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Research Article

Bioactivity-guided isolation and quantification of Chlorogenic acid from *Calystegia silvatica* (Kit.) Griseb. (Convolvulaceae)

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Abstract: This study aimed to investigate the bioactivity-guided isolation and quantification of chlorogenic acid from *Calystegia silvatica*, a plant traditionally utilized for its medicinal properties. The most cytotoxic aqueous sub-extract was subjected to open column chromatography to assess its cytotoxic activity against MCF-7 breast cancer cells using a resazurin reduction assay. The structure of the most active pure compound was determined to be chlorogenic acid (IC_{50}= 36.44 ± 2.18 µg/mL) using 1D and 2D NMR spectroscopy. A validated high-performance liquid chromatography-diode array detector (HPLC-DAD) method was employed for the quantification of chlorogenic acid, which resulted in a content of 10.05 mg/g crude extract. The results indicate the potential of *Calystegia silvatica* as a source of chlorogenic acid with cytotoxic activity.

1. INTRODUCTION

The use of medicinal plants in drug discovery has gained significant attention because of their rich bioactive and phytochemical compounds that have the potential to be used in the development of drugs for various diseases. These natural products have been recognized as important sources for the development of new pharmaceuticals and have traditionally been used in indigenous medicine. In this context, the Convolvulaceae family is an important group in drug research. *Calystegia silvatica*, also known as "large bindweed," is a medicinal plant belonging to the Convolvulaceae family that is traditionally used in Turkish folk medicine to alleviate knee pain (Sener *et al*., 2023). Although there has been limited phytochemical analysis of *C. silvatica*, various compounds such as fatty acids, phenols, sterols, ketones, hydrocarbons, and other organic compounds and volatile compounds in its essential oil were identified by gas chromatography–mass spectrometry (GC-MS) studies (Derbak *et al.*, 2023; Youssef *et al.*, 2023). Its sub-extracts exhibit cytotoxic effects against various cancer cell lines, with the stem

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extract showing moderate antitumor properties against cervical, prostate, and breast cancer cell lines, whereas the leaf extract exhibits weak antitumor properties (Youssef *et al*., 2023).

Cytotoxicity research is crucial for drug discovery, particularly because of the limitations of conventional chemotherapeutic agents in terms of their efficacy, side effects, and resistance. Preliminary investigations are essential to evaluate the potential of plant-derived compounds as alternative therapeutic options. In the context of breast cancer, which is the most common cancer in women, assessing the cytotoxic activity of plant extracts on various types of breast cancer cells could provide insights (Chen *et al*., 2018). In this study, we targeted the MCF-7 breast cancer cell line and conducted a bioactivity-guided isolation study to identify the cytotoxic effects. Chlorogenic acid, a significant secondary metabolite, was isolated and quantified by HPLC-DAD. We used a resazurin reduction assay to assess cytotoxic activity in the MCF-7 mammalian breast cancer cell line. Chlorogenic acid showed potential as an effective cytotoxic agent against MCF-7 cells.

2. MATERIAL and METHODS

2.1. Bioactivity-Guided Isolation

2.1.1. *Plant material*

The aerial parts of *Calystegia silvatica* were collected from Bostancı village in Trabzon (at an altitude of 85 meters) on June 23, 2016. The voucher specimen was deposited at the Herbarium of Ankara University Faculty of Pharmacy in Ankara, Türkiye, and assigned the identification number "AEF 26802." The species was identified by Prof. Dr. Ufuk Özgen, a professor of Pharmacognosy.

2.1.2. *Preparation of extract and subfractions*

The aerial parts of *Calystegia silvatica* (CS) were air-dried and powdered (433 g), and then extracted three times with methanol (2 liters for 8 hours each time). The resulting combined extracts were evaporated using a rotary evaporator at 40°C to obtain a crude residue. The dry methanolic extract (37 g) was then suspended in a mixture of water and methanol (9:1), and partitioned with chloroform (300 mL × 2) and ethyl acetate (300 mL × 2) to obtain the remaining aqueous phase. The resulting fractions were then lyophilized to obtain dry extracts, yielding chloroform (9.5 g), ethyl acetate (1.42 g), and the remaining aqueous phase (26 g), which was tested for its antiproliferative activity on MCF-7 mammalian breast cancer cell line (ER+ and PR+).

2.1.3. *Bioactivity-guided isolation studies*

Bioactivity-guided isolation led to the lyophilized aqueous phase, which exhibited the most cytotoxic activity among all sub-extracts. Consequently, the residual aqueous phase was dissolved in water and processed through reverse-phase silica gel column chromatography. Gradient elution was carried out using a solvent system consisting of H_2O: CH_3OH solvent system (99:1 to 0:100) for purification. A total of 53 fractions were obtained. Thereafter, the fractions were subjected to TLC using a solvent system of EtOAc:CH_3OH:H_2O (7:2:1), and similar fractions were combined into 4 main fractions. The results of the antiproliferative activity on MCF-7 cells enabled the identification of combination fractions 36-39 for further purification. The fraction was dissolved in methanol and separated on a Sephadex column through isocratic elution with 100% methanol. 19 fractions were obtained, and 3 subfractions were combined using thin-layer chromatography under the same conditions. The most effective fraction for cytotoxic activity was chlorogenic acid (CHA), which was purified from the combined fractions 11-15 (Figure 1). The structure of chlorogenic acid was determined using Nuclear Magnetic Resonance (NMR) spectroscopy. The compounds were dissolved in deuterated methanol (CD_3OH), and NMR spectra were obtained using a Bruker Ascend 400 MHz NMR spectrometer.

2.2. HPLC Analysis

2.2.1. *HPLC conditions*

Quantitative analysis was conducted utilizing a newly validated High-Performance Liquid Chromatography (HPLC) method that employed a C18 column (4.6 × 150 mm, 5 µm) in conjunction with a gradient program consisting of a two-solvent system: A (100% methanol) and B (2.5% v/v acetic acid in deionized water, adjusted to pH 2.65). The solvents were delivered at a constant flow rate of 1.2 mL/min, and the analysis was monitored using a diode array detector that scanned wavelengths between 240 and 320 nm. The gradient program was as follows: 0.01 min 15% A, 85% B; 4 min 30% A, 70% B; 7 min 40% A, 60% B; 12 min 50% A, 50% B; 0% B; 20 min 0% A, 100% B.

2.2.2. *Method validation*

The method was validated for linearity, recovery, precision, and selectivity in accordance with ICH guidelines (Singh, 1996) Five calibration curves were used to evaluate linearity, which included five different concentrations of CHA (6.25-100 g/mL) with five repetitive data points. To obtain a linear regression equation and determine the correlation coefficients (Table 1), the peak areas were plotted against different CHA concentration ranges. To measure the recovery, the percentage concentration of CHA was analyzed in triplicate at three different concentrations. Recovery was calculated by dividing the percentage of known quantities by the mean and standard deviation. Precision was evaluated by measuring intra- and inter-day precision for three different concentrations of CHA, and the relative standard deviation (% RSD) of retention times and % peak areas were determined for two separate days. The selectivity of the method was assessed by comparing the chromatograms.

2.2.3. *Quantitative analysis*

A calibration curve was used to determine the CHA content of the CS main methanol extract. The new HPLC method was used to run the extract in triplicate to perform quantitative analysis. The results were expressed as µg/mL.

2.3. Cytotoxic Activity Test

The cytotoxic effect of all sub-extracts and fractions was evaluated by bioactivity-directed resazurin induction assay on MCF-7 breast cancer.

Resazurin Reduction Assay

This assay is based on the conversion of resazurin to resorufin within viable cells. Non-viable cells cannot show blue staining because they lack metabolic capacity, which prevents the reduction of resazurin. To conduct this assay, 0.5 x 10^4 adherent cells were allowed to attach overnight, and 1 x 10^4 suspension cells per well were seeded in 96-well plates with varying concentrations of the test substance to obtain a total volume of 200 µL/well. After a 72-hour incubation period and the addition of resazurin (Sigma-Aldrich) for 4 hours, staining was measured using a plate reader (Infinite 200 M Plex-Tecan, Türkiye) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was performed independently three times (Kuete *et al*., 2016). Doxorubicin HCl (Sigma-Aldrich) was used as a positive control.

3. FINDINGS

3.1. Bioactivity-Guided Isolation Studies

NMR data indicated that the isolated compound is chlorogenic acid (Figure 1).1H-NMR (CD_3OD, 400 MHz): δ 7.59 (d, *J* = 16.2 Hz, H-7'), 7.07 (bs, H-2'), 6.96 (d, *J* = 7.3 Hz, H-6'), 6.79 (d, *J* = 6.3 Hz, H-5'), 6.31 (d, *J* = 15.9 Hz, H-8'), 5.39 (t, *J* = 10.1 Hz, H-5), 4.13 (bs, H-3), 3.69 (d, *J* = 9.6 Hz, H-4), 2.27-2.07 (m, H-6), 1.94-2.04 (m, H-2) (Figure 2). ^{13}C-APT NMR (CD_3OD, 100 MHz): δ 179.57 (C-7), 167.81 (C-9'), 148.12 (C-4'), 145.46 (C-7'), 145, 40 (C-

3'), 126.42 (C-1'), 121.54 (C-6'), 115.10 (C-5'), 114.15 (C-2'), 113.70 (C-8'), 76.32 (C-1), 73.75 (C-4), 71.70 (C-5), 71.15 (C-3), 39.23 (C-6), 37.72 (C-2) (Figure 3). NMR data were confirmed in previous studies (Yang *et al*., 2015).

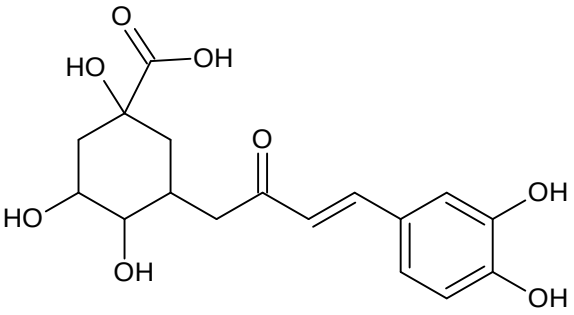

Figure 1. Molecular structure of chlorogenic acid.

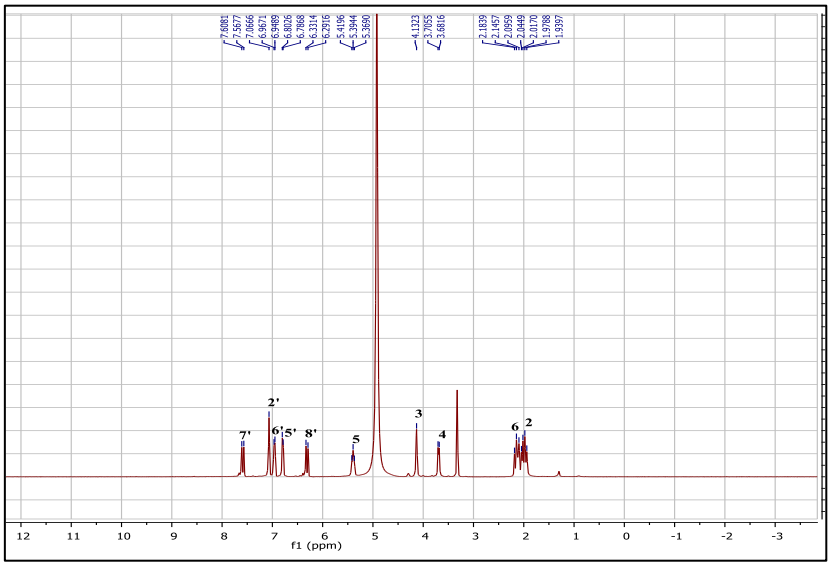

Figure 2. ^{1}H-NMR Spectrum of chlorogenic acid (CD_3OD, 400 MHz).

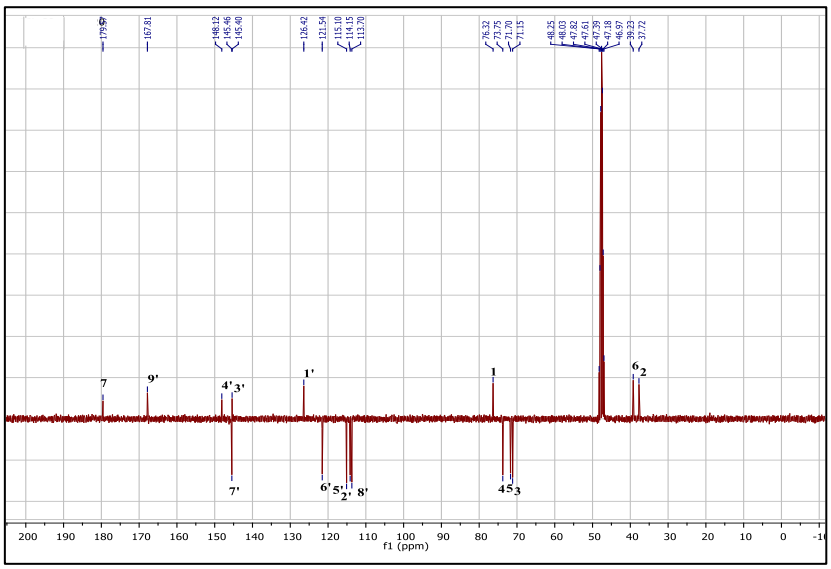

Figure 3. ^{13}C-APT NMR Spectrum of chlorogenic acid (CD_3OD, 100 MHz).

3.2. Results of HPLC Analysis

The different parameters of the new HPLC method for validation are shown in Table 1. The method showed good linearity (R^2>0.99) over the assayed concentration range (6.25-100 µg/mL) (Table 1, Figure 4). The relative standard deviation (RSD) values for intra- and inter-day precision were 0.20% and 0.83% for retention time and 0.33% and 0.35% for peak area, respectively. The accuracy for quality control varied from 98.77% to 101.78% (SD<0.81%) (see Table 1).

Table 1. Validation parameters of new HPLC method for detection of CHA in *Calystegia silvatica* methanol extract.

Retention time (min.)		Regression coefficient (R^2)	
3.23		0.9911	
Retention time (% RSD)		Peak area (% RSD)	
Intra-day	Inter-day	Intra-day	Inter-day
0.33	0.35	0.20	0.83
% Recovery (Mean ± SD)			
12.5 µg/mL	50 µg/mL	100 µg/mL	
101.78 ± 0.3010	98.77 ± 0.8177	100.83 ± 0.2013	

The quantitative analysis of CHA in the *C. silvatica* extract was performed based on the peak areas of the chromatograms of the sample using the calibration curve (Figure 3). Quantitative analysis by HPLC showed that the CHA content of the extract was expressed as mg/g crude extract (Figure 4). CHA content was detected as 10.05 mg/g crude extract.

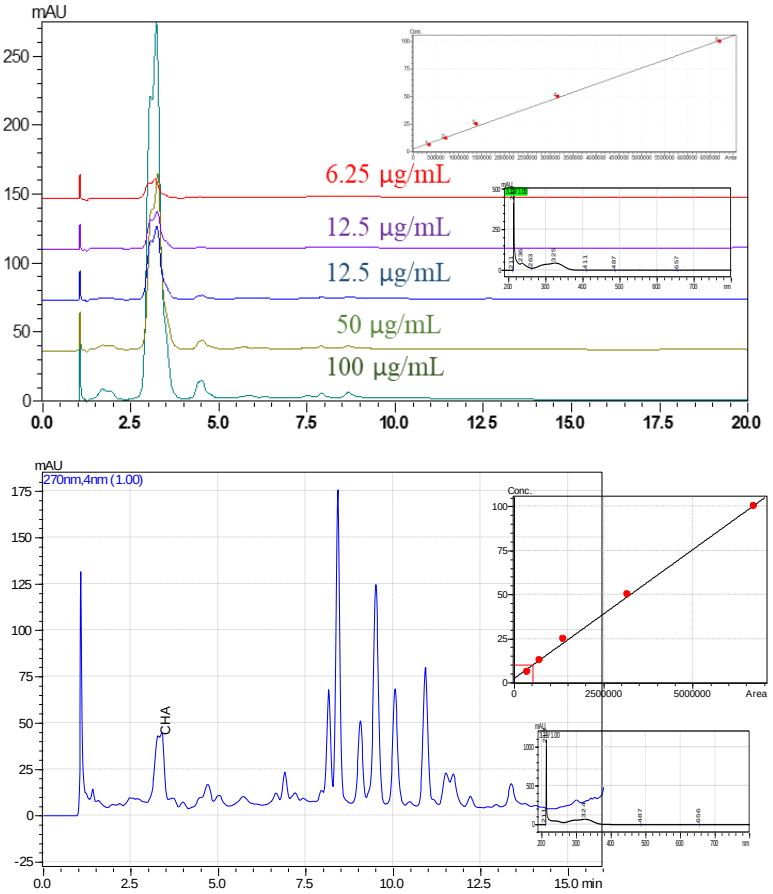

Figure 4. HPLC chromatograms and calibration curves for CHA, methanol extract of *Calystegia silvatica*.

3.3. Results of Cytotoxic Activity Test

The cytotoxic activity of the main methanol and sub-extracts of *Calystegia silvatica* was assessed based on IC_{50} values obtained through the resazurin method by measuring fluorescence. The main methanol extract displayed the lowest IC_{50} value of 124.70 ± 3.25 µg/mL, followed by the aqueous extract at 136.76 ± 5.36 µg/mL, while the ethyl acetate and chloroform extracts demonstrated higher IC_{50} values of 249.41 ± 2.58 µg/mL and 256.09 ± 3.98 µg/mL, respectively (Table 2). The aqueous extract was identified as the most active and suitable sub-extract for isolation studies. Subsequent fractionation of the active fractions obtained from column chromatography led to the isolation of chlorogenic acid compound with an IC_{50} value of 36.44 ± 2.18 µg/mL (Figure 5). The positive control doxorubicin demonstrated an IC_{50} value of 0.0522 ± 0.001 µg/mL.

Table 2. IC_{50} value of main methanol and sub-extracts of *Calystegia silvatica.*

	MeOH	*Aqueous*	*EtOAc*	*Chloroform*
IC_{50} (µg/mL)	124.70 ± 3.25	136.76 ±5.36	249.41 ± 2. 58	256.09 ± 3.98

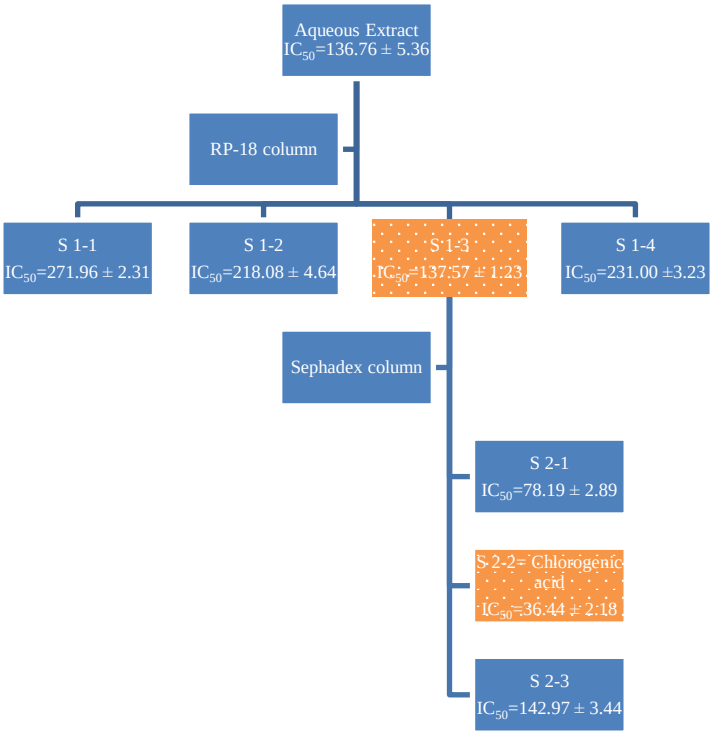

Figure 5. Bioactivity guided isolation scheme by using the cytotoxicity results on MCF-7 breast cancer cell line (IC_{50}: The concentration of a substance required to inhibit cell viability by 50%, µg/mL)

4. DISCUSSION and CONCLUSION

The study on the bioactivity-guided isolation and quantification of chlorogenic acid from *Calystegia silvatica* represents the first phytochemical isolation study on this plant, highlighting the presence of this important secondary metabolite. By focusing on the cytotoxic activity of the *C. silvatica* fractions against MCF-7 breast cancer cells and utilizing a bioactivity-guided isolation approach, the research sheds light on the bioactive potential of natural compounds for therapeutic applications. The quantification of chlorogenic acid using a validated HPLC-DAD method further strengthens the study's methodology and credibility.

MCF-7 breast cancer cells, known for their estrogen and progesterone receptor positivity (ER+ and PR+), are crucial in studying hormone-responsive breast cancer treatments. Previous studies have shown that the stem extract of *C. silvatica* exhibited an IC_{50} of 172 ± 15 µg/mL in

the same cancer cell line using the MTT method (Youssef *et al*., 2023). We evaluated the entire aerial part of the plant using the resazurin reduction assay, which has been demonstrated to be relatively inexpensive, sensitive, and non-invasive compared to tetrazolium assays (Petiti *et al.*, 2024). Our findings indicated that the main methanol extract of *C. silvatica* exhibited the lowest IC_{50} value of 124.70 ± 3.25 µg/mL, suggesting a potent inhibitory effect. Further investigation into the sub-fractions revealed that the aqueous extract was more active compared to the ethyl acetate and chloroform extracts, with IC_{50} values of 136.76 ± 5.36 µg/mL, 249.41 ± 2.58 µg/mL, and 256.09 ± 3.98 µg/mL, respectively. Consequently, our research focused on the aqueous extract for further isolation studies.

Subsequent fractionation of the active aqueous extract from column chromatography led to the isolation of chlorogenic acid, which showed a significantly lower IC_{50} value of 36.44 ± 2.18 µg/mL (102.9 ± 6.1 µM) according to the resazurin reduction assay. This high cytotoxic activity on MCF-7 breast cancer aligns with previous studies indicating its effectiveness in inducing apoptosis, DNA damage, reducing MMP levels, and activating the PKC signaling (Deka *et al.*, 2017; Suberu *et al.*, 2014). Chlorogenic acid has also been extensively studied for its various biological activities, including antioxidant properties (Sato *et al*., 2011), reducing cardiovascular disease and diabetes risk, and exhibiting antibacterial and anti-inflammatory effects (Farah & Lima, 2019). Considering its significant biological effects, quantification of this biologically active compound in the main methanol extract of *C. silvatica* was conducted using a newly developed and validated HPLC method. Notably, while green coffee is traditionally recognized as a primary source of chlorogenic acid (50-120 mg/g extract), our study demonstrates that *C. silvatica* also contains substantial levels of this compound (10.05 mg/g extract) (Ayelign & Sabally, 2013; Dado *et al.*, 2019; Farah *et al.*, 2008).

In conclusion, the research on chlorogenic acid from *C. silvatica* presents promising findings regarding its cytotoxic potential against breast cancer cells. Future studies could focus on the isolation and characterization of additional bioactive compounds from *C. silvatica*, as well as investigating the molecular mechanisms underlying its cytotoxic activity.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Merve Yüzbaşıoğlu Baran: Investigation, Methodology, Resources, Writing-Editing. **Sıla Özlem Şener:** Investigation, Resources, Methodology, Validation, Formal Analysis, Writing-original draft. **Şeyda Kanbolat:** Investigation, Methodology, Formal Analysis. **Merve Badem:** Investigation, Methodology, Formal Analysis. **Ufuk Özgen:** Resources, Methodology, Supervision, Writing-original draft.

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