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EVALUATION OF THE ANTICANCER ACTIVITY OF HYDATID CYST FLUID AND PROTEINS EXPRESSION VARIANTS ON MCF-7 HUMAN BREAST CANCER CELL LINES

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Abstract:
Background: In recent years, certain parasites, such as *Echinococcus granulosus*, have been thought of as agents of effect to immunological responses against both pathogens and tumors should be stimulated in order to avoid tumor escape and active monitoring by the tumor surveillance system. Therapeutics derived from Hydatid Cyst Fluid (HCF) have been show proven to be effective in the treatment of human cancer cell lines in experimental settings. The purpose of this study was to explore the influence of hydatid cyst fluid (HCF) crude on the cell activity of breast cancer (MCF-7) cell lines and the detection of gene expression. **Materials and Methods:** The fluid from the hydatid cyst was removed using a syringe and needle, then centrifuged at a speed of 2,000 g for two minutes. The supernatant was then air dried and kept at a temperature of 20 degrees Celsius to serve as a hydatid fluid antigen**.** MCF7 cancer cell lines were cultivated in RPMI-1640 media that was supplemented with 10% fetal bovine serum (FBS), 50 g/ml for ampicillin and streptomycin, and analyze the cytotoxic effect of HCF crude serial dilution (50, 25, 12.5, 6.25, and 3.125 g/ml) for 48 and 72 hours using an MTT test. Additionally, an RT-PCR test was utilized in order to identify the expression of several genes that are connected, such as EPCAM and KRT19**. Results:** The evaluations of the HCF crude Activity, the cells (MCF7) were treated for 48 and 72 hours using different concentrations for HCF. Also, our results showed up-regulation of KRT19 and done-regulation EPCAM genes**.**
Conclusion: According to the findings that we obtained in vitro, HCF crude is hazardous to MCF7 cell lines in a manner which is dependent upon both concentration and duration, as shown by an increase in the amount of apoptosis that is triggered by gene expression.
Keywords: Hydatid cyst fluid, Breast cancer, Gene expression, Cell line , MTT assay.

Introduction

It is the larval stage of the parasite known as *Echinococcus granulosus* that is known as the hydatid cyst. Worms mature and reside in the intestines of canines; humans are an unintentional intermediate host. The hydatid cyst fluid, also known as HCF, produced by *E. granulosus* is a complicated biological mixture that contains a large variety of proteins, glycoproteins, glycolipids, carbohydrates, and cyclophilin that are derived from both the parasite and the host (Aziz *et al*.,2011). In recent years, it has been established that protozoan parasites and the products produced from them have an anticancer effect. In many different animal models, some protozoan parasites are responsible for the activation and enhancement of the immune system, which in turn inhibits the growth of tumors, angiogenesis, and metastasis(Ding *et al*.,2022). Antigen B (AgB), Antigen 5, and the 78 KDa fraction are some of the antigens found in HCF. These antigens have been shown to have modulatory effects on the host immune response, which not only helps the parasite proliferate and remain viable, but also stimulates the cellular and humoral immune systems found in the host's body

(Zhang *et al*.,2008; Finlay *et al*.,2014). It has been demonstrated, both in cell cultures and in animal models, that HCF can suppress the progression of several malignancies, including melanoma, colon, lung, and adenocarcinoma(Yousofi Darani *et al*.,2012; Berriel *et al.,*2013; Ranasinghe, and McManus,2018).

Cancer is a disease that causes certain cells to reproduce and develop at an accelerated rate in comparison to other cells, resulting in the formation of an abnormal mass within healthy tissue . In addition to this, it poses a threat to human life on a global scale and continues to have a negative impact on both health and the economy each year. (Hanahan and Weinberg, 2000) Breast cancer is being one

of the most common cancers found in women worldwide, breast cancer also has a high mortality rate, making it the second most common type of cancer found in women (Sun *et al*.,2017). The Michigan Cancer Foundation7 (MCF-7) is a luminal molecular subtype that is frequently utilized in research and is noninvasive. It accounts for more than two thirds of all cancer cell line-related tests (Dai *et al*.,2017).At this time, cancer is typically treated with chemical medications and radiation, both of which have the potential to cause the patient long-term difficulties Abbas and Rehman, 2018) The current study aimed to investigate (1) the Sensitivity of breast cancer cells to biotherapy using hydatid cyst fluid. (2) Evaluation of the level of protein expression; (3) Analysis of the relative toxicity of hydatid cyst fluid at varying concentrations and over a period of time.

Materials and Methods:

HCF collection

Hydatid cysts were retrieved from the lungs and livers of diseased sheep carcasses obtained at the AL-Sadir slaughter in Baghdad. After being kept, these cysts were treated so that the protoscoleses could be extracted without disturbing the fluid. The fluid from the hydatid cyst was removed using a syringe and needle, then centrifuged at a speed of 2,000× g for two minutes. The supernatant was then air dried and kept at a temperature of- 20 degrees Celsius as crud hydatid fluid antigen. (Rahimi *et al*.,2011).

Cell line and Cell culture

The laboratory of AL-Nahrain University provided MCF-7 is a cancer cell line derived from human breast epithelial adenocarcinoma cells. Cells were cultured in a medium consisting of RPMI-1640 that had been complemented by a ten percent addition of fetal bovine serum. 100 U/ml of penicillin, and 100 mg/ml of streptomycin (all of which were manufactured by Gibco and distributed by Thermo Fisher Scientific, Inc.). The cell line was grown in an incubator that was humidified and supplied with 5% CO2 and maintained at 37 degrees Celsius. Cells were treated while they were in the exponential growth phase and passaged every three to four days.

The toxicity of HCF as measured by MTT

In order to conduct an analysis of cell proliferation, cells were seeded into 96-well plates (made by Sarstedt, Denmark) at a concentration of 200 l per well. The plates were then heated to 37 degrees Celsius. After diluting the HCF with RPMI-1640 medium free of serum, it was added to the wells at concentrations of 50, 25, 12,5, 6.25, and 3.125 g/ml respectively. The next step was to add 200 l of each dilution of HCF that had been described before to each well. The microplate was kept in an incubator at 37 degrees Celsius for a total of 48 and 72 hours [10]. After 48 and 72 hours of exposure, the vitality of the cells was evaluated, and treatment consisted of removing the media from the experiment, adding 100 l of a solution of
MTT with a concentration of 2 mg/ml, and incubating the mixture for 2 hours at 37 degrees Celsius. Finally, the insoluble formazan crystals were dissolved in 150 l of dimethyl sulfoxide (DMSO). A micro plate reader was used to determine the optical density (OD) of the sample at a wavelength of 570 nm. The formula for calculating the rate of cell growth inhibition is as follows: (IR) = (A-B) ./A*100. Where A is the mean of the optical density of wells that have not been treated, and B is the optical density of wells that have been treated.
(Al-Shammari *et al*.,2016; Alsabah *et al.,*2018)

Measurement of GAPDH, EPCAM and KRT19 Gene Expression

Lysates of cells that had been treated with the lowest possible doses as well as control cells that had not been treated were collected at regular intervals. (72 hours) and frozen at 86 degrees Celsius until they were utilized in the experiments to quantify the amounts of GAPDH, EPCAM, and KRT19 RNA. This was done so that the results could be interpreted more accurately. An RT-PCR analysis was performed, during which the RNA levels of GAPDH, EPCAM, and KRT19 were measured. Following the steps outlined in the manual provided by the manufacturer of the Trizol total RNA extraction kit (Anatolia Geneworks, Turkey), The complete amount of RNA that was contained in the cell lysate was successfully extracted. After the extracted RNAs had been reverse-transcribed to generate double-stranded cDNA by the reverse transcriptase polymerase enzyme utilizing the KAPA SYBR FAST One-Step qRT-PCR universal kit, these results were analyzed by real-time PCR utilizing the
MX3005 Real-Time PCR machine. (Kapa Biosystems, Cape Town, South Africa). (Agilent Technologies, USA).

As shown in Table (1-1), integrated DNA Technologies in Brussels, Belgium, developed gene-specific primers for the GAPDH, EPCAM, and KRT19 genes in order to detect the presence of these genes in the MCF7 cell lines. Amplification conditions for human GAPDH, EPCAM, and KRT19 were as follows: 42 degrees Celsius for five minutes, denaturation at 95 degrees Celsius for ten minutes, and amplification in forty cycles, with each cycle at a denaturation temperature of 95 degrees Celsius for three seconds, an annealing temperature of sixty degrees Celsius for twenty minutes, and an elongation temperature of seventy-two degrees Celsius for twenty minutes. Controls were included in every cycle of the real-time PCR assay, and for each primer pair, there was at least one sample that did not include any cDNA and instead contained just RNase-free water.

The findings from each sample were examined with the help of a relative quantitative analysis so that the distinction between the sample and the control could be determined. After calculating the mean CT values of the genes in each sample (with a replication done twice for each sample), the 11CT approach was applied in order to normalize the expression levels. This method was detailed in the preceding section.((Livak and Schmittgen,2001)

Table (1-1) The sequences of the primers that were utilized for the GAPDH, EPCAM, and KRT19 genes

Primer	Primer sequences	Product s (bp)	References
	F AAGCAACGCGAAGAACCTTA		
	R TGTCACCGGCAGTCAACTTA		
	F CCTGGCCGTGCTAGACAATGG		
	R GGGTTCCGCTGGATCAAGACC		
	F CAGATCGACAATGCCCGTCTGG		
	R TGCATCTCCAGGTCGGTCCTG		

Data analysis:

The results of the experiments were given as the mean accompanied with the application of the standard deviation. For the purpose of carrying out the statistical analysis, a one-way analysis of variance (ANOVA) was utilized. Multiple comparisons were carried out in order to demonstrate variations between studies, and the differences were deemed statistically significant when their values were less than 0.05 ($P<0.05$).

Results:

Toxicity of HCF by MTT the current work, MCF7 cell line were treated with different concentrations (3.125, 6.25, 12.5, 25 and 50) within 48 hours and 72 hours. The results showed that the cell activity was inhibited by HCF depending a dose- and time- dependent manner , as shown in **figures (1-1)(2.1)**. The statistical analysis revealed that, following 48 and 72 hours of exposure, there was a significant ($P<0.05$) inhibitory effect among the concentrations of HCF (3.125, 6.25, 12.5, 25 and 50) µm. Highest significant ($P\leq0.01$) inhibition rate were obtained at dose of 50 and 25 µg / ml after 48 and 72 hours comparing to other concentrations(3.125, 6.25 and 12.5) in same treated period.

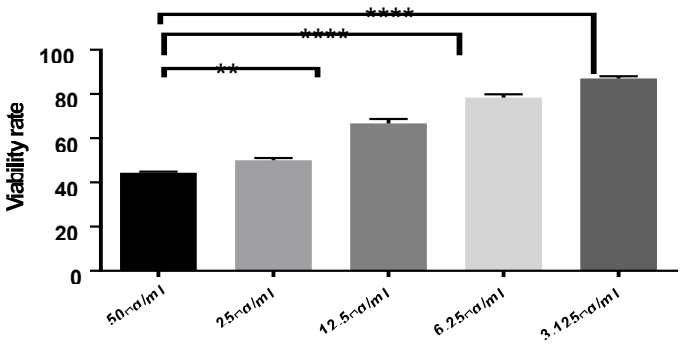

Figure (1-1): The effect of HCF crude on the percentage of MCF7 cancer cells that were able to survive. After 48 hours, the effects of HCF at doses of 50, 25, 12,5, 6.25, and 3.125 g/ml were observed in the cells that were treated with it. The MTT assay was used to determine the viability of the cells, and the results of this analysis are provided as a percentage of the growth inhibition rate (IR). (ns) A lack of significance, the data are presented as mean with a standard deviation.

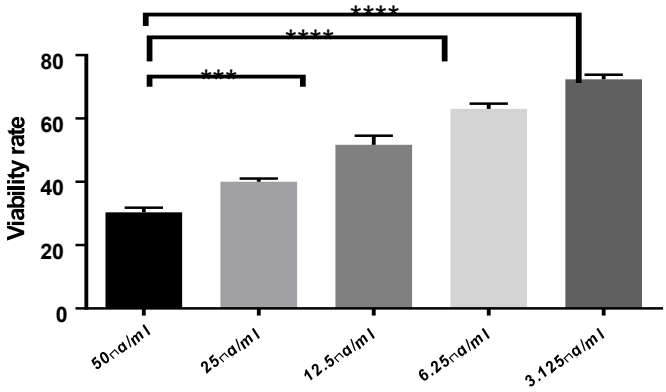

Figure (2-1): The effect that HCF crude had on the viability of the cancer cells MCF7. After 72 hours, the effects of administering HCF to the cells at doses of (50), 25, 12,5, 6.25, and 3.125 g/ml were evaluated. The MTT assay was used to determine the viability of the cells, and the results of this analysis are provided as a percentage of the growth inhibition rate (IR). (ns) A lack of significance, The data are presented as a mean with a standard deviation.

Measurement of KRT19 and EPCAM genes expression

We evaluated whether or whether the MCF-7 cells' exposure to HCF resulted in differential gene expression. In comparison to cells that had not been treated with anything, the expression of the KRT19 and EPCAM genes was significantly altered when the MCF-7 cells were subjected to varying concentrations of HCF over a period of 72 hours. We confirmed the transcriptional effects of HCF on KRT19 and EPCAM genes using real time PCR (RT-PCR). Specifically, we found that HCF administration led to a downregulation of KRT19 and an upregulation of EPCAM. According to our findings, the up-regulation of the KRT19 and EPCAM genes was found in MCF-7 cells 72 hours after treatment was administered in **figure (3-1).**

(4-1).

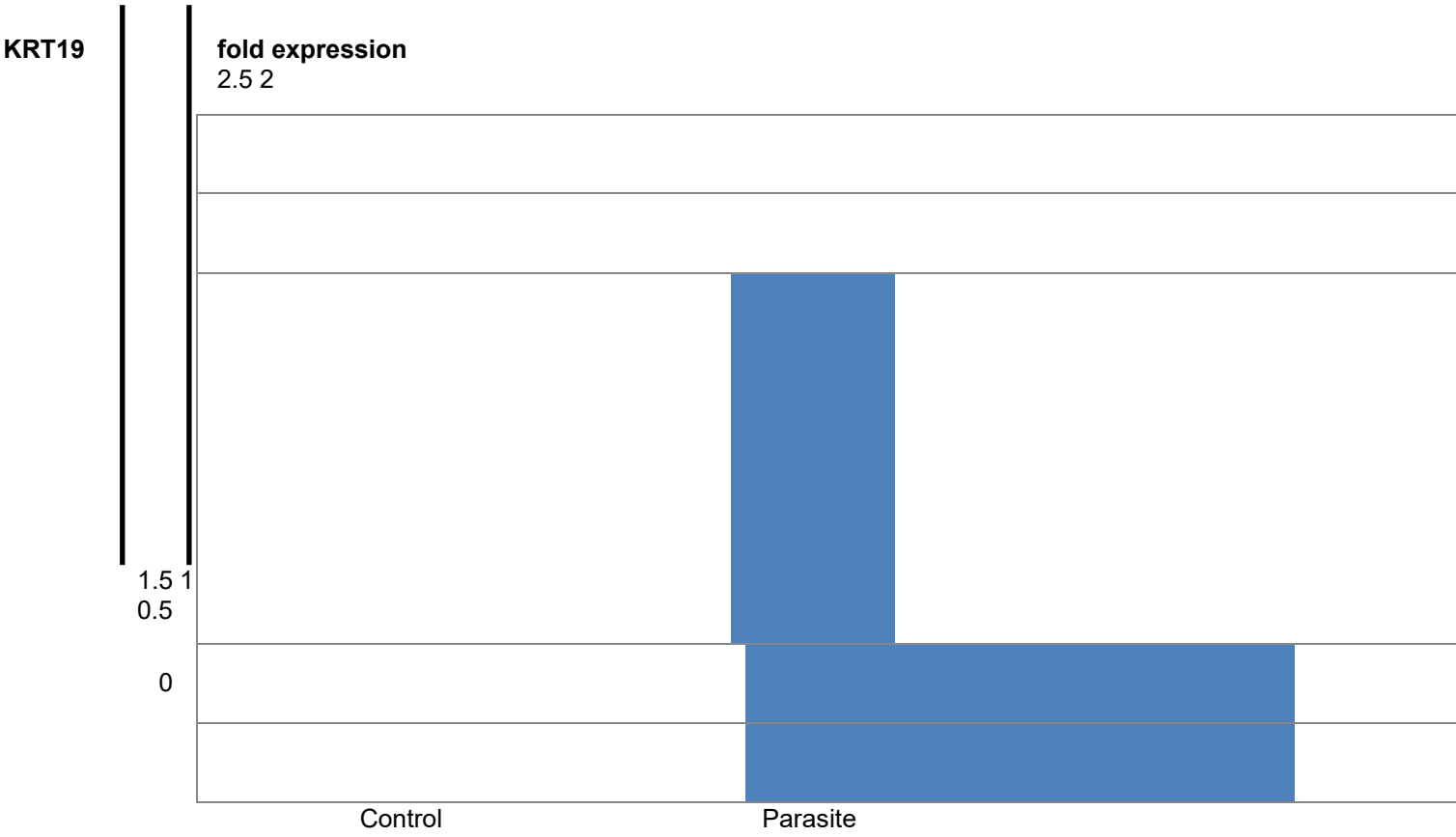

Figure (3-1): Exposition of MCF-7 cells to a dose of HCF for a period of three days, followed by the measurement of KRT19 gene expression in both treated and untreated cancer cells. Following exposure to HCF crude for 72 hours, there was an increase in the level of KRT19 expression in MCF-7 cells.

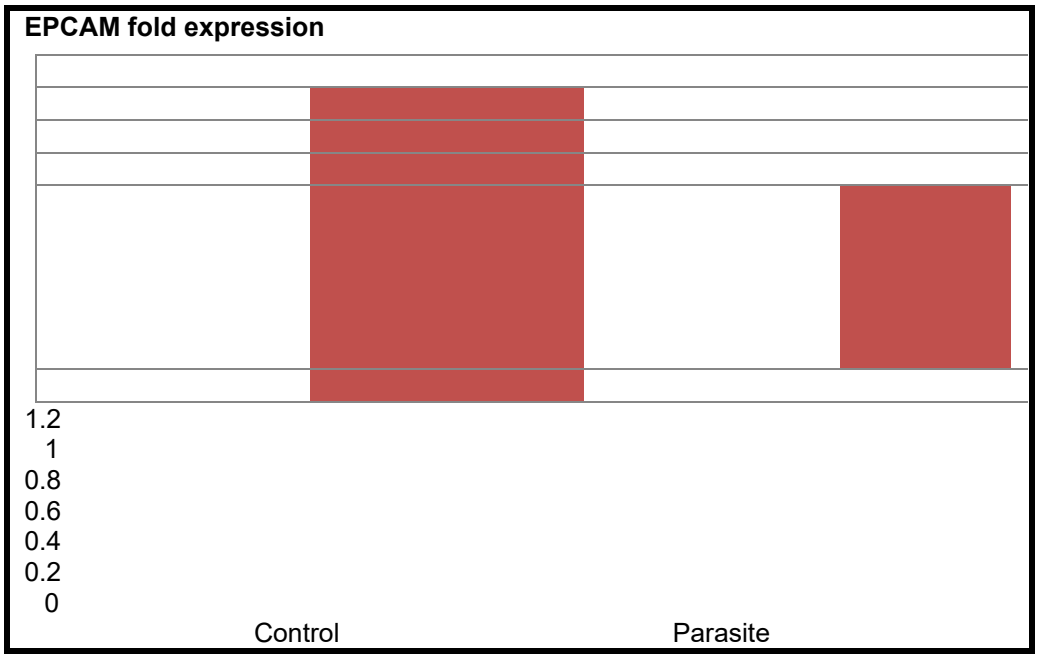

Figure (4-1): Exposition of MCF-7 cells to a dose of HCF for a period of three days, followed by the measurement of EPCAM gene expression in both treated and untreated cancer cells. After being exposed to HCF crude for 72 hours,

the level of EPCAM expression in MCF-7 cells was down-regulated.

Discussion:

Some parasites have been considered effectors because of their ability to produce immune responses that are anti-tumor and anti-pathogen. This can be helpful in preventing tumor escape and in maintaining an active tumor surveillance system.

In the current investigation, we employed cytotoxicity assay to examine the effect of HCF on the viability rate of (MCF-7) cancer cell lines in vitro. The findings of this work indicate that HCF crude can suppress the growth of (MCF-7) cell lines in a concentration and time dependent manner.

HCF is made up of a wide variety of immunomodulatory components, which have an effect on the cellular as well as the humoral immune responses in the body of the host (Siracusano *et al*.,2008). It was demonstrated that Both the antigens of the hydatid cyst and the excretory secretory products that cancer cells produce exhibited the capacity to cross react with one another(Daneshpour, *et al*.2016). Studies both in vitro and in vivo have shown that the HCF compound possesses anticancer properties. Clinicians have seen an extremely low incidence of incidental diagnosis of hydatid disease (HD) in patients who are undergoing surgery for a variety of solid tumors. (Akgül *et al*., 2003).Small cell lung cancer cells were killed in vitro when exposed to sera from patients with hydatid cysts(Karadayi *et al*.2013). According to Macintyre *et al*. (2001), hydatid fluid stimulates cell growth by upregulating CD25 and CD38 expression on human peripheral blood lymphoblast while downregulating CD28 (and other co-stimulatory molecules), which might lead to anergy or cell death. At 48 and 72 hours of treatment with 50, 25, 12,5, 6,25, and 3,125 g/ml of HCF resulted in substantial cytotoxicity (P< 0.001). Interestingly, HCF was more harmful at higher concentrations and for longer periods of time than it was at lower concentrations and shorter times.

Altun *et al*.(2015) indicated that hydatid disease might help avoid breast cancer Possible explanations include antigenic similarities between the parasite and cancer cells, as well as the ability of the parasite to stimulate the immune system to fight cancer.
The apoptosis of mouse breast cancer cells was triggered by hydatid cyst antigens. After being exposed to hydatid fluid, protoscoleces may produce poisons that trigger apoptotic pathways The controlled cell death known as apoptosis(Daneshpour, *et al*.,2019). Condensation of nuclear chromatin, alterations in membrane phosphatide symmetry, and enzymatic DNA cleavage all contribute to cell death, as does the subsequent division of cells into apoptotic components. (Singh *et al*.2019) in another study found that injecting mice with 300 ng/ml of HCF and 500 ng/ml of Antigen B decreased tumor volume and carcinogenesis in breast tissue. (Vafae Eslahi *et al*.,2022) . Furthermore, stimulation of pro-apoptotic Bax, Caspase-9, and Caspase-3, which are crucial to the intrinsic apoptotic pathway, may make miR-365 overexpression a negative regulator of melanoma growth .(Mohammadi *et al*.,2021).

Keratin 19 (also known as KRT19) is an essential component in the process by which MCF7 breast cancer cells proliferate.

that keratins, particularly when their expression levels are changed, can stimulate the formation of tumors The fact that K19 is necessary for the proliferation of MCF7 cells provides further evidence that cytoskeletal proteins as a family play an active role in the development of cancer cells. (Sharma *et al.*,2019) After being exposed to HCF, our research demonstrated that the expression of Keratin 19 was increased in MCF7 cancer cells.

Epithelial cell adhesion molecule, often known as EpCAM, is a type of transmembrane glycoprotein that is primarily recognized for its ability to mediate homotypic cell interactions in epithelial tissues. EpCAM has been utilized as a diagnostic marker for the purpose of identifying cancer cells in mesenchymal organs due to the fact that its expression is restricted to epithelia, both normal and malignant. (Keller *et al*., 2019)
After being exposed to HCF, the EPCAM gene was shown to be down-regulated in MCF7 cancer cells, according to our findings. Studies conducted in clinical settings demonstrated that EpCAM is overexpressed in a variety of malignancies, including breast cancer, and that this overexpression is associated with a poor prognosis. (Hiraga *et al*., 2016)

In another study, It was demonstrated that inhibiting EpCAM resulted in a chemosensitizing impact in MCF-7 cells by decreasing the expression of the anti-apoptotic protein Bcl-2. This resulted in the induction of apoptosis and cell cycle arrest at S phase when 5-FU was combined with the treatment, and this was accomplished through a mechanism that involved the modulation of the JNK and ERK1/2 signaling pathways. As a result of this, EpCAM has the potential to be a contender for the role of a chemosensor in the treatment of breast cancer. (Gao *et al*.,2014) Also demonstrated is that hydatid cyst fluid antigen is able to limit the growth of cancer cells by raising the rate of apoptosis in cancerous cells. Apoptosis can also be prevented by the HCF's ability to block genes that repress the process. Motavallihaghi *et al*.,(2023).

Conclusion:

Our findings shown that HCF contain hazardous antigens capable of suppressing the expansion of breast cancer cells (MCF7) by upregulating genes involved in apoptosis and causing cancer cell death through programmed cell death (apoptosis). In order to learn more about the anticancer process at play and the molecular mechanisms of apoptosis triggered by the parasite, more extensive and accurate tests are required.

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