Activity-guided Fractionation and in Vitro Anticancer Evaluation of Bioactive Phytoconstituents From Clitoria Ternatea L. Flowers Against HT-29 Human Colorectal Cancer

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ACTIVITY-GUIDED FRACTIONATION AND IN VITRO ANTICANCER EVALUATION OF BIOACTIVE PHYTOCONSTITUENTS FROM *CLITORIA TERNATEA* L. FLOWERS AGAINST HT-29 HUMAN COLORECTAL CANCER

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Abstract

Natural products continue to play a significant role in the discovery of anticancer agents because of their diverse bioactive constituents. *Clitoria ternatea* L. (Butterfly pea) is a medicinal plant rich in flavonoids, anthocyanins, phenolic compounds, and other phytochemicals with reported therapeutic potential, although the specific constituents responsible for its anticancer activity remain inadequately explored. In this study, the ethanolic flower extract was fractionated by liquid–liquid partitioning using n-hexane, chloroform, and ethyl acetate, followed by phytochemical screening and thin-layer chromatography (TLC) fingerprinting. The cytotoxic potential of the fractions was assessed against HT-29 human colorectal adenocarcinoma cells using the MTT assay (1–1000 µg/mL), and IC_{50} values were calculated with GraphPad Prism. Among the fractions, the ethyl acetate fraction showed the highest yield and was enriched with flavonoids, anthocyanins, phenolics, glycosides, and saponins. TLC analysis revealed five distinct bands, including one with an Rf value comparable to quercetin. This fraction also demonstrated the strongest cytotoxic effect (IC_{50} = 79.29 µg/mL), followed by the hexane (99.40 µg/mL) and chloroform (169.0 µg/mL) fractions, with concentration-dependent morphological changes in HT-29 cells. These findings indicate that activity-guided fractionation effectively enriched bioactive constituents, highlighting the ethyl acetate fraction as a promising source of anticancer phytochemicals for further isolation and structural characterization.

Keywords:

Clitoria ternatea L., Ethyl acetate fraction, Thin-layer chromatography, MTT assay, Colorectal cancer

Introduction

Cancer is one of the leading causes of morbidity and mortality worldwide and remains a major public health challenge despite remarkable advances in diagnosis and treatment. The disease is characterized by uncontrolled cellular proliferation, resistance to programmed cell death, sustained angiogenesis, and metastatic spread resulting from the accumulation of genetic and epigenetic alterations. Conventional therapeutic strategies, including surgery, chemotherapy, radiotherapy, immunotherapy, and targeted therapy, have significantly improved patient survival; however, their clinical application is frequently limited by severe adverse effects, multidrug resistance, poor selectivity toward cancer cells, and high treatment costs.[1-4]

Natural products have historically served as an important source of anticancer drugs. Approximately 60% of currently available anticancer agents have originated directly or indirectly from natural products or their derivatives.[5-9] Plant-derived compounds possess remarkable structural diversity and often exert anticancer effects through multiple molecular mechanisms, including induction of apoptosis, inhibition of angiogenesis, suppression of cell proliferation, regulation of oxidative stress, and modulation of intracellular signaling pathways.[10-13]

Among medicinal plants, *Clitoria ternatea* L. (Family: Fabaceae), commonly known as Butterfly pea, has attracted increasing scientific interest because of its broad spectrum of pharmacological activities. Traditionally, the plant has been used in Ayurvedic medicine as a memory enhancer, anti-inflammatory, antimicrobial, antioxidant, hepatoprotective, antidiabetic, and neuroprotective agent. The flowers are particularly rich in anthocyanins (ternatins), flavonoids, phenolic acids, triterpenoids, alkaloids, and other secondary metabolites that exhibit potent antioxidant and therapeutic activities.[14-20]

Several investigations have demonstrated that extracts of *Clitoria ternatea* possess cytotoxic activity against different cancer cell lines. These biological activities have largely been attributed to the presence of polyphenols and flavonoids capable of regulating oxidative stress, mitochondrial dysfunction, inflammatory mediators, and apoptosis-related signaling pathways. Flavonoids such as quercetin and kaempferol have been extensively reported to inhibit cancer cell proliferation by inducing cell-cycle arrest, activating intrinsic apoptotic pathways, and suppressing metastasis-associated signaling molecules.[21-23] Furthermore, flavonoids have also been identified as promising inhibitors of tubulin polymerization, thereby disrupting mitotic spindle formation and inducing apoptosis in rapidly dividing cancer cells.[24]

Although several studies have reported the biological activities of crude extracts of *Clitoria ternatea*, limited attention has been given to activity-guided fractionation for identifying the specific phytoconstituents responsible for anticancer activity. Crude plant extracts consist of complex mixtures of compounds with varying polarity, making it difficult to determine which constituents contribute most significantly to biological activity. Fractionation using solvents of increasing polarity facilitates the enrichment of chemically related compounds, enabling more precise evaluation of their pharmacological potential.

Thin-layer chromatography (TLC) remains a rapid and cost-effective analytical technique for phytochemical fingerprinting and preliminary identification of bioactive compounds. When combined with qualitative phytochemical screening and biological assays such as the MTT cytotoxicity assay, activity-guided fractionation represents a valuable strategy for identifying fractions enriched with potent anticancer constituents prior to advanced structural characterization using chromatographic and spectroscopic techniques.

Therefore, the present study was designed to perform activity-guided fractionation of the ethanolic flower extract of *Clitoria ternatea* using liquid–liquid partitioning, characterize the phytochemical profile of each fraction through qualitative chemical tests and TLC fingerprinting, and evaluate their in vitro anticancer potential against HT-29 human colorectal adenocarcinoma cells. The findings are expected to facilitate the identification of flavonoid-rich fractions with enhanced anticancer activity and provide a scientific basis for the future isolation and characterization of novel bioactive compounds from *Clitoria ternatea*.

Materials and Methods

Chemicals and Reagents

Analytical-grade chemicals and reagents were used throughout the study. n-Hexane, chloroform, ethyl acetate, dimethyl sulfoxide (DMSO), anisaldehyde reagent, ferric chloride, and NP/PEG reagent were procured from standard commercial suppliers. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin solution, and MTT reagent were used for cell culture experiments. HT-29 human colorectal adenocarcinoma cells were obtained from the National Centre for Cell Science, India. Cell culture reagents were purchased from HiMedia Laboratories, while antibiotics were obtained from Sigma-Aldrich.

Plant Material and Preparation of Crude Extract

Fresh flowers of *Clitoria ternatea* L. were collected from the Botanical Garden of Sant Gajanan Maharaj College of Pharmacy, Mahagaon, Maharashtra, India. The plant material was authenticated by botanist from Shivraj College Gadhinglaj affiliated to Shivaji University, Kolhapur, Maharashtra, India. The flowers were washed thoroughly with distilled water to remove impurities and shade-dried followed by drying in a hot-air oven at 40°C until constant weight was achieved. The dried flowers were pulverized into a coarse powder.

The powdered material was extracted with ethanol using an ultrasonication-assisted extraction technique. The extract was filtered through Whatman No. 1 filter paper, concentrated under reduced pressure using a rotary evaporator at 40°C, and stored at 4°C until further use.

Activity-Guided Liquid–Liquid Fractionation

The concentrated ethanolic extract was subjected to solvent–solvent partitioning according to increasing solvent polarity. Initially, the crude ethanolic extract was reconstituted in distilled water. Sequential extraction was carried out using n-Hexane, Chloroform and Ethyl acetate.[25]

Each solvent extraction was repeated three times to ensure complete partitioning of phytoconstituents. All fractions were collected separately and concentrated under reduced pressure using a rotary evaporator. The remaining aqueous layer was retained as the residual aqueous fraction.

The percentage yield of each fraction was calculated using the following equation:

Percentage Yield (%) = Weight of dried fraction ÷ Weight of crude ethanolic extract × 100

The obtained fractions were designated as: HF – Hexane Fraction; CF – Chloroform Fraction; EAF – Ethyl Acetate Fraction. The results were summarized in table 1 & figure 1.

Preliminary Phytochemical Screening

Qualitative phytochemical analysis of HF, CF, and EAF was performed using standard phytochemical procedures. The fractions were screened for: Alkaloids, Flavonoids, Phenolic compounds, Tannins, Anthocyanins, Steroids, Triterpenoids, Glycosides and Saponins. Phytochemical screening was performed according to standard pharmacogenetic procedures.[26]

The intensity of each phytochemical was expressed as abundant (+++), moderate (++), trace (+), or absent (−) in the table No 2.

Thin Layer Chromatography (TLC) Fingerprinting

Thin-layer chromatography (TLC) was performed on pre-coated silica gel 60 F_{254} aluminium plates to obtain the chromatographic fingerprint of the solvent fractions. The plates were activated at 100–110°C for 30 min before use. Each fraction was dissolved in its respective solvent, and aliquots were applied as spot using a capillary tube. The chromatograms were developed in saturated TLC chambers using optimized mobile phase systems selected according to the polarity of the compounds present in each fraction.[27]

Different mobile phases were employed to achieve optimum separation:

- Hexane fraction (HF): Hexane : Ethyl acetate (9:1, v/v)
- Chloroform fraction (CF): Chloroform : Methanol (9.5:0.5, v/v)
- Ethyl acetate fraction (EAF): Ethyl acetate : Methanol : Water (8:1:1, v/v/v)

After development, the plates were air-dried and visualized under UV light (254 nm) and after derivatization using anisaldehyde, ferric chloride, and NP/PEG reagents. The retention factor (Rf) values and the number of spots were recorded in the table no 3 & figure no 2, 3 & 4.

The retention factor (Rf) values were calculated using:

Rf=Distance travelled by compound / Distance travelled by solvent front

The chromatographic fingerprints were compared among fractions, and the EAF was further compared with an authentic quercetin standard.

In Vitro Cytotoxic Activity (MTT Assay)

The cytotoxic activity of the fractions was evaluated using the MTT assay, a colorimetric method based on the reduction of tetrazolium salt to purple formazan crystals by metabolically active cells.[28,29] The anticancer activity of the fractions was evaluated against HT-29 human colorectal adenocarcinoma cells using the MTT colorimetric assay. HT-29 cells were maintained

in DMEM supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Approximately 5×10^3 cells were seeded into each well of a sterile 96-well microplate and allowed to attach for 24 h. The cells were then treated with different concentrations (1–1000 μg/mL) of HF, CF and EAF.

Untreated cells served as the negative control, whereas doxorubicin served as the positive control. Following 24 h of incubation, MTT solution was added to each well and incubated for an additional 4 h. The resulting purple formazan crystals were dissolved using DMSO, and absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader. Percentage cell viability was calculated using the equation:

$$\% \text{ Cell\ Viability} = A_{\text{sample}} / A_{\text{control}} \times 100$$

IC_{50} values were determined using nonlinear regression analysis, Dose–response curves were generated and phase-contrast photomicrographs were taken and represented in table no 4, figure no 5& 6 respectively .

Results and Discussion:

Percentage Yield of Solvent Fractions

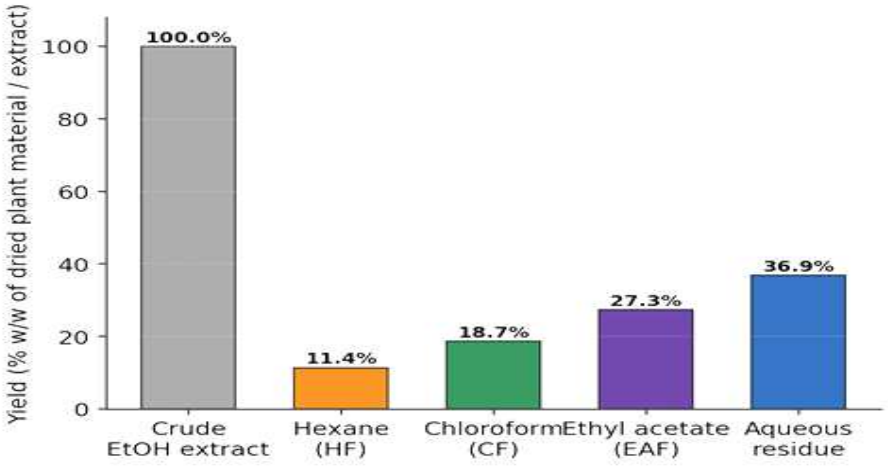
The crude ethanolic extract of *Clitoria ternatea* flowers was successfully fractionated using solvents of increasing polarity through liquid–liquid partitioning. Four fractions, namely hexane (HF), chloroform (CF), ethyl acetate (EAF), and the residual aqueous fraction, were obtained.

The percentage yields of the fractions are presented in table 1. The aqueous residue showed the highest recovery (36.9% w/w), followed by the ethyl acetate fraction (27.3% w/w), chloroform fraction (18.7% w/w), and hexane fraction (11.4% w/w). The relatively higher yield of the ethyl acetate fraction suggests that a considerable proportion of phytoconstituents present in *C. ternatea* flowers possess intermediate polarity.

Table 1. Percentage yield of solvent fractions obtained from the ethanolic extract of *Clitoria ternatea* flowers.

Fraction	Appearance	Yield (% w/w)
Crude ethanolic extract	Dark green-blue semisolid	100.0
Hexane fraction (HF)	Greenish waxy residue	11.4
Chloroform fraction (CF)	Dark green residue	18.7
Ethyl acetate fraction (EAF)	Brownish-green residue	27.3
Aqueous residue	Bluish aqueous mass	36.9

Figure 1. Percentage yield of the crude extract and solvent fractions.



Qualitative Phytochemical Screening

The qualitative phytochemical analysis revealed distinct chemical profiles among the three solvent fractions (Table 2). The hexane fraction was predominantly composed of non-polar constituents such as triterpenoids and steroids. The chloroform fraction contained moderate amounts of flavonoids, phenolics, alkaloids, and glycosides. In contrast, the ethyl acetate fraction exhibited abundant flavonoids, anthocyanins, phenolic compounds, glycosides, and saponins.

These findings indicate that solvent polarity plays an important role in the selective enrichment of phytochemicals, with ethyl acetate proving to be the most suitable solvent for concentrating flavonoid-rich compounds.

Table 2. Qualitative phytochemical profile of solvent fractions.

Sr. No	Phytoconstituent	HF	CF	EAF
1.	Triterpenoids	+++	++	–
2.	Steroids	+++	+	–
3.	Alkaloids	–	+	++
4.	Flavonoids	–	++	+++
5.	Anthocyanins	–	–	+++
6.	Phenolics/Tannins	–	+	+++
7.	Glycosides	–	+	++
8.	Saponins	–	–	++

(Note: +++: abundant; ++: moderate; +: trace; −: absent)

Thin Layer Chromatographic Fingerprinting:

Thin-layer chromatography was performed to characterize the phytochemical composition of each fraction. The hexane fraction produced two chromatographic bands with Rf values of 0.61 and 0.82, corresponding to non-polar constituents. The chloroform fraction produced three bands with Rf values of 0.30, 0.48, and 0.74, indicating moderately polar phytoconstituents. The ethyl acetate fraction exhibited the most complex chromatographic profile, producing five distinct bands with Rf values of 0.31, 0.41, 0.53, 0.63, and 0.74. The greater number of bands suggests that this fraction contains a diverse mixture of phytochemicals.

Visualization using NP/PEG reagent produced bright yellow, orange, and green fluorescent bands, confirming the presence of flavonoids.

Figure 2. TLC fingerprint (visible light)

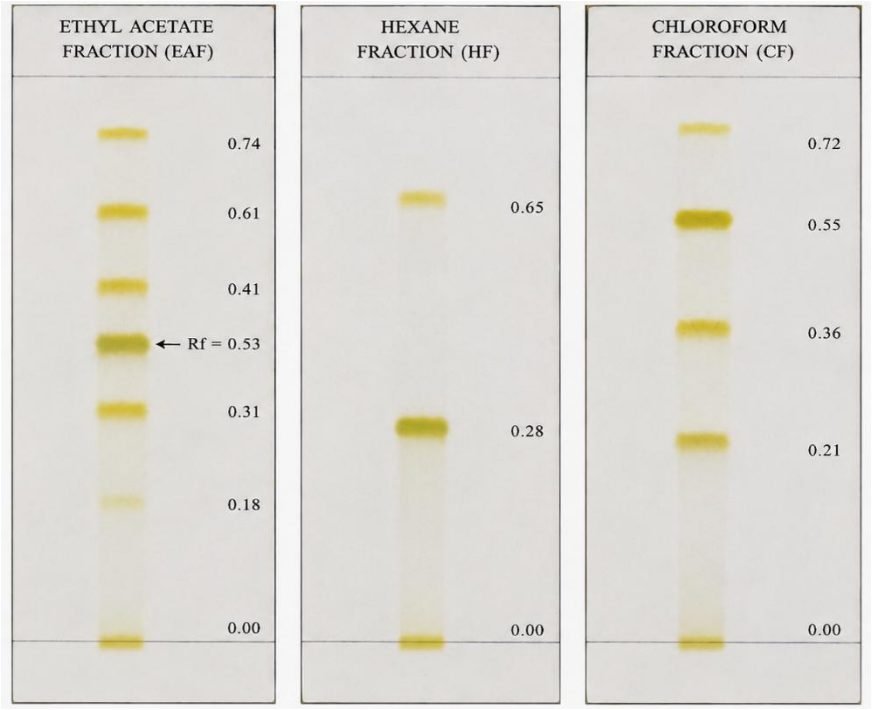


Figure 3. TLC fingerprint of the fractions visualized under UV 254 nm.

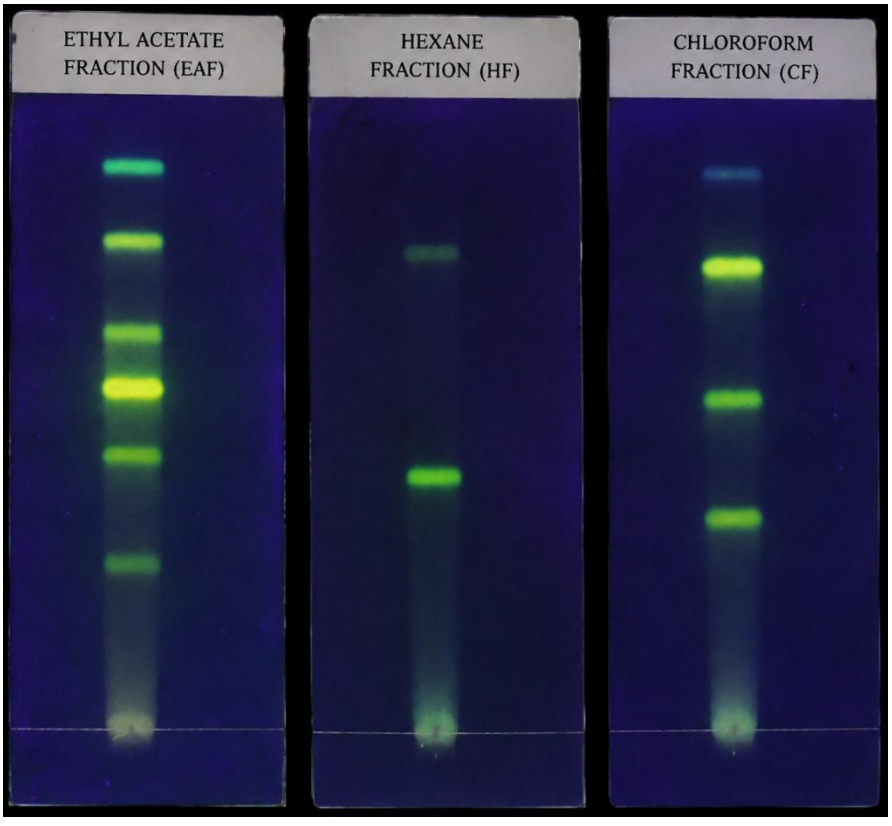


Figure 4. TLC comparison of the ethyl acetate fraction (EAF) with an authentic quercetin marker; the EAF band at Rf ≈ 0.53 co-migrates with quercetin.

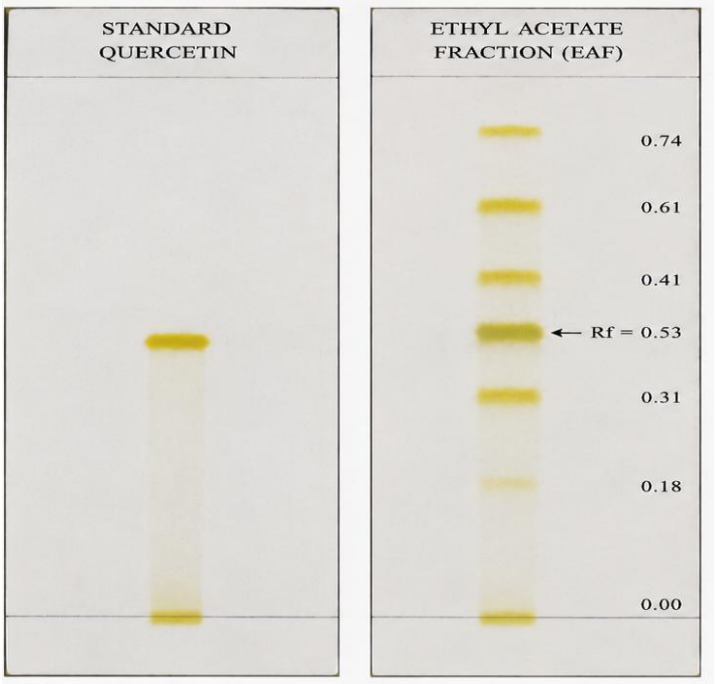


Table 3. Rf values of the principal bands resolved in each fraction.

Sr. No.	**Fraction**	**Number of Bands**	**Rf Values**	**Interpretation**
1.	HF	2	0.61, 0.82	Non-polar
2.	CF	3	0.30, 0.48, 0.74	Moderately polar compounds
3.	EAF	5	0.31, 0.41, 0.53, 0.63, 0.74	Flavonoid- and phenolic-rich fraction

In Vitro Cytotoxic Activity Against HT-29 Cells

The cytotoxic activity of the solvent fractions was evaluated using the MTT assay against HT-29 human colorectal adenocarcinoma cells. The dose–response curves demonstrated concentration-dependent inhibition of cell viability for all fractions.

Among the tested fractions, the ethyl acetate fraction exhibited the greatest cytotoxic activity, with an IC_{50} value of 79.29 µg/mL**,** followed by the hexane fraction **(**99.40 µg/mL**)** and the chloroform fraction **(**169.0 µg/mL**).** Doxorubicin, used as the positive control, exhibited an IC_{50} value of 71.33 µg/mL**.** The lower IC_{50} value observed for the ethyl acetate fraction indicates its superior anticancer potency, which may be attributed to its enrichment in flavonoids and phenolic compounds.

Table 4. Cytotoxic activity of solvent fractions against HT-29 cells.

Sr. No.	**Sample**	**IC_{50} (µg/mL)**	**R^2**	**Potency Rank**
1.	Doxorubicin	71.33	0.993	Reference
2.	Ethyl acetate fraction	79.29	0.932	1
3.	Hexane fraction	99.40	0.947	2
4.	Chloroform fraction	169.00	0.803	3

Morphological Changes in HT-29 Cells

Microscopic examination revealed distinct morphological alterations following treatment with the solvent fractions. Untreated HT-29 cells exhibited normal epithelial morphology with intact cell membranes and strong adherence to the culture surface.

Treatment with the hexane fraction resulted in moderate cell shrinkage and reduced cellular density. The chloroform fraction induced noticeable cellular deformation, although several viable cells remained attached.

The ethyl acetate fraction produced the most pronounced cytotoxic effects, characterized by marked cell shrinkage, membrane blebbing, loss of adherence, rounding of cells, and a significant reduction in cell population. These observations are consistent with apoptotic cell death and correlate well with the IC_{50} values obtained from the MTT assay.

Figure 5. Dose–response curves showing the cytotoxic activity of the fractions against HT-29 cells.

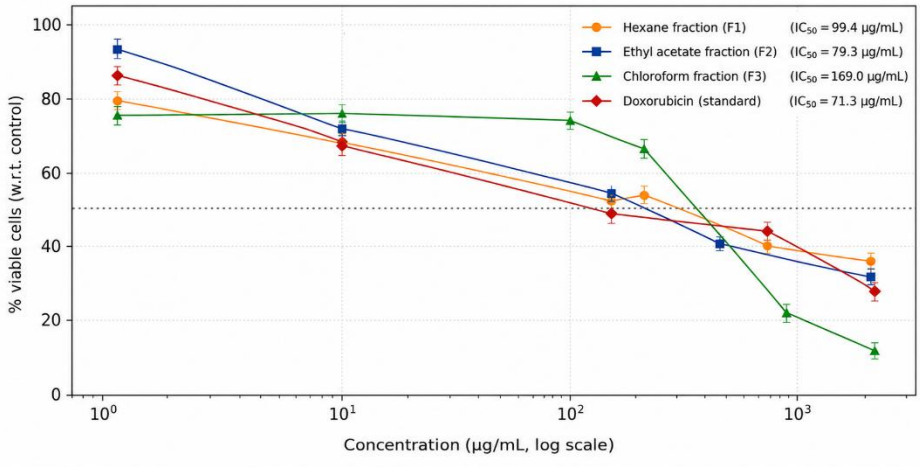
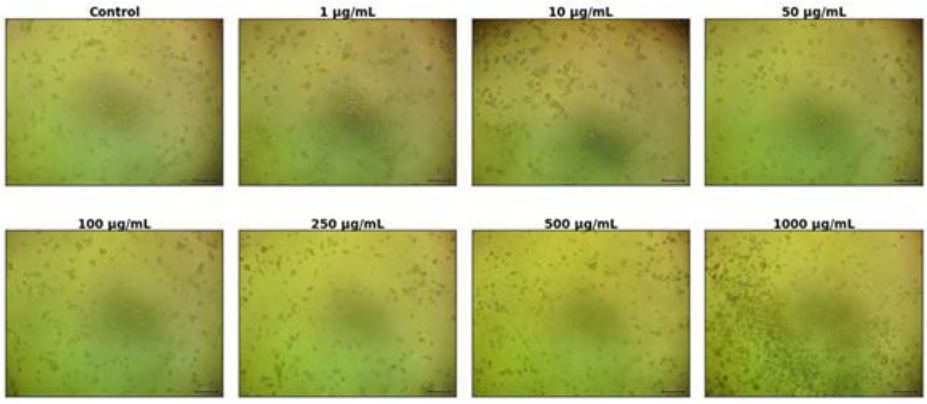
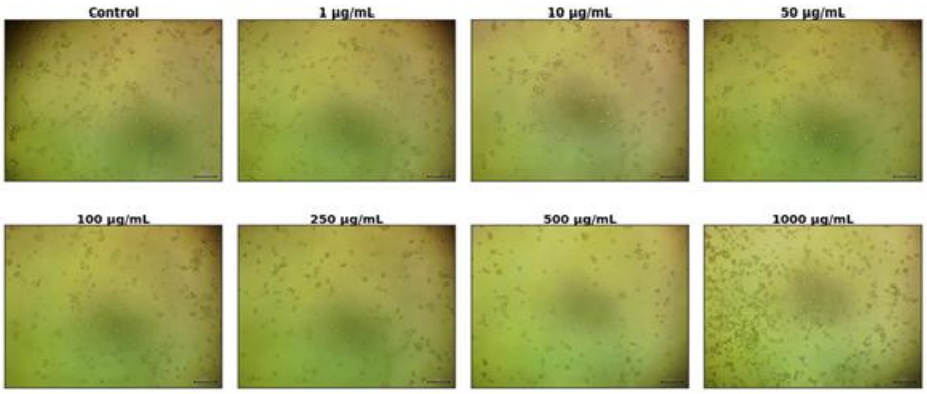


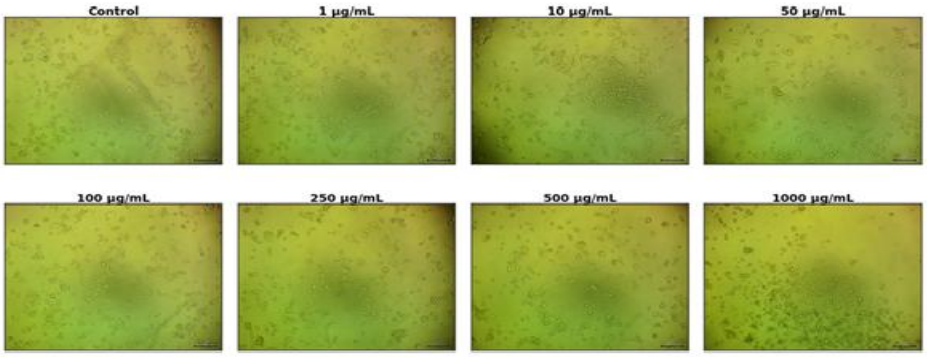
Figure 6. Representative phase-contrast photomicrographs of HT-29 cells treated with (A) hexane fraction, (B) ethyl acetate fraction, and (C) chloroform fraction.



(A)



(B)



(C)

Interpretation of the Results

The findings demonstrate that activity-guided fractionation effectively concentrated the anticancer phytochemicals of *Clitoria ternatea*. Among all solvent fractions, the ethyl acetate fraction displayed:

- The richest phytochemical profile.
- The highest number of TLC bands.
- A chromatographic band corresponding to quercetin.
- The lowest IC_{50} value against HT-29 cells.

- The most pronounced morphological evidence of apoptosis.

Collectively, these results indicate that the ethyl acetate fraction contains the major bioactive constituents responsible for the anticancer activity of *Clitoria ternatea* flowers and represents the most promising fraction for further purification and structural characterization.

Conclusion:

The present study successfully demonstrated that activity-guided fractionation is an effective strategy for enriching the anticancer phytoconstituents of *Clitoria ternatea* flowers. Sequential liquid–liquid partitioning of the crude ethanolic extract produced fractions with distinct phytochemical profiles and biological activities. Among the tested fractions, the ethyl acetate fraction exhibited the highest abundance of flavonoids, anthocyanins, and phenolic compounds, as confirmed by qualitative phytochemical screening and TLC fingerprinting.

The ethyl acetate fraction demonstrated the strongest cytotoxic activity against HT-29 human colorectal adenocarcinoma cells, with an IC_{50} value of 79.29 µg/mL. TLC analysis suggested the probable presence of a quercetin-type flavonoid, which may contribute to the observed anticancer activity. Morphological changes in treated HT-29 cells further supported the cytotoxic effects of the ethyl acetate fraction.

Overall, these findings indicate that the ethyl acetate fraction is the most promising source of bioactive phytoconstituents responsible for the anticancer activity of *Clitoria ternatea*. The study provides scientific evidence supporting the medicinal value of *Clitoria ternatea* flowers and establishes a foundation for the isolation, structural characterization, and mechanistic evaluation of individual bioactive compounds. Future investigations involving chromatographic purification, spectroscopic characterization, molecular mechanism studies, and

in vivo validation are warranted to facilitate the development of novel plant-derived anticancer agents.

Abbreviations:

CT, *Clitoria ternatea*; HF, hexane fraction; CF, chloroform fraction; EAF, ethyl acetate fraction; TLC, thin-layer chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; IC_{50}, half-maximal inhibitory concentration; SEM, standard error of the mean; ROS, reactive oxygen species; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography–tandem mass spectrometry; NMR, nuclear magnetic resonance.

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Conflict of Interest:

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Patient Consent:

Not applicable because it is *in vitro* activity

Ethical Approval:

This study involved only in vitro experiments using established human cancer cell lines and did not involve human participants or experimental animals. Therefore, institutional ethical approval was not required.

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