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Cytotoxicity of Ethanolic and Methanolic Extracts of *Medicago sativa* L. (Alfalfa) on K562 Myeloid Cancer Cell Lines

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Abstract

Background & Objectives: For centuries, the alfalfa (*Medicago sativa* L.) plant has been recognized for its versatile and active role in treating various diseases. Not only has it been utilized as a therapeutic agent, but it has also been served as a dietary component for both animals and humans. Given the distinctive attributes of this plant in ethnopharmacology, this study aimed to investigate the effects of ethanolic and methanolic extracts of *M. sativa* L. on the K562 myeloid cell line under *in vitro* conditions.

Materials & Methods: The phytochemical composition of *M. sativa* L. was determined through Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Cell viability was assessed using the MTT assay, wherein K562 cells were subjected to varying concentrations (50–100 µg/mL) of methanolic and ethanolic extracts over 24, 48, and 72-hour intervals to determine the IC50. Subsequently, the most promising IC50 result was employed in flow cytometry (Flow Jo Software) analysis.

Results: Active constituents identified included phytol, phenol, linolenic acid, and glycine. Statistical analysis revealed a time-dependent but not dose-dependent effect. It was noteworthy that the IC50 for the methanolic extract after 72 hours was 9.45 µg/mL, whereas it was 19.3 µg/mL for the ethanolic extract. Flow cytometry analysis indicated that the methanolic extract caused 49.16% and the ethanolic extract caused 15.42% of cell death.

Conclusion: The results demonstrated that the ethanolic extract of alfalfa is more effective than the methanolic extract on the K562 cell line. Therefore, *M. sativa* L. potential application in myeloid cancer therapy can be investigated in more details.

Keywords: *Medicago sativa* L. Chemical compound, Cancer cell line, K562, Herbal medicine

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Introduction

Medicago sativa, commonly known as alfalfa, has been utilized since antiquity, with its nomenclature in ancient Iran denoting "horse fodder" (1, 2). It is in diverse traditional medicinal practices worldwide, including those of China, India, North Africa, and America, that alfalfa has gained widespread recognition for its medicinal properties. This plant is replete with bioactive compounds, among which are phenols,

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flavonoids, alkaloids, saponins, phytoestrogens, coumarin, phytosterols, terpenes, and glycosides. Not only is alfalfa rich in these compounds, but it also harbors an abundance of essential vitamins, such as K, C, E, B12, B6, B2, niacin, folic acid, and pantothenic acid, as well as essential amino acids including valine, leucine, threonine, and lysine. Furthermore, it contains a diverse array of minerals, namely calcium, copper, iron, magnesium, manganese, phosphorus, zinc, and silicon compounds. It is due to this nutritional profile that the European Food Safety Authority (EFSA) has recognized alfalfa as a valuable food supplement, with numerous reported anticancer properties (3).

Chronic myeloid leukemia (CML), a clonal disorder originating from bone marrow stem cells, is characterized by an overproduction of mature granulocyte cells and their precursors (4). This myeloproliferative disorder is associated with the Philadelphia chromosome translocation, which occurs at the fusion of human chromosomes 9 and 22 (5). It is the resultant increase in Bcr-Abl tyrosine kinase inhibitor (TKI) activity that drives the uncontrolled proliferation and survival of hematopoietic cell lines (6). While laboratory animals are typically employed in *in vivo* research settings, they present challenges in controlling experimental conditions and elucidating causation. It is for this reason that *in vitro* studies often rely on cell line cultures, which offer advantages such as precise quantification, reproducibility, and controlled dosage assessments. Not only does this approach facilitate the determination of dose, concentration, and timing, but it also minimizes animal usage in cytotoxicity and drug screening studies (7, 8).

Research has documented the impact of alfalfa extract on various cancer cell lines, including MCF7, Hela, and Hacat, in *in vitro* studies (9). The anticancer mechanism involves several processes, among which are the modulation of oxidative stress, mitochondrial dysfunction, activation of oncogenes, and regulation of apoptotic pathways (10, 11). Given the pivotal role of selecting medicinal plants and appropriate solvents for their extraction, coupled with the widespread availability of alfalfa to both livestock and humans, this study aimed to identify alfalfa constituents using two different solvents and assess their efficacy against the K562 cell line. It is with the limited research on the effects of alfalfa on chronic myeloid cancer that these findings seek to shed light on potential herbal interventions for this malignancy.

Materials and Methods

Alfalfa leaves, harvested from agricultural fields in Kurdistan Province, Sanandaj, prior to flowering, were authenticated by the Agricultural Research Center of Kurdistan University (Voucher number 3879). All research procedures were conducted in accordance with the ethical guidelines approved by the ethics committee of the Islamic Azad University of Sanandaj (approval code: IR.IAU.SDJ.REC.1401.040). The primary objective of this study was to investigate the cytotoxic effects of ethanolic and methanolic alfalfa extracts on the chronic myeloid cancer cell line K562. Furthermore, the results of the MTT assay, which determined the optimal IC50 concentration, were utilized to assess cell apoptosis and necrosis through flow cytometry analysis.

Preparation of Ethanolic and Methanolic Extracts of Alfalfa

Alfalfa plants, sourced from farms in Fars province and harvested prior to the flowering stage, were processed upon approval from the Agricultural Research Herbarium Center. The green leaves were meticulously collected, cleansed with distilled water, and air-dried in a dark, cool environment for a period of three days. Subsequently, the dried leaves were thoroughly ground using an electric mill. Extraction was performed employing the maceration method with ethanol and methanol, followed by rotary

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evaporation under vacuum conditions (12).

Analysis of Extract's Active Ingredients Using Gas Chromatography Mass Spectrometry (GC-MS)

Given the absence of reactive components within the column and the extract's non-degradability at column temperatures, as established by previous studies, the GC-MS method was selected for analysis. The extract was analyzed to quantify active ingredients using a GC-MSD instrument manufactured by Agilent Technologies (USA).

Specifications of the GC-MS Device

The chromatograph is equipped with a 30-meter-long DBI column, featuring an internal diameter of 0.25 microns, coupled with a GC-MSD mass spectrometer. The column temperature is programmed to rise incrementally by 3°C per minute from an initial temperature of 20°C to a final temperature of 240°C. Helium, maintained at a pressure of 0.5 ml/min, serves as the column carrier gas, while the FID detector operates with an injection chamber temperature of 275°C and a detector temperature of 280°C. The Agilent mass spectrometer (model number G4513-80203) operates with an ionization energy of 70 electron volts. The stationary phase layer has a thickness of 0.25 microns. In the interop detectors, helium functions as the carrier gas with a flow rate of 50 mm/min. The thermal plan temperature ranges from 25°C to 50°C, increasing at a rate of 4 cm/min, while the injection chamber temperature is maintained at 360°C.

Preparation of MTT Solution

For the cytotoxicity test (MTT), three repetitions were conducted for each phase of the assay. To prepare the MTT solution, 212.5 mg of MTT powder was precisely weighed using a 000 scale, in accordance with calculations determining the appropriate volume of concentrated solution for each well and the total number of wells required. The MTT powder was transferred to a 15 mL falcon tube, to which 425 µL of RPMI 1640 medium was added, followed by the addition of 10 µL of MTT solution for each well. This procedure was repeated in its entirety three times to ensure reliability and reproducibility (13).

MTT Protocol with Extract

The experimental procedure commences with the establishment of a control group (solvent) alongside the selected concentrations of methanolic and ethanolic alfalfa extracts. Cells are prepared through a process of freezing, thawing, and passaging to achieve the desired population. Subsequently, the appropriate number of cells for seeding in each well of a 96-well plate is determined through cell counting. The cells are then subjected to treatment with various dilutions of methanolic and ethanolic alfalfa extracts at three distinct time points. After intervals of 24, 48, and 72 hours, MTT solution is introduced to each well. Following a 4-hour incubation period, the plate undergoes centrifugation, and the supernatant is removed. To facilitate the dissolution of formazan crystals formed by MTT, 200 µL of Dimethyl sulfoxide (DMSO) is added to each well, resulting in the development of a purple or amethyst coloration. The plate is then subjected to a 20-minute incubation period, followed by 20 minutes of shaking. Finally, absorbance measurements are obtained using an Enzyme-Link Immunosorbent Assay (ELISA) reader (14).

Cell Counting

The viability of cells was determined through staining with trypan blue solution, which allows for the differentiation between live and dead cells based on dye absorption. Cell counting was performed using methylene blue solution, Neubauer slides, and an inverted microscope. This method necessitated a cell density of at least 1×10^6 cells/mL, often requiring dilution of the cell suspension prior to centrifugation and re-suspension in a smaller volume (15).

Flow Cytometry Test Method

Flow cytometry was employed to measure

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cell apoptosis and necrosis, with the optimal IC50 value derived from the MTT test guiding the flow cytometry analysis. The procedural steps, cell preparation, and dilutions for the flow cytometry test mirrored those of the MTT test to ensure result validation. A 24-well plate was utilized for this purpose, with each dilution being repeated in triplicate. Flow cytometry analysis was conducted after a 72-hour period using the Santacruz dye, manufactured in the United States (16).

Statistical Analysis

Data analysis was performed using Prism 8.1 software, with results presented as mean ± standard deviation (SD). Differences between groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's test. Statistical significance was established at a P-value of <0.05.

Results

Gas Chromatographic Results

The findings of the gas chromatography test are presented in two formats. Firstly, a table detailing inhibition time and composition percentage in the library information section aids in identifying the key components of the extract. As evidenced in Table 1, the compounds Phytol and Phenol were identified as the primary active ingredients of the methanolic extract, collectively comprising 23.472% of the total composition.

Table 2 elucidates the composition of the ethanol extract. Among the identified compounds, 2-Methoxy-6-vinylphenol and Phytol collectively accounted for 0.833% of the total composition, thus establishing themselves as the most biologically active constituents of the extract. Conversely, Thiocyanic acid, while exhibiting the highest

Table 1. Gas chromatography analysis reveals the presence of active chemical elements in the methanolic extract of the alfalfa plant

Number	Compound	Percentage	Retention time
1	L-Alanin, 1,3-propane diamine	1.227	3.194
2	Glycine, Betain	17.601	3.318
3	2-pyrrolidinone,2(3H)-Furanone	10.151	13.218
4	2-piperidine carboxylic acid, Dicyandiamide	0.293	18.317
5	2-methyl piperidine	0.165	18.37
6	Methylphenidate	8.089	21.718
7	2-methoxy-4-vinyl phenol, 2,5-Diethyl-3methyl pyrazine	2.661	22.05
8	L-proline, 2-Hydroxyimidazole	6.535	25.979
9	1. piperidine ethanol	3.824	26.098
10	3-amino-s-triazol, 1-Butanamine	1.836	27.024
11	Methyl ester	2	37.672
12	Silan, Tetraethyl-	3.278	41.678
13	Tetraethylsilane	1.045	43.138
14	Thiocyanic acid, cyclopentane	0.855	44.883
15	Ethyl ester	0.2	45.934
16	Ethane, isothiocyanate, 1,3-Dioxane	2.102	46.409
17	9,12,15-octadecatrienoic acid, 1,3-cyclooctadiene	1.792	50.379
18	PHYTOL	18.474	50.682
19	Linolenic acid	7.45	52.029
20	Phenol, Hordenine, Dothiepin	2.916	54.398
21	PHYTOL	2.082	56.16
22	2-propenoic acid, Pholedrine	2.491	57.947
23	Glycerol 1-monopalmitate, Hexadecanedioic acid Stearic acid, monoglyceride	2.743	59.591

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concentration at 29.712% in the extract, was found to lack any significant active biological role.

Figure 1 presents chromatograms illustrating the active chemical elements present in the methanolic (A) and ethanolic (B) extracts of the alfalfa plant. These diagrams serve to complement the information provided in the gas chromatography table, offering a comprehensive visual representation of the composition of both extracts.

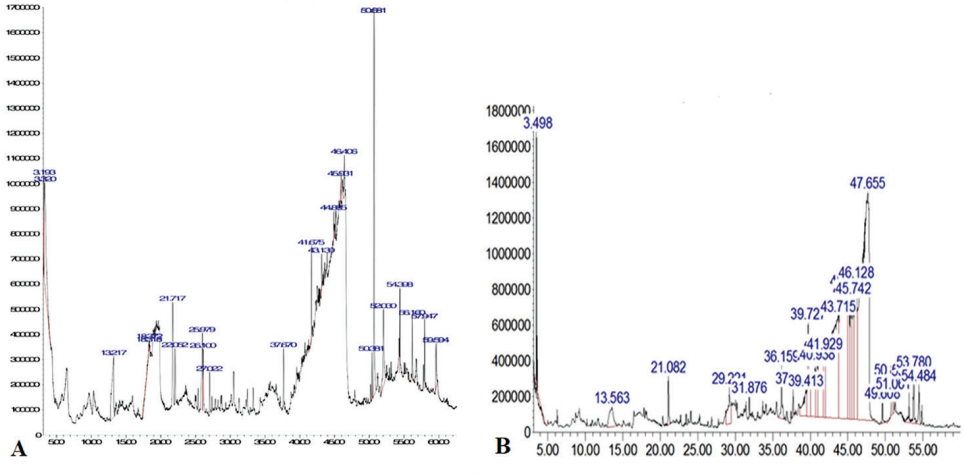


Figure 1. Comparison of gas chromatography graphs of active compounds in methanol (A) and ethanol (B) extracts of alfalfa

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MTT Test Results

The MTT test results were subjected to analysis using Prism 8.1 software, with the extract concentration serving as an independent variable. The impact of alfalfa methanolic and ethanolic extracts on the K562 cancer cell line was evaluated using one-way ANOVA followed by Tukey's post-hoc test, with statistical significance established at $P<0.05$. As evidenced in Chart 1, no significant differences in the average parameters were observed between the control and treatment groups at 24 or 48 hours ($P>0.05$). However, a significant difference emerged at the 72-hour time point ($P=0.013$) (Chart 1-D). Chart 1-E demonstrates that at concentrations of 75 µg/ml, while no significant differences were found between the control and treatment groups at 24 and 48 hours ($P>0.05$), a statistically significant difference became apparent after 72 hours ($P=0.027$). Furthermore, analysis of the data obtained at a dosage of 100 µg/ml across different time periods revealed no statistically significant changes ($P>0.05$) (Chart 1-F).

The results of the MTT test for the alfalfa ethanolic extract are depicted in Chart 2. It is noteworthy that significant differences were observed between the control and treatment groups at a dose of 50 µg/ml over a 72-hour period ($P=0.041$). However, these differences did not reach statistical significance at doses of 75 and 100 µg/ml ($P>0.05$).

The viability percentage of K562 cells following treatment with methanolic and ethanolic extracts of the alfalfa plant was determined based on ELISA findings. Analysis of the data presented in Table 3 reveals that the viability percentage of K562 cells is predominantly influenced by the duration of exposure to the extract rather than the concentration of the extract used for treatment.

It is noteworthy that the results indicated a time-dependent, rather than dose-dependent, effect on the viability percentage of K562 cells when treated with the extract (Table 4).

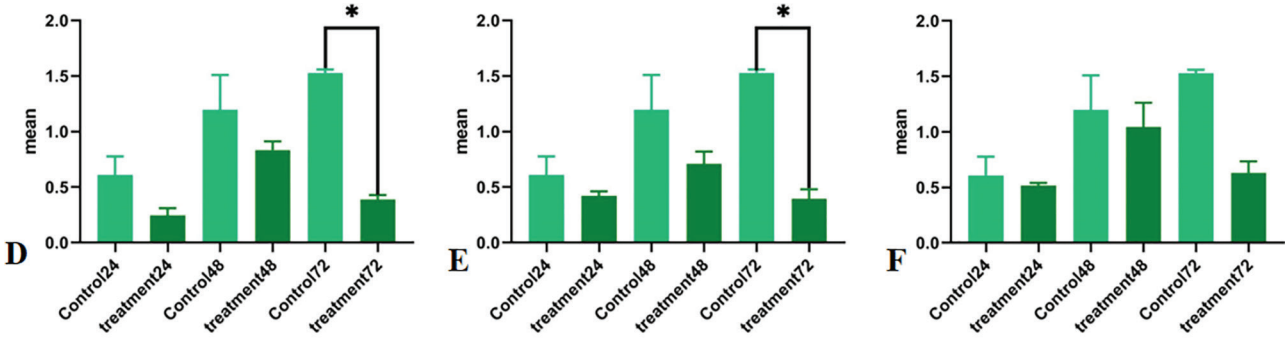


Chart 1. Comparison of the cell viability between the control group and the treatment with alfalfa methanol extract at doses of 50, 75, and 100 µg/ml (D, E, and F respectively) over 24, 48, and 72 hours in the MTT test

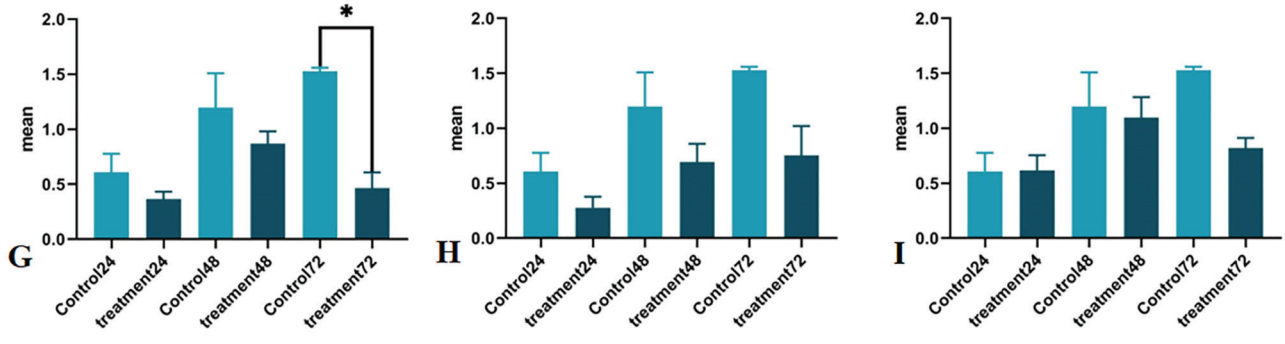


Chart 2. Comparison of the average live cells between the control group and the treatment with alfalfa ethanolic extract at doses of 50, 75, and 100 µg/ml (G, H, and I respectively) over 24, 48, and 72 hours in the MTT test

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Table 3. Viability Percentage of K562 Cells after Treatment with Alfalfa Methanolic Extract Based on Concentration and Time

Concentration (µg/mL)	24 h	48 h	72 h
50	40.2%	69.7%	35.6%
75	69.4%	59.3%	38.2%
100	85.2%	87.4%	57.7%

Table 4. Viability Percentage of K562 Cells after Treatment with Alfalfa Ethanolic Extract Based on Concentration and Time

Concentration (µg/mL)	24 h	48 h	72 h
50	45.6%	57.8%	42.6%
75	60.2%	72.7%	69%
100	89.1%	91.7%	75.2%

Table 5. IC_{50} Values for Alfalfa Ethanolic and Methanolic Extracts across Three Different Time Intervals

Time	IC_{50} methanolic Ex	IC_{50} ethanolic Ex
24 h	40.48	41.70
48 h	23.70	32.53
72 h	9.45	19.30

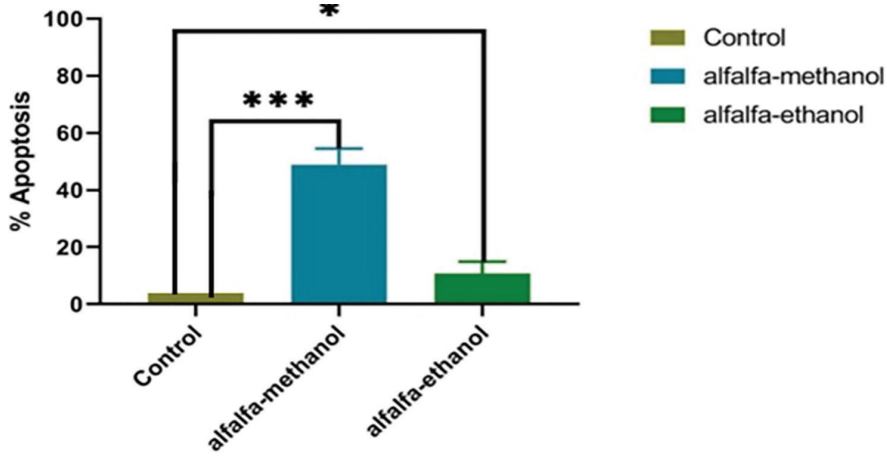


Chart 3. Comparative changes in the percentage of apoptosis in K562 cells with methanol and ethanol extracts of alfalfa
*Significance level P<0.05; *** Significance level P<0.001

Flow Cytometry Test Results

The IC50 of the extract was determined through triplicate dose-response analyses using data obtained from Prism 8.1 software. As evidenced by the results presented in Table 5, the IC50 values were attained following a 72-hour treatment period, with methanolic extract yielding a value of 9.45 µg/ml and ethanolic alfalfa extract yielding 19.3 µg/ml.

Cell culture and treatment were conducted in accordance with the established protocol, utilizing the optimal IC50 values obtained for both extracts. After a 72-hour incubation period, the plate was subjected to staining and outcome assessment. Subsequently, the flow cytometry test results underwent analysis using FlowJo software. The analysis revealed that the alfalfa methanolic extract induced early apoptosis in 49.16% of cells and late apoptosis in 15.42% of cells. These differences were found to be statistically significant in the treatment group compared to the control group for both extracts, as confirmed by statistical testing (Chart 3, Figure 2).

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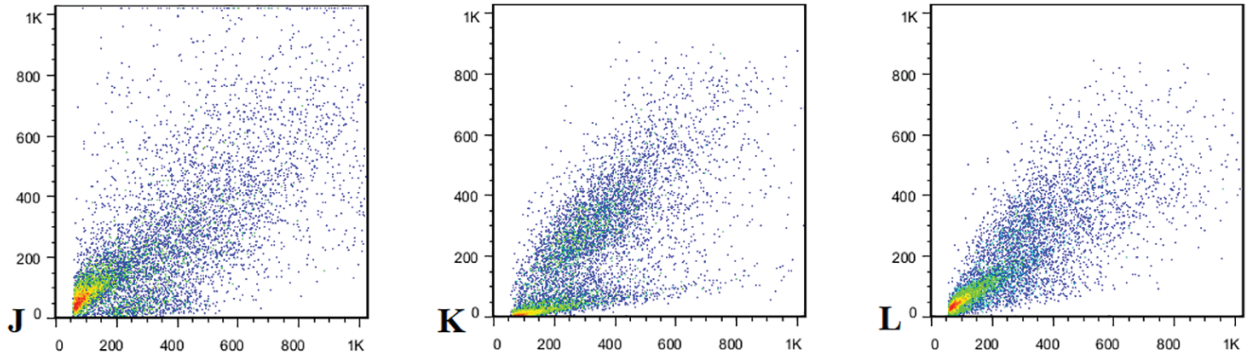


Figure 2. Comparison of the flow cytometry diagrams among the control (J), methanol (K), and ethanol (L) extracts of alfalfa

Discussion

CML, a genetic-blood abnormality, is significantly influenced by environmental factors in its onset and progression (17). Among these factors, oxygen free radicals (ROS) play a pivotal role in the pathways associated with cancer cell proliferation (18). Oxygen and nitrogen free radicals (RNS), generated as byproducts of cellular metabolism, are continually produced within biological systems, leading to damage of DNA, proteins, and lipids. Furthermore, ROS participate in enzymatic reactions, message transmission, and activation of nuclear transcription factors (19). Indeed, ROS modulate cellular metabolism and function by acting as secondary messengers in cellular signaling pathways, with effects including apoptosis induction, activation of host defense genes, and modulation of ion transport systems (20). Numerous plants contain phytochemicals that have been scientifically validated to inhibit the growth of cancer cells (21). Among these, phenols, terpenes, flavonoids, alkaloids, and other potent molecules have been identified as substances capable of exerting anticancer effects (22).

Throughout history, alfalfa has held a prominent position as one of the most widely utilized herbs. Hailed as the "king of cereals," it has served both as livestock feed and as a remedy for various ailments (23). Researchers have explored the effects of aqueous, alcoholic, and hydroalcoholic alfalfa extracts in several studies, with recent discussions focusing more on its potential impact in cancer research (24). Historically, however, research primarily centered on its anti-inflammatory and antioxidant properties, as well as its positive effects on various tissues (12). Despite its longstanding role and numerous benefits in both animal and human diets, the anticancer potential of alfalfa can only be fully realized through the correct extraction of its essential components, such as phenols and alkaloids, and their appropriate dosage (25). Alfalfa contains a plethora of compounds, including phenols, flavonoids, alkaloids, terpenes, glucosides, and saponins, all of which have demonstrated medicinal benefits. With its high protein content, alfalfa is poised to replace conventional protein sources in the future (26).

In our study, the predominant compounds identified in the ethanolic and methanolic extracts of alfalfa were silane, propionic acid, thiocyanic acid, and phytol. However, some components were unique to either the ethanol or methanol extracts. While the methanolic extract contained elements such as L-alanine, L-proline, linoleic acid, betaine, hydroxy imidazole, glycine, and phenols, the ethanol extract included compounds such as acetic acid, DL-homoserine, capric acid, arginine, isoquinoline, and cobalt. Furthermore,

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phytol and phenol, comprising approximately 23.5% of the methanolic extract of alfalfa, emerged as the most beneficial chemicals with significant biological roles. In contrast, only 0.83% of phytol was recovered from the ethanolic extract. Thiocyanic acid, known for its iodine chelation, antimicrobial, and anti-inflammatory properties, constituted the highest percentage of compounds (54.5%) isolated (27). This substance was detected in an extract with a methanol concentration of 9%. Consequently, we emphasize the biological significance of the methanolic extract *in vivo* research compared to the ethanolic extract. It is noteworthy that phytol exhibits cytotoxic, antioxidant, and autophagy- and apoptosis-inducing functions (28).

Studies by Yang and Weisburger have investigated the impact of antioxidants, phenolic compounds, isoflavonoids, and phytol on cancer cell proliferation. Furthermore, Frei et al. discovered that polyphenols possess antioxidant properties, mitigating DNA damage by inhibiting enzymes involved in oxidative stress and interrupting oxidation chain reactions through the formation of stable phenoxyl radicals (29, 30). The present study corroborates these findings. Due to its active chemical constituents, alfalfa has garnered attention for the treatment of various diseases. Nasir et al. examined the effects of alfalfa extract on liver, kidney, and ovarian function in rats, noting the optimal response at high extract doses (31). Similarly, Raeeszadeh et al. investigated the protective effects of alfalfa extract on the liver and kidney of rats exposed to nicotine and HgCl2 in a dose-dependent manner (32, 33). In contrast to recent studies, Raeeszadeh et al. reported their results in a dose-dependent manner, suggesting differential responses of cancer cells compared to healthy cells to drug compounds. It is worth noting that pharmaceutical agents play a pivotal role in targeted therapies for cancer cells (34).

In recent decades, there has been a significant expansion of *in vitro* research in cancer treatment, including investigations into various plant extracts. For instance, Moalemzadeh et al. studied the hydroalcoholic extract of Artemisia sieberi on the SKBR3 cancer cell line, revealing time-dependent rather than dose-dependent effects (35). Similarly, Kuti et al. (2017) demonstrated the efficacy of alkaloids and phytoestrogens in alfalfa for breast cancer treatment, while Babakhani et al. examined the effect of alfalfa ethanol extract on the MCF7 cancer cell line (36, 37). The time-dependent findings in the study by Babakhani et al. (2019) align with recent research. In a similar vein, Waziri Javid et al. investigated the effect of panicic acid from pomegranate seed oil on the K562 cell line, reporting the best IC50 value after 72 hours (38). Badrhadad et al. evaluated the effect of elderflower fractions on the K562 cancer cell line, noting the most favorable response after 72 hours and with higher extract concentrations (39). Aslani et al.'s study on the hydroalcoholic extract of oregano plant on the K562 cancer cell line also confirmed the time-dependent effects of the extract (40).

In this study, the transformation method was utilized to evaluate the viability of treated cells. One approach to determining the cell viability rate compared to the apoptosis rate of treated cells in a microtiter plate experiment is to estimate the cell count (41). The findings of the MTT test in our study revealed the most pronounced percentage of time-dependent and dose-independent effects of ethanolic and methanolic alfalfa extracts. The concentrations of alfalfa methanolic extract (IC50=9.45 µg/ml) and ethanolic extract (IC50=19.3 µg/ml) in the cells were determined. Thus, the methanolic extract exhibited greater efficiency compared to the ethanolic extract. It could be argued that halving the amount of ethanolic extract would double the IC50 level of the methanolic extract. According to the study results, apoptosis levels in cells treated with methanolic alfalfa extract were 49.16%, compared to 15.42% in cells treated with ethanolic

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alfalfa extract. This indicates that the methanolic extract is approximately 3.2 times more effective at inducing cellular apoptosis than the ethanolic extract. The efficacy of the extract is also time-dependent, with peak effectiveness observed at 72 hours, suggesting that prolonged exposure to the extract's active and apoptotic compounds enhances its effectiveness (37). However, the extract's response is not dose-dependent, likely due to the presence of toxic and ineffective compounds that can have harmful effects at higher concentrations. Saponins in alfalfa extract, at high doses, may inhibit oxidative stress-induced cell apoptosis through the MAPK signaling pathway (42, 43). Consequently, high doses of both ethanolic and methanolic extracts were ineffective in inducing apoptosis in the cell lines studied.

Conclusion

The study results demonstrated that the methanolic alfalfa extract was approximately 3.2 times more effective than the ethanolic extract in inducing apoptosis in K-562 cells, with its IC50 being half that of the ethanolic extract. The efficacy of the extract was found to be time-dependent but not dose-dependent for the K-562 cell line. Phytol, present in higher concentrations in the methanolic extract compared to the ethanolic extract, showed significant activity. Given the availability of alfalfa as a medicinal plant and its potent compounds, it is recommended for the prevention of CML. Further cellular and molecular research on the components of alfalfa extract, as well as studies investigating its mechanisms of action, are advised to facilitate compound purification and drug development.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Conflict of interest

None of the authors have stated a conflict of interest.

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Ethical Considerations

The ethical principles of working on cell lines were followed based on international standard guidelines.

Code of Ethics

All research procedures were approved by the ethics committee of the Islamic Azad University of Sanandaj under the code IR.IAU.SDJ.REC.1401.040.

Author Contributions

Conceptualization, data curation, formal analysis, investigation, methodology, and project administration: M. R and S. K, Software, supervision, validation, and visualization: M. R, A. A, Writing original draft: M. R, S. K, and H. K, Review and editing: M. R, H. K, and AA. A.

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