RESEARCH ARTICLE

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Molecular Insights into the Immunomodulating and Anticancer Mechanisms of *Eremina desertorum* (Forsskal, 1775) Mucin in HepG-2 and CACO-2 Cells

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Abstract

Background: The aim of the present research is to deeply investigate the cytotoxic and immunomodulatory activities of the mucin extracted from Ereminia desertorum snails´ mucus against two tumor cell lines; human hepatocellular carcinoma (HepG-2) and human colon adenocarcinoma (CACO-2) cells. Both cell lines were treated with Ereminia desertorum snails´ mucin and the anti-cancer potential of the mucin was evaluated by the crystal violet assay test and gene expression analysis using reverse transcription- polymerase chain reaction (RT-PCR). **Results:** The extract showed cytotoxic activity on both tumor cell lines however it was more pronounced CACO-2 (IC_{50}: 0.20±0.05 (µl/ml)) than HepG-2 (0.103±0.019 (µl/ml)). Gene expression levels (2^{-ddct}) of the transforming growth factor (TGF) β1, and tumor necrosis factor (TNF) α showed increased expression in HepG-2 and CACO-2 cells treated with the mucin extract by (18.38 and 19.14) and (14.38 and 10.69) folds respectively. Apoptotic gene expression (Cas3 and Cas9) in HepG-2 and CACO-2 cells showed increased expression after treatment with the mucin extract by (16.14, and 11.00) and (23.58 and 12.50) folds, respectively. Gene expression levels (2^{-ddct}) of Oncogenic markers (c-myc, Ras, β-catenin, and EGFR) in HepG-2 and CACO-2 cells treated with mucin extract decreased by (0.025, 0.025, 0.047 and 0.040) and (0.064, 0.183, 0.111 and 0.26) folds, respectively. **Conclusion:** The present study highlighted the anticancer and immunomodulatory activities of the mucin extracted from E. desertorum snails´ mucus. This could attract attention to such natural compound as a possible source of a therapeutic product against liver and colon cancers.

Keywords: *Eremina desertorum*- Mucin- Antioxidant- Anti-tumor- CACO-2- HepG-2

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Introduction

Molluscs are globally distributed and found in all habitats [1]. They were found in freshwater, marine and terrestrial habitats [2, 3]. This phylum contained a large number of invertebrates and considered as the second largest phylum after arthropods [4]. The extract of their soft body tissues contained high protein content and it has been used for long time as food resources and in folk medicine [5, 6]. Species of this Phylum had bioactive constituent that could be used as antioxidants, anticancer, antibacterial and antiviral agents [2, 4, 7].

Members of Mollusca secreted mucous that is used to help the animal in movement and protect it from desiccation and microbial infection [5, 8].The main components of this mucous are glycoproteins and mucopolysaccharides [9-11]. Mucin is a derivative of mucous that has many biomedical uses as it is a glycosylated protein that is widely used as hepatoprotective, anticancer, anti-aging, antioxidant, a wound healing and anti-microbial agents [1, 12-14]. *Eremina desertorum* (Forsskal, 1775) snails are desert snails that secrete mucous for their movement [9, 11]. Fatty acid esters, sesquiterpenes, quinolones and monoterpenes were the main bioactive component that were identified in their mucous by GC-MS/MS analyses [3]. *Eremina desertorum* mucin has antioxidant, anti-inflammatory, anticancer and antimicrobial properties [2-3].

Liver cancer is one of the most common cancers which resulted in deaths worldwide. It affected men more than women [15]. There are two types of the liver cancers which are hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma (ICC) [16]. HCC is a primary malignant tumor that affects hepatocytes [17].

Colorectal cancer represented the second most deadly cancer and the third most common malignancy in the gastrointestinal tract. It resulted in 0.9 million deaths worldwide in 2020 [18]. In spite of the curative methods

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available for handling these tumors but it stills a non-solvable and socioeconomic problem [19]. Therefore, huge efforts should be directed towards the necessity to investigate and evaluate novel natural preventive strategies to lower the occurrence and consequences of this disease.

Recently, mucin from terrestrial snails has been proven to be a good natural product to treat cancer and melanoma resistance [2]. Helix aspersa snail's mucin was used to treat human melanoma cells. It diminished the metastasis of the melanoma cells and reduced its viability [20]. Crystal violet (CV) cell cytotoxicity assay is one of the common methods used to detect cell viability or drug cytotoxicity. It is a non-enzymatic assay that is easy and simple that is used for the quantification of the viable adherent cells and colonies [21]. It relies on the affinity between the dye and the external surface of the Deoxyribonucleic acid (DNA) double helix where the amount of the dye absorbed depends on the total DNA content in the culture [22]. Therefore, the aim of the present work is to demonstrate the anticancer activity of E. desertorum mucin on HepG2 and CACO-2 cell lines using crystal violet assay and gene expression analysis.

Materials and Methods

Handling of Eremina desertorum snails, slime and mucin
a. Animals

Dechlorinated water was used to clean all individuals to remove excretions on the desert snails *Eremina desertorum* (Forsskal 1775) mantles and shells. Snails were raised in plastic boxes (16 x 11x 6 cm), temp., 26- 28 C°, kept under high soil moisture (80% relative humidity) and given access to fresh green lettuce leaves.

b. Snail slime and mucin extraction

The pure fresh slime samples of the cleaned foot epithelium were obtained according to [2]. The mucous was macerated in water at 40°C for 24 hours. Extraction of mucin was done according to [10]. Preparation of extract

Ereminia desertorum snails´ mucin was warmed to 37°C and filtered using syringe filter (45µm pore size) [2]. The sterile mucin was collected in sterile tube and used at once.

Anticancer activity evaluation
Cell line preparation

HepG2 and CACO-2 cell lines were obtained from our institute. Cells were revived and cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 1% penicillin/streptomycin (Sigma Aldrich), 1% L glutamine (Sigma Aldrich) and 10% fetal bovine serum (FBS) (Gibco). Cell culture flasks were incubated in 5% CO_2 humidified incubator (Thermo) till reaching confluency.

When cultured cells reached 80% confluency, media were discarded and flasks were washed with phosphate buffered saline (PBS) (Gibco) and cells were trypsinized by 0.25% Trypsin-EDTA solution (Sigma Aldrich). Flasks were incubated at 37°C for 2 min. Trypsin was deactivated by adding equal volume of culture media and Cells were collected and washed with PBS twice by centrifugation. HepG2 and CACO-2 cells were plated at a seeding density of $7x10^5$ viable cells/ml in 96 well culture plates and plane wells containing medium without cells were included. Plates were incubated in 5% CO_2 incubator (Thermo Scientific, USA) at 37°C allowing cells to grow into a monolayer.

Applying the extract to Cell lines

On full confluency of the wells, media were discarded and serial dilutions of extract (0.1, 0.05, 0.025…0.0015 mg/ml) were prepared in serum free RPMI. 200 µl of either concentration were added to each well of 96 well culture plate containing cell line, in triplicate. Negative control (wells with RPMI only) and positive control (wells with RPMI and lead nitrate 50mM/ml) were included. Plates were incubated overnight in 5% CO_2 incubator.

Morphological assessment of the culture cells

HepG-2 and CACO-2 cell plates were observed after 48 h of treatment using tissue culture inverted microscope (Olympus, USA). Detectable morphological changes such as loss of adherence character, loss of the spindle shape and gaining the round shape, shrinking, granulation or vacuolization were considered as indicators of cytotoxicity.

Crystal violet (CV) Cytotoxicity Assay

The culture medium was removed. Cells were washed gently with 200 µl of 1X Washing Solution to avoid disturbance of the cell monolayer. Wash solution was removed by pipetting. 50 µl of Crystal Violet Staining Solution was added to each well and stain for 20 min at RT. After incubation, the staining solution is removed. Use 200 µl of 1X Washing Solution (PBS+1% tween) to wash the cells. Wash the cells for 4 times. At the end of the 4th washing step, remove washing solutions as much as possible by pipetting and air-dry the plate if necessary.

100 µl of Solubilization Solution (10 Ethanol 99%: 9 DW: 2 Glacial acetic acid) was added to each well. The plate was applied to a shaker for 20 min at room temperature and the O.D. was measure at 590 nm. The inhibition activity (%) was calculating using graphpad prism 8.

Gene expression analysis
a. Cell trypsinization

Flasks were washed with phosphate buffered saline (PBS) (Gibco) and cells were trypsinized by 0.25% Trypsin-EDTA solution (Sigma Aldrich). Flasks were incubated at 37°C for 2 min. Trypsin was deactivated by equal volume of culture media and cells were collected, washed with PBS twice by centrifugation and subjected to gene analysis by RT-PCR.

b. Ribonucleic acid (RNA) extraction

RNA was isolated using the RNeasy Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. One microgram of cellular RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase and random hexamer primers (Invitrogen Life Technologies, USA).

c. RT-PCR

The PCR reaction was carried out in a volume of 10 µl in a mixture that contained appropriate sense- and anti-sense primers and a probe in TaqMan Universal PCR Master Mixture (Applied Biosystems, Foster City, California). We used the Assays-on-DemandTM Gene Expression products, which consist of a 20x mix of unlabeled PCR primers and TaqMan MGB probe (FAMTM dye-labeled). These assays are designed for the detection and quantification of specific human genetic sequences in RNA samples converted to complementary DNA (cDNA). The primers used are illustrated in Table 1. Real-time PCR amplification and data analysis were performed using the A7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was assayed in duplicate in a MicroAmp optical 96-well plate. The thermo-cycling condition consisted of 2 minutes at 50°C and 10 min incubations at 95°C, followed by 40 two-temperature cycles of 15 seconds at 95°C and 1 min at 60°C. Gene expression was normalized to internal controls and fold changes were calculated using the relative quantification method ($2^{-\Delta\Delta Cq}$).

Statistical analysis

One Way Analysis of Variance (ANOVA) test was used to test the effect of the extract on the measured parameters of tested cells in comparison to the other two groups.

Results

Assessment of Cytotoxic Activity of the Mucin
Morphological changes

Both HepG-2 and CACO-2 cells were evaluated by morphological changes; cells treated with Ereminia desertorum snails´ mucin showed detectable changes when compared to the negative control. Rounding and shrinking of the cells, disturbance of the cell monolayer, as well as characteristic changes of cell death including blebbing, granulation, and nuclear fragmentation were obvious in cells treated with Ereminia desertorum snails´ extract when compared to the characteristic adherent shiny spindle shaped normal cells (Figure 1).

Table 1. Primers Sequences for RT-PCR

Symbol	Gene	Primer sequence	Reference
Cas3	Caspase3	F: 5'-TGTTTGTGTGCTTCTGAGCC-3'	Thantip et al. (2017)
		R: 5'-CACGCCATGTCATCATCAAC-3'	
Cas9	Caspase9	F: 5'-CATTTCATGGTGGAGGTGAAG-3'	Thantip et al. (2017)
		R: 5'-GGGAACTGCAGGTGGCTG-3'	
TGFβ1	Transforming growth factor beta 1	5'-GGATACCAACTATTGCTTCAGCTCC-3'	Elsa et al.(2016)
		5'-AGGCTCCAAATATAGGGGCAGGGTC-3'	
TNFα	Tumor necrosis factor alpha	5'-AAAGCTTATGAGCACTGAAAGCATCAT-3'	Sayed et al.,2019
		5'-TGTAGATCACAGGGCAATGATCCCAAAG-3'	
N-RaS	Mammalian transforming ras genes	F: 5'-AGGACATACTGGATACAGCTGGA-3'	Sun et al. (2009)
		R: 5'-TGTCTGGTCTTGGCTGAGGT-3'	
β-Catenin	beta-catenin protein	F: 5'-GCGTGGACAATGGCTACTCAAG-3'	LA COSTE et al. (1998)
		R: 5'-TATTAACTACCACCTGGTCCTC-3'	
c-MYC	Proto-oncogen c-MYC	5'-CAG CAG AGCGAG CTG CAG CC-3'	CARRAMUSA et al.,2007
		5'-CTG TCT TTG CGC GCA GCCTG-3'	
EGFR	Epidermal growth factor receptor	F: 5'- AAGGAAATCCTCGATGAAGCCT-3'	Montero et al., 2006
		R: 5'- TGTCTTTGTTCCCGGACATA-3'	
β-actin	Beta-actin (reference gene)	F: 5-TCCCTGGAGAAGAGCTACG-3'	Sharula and Zhongjun. (2017)
		R: 5-GTAGTTTCGTGGATGCCACA-3'	

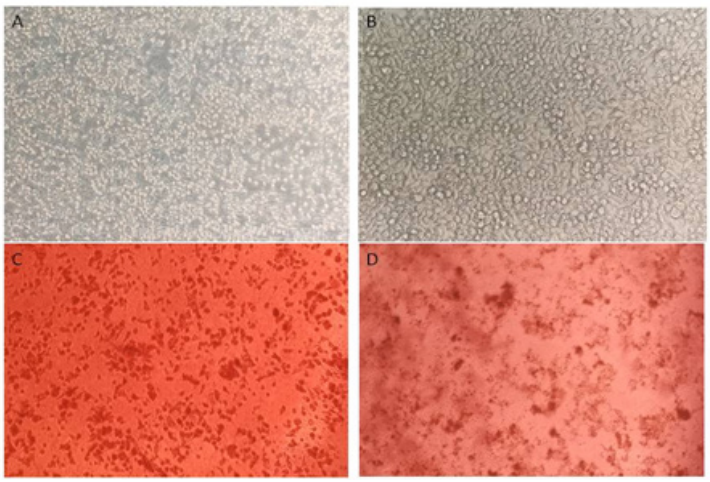

Figure 1. A HepG2 Anti-Proliferation Effects of Ereminia Desertorum Snails´ Mucin on HepG2 Cell Line

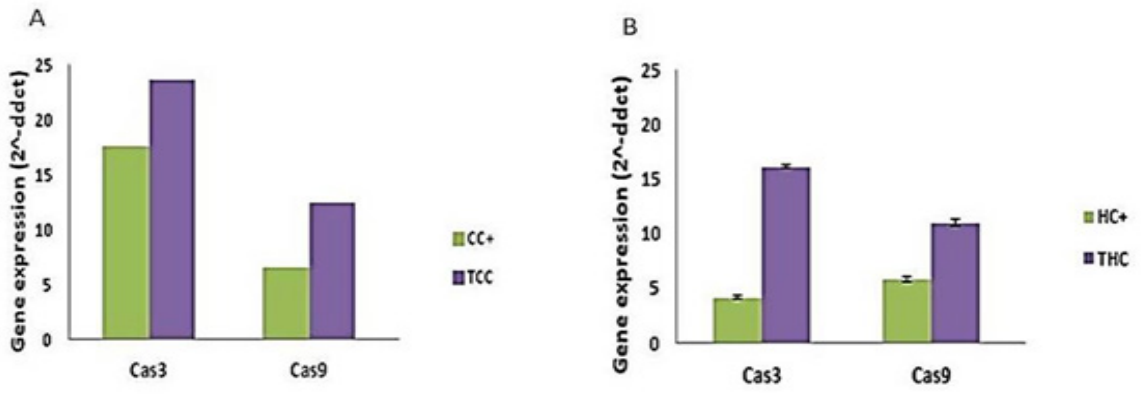

Figure 2. Apoptotic Genetic Expression Levels in HepG-2 (A) and CACO-2 (B) cell groups. Abbs; Cas3, Caspase3; Cas9, Caspase9.

Calculation of IC_{50}

The anticancer activity of extract was evaluated against HepG2 and CACO-2 cell lines using crystal violet cytotoxicity assay. As illustrated in Figures 2 and 3, the proliferation of HepG2 and CACO-2 cells was inhibited by using both Extract and chitosan in a concentration-dependent manner. Mucin was more effective in the inhibition of proliferation of HepG2 cells [IC50 = (0.20±0.05 µl/mL] (Figure 4A). Mucin could also inhibit the proliferation of CACO-2 cells [IC50 = 0.103±0.019 µl/ml] (Figure 4B).

Gene expression analysis

Immune markers

TGFβ1, and TNFα showed increased expression in HepG-2 and CACO-2 cells treated with the mucin extract by (18.38 and 19.14) and (14.38 and 10.69) folds respectively when compared to the cells treated with lead nitrate (3.01 and 4.60) and (9.67 and 6.06). Gene expression levels in untreated control cell groups equal (1) in data analysis and calculation. That means that the immune markers were up-regulated in HepG-2 and CACO-2 cells by treatment with mucin extract and controversial, down-regulated by treatment with lead nitrate (Figure 5).

Apoptotic genetic markers

Gene expression levels (2^{-ddct}) of Apoptotic genetic markers in HepG-2 and CACO-2 cells; Cas3, Cas9, showed increased expression in cells treated with the

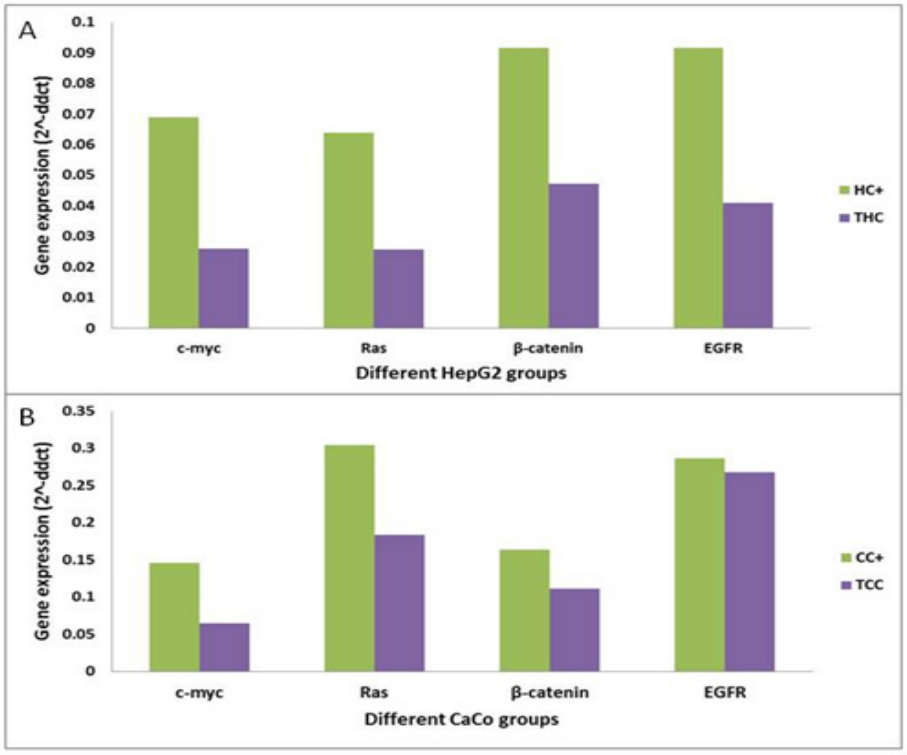

Figure 3. Oncogenic Genetic Expression Levels in HepG-2 and CACO-2 Cell Groups. Abbs; c-myc: Proto-oncogen c-MYC, Ras: Mammalian transforming ras genes, β-catenin: beta-catenin protein and EGFR: Epidermal growth factor receptor.

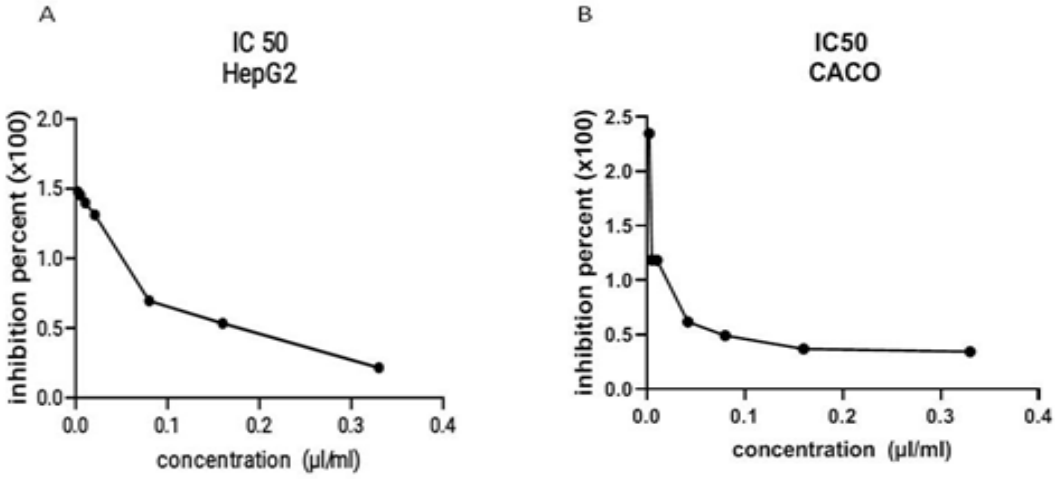

Figure 4. Anti-Proliferation Effects of Ereminia Desertorum Snails´ Mucin on HepG2 (A) and CACO-2 (B) cell lines.

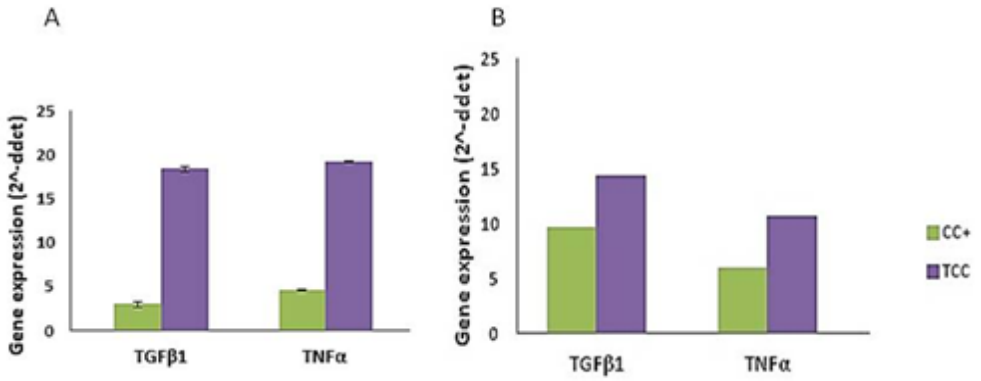

Figure 5. Immune Marker Genetic Expression Levels in HepG-2 (A) and CACO-2 (B) Cell Groups. Abbs; TGFβ1: Transforming growth factor beta1 and TNFα: Tumor necrosis factor alpha.

mucin extract by (16.14, and 11.00) and (23.58 and 12.50) folds, respectively when compared to the cells treated with lead nitrate (4.17, and 5.84) and(17.53, 6.54). Gene expression levels in untreated control cell group equal (1) in data analysis and calculation. That means that the Apoptotic genetic markers were up-regulated in HepG-2 and CACO-2 cells by treatment with mucin extract and controversial, down-regulated by treatment with lead nitrate (Figure 2).

Oncogenic genetic markers

On the contrary, Gene expression levels (2^{-ddct}) of Oncogenic markers in HepG-2 and CACO-2 cells; Proto-oncogen c-MYC (c-myc), Mammalian transforming ras genes (Ras), beta-catenin protein (β-catenin), and EGFR: Epidermal growth factor receptor showed increased expression in cells treated with the lead nitrate by (0.068, 0.063, 0.092 and 0.091) and (0.14, 0.30, 0.16 and 0.28) folds, respectively when compared to the cells treated with mucin extract (0.025, 0.025, 0.047 and 0.040) and (0.064, 0.183, 0.111 and 0.26). Gene expression levels in untreated control cell groups equal (1) in data analysis and calculation. That means that the oncogenic genetic markers were up-regulated in HepG-2 and CACO-2 cells by treatment with lead nitrate and controversial, down-regulated by treatment with mucin extract (Figure 3).

Discussion

Hepatocellular carcinoma (HCC) is the third malignant tumor cause of nearly 500,000 cancer-related mortality in males each year [23]. Recently, a huge number of natural products have gained a lot of attention because of their ability to protect and treat a lot of cancers [24-26]. In this study, the anticancer and immunomodulatory activities of Ereminia desertorum snails´ mucin was tested in vitro against HepG-2 and CACO-2 cells. E. desertorum snail mucin has antioxidant, anti-inflammatory and anticancer activities [27]. The present results showed that both HepG-2 and CACO-2 cells treated with E. desertorum snails´ mucin had detectable disturbance of the cell monolayer, rounding and shrinking of the cells, blebbing, granulation, and nuclear fragmentation which indication of the cell death when compared to the characteristic adherent shiny spindle shaped normal cells of the negative control. Also, Mucin was more effective in the inhibition of proliferation of HepG2 and CACO-2 cells. These results

were in a good accordance with (Fieber et al. 2012) who reported that the morphological changes in cells such as retraction, rounding, and granular appearance suggesting impending cell death [28]. Also, (Farshori et al. 2015) observed the morphological alterations in HepG2 cells after exposure to 400 mM of ethanol where cells lost their identical shape and shrank in size, while using Lavandula coronopifolia extracts reduced the cell viability of HepG2-induced ethanol and restored their original morphology [29]. Recently, the use of natural products to reduce the toxicity and cancers has been evolved [30]. Previous study has been reported that the mucous of Achatina fulica snails could decrease the viability and proliferation of triple-negative breast cancer (TNBC) cells with relatively lower cytotoxicity to normal breast epithelial cells and enhanced their response to chemotherapy [26], where it induced its death through an extrinsic apoptotic pathway and activation of Fas signaling. *Eremina desertorum* and Helix aspersa snail mucus has been reported to lack cytotoxicity on normal human skin fibroblast (HSF) cells where, IC50 > 300 µg/ml in both snails and this elucidated the disappearance of any toxic activity of the used concentration [27]. Transforming growth factor (TGF)-β1 is the inflammatory cytokine that promotes Epithelial to Mesenchymal Transition (EMT) [23, 31] and induces the invasion and migration of liver cancer cells [32-34]. The present investigation showed that treatment with mucin extract up-regulated the expression of the immune markers (TGFβ1, and TNFα) in HepG-2 and CACO-2 cells. Similarly, Liv.52; A herbal hepatoprotective and immunomodulatory drugs-has been confirmed to protect the liver from toxicity with ethanol through down regulation of TNFα expression [35]. Regarding the Apoptotic genetic markers expression levels in HepG-2 and CACO-2 cells (Caspase (cas) 3, 9); they were increased in cells treated with the mucin than the cells treated with lead nitrate. These Apoptotic genetic markers were up-regulated in HepG-2 and CACO-2 cells by treatment with mucin and controversial, down-regulated by treatment with lead nitrate. Clustered Regularly Interspaced Short Palindromic Repeats, (CRISPR)–Cas systems are RNA-guided nucleases that help in protection against viral infections. Type I systems are the most prevalent CRISPR–Cas systems in nature (endogenous CRISPR–Cas3 systems) that is used in genetic manipulation while for gene -editing applications was focused on single subunit Class 2 CRISPR systems (Cas9 and Cas12a) [36]. Previous study has been stated that snail mucus could has inhibits the viability of TNBC cells through induction of extrinsic apoptosis pathway through suppressing of nucleolin activation via Fas signaling [26]. Authors stated that the main component in snail mucous is a glycosaminoglycan which could bind to the plasma membrane nucleolin and suppresses the nucleolin-inhibited cell apoptosis. The over expression of intrinsic caspase-9pathway of apoptosis would increase the apoptosis in HepG2 cells treated by nisin [25]. The present results showed that the oncogenic genetic markers (c-myc, Ras, β-catenin, and EGFR) were down-regulated by treatment with mucin extract while up-regulated in HepG-2 and CACO-2 cells by treatment with lead nitrate. c-myc is a nuclear proto-oncogenes that overexpressed in more than 70% of human cancers [37]. The down-regulation of c-myc expression could be mandatory for the induction of apoptosis in cancers [38]. Many natural products might inhibit cell proliferation through the down regulation of c-myc expression [39]. These results in a good accordance with Endrini et al. [38] who confirmed the use of two plants, henna (Lawsonia inermis) and kejibeling (Strobilanthes crispus) as anticancer agents on (Caco-2), liver cancer cell lines (HepG2), and breast cancer cell lines through the down-regulation of c-myc expression. RAS Genes is of great significance for the development of cancers where it found in nearly about 30% of human cancers [17, 40]. It induced a large number of vacuoles and caspase-independent cell death called methuosis [41]. Efforts have been made to attenuate RAS activity through the therapeutic inhibition of downstream signaling molecules [40, 42, 43]. β-catenin is a major oncogene protein in Hepatocellular carcinoma (HCC) based on the mutations associated with aberrant Wnt/β-catenin signaling pathway that involved in the development and/or progression of tumorigenesis and tumor immune-evasion [44, 45]. It interacted with members of cadherin family to promote the signaling of the growth factor receptors like epidermal growth factor receptor (EGFR) and support HCC cell survival [46]. Tzanavaras et al. [47] reported that an EGFR tyrosine kinase inhibitor which is called Gefitinib could inhibit the growth of mouse liver cancer cells (H22).

Therefore, the present results confirmed the anticancer and immunomodulatory potencies of E. desertorum snails. These results were also confirmed by Atta et al. [2] who reported antioxidant and anti-cancer activities of E. desertorum snails´ mucin. Also, Mane et al. [48] concluded that the biogenically synthesized silver nanoparticles and mucus of Achatina fulica snails had an anticancer activity and resulted in more than 15% inhibition of Hela cells. Helix aspersa snails' mucus has ameliorated the induced colitis and has antioxidant and anti-inflammatory properties [49].

In conclusion, the present study elucidated the anticancer and immunomodulatory activities of Eremina desertorum snails´ mucin was tested in vitro against HepG-2 and CACO-2 cells. Further studies were needed to elucidate the best way for using these natural materials in the biomedical applications.

List of abbreviations

ANOVA: Analysis of Variance
CACO-2: human colon adenocarcinoma
cas: Caspase
cDNA: complementary DNA
c-myc: Proto-oncogen c-MYC
CRSPR: Clustered Regularly Interspaced Short Palindromic Repeats
CV: Crystal violet
DNA: Deoxyribonucleic acid
EGFR: Epidermal growth factor receptor
EMT: Epithelial to mesenchymal transition
FBS: fetal bovine serum
H22: mouse liver cancer cells

HCC: hepatocellular carcinoma
HepG-2: human hepatocellular carcinoma
HSF): human skin fibroblast
ICC: intrahepatic cholangiocarcinoma
PBS: phosphate buffered saline
Ras: Mammalian transforming ras genes
RNA: Ribonucleic acid
RPMI: Roswell Park Memorial Institute
RT-PCR: Reverse transcription polymerase chain reaction
TGF: transforming growth factor
TNBC: triple-negative breast cancer
TNF: tumor necrosis factor
β-catenin: beta-catenin protein

Author Contribution Statement

Amina Ibrahim, Shimaa Atta, Fayed Megahed put Conceptualization and performed the methodology. Shimaa Atta, Fayed Megahed performed the analysis and carried out data creation. Amina Ibrahim, Shimaa Atta, Fayed Megahed written and prepared original draft and reviewed and edited the manuscript. The authors read and approved to published version of the manuscript.

Acknowledgements

Not applicable.

Ethics statement

All experimental procedures were conducted according to guidelines of Ethics Committee of Theodor Bilharz Research Institute (TBRI).

Conflict of Interest

The authors declare that they have no conflict of interest.

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