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Reseacrh Article

Echinococcus granulosus Cyst Wall Antigens as Potential Anticancer Agents: Protein Characterization and Cytotoxic Effects on a Human Colorectal Cancer Cell Line

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Abstract

Colorectal cancer is among the most common cancers worldwide and is the second leading cause of cancer-related mortality. Early detection and screening are crucial for reducing both incidence and mortality. Parasitic diseases may induce anticancer effects through interactions between the parasite and its host. Accumulating scientific evidence has proven the validity of this concept, as some parasites have shown anticancer activity in vitro. This research aims to prepare and characterize hydatid cyst wall antigens by determining protein concentrations and analyzing them using electrophoresis, as well as evaluating their cytotoxic activity in a human colorectal adenocarcinoma cell line (HRT-18) compared with that in a normal human cell line (NHF). Methods: In this study, the Lowry method was used to measure protein concentration, and electrophoresis and MTT assays were used to determine the toxicity and IC50 values of the cyst wall antigens in both cancer and normal cell lines. The results revealed a dose-dependent cytotoxic effect that was significantly greater at the highest antigen concentration (100 µg) for colorectal cancer cells than for normal cells, while the effect was less pronounced on normal cells. The IC50 value of the cyst wall antigens on cancer cells was 24.0 µg/ml, whereas it was 100.8 µg/ml for normal cells, with a high selectivity index (SI) of 4.2, which indicated that the cyst wall antigens selectively target cancer cells while sparing normal cells. Conclusion: Antigens extracted from the cyst wall, including cyst wall antigens, are more cytotoxic to cancer cells than to normal cells; thus, these antigens are potential antitumor agents and warrant further investigation.

Keywords
Cyst wall antigens, Cytotoxicity, Echinococcus granulosus, MTT assay, SDS-PAGE

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Introduction:

Cystic echinococcosis caused by Echinococcus granulosus is a zoonotic disease, indicating that it can cause serious illness in both humans and animals. It is found on every continent except Antarctica. Infection with these parasites is a serious health problem, leading to high morbidity and mortality rates, as well as considerable economic losses in the livestock sector. [1] This disease is often neglected in many poor pastoral areas and affects various animal species, such as wild animals and livestock, leading to high rates of infection and mortality. In addition, the damage caused by tapeworm larvae poses a threat to human health. [2] It causes the formation of hydatid cysts in various organs of the body, with the liver and lungs being the most commonly affected. In severe cases, the infection can spread to the heart, brain, or bones. Symptoms vary from person to person, depending on the location and size of the cyst. [3] Most cases are asymptomatic. However, these cysts can compress organs and body structures, potentially displacing them and causing abdominal pain, discomfort, nausea, and vomiting. A rare complication of hydatid infection is the rupture of the cysts, leading to the spread of infection through implantation in the serous membranes of other organs. This can also cause anaphylactic shock due to leakage of fluid from the ruptured cyst. [4][5] Infected animals (definitive hosts) shed large numbers of E. granulosus eggs into the environment, such as through drinking water, cultivated vegetables, and farmland. These eggs can remain infectious for several months under suitable environmental conditions. [6] Dogs are the definitive host in the life cycle of Echinococcus granulosus, whereas ungulates are the primary intermediate hosts for this parasite. [7] Human infection occurs as an accidental host through direct contact with infected dogs, ingestion of food or water contaminated with parasite eggs, or transfer of eggs from hand to mouth. [6]

The hydatid cysts represent the larval stage of E. granulosus. [8] The hydatid cyst consists of an outer host-derived adventitial layer, a middle laminated layer secreted by the parasite, and an inner germinal layer [9] The innermost germinal layer of the hydatid cyst, measuring 20–25 micrometres in thickness, is the living part of the cyst wall. It is responsible for regulating cyst permeability, supporting asexual reproduction, and producing the fluid that fills the cyst cavity. [10]

Cancer is characterized by the uncontrolled growth of certain body cells, which can invade surrounding tissues and spread to other parts of the body. [11] It is the second leading cause of death worldwide. [12] Well-known risk factors for cancer include genetic predisposition, exposure to ionizing radiation, tobacco use, infections, unhealthy dietary patterns, alcohol consumption, physical inactivity and lack of exercise, obesity, and various environmental exposures. [13] Cancer continues to pose significant and costly challenges to the global healthcare system, necessitating the search for innovative approaches to identify anticancer agents that may help reduce the severity and spread of the disease in the future. Some scientific evidence suggests that parasitic infections may induce antitumor activity against certain types of cancer. The antitumor properties of hydatid cysts have attracted the attention of many researchers in recent years. [14] Multiple bioactive molecules present in Echinococcus antigens have exhibited notable anticancer effects in vivo. Research has demonstrated that the administration of these antigens can reduce tumor size and improve survival in in vivo models of breast cancer [15]. It has been shown that the immune response directed against lung cancer cells is more effective in mouse cancer models when E. granulosus antigens, such as antigen B, are used, as this contributes to a decrease in tumor growth and an increase in antitumor immunity [16].

Colorectal cancer is among the most common cancers worldwide and is the second leading cause of cancer-related mortality. In 2022, an estimated 1.9 million new cases and more than 900,000 deaths were reported globally, highlighting its significant burden. Early detection and screening are crucial for reducing both incidence and mortality [17]. Colon cancer cells have shown sensitivity to these antigens, enhancing the effectiveness of standard chemotherapy in suppressing tumor growth [18], but much remains to be learned about the biological activity, precise mechanisms of action of these antigens, and their clinical applicability.

Therefore, this study aimed to characterize the effects of hydatid cyst wall antigens on cancer and normal cell line growth and proliferation.

Materials and Methods:

Ethical approval:

This study was carried out in the Department of Biology/College of Education for Pure Sciences/University of Diyala/Diyala Province, Iraq. The protocols of this work were approved by the Scientific Ethics Committee of the Department of Biology, College of Education for Pure Sciences, University of Diyala, with reference number (CEPEC/030) at 23/2/2025. Additionally, the animal samples were handled in accordance with biosafety standards. Appropriate precautions were taken to ensure the safety of personnel and to maintain the integrity of the biological material throughout the study.

Materials:

An incubator (Cypress Diagnostics, Belgium), a microplate reader (Genix Lab, USA), a laminar flow cabinet (K&K Scientific Supplier, Korea), micropipettes (Cypress Diagnostics, Belgium), cell culture dishes (Santa Cruz Biotechnology, USA), MEM (US Biological, USA), fetal bovine serum (FBS) (Capricorn-Scientific, Germany), penicillin (100 IU) and streptomycin (100 µg) (Capricorn-Scientific, Germany), 96-well microplates (NEST Biotech, China), and an MTT assay kit (Elabscience, China) were used.

Methods:

Preparation and Isolation of Hydatid Cyst Wall Antigens (Germinal Layer and Lamellar Layer):

Thirty-six hydatid cysts were collected from the livers and lungs of naturally infected sheep that were slaughtered at a local abattoir in Diyala Province (Figure 1).

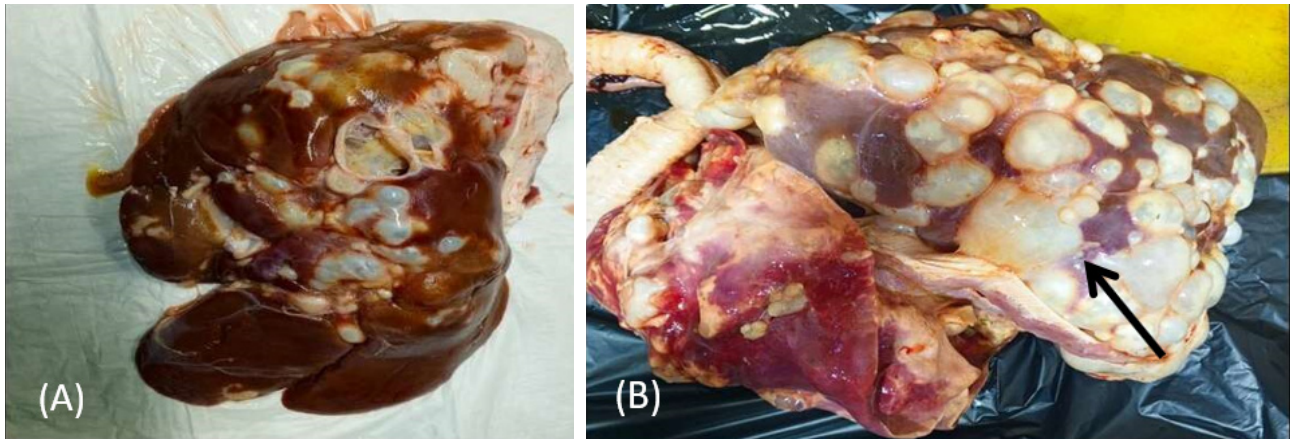

Figure (1): Liver and lungs of a sheep animal as a source for hydatid cyst cell wall protein isolation; (A) liver; (B) marker represents the lung.

In accordance with Steers et al. (2001) [19], all the samples were transported in clean, refrigerated containers to the laboratories at the College of Education for Pure Sciences, University of Diyala, where they were processed the same day. They were subsequently washed with physiological solution, and their surfaces were sterilized with 70% ethanol. Afterward, all surrounding tissues of the epididymal sac were removed using forceps and scissors, and occasionally a scalpel was used, and the maximum amount of fluid was aspirated using sterile 10 ml disposable plastic syringes with a G22 needle. The epididymal fluid was transferred to 100 ml plastic storage containers, the sacs were opened longitudinally with a blade, and the primary heads were collected along with the remaining epididymal fluid and placed in the same container. The germinal layer of the sac epithelium was isolated and added to the same container containing the epididymal fluid and then vigorously shaken to remove the attached primary heads. Afterward, components of the aqueous sac were used in the preparation of the antigens for the study.

The peritoneal fluid was gradually transferred to sterile 10 ml test tubes and sedimented using a centrifuge at 3000 rpm for 10 minutes at room temperature. The supernatant was poured off, the sediment of the primary oocytes was washed, and the volume was adjusted to 10 ml with saline solution. The mixture was centrifuged 4 times at 3000 rpm for 10 minutes, after which the supernatant was poured off, and the sediment was kept. The fertility of the sac was determined, after which the sediment was stored in the freezer after distilled water was added. The primary oocytes were examined by direct microscopic observation by placing a drop of the sediment on a glass slide, covering it with a cover slip, and inspecting it under a light microscope at 40X magnification to observe the fertility of the sac, as shown in Figure (2).

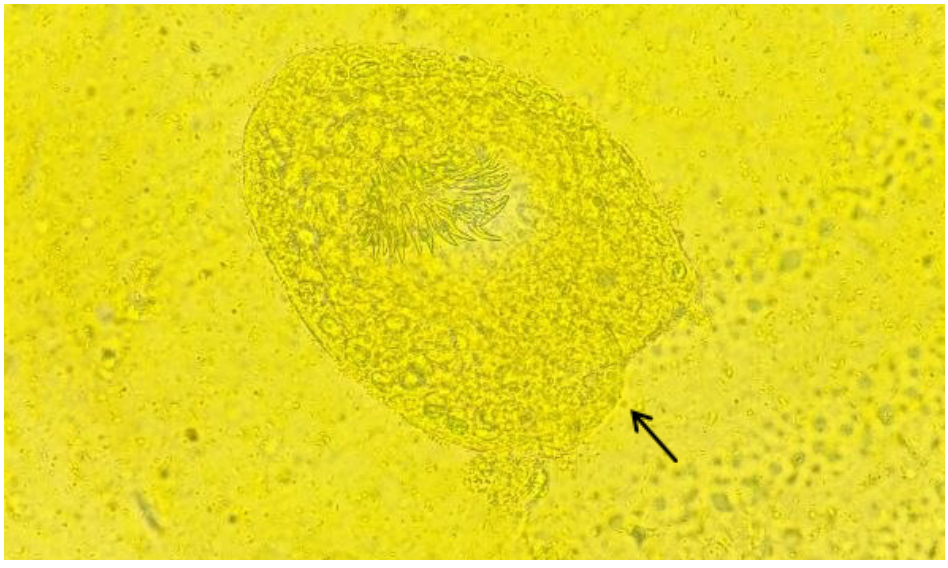

Figure (2): A light microscope image of the morphology of hydatid cell oocytes at 4X magnification.

Following aspiration of the cyst fluid, the lamellar layer (LL) was carefully separated from the germ layer (GL) by peeling and scraping with a scalpel under a light microscope. The isolated tissues were washed multiple times with phosphate-buffered saline (PBS) and inspected microscopically to confirm the absence of protoscoleces. Both the germ and lamellar layers were then frozen, thawed, and homogenized in a glass homogenizer with an equal volume of PBS. The homogeneous material was then subjected to ultrasonic treatment using a 150 W ultrasonic lithotripsy device (for 10 seconds on, then 5 seconds off) on ice for 25 minutes. The product (milky suspension) was collected and stored at -20°C until use in subsequent experiments [19].

Measuring the Protein Concentrations of Hydatid Cyst Wall Antigens:

The protein content of the hydatid cyst wall proteins was determined using the Lowry method [20] with a standard curve constructed with bovine serum albumin. This method relies on the measurement of freshly prepared alkaline copper reagent and bovine serum albumin (BSA) to create a titration curve with concentrations ranging from 0 to 100 µg/ml. The steps were as follows: 4 ml of alkaline copper reagent was added to 1 ml of the antigen solution, and the mixture was incubated for 10 minutes at room temperature. Afterward, 0.4 ml of diluted polyphenol reagent was added, the resulting mixture was thoroughly mixed, and the samples were incubated for 30 minutes. The absorbance was then read at 600 nm using a spectrophotometer. A protein-free tube served as the control.

The protein concentration of the antigen was calculated on the basis of the titration curve using the following equation:

$$y=ax+b.$$

where y = Absorbance, a = Slope, x = Concentration, and b = Intercept (20).

Electrophoretic Separation of Hydatid Cyst Wall Antigen Proteins Using SDS-PAGE:

A protein characterization assay was performed on hydatid cyst wall antigens to determine their different molecular weights. The SDS-PAGE method was used, following the procedure described in [21]. A 15% separation gel and a 5% concentration gel were prepared, and the antigen protein concentration was preadjusted on the basis of the prequantification of the protein. The samples were mixed 1:1 with a 2× SDS loading solution containing β-mercaptoethanol as a reducing agent, heated at 95°C for 3–5 minutes, and then centrifuged briefly for 1 minute. The gel was assembled in an electrophoresis apparatus filled with 1× running buffer, and equal amounts of hydatid cyst wall antigens were loaded into the wells alongside a prestained molecular weight marker. Electrophoresis was conducted under appropriate voltage conditions, and protein bands were visualized using Coomassie Brilliant Blue staining.

Cell line sources and culture conditions:

The two cell lines used in this study, which were provided by the Iraqi Center for Cancer and Medical Genetic Research, Mustansiriyah University, Baghdad, Iraq were authenticated, and Mycoplasma testing was performed in their cell bank unit. HRT-18 is a human colorectal adenocarcinoma cell line widely used as an in vitro model of colorectal cancer. It is an epithelial cell line originally derived from a 67-year-old Caucasian male patient with ileocecal adenocarcinoma. The HRT-18 cell line is widely utilized in cancer research, particularly in the study of metastasis, treatment response, and colorectal cancer pathogenesis https://www.atcc.org/products/ccl-244 [22]. The other cell line used was the normal human fibroblast (NHF) cell line, which is a primary fibroblast derived from human connective tissue [23].

The cells were cultured and maintained in minimum essential medium supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. The cultures were incubated in a humidified atmosphere at 37°C, and the cells in the exponential growth phase were used for all the subsequent experiments [24].

MTT procedure:

Cells were seeded at a density of 10,000 cells per well in a 96-well microplate and incubated at 37°C for 24 h until a confluent monolayer was established. Cytotoxicity was investigated through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were exposed to a range of concentrations (100, 50, 25, 12.5, 6.2, 3.1, and 0.00 µg/ml as negative controls), and the negative controls (cells without treatments) were exposed to phosphate-buffered saline (PBS); all tests and controls were repeated three times (as 3 technical replicates) for each concentration and incubated for 72 h. Afterward, 28 µL of MTT dye solution (2 mg/ml) was added to each well and incubated for 3 h, and after the exposure time, 100 µL of DMSO was added to each well and incubated for 15 min. Finally, the optical density of each well was measured at 492 nm using a microplate reader, and the percentage of cytotoxicity was calculated using the following formula:

Cytotoxicity % = (OD Control – OD sample)/OD Control × 100

where OD control represents the mean optical density of the untreated wells and OD sample denotes the optical density of the treated wells [25]. The median inhibitory concentration (IC50) was subsequently calculated by a nonlinear regression model, and the selectivity index (SI) was also calculated to evaluate the preferential cytotoxic effect of the cyst wall antigens on cancer cells compared with normal cells. The SI was determined using the following equation:

SI = IC50 (normal cells)/IC50 (cancer cells)

where SI represents the selectivity index, and the IC50 values were obtained from dose–response curves generated for both cancer and normal cell lines [26].

Statistical analysis:

The data obtained were statistically analyzed using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons between all treated and untreated concentrations using the GraphPad Prism 8.0 Program. The values are presented as the mean ± standard deviation of three replicate

measurements at $P < 0.01$ [27].

Results:

Protein concentration measurement and characterization by electrophoresis:

The results of the study, in which protein concentration was measured using the Lowry method, revealed that the protein concentration in the hydatid cyst wall antigens was 5.637 mg/ml. Determination of protein concentration enabled equal loading of protein samples during SDS-PAGE analysis. The SDS test results indicated the presence of distinct protein bands at molecular weights of 26 and 55 kDa, as shown in Figure (3).

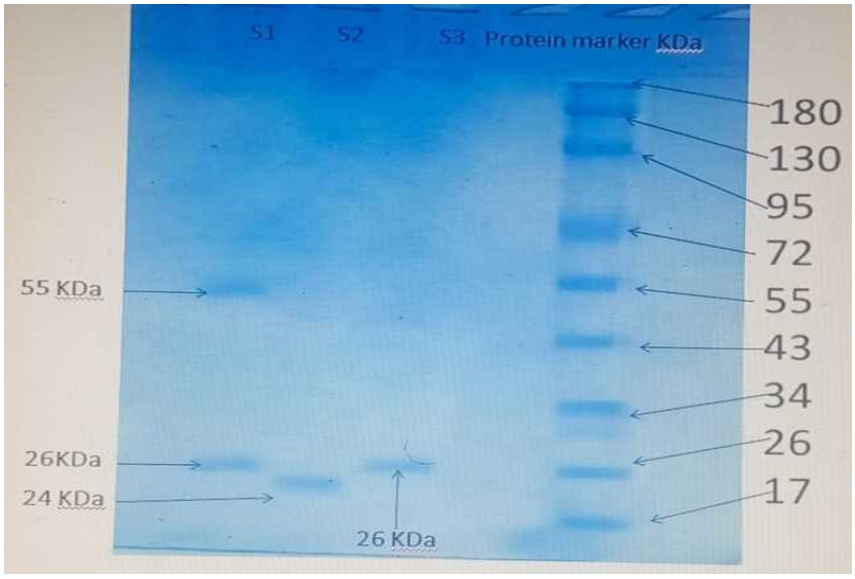

Figure (3): Characterization of the hydatid cyst wall antigens using electrophoresis, where (S 1) represents the cyst wall antigens.

MTT assay:

Morphological changes:

The cytotoxicity of hydatid cyst wall antigens was determined in colorectal cancer cells (HRT-18) and normal cells (NHF) using the MTT assay. The microscopy images in Fig (4, 5) show morphological changes in cells treated with hydatid cyst wall antigens compared with those in the untreated group. These morphological changes include cell shrinkage, membrane swelling, and morphological features suggestive of cell death. These findings suggest a cytotoxic effect of the antigens on cancer cells. and is consistent with the results of previous studies showing that components of the hydatid cyst wall possess anticancer activity against cancer cells.

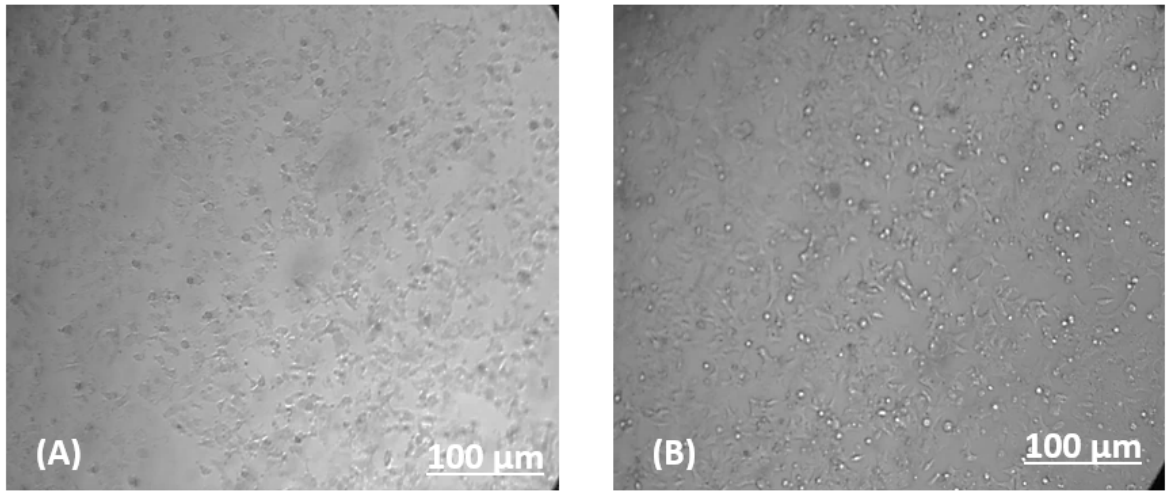

Figure (4): Proliferation of the colorectal cancer cell line HRT-18 before and after treatment with wall antigens, as shown by a decrease in cell proliferation under an inverted microscope at 10X; (A): HRT-18 cells (untreated); (B): HRT-18-treated cells.

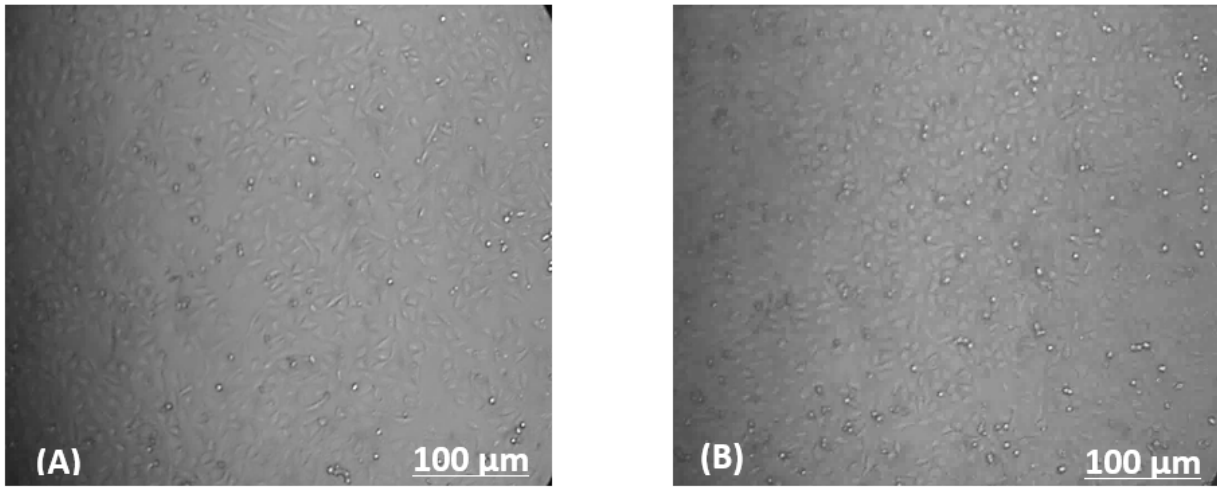

Figure (5): Proliferation of the normal NHF cell line before and after treatment with wall antigens, which had no effect on the normal cell line, as shown by an inverted microscope at 10X; (A): NHF cells (untreated), (B): NHF-treated cells.

Cytotoxicity Test:

Treatment with the hydatid cyst wall antigens at different concentrations (100, 50, 25, 12.5, 6.2, 3.1, and 0.00 µg) significantly affected the colorectal adenocarcinoma cell line in a dose-dependent manner compared with that in normal human cells, with the highest toxicity (48.39%) at $P < 0.01$, which was significantly greater than that in normal cells (21.18%) at the highest concentration, as illustrated in Fig (6). This selective effect may be attributed to structural and functional differences between cancer cells and normal cells, such as the increased rate of cell division and the disruption of apoptosis regulatory mechanisms in cancer cells, increasing their susceptibility to cytotoxic agents.

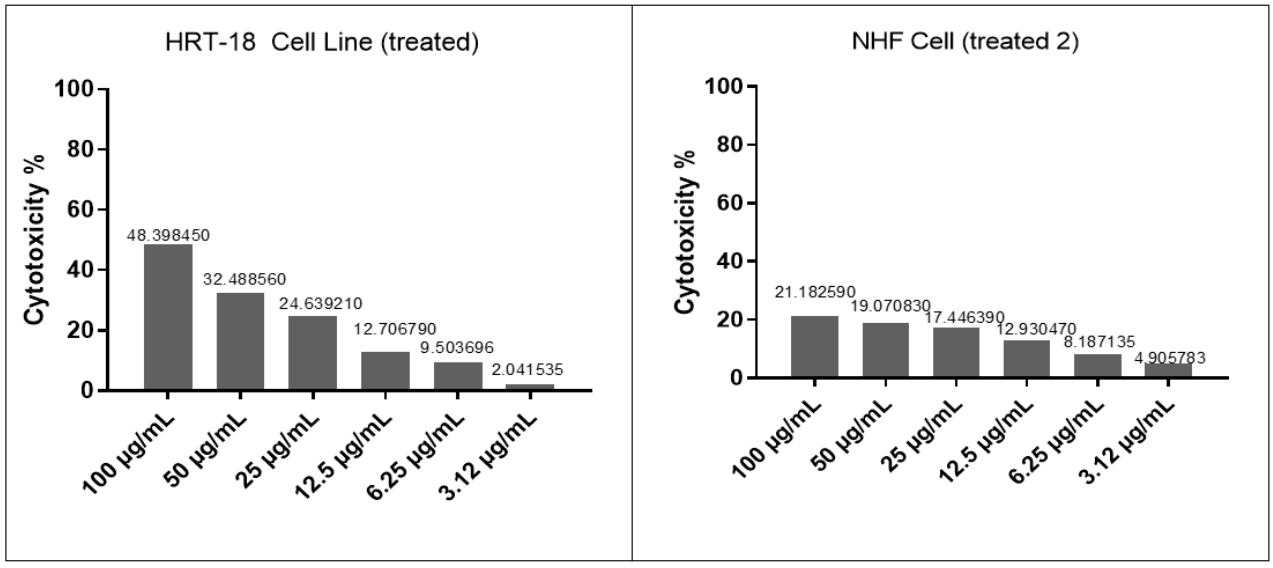

Figure (6): Inhibition curve showing the effect of the treated antigen on cell viability. The x-axis indicates the concentrations of cyst wall antigens (in micrograms) applied to the HRT-18 cancer cell line and the normal (NHF) cell line, while the y-axis indicates the percentage of cell death.

The IC50 value of the cyst wall antigens on the colorectal adenocarcinoma cell line was 24.00 µg/ml, whereas it was 100.8 µg/ml for normal cells treated with the same antigen (hydatid cyst wall antigens), as shown in Fig (7), and the selectivity index (SI) was calculated as the ratio of the IC50 in normal cells to that in cancer cells. Compared with normal cells, cancer cells exhibited an SI value of 4.2, indicating higher selective cytotoxicity toward cancer cells. This significant difference in sensitivity to the antigen between cancer cells and normal cells indicates selective activity, which is attributed to differences in the molecular properties of the cell surface. It has been proposed that cancer cells may overexpress specific glycoproteins that are absent or expressed at lower levels in normal cells, which could render them targets for parasite antigens. However, this hypothesis remains speculative and requires further mechanistic investigation.

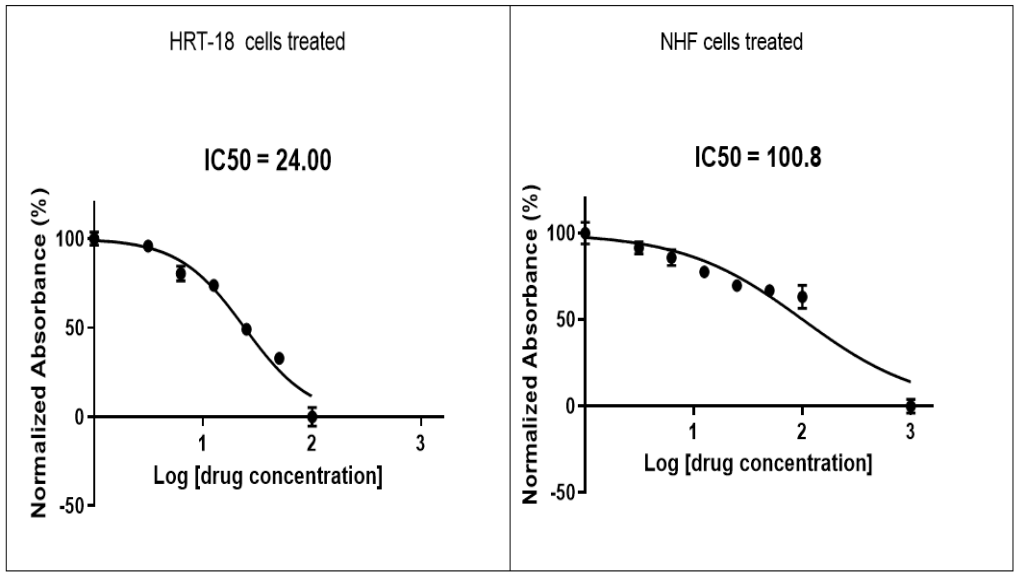

Figure (7): The dose–response curve used to determine the IC_{50} value for both HRT-18 cancer cells and normal (NHF) cells treated with cyst wall antigens for three replicates is shown in Figure 7. The x-axis represents the antigen concentration (in micrograms), while the y-axis represents the percentage of cell inhibition. The IC50 value is defined as the logarithm of the tested antigen concentration capable of inhibiting 50% of cell viability.

Discussion:

The external protection of Echinococcus tapeworm larvae by a lamellar (LL) layer, a carbohydrate-rich cellular structure, is not well understood. These data suggest that the carbohydrate structure of the LL is primarily composed of myosins, which are glycosylated proteins that form a protective network. E. granulosus, the causative agent of hydatid disease, has the largest lamellar layer in this genus. To date, no suitable methods exist for dissolving the E. granulosus LL layer and electrophoresis of the proposed structural myosins within it. It's reported that the reduction of disulfide bonds significantly aids in the dissociation of the lamellar layer, resulting in near-complete dissolution when mild ultrasonication is used. Structural myosins can subsequently be imaged using agarose gel electrophoresis[28]. These results suggest that hydatid cyst wall antigens contain multiple proteins with varying molecular weights. In this study, the crude hydatid cyst cell wall extract contained multiple proteins, and concentrating the extract prior to SDS-PAGE in future studies is recommended for visualizing all the protein bands.

These results are partially consistent with previous studies that recorded protein bands at 45 and 66 kDa [29] and partially consistent with the results of another study that recorded three bands (52, 41, and 22 kDa) for the antigen extracted from the germ layer [30]. The specific protein bands obtained indicate that the cyst wall antigens contain proteins or glycoproteins that play a role in the immune response. However, further specialized testing is needed to determine its functional nature more clearly and precisely. The lamellar layer is secreted by the germ layer, a component of the cyst wall of parasitic origin. The lamellar layer consists of different layers of mucopolysaccharides and chitin fibers and has been evolutionarily adapted to maintain somatic integrity (metacestode) and to protect germ layer cells from host immunity in intermediate hosts [31]. Its function is to maintain mechanical stability and avoid immune detection [32]. In addition, according to the results of the cytotoxicity and morphological changes described above, hydatid cyst wall antigens have anticancer effects, which may be due to their ability to cause cytotoxicity in cells. Many studies report that the parasite produces different glycoantigens in the lamellar layer that may contribute to reducing the effectiveness of the host's immune response and enhancing immune evasion mechanisms [33]. One study revealed that hydatid cyst wall antigens, in which 27- and 28-kDa protein bands were identified, inhibited breast tumor growth when they were applied to T14 breast cancer cells in mice. [34] In another study, exposure of the lung cancer cell line A549 to varying concentrations of laminated layer antigen resulted in a decrease in the number of viable cells, and cyst treatment induced cell death. [35]. Cancers and parasites share similar characteristics, as both express glycomolecules, which are not usually found on

the surfaces of healthy cells [36]. These sugar molecules expressed on cancer cells play critical roles in cell proliferation, adhesion, and invasion [18]. The second reason for selectivity is that cancer cells depend on different survival pathways, and treating them with an antigen may disrupt this balance and induce cell death, as in a study that reported an increased rate of programmed death in cells treated with B antigen compared with that in the untreated cell group; moreover, the expression of the BAX gene increased in the treated group, while the expression of BCL2 decreased in the untreated group [37]. Compared with normal cells, some antigens can activate an immune response more rapidly, leading to increased sensitivity of cancer cells. In a study supporting the pivotal role of immunity, inhibition of breast tumor growth and spread and improvement in survival rates in 4T1 mice immunized with hydatid cyst wall (HCW) antigens, particularly the lamellar layer and the 27 kDa protein bundle, were achieved through increased cytokine levels [33]. The findings of this study are in agreement with those reported in previous research, where IC50 values of 20, 30 and 30 micrograms/ml were reached when cells were treated with B antigen extracted from the fluid of aqueous cysts on three cancer cell lines, namely, the colon cancer cell lines C26, HCT116 and HEK293, respectively [18]. This study demonstrates for the first time that hydatid cyst wall antigens selectively induce cytotoxicity in colorectal cancer cells. Their complex proteomic profile, with diverse protein domains, suggests a potential mechanism underlying this selective anticancer activity. This study has several limitations. The absence of positive control, such as a standard chemotherapeutic agent, was detected. Protein identification (e.g., western blot or mass spectrometry) was not performed. Therefore, further studies are needed.

Conclusion:

The results of this study demonstrate that hydatid cyst wall antigens exhibit selective cytotoxic activity against colorectal cancer cells compared with normal cells. SDS-PAGE analysis revealed the presence of multiple protein bands, indicating the complex nature of these antigens. These findings suggest that hydatid cyst wall antigens may represent potential anticancer agents and warrant further investigation.

Author Declarations

Author Contributions

Mustafa Najim Abdalla, Nagham Y. Albayati, and Maeda H. Mohammad performed the experimental work. Mustafa Najim Abdalla conducted the statistical analysis. Mustafa Najim Abdalla, Maeda H. Mohammad, and Nagham Y. Albayati contributed to manuscript preparation, data interpretation, and final approval of the submitted version.

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Conflict of Interest

The authors declare that they have no conflicts of interest regarding the publication of this manuscript.

Data Availability Statement

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

Ethical Approval

This study was approved by the Scientific Committee and Ethics Committee of the Department of Biology, College of Education for Pure Sciences, University of Diyala, Iraq, under approval number CEPEC/030, dated 23 February 2025. All biological materials were handled according to institutional biosafety regulations and ethical guidelines.

Consent for Publication

All authors have read and approved the final manuscript and consent to its publication.

Human and Animal Rights

Hydatid cyst samples were obtained from naturally infected sheep collected from a local abattoir in Diyala Province, Iraq. Sample collection and handling were performed in accordance with institutional ethical and biosafety regulations.

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