

# Production and evaluation of secondary metabolites in callus culture of *Clitoria ternatea* L. by phytochemical screening and in vitro antioxidant and biological activities

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## Research Article

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# Abstract

The butterfly pea is a significant source of bioactive secondary metabolites. Callus cultures provide a viable option for the reliable synthesis of important secondary metabolites, overcoming the limitations of inconsistent yields from field-grown plants. The present results indicated that MS medium with 1.0 mg/L BAP and 1.0 mg/L 2,4-D produced a highly significant growth stimulation for callus induction in *Clitoria ternatea* L. after 40–45 days under darkness. The biomass of the callus augmented with each subsequent subculture, reaching its peak by the sixth subculture. The macronutrient content, antioxidant assays, flavonoids, phenolics, and HPLC analysis were assessed. Anti-diabetic, anticancer, and anti-inflammatory characteristics were evaluated. According to the HPLC analysis, the callus culture of *C. ternatea* L. comprises a range of flavonoid and phenolic compounds that demonstrated the most anti-diabetic, anti-inflammatory, and cytotoxic effects. In conclusion, using the callus culture of *C. ternatea* L. has shown promise in secondary metabolite synthesis. Moreover, callus cultures of *C. ternatea* L. have significant nutritional value, which increases antioxidant activity, in addition to their potential application as new natural secondary metabolites.

## 1. Introduction

Countries around the world are currently facing many crises that affect the process of securing food and medicine, from the coronavirus pandemic to the crisis of disruption of global food and medicine supply chains to the World War crisis (Roubík et al. 2023; Forgenie et al. 2024). In addition, natural phenomena such as desertification and water scarcity significantly impact the extinction of medicinal plants in many places worldwide. These phenomena cause ecosystem degradation, biodiversity loss, and a decline in vegetation cover, leading to the extinction and decline of many natural plants. Hence, the urgent need to double the efforts made to achieve food and medicine security for people has emerged (Eltaif et al., 2024). Egypt has treasures spread throughout the desert, waiting for someone to invest in them and care for them (Mouneer 2021). In the Egyptian desert, there are thousands of medicinal and aromatic plants that God has blessed it with, which are considered a huge wealth. This wealth, in its entirety, constitutes a great economic value that can contribute to bridging a large portion of the food gap that the country is currently facing (El-Demerdash, 2001). At a time when Egypt is moving towards building a strong economy that invests all its potential with a developed and creative mindset, it was necessary to look at these important resources with a new vision and benefit from them to reduce the import gap and encourage the localization of the pharmaceutical industry locally as a step towards self-sufficiency, which achieves drug security for citizens (Mouneer 2021). One of these medicinal plants is *C. ternatea* L., or 'Butterfly pea,' related to the legume family Fabaceae (Boulos 2009). It is a perennial, flowering, colonial plant that grows 5 to 2 meters tall. It is grown as a medicinal plant, ornamental fodder, and soil improver, as it adds nitrogen to agricultural soil. It is a plant that blooms in summer and winter (Rashid et al. 2020).

Given the pressing necessity to generate plant secondary metabolites purely and sustainably, tissue culture technology is employed to enhance their production relative to extraction from native plants. This

complements the pure active component, as it is devoid of fertilizer and pesticide residues encountered by plants under natural development conditions. It can also be provided sustainably throughout the year without being restricted to the plant's growth seasons. In this regard, the callus culture has the potential for biomass production all year round. DPPH scavenging activity and the ABTS assay were also used in *in vitro* antioxidant assays. Additionally, the research examined the potential of callus culture of *C. ternatea* L. to destroy Hep-G2 cancer cells and alleviate inflammation by testing their ability to prevent lipoxygenase and proteinase from performing their activities as well as evaluate the potential of bioactive compounds to reduce blood sugar levels or improve insulin sensitivity outside of a living organism through inhibiting enzymes involved in glucose metabolism, like  $\alpha$ -amylase and  $\alpha$ -glucosidase, or on evaluating the cells' absorption of glucose.

## 2. Materials and methods

### 2.1. Materials

In Egypt, *C. ternatea* L. seeds were purchased from the Baraem Company. The Biodiagnostic Company (Egypt) provided spectrophotometric kits for quantitative measurements. The hepatocellular carcinoma cell line (Hep-G2) was provided by the Naval American Research Unit in Egypt (NAmRU). Furthermore, the substances were purchased from Merck (Darmstadt, Germany).

### 2.2. Methods

#### 2.2.1. Obtaining an aseptic culture of *C. ternatea* L.

*C. ternatea* L. seeds were subjected to sterilization using 1.5% NaOCl (v/v) (Clorox with 5.25% sodium hypochlorite) for 15 minutes and subsequently cultivated on MS medium without plant growth regulators and were enriched with 3% (w/v) sucrose and 0.01% (w/v) Myo-inositol, as described by Murashige & Skoog (1962). After adjusting the pH to  $5.7 \pm 0.2$ , the 2.7 g/L gelling agent (phytagel) was added. After culturing, after a week of incubation at  $26 \pm 2^\circ\text{C}$  in the dark, all jars were switched to a schedule of 16 hours of light and 8 hours of darkness. Results were documented during a fifteen-day culture period, and the cotyledonary leaves served as explants for the next experiments.

#### 2.2.2. Effect of 2,4-D and BAP concentrations and combinations on *C. ternatea* L. callus induction.

Cotyledonary leaves were removed from one-month-old *in vitro* germinating seedlings to be used as explants. These were cultivated on MS medium with varied doses of 2,4-D (2,4-dichlorophenoxyacetic acid) at 0.5, 0.1, and 0.2 mg/L, either on their own or in combination with different concentrations of BAP (6-Benzylaminopurine) at 0.5, 0.1, and 0.2 mg/L. A control consisting of MS media devoid of plant growth regulators was added. Every culture was nurtured in an entirely dark environment. Callus biomasses (fresh weight in grams/ jar) were recorded 40 to 45 days after culture.

#### 2.2.3. Biomass accumulation of *C. ternatea* L. callus

Callus biomass was determined over six successive subcultures every 35 days on MS medium enhanced by 1.0 mg/L BAP and 1.0 mg/L 2,4-D. The callus biomass (growth rate) of FW and DW (g/jar) and morphological characteristics were recorded for all subcultures (Abou El-Dis et al. 2021). The growth rate (I) was calculated as:

$$\text{Growth rate (I)} = \frac{(W_{\max} - W_0)}{W_0} \quad \text{Eq1}$$

Where  $W_{\max}$ : final weight of callus (g),  $W_0$  – initial mass of callus (g).

## 2.2.4. Chemical composition analysis of *C. ternatea* L. callus

The chemical composition of a plant is a significant factor in determining its nutritional and medicinal value. This study assessed several important chemical properties of *C. ternatea* L. callus, such as iron (Fe) concentration, manganese (Mn) concentration, magnesium (Mg) concentration, copper (Cu) concentration, zinc (Zn) concentration, vitamin C concentration, total nitrogen (N), phosphorus (P) concentration, potassium (K) concentration, calcium (Ca) concentration. The vitamin C content (mg/kg) of callus tissue derived from plant tissue culture was assessed using the 2,4,6-di-chlorophenol indophenol titration method (Suliman and Saleh 2022). Total nitrogen (N) nitrate concentrations were measured using the Gerhardt Vapodest 20s apparatus and the Kjeldahl method (Suliman et al. 2025). The concentrations of nitrate and total nitrogen were measured using the Gerhardt Vapodest 20s apparatus and the Kjeldahl method. Using a flame photometer (PFP7). Cottenie et al. (1982) used the potassium and calcium content to measure, while Worsfold et al. (2016) used the phosphorus levels to evaluate. To calculate the levels of Mn, Mg, Cu, and Zn (in parts/ million), the American Public Health Association's (American Public Health Association, 1998; Abdelkader et al. 2024) standard methods were employed. The measurements were made using inductively coupled plasma optical emission spectroscopy (ICP-OES) to measure the iron concentration using the Perkin Elmer 4300DV device.

## 2.2.5. Measurement of total phenolic compounds (TP) and total flavonoids (TF) as well as antioxidant activities of the compounds in *C. ternatea* L. callus

### 2.2.5.1. Total phenolics

The total phenolic compounds in the dry matter particles was measured and determined spectrophotometrically (Waterhouse, 2002). 80% ethanol was used to extract the phenols. After mixing 70 ml of distilled water and 1 ml of the prepared sample, the quantity of phenol was measured by adding 15 ml of saturated sodium carbonate solution and the specialized reagent Folin-Ciocalteu. After 30 minutes of sitting at room temperature, the mixture was analyzed with a spectrophotometer set to 765 nm. The gallic acid calibration curve was produced according to the method by Nouman et al. (2014).

### 2.2.5.2. Total flavonoid

A 2% aluminum chloride ( $\text{AlCl}_3$ ) in methanol solution was added to 1.5 mL of extract to evaluate the total flavonoid content by Pai et al. (2015). For 10 minutes, the samples were left to incubate at 30°C. The absorbance was then measured at 368 nm using quercetin as the standard.

### **2.2.5.3. Antioxidant Assays**

#### **2.2.5.3.1. 2, 2-diphenyl-1-picrylhydrazyl (DPPH)**

The extracts' ability to scavenge free radicals was assessed using DPPH. Using Trolox, the standard curve was created. The DPPH test was carried out by the methods of Brand-Williams et al. (1995), and Suliman et al. (2025). 24 mg of DPPH was dissolved in 100 mL of methanol to create the stock solution, which was then kept at -20°C until it was needed. Using a spectrophotometer, 45 mL of methanol and 10 mL of stock solution were combined to create a solution. The absorbance was  $1.1 \pm 0.02$  units at 515 nm. For 5 minutes, the extracts (750  $\mu\text{L}$ ) and 1,500  $\mu\text{L}$  of the DPPH solution were left to react in the dark. The absorbance at 515 nm was then measured. The Trolox standard curve was linear from 25  $\mu\text{mol}$  to 800  $\mu\text{mol}$  and the results were quantified as mL of Trolox equivalent (mg TE) per gram (Sabry et al. 2024).

#### **2.2.5.3.2. 2, 2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS)**

The ABTS + free radical scavenging experiment used the procedures described by Abdelkader et al. (2024) to evaluate antioxidant activity. To put it briefly, distilled water was used to dissolve 7  $\mu\text{M}$  of ABTS. In order to generate the ABTS + radical, the ABTS solution was allowed to sit at room temperature in the dark for 16 hours after its reaction with 2.45 mM potassium sulfate. After that, distilled water was used to dilute the resultant ABTS + solution and left to equilibrate at 30°C until its absorbance at 734 nm was  $0.70 \pm 0.02$  (Mohammed et al. 2024).

### **2.2.6. High performance liquid chromatography analysis (HPLC) of secondary metabolites from the callus of *C. ternatea* L.**

The Agilent 1260 series instrument was used to conduct the HPLC analysis. The separation procedure used a 4.6 mm x 250 mm i.d. Zorbax Eclipse Plus C8 column with a 5  $\mu\text{m}$  particle size. Water (A) and a 0.05% trifluoroacetic acid solution in acetonitrile (B) made up the mobile phase, which had a flow rate of 0.9 mL/minute. 0 minutes (82% A), 0–1 minute (82% A), 1–11 minutes (75% A), 11–18 minutes (60% A), 18–22 minutes (82% A), and 22–24 minutes (82% A) comprised the linear gradient programming for the mobile phase. There was a multi-wavelength detector operating at 280 nm. 5  $\mu\text{L}$  of each sample solution were injected, and the column was kept at a steady 40°C. The resulting chromatograms in the column were carefully examined for distinct peaks and retention periods, and the analysis was planned to ensure that the chemicals were well separated. In order to measure the analyte concentrations in the sample solutions, calibration curves were also produced (Mohammed et al. 2024).

### **2.2.7. *In vitro* anti-diabetic activity evaluation**

### 2.2.7.1. $\alpha$ -amylase inhibition assay

The process created by Pant et al. (2013) and Ramachandran et al. (2013) was used with few modifications. Along with 1.5 ml of the enzyme (pH-7.2), 1.5 ml of sodium acetate buffer, and 1.5 ml of the enzyme (1%), the standard acarbose (100–400 g/ml) and 1.5 ml of *C. ternatea* L. callus culture were added. After letting the mixture remain at room temperature for 20 minutes, 2 ml of a 1% starch solution was added. The aforementioned mixture is incubated for 30 minutes at 37 °C. Next, 1.5 ml of the 3,5-dinitrosalicylic acid reagent is added to the mixture. The mixture is submerged in a bath of hot water for 5 minutes. At 540 nm, the absorbance is detected using a UV-visible spectrophotometer. Three duplicates of each experiment were conducted before the average was determined. Ultimately, the  $\alpha$ -amylase inhibition (%) was expressed as:

$$\% \text{ Inhibition} = (A_0 - A_1 / A_0) \times 100$$

Where  $A_0$  = The absorbance of control.  $A_1$  = The absorbance of the sample or standard.

### 2.2.7.2. $\alpha$ -glucosidase inhibition assay

A modified method of Vennila and Pavithra (2015) was used to assess the inhibitory action. 1.5 ml of the *C. ternatea* L. callus culture was combined with 1 ml of 2% w/v maltose starch solution in 0.2 M tris buffer (pH8). At 37 °C, the reaction mixture was incubated for 10 minutes. The reaction was initiated by adding 1 ml of  $\alpha$ -glucosidase enzyme (1U/ml), and it was then incubated for 40 minutes at 35 °C. The reaction was then stopped by adding 2 ml of 6 N HCl. The intensity of the color at 540 nm was measured using a spectrophotometer. The % inhibition was computed as:

$$\% \text{ Inhibition} = (A_0 - A_1 / A_0) \times 100$$

Where  $A_0$  = The absorbance of control.  $A_1$  = The absorbance of the sample

### 2.2.8. Anticancer activity for callus culture of *C. ternatea* L.

Through the application of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, cell viability was accustomed to measure the cytotoxicity activity of callus culture of *C. ternatea* L. against cancer cells (Hep-G2) by percentages of cell viability. In conclusion, 96-well microplates were seeded with  $3 \times 10^3$  cells/well using RPMI-1640 culture media (100  $\mu$ l). The cells were exposed to a 5% carbon dioxide concentration at 37°C overnight. Staurosporine was added to the cells at different doses (0.39, 1.56, 6.25, 25, and 100  $\mu$ g/ml) and the callus culture of *C. ternatea* L. After that, the cells were cultured for 24 hours. The cells were then cultivated for a whole day. Each well was added to a 100  $\mu$ l MTT solution with a 0.5 mg/ml concentration. In order to create purple formazan crystals, the cells were kept in an incubator overnight for a long time. Once the medium had been discarded, a 100  $\mu$ L solution of DMSO was added to dissolve the crystals. An automated microplate reader was applied to measure the dissolved formazan's optical density (OD) at 570 nm (Abou Baker and Mohammed 2022; Mohammed et al. 2024). Following the equation outlined below, the cell viability percentages were calculated:

$$\% \text{ Cell viability} = [A_{\text{sample}} / A_{\text{control}}] \times 100$$

## 2.2.9. Evaluation of anti-inflammatory efficacy for callus culture of *C. ternatea* L.

### 2.2.9.1. Albumin denaturation assay

The albumin denaturation experiment was assessed with the method outlined by Pieroni et al. (2011). 2 ml of the callus culture of *C. ternatea* L., 200 ml of fresh egg albumin, and 2.8 ml of a phosphate buffer solution with a pH of 6.4 were combined to create a reaction mixture that included 5 ml. For 15 minutes, the reaction mixture was incubated at 37°C in an incubator. The temperature then increased for 5 minutes to 70°C. A wavelength of 660 nm was then used to test the combination's absorbance. The negative control was deionized water, whereas Diclofenac sodium was the positive control. In the albumin denaturation test, the expected percentage of inhibition was: Inhibition =  $100 \times (A_{\text{sample}} / (A_{\text{control}} - 1))$

### 2.2.9.2. HYA Inhibitory Activity Evaluation

The modified technique described by Sahasrabudhe and Dedhar (2010) was used to assess the inhibition of HYA. After incubating various callus cultures of *C. ternatea* L. concentrations (50 µL) and bovine HYA (100 µL; 400 U/mL) in acetate buffer (0.1 M) for 20 minutes at 37°C, CaCl<sub>2</sub> 12.5 mM (100 µL) was added, and the combination was again incubated for 20 minutes at 37°C. The reaction was initiated by adding 250 µL of sodium hyaluronate (1.2 mg/mL), and it was then incubated for 40 minutes at 37°C. The reaction mixture was then incubated for 3 minutes in water at 90°C, then potassium borate (0.4 M; 100 µL) and sodium hydroxide solution (0.4 M; 100 µL) were added. The absorbance at 585 nm was evaluated 20 minutes after the cool reaction mixture was mixed with 3000 µL of 10% p-Dimethyl-aminobenzaldehyde. Ibuprofen was used as the standard.

### 2.2.9.3. Lipoxxygenase Inhibition Activity Evaluation

Using the method developed by Shrivastava et al. (2023), the callus culture of *C. ternatea* L. was evaluated for lipoxxygenase inhibitory activity. 5-lipoxidase was the enzyme used, while linoleic acid was the substrate. A solution was prepared by mixing 10 µl of lipoxxygenase (final concentration: 8000 U/ml) with 1 ml of sodium borate buffer (0.1 M) (pH: 8.8). Then, 1 ml of this mixture was incubated with the callus culture of *C. ternatea* L. varying in concentration from 100 to 2000 g/ml. The temperature of the reaction mixture was kept constant at  $30 \pm 2^\circ\text{C}$  for 5 minutes. To begin the reaction, 10 linoleic acid µl (10 mmol) were introduced. Spectrophotometer readings were taken at 234 nm to determine the absorbance. The percentage of lipoxxygenase inhibition was calculated as:

$$\% \text{ inhibition of proteinase activity} = \left[ 1 - \frac{A_2 - A_1}{A_1} \right] \times 100$$

where A1 represents control absorbance and A2 represents sample absorbance

## 2.2.9.4. Proteinase Inhibitory Activity Evaluation

The proteinase inhibitory activity of the callus culture of *C. ternatea* L. was assessed using this technique by Truong et al. (2021). 1 ml of the *C. ternatea* L. callus culture was mixed with a solution containing 0.06 mg of trypsin and 1 ml of a pH 7.4 Tris-HCl buffer with concentrations ranging from 100 to 2000 µg/ml. The mixture was incubated at 37°C for 5 minutes. 0.7% (w/v) casein protein was added to a 1 ml solution. For a further 20-minute period, the reaction mixture was incubated. To halt the reaction, 1 ml of 70% perchloric acid was added. The liquid was centrifuged with a 1968 G-force for 10 minutes at 4°C. The spectrophotometer will use the blank as a buffer solution to determine the absorbance of the supernatant at 210 nm. The proportion of proteinase activity inhibition was determined as follows:

$$\% \text{ inhibition of proteinase activity} = \left[ 1 - \frac{A_2 - A_1}{A_1} \right] \times 100$$

where A1 represents control absorbance and A2 represents sample absorbance

## 2.2.10. Statistical analysis

The mean ± SD was used to display the data. Tukey post-hoc analysis and a one-way ANOVA test were used to evaluate the assays under research for mean and difference values. The experiment was conducted using SPSS software (Chicago, IL, USA) version 22.0 ( $p \leq 0.05$ ).

## 3. Results and Discussion

### 3.1. Obtaining of an aseptic culture of *C. ternatea* L.

The maximum survival rate (100%) was achieved without contamination; seeds were meticulously cleansed and subjected to 30 minutes of running water to eliminate dirt. Seeds were subjected to a 15-minute treatment with 1.5% NaOCl, followed by three washes with sterilized distilled water to eliminate residual sterilizing agents. Under carefully monitored circumstances, sterilized seeds were grown on MS medium without additional plant growth regulators and nurtured till germination. The maximum germination rate (100%) was observed after 15 days of cultivation. All the developing plantlets are robust and will endure for utilization in the subsequent studies (Fig. 1).

### 3.2. Different Concentrations of 2,4-D and BAP on *C. ternatea* L. Callus Induction.

The results shown in **Figure (2)** reveal that an MS medium without any plant growth regulators does not produce any callus, and a medium with only different amounts of BAP does not lead to callus formation. However, some callus is produced by MS medium having varying concentrations of 2,4-D; the weights of the media containing 0.5 mg/L and 1.0 mg/L of 2,4-D range from 0.43 g/jar to 0.48 g/jar. A significant

quantity of callus was discovered on MS medium containing different concentrations and combinations of 2,4-D and BAP. Fresh weights for MS medium containing 0.5 mg/L 2,4-D and 1.0 mg/L 2,4-D range from 0.43 g/jar to 0.48 g/jar, indicating that the medium efficiently supports moderate callus development. When the MS medium was enhanced with different concentrations and combinations of 2,4-D and BAP, a significant frequency of callus formation was observed. 1.0 mg/L 2,4-D and 1.0 mg/L BAP treated MS medium produced the highest amount of callus, 0.66 g/jar (fresh weight). Additionally, it was found that when the amounts of BAP and 2,4-D were higher, the amount of callus produced was lower, with a measured weight of 0.48 g/jar on MS medium that had 2.0 mg/L of both 2,4-D and BAP.

This aligns with Park et al. (2023a), who established that acquiring a rapidly proliferating, friable soybean callus is regulated by the auxin-cytokinin balance, influencing the differentiation or dedifferentiation of the explant. The presence of auxin, compared to cytokinin, promotes root formation, while the presence of cytokinin promotes bud regeneration. Therefore, an adequate ratio of auxin and cytokinin is necessary to facilitate callus formation. The injection of 2,4-D, a potent synthetic auxin, stimulates auxin response genes by the interaction of auxin response factors (transcription factors) with auxin-responsive elements, as shown by Ikeuchi et al. (2013) and Song (2014).

The explants expanded as a consequence of the tissues within them undergoing rapid cell division, which led to callus development. According to Hemmati et al. (2020), 2,4-D and BAP have beneficial effects. By controlling gene expression, cytokinin may increase auxin's efficiency in callus development, directly influencing auxin's function in this process. MS medium supplemented with BAP, in conjunction with NAA or 2,4-D, can facilitate callus production from *Origanum vulgare in vitro*, with lower concentrations of 2,4-D demonstrating greater efficacy (Zakaria et al. 2024), *Silybum marianum* (Hassanen et al. 2021), and *Thymus decussatus* (Mashal et al. 2024). It has been shown in several studies that when auxins and cytokinins are properly balanced in the culture media, callus development and cell division accelerate (Sagharyan et al. 2020; Abdelazeez et al. 2022). After 40–45 days of development in total darkness, the most efficient way to increase callus biomass from cotyledonary leaf explants of *C. ternatea* L. was to use 2,4-D (1.0 mg/L) and BAP (1.0 mg/L) combined.

### **3.3. Biomass Accumulation and Growth Rate of *C. ternatea* L. callus through Six Successive Subcultures on MS Medium Fortified with 1.0 mg/L of 2,4-D and 1.0 mg/L of BAP**

The data presented in **Table (1)** and **Figure (3)** illustrate the impact of six successive subcultures on the biomass accumulation of *C. ternatea* L. callus on MS medium with 1.0 mg/L 2,4-D and BAP. This process was conducted every 30–35 days, depending on the vitality of the callus and its condition within the jars. It involved transferring a portion of the actively growing callus to a fresh culture medium with the same composition. The results indicated a gradual increase in biomass accumulation, with friable white callus—indicative of actively dividing cells—observed by the third subculture (**Fig. 3C**).

Commencing with the third subculture, friable white callus clones were chosen and transferred to fresh medium with identical hormone concentrations, while brown, dead, and compact portions of the callus were discarded. The sixth subculture demonstrated homogeneity in both appearance and growth. The

results indicated that the rate of callus biomass, measured in terms of FW and DW in grams, increased significantly from  $6 \pm 0.7$  and  $1.2 \pm 0.14$  g/jar, respectively, in the first subculture to  $16.4 \pm 0.54$  and  $3.28 \pm 0.1$  g/jar, respectively, in the sixth subculture, representing an increase of approximately tenfold (**Fig. 3F**). These findings align with Wahyuni et al. (2020), who noted that repetitive subculturing is a crucial procedure in plant tissue culture, necessitating consideration of the frequency of subculturing. Repeated subcultures affected the callus morphology, which was reflected in its texture and color. The callus color was deeper during the first growing phase but became lighter with the rising number of subcultures. The alteration in color and texture of the callus signified cellular activity during division; the callus subculture was crucial for facilitating proper growth and development. Abou El-Dis et al. (2021) advised that callus be subcultured every 4 to 6 weeks. The growth rate was determined using Eq. 1.

### **3.4. Macronutrient content of *C. ternatea* L. callus culture**

**Table (2)** presents the results, detailing the percentages of mineral elements found in the *C. ternatea* L. callus sample. The analysis indicates high concentrations of calcium, iron, magnesium, manganese, copper, zinc, phosphorus, potassium, and total nitrogen. These mineral salts are considered highly beneficial to overall health and wellness. Mineral-rich plants are a good source of medicinal properties. Mineral analysis can infer the feasibility of using a plant for therapeutic purposes. This investigation shows copper has the lowest abundance (2.41 mg). Nitrogen and potassium levels in this study were exceptionally high (2.38 and 1.64/100g dry weight). People with diabetes who take diuretics to regulate their hypertension and have trouble excreting potassium through bodily fluids can benefit from the high potassium content in their diets.

Furthermore, growth, skeletal development, and other essential bodily functions depend on minerals like calcium and magnesium. According to the current investigation, the callus of *C. ternatea* L. has an iron content of 5.99 mg/100g DW. Anemia and other related disorders can be avoided with iron (Swati and Varsha, 2014). The current study found zinc at 3.95 mg/100 g DW, which boosts immunity and lowers cancer risk. Zinc impacts protein synthesis, healthy bodily development, and sickness healing. The manganese content of *C. ternatea* L. callus was 6.2 mg/100 g DW. Energy production and immune system support are two functions of manganese. Additionally, it supports blood coagulation by working with vitamin K and controls the effects of stress by working with B complex vitamins (Muhammad and Rabeta 2018; El-shiekh et al. 2023).

### **3.5. Total antioxidant activity, DPPH, ABTS, total flavonoids, and total phenolics**

Additionally, *C. ternatea* L. has antibacterial, anthelmintic, hepatoprotective, antiasthmatic, antidiabetic, and antioxidant qualities. Phenolic chemicals, Tannins, ternatins, anthocyanins, alkaloids, flavonoids, taraxerol, and taraxerone are the main components of *C. ternatea* L. callus (Muhammad and Rabeta 2018; Jayanti et al. 2021). In tissue culture, phenols, flavonoids, and antioxidant activity are observed. The key natural compounds in *Scutellaria* roots that give Huang-Qin its cancer-fighting effects are the

flavones wogonoside, baicalin, and their less complex counterparts wogonin and baicalein (Chou et al. 2003; Li-Weber 2009).

Flavonoids exhibit strong antioxidant properties. The largest class of phytochemicals, flavonoids, are thought to be the most prevalent type of polyphenol found in fruits and vegetables. By scavenging free radicals and ROS, chelating metals, and halting the oxidation of low-density lipoproteins, flavonoids can demonstrate their exceptional antioxidant qualities. (LDLs) (Abdelkader et al. 2024; Fodail et al. 2025). Baicalein and baicalin, two flavonoids found in the radix of *C. ternatea* L. callus that have an o-di-hydroxyl group in the A ring, may be effective free radical scavengers and may be used to treat head damage caused by attacks by free radicals (Elmoslemany et al. 2024).

The analyzers demonstrated increased antioxidant activity using the DPPH technique (Table 3). Significant values in the association between the investigated phenolic components and the decline in extract strength were discovered using correlation analyses. The DPPH method found a moderately good correlation between the sample's antioxidant activity and phenolic component content (Mohammed et al. 2024). As a result, analyses of a *C. ternatea* L. sample revealed strong antioxidant activity that applies to further studies, especially those including pharmacokinetics. This outcome is in line with Wahyu et al. (2022).

Table 1  
Features of *C. ternatea* L. callus on MS supplemented with 1.0 mg/L 2,4-D and BAP across six subcultures

| No of subculture                                                                                                 | Fresh weight g/jar        | Dry weight g/jar         | Morphological Characteristics                                                   |
|------------------------------------------------------------------------------------------------------------------|---------------------------|--------------------------|---------------------------------------------------------------------------------|
| 1st subculture                                                                                                   | 6 ± 0.7 <sup>e</sup>      | 1.2 ± 0.14 <sup>e</sup>  | Compact - light brownish, with some white colonies                              |
| 2nd subculture                                                                                                   | 8.4 ± 0.9 <sup>e</sup>    | 1.7 ± 0.17 <sup>e</sup>  | Compact - light brownish, with some slightly white colonies                     |
| 3rd subculture                                                                                                   | 11.8 ± 0.83 <sup>d</sup>  | 2.36 ± 0.16 <sup>d</sup> | Some friable with some yellowing, some compact light brownish-red colonies      |
| 4th subculture                                                                                                   | 13.40 ± 0.54 <sup>c</sup> | 2.68 ± 0.10 <sup>c</sup> | More friable with more yellowing patches few reddish yellow spots, some compact |
| 5th subculture                                                                                                   | 15.2 ± 0.83 <sup>b</sup>  | 3.04 ± 0.16 <sup>b</sup> | More friable with yellowing patches, few compact                                |
| 6th subculture                                                                                                   | 16.4 ± 0.54 <sup>a</sup>  | 3.28 ± 0.10 <sup>a</sup> | Completely friable yellowish callus                                             |
| Mean values followed by the same superscript letters within a column are not significantly different at P ≤ 0.05 |                           |                          |                                                                                 |

Table 2  
Macronutrient content of *C. ternatea* L. callus culture

| Mineral elements       | <i>C. ternatea</i> L. callus |
|------------------------|------------------------------|
| Vitamin C (mg/100 g)   | 18.27 ± 2.62                 |
| Total nitrogen (N) (%) | 2.38 ± 0.05                  |
| Phosphorus (P) (%)     | 0.66 ± 0.02                  |
| Potassium (K) (%)      | 1.64 ± 0.17                  |
| Calcium (Ca) (%)       | 0.73 ± 0.03                  |
| Iron (Fe) (ppm)        | 5.99 ± 0.28                  |
| Manganese (Mn) (ppm)   | 3.31 ± 0.21                  |
| Mg (ppm)               | 6.2 ± 0.13                   |
| Cu (ppm)               | 2.41 ± 0.16                  |
| Zn (ppm)               | 3.95 ± 0.12                  |

Table 3  
Total antioxidant activity, DPPH, ABTS, total flavonoids, and total phenolics.

| Antioxidant capacity            | <i>C. ternatea</i> L. callus |
|---------------------------------|------------------------------|
| Total Phenolics (TP) (mg/g Dw)  | 81.37 ± 3.65                 |
| Total Flavonoids (TF) (mg/g Dw) | 4.63 ± 1.03                  |
| DPPH (mg/g Dw)                  | 15.52 ± 1.17                 |
| ABTS ( mg/g Dw)                 | 2.32 ± 0.09                  |

## 4.5. High-Performance Phenolic Extract Analysis Using Liquid Chromatography *C. ternatea* L. callus Phenolic Extract

**Table (4)** displays the HPLC profiles' quantitative results. For HPLC analysis, a random sample was chosen. The compounds and active ingredients in them emerged as a result of the analysis. These substances preserve human health (Pengelly 2020; Mohammed et al. 2024). The plant contains many important compounds (such as Hesperetin, Daidzein, Quercetin, Cinnamic acid, Kaempferol, Rutin, Vanillin, Ferulic acid, Rosmarinic acid, Chlorogenic acid, Ellagic acid, Coumaric acid, and Gallic acid) in high concentrations. Several fruits, herbs, and nuts are often used to isolate gallic acid (GA), 3,4,5-trihydroxybenzoic acid, and chlorogenic acid, which are naturally occurring secondary metabolites. The potent anti-inflammatory properties of gallic acid have garnered more interest in recent years. According

to numerous literature evaluations, the inadequate extraction rate of gallic acid restricts its use in development, even though it is a rich plant source. It is important to remember that gallic acid can be made in vast quantities by chemical and biological synthesis in addition to being extracted from various plants. Pharmacological research indicates that gallic acid is rapidly absorbed and excreted orally. According to pharmacological studies, Gallic acid, found in the callus of *C. ternatea* L., a plant that grows in China and some parts of Russia, is quickly absorbed and eliminated when taken orally. It is applied in traditional medicine to treat allergies, inflammation, headaches, and infections. It may also have antiviral and antifungal properties. The results show that the MAPK and NF $\kappa$ B signaling pathways are the primary drivers of GA's anti-inflammatory effects. As a result, it lessens the inflammatory response by lowering the synthesis of adhesion molecules, chemokines, inflammatory cytokines, and cell infiltration (Mohammed et al. 2025).

Ellagic acid is a chemical that inhibits, delays or stops things from oxidizing, trapping free radicals, and reducing oxidative stress. Additionally, ellagic acid is an ingredient in several commercial goods with antioxidant properties. These compounds' anti-mutagenic, anti-microbial, and antioxidant qualities, along with their status as HIV inhibitors, confer several advantages (Sepúlveda et al. 2011). One phenolic component in plants is rosmarinic acid, crucial for supporting plants' growth and defense systems. Rosmarinic acid lowers the risk of certain cancer types by avoiding free radical-induced cell damage. It possesses anti-cancer, anti-angiogenic, antioxidant, anti-inflammatory, and antibacterial qualities (Roszkowski 2023).

The antioxidant and anti-inflammatory qualities of this beverage may improve endothelial function. Additionally, consuming much tea is associated with higher concentrations of bioactive substances like polyphenols (Pacheco-Coello et al. 2020).

### 3.5. *In vitro* antidiabetic activity

Outside of a live organism, *in vitro*, antidiabetic research assesses the ability of bioactive substances to minimize blood sugar or promote insulin sensitivity. These investigations often concentrate on measuring glucose absorption by cells or blocking enzymes like  $\alpha$ -amylase and  $\alpha$ -glucosidase involved in glucose metabolism.

By blocking important enzymes linked to type 2 diabetes, herbal remedies provide a cost-effective way to treat the condition (Mohammed et al. 2023; Soliman et al. 2024). **Table (5)** displays the crude and concentrated extracts' ability to inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase. Compared to the positive control acarbose, *C. ternatea* L. callus culture and a substantial inhibitory impact of concentrated extracts on the  $\alpha$ -amylase enzyme were noticed. Additionally, the concentrated extracts of *C. ternatea* L. callus culture demonstrated a considerable  $\alpha$ -glucosidase inhibitory activity more than 3 times higher than that of the standard acarbose. According to our findings, the callus culture of *C. ternatea* L. exhibited the strongest inhibitory activity for both  $\alpha$ -amylase ( $IC_{50} = 850.5 \pm 0.54 \mu\text{g/mL}$ ) and  $\alpha$ -glucosidase ( $IC_{50} = 198.63 \pm 0.67 \mu\text{g/mL}$ ). The primary chemicals found in *C. ternatea* L. callus culture have inhibitory activity greater

than those of acarbose ( $IC_{50} = 269.53 \pm 1.06 \mu\text{g/mL}$  for  $\alpha$ -glucosidase and  $18.36 \pm 0.99 \mu\text{g/mL}$  for  $\alpha$ -amylase).

Nevertheless, the inhibitory actions of  $\alpha$ -amylase and  $\alpha$ -glucosidase on the callus culture of *C. ternatea* L. were not documented in any of the published works. As the primary antioxidant molecules among the chemical ingredients, polyphenols have shown promise in the treatment of diabetes mellitus because they influence fat metabolism in addition to regulating carbohydrate metabolism and stimulating insulin release (Paun et al. 2020; Mohammed et al. 2025).

### 3.6. Anticancer activity

Using a viability test, **Figure (5)** illustrates the *in vitro* cytotoxic activity of the callus culture of *C. ternatea* L. against HepG2 cell lines using a viability test. The capacity of a cell to regain or sustain a state of living is assessed by a viability test. Using varying dosages of the adventitious root culture of *C. ternatea* L. to examine the capacity of hepatocellular carcinoma (HepG2) cancer cells to recuperate post-therapy. Staurosporine is a positive standard often employed in antitumor treatment because of its anticancer action in terms of its half-maximal inhibitory concentration ( $IC_{50}$ ).

Moreover, after treatment with  $1.56 \mu\text{g/ml}$  of *C. ternatea* L., 83.5% of the hepatocellular carcinoma (HepG2) cancer cells remain healthy (Fig. 5). However, the viability percentage dropped to 50% when the concentration was raised to  $100 \mu\text{g/ml}$  (48.4%). For  $100 \mu\text{g/ml}$  staurosporine (positive standard), the viability percentage was 29.2% and 55.5% at  $1.56 \mu\text{g/ml}$ . According to the present data, the *C. ternatea* L. callus culture demonstrated the strongest anticancer activity due to containing elevated levels of phenolic and flavonoid compounds which were observed in the HPLC chromatography (Table 4 and Fig. 4). Flavonols, the most important subclass of flavonoids, are substances present in the callus culture of *C. ternatea*. Biochemical properties of flavonols, including hepatoprotective, cardiovascular, and anti-inflammatory actions, have been demonstrated (Hassan et al. 2020; Elmoslemany et al. 2024). Furthermore, *C. ternatea* L. callus culture secondary metabolites have potent anti-inflammatory and anti-carcinogenic properties (Abdelazeez et al. 2025; Aboueldis et al. 2025).

Table 4  
High-Performance Phenolic Extract Analysis Using Liquid Chromatography of *C. ternatea* L. callus

| Compounds        | Area  | Conc. (µg/ml) | Conc. (µg/g) |
|------------------|-------|---------------|--------------|
| Gallic acid      | 79.41 | 6.94          | 346.92       |
| Chlorogenic acid | 46.12 | 6.58          | 329.22       |
| Catechin         | 0.83  | 0.20          | 10.25        |
| Methyl gallate   | 7.46  | 0.42          | 20.82        |
| Coffeic acid     | 21.05 | 1.85          | 92.62        |
| Rutin            | 11.13 | 1.99          | 99.44        |
| Ellagic acid     | 6.41  | 0.68          | 33.83        |
| Coumaric acid    | 1.10  | 0.04          | 2.05         |
| Vanillin         | 1.79  | 0.07          | 3.66         |
| Ferulic acid     | 9.70  | 0.60          | 30.10        |
| Naringenin       | 4.29  | 0.43          | 21.47        |
| Rosmarinic acid  | 34.49 | 3.77          | 188.64       |
| Daidzein         | 12.00 | 0.84          | 42.07        |
| Quercetin        | 1.11  | 0.07          | 3.54         |
| Cinnamic acid    | 31.20 | 0.60          | 30.07        |
| Kaempferol       | 12.95 | 0.96          | 47.92        |
| Hesperetin       | 4.09  | 0.19          | 9.67         |

Table 5  
 $\alpha$ -Amylase and  $\alpha$ -Glucosidase inhibitory activities of callus culture of *C. ternatea* L.

| Samples                                            | $\alpha$ -amylase IC <sub>50</sub><br>(µg/mL) | $\alpha$ -glucosidase IC <sub>50</sub><br>(µg/mL) |
|----------------------------------------------------|-----------------------------------------------|---------------------------------------------------|
| Acarbose                                           | 18.36 ± 0.99                                  | 269.53 ± 1.06                                     |
| Callus culture of <i>C. ternatea</i> L.            | 850.5 ± 0.54                                  | 198.63 ± 0.67                                     |
| The values are the IC <sub>50</sub> values (n = 6) |                                               |                                                   |

### 3.7. Evaluation of anti-inflammatory activities

All the bioactive phenolic and flavonoid components of the *C. ternatea* L. callus culture presented in **Table (4)** provide anti-inflammatory properties. Furthermore, there is a strong link between oxidation and inflammation since inflammation results from free radicals, which harm cells. Inflammation is a protective mechanism organisms utilize to remove detrimental stimuli and a signal to promote the healing process (Salem et al. 2024).

### 3.7.1. Albumin denaturation assay

The callus culture of *C. ternatea* L. was evaluated for its anti-inflammatory properties *in vitro* using the albumin denaturation technique. According to Acharya and Chaudhuri (2021), denaturation alters the electrostatic, hydrogen, hydrophobic, and disulfide bonds that keep proteins in their three-dimensional structure. By producing self-antigens *in vivo*, this denaturation triggers the inflammatory response.

This study compared the anti-inflammatory properties to those of diclofenac sodium. In the albumin denaturation test, the traditional diclofenac sodium's stability range was statistically significant ( $p < 0.05$ ) ( $59.25 \pm 0.48$ – $96.68 \pm 0.62\%$ ). The proportion of albumin denaturation inhibition and its concentration are directly correlated, according to the data shown in **Figure (6)**. The percentage varied from  $47.9 \pm 1.1$  to  $91.73 \pm 0.95$  in the *C. ternatea* L. callus culture. Polyphenols may account for the beneficial effects of *C. ternatea* L. on inflammation (Alshafei et al. 2023). Moreover, polyphenols influence many molecular targets implicated in inflammatory signaling pathways and lower indices of inflammation (Abdoon et al. 2024).

### 3.7.2. HYA, Lipxygenase and Proteinase inhibition assays

Peptide linkages may be broken, and they can hydrolyze other proteins. Additionally, they may induce inflammation by regulating the synthesis and action of inflammatory cytokines, chemokines, and other immune components (Al-Qahtani et al. 2024). The study examined the proteinase inhibition of the *C. ternatea* L. callus culture using ibuprofen, aceclofenac, and indomethacin as reference drugs (Table 6). According to the findings, the callus culture of *C. ternatea* L. has an IC50 value of  $35.37 \pm 0.63$ .

Table 6  
Lipoxygenase and Proteinase Inhibitions of callus culture of *C. ternatea* L.

| Samples                                 | HYA Inhibition   | Lipoxygenase Inhibition | Proteinase inhibition |
|-----------------------------------------|------------------|-------------------------|-----------------------|
| Aceclofenac                             | $16.34 \pm 0.37$ | $13.78 \pm 1.06$        | $20.52 \pm 1.05$      |
| Indomethacin                            | $8.67 \pm 0.89$  | $9 \pm 0.93$            | $9.42 \pm 0.92$       |
| Ibuprofen                               | $15.5 \pm 0.67$  | $18.17 \pm 1.17$        | $17.37 \pm 0.99$      |
| Callus culture of <i>C. ternatea</i> L. | $53.41 \pm 0.66$ | $47.35 \pm 0.98$        | $35.37 \pm 0.63$      |
| The values are the IC50 values (n = 6)  |                  |                         |                       |

This is the first study that investigated the *in vitro* HYA, lipoxygenase, and proteinase activities of the callus culture of *C. ternatea* L. The callus culture of *C. ternatea* L. demonstrated elevated HYA inhibition level ( $IC_{50} = 53.41 \pm 0.66 \mu\text{g/mL}$ ) instead of the standards. Moreover, HYA inhibitory activity was closely associated with radical scavenging activity. Additionally, the concentrated extract's anti-inflammatory properties may be related to its higher polyphenolic content, particularly gallic acid, chlorogenic acid, rosmarinic acid, caffeic acid, cinnamic acid, and kaempferol. This is consistent with the information we have in **Table (4)**.

The manufacture of leukotrienes, or hydroperoxides, crucial to the pathogenesis of several inflammatory disorders, depends on the enzyme lipoxygenase. Inhibiting lipid hydroperoxide production during enzymatic peroxidation is one method of antioxidant activity. This may restrict the lipidic substrate available for the lipoxygenase catalytic cycle. Furthermore, using lipoxidase as the enzyme and linoleic acid as the substrate, minor modifications were made to demonstrate the efficacy of lipoxygenase inhibition (Medina et al. 2024). This activity was conducted because the lipoxygenase enzyme pathway plays a significant role in developing inflammatory disorders. It is well established that non-inflammatory medications promote tissue regeneration and reduce lipoxygenase activity. Arachidonic acid, linoleic acid, and linolenic acid are polyunsaturated fatty acids oxidized to form hydroperoxidase, producing the single-unit enzyme known as lipoxygenase (LOX). Mammals often include 5-lipoxygenase, which is produced from arachidonic acid's 5-carbon position. Many immune, epithelial, and cancerous cells have the LOX protein. Numerous physiological conditions, including stroke, neurological disorders, skin conditions, cardiovascular problems, and cancer, depend on it. Significantly, it is a precursor to inflammation (Gunathilake et al. 2018; Truong et al. 2021). The bioactivity of the callus culture of *C. ternatea* L. may affect its anti-inflammatory properties. The study findings shown in **Table (6)** make this evident.

## Conclusion and Future Perspective

The research examines the production of secondary metabolites in callus cultures of *C. ternatea* L., a plant recognized for its therapeutic qualities. Plant tissue culture is considered one of the most important biotechnological methods for large-scale production of pharmaceutical compounds from natural resources, which in turn helps conserve natural resources, especially endangered plants. Plant growth regulators are essential for callus induction from different explants, a critical step in our study. In addition, many factors affect the callus productivity of *C. ternatea* L., including environmental factors, such as the number of subcultures and the incubation period. Furthermore, the current study demonstrates the potent and encouraging anti-diabetic, anti-inflammatory qualities, anticancer, and antioxidant qualities of callus culture of *C. ternatea* L. By using these attributes; it may be possible to avoid chronic diseases, such as diabetes, heart disease, and certain forms of cancer. In light of this, the current study shows that various biological and nutritional benefits are attributed to the bioactive components found in the callus culture of *C. ternatea* L. These outcomes could provide valuable perspectives into the fields of biotechnology, biodiversity, and medical research about future investigations of the callus culture of *C. ternatea* L. Our future objectives involve enhancing callus and

cell suspension cultures of *C. ternatea* L. by bioreactor implementation and increasing its efficacy for phytoremediation studies.

## References

1. Abdelazeez WMA, Anatolievna KY, Zavdetovna KL, Damirovna AG, Abou El-Dis, GR, Arnoldovna, TO (2022) Enhanced productivity of atropine in cell suspension culture of *Hyoscyamus muticus* L. *In Vitro Cell Dev Biol Plant* 58:593–605. <https://doi.org/10.1007/s11627-022-10273-w>
2. Abdelazeez W, Aboueldis GR, Suliman AA, Mohammed DM (2025) Production of secondary metabolites in callus cultures of *Scutellaria baicalensis* L. and assessment of their anti-inflammatory and antioxidant efficacy in ulcerative colitis rats. *Plant Cell Tiss Organ Cult* 160, 80 (2025). <https://doi.org/10.1007/s11240-025-02996-6>
3. Abdelkader M, Elkhawaga FA, Suliman AA, Puchkov M, Kuranova KN, Mahmoud MH, Abdelkader MF (2024) Understanding the Regular Biological Mechanism of Susceptibility of Tomato Plants to Low Incidences of Blossom-End Rot. *Horticulturae* 10(6): 648. <https://doi.org/10.3390/horticulturae10060648>
4. Abdelkader M, Suliman AA, Salem SS, Assiya A, Voronina L, Puchkov M, Loktionova E, Bhuker A, Ataya FS, Mahmoud MH, Abdelkader MFM (2024) Studying the combined impact of salinity and drought stress-simulated conditions on physio-biochemical characteristics of lettuce plant. *Horticulturae* 10(11): 1186 <https://doi.org/10.3390/horticulturae10111186>
5. Abdoon ASS, Hegazy AM, Abdel-Azeem AS, Al-Atrash AM, Mohammed DM (2024) The protective effects of some herbs on mitigating HFD-induced obesity *via* enhancing biochemical indicators and fertility in female rats. *Heliyon* 10(9): e30249 <https://doi.org/10.1016/j.heliyon.2024.e30249>
6. Abou Baker DH, Mohammed DM (2022) Polyphenolic rich fraction of *Physalis peruviana* calyces and its nano emulsion induce apoptosis by caspase 3 up-regulation and G2/M arrest in hepatocellular carcinoma. *Food Biosci* 50:102007. <https://doi.org/10.1016/j.fbio.2022.102007>
7. Abou El-Dis GR, Zavdetovna KL, Nikolaevich AA, Abdelazeez WMA, Arnoldovna TO (2021) Influence of light on the accumulation of anthocyanins in callus culture of *Vaccinium corymbosum* L. cv. Sunt Blue Giant. *J Photochem Photobiol* 8: 100058. <https://doi.org/10.1016/j.jpap.2021.100058>
8. Aboueldis GR, Abdelazeez WM, Suliman AA, Mohammed DM (2025) Therapeutic efficacy of secondary metabolites produced from cell suspension culture of *Vaccinium corymbosum* L. mitigates high-fat-diet-induced metabolic syndrome in rat model. *Food Biosci* 68: 106795. <https://doi.org/10.1016/j.fbio.2025.106795>
9. Acharya VV, Chaudhuri P (2021) Modalities of protein denaturation and nature of denaturants. *Int J Pharm Sci Rev Res* 69(2):19–24. <https://doi.org/10.47583/ijpsrr.2021.v69i02.002>
10. Al-Qahtani AA, Alhamlan FS, Al-Qahtani AA (2024) Pro-inflammatory and anti-inflammatory interleukins in infectious diseases: a comprehensive review. *Trop Med Infect Dis*. 9(1): 13. <https://doi.org/10.3390/tropicalmed9010013>

11. Alshafei MM, Mohammed D, Hanafi EM, Kassem SS, El-Messery TM, Korany R, Elsaid MM (2023) Red beet Peel-loaded Liposome's impact on metabolic syndrome rats and its use as fortifier in yoghurt powder. *Egypt J Chem* 66(2):517–533 <https://doi.org/10.21608/ejchem.2022.129208.5714>
12. American Public Health Association. (1998). *Standard methods for the examination of water and wastewater* (20th ed.). Washington, DC: APHA.
13. Boulos L (2009) *Flora of Egypt Checklist, Revised Annotated Edition*. Al Hadara Publishing, Egypt. pp 23
14. Rashid SA, Tong WY, Leong CR, Ghazali NMA, Taher MA, Ahmad N, Tan W, Teo SH (2020) Anthocyanin Microcapsule from *Clitoria ternatea*: Potential Bio-preservative and Blue Colorant for Baked Food Products. *Arab J Sci Eng* 46(1):65–72. <https://doi.org/10.1007/s13369-020-04716-y>
15. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. *LWT - Food Sci and Tech* 28 (1): 25–30 [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
16. Chou CC, Pan SL, Teng CM, Guh JH (2003) Pharmacological evaluation of several major ingredients of Chinese herbal medicines in human hepatoma Hep3B cells. *Eur J Pharm Sci* 19:403–412. [https://doi.org/10.1016/S0928-0987\(03\)00144-1](https://doi.org/10.1016/S0928-0987(03)00144-1)
17. Cottenie A, Verloo M, Kiekens L, Velghe G, Camerlynck R (1982) Chemical analysis of plant and soil. laboratory of analytical and agrochemistry, State Univ. Ghent Belgium, pp 100–129
18. El-Demerdash M (2001) Medicinal Plants of Egypt. In: Saxena PK (eds) *Development of Plant-Based Medicines: Conservation, Efficacy and Safety*. Springer, Dordrecht. [https://doi.org/10.1007/978-94-015-9779-1\\_4](https://doi.org/10.1007/978-94-015-9779-1_4)
19. Elmoslemamy AM, Elzallat M, Abd-Elfatah MH, Mohammed DM, Abd Elhady EE (2024) Possible therapeutic effect of frankincense (*gum olibanum*) and myrrh (*Commiphora Myrrha*) resins extracts on DEN/CCL4 induced hepatocellular carcinoma in rats. *Phytomedicine Plus* 4(1):100517. <https://doi.org/10.1016/j.phyplu.2023.100517>
20. El-shiekh AF, Omar MA, Mansour AI, Ahmed MA, Mohammed DM, El-Messery TM (2023) Physicochemical properties of functional yoghurt fortified with microencapsulated moringa and black cumin oils. *Al-Azhar j Agri Res* 48(3): 564-574. <https://doi.org/10.21608/ajar.2024.237090.1265>
21. Eltaif NI, Gharaibeh MA, Fadhil Al-Quraishi AM (2024) Impact of Climate Change on Iraq: Severe Water Scarcity and Desertification. In: Al-Quraishi A., Negm A., Benzougagh B. (eds) *Climate Change and Environmental Degradation in the MENA Region. The Handbook of Environmental Chemistry*, vol 136. Springer, Cham. [https://doi.org/10.1007/698\\_2024\\_1100](https://doi.org/10.1007/698_2024_1100)
22. Fodail MM, Ashour AM, Badr H, Ashmawy T, Abouelghait H (2025) Impact of Dietary Supplementation of Date Palm (*Phoenix Dactylifera* L) Pollen on Hemato-Biochemical Parameters, Antioxidant Status, Follicular Dynamics, and Litter Size of Rahmani Ewes during breeding season. *Al-Azhar J Agri Res* 50(1): 15-23. <https://doi.org/10.21608/ajar.2025.339368.1387>
23. Forgenie D, Hutchinson SD, Mahase-Forgenie M, Khoiriyah N (2024) Analyzing per capita food consumption patterns in net food-importing developing countries. *J Agric Food Res* 18, 101278.

<https://doi.org/10.1016/j.jafr.2024.101278>

24. Gunathilake KDPP, Ranaweera KKDS, Rupasinghe HP (2018) *In vitro* anti-inflammatory properties of selected green leafy vegetables. *Biomedicines* 6:107.  
<https://doi.org/10.3390/biomedicines6040107>
25. Hassan AY, Mohamed MA, Abdel-Aziem A, Hussain AO (2020) Synthesis and anticancer activity of some fused heterocyclic compounds containing pyrazole ring. *Polycycl Aromat Compd* 40:1280–1290. <https://doi.org/10.1080/10406638.2020.1764984>
26. Hassanen S, Diab M, Hegazi G, Abou El-Dis, GR (2021) Silymarin accumulation in *Silybum marianum* suspension culture *via* precursor feeding. *Catrina: Int J Environ Sci* 24 (1): 15-23  
<https://doi.org/10.21608/cat.2022.94562.1102>
27. Hemmati N, Cheniany M, Ganjeali A (2020) Effect of plant growth regulators and explants on callus induction and study of antioxidant potentials and phenolic metabolites in *Salvia tebesana* Bunge. *Bot Serb* 44(2):163–173. <https://doi.org/10.2298/BOTSERB2002163H>
28. Ikeuchi M, Sugimoto K, Iwase A. (2013) Plant callus: mechanisms of induction and repression. *Plant Cell* 225(9):3159–73. <https://doi.org/10.1105/tpc.113.116053>
29. Jayanti M, Ulfa AM, Saputra Yasir, A (2021) The Formulation and Physical Evaluation Tests of Ethanol in Telang Flower (*Clitoria ternatea* L.) Extract Losio Form as Antioxidant. *Biomed J Indo* 7(1): 488–495. <https://doi.org/10.32539/BJI.v7i3.543>
30. Li-Weber M (2009) New therapeutic aspects of flavones: the anticancer properties of *Scutellaria* and its main active constituents Wogonin, Baicalein and Baicalin. *Cancer Treat Rev* 35:57–68.  
<https://doi.org/10.1016/j.ctrv.2008.09.005>
31. Mashal DS, Abdelazeez WMA, Ghareb HES, Ebad FA (2024) Callus Induction and Enhancing the Production of Biomass and Pharmaceutical Components of *Thymus decussatus* as an Endangered Medicinal Plants in Egypt. *Al-Azhar J Agri Res* 49(2):176-195.  
<https://doi.org/10.21608/ajar.2024.294030.1357>
32. Medina AV, Nazareno MA, Chaillou LL (2024) Inhibitory effect of phenolic compounds on lipoxygenase activity in reverse micellar systems. *Food Biosci* 62: 105039.  
<https://doi.org/10.1016/j.fbio.2024.105039>
33. Mohammed DM, Abdelgawad MA, Ghoneim MM, El-Sherbiny M, Mahdi WA, Alshehri S, Ebrahim HA, Farouk A (2023) Effect of nano-encapsulated food flavorings on streptozotocin-induced diabetic rats. *Food Funct* 14(19): 8814–8828. <https://doi.org/10.1039/D3FO01299A>
34. Mohammed DM, El-Messery TM, Baranenko DA, Hashim MA, Tyutkov N, Marrez DA, Elmessery WM, El-Said MM (2004) Effect of *Spirulina maxima* microcapsules to mitigate testicular toxicity induced by cadmium in rats: Optimization of *in vitro* release behavior in the milk beverage. *J Funct Foods* 112:105938. <https://doi.org/10.1016/j.jff.2023.105938>
35. Mohammed DM, Maan SA, Abou Baker DH, Abozed SS (2024b). *In vitro* assessments of antioxidant, antimicrobial, cytotoxicity and anti-inflammatory characteristics of flavonoid fractions from flavedo

- and albedo orange peel as novel food additives. Food Biosci 62: 105581.  
<https://doi.org/10.1016/j.fbio.2024.105581>
36. Mohammed DM, Salem MB, Elzallat M, Hammam OA, Suliman AA (2024) Moringa oleifera L.-mediated zinc oxide nano-biofertilizer alleviates non-alcoholic steatohepatitis *via* modulating de novo lipogenesis pathway and miRNA-122 expression. Food Biosci 60: 104286.  
<https://doi.org/10.1016/j.fbio.2024.104286>
  37. Mohammed DM, Yang X, El-Messery TM, Jiang X, Zahran HA, Gebremeskal YH, Farouk A (2025) Bioactive Moringa Oleifera and Nigella sativa oils microcapsules alleviate high-fat-diet induced hepatic oxidative damage and inflammation in rats. Food Biosci 64:105873  
<https://doi.org/10.1016/j.fbio.2025.105873>
  38. Mouneer TA (2021) Sustainable Development Importance in Higher Education for Occupational Health and Safety Using Egypt Vision 2030 under COVID-19 Pandemic. J Geosci Environ Prot 9: 74-112. <https://doi.org/10.4236/gep.2021.94006>
  39. Muhammad ER, Rabeta MS (2018) A potential of telang tree (*Clitoria ternatea*) in human health. J Food Res 2 (5): 415–420. [https://doi.org/10.26656/fr.2017.2\(5\).073](https://doi.org/10.26656/fr.2017.2(5).073)
  40. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497. <https://doi.org/10.1111/j.1399-3054.1962.tb08052.x>
  41. Nouman W, Basra SMA, Yasmeen A, Yasmeen A, Gull T, Hussain SB, Zubair M, Gul R (2014) Seed priming improves the emergence potential, growth and antioxidant system of *Moringa oleifera* under saline conditions. Plant Growth Regul 73: 267–278. <https://doi.org/10.1007/s10725-014-9887-y>
  42. Pacheco-Coello F, Peraza-Marrero M, Orosco-Vargas C, Ramirez-Azuaje D, Pinto-Catari I (2020) Determination of Total Phenolic Compounds and Evaluation of the Antioxidant Activity of Commercial and Artisanal Green Tea Traded in Maracay, Venezuela. Rev Bol Quim 37(1): 28–33. <https://doi.org/10.34098/2078-3949.37.1.4>
  43. Pai TV, Sawant SY, Ghatak AA, Chaturvedi PA, Gupte A, Desai NS (2015) Characterization of Indian beers: chemical composition and antioxidant potential. J Food Sci Technol 52: 1414–1423  
<https://doi.org/10.1007/s13197-013-1152-2>
  44. Pant, G.; Sai, K.; Babasaheb, S.; Reddy, R.; Sibi, G. (2013) *In vitro*  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitor activity of abutilon indicum leaves. Asian J. Pharm. Clin. Res, 6(5): 22–24.
  45. Park J, Choi Y, Jeong M, Jeong Y, Han J, Choi H (2023a) Uncovering transcriptional reprogramming during callus development in soybean: insights and implications. Front Plant Sci 14, 1239917. <https://doi.org/10.3389/fpls.2023.1239917>
  46. Paun G, Neagu E, Albu C, Savin S, Radu GL (2020) *In vitro* evaluation of antidiabetic and anti-inflammatory activities of polyphenolic-rich extracts from *Anchusa officinalis* and *Melilotus officinalis*. ACS Omega. 5:13014–13022 <https://dx.doi.org/10.1021/acsomega.0c00929>
  47. Pengelly A (2020) The constituents of medicinal plants: An introduction to the chemistry and therapeutics of herbal medicine 2nd ed. London, Routledge: CABI Publishing.  
<https://doi.org/10.4324/9781003117964>

48. Pieroni LG, de Rezende FM, Ximenes VF, Dokkedal AL (2011) Antioxidant activity and total phenols from the methanolic extract of *Miconia albicans* (Sw.) Triana leaves. *Molecules* 16:9439–9450. <https://doi.org/10.3390/molecules16119439>
49. Ramachandran S, Rajasekaran A, Adhirajan N (2013) *In vivo* and *in vitro* antidiabetic activity of *Terminalia paniculata* bark: an evaluation of possible phytoconstituents and mechanisms for blood glucose control in diabetes. *ISRN Pharmacol* (1): 484675. <https://doi.org/10.1155/2013/484675>
50. Roszkowski S (2023) Application of polyphenols and flavonoids in oncological therapy. *Molecules* 28(10):4080. <https://doi.org/10.3390/molecules28104080>
51. Roubík H, Lošťák M, Ketuama CT, Soukupová J, Procházka P, Hruška A, Hakl J, Pacek L, Karlík P, Mensiková LK, Jurasová V, Ogbu CA and Hejcman M (2023) COVID-19 crisis interlinkage with past pandemics and their effects on food security. *Glob. Health* 19, 52 (2023). <https://doi.org/10.1186/s12992-023-00952-7>
52. Sabry BA, Badr AN, Mohammed DM, Desoukey MA, Farouk A (2024) Validating the protective role of orange and tangerine peel extracts for amending food safety against microorganisms' contamination using molecular docking. *Heliyon* 10(6):e27737. <https://doi.org/10.1016/j.heliyon.2024.e27737>
53. Sagharyan M, Ganjeali A, Cheniyani M, Mousavi-Kouhi M (2020) Optimization of callus induction with enhancing production of phenolic compounds production and antioxidants activity in callus cultures of *Nepeta binaloudensis* Jamzad (Lamiaceae). *Iran J Biotechnol* 18(4):47–55. <https://doi.org/10.30498/IJB.2020.2621>
54. Sahasrabudhe A, Deodhar M (2010) Anti-hyaluronidase, anti-elastase activity of *Garcinia indica*. *Int J Botany* 6:299–303. <https://doi.org/10.3923/ijb.2010.299.303>
55. Salem MB, Mohammed DM, Hammam OA, Elzallat M (2024) Mitigation of intrahepatic cholestasis induced by 17 $\alpha$ -ethinylestradiol *via* nanoformulation of *Silybum marianum* L. *BMC Complement Med Ther* 24(1):51. <https://doi.org/10.1186/s12906-024-04351-2>
56. Sepúlveda L, Ascacio A, Rodriguez-Herrera R, Aguilera-Carbó A, Aguilar CN (2011) Ellagic acid: biological properties and biotechnological development for production processes. *Afri J Biotechnol* 10(22):4518–4523 .
57. Shrivastava AK, Keshari M, Neupane M, Chaudhary S, Dhakal PK, Shrestha L, Palikhey A, Yadav CK, Lamichhane G, Shekh MU, Yadav RK (2023) Evaluation of antioxidant and anti-inflammatory activities, and metabolite profiling of selected medicinal plants of Nepal. *J Trop Med*. 2023:6641018. <https://doi.org/10.1155/2023/6641018>
58. Soliman TN, Karam-Allah AAK, Abo-Zaid EM, Mohammed DM (2024) Efficacy of nanoencapsulated *Moringa oleifera* L. seeds and *Ocimum tenuiflorum* L. leaves extracts incorporated in functional soft cheese on streptozotocin-induced diabetic rats. *Phytomed Plus* 4(3):100598. <https://doi.org/10.1016/j.phyplu.2024.100598>
59. Song Y (2014) Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. *J Integr Plant Biol* 56:106–113. <https://doi.org/10.1111/jipb.12131>

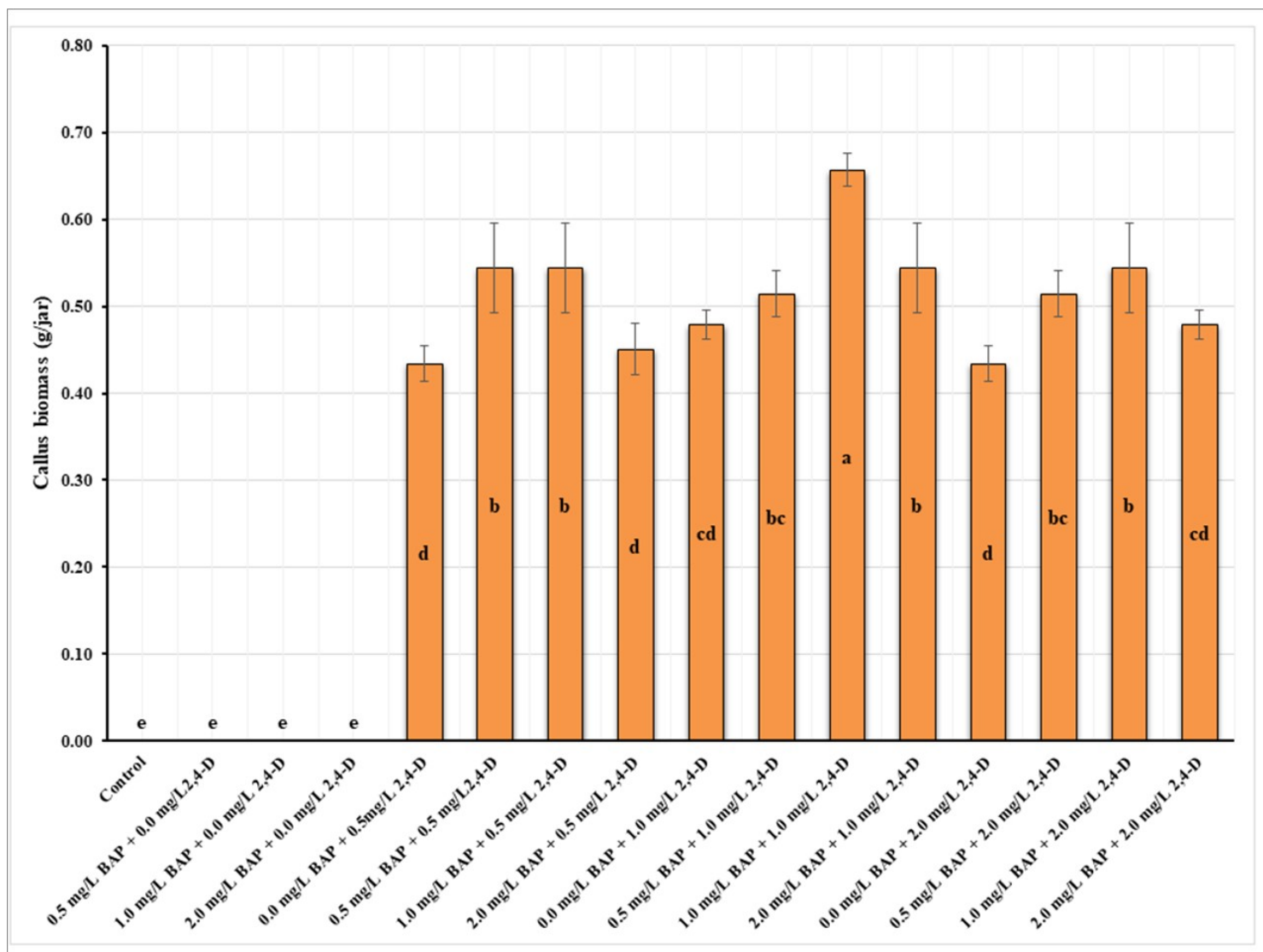
60. Suliman AA, Saleh S (2022) Effect of chloromequate chloride and indole-3-butyric acid as chemical growth regulators on tomato productivity and its chemical composition. *Egy J Chem* 65(9) 617-623. <https://doi.org/10.21608/ejchem.2022.129390.5716>
61. Suliman AA, El-Dewiny CY, Soliman MK, Salem SS (2025) Investigation of the effects of applying Bio-Magnesium oxide nanoparticle fertilizer to *Moringa Oleifera* plants on the chemical and vegetative properties of the plants' leaves. *Biotechnol J.* 20(3):e202400536. <https://doi.org/10.1002/biot.202400536>
62. Swati D, Varsha J (2014) Bromatological and mineral assessment of *Clitoria ternatea* Linn. leaves. *Int J Pharm Pharm Sci* 6 (3): 244–246.
63. Truong DH, Ta NT, Pham TV, Huynh TD, Do QT, Dinh NC, Dang CD, Nguyen TK, Bui AV (2021) Effects of solvent–solvent fractionation on the total terpenoid content and *in vitro* anti-inflammatory activity of *Serevenia buxifolia* bark extract. *Food Sci Nutr* 9:1720–1735. <https://doi.org/10.1002/fsn3.2149>
64. Vennila V, Pavithra V (2015) *In vitro* alpha amylase and alpha glucosidase inhibitory activity of various solvent extracts of *Hybanthus enneaspermus* Linn. *World J. Pharm. Pharm. Sci.* 4 (4): 1425–1437.
65. Wahyu W, Wargasetia TL, Zakaria TM, Meganitha MSG, Halim N, Dewi NSM, Santiadi S (2022) Antioxidant activities of ginger (*Zingiber officinale*) and telang flower (*Clitoria ternatea* L.) combination tea. *Maj Kedokt Bandung* 54 (3): 154–160. <http://localhost:8080/xmlui/handle/123456789/8692>
66. Wahyuni DK, Huda A, Faizah S, Purnobasuki H, Wardoyo BPE (2020) Effects of light, sucrose concentration and repetitive subculture on callus growth and medically important production in *Justicia gendarussa* Burm.f. *Biotechnol Rep.* <https://doi.org/10.1016/j.btre.2020.e00473>
67. Waterhouse AL (2003) Determination of total phenolics. In: *Current protocols in food analytical chemistry*. Wiley, Hoboken, pp 130–143. <https://doi.org/10.1002/0471142913.fai0101s06>
68. Worsfold P, Mckelvie I, Monbet P (2016) *Analytica chimica acta* determination of phosphorus in natural waters: A historical review. *Anal Chim Acta* 918:8–20. <https://doi.org/10.1016/j.aca.2016>
69. Zakaria TNAAT, Tan HS, Hassan Z, Subramaniam S, Chew BL (2024) The Effects Of 2,4-D, BAP, and Sucrose Concentrations in The Callus Induction of White (*Clitoria ternatea* var. Albiflora) and Blue Butterfly Pea (*Clitoria ternatea*). *Malaysian Appl Biol* 53(4): 53–63. <https://doi.org/10.55230/mabjournal.v53i4.3087>

## Figures



Figure 1

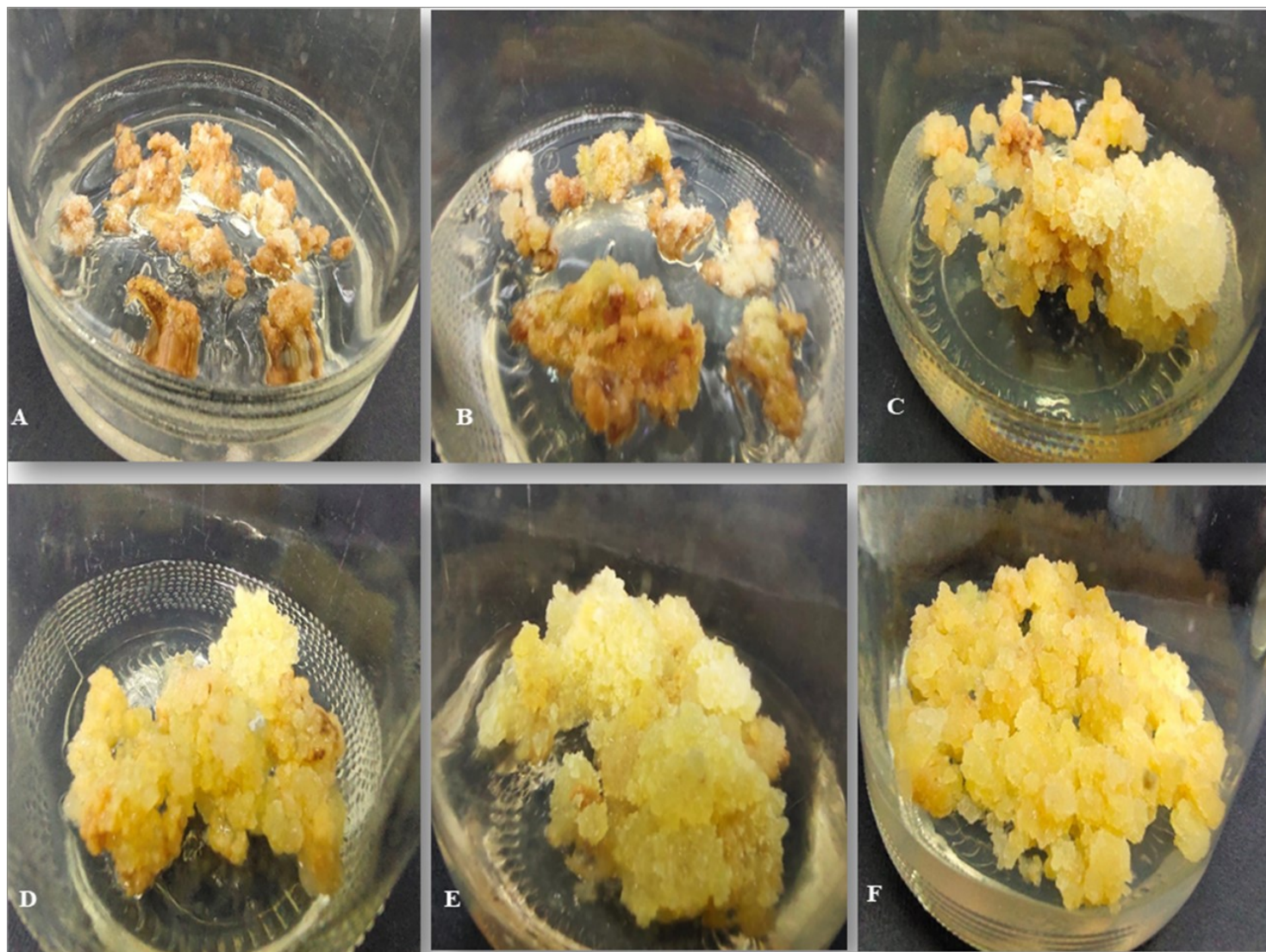
Aseptic culture of a *C. ternatea* L.



**Figure 2**

***C. ternatea* L. callus induction from cotyledonary leaf explant after 40–45 days of culture with varied doses and combinations of 2,4-D and BAP**

Mean values followed by the same superscript letters within a column are not significantly different at  $P \leq 0.05$ , callus biomass represented fresh weight in gram/ jar.



**Figure 3**

**Biomass accumulation of callus *C. ternatea* L. on MS supplemented with 1.0 mg/L of 2,4-D and 1.0 mg/L of BAP through six successive subcultures**

A, B, C—The first, second, and third subcultures. D, E, F—the fourth, fifth, and sixth subcultures.

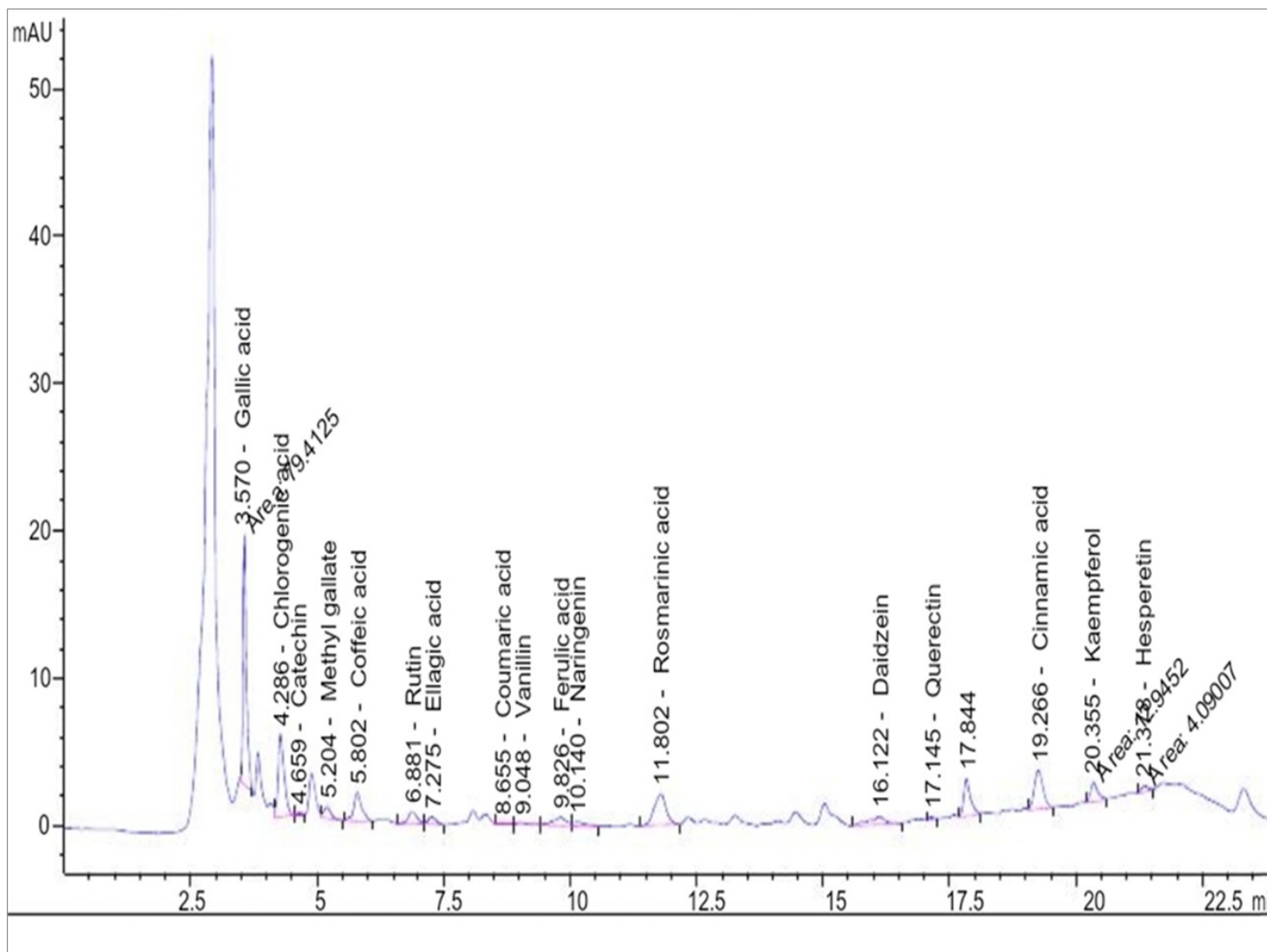


Figure 4

HPLC examination of *C. ternatea* L. callus

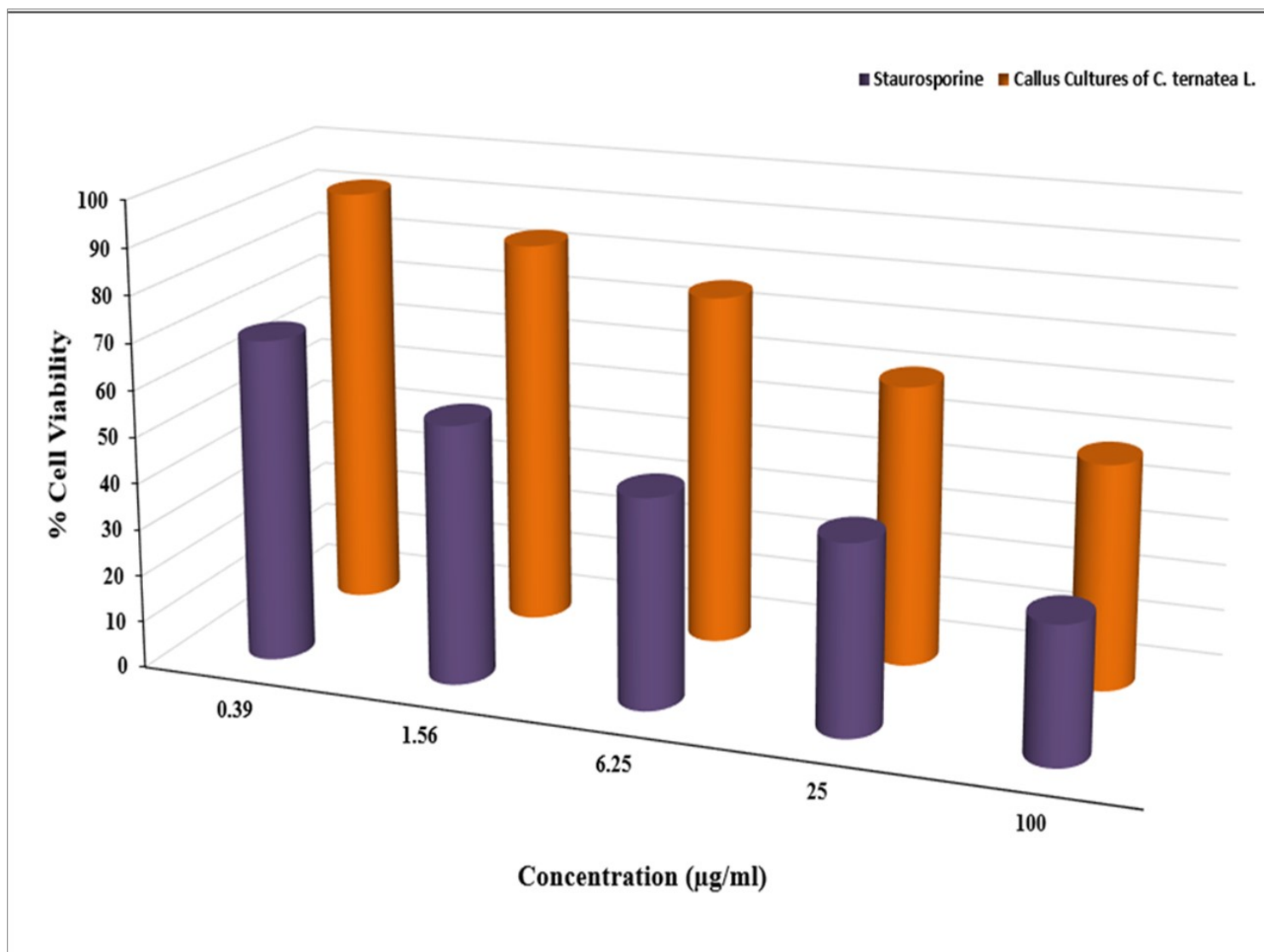


Figure 5

Anticancer activity of callus culture of *C. ternatea* L.

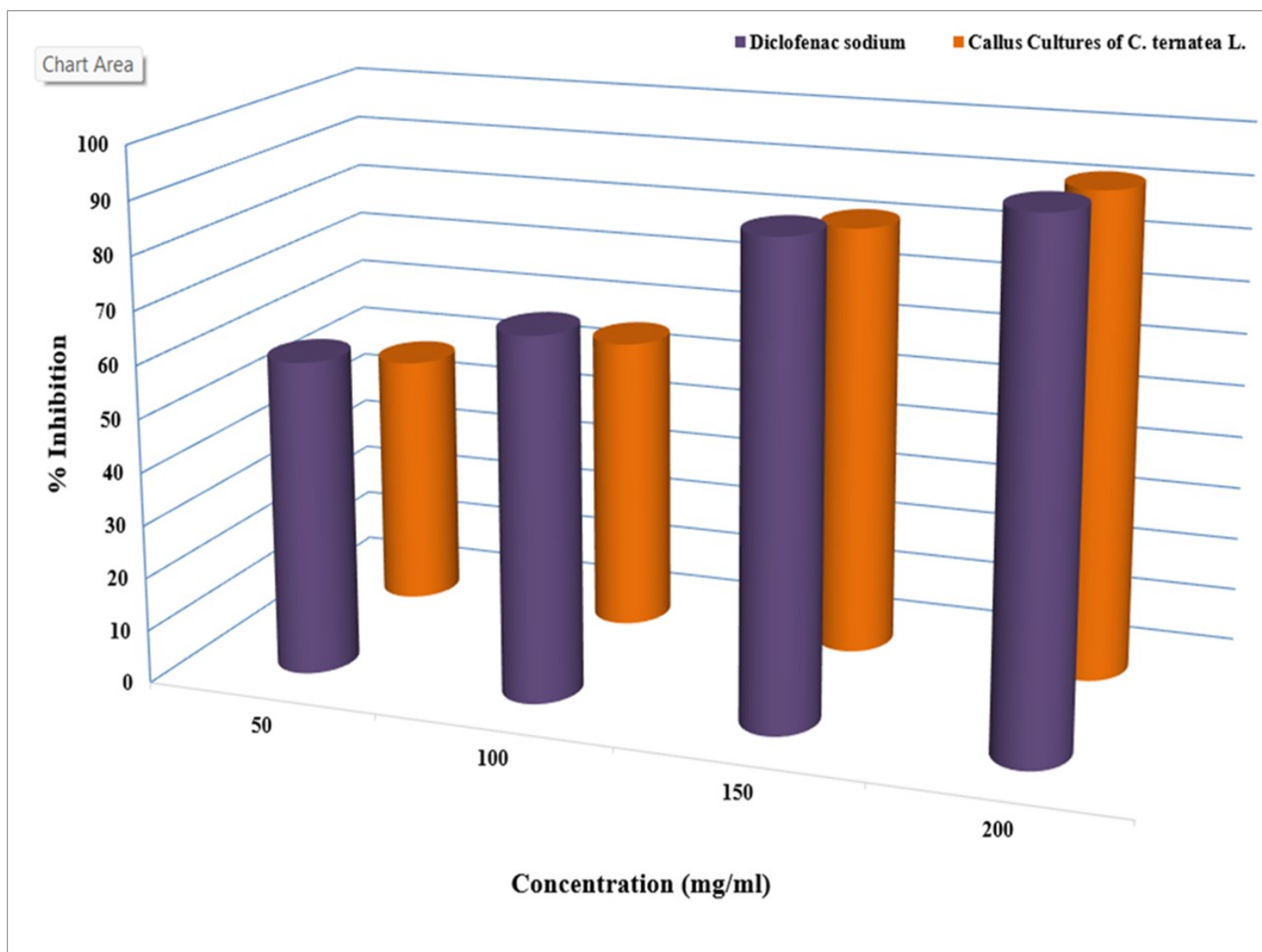


Figure 6

Albumin denaturation assay