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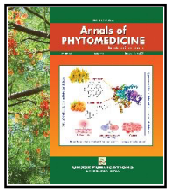

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Optimizing bioactive compound extraction from *Clitoria ternatea* L. blue flowers: A comparative solvent study

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Abstract

The present study aimed to assess the maximum extraction efficacy of phenolic compounds, flavonoids, anthocyanins, and tannins from the blue flowers of *Clitoria ternatea* L. using five solvents (water, methanol, ethanol, acetone, citrate buffer) across four durations (0, 4, 8, 12 h). A comparative evaluation of antioxidant activities (DPPH, ABTS, FRAP, superoxide anion scavenging) and antioxidant enzyme activities (peroxidase, superoxide dismutase, guaiacol peroxidase, polyphenol oxidase) was conducted. Results demonstrated ethanol as the optimal solvent for phytochemical recovery, with 8 h extraction yielding peak concentrations: phenolics (193.0 ± 0.95 mg GAE/g Fw), flavonoids (469.34 ± 0.61 mg catechine/g Fw), anthocyanins (178.33 ± 0.88 mg CGE/g Fw), and tannins (372.0 ± 0.89 mg TAE/g Fw). Ethanol extracts also exhibited superior antioxidant capacity, including DPPH (92.43 ± 0.47 mg ascorbic acid/g Fw), ABTS (89.36 ± 0.67 mg gallic acid/g Fw), FRAP (294.8 ± 0.74 mg BHT/g Fw), and superoxide anion scavenging (89.33 ± 1.22 mg ascorbic acid/g Fw). Enzymatic activities were highest in ethanol: peroxidase (391.20 ± 0.70 U/ml), superoxide dismutase (513.4 ± 0.67 U/ml), guaiacol peroxidase (513.4 ± 0.9 U/ml), and polyphenol oxidase (812.16 ± 1.29 U/ml). Statistical analysis confirmed significance ($p<0.05$) across all parameters. These findings position ethanol as the preferred medium for maximizing phytochemical yield from *C. ternatea*, offering applications in natural antioxidant production, pharmaceutical formulations, herbal therapeutics, and food-grade colorants. The study provides a methodological framework for optimizing solvent selection and extraction timelines in plant-based bioactive compound recovery.

1. Introduction

Clitoria ternatea L., a perennial twining herbaceous plant of the Fabaceae family, is widely cultivated in Asian countries for its herbal and culinary uses (Makasana *et al*., 2017). Beyond its culinary uses, this plant has numerous health benefits with various parts used for therapeutic purposes. The plant's versatility is evident in its multiple applications, including seeds, and roots. The leaves used as poultices for inflamed joints (Abubakar and Haque, 2020), and flower juice for skin conditions, irritated eyes, and insect bites. *C. ternatea's* roots also treat ailments like pulmonary TB, infections, asthma, and body aches (Zingare *et al.,* 2013). The plant possesses several biological properties, including hepatoprotective, antibacterial, antidiabetic, antioxidant, and antihyperlipidemic effects (Escher *et al.,* 2020).

Antioxidants in butterfly pea flower enhance the body's defenses against illness. The study by Srichaikul (2018) reveals that the drink made of extracts of its flowers helps to prevent wrinkles and slow down skin ageing due to their antioxidant properties. Studies have also shown that when floral extract was subjected to total phenolic, ferric reducing antioxidant power (FRAP), and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) tests for scavenging free radicals, enhances both antioxidant qualities and polyphenol amounts (Pasukamonset *et al.,* 2018). These findings highlight *C. ternatea's* potential as a valuable resource for promoting health and well-being.

Polyphenols, a common type of secondary metabolite in plants, play a crucial role in disease resistance and UV protection (Ganesan and Xu, 2017). They also contribute to food colouring, astringency, and bitterness in the food industry. As potent antioxidants, polyphenols provide significant health benefits, including protection against diseases and cancers (Maqsood *et al.,* 2013), and enable plants to scavenge radicals (Alvarez *et al.,* 2016). However, the use of organic solvents in extraction poses environmental and health concerns (Pena *et al.,* 2015). To address this, effective solvents like ethanol can achieve optimal extraction with high yields, superior purity, and minimal waste (Chemat *et al*., 2012). Ethanol's broad polarity index makes it suitable for eliminating polar and nonpolar materials from natural products (Gulcin *et al.,* 2010), and its favourable polarity and ability to dissolve bioactive compounds make it an optimal solvent in cosmetics and pharmaceuticals.

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The objective of this research study was to assess the ideal extraction solvents by utilizing five distinct solvents water, methanol, 60 per cent ethanol, acetone, and citrate buffer with varying times (0, 4, 8, and 12 h) at room temperature by measuring the amounts of total phenols, flavonoids, tannins, and anthocyanin. The evaluation of antioxidants, such as superoxide radical scavenging activity, ABTS, FRAP, and 2,2-diphenyl-1-picrylhydrazyl-hydrate (DPPH), as well as antioxidant enzymes like peroxidase, SOD, guaiacol peroxidase, and polyphenol oxidase of *C. ternatea*, was also conducted using these chosen extraction solvents. Furthermore, the researchers conducted a systematic assessment of *C. ternatea's* phytochemical content and antioxidant activities in comparison to other research investigations.

The publications were found using the NCBI (National Centre for Biotechnology Information, PubMed, and PubMed Central databases) and the SCOPUS database. 206 original research publications, all were obtained from various research publications. Every article's title, abstract, and primary contents were carefully reviewed to make sure they were pertinent to this subject.

2. Materials and Methods

The study was conducted on *C. ternatea*, a healthy blue-flowering shrub. The blue flowers of *C. ternatea* were collected from the University Campus at Pusa (Figure 1). All analytical work was carried out at the Department of Botany, Plant Physiology, and Biochemistry, CBS and H, Dr. Rajendra Prasad Central Agricultural University, Pusa, Samastipur, Bihar. The plant species, *C. ternatea* was authenticated by the CSIR-National Botanical Research Institute (NBRI), Lucknow, in accordance with the classification system having an Authentication Number, PDSH/LWG/Authentication/2025-26/07.

Figure 1: Flower of *Clitoria ternatea*.

2.1 Extraction methods

The fresh and mature blue flowers of *C. ternatea* were collected from the garden. Both fresh and distilled water were used to wash it. The blue blooms were broken into tiny fragments. Finally, they added to the different flasks. Five distinct solvent types were used, including acetone, water, methanol, 60 per cent ethanol, and citrate buffer (10 mM 1:1v/v ratio, pH 3.0). The pieces of flowers were individually dipped into each solvent. Following that, each flask was left at room temperature for 0, 4, 8, and 12 h before being placed on a rotary shaker set to 100 rpm.

After that, each extract was centrifuged individually for 15 min at 5000 rpm to extract only the supernatant. Finally, the same volume of solvent was used to make all extract solvents. Following that, aluminium foil was placed over each extracted sample to stop the solvent from vaporizing. Lastly, for additional biochemical investigation, the sample extracts were stored at 40°C in the refrigerator. Every sample was examined three times.

2.2 Evaluation of secondary metabolites and antioxidants of *C. ternatea*

2.2.1 Total phenol estimation

The Folin Ciocalteu reagent was used to determine the sample extracts, and total phenol concentration of 2.5 ml of 0.2 N Na_2CO_3 and 0.1 ml of Folin Ciocalteu reagent were added to each solvent extract by the using Singleton method (Singleton and Rossi, 1965). For half an hour, this was incubated at room temperature. Absorbance at 760 nm was measured using a spectrophotometer. The fresh weight of the sample was measured in milligrams using gallic acid as a reference (mg GAE/g Fwt).

2.2.2 Flavonoids estimation

The $AlCl_3$ technique was used to calculate the total flavonoid content (Kumaran and Karunakaran, 2007). Each 0.3 ml extract solvent was mixed with 3.4 ml of methanol (30%), $AlCl_3$ (0.15 ml, 0.3 M), and $NaNO_2$ (0.15 ml, 0.5 M) in a test tube. 1 ml of NaOH was added after 5 min. At 506 nm, absorbance was measured. The whole flavonoid content was expressed as catechin equivalents mg/g fresh weight.

2.2.3 Anthocyanin estimation

The total anthocyanin pigment was assayed by the pH differential method (Gao and Mazza, 1995). In this method, KCl buffer (0.025 M, pH 1.0) and CH_3COONa buffer (0.4 M, pH 4.5) were used. To equilibrate, 4 ml of buffers and 1 ml of the solvent extract aliquot were also added. The mixture was incubated at 28°C for 15 min. Blank was prepared with distilled water and absorbance was read at 510 and 700 nm. The result was calculated as milligrams of cyanidin-3 glucoside equivalents (CGE) per gram of fresh weight.

Absorbance (A) = [(A520 nm × A700 nm) pH 1.0 – (A520 nm 6 A700 nm) pH 4.5]

The total anthocyanin was expressed as cyanidin-3glucoside equivalent as shown in the equation:

Total anthocyanin mg/g = A × MW × DF × 1000/ e × l

where,

MW = Molecular weight (449.2 g/mole for cyanidin-3 glucoside)

DF = Dilution factor,

l = path of length (1 cm)

e = Molar extinction coefficient (26900 molar extinction coefficient in L/mole/cm for cyanidin-3- glycoside)

2.2.4 Tannins estimation

The tannin content was determined using the Folin-Denis reagent (Moonmun *et al.*, 2017). 5 ml of Folin-Denis reagent was combined with 1 ml of solvent extract. The mixture was shaken constantly for 3 min. After mixing, a 10 ml solution of sodium carbonate was added.

The solvent extract was left to stand for two hours. The extract was then centrifuged until all components had settled to the bottom of the centrifuge tube. The optical density (OD) at 700 nm was measured using only the supernatant. A blank was prepared using the same technique. A standard curve for tannic acid was created using multiple dilutions of tannin solutions (1:250, 1:50, 1:40, 1:25, and 1:10) to determine milligram equivalents of tannic acid (mg TEA/g Fwt.).

2.2.5 Assay of DPPH scavenging activity

With a minor modification in the method of Sunil *et al.* (2014), conducted a DPPH radical scavenging activity assay. 1.0 ml of DPPH solution (0.1 mM) was mixed with 1.0 ml of the extract solvent aliquot. For the blank, ethanol and DPPH were used in equal amounts. The mixture was shaken thoroughly and left to stand in the dark for 30 min. The absorbance was then measured at 517 nm. The percentage of inhibition was calculated to determine the DPPH radical scavenging activity, which was expressed as milligrams of ascorbic acid equivalent per gram of fresh sample weight (mg AA/g FW).

Inhibition% = [(A control 6 A sample)/A control] × 100%

IC_{50} values were used to measure the extract's ability to scavenge DPPH radicals. Plotting the scavenging activity versus sample concentration allowed for the determination of the extract's effective concentration (IC_{50}) required for 50 per cent DPPH radical scavenging.

2.2.6 Assay of ABTS scavenging activity

The protocol for measuring ABTs was established on the method described by Stratil *et al.* (2007). To generate the radical cations ABTs+, a solution consisting of a 1:1 (v/v) mixture of ABTs (7 mM) and potassium persulfate (4.95 mM) was left overnight under dark conditions at room temperature. Subsequently, 1.0 ml of extract, 3.9 ml of the ABTS solution, and 0.1 ml of phosphate buffer (pH 7.4) were added to the reaction mixture. The absorbance was measured at 745 nm. An identical process was used to prepare the blank, except that no extract was included in the reaction mixture. The inhibition percentage was determined using the formula below. The ABTS assay was calibrated against gallic acid concentrations in mg/ml.

Inhibition % = [(A control 6 A sample)/A control] × 100%

IC_{50} values were used to illustrate the extract's capacity to scavenge ABT radicals. The IC_{50} values, which are presented as percentages, were calculated using a graph that displays the concentration of the sample needed to scavenge half of the free radicals generated by 50 per cent ABT.

2.2.7 Assay of FRAP activity

Ferric-reducing antioxidant power activity (FRAP) was determined by using the modified method of Athukorala *et al.* (2006). In the reaction mixture, 2.5 ml of phosphate buffer, 2.5 ml of potassium ferricyanide, and 1.0 ml of each extract solvent. Left for incubation at fifty degrees centigrade for 20 min and then after 2.5 ml of $FeCl_2$ was added. Lastly, the reaction mixture was centrifuged again at 3000 rpm for 10 min. 2.5 ml distilled water was mixed and then the top layer of solution was removed by a sucking tube. Finally, v was measured at 700 nm.

A greater reducing power was indicated by an increase in the absorbance of the reaction mixture. The FRAP assay was calibrated using Butylated Hydroxy Toluene (BHT) in milligrams per milliliter. The IC_{50} measurements were used to quantify the extract's capacity to scavenge FRAP radicals. A graph showing the sample concentration required to scavenge 50 per cent of the FRAP free radicals was used to calculate the IC_{50} values. The inhibition percentage was also calculated using the following formula:

Inhibition % = [(A control 6 A sample)/A control] × 100%

2.2.8 Assay of superoxide radical scavenging activity

The method of Kakkar *et al*. (1984) was adopted to assess the superoxide anion scavenging activity of *C. ternatea* blue flowers using nitroblue tetrazolium (NBT). A solution containing 1 mM hydroxylamine hydrochloride and 24 mM NBT was prepared. The reaction mixtures consisted of 100 ìl of NBT (24 mM), 0.2 µl of 0.1 mM ethylene diamine tetra acetic acid (EDTA) solution, 1 ml of distilled water, and varying volumes (50-250 µl) of the extract. The reaction was initiated by adding approximately 0.1 ml of hydroxylamine hydrochloride (1 mM) to the reaction mixture.

The decrease in NBT absorbance was measured at 560 nm after incubating the reaction mixture for 20 min at 25°C. A control experiment was conducted using the same procedure but without the extract. The sample's antioxidant activity was expressed as mg ascorbic acid per gram of fresh sample weight, using a standard curve based on ascorbic acid (0.1 mg/ml). The percentage inhibition of superoxide radical scavenging activity was calculated using a specific formula:

Superoxide scavenging % = [(A control × A sample)/A control] × 100

2.2.9 Assay of peroxidase (POX) activity

To measure peroxidase activity, the method described by Putter (1974) was used. The extract from *C. ternetea* was kept on ice. In a cuvette, 3.0 ml of the assay mixture was prepared, consisting of 0.10 ml of phosphate buffer, 0.03 ml of hydrogen peroxide (0.042%, 12.3 mM), 0.1 M buffer at pH 7.0, 0.05 ml of guaiacol solution (20 mM), and 0.10 ml of extract. Using a UV-VIS spectrophotometer, the absorbance was measured at 430 nm. The same technique was used to prepare a blank but without the extract. The spectrophotometer was set to zero at a wavelength of 430 nm, and readings were taken at 20-second intervals.

Peroxidase activity was expressed in units per milliliter or micromoles per gram per minute of fresh weight of *C. ternetea*. The formula used to determine peroxidase activity is given below:

Unit/ml = [(A 430 per 20 sec Test sample 6 A430 per 20 sec Blank) × total volume × D. factor/6.39 × volume (ml) of enzyme used in the reaction mixture

where,

6.39 = Guaiacol dehydrogenase product's extinction coefficient at 436 nm

Units/mg protein = Unit per ml enzyme/mg protein per ml enzyme

D.F = dilution factor

2.2.10 Assay of superoxide dismutase (SOD) activity

The SOD assay was determined by Kakkar *et al*. (1984). For this analysis, the reaction mixture consisted of 0.2 ml of the extract

prepared for the test combination, 0.3 ml of NBT (300 µM), 0.1 ml of PMS (186 µM), and 1.2 ml of sodium pyrophosphate buffer (50 mM, pH 6.4). To initiate the reaction, 0.2 ml of NADH (780 µM) was added, bringing the total volume to the required amount. Following 90-second incubation at 30°C, 1.0 ml of glacial acetic acid was added to the mixture to stop the reaction. Subsequently, 4.0 ml of n-butanol was added, and the reaction mixture was thoroughly shaken and left to stand for 10 min.

The chromogenic intensity of the butanol layer was measured at 560 nm after separation. Standardization was facilitated by using butanol as the blank. One unit of enzyme activity is defined as the quantity of enzyme that causes a 50 per cent inhibition of NBT reduction per minute. To measure the activity, begin by recording the optical density (OD) of the blank and sample at 560 nm in a spectrophotometer for 1 min.

NBT reduction % = [(A control/min × A sample/min)/A control/min] × 100%

On the SOD standard inhibition curve, the inhibition percentage vs. log (SOD concentration) was plotted according to the inhibition percentage of each SOD standard.

2.2.11 Assay of guaiacol peroxidase (GPO) activity

To measure guaiacol peroxidase, the technique described by Egley *et al*. (1983) was employed. The assay mixture consisted of 0.05 ml of sample extract, 2 ml of phosphate buffer (40 mM, pH 6.1), and 0.2 ml of 2 mM H_2O_2, making a total volume of 5.0 ml. An increase in absorbance at 420 nm was observed at 30-second intervals for up to 1 min (extinction coefficient of 26.6 mM^{-1} cm^{-1}). The decrease in micromoles of H_2O_2 per min was used as one method to express enzyme-specific activity.

2.2.12 Assay of polyphenol oxidase (PPO) activity

The method developed by Esterbauer *et al*. (1977) was used to quantify polyphenol oxidase activity. In this approach, spectrophotometry was employed to assess the activities of laccase and catechol oxidase simultaneously. The extraction medium consisted of 4.0 ml of a solution containing 50 mM Tris HCl (pH 7.2), 0.4 M sorbitol, and 10 mM NaCl. The spectrophotometer was set to measure absorbance at 495 nm. The cuvette was filled with 2.5 ml of 0.1 M phosphate buffer and 0.3 ml of catechol solution (0.01 M catechol dissolved in 0.1 M phosphate buffer, pH, 6.5). After adding 0.2 ml of the enzyme extract to the spectrophotometer, the absorbance change was recorded every 0 to 5 min.

Under the test conditions, one unit of PPO activity was defined as the quantity of enzyme required to convert one micromole of dihydrophenol into 1.0 µmol of quinone per min. The unit of PPO activity was expressed as units per milliliter (U/ml). The following calculation was used to determine PPO activity.

Enzyme unit = K × absorbance per minute

where,

K for catechol oxidase = 0.272 and

K for laccase = 0.242

2.3 Statistical analysis

The antioxidant profile and enzyme activity data of *C. ternatea* extracts were analyzed through a t-test, completely randomized block design, and one-way analysis of variance (ANOVA). Each parameter of each sample was analyzed three times. Version 27 of the IBM SPSS statistics program was utilized for the statistical analysis.

3. Results

The results for all parameters, including total phenol content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and total tannin content (TTC), were determined using equations derived from standards. Folin-Ciocalteu reagent was employed to measure total phenols, with gallic acid serving as the standard in the tested CT flower extracts dissolved in water, methanol, ethanol, acetone, and citrate buffer. These were calculated in gallic acid equivalents using the standard curve equation ($y = 0.008x + 0.112$; $R^2 = 0.997$).

The total flavonoid content in extracts using water, methanol, ethanol, acetone, and citrate buffer was calculated using a catechin standard curve ($y = 0.0127x - 0.0767$; $R^2 = 0.995$). Total anthocyanin content was determined using the pH differential technique. The total anthocyanin content of water extracts was expressed in cyanidin-3-glucoside equivalents (CGE) per gram of fresh weight, represented in mg CGE/g fresh weight. This was calculated using the CGE standard curve ($y = 0.226 + 0.014x$; $R^2 = 0.995$). Similarly, the total tannin content of selected solvent extracts was determined using the Folin-Denis reagent technique and expressed in tannic acid equivalents. The standard curve equation used was $y = 0.0094x + 0.0142$ ($R^2 = 0.9972$).

3.1 Extraction of *C. ternatea* flower in water for 0, 4, 8 and 12 h

10 ml of water was used to dissolve 0.2 g of fresh *C. ternatea* flowers. The extraction time for the water extract was set at 0, 4, 8, and 12 h to optimize phytochemical extraction. For comparison with this study, Table 1 systematically lists the phytochemical content and antioxidant characteristics of *C. ternatea* extracts in aqueous, ethanol, methanol, acetone, and citrate buffer solvents. The total phenol content (117-130 mg GAE/g Fw), total flavonoid content (201-219 mg catechin/g Fw), total anthocyanin content (87-111 mg CGE/g Fw), and total tannin content (189-233 mg TAE/gFw) of the flower water extracts of *C. ternatea* at 0, 4, 8, and 12 h are shown in Table 1.

Table 1: Comparison between total phenols, total flavonoid, total anthocyanins, and total tannins in *C. ternatea* in water extract in 0, 4, 8, and 12 h

S.No.	Hours	TPC (mg GAE/g) Fw	TFC (mg catechine/g) Fw	TAC (mg CGE/g) Fw	TTC (mg TAE/g) Fw
1.	0	121.2 ± 0.11	201.15 ± 0.13	087.09 ± 0.21	189.0 ± 0.07
2.	4	122.3 ± 0.17	211.49 ± 0.18	091.49 ± 0.3	222.1 ± 0.09
3.	8	130.1 ± 0.15	219.44 ± 0.11	111.33 ± 0.82	233.0 ± 0.17
4.	12	117.4 ± 0.45	213.37 ± 0.18	090.09 ± 0.31	229.0 ± 0.11

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) ($p<0.05$).

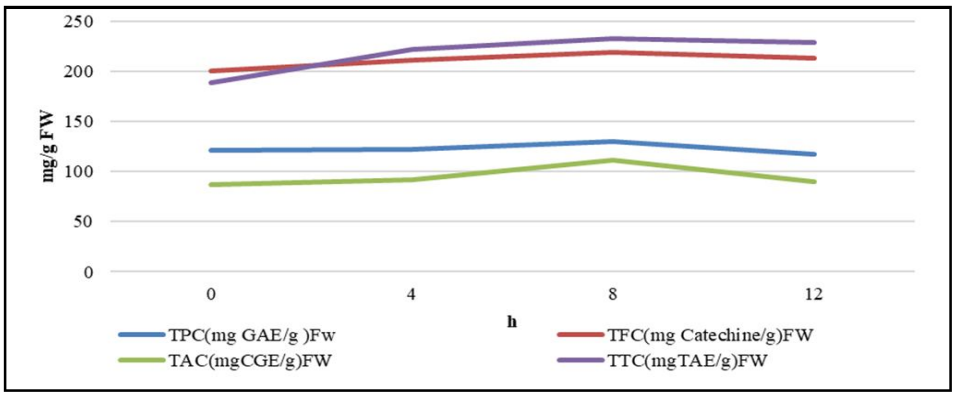

Figure 2: **Water extracts of *C. ternatea* flower in 0, 4, 8, and 12 h.**

When comparing the data for the duration time of *C. ternatea* flower water extract, it was observed that the extract had the highest values for all parameters at 8 h duration. These parameters included total phenol content (130 mg GAE/g Fw), total flavonoid content (219 mg catechin/g Fw), total anthocyanin content (111 mg CGE/g Fw), and total tannin content (233 mg TAE/g Fw), which were higher than those at other durations such as 0, 4, and 12 h. The contents of TPC, TFC, TAC, and TTC showed the lowest values at zero hours (Figure 2). The results indicated that the concentrations of TPC, TFC, TAC, and TTC in the flower water extract at 8 h were significantly lower (p<0.05).

3.2 Methanol extracts of *C. ternatea* flower 0, 4, 8, and 12 h

10 ml of 100 per cent methanol was used as the solvent for 0.2 g of fresh *C. ternatea* flowers. For optimal phytochemical extraction, the methanol extraction time was set at 0, 4, 8, and 12 h. It was established that a higher anthocyanin concentration was linked to the blue colour of the petals. For *C. ternatea* flowers, the methanol extraction ranges were 129-177 mg GAE/g FW for TPC, 362-399 mg catechin/g Fw for TFC, 135-158 mg CGE/g Fw for TAC, and 313-333 mg TAE/g Fw for TTC at 0, 4, 8, and 12 h (Table 2).

Table 2: Comparison between total phenols, total flavonoid, total anthocyanins, and total tannins in *C. ternatea* in methanol extract in 0, 4, 8, and 12 h

S.No.	Hours	TPC (mg GAE/g) Fw	TFC (mg catechine/g) Fw	TAC (mg CGE/g) Fw	TTC (mg TAE/g) Fw
1.	0	129.02 ± 0.13	362.03 ± 0.81	135.03 ± 0.12	313.17 ± 0.08
2.	4	137.20 ± 0.29	391.14 ± 0.01	148.03 ± 0.02	323.13 ± 0.17
3.	8	177.20 ± 0.18	399.34 ± 0.21	158.33 ± 0.32	333.03 ± 0.87
4.	12	131.20 ± 0.16	389.03 ± 0.11	135.13 ± 0.39	327.03 ± 0.07

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) (p<0.05).

Additionally, the data indicated that the maximal solubility of phytochemicals (TTC 177 mg/g, TFC 399 mg/g, TAC 158 mg/g, and TTC 333 mg/g) was observed over an 8 h period (Figure 3). The findings suggested that an 8 h soaking duration is ideal for practical applications. Among all the assays conducted, only TAC showed a significant difference between the accessions.

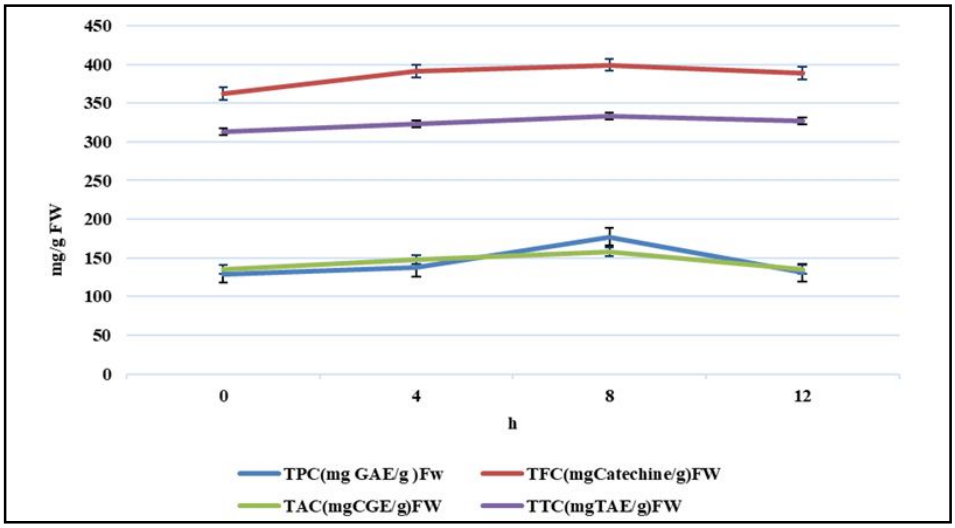

Figure 3: Methanol extracts of *C. ternatea* flower in 0, 4, 8, and 12 h.

3.3 Ethanol extracts of *C. ternatea* flower 0, 4, 8 and 12 h

10 ml of 60 per cent ethanol was used as the solvent for 0.2 g of fresh *C. ternatea* flowers. For optimal phytochemical extraction, the extraction time for the ethanol extract was set at 0, 4, 8, and 12 h. The results showed that the total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and total tannin content (TTC) of the ethanol extract of *C. ternatea* flowers ranged from 178 to 193 mg GAE/g FW, 461 to 469 mg catechin/g Fw, 118 to 178 mg CGE/g Fw, and 327 to 372 mg TAE/g Fw, respectively, over the 0, 4, 8, and 12 h periods (Table 3).

Table 3: Comparison between total phenols, total flavonoid, total anthocyanins, and total tannins in *C. ternatea* in ethanol extract in 0, 4, 8, and 12 h

S.No.	Hours	TPC (mg GAE/g) Fw	TFC (mg catechine/g) Fw	TAC (mg CGE/g) Fw	TTC (mg TAE/g) Fw
1.	0	180.0 ± 0.09	461.04 ± 0.70	118.03 ± 0.25	327.0 ± 0.01
2.	4	182.1 ± 0.15	463.04 ± 0.06	148.05 ± 0.91	361.0 ± 0.01
3.	8	193.0 ± 0.95	469.34 ± 0.61	178.33 ± 0.88	372.0 ± 0.89
4.	12	178.0 ± 0.69	466.01 ± 0.21	144.33 ± 0.80	352.2 ± 0.31

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) (p<0.05).

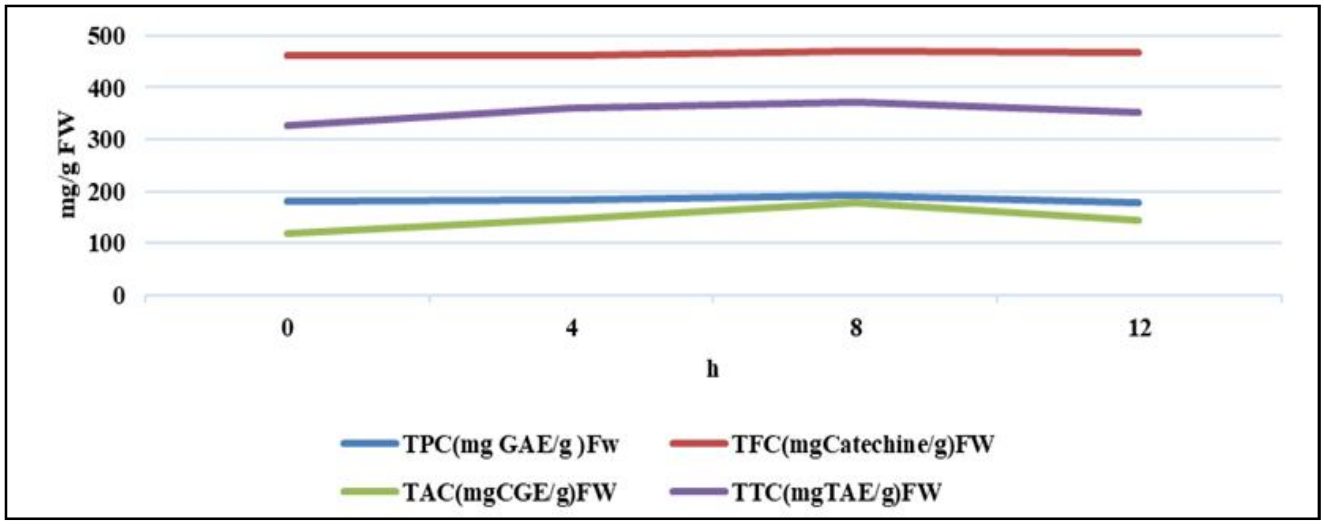

Figure 4: Ethanol extracts of *C. ternatea* flower in 0, 4, 8, and 12 h.

However, after eight hours, the ethanol extracts showed the highest results for total phenolic content (TPC), total flavonoid content (TFC), total antioxidant content (TAC), and total tannin content (TTC) at 193 mg/g, 469 mg/g, 178 mg/g, and 372 mg/g, respectively. This could be due to ethanol's high polarity, which makes it easier for phytochemicals to dissolve in it compared to other solvents (Table 3 and Figure 4). Overall, the results indicate that after eight hours, the phytochemicals in the ethanol extract reach their peak potency.

3.4 Acetone extracts of *C. ternatea* flower in 0, 4, 8 and 12 h

Acetone (10 ml) was used as the solvent for 0.2 g of fresh *C. ternatea* flowers. For optimal phytochemical extraction, the duration of the acetone extract was set at 0, 4, 8, and 12 h. The results of the acetone extraction of *C. ternatea* at these time points were represented as mean values: 97-116 mg GAE/g Fw for TPC, 333-419 mg catechin/g Fw for TFC, 119-149 mg CGE/g Fw for TAC, and 256-318 mg TAE/g Fw for TTC.

Table 4: Comparison between total phenols, total flavonoid, total anthocyanins, and total tannins in *C. ternatea* in acetone extract in 0, 4, 8, and 12 h

S.No.	Hours	TPC (mg GAE/g) Fw	TFC (mg catechine/g) Fw	TAC (mg CGE/g) Fw	TTC (mg TAE/g) Fw
1.	0	097.02 ± 0.17	333.02 ± 0.08	119.37 ± 0.71	256.09 ± 0.29
2.	4	111.02 ± 0.89	379.12 ± 0.09	131.04 ± 0.33	291.90 ± 0.31
3.	8	116.32 ± 0.18	419.14 ± 0.41	149.34 ± 0.61	318.04 ± 0.17
4.	12	096.31 ± 0.88	356.92 ± 0.78	119.38 ± 0.97	297.09 ± 0.35

Values are presented as mean ± S.D, n=4 (Experiments were made as 3 parallel) (p<0.05).

The highest recovery of the content of phenols, flavonoids, anthocyanins, and tannins were also demonstrated to occur within 8 h (116 mg/g, 419 mg/g, 149 mg/g, and 318 mg/g, respectively) than the other time duration like 0, 4, 12 h in ethanol (Table 4 and Figure 5).

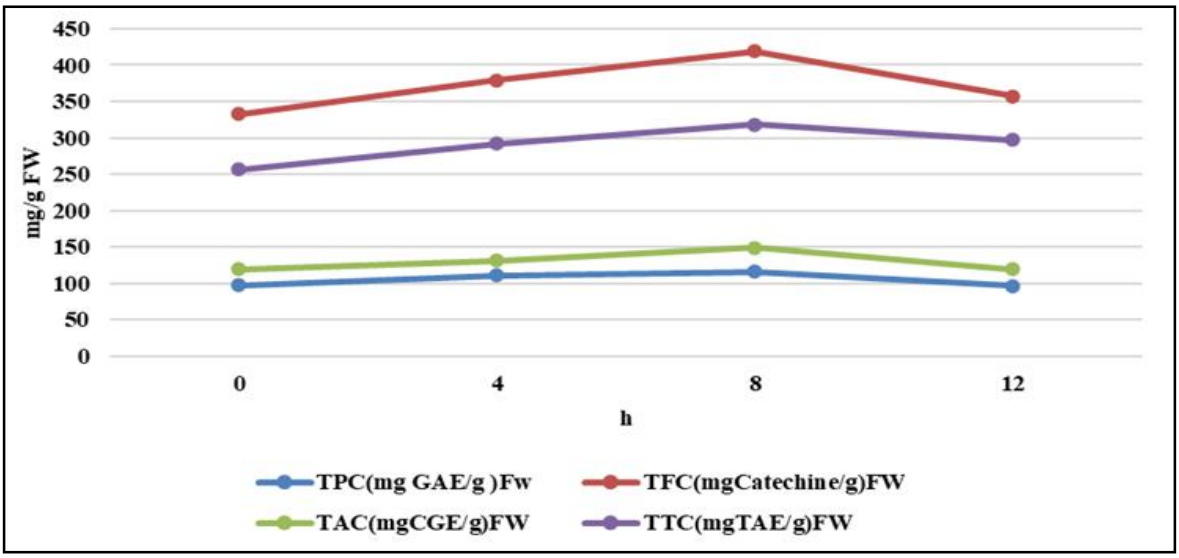

Figure 5: Acetone extracts of *C. ternatea* flower in 0, 4, 8, and 12 h.

3.5 Citrate buffer extracts of *C. ternatea* flower 0, 4, 8, and 12 h

Ten milliliters of citrate buffer (10 mM, pH 3.0) was used as the solvent for 0.2 g of fresh *C. ternatea* flowers. For optimal phytochemical extraction, the extract's time length was set at 0, 4, 8, and 12 h. The phytochemical data showed that the total phenol, total flavonoid content, total anthocyanin content, and total tannin content ranged from 88-144 mg/g, 209-249 mg/g, 101-141, and 167-213 mg/g, respectively.

Table 5: Comparison between total phenols, total flavonoid, total anthocyanins, and total tannins in *C. ternatea* blue flower in citrate buffer extract in 0, 4, 8, and 12 h

S.No.	Hours	TPC (mg GAE/g) Fw	TFC (mg catechine/g) Fw	TAC (mg CGE/g) Fw	TTC (mg TAE/g) Fw
1.	0	088.0 ± 0.18	209.04 ± 0.37	101.69 ± 0.02	167.9 ± 0.17
2.	4	127.0 ± 0.01	231.37 ± 0.91	131.09 ± 0.18	202.9 ± 0.25
3.	8	144.2 ± 0.19	249.34 ± 0.21	141.73 ± 0.62	213.4 ± 0.87
4.	12	122.6 ± 0.11	229.24 ± 0.81	129.73 ± 0.42	187.3 ± 0.53

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) (p<0.05).

The 8 h period yielded the highest value of findings when compared to the other durations (TPC, TFC, TAC, and TTC for 144.2 ± 0.19, 249.34 ± 0.21, 141.73 ± 0.62, and 213.4 ± 0.87, respectively) (Table 5 and Figure 6). The mean value of data was statistically significant at p<0.05 at 5 per cent level.

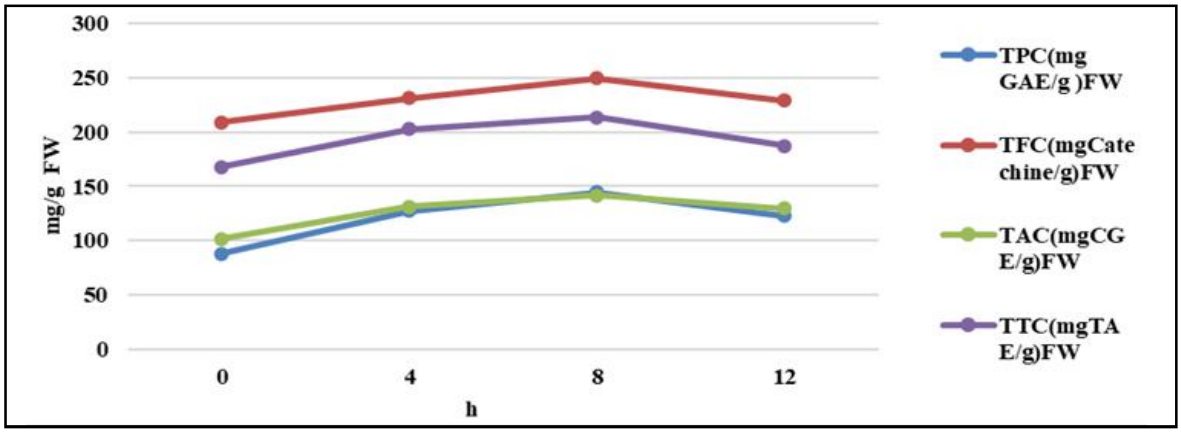

Figure 6: Citrate Buffer extracts of *C. ternatea* flower 0, 4, 8, and 12 h.

3.6 Antioxidant activities of *C. ternatea* flower in different solvents

Antioxidant tests, including ferric reducing antioxidant power (FRAP), superoxide anions, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), were evaluated in water, methanol, ethanol, acetone, and citrate buffer. The assays were calculated against standards using the following equations: DPPH (standard ascorbic acid, $y = 0.0103x + 0.076$; $R^2 = 0.9988$), ABTS (standard gallic acid, $Abs = 0.0109\ (GA)x + 0.002$; $R^2 = 0.998$), FRAP (standard BHT, $y = 0.009x + 0.023$; $R^2 = 0.985$), and superoxide anion radical (standard ascorbic acid, $l = 37.7x\ [O^-]$; $R^2 = 0.993$). The mean values for DPPH, ABTS, FRAP, and superoxide were 92.43 ± 0.47, 89.36 ± 0.67, 294.8 ± 0.74, and 89.33 ± 1.22 mg/g fresh weight, respectively. The results showed that the ethanol extract had the highest value, while water extracts had lower mean values compared to other solvents when compared to the positive standards (Table 6 and Figure 7).

Table 6: Comparison between DPPH, ABTs, FRAP, and superoxide anion in *C. ternatea* blue flower extract in water, methanol, ethanol, acetone, and citrate buffer

S.No.	Solvent	DPPH (mg ascorbic acid/g, Fw)	ABTs (mg gallic acid/g, Fw)	FRAP (mg BHT/g, Fw)	Superoxide anion (mg ascorbic acid/g, Fw)
1.	Water	72.03 ± 0.47	43.26 ± 0.87	204.5 ± 1.14	48.39 ± 1.62
2.	Methanol	88.13 ± 0.16	80.77 ± 1.56	248.7 ± 0.94	69.61 ± 0.29
3.	Ethanol	92.43 ± 0.47	89.36 ± 0.67	294.8 ± 0.74	89.33 ± 1.22
4.	Acetone	81.43 ± 0.14	65.96 ± 1.67	224.1 ± 0.84	51.38 ± 1.92
5.	Citrate Buffer	78.04 ± 0.24	55.16 ± 0.74	234.3 ± 0.56	59.03 ± 0.29

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) ($p<0.05$).

The results also observed that maximum antioxidant scavenging activity was found in ethanol solvent followed by methanol, acetone, citrate buffer and water. Here, citrate buffer as a solvent was also better solvent than water and it may be due to buffer as pH dependent.

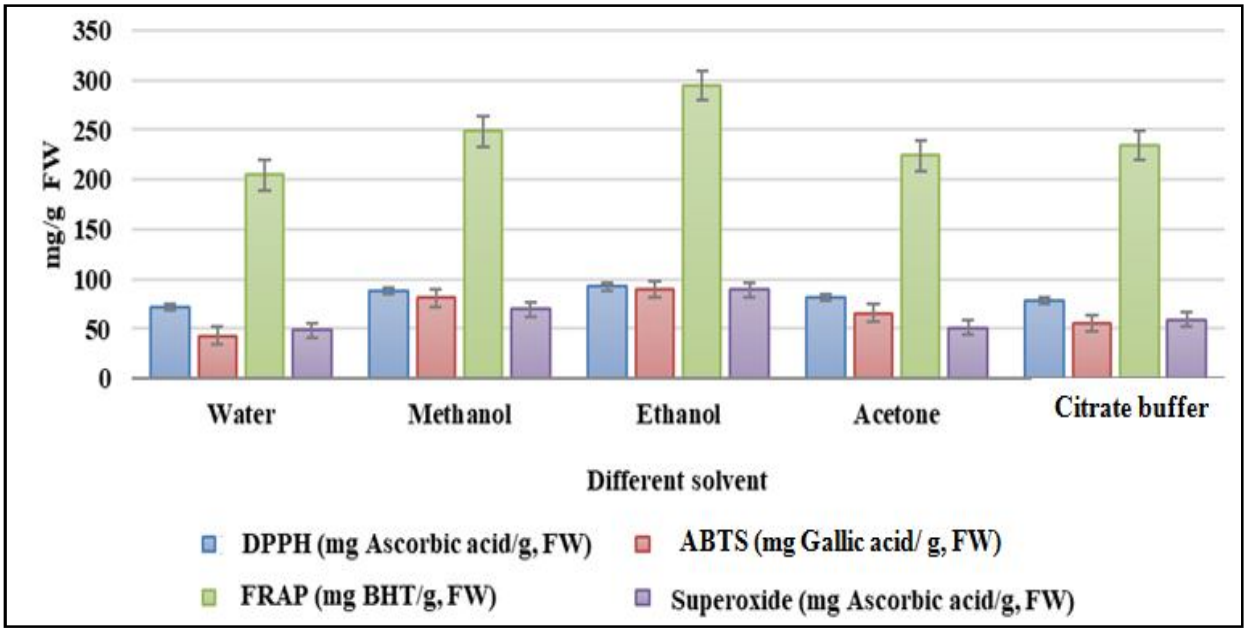

Figure 7: Comparison between DPPH, ABTS, FRAP, and superoxide anion in *C. ternatea* in different solvents.

DPPH, ABTs, FRAP, and superoxide anions are artificial radical scavengers that are generally used in the laboratory for measuring free radicals which is trap free radicals in the solvent.

3.7 Enzyme activities of *C. ternatea* flower in different solvents

A range of solvents, including acetone, ethanol, methanol, water, and citrate buffer, were used to assess the antioxidant enzyme activities of *C. ternatea* blue flowers. The activity of the superoxide dismutase enzyme was calculated against a standard using the equation $y = 2.77\times + 1.102$, with an R^2 value of 0.981. Other enzyme activities, such as peroxidase, guaiacol peroxidase, and polyphenol oxidase, were calculated using specific formulas. The results of the study indicate that *C. ternatea* blue flowers exhibit the highest levels of enzyme activities in ethanol extracts. The mean values for these activities were 391.20 ± 0.70 U/ml for peroxidase, 772.33 ± 0.67 U/ml for superoxide dismutase, 513.4 ± 0.9 U/ml for guaiacol peroxidase, and 812.16 ± 1.29 U/ml for polyphenol oxidase (Table 7 and Figure 8).

Table 7: Comparison between DPPH, ABTS, FRAP, and superoxide anion in *C. ternatea* blue flower extract in water, methanol, ethanol, acetone, and citrate buffer

S. No.	Solvent	Peroxidase (U/ml)	SOD (U/ml)	Guaiacol peroxidase (U/ml)	Polyphenol oxidase (U/ml)
1.	Water	360.00 ± 0.41	683.20 ±.27	478.14 ± 1.11	665.19 ± 0.90
2.	Methanol	379.32 ± 0.49	768.48 ± 0.61	501.30 ± 0.50	776.80 ± 0.80
3.	Ethanol	391.20 ± 0.70	772.33 ± 0.67	513.40 ± 0.90	812.16 ± 1.29
4.	Acetone	385.00 ± 0.20	745.13 ± 0.27	493.10 ± 0.70	789.13 ± 0.80
5.	Citrate buffer	272.10 ± 0.72	702.32 ± 0.13	489.90 ± 1.15	698.60 ± 0.56

Values are presented as mean ± S.D, n=4. (Experiments were made as 3 parallel) (p<0.05).

The lowest standard mean of all enzymes activities was found in citrate buffer (272.10 ± 0.72, 702.32 ± 0.13, 489.9 ± 1.15, and 698.60 ± 0.56 for peroxidase, SOD, GP and PPO, respectively). The results of decreased antioxidant enzyme activities in the citrate buffer were somewhat lower because the buffer's pH might not have been optimal for enzyme activity. All results are statistically significant at p<0.05.

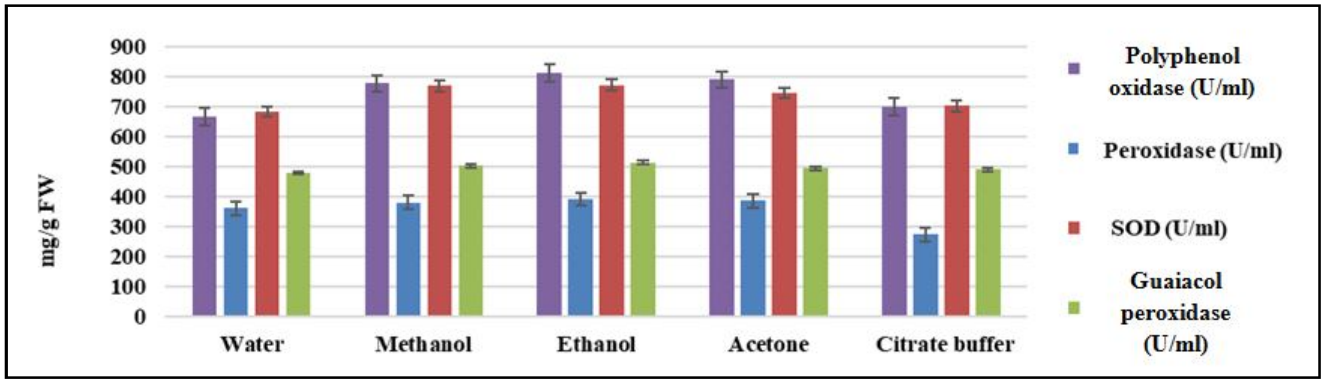

Figure 8: Enzyme activities of *C. ternatea* flower in different solvents.

4. Discussion

Water, the all-purpose solvent, is typically chosen for *C. ternatea* flower extraction because of its strong polarity. It can be used to extract a wide variety of polar molecules because it is one of the most polar solvents (Kaufmann and Christen, 2002). TPC was observed in an initial increase (from 0 to 8 h 121.2 to 130.1 mg GAE/g) may be due to the release of bound phenolic or biosynthesis stimulation and a decrease at 12 h could result from degradation or oxidation of phenolic compounds over prolonged exposure. Like TPC, TFC was also recorded that the initial enhancement (0 to 8 h, 201.15 to 219.44 mg catechin/g) could be due to enzymatic activities or stress responses. Decline after 8 h might be due to flavonoid breakdown or polymerization (at 12 h, 219.44 to 213.37 mg catechin/g). Total anthocyanin was also following the same trend of data. Higher antioxidant activity at 8 h may reflect increased bioactive compound availability (Peaks at 0 to 8 h, 87.09 to 111.33 mg CGE/g). The decline may be from antioxidant degradation or compound instability (111.33 to 90.09 mg CGE/g). TTC, accumulation over time (from 0 to 8 h, 189.0 to 233.0 mg TAE/g) is likely due to continued biosynthesis, while the slight decline at 12 h could be due to less polymerization or loss of extractable tannins. The possible explanation is that the bioactive substances found on the blue CT flower would deteriorate over longer periods.

Methanol is a solvent that generally makes bioactive substances more soluble and facilitates the solvent's entry into the plant components during the extraction procedure (Wong and Tan, 2020). Through the data, the TPC value was increased (0 to 8 h) from 129.02 to 177.20 mg GAE/g and decreased at 12 h to 131.20 mg GAE/g. The biochemical reason is that the increase up to 8 h is likely due to enhanced phenolic biosynthesis as a stress or defence response, potentially mediated by increased enzyme activity (e.g. phenylalanine ammonia-lyase, PAL). The decrease at 12 h suggests phenolic degradation or oxidation, possibly due to prolonged exposure to stress or oxidative enzymes like polyphenol oxidase (PPO). The TFC content was also increased in 0 to 8 h (362.03 to 399.34 mg catechin/g) reflecting enhanced flavonoid biosynthetic pathway activity, possibly involving chalcone synthase (CHS). The drop at 12 h (389.03 mg catechin/g) could be from flavonoid oxidation or conversion into more complex forms (*e.g.*, proanthocyanidins), making them less extractable. Similarly, TAC and TTC were recorded with the same trend. Tannins may increase in 8 h might be defensive polyphenol responses. The modest increase could be linked to condensed tannin synthesis. The later decrease may result from polymerization into higher molecular weight forms, which are less soluble and thus less detectable in extraction. Flavonoid and phenolic chemicals were successfully obtained in earlier extraction studies using methanol or aqueous methanol on several other plant flowers (Mehla, 2013).

Ethanol, a polar solvent, is therefore expected to recover anthocyanin, flavonoids, tannins, and phenols more quickly than other solvents. The TPC increase up to 0 to 8 h (180.0 to 193 mg) suggests activation of the phenylpropanoid and then decreased to 178.0 mg at 12 h due to oxidative degradation of phenolic compounds. However, TFC increase in 0 to 8 h (461.04 to 469.34 mg catechin/g) suggested this claim to increase flavonoid biosynthesis and decline (466.01 mg/g) at 12 h may be due to flavonoid oxidation, enzyme inactivation or conversion into complex forms like anthocyanins or proanthocyanidins (Lopez *et al*., 2019). Total anthocyanin and total tannin were also observed the same and the reason might be the increased biosynthetic activity in 0 to 8 h. Overall, the results indicate that after 8 h, the phytochemicals in the ethanol extract are at their peak potency.

Acetone is a regular solvent used to extract bioactive substances from many plants and is usually thought to be a good choice for maximizing extraction yields (Pengkumsri *et al*., 2019). The data revealed that the TPC, increased from 97.02 mg GAE/g at 0 h to a peak of 116.32 mg at 8 h, followed by a decrease to 96.31 mg at 12 h, the reason might be an increase in bioactive enzymatic activity with duration 0 to 8 h and decline at 12 h due to degradation of some phenolic compounds. Similarly, other results followed as same trends in total flavonoids, total anthocyanin, and total tannins. Increasing trends of activity showed (0 to 8 h) that TPC, TFC, TAC, and TTC typically accumulate as part of the plant's defensive response.

Citrate buffer is also used as an extraction solvent in many biological experiments because it has chelating properties and pH stability. At stable acidic pH, citrate buffer might enhance the extraction of bioactive compounds in the solvent. The results of TPC disclosed that TPC increased from 88.0 mg to 144.2 mg at 0 to 8 h, then decreased to 122.6 mg at 12 h, the reason could be an increase in enzymatic activity in particular pH and after four hours their catalytic activity might be lost. Total flavonoid content was also increased from 209.04 mg catechin/g to 249.34 mg at 0 to 8 h, and then declined to 229.24 mg at 12 h, the biochemical reason could be the same as other solvents. Similarly, TAC and TTC were also the same reason found with other selected parameters.

The antioxidant capacities of plant extracts, expressed in terms of DPPH, ABTS, FRAP, and superoxide anion scavenging assays, varied significantly depending on the solvent used for extraction. This variation can be attributed to the differences in polarity and solubility properties of each solvent, which affect the extraction efficiency of phenolic and other antioxidant compounds (Gulcin *et al*., 2010). Ethanol demonstrated the highest antioxidant activity across all assays such as DPPH (92.43 mg ascorbic acid/g Fw), ABTS (89.36 mg gallic acid/g Fw), FRAP (294.8 mg BHT/g Fw), and superoxide anion scavenging (89.33 mg ascorbic acid/g Fw). This is likely due to ethanol's moderate polarity, which allows it to solubilize both polar and moderately non-polar bioactive compounds, including flavonoids and phenolic acids (Rabeta *et al*., 2013). Methanol showed slightly lower but still significant antioxidant activity, suggesting that methanol is also efficient in extracting a wide range of phenolic compounds. Methanol's smaller molecular size and higher polarity compared to ethanol may contribute to its ability to penetrate plant tissues and extract intracellular antioxidants effectively. Acetone and citrate buffer extracted moderate levels of antioxidant compounds. Acetone (e.g., DPPH: 81.43 mg AA/g Fw) is less polar than methanol and ethanol, which might reduce its effectiveness in extracting highly polar antioxidant compounds. However, it still solubilizes some moderately polar compounds, which explains its intermediate antioxidant values. Citrate buffer, a polar aqueous solvent, extracted mostly hydrophilic antioxidants like ascorbic acid and certain phenolic, which explains its moderate but lower efficacy. Water, being the most polar solvent, showed the lowest antioxidant activities across most assays (*e.g*., DPPH: 72.03 mg AA/g FW: ABTS: 43.26 mg GA/g FW). The reason might be the presence of polar substances like the OH group in the flavonoids and phenolic acids that easily dissolve in solvents which may be the cause of increased antioxidant activity (Yi *et al*., 2020).

The activity of antioxidant enzymes such as peroxidase, superoxide dismutase (SOD), guaiacol peroxidase, and polyphenol oxidase is varied with the extraction solvent due to differences in polarity, enzyme stability, and solubility of protein complexes. Ethanol extracted the highest levels of all four enzymes, including peroxidase (391.20 U/ml) and SOD (772.33 U/ml), likely due to its moderate polarity, which preserves enzyme structure while efficiently solubilizing both hydrophilic and moderately hydrophobic proteins. Methanol and acetone also showed high enzyme activities, though slightly lower than ethanol. Their polarity allows effective extraction of protein-bound enzymes but may induce mild denaturation compared to ethanol (Rajamanikam *et al*., 2015). Water showed moderate enzyme activity, especially for SOD (683.20 U/ml), as it stabilizes hydrophilic enzymes well but is less effective at extracting membrane-bound or less polar proteins. Citrate buffer showed the lowest Peroxidase activity (272.10 U/ml), possibly due to pH-dependent enzyme degradation or reduced extraction efficiency for certain proteins, despite maintaining good SOD and polyphenol oxidase levels.

5. Conclusion

The study compared the efficiency of various solvents in extracting bioactive compounds from *C. ternatea*. Ethanol was found to be the most efficient solvent, outperforming methanol, acetone, citrate buffer, and water. The highest yield of bioactive compounds was observed after eight hours of extraction, with a decrease noted at twelve hours, possibly due to degradation of compounds over longer extraction periods. The ethanol extract exhibited high levels of enzyme activity, including peroxidase, superoxide dismutase (SOD), guaiacol peroxidase, and polyphenol oxidase (PPO). It also demonstrated strong radical scavenging capabilities, as measured by DPPH, ABTS, and FRAP assays. Furthermore, the ethanol extract had elevated levels of total phenolic content (TPC), total flavonoid content (TFC), total anthocyanin content (TAC), and total tannin content (TTC) after eight hours of extraction. The study concludes that ethanol is the most effective solvent for extracting phytochemicals from *C. ternatea*, making it a potential natural antioxidant enhancer. The efficacy of solvents can be ranked as follows: ethanol > methanol > acetone > citrate buffer > water. The findings suggest that *Clitoria ternatea L*. extract, particularly when using ethanol as a solvent, could be effectively utilized as a natural antioxidant enhancer in the development of food colorants, cosmeceutical products (such as skin care herbal gel, antiageing creams, body lotion, and soap), and pharmaceutical industries.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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