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RESEARCH

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In vitro anticancer activity of hydatid cyst fluid on colon cancer cell line (C26)

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Abstract

Background Colon cancer is the third most common cancer and the fourth leading cause of death from cancer. Some parasites are introduced as an antineoplastic agents that can inhibit the progress of some cancers. The aim of this study was to investigate the effect of crude hydatid cyst fluid (HCF) on clone cancer cell line (C26).

Methods HCF was isolated from hydatid cysts by syringe, and at the first, its toxicity was obtained by 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cell cycle analysis and apoptosis were measured by flow cytometer, and also the expression of Bcl-2 Associated X-protein (BAX) and B-cell lymphoma-2 (BCL2) genes was measured by quantitative reverse transcription PCR.

Results The amount of apoptosis was increased in B antigen-treated cell lines in comparison with the control group. Also, the expression of BAX was increased in the treated group, while the BCL2 expression was decreased in comparison with the control one. Cell cycle analysis in the antigen-treated group compared to the other groups showed that the cells were more in the G0/G1 phase, as well as in the G2/M phase, and fewer cells were in the synthesis phase.

Conclusion Our finding showed that HCF possibly contains active compounds and can limit the growth and development of C26 cell line by reducing or increasing the genes involved in apoptosis and finally the effect on the cell cycle.

Keywords Colon cancer, C26, Hydatid cyst fluid, Apoptosis, Cell cycle

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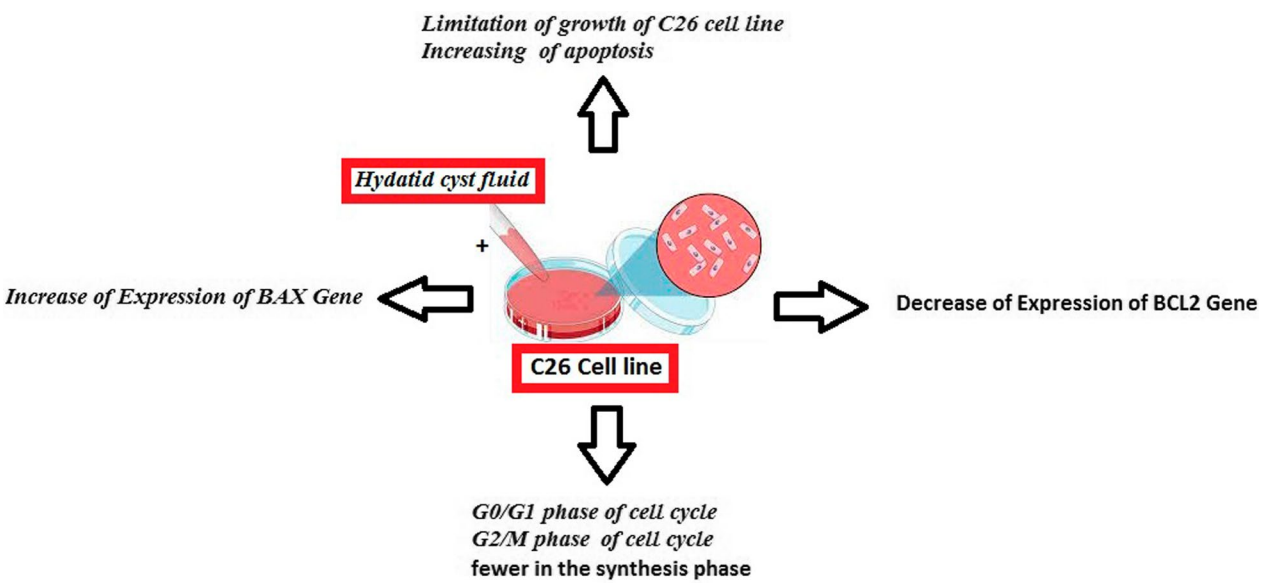
Graphical Abstract



Introduction

Cancer is still a major hazard to health, even after much research works worldwide. Colon cancer is the third most common cancer in the world, and the fourth leading cause of death from cancer. About 60% of patients with colon cancer are stage II/III disease and surgery is the only treatment for these patients [1]. The current therapies for cancer patients are radiotherapy, chemotherapy, and eventually surgery. Radiotherapy and chemotherapy destroy normal cells with many side effects and also drug resistance and recurrence cause a poor prognosis in the treatment [2, 3]. Humans have always looked for less risky ways to treat cancers with higher efficiency [4, 5]. Several infectious causes of cancer in humans are Helicobacter pylori bacterium, the human papilloma viruses (HPV), and the hepatitis B and C viruses [6, 7], but it has been hypothesized that some parasitic infections may develop innate immune responses that show antineoplastic activity. Retrospective studies show that in patients with hydatid disease, the prevalence of cancer is significantly lower than in normal ones [8]. A hydatid cyst contains the larvae of the *Echinococcus granulosus*, a parasitic tapeworm responsible for echinococcosis [9, 10]. The hydatid cyst fluid of *E. granulosus* is a mixture of glycoprotein and glycolipid, carbohydrates, cyclophilin, and ferritin [11]. HCF contains different antigens such as Antigen B (Ag B), Antigen A and 78 KDa fraction [12–15]. According to the literature, HCF can inhibit the growth of some cancers both in cell cultures and animal models [9, 14, 16–18]. Hydatid cyst antigens induced apoptosis on mouse breast cancer cells [4]. In fact, antigenic similarities between *E. granulosus* and some tumors have been shown, for example, mucin-type O-glycan antigens of some cancers are expressed by some helminth parasites [19]. The mentioned similarities are responsible for the induction of a cross-reactive immunity which could inhibit cancer growth [14]. Apoptosis, which is the programmed death of cells, is caused by condensation of nuclear chromatin, changes in the symmetry of membrane phosphatides, and enzymatic cutting of DNA, and finally, the division of cells into apoptotic components leads to cell death. BCL2 family proteins are the main regulators of apoptotic cell death and so far about 25 members of this family have been identified based on functional studies and protection of BH domains that contribute to BCL2 function in cell death and survival [20]. They are classified into three subgroups. One of these subgroups contains BH3 pro-apoptotic proteins, they can interact with anti-apoptotic proteins or pro-apoptotic members, and this subgroup can inhibit anti-apoptotic molecules or directly BAX pro-apoptotic to activating apoptosis induction. Although it is not fully understood how BCL2 family proteins regulate the apoptotic pathway, it has been shown that the biological functions of this protein family are dependent on protein–protein interactions. High expression of BCL2 gene in human cancers has led to cancer resistance to

chemotherapy drugs that act by inducing apoptosis in cancer cells. Therefore, blocking BCL2 can restore the apoptosis process in cancer cells [21]. In this study, by examining the rate of apoptosis and genes of the apoptotic pathway, as well as cell cycle phase changes, the effect of Ag B on C26 cell line was investigated.

Materials and methods

HCF collection

HCF was collected from the livers of infected sheep at a slaughterhouse in Hamadan, Iran. At first, the cysts were examined for the presence of infection, as well as the presence of protoscolex, and cysts that had protoscolex and were also free of infection were included in the study. The fluid was aspirated by a syringe and needle and finally collected at − 20 °C [22].

Cell culture

Colon cancer (C26), human embryonic kidney (HEK293) and human colorectal carcinoma (HCT116) cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 100 ng/mL of streptomycin and 100 units/mL of penicillin and kept at 37 °C in a 5% CO2 incubator [23, 24].

Toxicity of HCF by MTT

About 5×10^3 C26 Cells/Well in culture medium was incubated in a 96-well plate for 24 h at 37 °C and 5% CO2. The HCF was added to the wells with concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM for 24 h, in the control group, DMEM with 10% FBS was added to the wells. Then, 5 mg/ml MTT solution was added to the parasite medium and incubated for 3–4 h in the humidity incubator. By adding 100 µl of Dimethyl sulfoxide (DMSO) and 25 µl of NaCl glycine buffer (pH 10.5) to the medium, formazan salt was dissolved, and finally, the amount of light absorption was measured at a wavelength of 570–570 nm with a spectrophotometer [25]. All the tests were performed in triplicate. The cell viability was calculated by ELISA Plate Reader at 570 nm with below formula:

$$\text{viability} = \frac{\text{OD test}}{\text{OD control}} \times 100$$

Cell cycle analysis

By flow cytometry (Life Tech Attune NxT flow cytometer, German) method, the cell cycle can be examined in three main cellular phases (G1, S, G2/M). The mediums containing C26 three cell lines of C26, HEK293 and HCT116 were centrifuged at 2500 rpm for 15 min, after draining of the supernatant, 5 ml of formalin 10% was added to the cells and incubated for 10 min at room temperature in the dark. After centrifugation and draining the supernatant, 0.1% Triton was added to the sediment containing C26 cells and incubated for 40 min in the dark at room temperature and then centrifuged and finally 3 ml phosphate-buffered saline (PBS), 2 µl RNAse and 2 µl Propidium Iodide (PI) were added to sediment and incubated for 30 min in the dark at room temperature and read by flow cytometry [26].

Apoptosis assay

The rate of cellular apoptosis in normal and cancer cells exposed to B antigen and non-exposed is measured and compared. Using the annexin V-fluorescein isothiocyanate (V-FITC) apoptosis kit (eBioscience Annexin V Apoptosis Invitrogen Detection Kit FITC, Invitrogen by Thermo Fisher Scientific, USA) and flow cytometry method, apoptosis rates of three cell lines were determined in the presence of HCF [27]. The rate of apoptosis was estimated by the green fluorescence of annexin V-FITC-phosphatidylserine. After washing in PBS, 10^6 cells were rinsed and re-suspended in the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. After incubation at room temperature for 5–15 min in the dark, annexin V-FITC (5 µl) was added. Before the Flow cytometry analysis, PI Staining Solution (5 µl) was added to the cell suspension to detect necrotic cells [28, 29].

Gene expression of BAX and BCL2

To confirm the apoptosis process, the expression of the BAX and BCL2 genes in the C26 cells was determined. BAX is a pro-apoptotic gene and BCL2 is an anti-apoptotic gene and the GAPDH gene was considered as the reference gene [30]. The primers used in the study are as follows: (Table 1).

RNA extraction from C26 cells exposed to antigen

At first C26, HCT116, and HEK239 cells were treated with 20 mM, 30 mM, and 30 mM of B antigen for 24 h, respectively. Then, 1 ml of cold RNX plus solution (Sinacolon Company, Iran) was added to the cell-containing microtube and pipetted several times, vortexed for 5–10 s, and incubated at room temperature for 5 min. In the next step, 200 µl of cold chloroform was added to the mixture. The mixture was vortexed for 15 s and then placed on ice for 5 min. It was then centrifuged at 12,000 rpm at 4 °C for 15 min. The upper phase was transferred to another tube, and cold isopropanol was added with the same volume of the mixture. The mixture was centrifuged at 12,000 rpm at 4 °C for 15 min to show RNA as a white plug at the bottom of the microtube.

Table 1 The sequence of primers used for BAX, BCL2 and GAPDH genes

Gene	Sense strand	Antisense strand	Accession number	Product size (bp)
BAX	TTTTGCTACAGGGTTTCATCC	TATTGCTGTCCAGTTCATCTC	NM_007527	147
BCL2	TGGTGTGGTTGCCTTATG	GGTATATCCGCTACAAGTTAC	NM_009741.5 NM_177410.3	112
GAPDH	CAAATTCAACGGCACAGTCAAGG	GACTCCACGACATACTCAGCAC	NM_001289726.1 NM_008084.3	132

Isopropanol was then discarded and 1 cc of 75% ethanol was poured on it for washing. The mixture was centrifuged at 7500 rpm at 4 °C for 8 min. The supernatant was drained and the microtube was placed under the hood at room temperature to dry the ethanol. 50 µl of DEPC Water was added to the tube to dissolve the RNA precipitate [31]. The samples were placed in an incubator at 55 °C for 10 min to dissolve the RNA. Then, 2 µl of RNA was removed and used for qualitative and quantitative evaluation of the extracted RNA. From this stage, the RNA must be stored on the ice to be used to make cDNA, and the rest of the RNA was stored at − 70 °C [32].

Quantitative evaluation of the extracted RNA

RNA concentration and purity were determined according to the optical absorption of the sample at 260 and 280 wavelengths using a NanoDrop device (Thermo Fisher Scientific, MA). The absorption ratio of 260/280 indicates the purity and contamination of RNA [33].

cDNA synthesis from extracted RNA

cDNA was synthesized according to the protocol provided by Fermentase Company (K1631). At this stage, the RNA was converted to DNA during the reverse transcription reaction. To test all RNA samples, different volumes were taken everyone contain 2 µg of RNA and poured into a 0.2 ml RNase-free microtube on ice. Then, 1 µl of random hexamer primer was added to the microtube and the volume of the mixture reached 12 µl with nuclease-free water. Then, the mixture was mixed and spun and incubated at 65 °C for 5 min and immediately placed on ice, and the following ingredients were added to it, respectively: 1—Deoxyribonucleotide triphosphates (dNTP) 10 mM (4 µl), 2—Reverse transcriptase enzyme (2 µl), 3—RNAase inhibitor (Ribolock) (2 µl) [34].

Determination of expression of BAX and BCL2 genes in prepared cDNA

The steps of qRT-PCR were performed by SYBR Green (YTA, Iran) and according to the method of the company. First, a master was prepared with the following concentration and components. For each sample, 10 µl of master mix and 7 µl of nuclease-free water were added to the microtube. Then, 1 µl of each of Forward and Reverse primers was added (the concentration of primers was 8 pmol). Finally, a volume of 1 µl of cDNAs was added to the strip tubes with a dilution of 1.10 and 19 µl of the master mix to a total volume of 20 µl. For the negative control sample, instead of cDNA, the same volume of nuclease-free water was added. Temperature cycles at different stages of qRT-PCR are specific to each gene and must be determined. The number of cycles in all reactions was considered 40 times. In this study, the GAPDH gene was used as a reference gene for normalization [35].

Statistical analysis

Data were analyzed using ANOVA and Student's *t* tests, and the differences were statistically significant at $P < 0.05$. Data were reported as mean ± standard deviation from three independent experiments [36].

Results

Toxicity of HCF by MTT

The IC_{50} of B antigen was measured for all three types of cancer cell lines. The IC_{50} for C26, HCT116 and HEK239 cell lines were calculated 20, 30 and 30 µM, respectively.

Cell cycle analysis

Results of flow cytometry showed the cell cycles in three main cellular phases (G1, S, G2/M). As the results of cell cycle analysis show, most of the cancer cell populations in C26 and HCT 116 cell lines were normally observed in the synthesis phase, which was significantly higher than other groups (C **, D **) ($P < 0.05$). While when these cell lines were exposed to B antigen, most of the cell population was in phase G0/G1 and (A ***, B ***) ($P < 0.001$) and the cell population was in division phase. Mitosis was significantly lower than in other groups (E ***, D ***) ($P < 0.001$) (Table 2).

Apoptosis assay

According to Fig. 1. the amount of cell apoptosis in cancer cell lines are increased in Ag B-treated groups

Table 2 Results of cell cycle analysis in cell lines

Cell line	G0/G1 (Quiescent phase/cell growth)	S (DNA synthesis)	G2/M (Cell growth/mitotic)
HEK239	29.2 ± 1.03	40.3 ± 2.63	30.5 ± 1.65
HEK293 + HCF	33.1 ± 2.63	42.4 ± 1.05	24.5 ± 1.95
C26	28.8 ± 1.71	56.3 ± 2.13^{C**}	14.9 ± 1.83
C26 + HCF	59.6 ± 1.15^{A***}	34.7 ± 1.83	5.7 ± 2.81^{E***}
HCT116	20.1 ± 1.73	60.1 ± 1.01^{D**}	80.2 ± 3.11
HCT116 + HCF	46.5 ± 3.18^{B***}	37.6 ± 2.83	15.9 ± 2.14^{F***}

A, B, E, D*** ($P < 0.001$), C, D** ($P < 0.05$)

(HCT116, C26 and HEK293 cell lines) in comparison with the untreated group ($P < 0.001$) (Table 3) (Fig. 2).

Gene expression of BAX and BCL 2

In HCF treated group, the expression level of BAX was increased nine times in comparison with control, while the level of BCL2 expression was decreased (Table 1, Fig. 3) ($P < 0.001$).

Discussion

Several helminths have been confirmed to be carcinogenic in humans, such as the liver fluke *Clonorchis sinensis* and *Opisthorchis viverini* (reason for cholangiocarcinoma) [37]. On the other hand, certain helminth infections could induce anticancer activities, such as the pork worm *Trichinella spiralis*, which protects infected mice against tumor growth and metastasis [38]. The relationship between *E. granulosus* and cancer has been unclear until an epidemiological study on patients with CE found a negative correlation between CE and solid tumors [8] and has led to a theory that infection of *E.*

Table 3 Results of apoptosis in different groups

Cell line	Apoptosis
HEK293	1.0%
HEK293 + HCF	1.32%
C26	5.5%
C26 + HCF	11.6%***
HCT116	6.7%
HCT116 + HCF	9.43%***

***($P < 0.001$)

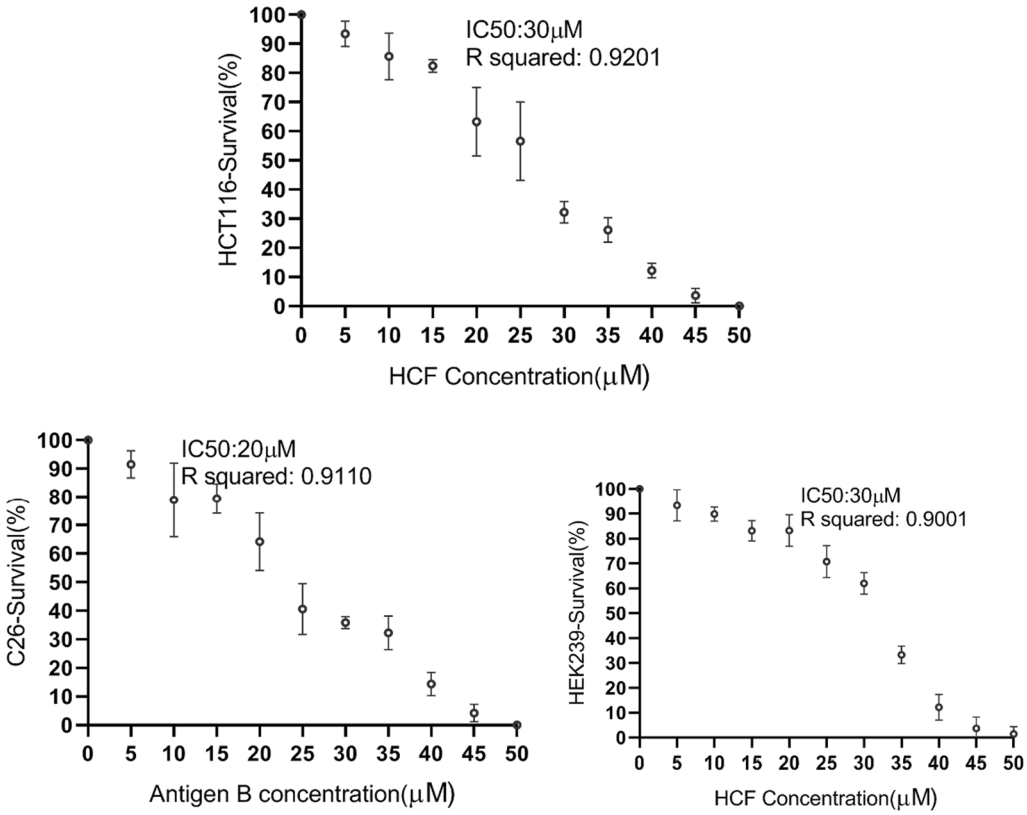


Fig. 1 Survival of C 26, HCT 116 and HEK 239 cells exposed to different concentrations of HCF in 24 h

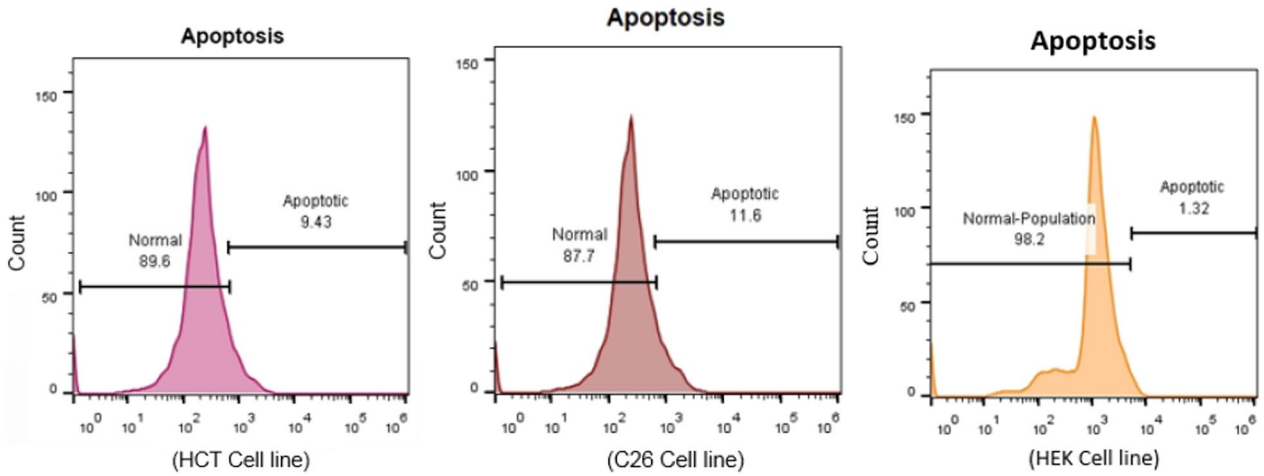


Fig. 2 An example of a measurement of apoptosis in HCT116, C 26 and HEK293 cell lines exposed to HCF

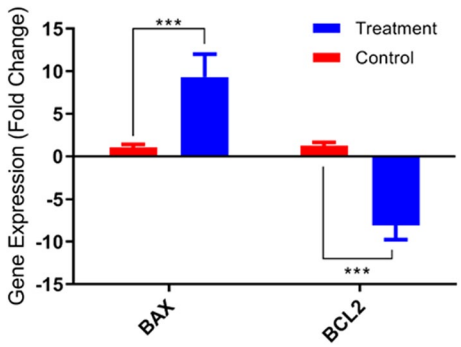


Fig. 3 Gene expression of BAX and BCL2. (***$P < 0.001$)

granulosus may cause a protective effect against cancer. Protoscolices in hydatid cysts (the larval stage of *E. granulosus*) could induce cell death in WEHI164 fibrosarcoma cell in vitro [39]. Moreover, vaccination with hydatid fluid induced tumor regression in mice with experimental C26 colon cancer [14]. Altogether, these evidence suggested that *E. granulosus* may show a protective effect against some cancer types in vitro and in vivo or the prevalence of cancer is significantly lower in people with hydatid infection than in normal ones [6]. Despite the great investigation of researchers, the mechanisms of the anticancer effect induced by *E. granulosus* are unknown [40]. Several probable mechanisms have been proposed, including the direct anticancer effect of parasite molecules and the indirect anticancer effect through stimulation of the host immune response. In the acute stage, the oncosphere releases EgKI-1 (Kunitz-type protease inhibitor) which potently null the neutrophil elastase and could inhibit some human cancers from growth and migration, probably through cell cycle disruption and induction of apoptosis in cancer cells, without affecting normal cell growth in vitro [41]. Meanwhile, the host immune system recognizes the mucin-type O-glycan of the parasite, which leads to activating innate and Th1-polarized immune responses, which are protective against cancer. But in the chronic stage, when the cyst dies or ruptures, the content will be released into liver or other infection sites which quickly activates innate immune system and converts Th2 response to Th1 response. The Th1 response is protective against cancer. Ag B, a potent neutrophil elastase inhibitor highly expressed in the hydatid cyst, may show an anticancer effect through inhibition of neutrophil elastase and neutrophil chemotaxis. In addition, the protoscolex may also have a role in the anticancer effect [40].

The indirect mechanism of anticancer effect is through activation of the host immune system. Cancer cells could activate innate and adaptive immunity the same as parasite infection [42]. Therefore, it has been hypothesized that parasites chronically with low-density infection may induce indirect anticancer activities by boosting the host immune system [39].

The similarity between several parasites antigens and certain cancer types has been also reported, mainly the cancer-associated mucin-type O-glycans [19], which causes crosstalk between parasites and carcinomas. In the case of *E. granulosus*, the initial evidence of common antigen came from a report in 1970s, which expressed that an immunoelectrophoresis test with hydatid fluid and serum of a patient with a pulmonary carcinoma led to an intense precipitin band [43].

The immunity induced by the parasite depends on infection stages: (1) during oncosphere invasion stage, a Th1-polarized response is dominant; (2) in the phase of cyst formation and growth, a Th2-polarized response will start; (3) when the cyst dies and ruptures, the Th2-polarized response will quickly replace by Th1-polarized

response [44, 45]. It seems that the Th1-polarized response induced the anticancer effect at specific stages of infection [46]. Besides adaptive immunity, the studies also show natural killer cell activation, which indicates the role of innate immunity in the anticancer effect [47].

Conclusion

The findings of this study elucidate some of the mechanisms of the effect of hydatid cyst fluid antigens on the prevention of cancer cell progression, and our results clearly show some hypotheses about the effect of parasite antigens on cancer cells. It was shown that hydatid cyst fluid antigen can inhibit the progression of cancer cells by increasing the rate of apoptosis. Also, the HCF can inhibit genes that suppress apoptosis.

Abbreviations

C26	Colon cancer
HCF	Hydatid cyst fluid
MTT	2,5-Diphenyl-2H-tetrazolium bromide
BAX	Bcl-2 Associated X-protein
BCL2	B-cell lymphoma-2
qRT-PCR	Quantitative reverse transcription PCR
Ag B	Antigen B
HEK293	Human embryonic kidney
HCT116	Human colorectal carcinoma
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
DMSO	Dimethyl sulfoxide
PI	Propidium iodide
PBS	Phosphate-buffered saline
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
dNTP	Deoxyribonucleotide triphosphates

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Author contributions

We declare that we contributed significantly toward the research study; SM, NB designed the experiments. HT, SS, and AS performed the experiments. SM, AS, and MY wrote the manuscript, and AS revised the manuscript. SM, NB carried out the data analysis. All authors reviewed, considered, and approved the manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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