Comparative Assessment of the Cytotoxic Effects of Aqueous and Ethanolic Extracts of Artemisia herba-alba on HBL100 Normal Cells and HCAM Hepatocellular Carcinoma Cells

Rasha Hattab AL-Asadie 1*., Nasir AbdAli AL-Mansour., Lina Abbas Naser 2*

.1 Department of Biology , College of Science , University of Basrah, Iraq

.2 Dpartment of Microbiology,College of Medicine, University of Basrah, Iraq

Email: rasha.hattab@uobasrah.edu.iq

Abstract

This study investigates the differential cytotoxic effects of aqueous and ethanolic extracts of Artemisia herba-alba against hepatocellular carcinoma (HCAM) cells in comparison with normal epithelial HBL100 cells , Both plant extracts were prepared using standard aqueous and ethanol-based extraction procedures to evaluate how solvent polarity influences the biological activity of the resulting extracts , Cytotoxicity was assessed using the MTT assay across two concentrations (2000 and 4000 µg/ml) , The findings demonstrated that both extracts inhibited HCAM cell proliferation in a concentration-dependent manner; however, the aqueous extract exhibited markedly stronger cytotoxicity than the ethanolic extract , Conversely, both extracts showed minimal inhibitory activity toward the normal HBL100 cell line, indicating a degree of selectivity toward malignant cells , These results suggest that Artemisia herba-alba particularly its aqueous extract contains bioactive phytochemicals with promising anticancer potential and may serve as a useful candidate for developing natural therapeutic agents targeting liver cancer.

Keywords: Artemisia herba-alba, aqueous extract, ethanolic extract, hepatocellular carcinoma, HCAM cell line, HBL100, cytotoxicity, antioxidant phytochemicals.

Introduction

Liver cancer remains one of the most prevalent and life-threatening malignancies worldwide, and its management continues to pose significant clinical challenges due to limited treatment options, high recurrence rates and poor prognosis (10) , Consequently, there is growing scientific and pharmaceutical interest in exploring natural products as alternative or complementary anticancer agents (8) , Among medicinal plants species belonging to the genus Artemisia (family Asteraceae) have gained considerable attention owing to their rich phytochemical profiles and documented pharmacological activities , Several Artemisia species have demonstrated potent cytotoxic, antiproliferative, and pro-apoptotic properties across various cancer models (7) These biological activities are largely attributed to the plant's rich content of active compounds, particularly sesquiterpene lactones such as artemisinin and artemisinic acid, as well as flavonoids, phenolic acids, and essential oils, which have been shown to induce apoptosis, generate reactive oxygen species, and suppress the proliferation of cancer cells (14) , Prior studies have reported that ethanolic extracts of Artemisia herba-alba possess notable anticancer activity against hepatocellular carcinoma and cholangiocarcinoma cell lines , primarily through mechanisms involving oxidative stress induction, mitochondrial dysfunction, and apoptosis activation (21) , Furthermore, aqueous and ethanolic extracts

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of A. herba alba have exhibited selective cytotoxicity against cancer cells while sparing normal cell lines, suggesting the presence of bioactive compounds with targeted anticancer effects (20) , Despite the promising evidence comparative evaluations of aqueous versus ethanolic extracts of A. herba alba particularly regarding their selective effects on liver cancer cell lines and corresponding normal cells remain limited , Therefore, the present study aims to assess the cytotoxic and selective inhibitory effects of aqueous and ethanolic extracts of A. herba alba against HCAM hepatocellular carcinoma cells and HBL100 normal epithelial cells , This comparison provides valuable insight into the influence of extraction solvent on phytochemical potency and cancer cell selectivity (15. (

Figure (1): Artemisia herba alba

Materials and Methods

Plant Material and Preparation of Extracts

Fresh aerial parts (leaves) of Artemisia herba-alba were collected dried at room temperature and finely powdered , Two extraction procedures were conducted to examine the effect of solvent polarity on the bioactivity of the obtained extracts.

.1Aqueous Extract Preparation

The aqueous extract was prepared following the method of (5) with slight modifications , Briefly, 10 g of dried plant powder were mixed with 500 ml of cold distilled water and stirred for 15 minutes using a magnetic stirrer , The mixture was allowed to stand for 30 minutes to facilitate sedimentation , The suspension was filtered sequentially through medical gauze and Whatman filter paper to remove debris , The filtrate was centrifuged at 3000 rpm for 15 minutes and the supernatant was concentrated under reduced pressure using a rotary evaporator at 45°C until a semi-solid extract was obtained , The final extract was stored at 4°C in sterile containers until use.

.2Ethanolic Extract Preparation

Ethanolic extraction was carried out using a Soxhlet apparatus. A total of 20 g of dried A. herba-alba powder were placed in a thimble and extracted with 400 ml of 95% ethanol for 24 hours at a temperature not exceeding 45°C , The resulting extract was evaporated at 45°C using a rotary evaporator to remove ethanol , The crude extract was dried completely weighed and stored at 4°C for subsequent experiments , Extraction was repeated when necessary to obtain sufficient extract quantities.

Culturing of Cell Lines and Cell Toxicity Bioassays

Special Solutions for Primary and Secondary Tissue Culture

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Stock Solution Used for Tissue Culture and Subculture

• Rosswell Park Memorial Institute (RPMI-1640) Medium

• RPMI-1640 with buffer, L-glutamine (10.4 g(

• Sodium bicarbonate (4.4%) – 15 mL

•)used to adjust the pH to 7.2(

• Penicillin – 0.5 mL

• Streptomycin – 0.5 mL

• Fetal Bovine Serum (FBS) (100 mL(

Complete the solution to one liter by adding distilled water, then sterilize with a 0.22 micron filter.

• Antibiotics

a- Penicillin solution containing 1 gram of penicillin in 5 mL of distilled water, then take 0.5 mL of it and add to one liter of culture medium and store the remainder at -20°C.

b- Streptomycin solution containing 1 gram of streptomycin in 5 mL of distilled water to bring the final concentration to 200 mg/mL, then take 0.5 mL of it and add to one liter of culture medium and store the remainder at -20°C.

• Phosphate Buffer Saline (PBS(

Nacl 8 gm

KCL 0.20 gm

NaHPO4 0.92 gm

KH2PO4 0.20 gm

D.W. 100 µl

Sterilize in an autoclave at 121°C for 15 minutes, then store at 4°C until use.

• Trypsin - Versene solution

Trypsin solution 20 µl

Versene solution 10 µl

PBS 370 µl

Mix before use under sterile conditions and store at 4°C.

Cell Lines Used

Two cell lines were used in this study:

HBL-100: A normal human cell line derived from human milk epithelial cells during early lactation, showing no malignant features and maintaining a normal diploid karyotype.

HCAM (Hepatocyte carcinoma cell line): A liver cancer cell line isolated from hepatocellular carcinoma tissue .

Both cell lines were routinely maintained and subcultured to preserve their growth and viability.

Cell Culture Procedure

The HBL-100 and HCAM cell lines were obtained from the Iraqi Biotechnology Cell Bank (IRAQ Biotech) in Basrah Governorate. Upon arrival at the laboratory, the cells were subcultured at the sub-confluence stage using a 0.25% trypsin–EDTA solution for cell detachment. The cells were then transferred to sterile culture flasks containing 15 ml of RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml of penicillin, and 100 µg/ml of streptomycin until they reached 80–90% confluence the mixture was gently mixed and then sterilely transferred to culture plates or sterile flasks, upon reaching full confluence (80-90%) , The culture medium was then aspirated, and the cell monolayer was washed with phosphate-buffered saline (PBS) Afterward, 1 ml of trypsin–EDTA was added, and the cells were incubated at 37°C for 2–5 minutes to allow complete detachment. The suspension was centrifuged, the supernatant discarded, and the cell pellet resuspended in fresh medium and

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redistributed into new culture flasks or plates for further subculturing , The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (12 . (

Maintenance of Cell Cultures

The cell lines were routinely maintained to ensure their viability and stability The growth and morphology of the cells were monitored daily under an inverted microscope to assess cellular shape, confluence, and the absence of contamination indicators, The culture medium was replaced every 2–3 days, or whenever the color of the phenol red indicator changed, signaling nutrient depletion. Once the cells reached the optimal growth stage (subconfluence), they were subcultured following standard aseptic protocols (1. (

Cell Counting and Determination of Cell Density

Cell viability and density were estimated according to the protocol described by (16) , A 20 µl aliquot of the cell suspension was mixed with 20 µl of 0.4% trypan blue stain, and viable and non-viable cells were counted using a Neubauer hemocytometer under an inverted microscope at ×10 and ×40 magnifications. Viable cells appeared colorless, whereas non-viable cells were stained blue The cells were then seeded at a density of 1×10^5 cells/ml (100 µl per well) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24–48 hours until a uniform monolayer was formed, suitable for subsequent .

Cytotoxicity test using MTT

)a) culture phase (Seeding(

The cells were harvested after reaching a homogeneous layer, washed twice with PBS solution, then 1 ml of trypsin was added to remove it, and the reaction was stopped by adding growth medium containing FBS. The cells were resuspended and distributed into 96-well plates at a density of 1×10^6 cells per well in 100 µL per well.

) b) Exposure Phase

Cells were exposed to aqueous and alcoholic extracts of both artichoke and wormwood at specified concentrations 2000 and 4000 µg (mL) to evaluate their toxic effects, Each compound was applied separately to cells of each cell line.

MTT Test Protocol

After 72 hours of exposure to the compounds, the medium was discarded and the cells were washed with 100 µL of PBS, The MTT solution (mg/ml) was counted in filtered PBS, and 10 µL of it was added to each well, along with 90 µL of serum-free medium, The plates were incubated for 2 hours at 37°C. The medium was then replaced with 130 µL of DMSO to dissolve the Formazan crystals, and incubation was carried out for 20 minutes at room temperature (6) (Absorbance was measured at 620 nm using a plate reader (BioTek), USA), The assay was performed in triplicate, and the cell growth inhibition rate (cytotoxicity ratio) was calculated as follows:

) PR)= A/B*100

)PR) : Proliferation rate

A: Average optical density of untreated wells

B: Optical density of treated wells

The inhibition rate can be calculated from: IR=100-PR

Results:

The Effect of Aqueous and Ethanolic Extracts of Artemisia herba alba on the Normal Cell Line HBL100

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The cytotoxic effects of the aqueous and ethanolic extracts of Artemisia herba alba leaves on the normal cell line HBL100 were evaluated using the MTT assay at two concentrations (2000 and 4000 µg/ml), with four replicates for each concentration. Absorbance was measured at a wavelength of 620 nm, and untreated cells were used as the control group, which recorded an absorbance value of 0.92586 nm.

The effect of Aqueous and Ethanolic extracts of Artemisia herba-alba on normal cell line HBL100:

Table (1) presents the effect of aqueous and ethanolic extracts of Artemisia herba-alba at two concentrations (2000 and 4000 µg/ml) on normal breast cell line HBL100, using the MTT assay, which measures the viability of cells through absorbance at 620 nm.

Aqueous Extract: The results show that the absorbance rate decreased from 0.74875 at 2000 µg/ml to 0.50375 at 4000 µg/ml, indicating an increase in the inhibitory effect with increasing concentration , Accordingly, the inhibition rate rose from 19.129% to 45.591%, suggesting that the aqueous extract exhibited a concentration-dependent reduction in cell viability , The low standard deviation values (0.0814 and 0.0791) indicate good reproducibility and consistency among the experimental replicates.

Ethanolic Extract: For the ethanolic extract, the absorbance values were 0.8325 and 0.725 at 2000 µg/ml and 4000 µg/ml, respectively , Although a slight decrease in absorbance was observed with increasing concentration, the corresponding inhibition rates (10.084% and 21.694%) were lower than those recorded for the aqueous extract , This finding implies that the ethanolic extract exerts a weaker inhibitory effect on normal cells ,The standard deviation values (0.0617 and 0.0656) further confirm the reliability of the data.

Comparison Between Extracts

A comparative analysis indicates that the aqueous extract produced higher inhibition rates than the ethanolic extract at both tested concentrations, particularly at 4000 µg/ml (45.591% and 21.694%) , This difference could be attributed to the distinct phytochemical profiles of the two extracts; water may extract more hydrophilic bioactive compounds responsible for stronger cytotoxic or antiproliferative activity

Table (1): The effect of Aqueous and Ethanolic extracts of Artemisia herba alba on normal cell lin HBL100

Extract type	Aqueous Extract		Ethanolic Extract	
Concentration	2000	4000	2000	4000
Rate of absorption	0.74875	0.50375	0.8325	0.725
Rate of inhibition	19.129	45.591	10.084	21.694
SD±	0.0814	0.0791	0.0617	0.0656
Control	0.92586			
Type of test	MTT assay			
Absorbances	620 nm			
Cell line	Normal Cell Line			HBL100

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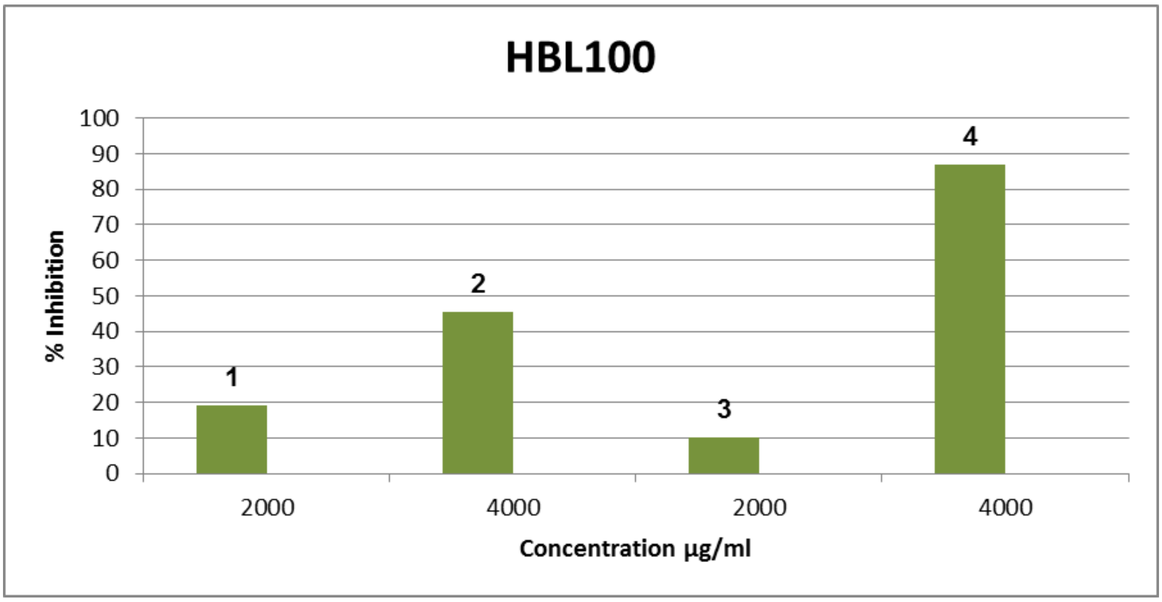

Figure (2): The effect of Aqueous and Ethanolic extracts of Artemisia herba alba on normal cell lin HBL100 : 1 and 2 aqueous extract , 3 and 4 ethanolic extract .

The Effect of Aqueous and Ethanolic Extracts of Artemisia herba alba on the Hepatocellular Carcinoma Cells HCAM .

The presented data in Table (2) illustrate the cytotoxic effects of both aqueous and ethanolic extracts of Artemisia herba alba at two concentrations (2000 and 4000 µg/ml) on the Hepatocyte Cancer Cell Line (HCAM) using the MTT assay at 620 nm.

Aqueous Extract: At a concentration of 2000 µg/ml, the aqueous extract showed an absorbance of 0.46300, corresponding to an inhibition rate of 14.84%, indicating a mild cytotoxic effect. However, increasing the concentration to 4000 µg/ml markedly decreased the absorbance to 0.23275, resulting in a significantly higher inhibition rate of 57.19% , This demonstrates a dose-dependent inhibitory effect, suggesting that higher concentrations of the aqueous extract possess greater anticancer activity , The corresponding standard deviations (SD± = 0.0084 and 0.0325) indicate good reproducibility of the data.

Ethanolic Extract: For the ethanolic extract, the absorbance at 2000 µg/ml was 0.48800, with a relatively low inhibition rate of 10.24%, showing limited cytotoxicity at lower concentration , When the concentration was doubled to 4000 µg/ml, the absorbance decreased to 0.33475, with an inhibition rate of 38.43%. Although this also reflects a concentration-dependent increase in cytotoxicity, the ethanolic extract exhibited lower overall inhibitory potential compared with the aqueous extract at equivalent concentrations. The standard deviations (SD± = 0.0328 and 0.0526) further support the reliability of these measurements.

Comparative Analysis :

Comparing both extracts, it is evident that the aqueous extract exhibited stronger cytotoxic activity against the HCAM cell line than the ethanolic extract, particularly at higher concentration (4000 µg/ml) , The marked difference in inhibition rates (57.19% and

38.43%) suggests that water-soluble bioactive compounds in Artemisia herba alba may be more effective in suppressing the proliferation of hepatocellular carcinoma cells than those extracted in ethanol.

Table (2): The effect of Aqueous and Ethanolic extracts of Artemisia herba alba on Hepatocyt Cancer Cell Line HCAM.

Extract type	Aqueous Extract		Ethanolic Extract	
Concentration	2000	4000	2000	4000
Rate of absorption	0.46300	0.23275	0.48800	0.33475
Rate of inhibition	14.840	57.190	10.2425	38.4290
SD±	0.0084	0.0325	0.0328	0.0526
Control	0.54368			
Type of test	MTT assay			
Absorbances	620 nm			
Cell line	HEPATOCYT Cancer cell line			HCAM

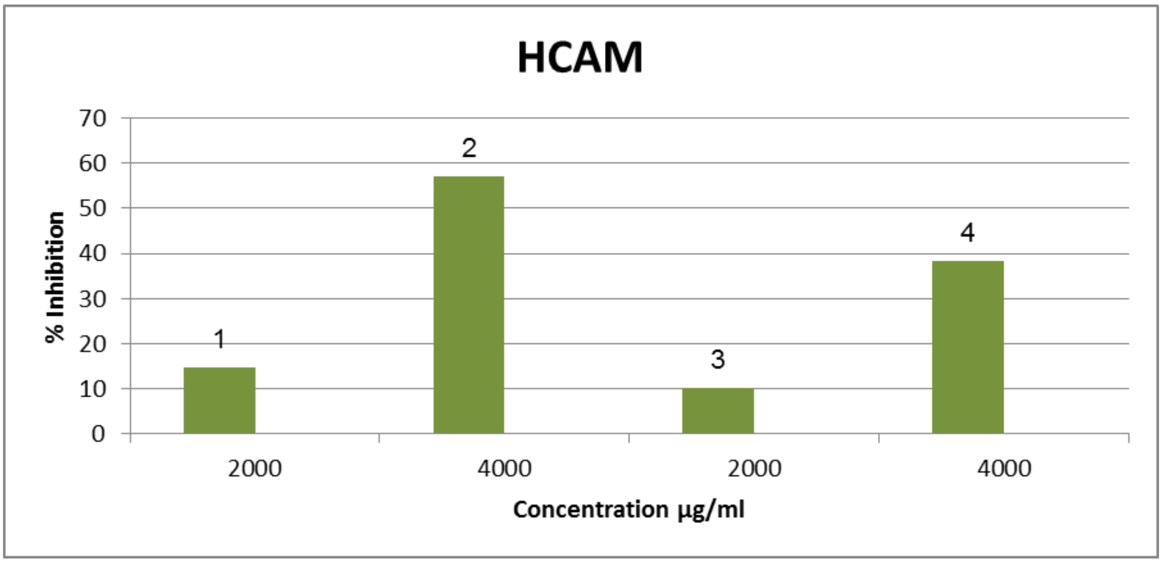

Figure (3): The effect of Aqueous and Ethanolic extracts of Artemisia herba alba on Hepatocyt cancer cell line HCAM : 1 and 2 aqueous extract , 3 and 4 ethanolic extract .

The results indicate that Artemisia herba alba possesses promising anticancer potential, especially in its aqueous form, which demonstrated a dose-dependent cytotoxic effect against hepatocyte cancer cells. This suggests that polar phytochemical constituents, such as flavonoids, phenolic acids, or other hydrophilic compounds, may play a major role in the observed inhibitory activity, These findings suggest that the bioactive constituents responsible for the cytotoxic activity of Artemisia are predominantly polar in nature , which explains the superior performance of the aqueous extract compared with the ethanolic extract , Polar phytochemicals particularly phenolic acids, flavonoids, Terpenoids and glycosidesare known to dissolve more efficiently in water resulting in higher extraction yields and enhanced biological potency , The elevated concentrations of these hydrophilic compounds in the water extract may therefore account for its stronger anticancer activity , Moreover, a possible synergistic interaction among these molecules could further enhance the extract's ability to induce cytotoxicity in hepatocellular carcinoma cells.

Discussion

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The findings of the present study demonstrate that Artemisia herba alba extracts possess pronounced cytotoxic activity against hepatocellular carcinoma (HCAM) cells, with a clear dependence on extract type and concentration , notably the aqueous extract exhibited stronger inhibitory effects on cancer cells compared with the ethanolic extract suggesting that solvent polarity plays a crucial role in determining the extraction efficiency of bioactive compounds with anticancer properties (14) , The enhanced cytotoxicity of the aqueous extract aligns with previous reports indicating that water soluble phytochemicals particularly phenolic acids (ex: Caffeic acid, Chlorogenic acid, Rosmarinic acid, Ferulic acid) and flavonoids (ex: Quercetin ,Rutin,Luteolin,Apigenin) and Terpenoids (Artemisinin) and glycosylated derivatives contribute substantially to the anticancer potential of Artemisia species (2) , Such compounds are known to exert multiple biological effects, including modulation of oxidative stress responses, inhibition of tumor proliferation, and induction of apoptosis , The markedly higher inhibition rate observed at 4000 µg/ml supports the hypothesis that increasing concentrations of polar constituents intensify the disruption of cancer cell viability (19) , A key mechanism underlying the observed cytotoxicity may involve redox imbalance within cancer cells Polyphenolic compounds can act as antioxidants under physiological conditions; however, within cancer cells where basal reactive oxygen species (ROS) levels are already elevated these compounds may shift toward a pro-oxidant role (21) , This transition triggers mitochondrial dysfunction, promotes oxidative damage, and activates intrinsic apoptotic pathways such dual antioxidant pro-oxidant behavior has been widely linked to the selective cytotoxicity of many plant-derived extracts , The reduced sensitivity of the normal HBL100 cell line to both extracts particularly at lower concentrations reinforces the selectivity of A. herba alba constituents toward malignant cells (15. (

In addition to oxidative stress–mediated apoptosis, several other mechanisms may contribute to the anticancer potential of A. herba-alba phytochemicals such as sesquiterpene lactones, coumarins, and flavonoids have been reported to modulate critical molecular pathways involved in tumor progression (4) , These include inhibition of PI3K/AKT/mTOR signaling, suppression of NF-κB activation, and attenuation of inflammatory cytokine production all of which are frequently upregulated in hepatocarcinogenesis (11) , Downregulation of these pathways can reduce tumor cell proliferation, limit angiogenesis, and enhance sensitivity to apoptotic signals , Another important aspect is the potential of A. herba-alba extracts to induce cell cycle arrest, particularly at the G1 or G2/M checkpoints by interfering with cyclin-dependent kinases and checkpoint regulators (18) , the extracts may halt DNA replication or mitotic progression, thereby reducing overall proliferation rates. This mechanism has been described in related Artemisia species and is consistent with the dose-dependent inhibition observed in the current study (17. (

The differential cytotoxic profiles of the aqueous and ethanolic extracts further underscore the significance of solvent selection in plant-based anticancer research , aqueous extraction typically enriches hydrophilic compounds with strong

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antioxidant and cytotoxic properties whereas ethanol extracts tend to concentrate terpenoids, sterols, and other lipophilic constituents that may exert weaker direct cytotoxicity or act through alternative pathways (3) , Similar solvent-dependent variations have been reported in multiple studies evaluating Artemisia species highlighting the need for comprehensive phytochemical analyses to elucidate the active constituents responsible for the observed effects , Overall, the current findings support the potential of A. herba-alba as a promising natural source of anticancer agents, particularly in the context of liver cancer (13) , The selective cytotoxicity toward HCAM cells, combined with minimal effects on normal HBL100 cells, underscores its therapeutic relevance. Nevertheless, further research is required to clarify the precise molecular mechanisms underlying its activity, identify the specific bioactive compounds involved, and assess in vivo efficacy and safety in animal models , additionally future studies evaluating extract combinations or synergistic interactions with existing chemotherapeutic agents may broaden the clinical applicability of this medicinal plant (9. (

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