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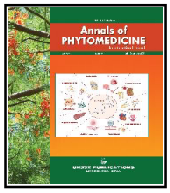

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Phytochemical screening and pharmacological benefits of *Parthenium hysterophorus* L.: *In vitro* anticancer cell line evaluation

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Abstract

This study explores the phytochemical profile and anticancer potential of vegetative aerial *Parthenium hysterophorus* L., (PHv) powder extracts using supercritical carbon dioxide extraction (SC-CO_2). Objectives include optimizing extraction, fractionating crude extracts, identifying parthenin, *viz*., TLC and HPLC, and evaluating test drug doses on MCF-7, HeLa, and HepG2 cancer cell lines. The extraction efficiencies ranged from 83.64% to 94.50%, with the highest yield (94.50%) achieved at 200 bar and 60°C. This yield was significantly higher than the Soxhlet extraction efficiency, which was 46.30%. Analysis of variance showed a significant effect of pressure levels on SC-CO_2 extraction efficiency ($F = 8040.23 > F_{crit} = 5.14$, $p < 0.01$). Phytochemical screening in ethanol fractions exposed a high presence of flavonoids, phenols, tannins, alkaloids, and terpenoids, particularly in T_3, and moderate levels in T_1, and T_0. Parthenin, identified in the ethanol fractions of PHv extract through TLC and validated by HPLC. The optimized ethanol fraction (T_3) test drug from PHv powder extract, demonstrated dose-dependent inhibition in MTT assays, with inhibition rates of 67.34% (MCF-7), 57.88% (HeLa), and 65.16% (HepG2) at 100 µg/ml concentrations. The quadratic regression model indicated that drug dose (A), cancer cell lines (B), and their interactions (AB) significantly influenced cell inhibition and viability, with $F_{8,18}$ value of 1805.39, $p<0.05$. The findings presented here bolster the case for PHv powder extracts potential as a naturally derived cancer fighting substance, highlighting the need for additional in vivo experiments and investigations into its mode of action.

1. Introduction

Plant flora has long been a vital resource for natural medicines and remedies, particularly in addressing chronic human diseases in the modern era. This is largely attributed to secondary metabolites (allelochemicals), novel chemotypes, and bioactive molecules that are essential for drug development (Chun and Hongjie, 2019; Kumar *et al*., 2013; Veeresham, 2012). Furthermore, advancements in medicinal and aromatic plants continue to support traditional healthcare in remote and rural areas, with the Ministry of AYUSH promoting the evidence-based integration of Indian medicinal plant-derived drugs into clinical practice to enhance healthcare (Máthé and Khan, 2022).

Chronic illnesses, such as cardiovascular, cancers, respiratory disorders, and diabetes, are the leading causes of mortality worldwide, responsible for nearly 70% of global deaths (Jiménez *et al*., 2021). Among these, cancer is a second leading cause of death, with its prevalence rising and approximately 9.6 million fatalities reported in 2018 (Sharma *et al*., 2015). In India, breast and cervical cancers are the most common among females, with recorded incidences of 7.13 lakhs (Flama Monteiro *et al*., 2024). Around 60% of anticancer drugs originate from plant sources, largely due to the challenges associated with existing treatments, like toxicity to healthy cells, the development of drug resistance from genetic mutations, high costs, and limited access to specific medications (Sharma *et al*., 2015; Kumar *et al*., 2013a). These challenges underscore the critical need for innovative, affordable, and more effective therapeutic strategies (Máthé and Khan, 2022; Sharma *et al*., 2015).

Parthenium hysterophorus L., (family: Asteraceae) is an invasive herb that thrives within harsh climates due to its potential secondary metabolites, particularly allelochemicals parthenin, phenols, flavonoid *etc*. (Patel, 2011; Kumar *et al*., 2013; Kumar *et al*., 2014). Sesquiterpene lactones (STLs), parthenin, found in its leaves, stems, and roots have various pharmacological, therapeutic, and ecological roles, including plant defense and cytotoxicity (Patel, 2011; Ahmad *et al*., 2018). This herb also contains elevated levels of secondary

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metabolites, including sesquiterpene lactones, *viz*., parthenin, with the highest concentrations found in the plant (Das *et al*., 2018; Rice *et al*., 2021). Furthermore, global research has revealed the exceptional nutritional composition of the plant, which is abundant in protein, carbohydrates, fats, and fiber (Begum *et al*., 2020; Mishra *et al*., 2012), highlighting its potential for various beneficial uses. The overall plant composition includes 17% terpenes, 32% fatty acids, 4% hydrocarbons, 1.5% alcohols, 4% phytosterols, and 12% miscellaneous metabolites (Ahmad *et al*., 2018).

The extracts of *P. hysterophorus*, obtained *via* hydrodistillation and soxhlet methods, exhibit notable anticancer activity against HeLa (Anup *et al*., 2015; Sharma *et al*, 2015), MCF-7 (Anup *et al*., 2015; Kumar *et al*., 2013a), PC-3 (Haq *et al*., 20111), A-498, MDA-MB-231(Panwar *et al*., 2015*)*, lung, colon, ovarian (Kumar *et al*., 2014), THP-1, and DU-145 cells (Kumar *et al*., 2013a). Furthermore, silver nanoparticles (AgNPs) synthesized from parthenium leaves show cytotoxicity against HeLa (Sivakumar *et al*., 2021), HepG2 (Ahsan *et al*., 2020; Sivakumar *et al*., 2021), and B16F10 cells (Ahsan *et al*., 2020) *in vitro*. Decoctions of *P. hysterophorus* have long been utilized in traditional medicine to treat various health conditions like fever, anemia, inflammatory skin disorders, neurological ailments, and female reproductive issues (Patel, 2011; Sharma *et al*, 2015).

Studies also reveal that *P. hysterophorus* extracts exert dose-dependent cytotoxic effects with increasing concentrations of leaf extract (Kumar *et al*., 2013; Anup *et al*., 2015; Malarkodi *et al*., 2024). Some researchers have reported the isolation of parthenin using thin layer chromatography (TLC), with a spot exhibiting an Rf value of 0.60 (Hernandez *et al*., 2011). Additionally, the majority of polyphenols, flavonoids, and parthenin compounds were detected at lower Rf values on TLC plate (Basarkar and Saoji, 2013; Kumar *et al*., 2013; Jaiswal *et al*., 2022).

Supercritical carbon dioxide (SC-CO_2) extraction, which employs CO_2 in its supercritical state, offers an efficient and environmentally friendly method for obtaining bioactive compounds. This method enables the targeted extraction of biologically active compounds by exploiting the adjustable solubility of carbon dioxide under different temperature and pressure conditions. The process is non-toxic, produces pure extracts, and has minimal environmental impact, making it widely applicable in the food, pharmaceutical, and cosmetic industries (Bezerra *et al*., 2020; Lorigooini *et al*., 2020).

Research on SC-CO_2 extraction and the isolation of bioactive compounds from *P. hysterophorus* remains limited, despite its potential applications. Furthermore, investigations into the plant's biological activities, such as phytochemical profiling and anticancer effects, are also lacking. This research is meant to explore the optimization of *P. hysterophorus* extraction efficiency using the SC-CO_2 technique at pressures of 100, 150, as well as 200 bar at temperatures of 60°C. The study further includes the fractionation of crude extracts using solvents ranging from non-polar to polar, phytochemical screening, and the evaluation of selected parthenin-based extracts for their potential anticancer activity.

2. Materials and Methods

2.1 Collection of vegetative stage *P. hysterophorus*

P. hysterophorus (PHv), which were at fresh vegetative stages have been collected from different places around local waste and barren lands (16.2160° N, 77.3566° E). The authentication of plant was done by the from the Department of Horticulture, Agriculture College, Aswaraopet (ACA/PJTSAU/HORT/Weed/Parthenium/03). The plants were washed with tap water, roots were removed, along the remaining parts were chopped. These parts dried using a dehumidified air dryer at 40°C and 15% relative humidity, then ground into a fine powder and passed through a 250-micron sieve. This powder is stored in a freezer for subsequent extraction and analysis. The chemicals and reagents of analytical grade including the required standards were obtained from M/s. Sigma Aldrich Chemicals, Hyderabad.

Figure 1: Fresh *P. hysterophorus*.

2.2 Experimental setup for supercritical carbon dioxide (SC-CO_2) extraction of vegetative stage *P. hysterophorus* powder

Supercritical carbon dioxide (SC-CO_2) extraction is performed by utilizing the Waters Thar SFE 500 system with CO_2 as the primary solvent at a flow rate of 2 g/min and ethanol as a co-solvent at a flow rate of 20 g/min. The extraction process was conducted under varying pressure conditions set at 100, 150, and 200 bars.

Table 1: Treatment combinations for SC-CO_2 extraction of vegetative stage *P. hysterophorus* powder

Treatments	Temperature, °C	Pressure, bar
T_1	60	100
T_2	60	150
T_3	60	200
T_0 (Soxhlet extraction)	85	120 min

The choice of 100 bar, slightly above the critical pressure of CO_2 (73 bar), was founded on previous studies (Liza *et al*., 2010; Wang *et al*., 2008). To prevent the potential degradation of heat-sensitive compounds, the maximum extraction temperature was limited to 60°C, as specified in the study by Cossuta *et al*. (2008). The extraction time was kept constant at 120 min for all trials.

2.2.1 Soxhlet extraction

Bioactive compounds were extracted from PHv powder employing the soxhlet extraction technique with a SOCS-PLUS device (Pelican equipment; SCS-08), employing ethanol as the solvent. A precise amount of 100 g of PHv powder was placed in the extractor's thimble within the sample compartment. This compartment is connected to a 500 ml round bottom flask containing 300-350 ml of ethanol. The extraction process has conducted for 120 min. At 85°C, the remaining solvent was detached utilizing a rotary flash vacuum evaporator (Superfit, Rotavap; PBU-6D) (Martinez *et al*., 2010).

2.2.2 Extraction efficiency of SC-CO_2 unit

The withdrawal efficiency of bioactive compounds from PHv powder using an SC-CO_2 unit was evaluated to assess its performance. The method outlined by Olawale *et al*. (2012) was employed for regulating the extraction efficiency. It is the ratio of the quantity of extract recovered during extraction to the original amount of extract present in powder, V (mg/ml) in below equation:

$$\text{Extraction efficiency, \%} = \frac{\text{Quantity of extract recovered during extraction}}{\text{Quantity of extract recovered during extraction}} \times 100$$

2.3 Phytochemical screening of solvent fractionated vegetative stage *P. hysterophorus* powder extracts

Standard procedures, with slight modifications as described by Jaiswal *et al*. (2022), were employed to qualitatively analyze plant molecules such as phenols, flavonoids, alkaloids, tannins, terpenoids, and saponins present in n-hexane, chloroform, and ethanol fractions of PHv powder extracts.

2.4 Isolation of parthenin from *P. hysterophorus* leaf

A quantity of 100 g of powdered material, dried in the shade, was soaked in 600 ml of methanol for 24 h. The resulting extract has been summarized to approximately half its initial volume under low pressure. An equivalent amount of water has been introduced. Following filtration, the solution underwent three extractions using 60 ml of chloroform each time. The chloroform extract was then evaporated under reduced pressure, yielding 5.1 g of a yellowish, oily substance. This concentrated chloroform fraction was then subjected to column chromatography using a silica gel column (50 cm × 2.5 cm) and eluted with a 3:1 mixture of dichloromethane and methanol. Fractions of 3 ml was collected and analyzed *via* TLC on Merck's silica gel GF 60 R plates, using 3:1 chloroform: ethanol mixture as the mobile phase. The spots as identified under UV light at 254 nm and after applying vanillin reagent (5 g/l in a 4:1 mixture of sulphuric acid and anhydrous ethanol). Fractions containing parthenin, identifiable by a violet zone with an Rf value of approximately 0.6, were gathered. Evaporation under reduced pressure produced 1.3 g of an oily substance, which was then dissolved in a minimal amount of ethanol (about 1 ml). Upon adding 5 ml of ether: n-hexane mixture (1:4), the solution was refrigerated for 24 h. The resulting solid is recrystallized utilizing a minimal quantity of acetone, with n-hexane added dropwise until turbidity appears (Hernandez *et al*., 2011). Figure 2 illustrates the detailed isolation process.

2.5 Thin layer chromatography (TLC) based assessment of parthenin in solvent fractionated vegetative stage *P. hysterophorus* extracts

Aluminum plates that had been precoated (silica gel 60, Merck, 0.2 mm thickness) are utilized for thin-layer chromatography. After being dried and activated for an h at 110°C, the plates were left to cool. Solvent fractions of vegetative stage *P. hysterophorus* powder extracts obtained through supercritical extraction (n-hexane, chloroform, and ethanol) were labeled as T_1 to T_3, T_0 (control) dissolved in their respective solvents, and applied as spots using fine capillary tubes. After optimizing the conditions, a mobile phase comprising a mixture of chloroform and ethanol in a 3:1 ratio was selected. The chromatogram showed the phytochemicals in the extracts as eluted marks, and equation was used to get the retention factor (Rf value). The marks were visible to the naked eye upon direct observation. The developed chromatogram was analyzed under a UV lamp at 254 nm and treated with vanillin reagent to identify purple bands indicative of parthenin content. The solvent fraction extracts exhibited a lower diversity of phytochemicals (Hernandez *et al*., 2011). The formula was used to determine the Rf value:

$$\text{Rf} = \frac{\text{Distance traveled by solute from origin}}{\text{Distance traveled by solvent from the origin}}$$

2.6 Quantification of parthenin in the isolated and solvent fractionate of vegetative stage *P. hysterophorus* powder extract using high-performance liquid chromatography (HPLC)

The parthenin sample was analyzed by using HPLC with a Li Chrospher 100 RP-185 m column (250 mm × 4.6 mm). The chromatogram was obtained using a gradient elution method with water (W) and acetonitrile (A) as mobile phases. The flow rate was set at 1.0 ml/min, and the gradient composition that is maintained at 60:40 (W:A) for 15 min.

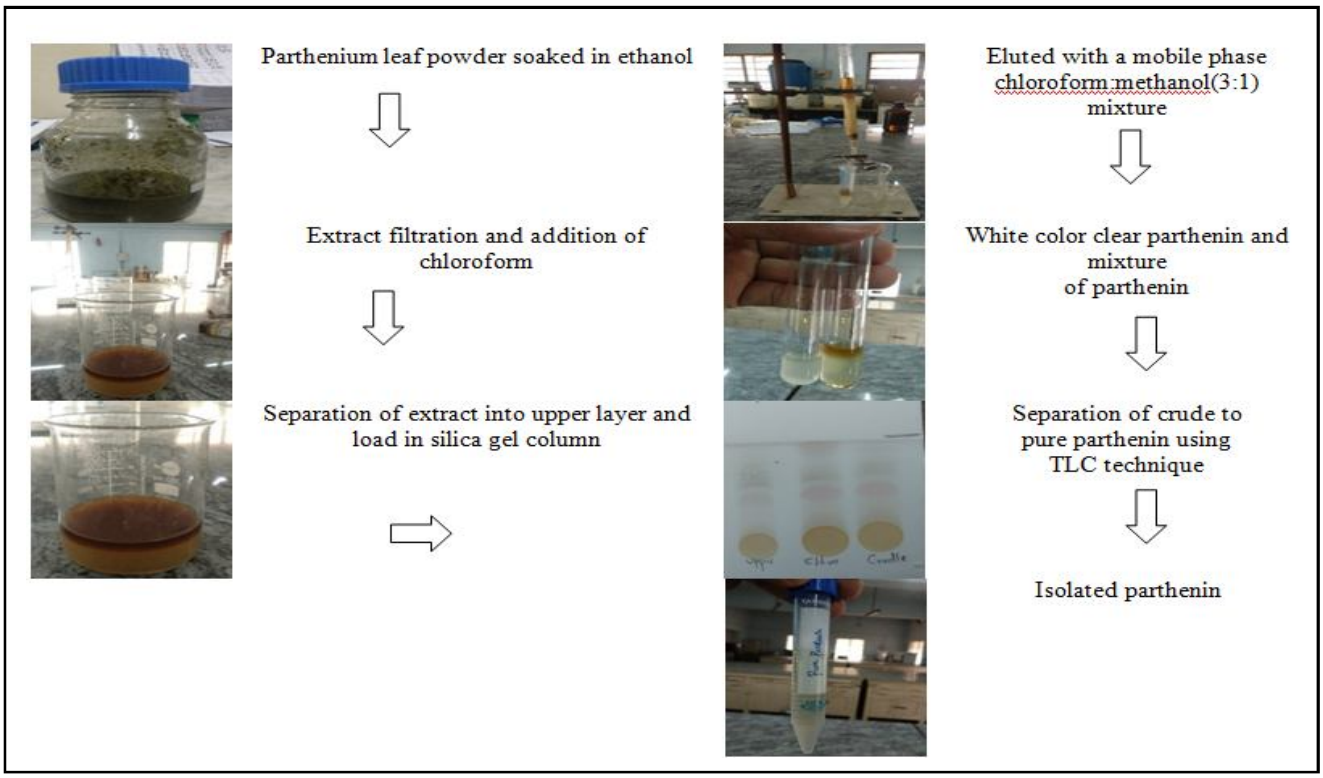

Figure 2: Extraction and isolation of parthenin from *P. hysterophorous* leaves by TLC.

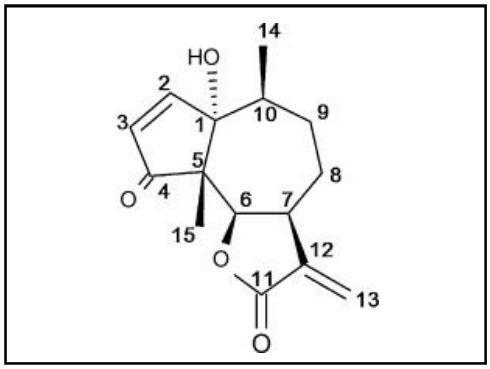

Figure 3: Chemical structure of parthenin.

2.6.1 Reference sample

In a 2.0 ml measuring flask, 0.2 mg of parthenin sample was precisely weighed and dissolved using ethanol. Subsequently, 120 µl of this prepared stock solution is pipetted into another 2.0 ml measuring flask and further diluted with a mixture of acetonitrile and water in a 3:1 ratio.

2.6.2 Test sample

The test drug fractionate from PHv powder extract and isolated parthenin, weighing 0.2 mg, was dissolved in ethanol and placed in a 20.0 ml measuring flask, which was then filled. The solution was further diluted by taking 1.0 ml and bringing it to a total volume of 10.0 ml using a mobile phase. This mobile phase was composed of water and acetonitrile mixed in a 3:1 proportion. The resultant mixture was filtered *via* a membrane with a 0.45 µm pore size before HPLC injection.

2.6.3 Chromatographic setting

The parthenin and test drug sample solutions were introduced into the system in 10 µl volumes and separated using a gradient elution method. The mobile phase contained of water (W) as well as acetonitrile (A), flowing at a rate of 1.0 ml/min. The gradient program is as follows: initial conditions of 60:40 (W: A) were maintained for 10 min, followed by a shift to 0:100 at 13 min, which was held until 15 min. The system then returned to the initial conditions of 60:40 by 18 min and was equilibrated until 20 min. Compound detection was performed at a wavelength of 250 nm (Hernandez *et al*., 2011).

2.7 Evaluation of *in vitro* anticancer activity using solvent fractionated vegetative stage *P. hysterophorus* powder extract test drug concentrations

Three cancer cell lines - MCF-7 (breast), HeLa (cervical), and HepG2 (liver) - are cultured in appropriate media (DMEM and RPMI-1640) enriched with 10% FBS and 1% antibiotic solution. Cell cultivation occurred in a Heraeus CO_2 incubator at 37°C, 90% humidity, and 5% CO_2. For experimentation, cells remained seeded in triplicate at 3 × 10^4/well in 96-well plates and incubated for 24 h. The cells are then treated with test drug fractionated SO-CO_2 extract of PHv powder at concentrations of 25, 50, and 100 µg/ml for 24 and 48 h post-treatment, the medium is replaced with fresh medium containing 100 µl MTT (5 mg/ml in PBS, pH 7.4) and incubated for 4 h at 37°C in darkness. The subsequent formazan crystals have been solubilized with 150 µl DMSO per well. Absorbance has measured at 570 nm employed by a Spectramax microplate reader. Control groups consisted of untreated cells (Ahsan *et al*., 2020; Kumar *et al*., 2013).

The percentage of cell viability was determined using the formula:

$$\text{Cell viability (\%)} = (A_t - A_b)/(A_c - A_b) \times 100$$

The "A_t" represents absorbance value of test compound; "A_b" represents absorbance value of blank and "A_c" represents absorbance value of control.

The percentage of cell inhibition was determined using the formula:

$$\text{Cell inhibition (\%)} = (100 - \text{\% cell viability}) \times 100$$

2.8 Statistical analysis

Three triplicates of each experiment were carried out, and the outcomes were examined using MS Excel at a significance level of $p<0.01$. Additionally, a Factorial Completely Randomized Design (FCRD) was employed for evaluation by Design-Expert software (v7.7.0, Statease Inc., USA). Significant quadratic terms ($p<0.05$) were identified *via* ANOVA, and model adequacy was validated using R^2 (Montgomery, 2001).

3. Results

The vegetative-stage *Parthenium hysterophorus* L. (PHv) powder underwent supercritical carbon dioxide extraction (SC-CO_2) to recover bioactive compounds, employing varying pressures (100, 150, and 200 bar) at 60°C as outlined in the methodology. The solvent extraction method was used as a control for comparison. The crude extracts (T_1 to T_3) obtained from SC-CO_2 were analyzed for extraction efficiency. Additionally, the T_0 to T_4 crude extracts were fractionated by solvents of increasing chloroform, polarity-n-hexane, and ethanol. The fractionated extracts underwent qualitative analysis, thin-layer chromatography (TLC), HPLC analysis to identify parthenin, and selected solvent fractions which estimated for their activity against *in vitro* cancer cell lines. The detailed findings and discussions are presented below.

3.1 Effect of SC-CO_2 extraction pressure on extraction efficiency of vegetative stage *P. hysterophorus* powder

The results of the SC-CO_2 extraction efficiencies for PHv powder at various temperatures and pressures are detailed in Table 2. Based on the data from the table, the efficiency was observed to range from 83.64% to 94.50% at 60°C. The highest efficiency (94.50%) recorded at 200 bar and 60°C, while the lower efficiency (83.64%) was noticed at 100 bar and 60°C. Soxhlet extraction (control) was 46.32% less than SC-CO_2 extraction.

As it has been experiential in the table, the extraction efficiency improved an increase in pressure (100–200 bar). This can be attributed to the higher CO_2 density, which enhances its solvent strength and the solubility of analytes (Bezerra *et al*., 2020; Liza *et al*., 2010; Lorigooini *et al*., 2020). Additionally, pressure also temperature significantly affect thermodynamic properties, promoting improved convective mass transfer and higher extraction yields (de Miranda *et al.,* 2014).

The one-way ANOVA showed a substantial effect of pressure levels on SFE extraction efficiency ($F = 8040.23 > F_{crit} = 5.14$, $p<0.01$), indicating significant differences in mean extraction efficiencies across the pressure levels.

Table 2: Effect of pressure levels on extraction efficiency of vegetative stage *P. hysterophorus* powder extracts

Pressure	Treatments	Extraction efficiency, %
100	T_1	83.64 ± 0.087*
150	T_2	92.13 ± 0.092*
200	T_3	94.50 ± 0.091*
Soxhlet extract	T_0	46.32 ± 0.516

Each value is expressed as the mean ± SD (n = 9). *Statistical significance at $p<0.01$ using one-way ANOVA.

3.2 Solvent fractionation and qualitative phytochemical screening of vegetative stage *P. hysterophorus* powder extracts

The SC-CO_2 crude extracts (T_1 to T_3) and the T_0 control from PHv powder underwent sequential solvent fractionation using solvents of increasing polarity: n-hexane, chloroform, and ethanol. This solvent fractionation technique enables the isolation and concentration of selective bioactive compounds found to be in crude extract. Such targeted extraction increases the potential efficacy of the isolated compounds for anticancer activities, enhancing their pharmacological use.

The consequences of the phytochemical screening have been presented in Table 3. Phenols were detected in the n-hexane solvent only in T_3 (low). In the chloroform solvent, phenols were present in T_1, and T_3, with low levels. In the ethanol solvent, the highest concentration of phenols was found in T_3 (high), followed by moderate levels in T_1, and T_0. The flavonoids in the ethanol fraction demonstrated a dark color (+++), indicating rich content and strong extraction efficiency for polar flavonoids. Alkaloids showed a moderate presence in the n-hexane and ethanol fractions among treatments. Tannins in the n-hexane and ethanol fractions also displayed a dark color (+++), T_3 and T_0, indicating rich content. Terpenoids were abundant in both the n-hexane and chloroform fractions, particularly in T_2 and T_3. Lastly, the n-hexane fraction showed a high presence of saponins (+++), indicating a positive color.

Relevant studies on the phytochemical screening of *P. hysterophorus* have shown varied findings. Sequential extraction of *P. hysterophorus* flowers exposed that n-hexane extracts lacked tannins and saponins but contained flavonoids, terpenoids, and alkaloids. Chloroform extracts included tannins, flavonoids, terpenoids, and alkaloids but lacked saponins, while ethanol extracts contained all phytochemicals except saponins (Kumar *et al*., 2013a). Further, sequential hexane extracts of *P. hysterophorus* leaves revealed the absence of flavonoids, saponins, and tannins, with trace amounts of alkaloids. Phenols and terpenes, however, were abundant in low-temperature extracts (Jaiswal *et al*., 2022). Additionally, ethanol leaf extracts contained alkaloids, flavonoids, phenols, tannins, and terpenoids but no saponins (Mishra *et al*., 2012). The quantity and quality of secondary metabolites in plants are influenced by species, genotype, developmental stage, and environmental factors Shahhoseini and Daneshvar (2023).

Table 3: Screening of phytochemical in n-hexane, chloroform, and ethanol fraction of vegetative stage *P. hysterophorus* powder extracts (T_1 to T_3, T_0)

Test type	Type of solvent	T_1	T_2	T_3	T_0
Phenols	n-Hexane	-	-	++	-
	Chloroform	+	-	+	-
	Ethanol	++	-	+++	++
Flavonoids	n-Hexane	-	+	-	-
	Chloroform	-	+	-	+
	Ethanol	-	+	+++	-
Alkaloids	n-Hexane	-	++	-	+
	Chloroform	-	-	-	-
	Ethanol	-	+	++	-
Tannins	n-Hexane	++	-	+++	+
	Chloroform	+	-	+	-
	Ethanol	++	-	++	+++
Terpenoids	n-Hexane	++	+++	-	+
	Chloroform	-	+	-	++
	Ethanol	-	+	-	-
Saponins	n-Hexane	-	+++	-	-
	Chloroform	+	-	+	-
	Ethanol	-	-	-	-

3.3 Thin layer chromatograph (TLC) analysis of parthenin in the n-hexane, chloroform, and ethanol fractions of vegetative stage *P. hysterophorus* powder extracts

The presence of parthenium in the n-hexane, chloroform, as well as ethanol fractions of SC-CO_2 extracts from PHv powder was evaluated using TLC. Violet spots with an Rf value of 0.6, indicative of parthenin, were detected using valine reagent and a chloroform: ethanol (3:1) solvent system.

As showed in Figure 4(a), the TLC study in the n-hexane fractions (T_0 to T_3) showed no parthenin band at the calculated Rf value under UV light at 254 nm. Similarly, no violet spots corresponding to parthenin were observed in the chloroform fractions (T_0 to T_3), confirming the absence of parthenin in these samples as observed Figure 4(b).

In contrast, the ethanol fractions of PHv powder extracted with SC-CO_2 confirmed the presence of parthenin. The chromatogram in Figure 4(c) showed violet spots corresponding to parthenin in spots in T_1, T_2, T_3, and T_0, with a calculated Rf value of approximately 0.6 listed in Table 4. These spots were also observed with the naked eye and identified based on their respective Rf values.

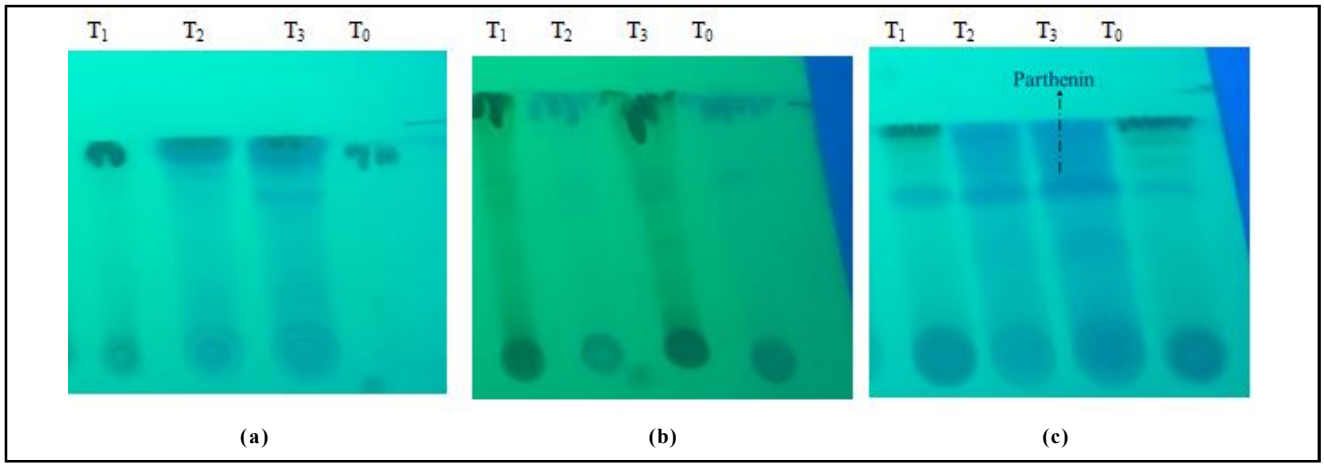

Figure 4: Detection of parthenin compound in (a) n-hexane, (b) chloroform, and (c) ethanol fractionates of vegetative stage *P. hysterophorus* powder extract at UV light of 254 nm.

Table 4: Thin layer chromatography of ethanol fractions of vegetative stage *P. hysterophorus* powder extracts

Test extract	Solvent system	Detecting agent	Number of spots	Rf value
Ethanol fractionates of PHv extract	Chloroform: Ethanol (3:1)	Valinin reagent	T_1, T_2, T_3, and T_0	0.6

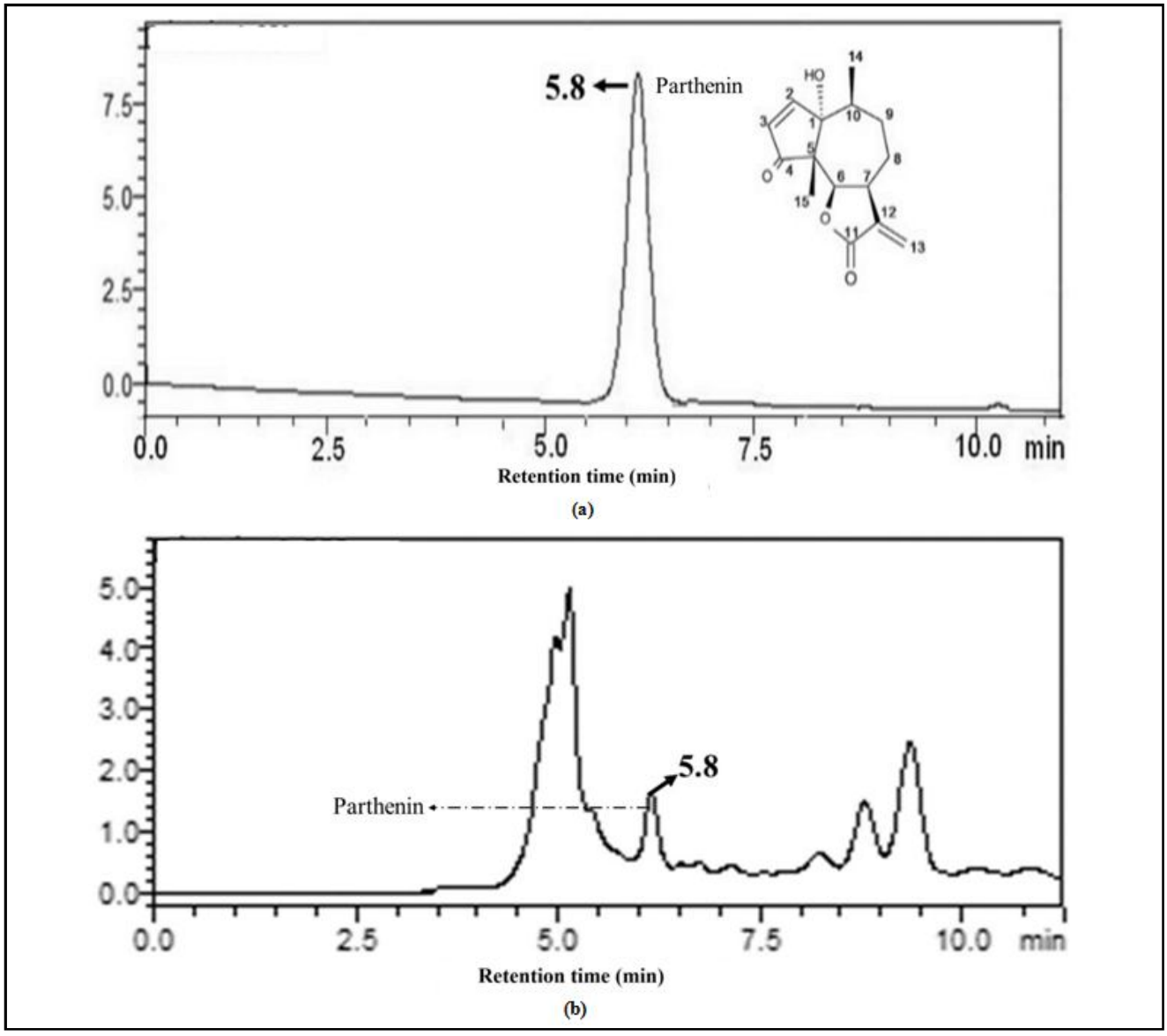

Figure 5: HPLC chromatogram of the (a) Isolated parthenin sample; (b) Ethanol fractionate test drug from vegetative stage *P. hysterophorus* powder extract, employing a LiChrospher 100 RP-18 5 m (250 mm 4.6 mm) gradient with (W) water and (A) acetonitrile at a flow rate of 1.0 ml/min and detection at 250 nm: 0 min, 60:40 (W: B); 10 min, 60:40; 13 0:100; 15 0:100; 18 min, 60:40; 20 min, 60:40.3.5.

The findings are consistent with previous reports indicating that parthenin elutes with an Rf value of approximately 0.6 in chloroform: ethanol (3:1) solvent system (Hernandez *et al*., 2011). Polar compounds, like flavonoids, phenols, carbohydrates and glycosides generally show lower Rf values, while nonpolar compounds like steroids, prostaglandins, essential oils, and terpenes exhibit higher Rf values. Compounds within intermediate Rf values, like saponins and mycotoxins, possess both polar and nonpolar properties, resulting in mixed migration behavior (Jaiswal *et al*., 2022). This is further supported by the phenolic content analysis of *P. hysterophorus* flower and root extracts, where hexane and ethanol fractions demonstrated lower Rf values, reaffirming that polar compounds are typically identified at lower Rf values (Kumar *et al*., 2013a).

3.4 Validation of isolated parthenin and ethanol fraction from vegetative stage *P. hysterophorus* powder extract by HPLC method

Parthenin, the primary bioactive compound and a major constituent of PHv, was successfully isolated from its leaf using thin-layer

chromatography (TLC), as outlined in the material and method section. The step-by-step isolation process is illustrated in Figure 2, and the isolated parthenin displayed an Rf value of 0.6. These spots were also observed with the naked eye and identified based on the Rf values.

To confirm its identity, the isolated compound underwent HPLC analysis following the specified protocol of the above methodology. The HPLC analysis produced a distinct peak with a retention time of 5.8 min, validating the presence of parthenin, as shown in Figure 5a.

The main aim of isolating parthenin is being establish a reliable reference standard for analyzing and validating the TLC method for detecting parthenin in SC-CO_2 extract solvent fractions. These fractions, derived from n-hexane, chloroform, and ethanol, were systematically analyzed across samples T_1 to T_3 and T_0 using TLC.

Post-analysis of all solvent-partitioned fractions (T_0 to T_3) of *PHv* powder using SC-CO_2 and Soxhlet extraction methods through TLC revealed that parthenin spots were absent in the n-hexane and chloroform fractionated extracts. In contrast, the ethanol fractions, a polar solvent, exhibited multiple bioactive compound-rich spots, predominantly containing parthenin (Figure 4c). These spots were also observed with the naked eye and identified based on their respective Rf values.

The experimental data demonstrated that treatment T_3 exhibited the highest extraction efficiency among all treatments (T_1 to T_3). TLC analysis confirmed the presence of parthenin in the ethanol-fractionated T_3 extract. This finding was further validated through HPLC analysis, which identified parthenin with a retention time of 5.8 min, as illustrated in Figure 5b. These results strongly support previous studies on TLC analysis and HPLC validation of parthenin (Hernandez *et al*., 2011).

3.5 Evaluation of *in vitro* cytotoxic effects of ethanol fraction test drug from vegetative stage *P. hysterophorus* powder extract on HeLa cervical cancer, MCF-7 breast cancer, and HepG2 liver cancer cell lines

It is evident which are present in significant proportion of principal bioactive compounds resultant by herbal plants, such as taxol and *camptothecin*-isolated within *Taxus brevifolia* and *Cuscuta reflexa*, respectively for nearly 60% of anticancer drugs used in cancer treatment (Kumar *et al*., 2013). The ideal anticancer drug has low side effects, induces apoptosis, and targets specific cytotoxicity to cancer cells. In this context, in cytotoxic potential of ethanol-fractionated SC-CO_2 extracts from aerial vegetative parts of *P. hysterophorus* powder (T_3) was tested against MCF-7, HeLa, and HepG2 cell line at a dose of 25 µg/ml, 50 µg/ml, and 100 µg/ml, with isolated parthenin applied as a control at 100 µg/ml.

3.5.1 Evaluation of *in vitro* cytotoxic effects of ethanol fraction test drug from vegetative stage *P. hysterophorus* powder extract on MCF-7 breast cancer cell lines: Per cent viability and inhibition

The important effect of the test drug at various concentrations on MCF-7 breast cancer cells is demonstrated in Figure 6 (% cell viability) and Figure 7 (% cell inhibition). As illustrated in the figure, the cytotoxic effect on breast cancer cells was substantial, with cell inhibition increasing significantly as the drug concentration rose ($p<$ 0.0001). Conversely, Figure 6 shows a corresponding decrease in cell viability. According to Table 5, at a low concentration (25 µg/ml), the ethanol fraction drug preserved a high cell viability of 59.47%, with a corresponding low inhibition rate of 40.53% in MCF-7 cells. In contrast, at a higher dose of 100 µg/ml, MCF-7 cell inhibition increased to 67.34%, while cell viability dropped to 32.66%. This significant inhibition was comparable to that observed with the isolated compound parthenin, which demonstrated 73.58% inhibition at the same concentration.

3.5.2 Evaluation of *in vitro* cytotoxic effects of ethanol fraction test drug from vegetative stage *P. hysterophorus* powder extract on HeLa cervical cancer cell lines: Per cent viability and inhibition

The anticancer activity exhibited a clear dose-dependent response, as demonstrated by the present results from the HeLa cervical cancer cell line. Treatment with an ethanol fraction test drug dose of 25 µg/ml resulted in cell viability of 82.90% and an inhibition rate of 17.10%. As the drug dose was increased to 100 µg/ml, the inhibition rate significantly rose to 57.88%, while cell viability decreased to 42.12%, as detailed in Table 5. Furthermore, Figure 6 illustrates a significant decrease in cervical cancer cell viability with increasing drug concentration, with results showing statistical significance at $p<$ 0.0001. Conversely, Figure 7 shows a corresponding significant increase in per cent cell inhibition across all doses of the test drug. This suggests that the ethanol fraction (T_3), despite its slightly reduced potency compared to isolated parthenin (66.21% inhibition), retains substantial efficacy in targeting HeLa cells, indicating its potential as a promising therapeutic agent in cervical cancer treatment.

3.5.3 Evaluation of *in vitro* cytotoxic effects of ethanol fraction test drug from vegetative stage *P. hysterophorus* powder extract on HepG2 Liver cancer cell lines: Per cent viability and inhibition

The effect of the ethanol-fractionated drug (T_3) of PHv powder at dosage of 25 µg/ml, 50 µg/ml, and 100 µg/ml on HepG2 human liver cancer cell lines is detailed in Table 5. The per cent cell viability of HepG2 cancer cells reduced with increasing drug concentrations, recorded as 56.71%, 43.75%, and 34.84%, respectively. Correspondingly, the per cent cell inhibition increased to 43.29%, 56.25%, and 65.16%, respectively. This anticancer activity exhibited a clear dose-dependent response, as demonstrated by the experimental results, and is illustrated in Figure 6 (cell viability) and Figure 7 (cell inhibition). Although, slightly less effective than parthenin, which achieved a 74.34% inhibition rate at 100 µg/ml, the T_3 drug demonstrated significant cytotoxic potential against HepG2 cells.

Supporting these studies, parthenium extracts obtained through solvent extraction, sequential solvent extraction, and nanoparticle synthesis techniques were reported to demonstrate anticancer activity in various cell line studies. Findings demonstrate significant dose-dependent inhibition of cancer cell lines (Kumar *et al*., 2013; Sharma *et al*., 2015; Haq *et al*., 2011; Anup *et al*., 2015; Ahsan *et al*., 2020). For the MCF-7 (breast cancer) cell line, sequential ethanol extracts achieved 81% inhibition at 100 mg/ml (Kumar *et al*., 2013), while ethanol and methanol extracts inhibited growth by 77% (Anup *et al*., 2015) and up to 82% (Haq *et al*., 2011), respectively. Methanolic extracts inhibited HeLa cell growth by 80–90% at 125–1000 µg/ml (Sharma *et al*., 2015) and 80% at 15 µM (Anup *et al*., 2015). Similarly, ethanolic extracts of *M. philippensis* reduced HeLa cell viability from 46.15% at 25 µg/ml to 26.55% at 100 µg/ml

(Malarkodi *et al*., 2024). HepG2 (liver cancer) cell line growth was reduced by 70% at 100 µg/ml (Ahsan *et al*., 2020) and 50% at 53 µg/ml (Sivakumar *et al*., 2021) using nanoparticle-synthesized extracts. These results confirm the efficacy of *Parthenium* extracts in inhibiting cancer cell growth and highlight their potential for anticancer applications. These studies collectively highlight the consistent efficacy of *P. hysterophorus* across MCF-7, HeLa, and HepG2 cell lines, suggesting its utility in cancer therapy research.

The ANOVA analysis of the quadratic regression model reveals that the ethanol fraction dose (A) and cancer cell lines (B)-as well as their interactions (AB), significantly impact cell inhibition and viability through linear, quadratic, and interaction terms. The model's F-value of 1805.39 is highly significant (p<0.0001), confirming that these factors and their interactions strongly influence the outcomes. With an R^2 value of 0.9988, the model demonstrates an excellent fit, explaining 99.88% of the variation in cell inhibition and viability.

The optimized predictive model for percentage of cell viability was determined using the formula:

Cell viability (%) = 53.58 + 12.78*A - 0.9911*A^2 – 8.13*B + 16.61*B^2 + 1.23*AB – 0.2278*A^2B – 0.0644*AB^2 + 0.5867* A^2B^2

The optimized predictive model for percentage of cell inhibition was determined using the formula:

Cell inhibition (%) = 46.42 – 12.78*A + 0.9911*A^2 + 8.13*B – 16.61*B^2 – 1.23*AB + 0.2278*A^2B + 0.0644*AB^2 – 5867* A^2B^2

The "A" represents ethanol fraction drug concentration and "B" represents respective tested cancer cell lines viability and inhibition.

Table 5: Effect of *in vitro* anticancer activity of ethanol fractionate test drug from vegetative stage *P. hysterophorus* powder extract on cancer cell lines

Cancer cell lines	Test drug concentrations (µg/ml)	Per cent viability	Per cent inhibition
MCF-7	25	59.47 ± 0.81*	40.53 ± 0.81*
	50	44.23 ± 0.37*	55.77 ± 0.37*
	100	32.66 ± 0.56*	67.34 ± 0.56*
HeLa	25	82.90 ± 0.67*	17.10 ± 0.67*
	50	69.78 ± 0.79*	30.22 ± 0.79*
	100	57.88 ± 0.35*	42.12 ± 0.35*
HepG2	25	56.71 ± 0.24*	43.29 ± 0.24*
	50	43.75 ± 0.86*	56.25 ± 0.86*
	100	34.84 ± 0.98*	65.16 ± 0.98*
MCF-7	100	26.42 ± 0.37#	73.58 ± 0.37
HeLa	100	33.69 ± 0.17#	66.28 ± 0.19
HepG2	100	25.68 ± 0.56#	74.34 ± 0.56
Control	0	100	0
	Mean	53.58	46.42
	SEm (±)	0.519	0.519
	CD @ 1 %	2.325	2.325
	R^2	0.9988	0.9988

Each value is expressed as the mean ± SD (n = 27). *Statistical significance at p<0.05. # Isolated parthenin.

Table 5a: Analysis of variance (ANOVA) i*n vitro* anticancer activity of ethanol fractionate test drug, T_3, from vegetative stage *P. hysterophorus* powder extract on cancer cell lines

Source	Some of squares	df	Mean square	F value	*p*-value
Model	6475.35	8	809.42	1805.39	<0.0001
A-Drug dose	2729.6	2	1364.8	3044.16	<0.0001
B-Cancer cell lines	3724.61	2	1862.31	4153.84	<0.0001
AB	21.14	4	5.28	11.79	<0.0001
Pure error	8.07	18	0.4483		

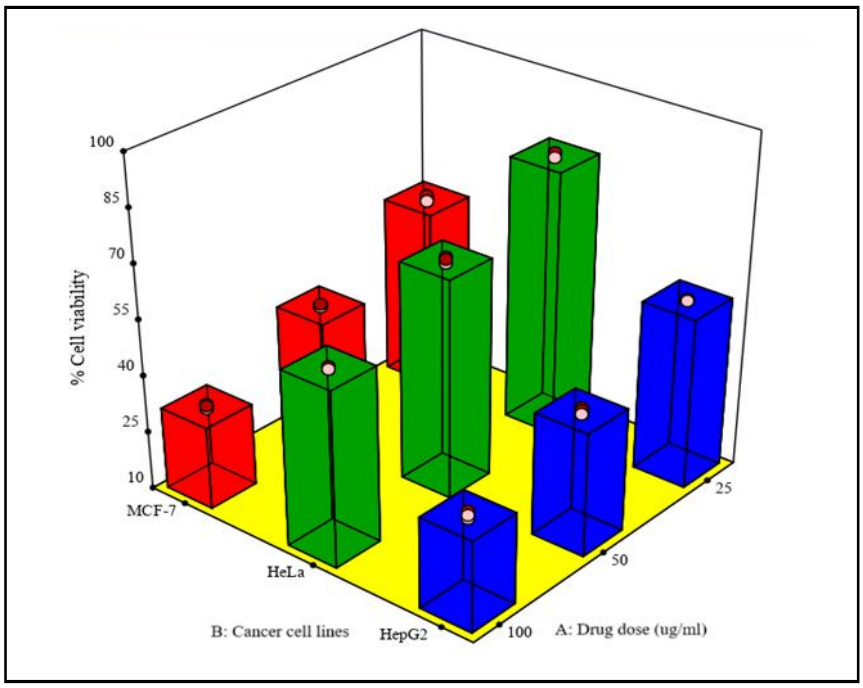

Figure 6: Effect of vegetative stage *P. hysterophorus* powder extract ethanol fraction test drug concentration on per cent cell viability of MCF-7, HeLa, and HepG2 cell lines.

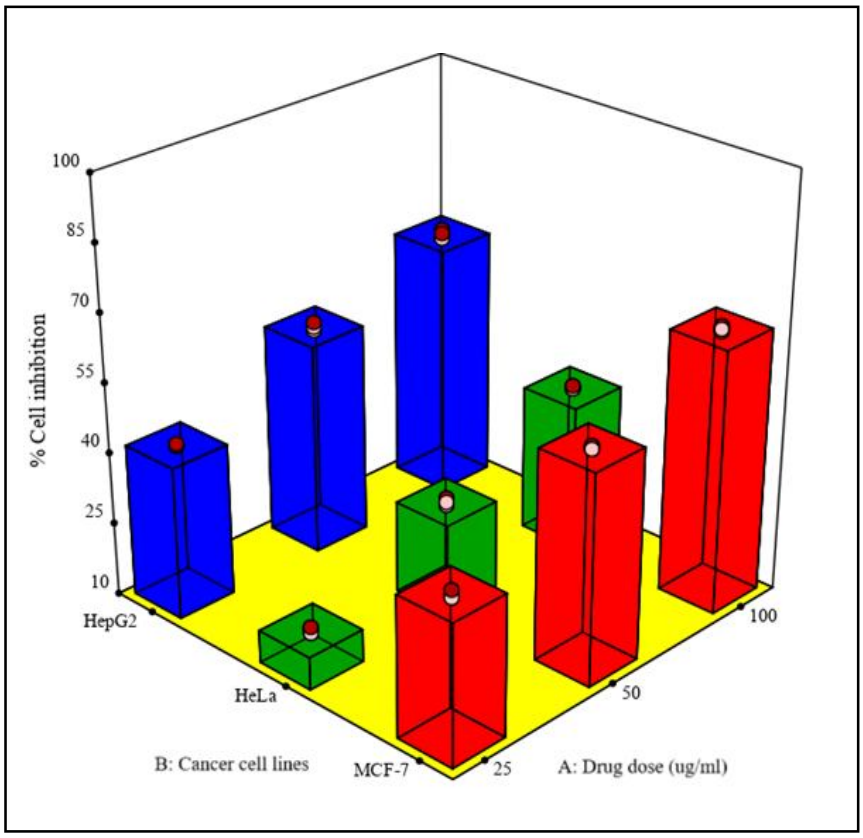

Figure 7: Effect of vegetative stage *P. hysterophorus* powder extract ethanol fraction test drug concentrations on per cent cell inhibition of MCF-7, HeLa, and HepG2 cell lines.

4. Discussion

Globally, chronic diseases account for approximately 70% of deaths, with cancer ranking as the second leading source, responsible for an estimated 9.6 million fatalities in 2018 (Kumar *et al*., 2013a). The limitations of modern medicine, including undesirable side effects, have intensified the search for novel herbal drugs to address chronic illnesses (Rajendran *et al*., 2024). Notably, nearly 60% of anticancer drugs in use today are derived from plant sources, such as *Taxus brevifolia* and *Cuscuta reflexa* (Kumar *et al*., 2013).

The term *Parthenium* originates from the Latin word *Parthenice*, reflecting its ancient reputation for medicinal properties (Bailey, 1960). Folkloric and recent scientific literature highlights the potential therapeutic benefits of *P. hysterophorus*, including antimicrobial, antioxidant, and anticancer activities (Patel, 2011; Jaiswal *et al*., 2022).

The current investigation evaluates the anticancer activity of an ethanol fraction targeting parthenin, derived from *P. hysterophorus* powder extracted at its vegetative stage using SC-CO_2 technology. The investigation focuses on the bioactive compound's effects against MCF-7 (breast cancer), HepG2 (liver cancer) and HeLa (cervical cancer), cell lines. Parthenin, a sesquiterpene lactone (STL) and the plant's primary bioactive compound, belongs to a class of naturally occurring substances prevalent in the Asteraceae family. These compounds are renowned for their pharmacological and therapeutic properties, including plant defense and potent cytotoxic effects, making them promising candidates for cancer therapy (Ahmad *et al*., 2018; Kumar *et al*., 2014; Patel, 2011).

Our findings highlight the influence of SC-CO_2 extraction parameters, particularly pressure and temperature, on the extraction efficiency of crude extracts of PHv powder. The results indicate a notable increase in extraction efficiency with rising temperature and pressure. Extraction of PHv powder using the SC-CO_2 and Soxhlet methods yielded extraction efficiencies of $94.50 \pm 0.091\%$ and $46.32 \pm 0.516\%$, respectively.

This improvement is attributed to the positive correlation between pressure and temperature, which enhances the solvent properties of CO_2 in its supercritical state (Liza *et al*., 2010**)**. Under these conditions, CO_2 exhibits reduced viscosity, increased density, and superior extractive capabilities, facilitating the effective extraction of bioactive compounds.

Furthermore, the selected temperature of 60°C did not significantly degrade the bioactive compounds, as the extraction was conducted close to CO_2s critical point (31.1°C at 73.8 atm). This operating condition preserves the natural structure and activity of the bioactive compounds, ensuring their stability and functionality throughout the extraction process.

The qualitative analysis of solvent fractions, based on TLC Rf values compared to isolated parthenin, confirmed that most bioactive compounds were eluted in the ethanol fraction of SC-CO_2 extracts across the majority of treatments, with an Rf value of 0.6. This observation was further validated through HPLC analysis of the optimized ethanol fractionate (T_3). Phytochemical screening of ethanol fraction revealed in presence of key secondary metabolites, including phenols, flavonoids, alkaloids, terpenoids, tannins, and saponins.

Previous studies have reported that isolated parthenin elutes at 5.8 min in HPLC and exhibits an Rf value of 0.6 in TLC (Hernandez *et al*., 2011). These findings are consistent with earlier observations indicating that polar bioactive compounds, such as phenols, flavonoids, and alkaloids, typically elute at lower Rf values (below 0.6) due to shorter travel distances and faster appearance on TLC (Kumar *et al*., 2014; Jaiswal *et al*., 2022).

Most of the literature indicates that solvent-based extractions of *P. hysterophorus* have predominantly been used to evaluate its anticancer potential. These extracts have demonstrated significant activity against cancer cell lines like MCF-7, HeLa, HepG2, and even HIV, highlighting the promising therapeutic potential of this immortal plant. However, no studies were found exploring the innovative green supercritical carbon dioxide extraction method for *P. hysterophorus* at its vegetative stage. Therefore, this study aims to evaluate the purified SC-CO_2 extract for anticancer activity.

The results of the per cent cell viability and inhibition experiments conducted on MCF-7, HeLa, and HepG2 cell lines at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml using MTT assay, with isolated parthenin as the reference drug at 100 µg/ml, demonstrated dose-dependent anticancer activity, as described by Kumar *et al*. (2013).

The ethanol-fractionated drug showed higher cell survival rates at lower concentration of 25 µg/ml, on viability percentages of 59.47%, 82.90%, and 56.71% for MCF-7, HeLa, and HepG2 cells, respectively. In contrast, which is on highest concentration of 100 µg/ml, cell viability decreased significantly to 32.66%, 57.88%, and 34.84% for MCF-7, HeLa, and HepG2 cells, respectively.

Overall, the results suggest that *P. hysterophorus* (PHv) SC-CO_2 extracts hold significant promise as effective agents for anticancer cell drugs, contributing to sustainable and eco-friendly therapeutic practices.

5. Conclusion

The term *Parthenium* originates from the Latin word *Parthenice*, historically associated with medicinal properties. While this invasive plant has an impact on crop yields and health, its resilience is attributed to potent allelopathic bioactive compounds with traditional health benefits. Recent research focuses on its applications as an antimicrobial, anticancer agent, biopesticide, industrial dye cleaner, and a source of bioactive molecules. Unlike conventional solvent and hydrodistillation methods, this study pioneers the use of supercritical carbon dioxide (SC-CO_2) extraction in *P. hysterophorus* at its vegetative stage. This innovative approach shall be facilitating the precise extraction of bioactive components in their unaltered, natural state, avoiding any chemical modifications. Phytochemical screening revealed that the ethanol fractionates from PHv powder extracts contained the highest concentration of secondary metabolites. SC-CO_2 extraction efficiency increased with increasing pressure. Crude extracts were fractionated using n-hexane, chloroform, and ethanol, with parthenin identified in the ethanol fraction (Rf 0.6), *via.,* TLC and same validated through HPLC. The ethanol fraction, optimized at 200 bar and 60°C, showed significant dose-dependent anticancer activity against MCF-7, HeLa, and HepG2 cell lines.

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Conflict of interest

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

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