of the floral samples confirmed the presence of phenolic compounds, flavonoids and anthocyanins. The total phenolic, flavonoid and anthocyanin contents were found to be 16.66 mg GAE/g, 40 mg QE/g and 71.3 mg C3G/L, respectively, indicating the presence of these bioactive secondary metabolites in the flowers. In the subsequent stage, the established callus will be treated with different concentrations of a chemical mutagen to induce genetic variation. The mutagen-treated callus will subsequently be maintained and compared with untreated control callus through phytochemical analysis to investigate possible changes in the metabolite profile.

Keywords: *Clitoria ternatea*, callus culture, phytochemical analysis, anthocyanin

I. INTRODUCTION

Changes in modern lifestyle have contributed to a number of health problems among students and adults, including chronic anxiety, depression, memory decline, hypertension, high cholesterol, diabetes, respiratory problems, skin disorders and cancers (Jadhav et al., 2025). Butterfly Pea has drawn attention because of its flowers are rich in antioxidant and anticancer compounds, and the plant has been linked with brain health in young people, skin care and protection of cells.

Clitoria ternatea, known as butterfly pea, is a perennial herbaceous plant belonging to the family Fabaceae. Being a legume, it hosts nitrogen-fixing bacteria in its roots, which add nitrogen to the surrounding soil naturally (Oguis et al., 2019). The flowers are famous for their intense blue colour, which comes from an anthocyanin pigment called ternatin and changes with pH, making the flower a source of natural blue food colorant. Anthocyanins have good market value as natural colorants in the cosmetic, food and textile industries (Handayani et al., 2024; Jeyaraj et al., 2022). The plant also contains the flavonol glycosides quercetin and myricetin, which act as strong anti-glycation agents and promote collagen synthesis (Multisona et al., 2023). In addition, it has cyclotides, which are reported to help suppress cellular mutations (Oguis et al., 2019), and the pentacyclic triterpenoids taraxerol and taraxerone, which raise acetylcholine levels and may therefore improve focus, memory and muscle movement (Jadhav et al., 2025; Mukherjee et al., 2008).



Despite this therapeutic and commercial promise, *C. ternatea* does not give a consistent yield under natural conditions. Wild plants are exposed to changing environmental, seasonal and geographical factors, affects production of secondary metabolites fluctuates (Mohammed et al., 2025). Plant tissue culture offers a way of growing plants in vitro under controlled environmental and nutritional conditions (Mohammed et al., 2025). By adjusting the culture medium, growth regulators, environmental conditions and subculture intervals, a tissue culture system can be designed for rapid multiplication, callus formation, regeneration and the study of secondary metabolite production (Mohamed & Taha, 2011; Teoh et al. 2023). Embryo culture in vitro also avoids the risk of soil-borne pathogens and the problem of early seed dormancy. Callus culture is especially useful for studying secondary metabolism, since it provides an actively dividing and fairly uniform supply of plant biomass.

The present study therefore aimed to establish *C. ternatea* under in vitro conditions, to induce and propagate callus cultures, and to characterise selected phytochemicals of the plant material.

II. LITERATURE REVIEW

1. Mukherjee et al. (2008) reviewed the traditional medicinal uses and phytochemical importance of *Clitoria ternatea*. The review discussed compounds such as triterpenoids, flavonoids, anthocyanins and steroids, along with their reported biological activities and therapeutic potential.

2. Kumar and Thomas (2012) developed an in vitro regeneration system for *C. ternatea* using cotyledonary explants. They successfully induced embryogenic callus, regenerated plants through somatic embryogenesis and demonstrated synthetic seed production, highlighting the potential of tissue culture for plant propagation.

3. Jaafar et al. (2020) investigated the extraction of phenolic, flavonoid and anthocyanin compounds from *C. ternatea* flowers. Their findings showed that extraction conditions can significantly influence phytochemical recovery and antioxidant activity, supporting the use of TPC, TFC and anthocyanin assays.

4. Teoh et al. (2023) studied callus induction from cotyledon and hypocotyl explants of *C. ternatea* using different concentrations of 2,4-D. Cotyledon explants showed a better response under suitable conditions, with friable callus formation, providing a useful basis for further tissue culture studies.

5. Nguyen et al. (2026) investigated a sealed-vessel extraction method for recovering phenolic compounds and anthocyanins from *C. ternatea* flowers. They found that factors such as temperature, ethanol concentration and extraction time influenced phytochemical analysis to characterize individual anthocyanins.

III. RESEARCH METHODOLOGY

This study deals with the in vitro establishment and phytochemical analysis of *Clitoria ternatea*. It aims to follow growth and metabolite changes under a controlled environment and to set up a standard protocol for callus regeneration.

1. Explant Selection and Collection

Explants were obtained from the PIET building at PCE, Nagpur. Healthy mature seeds showing no visible damage were selected. The seeds were soaked in water overnight and then transferred to the Plant Tissue Culture Laboratory for surface sterilization.

2. In Vitro Seed Germination and Embryo Culture

Healthy *Clitoria ternatea* seeds were selected as explants and, after surface sterilization, were aseptically inoculated onto sterile MS medium (Kumar & Thomas, 2012). Sterilization involved a mild detergent, 70% ethanol and a surface-sterilizing agent (Kumar & Thomas, 2012). Inoculation was performed inside a biosafety cabinet under aseptic conditions (Teoh et al., 2023). Cultures were maintained at approximately $25 \pm 2^\circ\text{C}$ with a 16 h light/8 h dark photoperiod.

3. In Vitro Embryo Culture

After cleaning and surface sterilization, embryos were carefully excised from the seeds and aseptically transferred to half-strength MS medium. The cultures were maintained at $25 \pm 2^\circ\text{C}$ under a 16 h light and 8 h dark photoperiod.



IV. CALLUS INDUCTION WITH DIFFERENT HORMONE COMBINATIONS

Callus induction and maintenance depend on factors such as explant type, genotype, basal-medium composition, the auxin-to-cytokinin balance, and culture conditions including temperature, photoperiod and subculture timing (Mohammed et al., 2025). Sterile cotyledonary leaf and hypocotyl explants were excised from seedlings established in vitro and used for callus induction (Mohammed et al., 2025; Teoh et al., 2023). The explants were cultured on MS medium containing 2,4-D, IAA or NAA at 0.5, 1.0 or 2.0 mg/L, together with kinetin or BAP at the corresponding concentrations. Cultures were maintained at approximately $25 \pm 2^\circ\text{C}$ in complete darkness (Mohammed et al., 2025).

V. PHYTOCHEMICAL ANALYSIS OF NATURALLY GROWN FLOWERS AND LEAVES

5.1 Solvent Extraction

Because anthocyanins are sensitive to heat, the samples were extracted by cold maceration. Dried *Clitoria ternatea* flowers and leaves were immersed in 70% ethanol at a 1:10 solid-to-solvent ratio and kept in darkness at room temperature for 48 h. The extracts were filtered through Whatman No. 1 filter paper, and the filtrates were stored at 4°C until analysis (Sutarkar et al., 2025).

5.2 Determination of Total Phenolic Content (TPC)

The TPC of dried *Clitoria ternatea* flowers and leaves was determined by spectrophotometry. A 0.5 mL portion of fresh extract was combined with 2.5 mL of Folin-Ciocalteu reagent diluted 1/10 and 70% ethanol, then kept at room temperature for 2–5 min. Next, 2 mL of 7.5% w/v sodium carbonate was added and the mixture was allowed to react for 30 min. Absorbance was measured at 765 nm using a UV-visible spectrophotometer. Results were expressed as mg gallic acid equivalents per g of dry sample (Bakar et al., 2009; Singleton and Rossi, 1965).

5.3 Determination of Total Flavonoid Content (TFC)

For TFC estimation, 1 mL of dried *C. ternatea* extract was mixed with 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, 70% ethanol and distilled water. The reaction mixture was kept at room temperature for 30 min. Quercetin was used as the reference standard, and absorbance was measured at 415 nm with a UV-visible spectrophotometer. Results were expressed as mg quercetin equivalents per g of dry sample (Jaafar et al., 2020).

5.4 Determination of Total Anthocyanin Content (TAC)

TAC was determined by the pH-differential method (Lee et al., 2005). The dried-sample extract was prepared in 0.1 N HCl because acidic conditions help maintain anthocyanin stability. For the assay, 0.5 mL of extract was separately combined with 4.5 mL of pH 1.0 potassium chloride buffer and 4.5 mL of pH 4.5 sodium acetate buffer. Both preparations were kept in the dark at room temperature for 25 min. Absorbance was recorded at 520 and 700 nm against a distilled-water blank using a Shimadzu UV-visible spectrophotometer. The net absorbance was calculated as:

$$\text{Absorbance (A)} = [(A_{520\text{nm}} - A_{700\text{nm}}) \text{ pH} 1.0] - [(A_{520\text{nm}} - A_{700\text{nm}}) \text{ pH} 4.5]$$

The total anthocyanin concentration was then determined from the net absorbance using the Beer–Lambert relationship:

$$\text{TAC (mg/L)} = A \times \text{MW} \times \text{DF} \times 1000 / \epsilon \times l$$

where:

MW = molecular weight (448.8 g/mol for cyanidin-3-glucoside)

DF = dilution factor

ϵ = molar extinction coefficient (26,900 L/mol/cm for cyanidin-3-glucoside)

l = path length (1 cm)

The calculated TAC was reported as milligrams of cyanidin-3-glucoside equivalents per litre (mg C3G/L).

5.5 UV-Visible Detection of Anthocyanins in the Extract

UV-visible scanning was performed to verify anthocyanin absorption and identify the wavelength of maximum absorption. Optical density (OD) was recorded for both natural and acidified extracts across the selected wavelength range using a Shimadzu UV-Vis spectrophotometer.



VI. RESULTS AND DISCUSSION

6.1 In Vitro Establishment of *Clitoria ternatea*

Successful seed germination enabled establishment of the in vitro cultures for subsequent callus induction. Embryos served as an effective starting material because their actively growing tissues were capable of forming callus. Germination of intact seeds took approximately three weeks, whereas isolated embryos produced a hypocotyl in about seven days.

6.2 Effect of Hormone Composition on Callus Formation

Callus formation occurred after the selected explants were cultured on MS medium containing plant growth regulators. The treatment containing 2.0 mg/L 2,4-D with 0.5 mg/L kinetin produced callus rapidly, while 2.0 mg/L 2,4-D combined with 1.0 mg/L BAP also showed a strong induction response, consistent with the findings of (Mohamed and Taha et al., 2011). The maximum callus response was observed 20–25 days after inoculation.

Table 1. Callus induction response under different hormone combinations

Sr. No.	Hormone Concentration (mg/L)		Callus induction
	Auxin	Cytokinin	
1.	2.0 mg/L 2,4-D	0.5 mg/L Kinetin	+
2.	1.0 mg/L 2,4-D	1.0 mg/L Kinetin	-
3.	2.0 mg/L 2,4-D	1.0 mg/L Kinetin	-
4.	1.0 mg/L 2,4-D	1.0 mg/L BAP	-
5.	2.0 mg/L 2,4-D	1.0 mg/L BAP	+
6.	1.5 mg/L IAA	1.5 mg/L BAP	+
7.	2.0 mg/L NAA	1.0 mg/L BAP	+
8.	1.0 mg/L NAA	1.0 mg/L BAP	-

In Table 1, (+) represents callus formation on the explant under the specified hormone treatment in MS medium, whereas (–) represents the absence of prominent callus development.



Figure 1. (a)



Figure 1. (b)



Figure 1. (c)

Figure 1. (a) Collection of *C. ternatea* samples at the PIET building, Nagpur;

Figure 1. (b) In vitro establishment of *Clitoria ternatea* on MS medium;

Figure 1. (c) Callus formation from *C. ternatea* explants cultured on MS medium supplemented with PGRs.

6.3 Phytochemical Analysis

Phytochemical analysis confirmed that phenolic compounds, flavonoids and anthocyanin are present in the flowers and leaves of *C. ternatea*. The quantitative results given in Tables 2 and 3.

Table 2. Quantitative phytochemical profile of *Clitoria ternatea* flower sample

Phytochemical parameter	Result	Expression
Total phenolic content	16.66	mg GAE/g



Total flavonoid content	40.00	mg QE/g
Total anthocyanin content	71.30	mg C3G/L

Table 3. Quantitative phytochemical profile of *Clitoria ternatea* leaf sample

Phytochemical parameter	Result	Expression
Total phenolic content	48.43	mg GAE/g
Total flavonoid content	292.48	mg QE/g
Total anthocyanin content	34.1	mg C3G/L

The phytochemical analysis showed clear differences in total phenolic, flavonoid and especially anthocyanin content between the two samples. The flower extract had lower TPC and TFC than the leaf extract, whereas the leaf sample contained only a very small amount of anthocyanin. In both samples TFC was much higher than TPC. This agrees with Jaafar et al. (2020), who reported TFC (187.05 mg QE/g) above TPC (41.17 mg GAE/g) in *C. ternatea* flower extract, but contrasts with Divya et al. (2018), who reported higher TPC than TFC in leaf extracts. The leaf sample (TPC = 48.43 mg GAE/g; TFC = 292.48 mg QE/g) was well above the 25 mg GAE/g TPC and 10 mg QE/g TFC reported for blue-flowered leaves by Divya et al. (2018). The flower sample (TPC = 16.66 mg GAE/g; TFC = 40.00 mg QE/g) had lower TPC and TFC than the flower values reported by Jaafar et al. (2020), which may reflect differences in solvent and extraction method.

6.4 Anthocyanin Detection in Extract using U V-Vis Spectrophotometer

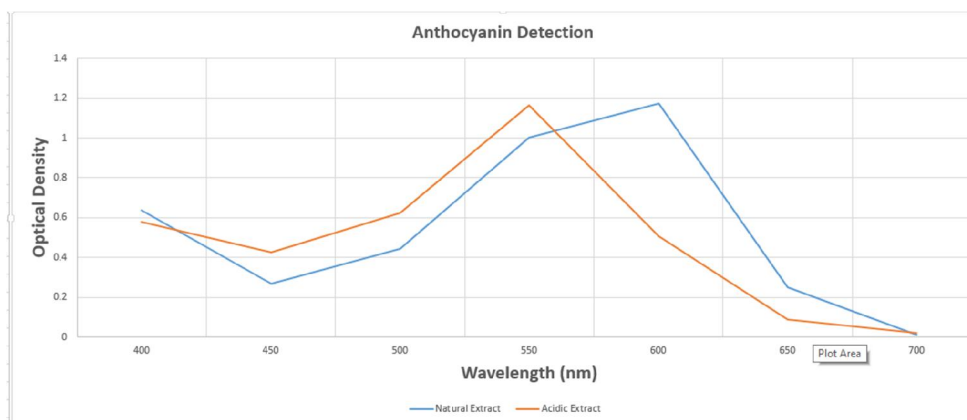


Figure 2. Anthocyanin absorption profile recorded using a Shimadzu UV-Vis spectrophotometer

Anthocyanin absorption varies with the pH of the pigment extract. Acidic extracts within pH 1–3 generally show maxima between 520 and 550 nm, whereas natural blue extracts can exhibit maxima at longer wavelengths of 570–620 nm (Handayani et al., 2024). In Figure 2, the acidic extract reached maximum absorbance at 550 nm and the natural extract at 600 nm. Both values are within the reported ranges and support the detection of anthocyanins in the extracts.

VII. CONCLUSION

The study established in vitro callus cultures of *Clitoria ternatea* and quantified selected phytochemicals in flowers and leaves from the wild ecotype. Explants were selected and processed using plant tissue culture (PTC) procedures, with cultures maintained aseptically on MS medium containing different combinations of 2,4-D, kinetin, BAP, NAA and IAA. Maceration was used for solvent extraction, followed by spectrophotometric quantification of the selected compounds. Embryo culture provided the most effective starting material for in vitro establishment, while the strongest callus responses occurred with 2.0 mg/L 2,4-D plus 0.5 mg/L kinetin and 2.0 mg/L 2,4-D plus 1.0 mg/L BAP. The flower extracts contained 16.66 mg GAE/g TPC, 40 mg QE/g TFC and 71.3 mg C3G/L TAC, whereas the leaf extracts contained 48.43 mg GAE/g TPC, 292.48 mg QE/g TFC and 34.1 mg C3G/L TAC. These findings demonstrate tissue-dependent differences in secondary-metabolite concentrations. TFC exceeded TPC in both tissues, agreeing with Jaafar



et al. (2020) for *C. ternatea* flower extract but differing from the pattern reported by Divya et al. (2018) for leaf extracts. Thus, controlled callus cultures may serve as a useful system for further study and production of secondary metabolites with pharmaceutical and industrial applications.

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