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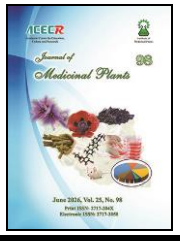

Research Article

Phytochemical profiling and *in vitro* cytotoxic evaluation of alkaloids isolated from *Equisetum arvense* L. native to northern Iraq against human cancer cell lines

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ABSTRACT

Background: *Equisetum arvense* L. (Horsetail) is a medicinal perennial herb widely distributed across the Northern Hemisphere. Traditionally utilized for gastrointestinal, renal, and musculoskeletal disorders, its specific alkaloid profile and associated anticancer properties (particularly regarding regional chemotypes) remain understudied. **Objective:** This study evaluated the phytochemical composition of alkaloids isolated from *E. arvense* growing in northern Iraq and evaluated their cytotoxic potential against three human cancer cell lines (HePG2, WRL68, and SK-GT2) alongside a normal cell line (REF). **Methods:** Purified alkaloids were extracted from *E. arvense* and subjected to qualitative and quantitative chemical profiling. Cytotoxicity was evaluated across a concentration range of 25 to 1600 µg/ml at multiple exposure intervals using standard cell viability assays. **Results:** Chromatographic analysis identified five distinct alkaloids: hordenine (426.4 µg/g), caulerpin (324.1 µg/g), martensine (139.3 µg/g), isoquinoline (139.1 µg/g), and almazolone (77.6 µg/g). The total alkaloid extract demonstrated concentration- and time-dependent cytotoxicity across all cancerous cell lines ($P < 0.05$). Following 24 hours of exposure at 1600 µg/ml, maximum growth inhibition was observed in HePG2 (78.90 %) and SK-GT2 (77.40 %) cells, with moderate inhibition in WRL68 cells (39.90 %). Notably, no significant cytotoxic effect was observed against the normal REF cell line. **Conclusion:** The alkaloid fraction of *E. arvense* native to northern Iraq exhibits selective cytotoxic activity against human cancer cell lines while sparing normal cells, supporting its potential as a source for natural lead compounds in oncology drug discovery.

Abbreviations: HePG2, WRL68 and SK-GT2, cancerous cell lines; RFF, Normal cells line; BHA, [butylated hydroxyl anisole; BHT, butylated hydroxyl toluene; REF, Rat Embryo Fibroblast; ICCMGR, Iraqi Center for Cancer and Genetic Research; FVS, Fetal Bovine Serum

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1. Introduction

Ferns have many species which have been used in dietary consumption as a vegetable or as folk medicine. In recent years, considerable research has focused on the identification and characterization of secondary metabolites present in ferns. They have also investigated the potential medical applications of these compounds [1]. *Equisetum arvense* is a member of the fern family. It is a vascular plant that belongs to the Equisetaceae family [2] distributed in Asia and Africa. The plant spreads in northern Iraq [3]. Traditionally, *Equisetum arvense* has been used to manage gastric bleeding, promote wound healing, relieve menstrual disorders, and treat rheumatic conditions. [4]. It is also used to treat various conditions such as oral infections, alcohol addiction, and thiamine deficiency [5]. Additionally, *E. arvense* is used to manage diarrhoea, urethritis, epidemic conjunctivitis, joint pain, kidney stones, and urinary tract infections. [6].

The plant has antioxidant, antibacterial, antifungal, and anticancer properties [4]. It also helps with inflammation, dizziness, headaches, tremors, and paralysis. Moreover, it is used for hyperglycemia, cerebral ischemia, memory disorders, and hepatitis [7]. The plant contains many secondary metabolites. These include flavonoids, phenols, cinnamic acid, and caffeic acid [8]. It also has essential oils, tannins, triterpenoids, and steroids. In addition, it contains various types of alkaloids. [9, 10].

Alkaloids are bioactive compounds found in plants. They are widely used as anticancer agents. The *E. arvense* plant contains several types of alkaloids. These include soquinoline, isoquinoline, hordenine, caulerpin, almazolone, and martensine. These compounds can produce strong anticancer effects. They work by stopping the cell cycle. They also trigger apoptosis. This process leads to the death of cancer cells [11].

Reactive oxygen species (ROS) are chemically reactive molecules. That are naturally produced during cellular metabolism. When the balance between free radical production and antioxidant defenses is disturbed, oxidative stress occurs [4, 12]. Synthetic antioxidants, including butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), and α-tocopherol are widely used to control ROS levels in the food and pharmaceutical industries. However, concerns regarding the potential toxicity of synthetic antioxidants have stimulated increasing interest in natural products with antioxidant activity.

Phenolic compounds can neutralize free radicals. This ability depends on their chemical structure, molecular weight, and functional groups such as hydroxyl or methoxy [12]. The biosynthesis of these secondary metabolites is a dynamic process. It largely depends on environmental conditions. It also depends on the specific traits of the plant. One important factor is the type of plant parts or organs used. Extraction is an important first step in isolating, quantifying, and identifying secondary metabolites from plants. The choice of solvent system is critical. Factors such as solvent type, purity, and polarity affect how well polyphenols and their antioxidant activities are extracted [13, 14]. Phenolic compounds have different levels of stability and solubility. These can vary depending on the solvent used. This makes selecting the right solvent a complex task. It requires careful thought and cannot be taken lightly. Therefore, it is necessary to investigate which solvents work best for extracting polyphenols from each plant species.

In this study, the aim was threefold. First, to evaluate how well different solvents recover phenolic compounds from two parts of S. tebesana. Second, to measure antioxidant properties and the content of phenolic subgroups using these solvents. Third, to analyze and quantify individual flavonoids using HPLC for the first time.

2. Materials and methods

2.1. Plant collection

Plant materials were collected from northern Iraq, dried and ground before use. The samples were confirmed and identified by Prof. Dr. Bahram Khidhr Maulood, a botanist in the College of Science at the University of Salahaddin. Voucher specimens (No. 4, 9, 5, E.ar.) were deposited in the Kurdistan Herbarium, Howler Botanical Garden, Erbil Governorate.

2.2. Plant extraction

The extraction was performed according to the method described by [15]. Plant powder was macerated in absolute ethanol at a ratio (1:10) (w/v) for 3 days. Subsequently, the extract was filtered and concentrated to 50 ml. The concentrate was acidified with 1 ml of 0.1 N HCl and extracted with 10 ml of ethyl acetate. Ammonia (3 ml) was then added and the mixture was re-extracted with 20 ml of ethyl acetate. Finally, the ethyl acetate layer collected and evaporated to dryness.

2.3. Qualitative analysis of alkaloids

Alkaloids were detected in the extract using two different reagents:

Mayer's reagent: A few drops of Mayer's reagent were added to 1 ml of the extract in a watch glass. The formation of a white precipitate indicated the presence of alkaloids.

Wagner's reagent: Several drops of Wagner's reagent were added to 1 ml of the extract in a watch glass. The appearance of an orange precipitate suggested the presence of alkaloids [9].

2.4. Quantitative analysis of alkaloids

Quantitative HPLC analysis was performed according to the method described by [10]. Purified alkaliods were analyzed using a sykam (Germany) HPLC system equipped with a phenomenex C-8 (50 x 4.6 mm I.D). The mobile phase consisted of acetonitrile: Methanol: Ortho phosphoric acid (55:45:1 v/v), with UV detection at 280 nm and a flow rate of 1.0 ml/min. A 100 µm of sample was injected.

2.5. Cell lines cytotoxicity analysis

Four cell lines were used in the study. These three malignant cell lines (HePG2, WRL68, and SK-GT2) and one normal cell line, Rat Embryo Fibroblast (REF). The cell lines were obtained from the Iraqi Center for Cancer and Genetic Research (ICCMGR). They were cultured in RPMI1640 medium supplemented with 10 % Fetal Bovine Serum (FBS) and 1 % penicillin-streptomycin. The cells were incubated at 37 °C with 5 % CO_2 [16]. Upon reaching approximately 80-90 % confluence, the cells were treated with *Equisetum arvense* extract at concentrations 25, 50, 100, 200, 400, 800, and 1600 µg/ml. Cytotoxicity was evaluated after 24 hours of treatment. The cells were subsequently stained with 5 % crystal violet. The percentage of growth inhibition rate was calculated using following equation:

$$\text{Inhibition ratio} = \frac{A-B}{A} \times 100$$

Where, **A** represents the optical density of the untreated control cells, and **B** is the optical density of the treated cells [11].

2.6. Statistical analysis

Statistical analysis was performed using t-test to compare treated and untreated (control) cells. A $P \leq 0.05$ was considered statistically significant [17].

3. Results

3.1. Phytochemical screening for alkaloids

The results of the alkaloids extracted from the Equisetum arvense plant are presented in Table 1.

3.2. The quantitative analysis of equisetum arvense alkaloids

The results presented in Table 2 demonstrate the presence of several alkaloids in the *Equisetum arvense* extract. The identified alkaloids included isoquinoline, a heterocyclic alkaloid; hordenine, a non-heterocyclic alkaloid; and caulerpin, almazolone, and martensine, which are classified as indole alkaloids. The HPLC chromatogram of the identified alkaloids is shown in Fig. 1, and the corresponding standard chromatogram is presented in Fig. 2.

3.3. Cytotoxicity analysis of Equisetum arvense alkaloids on cell lines

The cytotoxic effects of alkaloids isolated from *Equisetum arvense* were evaluated against three cell lines (HepG2, WRL68, and SK-GT2) and the normal REF cells. The inhibitory effects of the plant extract are presented in Tables 3, 4, 5, and 6, respectively.

The results demonstrated a concentration-dependent inhibitory effect, with the percentage of growth inhibition increasing from 19.97 % at 25 µg/ml to 70.90 % at 1600 µg/ml. All concentrations tested in the experiment showed significant inhibition compared to the control, which exhibited an inhibition rate of only 3.23 % for the HepG2 cell line (as shown in Fig. 3). Notably, the SK-GT2 cell line demonstrated the highest inhibition rate, reaching 77.40 % at a concentration of 1600 µg/ml of *Equisetum* alkaloids.

Table 1. Alkaloids phytochemical screening in *Equisetum arvense* extract.)

Phytochemical	Mayer's reagent	Wagner reagent
Alkaloids	+ / white precipitate	+ / brown precipitate

Table 2. Types and quantity of *Equisetum arvense* alkaloids

Alkaloids compounds	Quantity of alkaloids (µg/ml)
Isoqurnoline	139.1
Hordinie	426.4
Caulerpin	324.1
Almazolone	77.6
Martensine	139.3

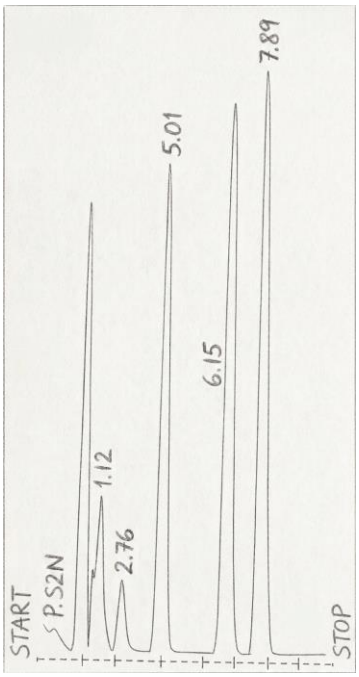


Fig. 1. HPLC chromatography of *Equisetum arvense* purified alkaloids

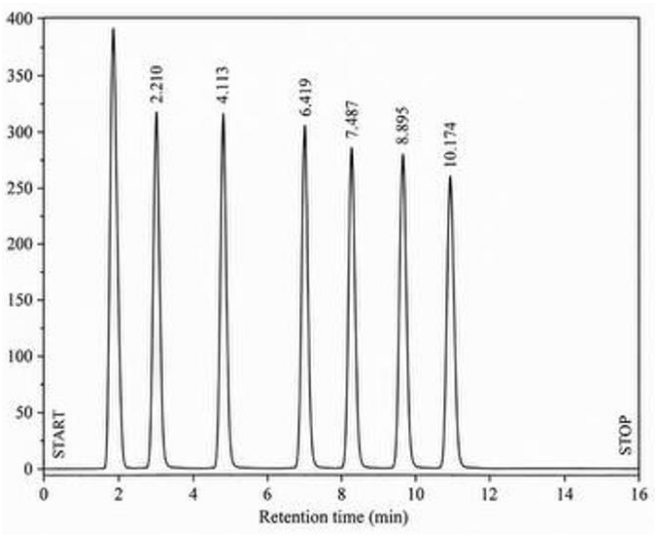


Fig. 2. HPLC chromatography of standards alkaloids

Table 3. Inhibition rate in HepG2 cell line influenced by different concentrations of extract of *E. arvense* plant after exposure for 24 h

Alkaloids *Concentration* µg/ml	**Inhibition rate % ± Standard error**
Control	3.23 ± 0.00^{j}
25	$19.97 \pm 0.81^{g*}$
50	$22.12 \pm 0.30^{f*}$
100	44.65 ± 0.23^{e}
200	$46.89 \pm 0.33^{d*}$
400	$60.97 \pm 1.12^{c*}$
800	$75.46 \pm 0.37^{b*}$
1600	$78.90 \pm 0.60^{a*}$

Values are expressed as Mean ± Standard Error. Asterisks (*) indicate a statistically significant difference compared to the untreated control group ($P \leq 0.05$, Student's t-test). Different superscript letters (s, f, e) indicate statistically significant differences between the treated groups ($P \leq 0.05$, Student's t-test)

Table 4. Inhibition rate of *E. arvense* alkaloids plant on SK-GT2 cell line influenced at 24h. exposure

Concentration µg/ml	**Inhibition rate % ± Standard error**
Control	1.15 ± 0.00^{j}
25	$15.90 \pm 0.36^{h*}$
50	$17.41 \pm 0.31^{g*}$
100	$17.09 \pm 0.79^{f*}$
200	23.16 ± 0.63^{e}
400	38.11 ± 1.36^{d}
800	$61.87 \pm 1.20^{c*}$
1600	$77.40 \pm 0.59^{b*}$

The different letters indicate that there are statistical differences at the level of $P \leq 0.05$

Values are expressed as Mean ± Standard Error. Asterisks (*) indicate a statistically significant difference compared to the untreated control group ($P \leq 0.05$, Student's t-test). Different superscript letters (s, f, e) indicate statistically significant differences between the treated groups ($P \leq 0.05$, Student's t-test)

Table 5. Inhibition rate of *E*. arvense alkaloids plant on WRL68 cell line influenced at 24h exposure

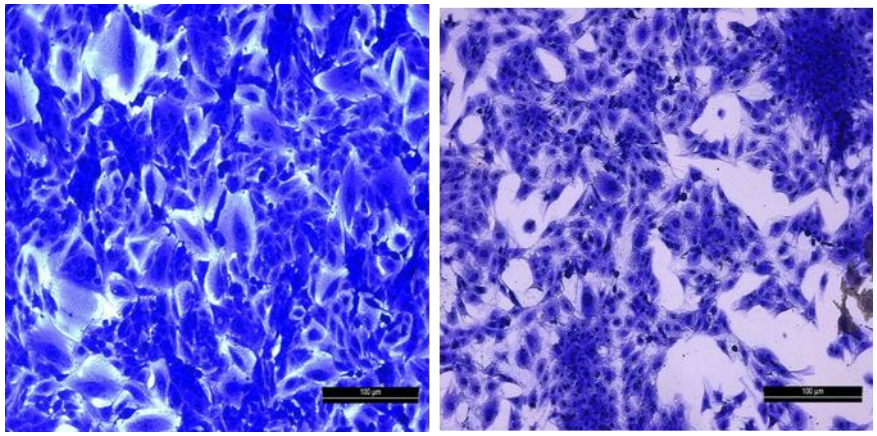
Concentration (µg/ml)	Inhibition rate % % ± Standard error
Control	0.00 ± 16.24^{c}
25	6.09 ± 2.25^{bc}
50	8.40± 6.99^{c}
100	25.13 ±1.19^{ab*}
200	36.01 ± 7.55^{ab}
400	31.64 ±9.52^{a*}
800	37.58 ± 6.61^{a*}
1600	39.90 ± 5.51^{a*}

Values are expressed as Mean ± Standard Error. Asterisks (*) indicate a statistically significant difference compared to the untreated control group ($P \leq 0.05$, Student's t-test). Different superscript letters (s, f, e) indicate statistically significant differences between the treated groups ($P \leq 0.05$, Student's t-test)

Table 6. Inhibition rate of *E. arvense* alkaloids plant on REF cell line influenced at 24h exposure

Concentration µg/ml	Inhibition rate % % ± Standard error
Control	0.0 ± 0.0^{c}
25	0.0 ± 0.0^{c}
50	0.0 ± 0.0^{c}
100	0.0 ± 0.0^{a*}
200	1.15 ± 14.50^{ab*}
400	4.0 ± 6.77^{a}
800	7.2 ± 8.33^{a*}
1600	10.11 ± 9.44^{a*}

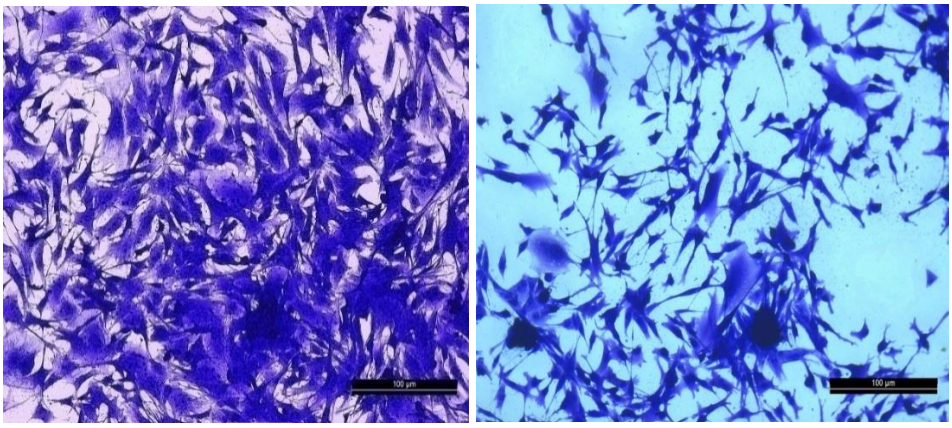
Values are expressed as Mean ± Standard Error. Asterisks (*) indicate a statistically significant difference compared to the untreated control group ($P \leq 0.05$, Student's t-test). Different superscript letters (s, f, e) indicate statistically significant differences between the treated groups ($P \leq 0.05$, Student's t-test)



A **B**

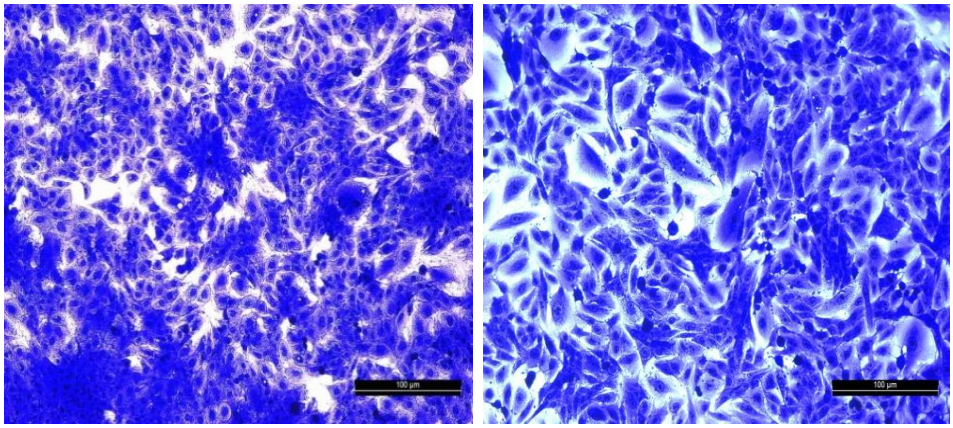
Fig. 3. Effect of alkaloid *E. arvense* on HepG2 cancer cell line after 24h. exposure (crystal violate, 100X). A: HepG2 as control showed monolayer confluent. B: HepG2 treated with extract at 1600 µg/ml, showed loss of confluent and dead of cells.

All the remaining concentrations show a significant difference from the control group (Fig. 4). A similar concentration-dependent inhibitory effect was observed in the WRL68 cell line, where growth inhibition increased with increasing alkaloid concentration and differed significantly from the control group (Fig. 5). Furthermore the alkaloid extract exhibited the greatest cytotoxic activity against the HepG2 cell line, followed by the SK-GT2 and WRL68 cell lines. In contrast, the REF cell line showed minimal sensitivity to the treatment, with a maximum growth inhibition of only 10 % at the highest tested concentration (1600 µg/ml) (Fig. 6).



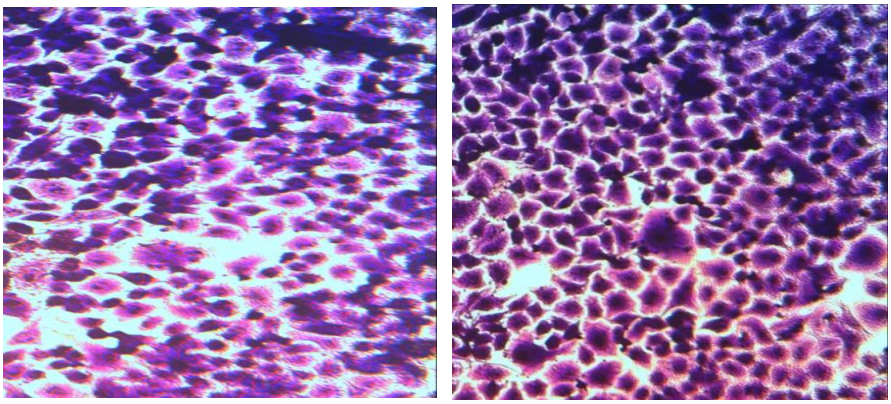
A **B**

Fig. 4. Effect of alkaloid *E. arvense* on SK-GT 2 cancer cell line after 24h. exposure (crystal violate, 100X). A: SK-GT2 as control showed monolayer confluent. B: SK-GT 2 treated with extract at 1600 µg/ml, showed loss of confluent and dead of cells.



A **B**

Fig. 5. Effect of alkaloid *E. arvense* on WRL68 cancer cell line after 24h. exposure (crystal violate, 100X). A: WRL68 as control showed monolayer confluent. B: WRL68 treated with extract at 1600 µg/ml, showed loss of confluent and dead of cells.



A **B**

Fig. 6. Effect of alkaloid *E. arvense* on REFcell line after 24h. exposure (crystal violate, 100X). A: REF as control showed monolayer confluent. B: REF treated with extract at 1600 µg/ml, showed all cells preserved with shape and number.

4. Discussion

Alkaloids are widely distributed in higher and lower plants, algae, and other marine plants [18]. Their content varies according to several factors. These include the plant's age, geographical location, and seasonal variation. Understanding these factors is essential for evaluating the biological properties and potential applications of medicinal plants [19].

The present findings demonstrated that the cytotoxic activity of *E. arvense* alkaloids increased in a concentration- and time-dependent manner, with higher extract concentrations producing greater growth inhibition. Similar findings have been reported in previous studies [20, 21], which showed that *Equisetum* alkaloid extracts inhibit the growth of several cancer cell lines, including pancreatic carcinoma (AsPC-1), human colon carcinoma, MCF-7, and HeLa cells. The observed cytotoxic activity is likely attributable to the selective targeting of cancer cells by the bioactive alkaloid constituents.

Certain active compounds inhibit cancer cells by binding strongly to their DNA. Cancer cell DNA is more relaxed and unstable because it has many broken hydrogen bonds. This allows active compounds to attach more easily to damaged DNA regions [22]. Some researchers suggest another mechanism. Secondary metabolites selectively adhere to cancer cells because these cells have higher iron levels. Cancer cells need more iron for rapid DNA multiplication during frequent cell division. This higher iron content attracts active secondary compounds [23].

Beljanski [18] explains that alkaloids reduce the metabolic activity of cancer cells. They affect dehydrogenase enzymes and lower NAD and phosphate levels. Other researchers [24] emphasize the antioxidant and antiproliferative effects of Equisetum extract. The extract is effective against HeLa, HT-29, and MCF cell lines. Alkaloids can stop cancer cell proliferation by arresting the cell cycle in G1 or G2/M phases. This arrest promotes apoptosis. Alkaloids also inhibit microtubule formation. Alkaloids from Equisetum significantly affect MCF7 cell growth but have little impact on REF cell lines [30]. Al-Marzook and Omran [25] highlight that Equisetum extract can inhibit A549 cell proliferation. This shows the extract's potential for therapeutic use.

The aqueous extract of *Equisetum* significantly inhibits metabolic activity in WM-2266-4 melanoma cells. It reduces activity by 70 % compared to controls. The aqueous extract also shows cytotoxic effects against the U937 cell line [3, 26]. Reference [19] reports that ethanolic Equisetum extract reduces ASPC-1 cell proliferation.

5. Conclusion

The extraction of active compounds from *Equisetum arvense*, also known as horsetail, holds great potential for medicine development. These compounds are rich in bioactive substances. They have many health benefits, such as anti-inflammatory, diuretic, and antioxidant effects. More scientific research is needed to understand how these compounds work and how effective they are. Thorough studies will help researchers use the plant's potential to create effective treatments. These treatments could improve patient outcomes. This research offers a valuable opportunity for the pharmaceutical industry. It can help connect traditional herbal remedies with modern healthcare. By combining the benefits of *E. arvense* with current medication formulations, the industry can develop innovative and scientifically supported therapies.

Authors contributions

The laboratory experiments were conducted by H. M. H., S. F. H., and S. Al-Majmaie. The statistical analysis was performed and the draft was edited by Gh. S. S. and Z. A. I. A. M. and I. H. provided invaluable advisory support and reviewed the project.

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Conflict of interest

The authors declare that there is no conflict of interest.

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