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Original Article

COMPARATIVE STUDY OF TWO *HELIX ASPERSA* EXTRACTS ON TUMOR CELL LINES (HUT-78 AND SEAX) PROLIFERATION AND MMP-9 SECRETION

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ABSTRACT

Objective: Despite the progress in cancer research, current therapies are ineffective and cause many adverse effects. The discovery of new natural anti-tumor agents that can act on multiple mechanisms of growth and tumor invasion with minor side effects and which can be safe for patients. Therefore, we sought new natural products from an invertebrate organism belonging to the phylum of mollusks: a land snail, *Helix aspersa.* This study was aimed to evaluate the cytotoxic activity of two extracts prepared from *Helix aspersa*, on two cutaneous T cell lymphoma cell line (HUT-78 and SeAx). Their effect on MMP-9 expression was also tested.

Methods: We prepared from the snail *Helix aspersa*: an aqueous (AE) and a hydroalcoholic extracts (HAE). We have evaluated the concentration of total proteins and total phenols in these extracts. The percentage of cell mortality was evaluated after incubating the cell lines with the two extracts at different concentrations, by using trypan blue exclusion method. Finally, the tumor cells expression of metalloproteinase MMP-9 was examined by zymography analysis.

Results: We have found that 1 mg/ml of AE contains (4.53±0.48 mg) of total proteins and (2.44±0.11 mg) GAE $g^{-1}$of phenols. 1 mg/ml of HAE contains (1.83±0.23 mg) of total proteins and (2.81±0.16 mg) GAE g^{-1} of phenols. On one hand, both extracts exerted a toxic effect on these tumor cells. Indeed, aqueous extract induced 44.09 % mortality in HUT-78 and 31.47 % mortality in SeAx tumor cell line with 50 µg/ml. While, the hydroalcoholic extract induced 29.90 % in HUT-78 and 25 % in SeAx. On the other hand, the result showed no changes of MMP-9 expression.

Conclusion: *Helix aspersa*'s extracts had a toxic effect on cutaneous T cell lymphoma (CTCL), but did not inhibit the production of the protease by these two cell lines in culture.

Keywords: *Helix Aspersa* extracts, HUT-78 cell line, SeAx cell line, Cytotoxicity, MMP-9 expression

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INTRODUCTION

Helix aspersa is a terrestrial pulmonate gastropod of the helicidae family. It is the most widely cultivated specie and most profitable economically. It is an important source of protein and microelements (salt, copper, iron, phosphor, and selenium) [1, 2]. Deficiency of certain microelements can be responsible for an increased risk in many diseases, such as: cardiovascular diseases, several forms of cancer, immunodeficiency, and allergies. Many studies confirmed in human nutrition, the potential quality of the protein content issued from snail. However, its importance in human therapeutics is poorly documented.

Helix aspersa is widespread in the world because of its adaptability against climatic and environmental stress conditions. This assumes that the snail would produce chemical substances against many dangers it would face mobile predators, invading microorganisms and parasites. Therefore, we explored and evaluated the effects of two snail's extracts against proliferation of two tumor cell lines of cutaneous lymphomas named HUT-78 and SeAx.

During the various stages of tumor development, the malignant cells acquire several mechanisms that favor tumor growth and invasion to other tissues. The ability of tumor cells to migrate through the extracellular matrix (ECM) has been partly attributed to their capacity to secret some proteases called matrix metalloproteinase (MMPs). MMPs are a family of which the first enzyme was discovered in 1962 [3]. These enzymes are endopeptidase zinc-dependent and have high-affinity for binding sites of ECM components. They are mainly produced by inflammatory cells such as macrophages, neutrophils, mast cells, dendritic cells and T cells. [4, 5]. They are also associated with invasion and metastasis [6]. [6]. Many studies showed the link between the irregularity of the expression of MMPs and cancer development [7-9]. They have received close attention since their production increased and appeared to correlate with the malignant potential of several types of cancer, including cutaneous lymphoma. Especially, an increase of MMP-9 mRNA, a type IV collagenase of 92 kDa à éliminer [10]. In the tumor cells of non-Hodgkin lymphoma [10, 11].

It is known that the growth and migration of tumor cells is the result of a dysfunction of the molecular machinery leading to the resistance of the tumor cell to apoptosis. This results in an increased-proliferative capacity, which associated with an increased MMP secretion favors, metastasis dissemination.

In this study we investigated in the first step the capacity of snail extracts (AE and HAE) to inhibit the proliferation of two cutaneous lymphomas cell lines HUT-78 and SEAX. In the second step we checked the effects of snail extracts on "MMP-9" secretion from these cell lines, using zymography technique.

MATERIALS AND METHODS

Preparation of *Helix aspersa* extracts

Helix aspersa was collected during the spring season in different regions of eastern Algeria. The animals were kept in the laboratory at room temperature (25 °C), and fed lettuce, cucumber and carrot. The extraction was performed according to the protocol elaborated by El-Wahidi M *et al.*, 2011 [12] and modified by us. Briefly, the gastropod *Helix aspersa* is stripped of its shell, and its visceral mass

is removed. The foot and the head were recovered, crushed and homogenized using a homogenizer (DAIHAN scientific-HG15AO90331601) at a rate of 20 t/min for 20 min. The homogenate obtained was lyophilized (Freeze drayer Labconco, France) (heat at 56 °C, Vacuum 0.9 mbar). The freeze-dried *Helix aspersa* was extracted three times in distilled water, and filtered on büchner. The filtrates obtained from the aqueous extract (AE) were frozen at-30 °C until use. The resulting pellet was extracted once with ethanol 80% and twice with absolute ethanol, filtered on büchner. The filtrates were combined and alcohol was fully evaporated using a rotary evaporator (at 40 °C). The suspension obtained was adjusted with distilled water to a volume of 55 ml then, extracted three times with the same volume of dichloro-methane.

The two phases, aqueous and organic, were separated using a separatory funnel. The aqueous phase was frozen at-30 °C and constitutes the extract HAE. The aqueous extract AE and hydroalcoholic extract HAE were lyophilized, to be subsequently diluted in culture medium RPMI 1640 and sterile buffer (PBS) respectively.

Estimation of total proteins and total phenolic content in the aqueous extract and hydroalcoholic extract

Total proteins content in aqueous extract and hydroalcoholic extract was determined by the Bradford method [13]. Total phenolics content in aqueous and hydroalcoholic extracts were determined by the method elaborated by Vermerris and Nicholson 2006 [14].

Cell culture

HUT-78 and SeAx cell lines were kindly provided by Dr Michel L (INSERM S 976, France). HUT-78 and SeAx are cutaneous T cell lymphoma, chosen because they are defective in apoptosis. Both HUT-78 and SEAX were maintained culture in RPMI 1640 medium, Gluta MAX supplement (Gibco-61870044), supplemented with 10 % decomplemented fetal bovine serum (Gibco-10270-106) and 1 % of antibiotics (penicillin and streptomycin). All cell lines were grown in 24-well tissue culture plates at 37 °C in a humidified atmosphere with 5 % CO_2 at 2 × 10^5 cells/ml in complete medium. For activation experiments, cell cultures were treated with snail extracts for 24 h.

Toxicity test

To evaluate the number of alive and dead cells, the cells were stained with 0.2 % trypan blue (Gibco-15250061) and counted on a hemocytometer. The cytotoxicity assay was performed to test the ability of the two extract "AE" and "HAE" to kill cancer cells. Cells stained in blue following trypan blue uptake were interpreted as nonviable.

Cell viability experiments were performed as follow: 250 µl of the cell suspension of the two lines at a concentration of 2 million cells per ml were incubated 24 h with different concentrations of each extracts previously filtered on a 0.2 µm filter. After 24 h of incubation, the cells were harvested. 10 µl of trypan blue at 0.8 % was added to 90 µl of each cell suspension. After 2 min, the number of stained cells and the total number of cells were counted to determine the percentage of death in each condition of stimulation.

Hoechst test (evaluation of chromatin condensation)

It is possible to detect apoptosis, through the dye Hoechst 33342 (Trihydrochloride, Trihydrate, 10 mg/ml Solution in Water-molecular probes, H3570), a fluorescent dye that stains DNA. Apoptosis was evaluated by the degree of chromatin condensation determined by fluorescence staining of living and dead cells according to the protocol given by the manufacturer (Molecular probes). The Hoechst staining method was as following:

- After stimulation of the two lines HUT-78 and SeAx with the respective extracts at a concentration of 50 µg/ml for 24 h, cells were pelleted and re-suspended in 100 µl with the diluted Hoechst reagent (1:50 in RPMI 1640 with 1 % antibiotic).
- Hoechst staining was visualized on 20 µl of cell suspension deposited on a slide.
- Finally, cells nuclei were observed under a fluorescence microscope (Leica DM2500).

Gel zymography

T-cell lymphoma HUT-78 and SeAx cells were seeded at a concentration of 2 million cells per ml on plastic plates and stimulated in a serum-free conditioned medium, with snail extracts. Cultures media were harvested after 24 h and separated through a 10 % sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 1 mg per ml gelatin as substrate. Following electrophoresis (PROTEAN® Tetra Vertical Electrophoresis Cell, biorad), the gels were washed in 2.5 % Triton X-100 for 1 h at room temperature; further incubated at 37 °C overnight in 50 mmol Tris-HCl, 5 mmol $CaCl_2$, 200 mmol NaCl (pH 7.6); stained with Coomassie blue G-250 (0.1 %); and destained in methanol/acetic acid/water (20/10/70; vol/vol/vol). MMP gelatinolytic activity was evidenced as white zones of lysis.

Aqueous and hydroalcoholic extracts from the terrestrial mollusk were tested at the following concentrations: 1 µg/ml, 5 µg/ml, 10 µg/ml and 50 µg/ml. Supernatant of untreated tumor cells was considered as control.

Statistical analysis

The values are expressed as mean±standard error of the mean. For comparison of different groups, *p*-values were calculated with student's t-test. In all cases $p<0.05$ was considered to be statistically significant.

RESULTS

Estimation of proteins and total phenolic in aqueous extract and hydro alcoholic extract

Total protein and total phenolic contents of *Helix aspersa* extracts tested for each extract are summarized in fig. 1. As can be observed, the quantity of proteins in aqueous extract was (1.83+-0.23 mg/ml, p=0.005) GAE g^{-1} in hydroalcoholic crude extract (fig. 1).

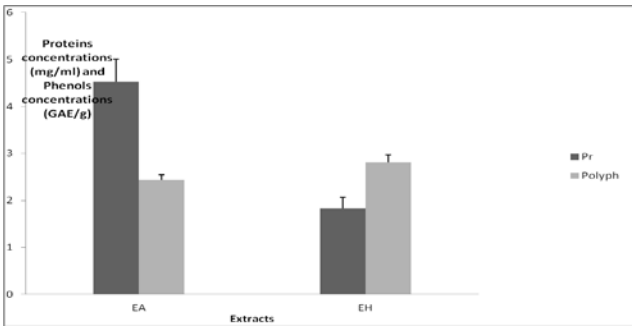

Fig. 1: Level of protein and total phenol content in aqueous and hydroalcoholic extracts of *Helix aspersa*

Pr: proteins, polyph: polyphenols

Cytotoxic effects of *Helix aspersa*'s extracts

Effects of aqueous extract on two lymphoma cell lines

HUT-78 and SEAX cells were cultured at a concentration of 2 million cells/ml. The mortality percentage of cancer cells was evaluated using trypan blue following cell stimulation with aqueous and hydroalcoholic extracts at different concentrations for 24 h. Effects of extracts were evaluated in triplicate. The number of dead cells was determined in each well. The cytotoxicity assay showed a significant increase in the number of dead cells in the culture medium supplemented with AE compared to control cultures. Compared with untreated cells (14.44±0.69%), the mean percentage of cell death at the end of incubation increased up to (17.59±2.42%, p=0.006) with1 µg/ml, (25.82±3.26%, p=0.005) with 5 µg/ml,

(25.19±5.44%,p=0.004) with 10 µg/ml and finally (44.09±3.89%, p=0.003) with 50 µg/ml of AE in HUT-78 (fig. 2), and (18.21±3.46%, p=0.012) with 1 µg/ml, (24.11±5.06%, p=0.014) with 5µg/ml, (27.67±5.44%, p=0.013) with 10 µg/ml, and (31.47±2.96%, p=0.003) in SeAx cell line compared to untreated cells (12.14±1.05%) (fig. 2).

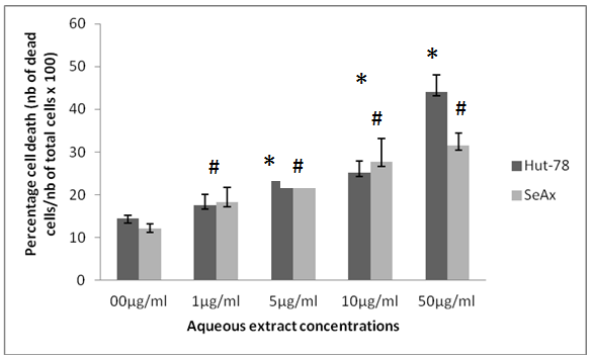

Fig. 2: Effects of different concentrations of aqueous extract on Hut-78 and SeAx cell death after 24 h of incubation, *p≤0.05 compared to control cultures of HUT-78. #p≤0.05 compared to control cultures of SeAx

Effects of hydro alcoholic extract on two lymphoma cell lines

We also observed a significant increase in cell mortality of the two lines upon treatment with HAE compared to untreated cells (14.44±0.69%); HAE treatment enhanced cell mortality up to (20.47±5.53%, p=0.023), (20.81±3.44%, p=0.009), (24.24±6.17%, p=0.021) and, (29.90±5.98%, p=0.013), when used at 1 µg/ml, 5 µg/ml, 10 µg/ml and 50 µg/ml respectively in HUT-78 cell line (fig. 3). However, the observed cell mortality was of (18.84±1.47%, p=0.002) with 1 µg/ml, (21.31±2.21%, p=0.004) with 5 µg/ml, (23.41±2.25%, p=0.003) with 10 µg/ml and (25±5.57%, p=0.016) with 50 µg/ml as compared to untreated SeAx cells (12.14±1.05) (fig. 3).

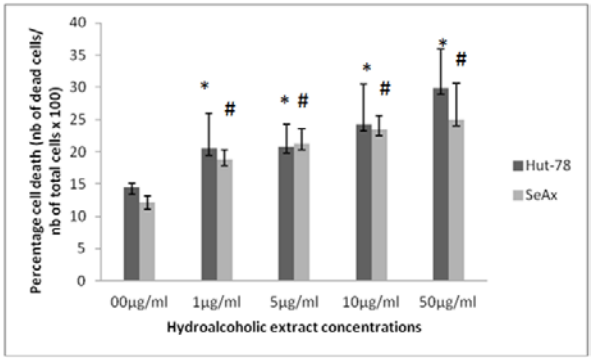

Fig. 3: Effects of different concentrations of alcoholic extract on Hut-78 and SeAx cell death after 24 h of incubation, *p ≤0.05 compared to control cultures of HUT-78. #p≤0.05 compared to control cultures of SeAx

Chromatin condensation

To further the type of cell cytotoxicity induced by the aqueous extract corresponded to cells necrosis or apoptosis, a marking of the cell nuclei was performed with the DNA counter stain hoechst dye. Cell staining observed by fluorescence microscopy showed that treatment of the 2 cell lines HUT-78 and SEAX stimulated with AE at 50 µg/ml for 24 h caused a fragmentation of nuclei (yellow arrows, fig. 4B and 4D) and chromatin condensation (fig. 4 B and 4 D) respectively; as compare with untreated tumor cells HUT-78 and SeAx (fig. 4 A and 4 C) that exhibit normal morphology of nuclei except few cells of HUT-78 cell line (red arrow in fig. 4 A).

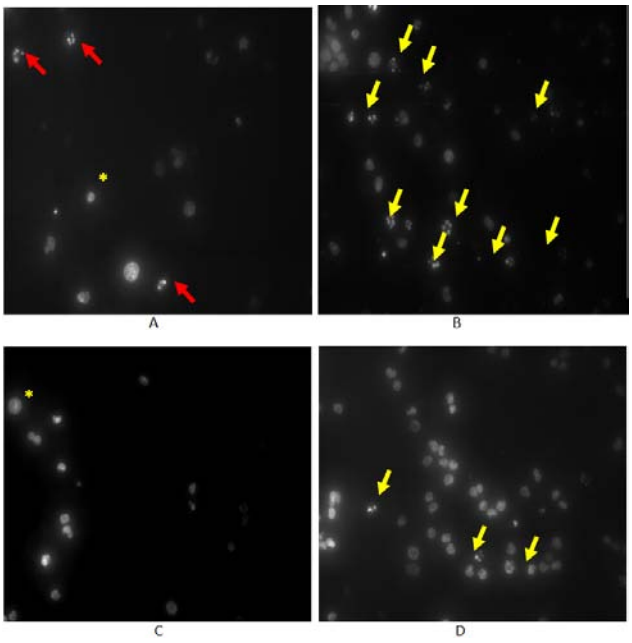

Fig. 4: Marking of nuclei of tumors cells incubated with A dans une nvl ligne. Marking of nuclei of the tumor cell line HUT-78 by the hoechst dye in the absence of sample (control). Note the presence of few cells with nuclear fragmentation (red arrow). Other cells had normal morphology. B: Marking of nuclei of the tumor cell line HUT-78 by the hoechst dye in the presence of the aqueous extract 50 µg/ml: The apoptotic cells appeared condensed and fragmented. We noticed a very bright color corresponding to condensed/fragmented chromatin. Percentage of apoptotic nuclei identified by hoechst staining was about 42 %. Cells that did not fluoresce had normal morphology. C: Marking of nuclei of the tumor line SEAX by the hoechst dye in the absence of sample (control). The cells had normal morphology. D: Marking of nuclei of the tumor line SEAX by the hoechst dye in the presence of the aqueous extract at 50 µg/ml: we revealed the presence of cells with a very bright condensed chromatin and fragmented nucleus. The average rate of apoptotic cells was around ajouter un point

Effects of the two extracts on the expression of the metalloproteinase MMP-9

To assess whether the nuclear textural and DNA changes were associated with modifications in MMP secretion, cells were incubated with the gastropod extracts as described above and the cultured media harvested at 24 h tested for MMP-9 activities by gel zymography (fig. 5). HUT-78 naturally expressed the MMP-9 protease under the pro-form of 92 kDa, whereas no protease activity was observed in the cultured media from untreated SeAx cells. Furthermore, no changes in MMP-9 secretion were observed in both HUT-78 and SeAx cells treated with the AE and HAE extracts whatever the concentration used (fig. 5A/B and 5C/D respectively).

To further confirm this lack of effects of the AE and HAE extracts, MMP-9 secretion from HUT-78 and SeAx cells was also analyzed after treatment with two cytokines used as positive control (CXCL10: Recombinant Human CXCL10/IP-10 Protein-RandD systems-266-IP and IL-17: Recombinant Human IL-17/IL-17A-RandD systems-317-ILB). CXCL10 induced a mild increase of MMP-9 secretion from HUT-78 cells, while the effects of IL-17 on MMP-9 secretion were more pronounced in these cells,

definitively suggesting that the secretion of MMP-9 was not affected by the gastropod extracts (fig. 5E). Nevertheless, none of the positive controls was able to induce MMP-9 secretion from the SeAx cell type (fig. 5E) showing that this protease is not expressed by these cells.

Cells were cultured for 24h and conditioned media were used for the measurement of MMP-9 activities by gelatin zymography. A and B are representative gel zymography performed with HUT-78 while C and D are representative of experiments performed with SeAx cells.

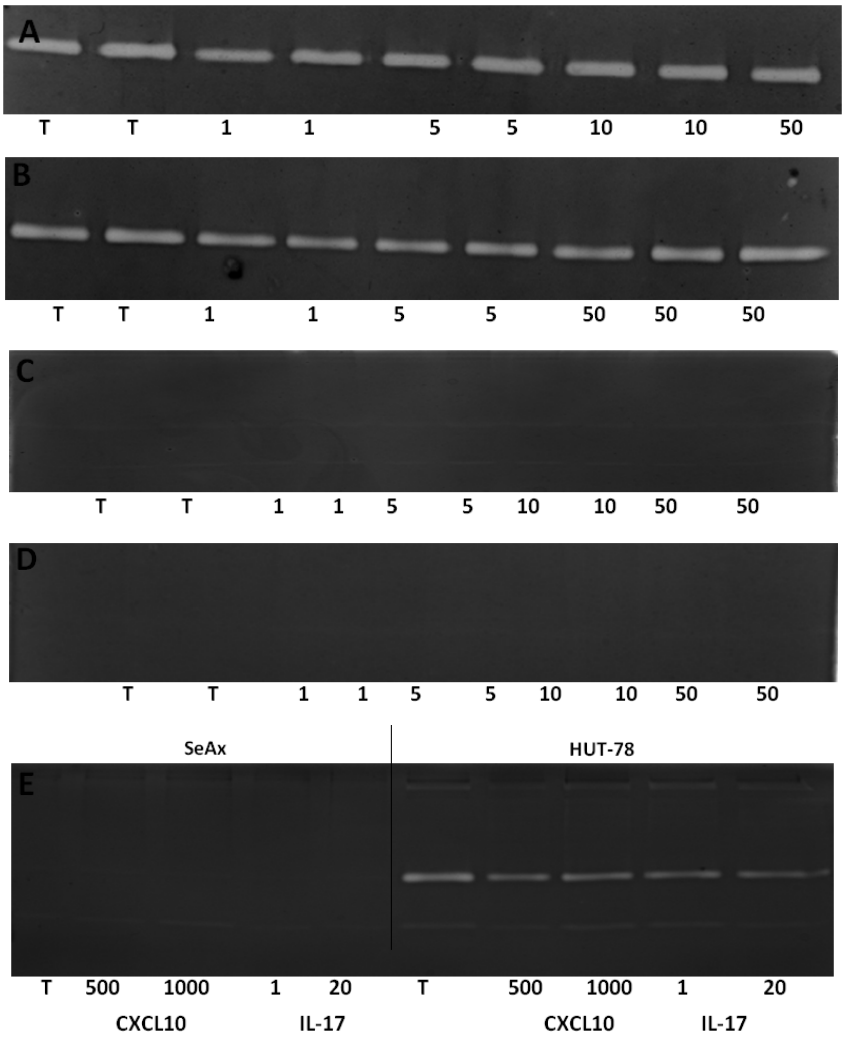

Fig. 5: Gelatin zymography analysis of MMP-9 in different groups, A: Wells 1-2: HUT-78 control, wells 3-4: HUT-78/aqueous extract 1 µg/ml, wells 5-6: HUT-78/aqueous extract 5 µg/ml, wells 7-8: HUT-78/aqueous extract 10µg/ml, wells 9-10: HUT-78/aqueous extract 50 µg/ml. B: Wells 1-2: HUT-78 control, wells 3-4: HUT-78/hydroalcoholic extract 1 µg/ml, wells 5-6: HUT-78/hydroalcoholic extract 5 µg/ml, wells 7-8-9: HUT-78/hydroalcoholic extract 50 µg/ml. C: wells 1-2: SeAx control, wells 3-4: SeAx/aqueous extract 1 µg/ml, wells 5-6: SeAx/aqueous extract 5 µg/ml, wells 7-8: SeAx/aqueous extract 10 µg/ml, wells 9-10: SeAx/aqueous extract 50 µg/ml. D: Wells 1-2: SeAx control, wells 3-4: SeAx/hydroalcoholic extract 1µg/ml, wells 5-6: SeAx/aqueous extract 5 µg/ml. wells 7-8: SeAx/hydroalcoholic extract 1 µg/ml, wells 9-10: SeAx/hydroalcoholic extract 50 µg/ml. E: Well 1: SeAx Control, well 2: SeAx/CXCL10 500 pg/ml, well 3: SeAx/CXCL10 (1000 pg/ml), well 4: SeAx/IL 17 (1 ng/ml), well 5: SeAx/IL-17 (20 ng/ml), well 6: HUT-78 control, well 7: Hut-78/CXCL10 (500 pg/ml), well 8: HUT-78/CXCL10 (1000 pg/ml), well 9: HUT-78/IL-17 (1 ng/ml), well 10: HUT-78/IL-17 (20 ng/ml).

DISCUSSION

The two extracts AE and HAE prepared from the gastropod *Helix aspersa*, showed a toxic activity against both tumor lines analyzed. Indeed, the counting using trypan blue showed that the aqueous extract with the dose of 50 µg/ml induced 44.09±3.89 %, (p=0.003) mortality in the tumor line HUT-78 and 31.47±2.96 %, (p=0.003) in the tumor line SEAX. While the alcoholic extract with the same concentration of 50 µg/ml induced 29.90±5.98 %, (p=0.013) death of HUT-78 line and 25±5.57 %, (p=0.016) for the second SEAX cell line. These results showed that both extracts of the mollusk have similar toxic activity against these two tumor cell lines. The aqueous extract induced apoptosis in tumor cells. This was demonstrated by typical changes in morphology of the cells incubated with the aqueous extract such as condensation of chromatin and its fragmentation observed by hoechst dye. Apoptosis can be induced through the activation of death receptors which in turn results in the recruitment of specialized adaptor proteins and activation of caspase cascades. El ouar *et al*, 2013, found that the aqueous extract of *Helix aspersa* was highly toxic against tumor cells of breast cancer; the same extract decreased the Bcl-2 levels in the tumor line Hs578T. They found that the extract induces necrosis in these tumor cells [15].

The aqueous extract contains highly polar molecules such as proteins, salts and peptides. Whereas, the hydroalcoholic extract contains less polar products such as alcohols, phenols and amines. For instances, that both extract contains different active molecule that alter cancer cell viability. The determination of total protein and

total phenols content in the two extracts showed that the aqueous extract contained 4.53±0.48 mg/ml of protein and 2.44±0.11 mg GAE g-1 of phenols while the hydroalcoholic extract contained 1.83±0.23 mg/ml of protein and 2.81±0.16 mg GAE g-1of phenols. Polyphenols are secondary metabolic products belonging to a family of organic molecules characterized as their name suggests, by the presence of several phenolic groups. The data regarding total phenolic content in the foot of *Helix aspersa* is relatively limited.

Those results showed that the cytotoxic effect exerted by the two extracts may be due to proteins, peptides or phenols contained in theses extracts and that are able to induce apoptosis in the tumor cell lines (HUT-78 and SeAx).

A research team has isolated and identified peptides from several shellfish of marine snail belonging to the genus *Conus ananya* such as the contoxine [16]. These peptides had several effects; they display anti mitotic effect by inhibiting microtubule assembly and polymerization blocking cancer cells in metaphase [17], an antitumor effect by inducing a disturbance in the lysosomal function which leads cell to cell death [18]. They also block the expression of some enzymes involved in DNA replication and cellular proliferation and therefore they inhibit the growth and tumor invasion [19]. Another research team has identified a glycosaminoglycan in the flesh of the african snail: acharan sulfate [20]. This molecule with a molecular weight of 114 kDa is secreted in response to stimulation [21]. It possesses various biological activities: it inhibits the growth of the tumor in mice implanted with sarcoma cells. This tumor activity may be related to its inhibition of angiogenesis. It inhibitates the expression of fibroblast growth factor and the formation of tubes *in vivo* induced by VEGF-[22, 23]. Acharan sulfate can also increase the production of cytokines (TNF and IL-1β), the expression of molecules of co-stimulation B7-1 and B7-2, CD_{40} and major histocompatibility complex II (MHC II) *in vitro* by immature dendritic cells [24]. This could explain the anti tumor activity of this molecule *in vivo*. Others researchers were interested to study biomolecules isolated from marine sponges which the majority of them are cyclic peptides. These molecules are secondary metabolites that possess numerous biological activities including anti-cancer activity [16, 25]. One of these peptides was tested on tumor lines. Research teams had found that this compound induces apoptosis in these tumor lines by activating caspase-3 and decreasing the expression of bcl-2 [26, 28]. This same compound had anti mitotic effect by binding to tubulin (microtubules protein, a major component of the cytoskeleton) [29, 30].

We know that vascular endothelial growth factor (VEGF) has a role in tumor angiogenesis and is implicated in formation of new vessels, this need a degradation of basal membrane with MMP. The production of MMP-9 by the two tumor lines HUT-78 and SEAX stimulated by aqueous and hydroalcoholic extracts of *Helix aspersa* is not modified by the two extracts used at different concentrations relative to the control. MMP-9 is a protease expressed in several cancers such as: renal cell carcinoma, [31] and hematological cancers, [32, 33]. There are several transcription factors that regulate the expression of MMP-9: include NFkB, AP-1, AP-2 and SP-1 [34]. The inhibition of the production of MMP-9 is regulated by several cytokines via transduction cascade of the JAK/STAT [35].

Currentcurrently our team is interested in evaluating the *in vivo* effects of our other extracts such as anti-ulcer effects experienced by other researchers, [36] and also protective effects against chemo-induced hepatotoxicity in animal models [37].

The lack of *in vitro* effects of extracts indicates that the different molecules contained in the two extracts did not induce any changes at the molecular level in the transduction cascades which can induce an increase in expression of MMP-9 in line SEAX or which inhibit the production of this protease in tumor line HUT-78. We could say that our extracts have not an angiogenic effect by activation of MMP-9.

CONCLUSION

In conclusion, our results demonstrate that both extracts prepared from the terrestrial gastropod *Helix aspersa* had a cytotoxic effect on CTCL studied by increasing mortality. It is possible that the extracts induce apoptosis in tumor cells by stimulating the NFkB pathway, activating the Bax gene, reducing the intensity of the intracellular survival signals or by inhibiting the phosphorylation of signaling pathway proteins cell survival. However, these extracts had no effect on the expression of MMP-9 in these CTCL.

Our work is only a preliminary study to test *in vitro* toxic effects of extracts of terrestrial snail *Helix aspersa.* Others works *in vivo* are in progress. Another comprehensive study will be interesting to show the mechanism of action of bioactive molecules after purification. For instance, their effects on the expression of protein kinases of different signal transduction pathways that are involved in expression of an antiapoptotic or proapoptotic process such as Bcl-2 and Bax, NFkB, P53 in tumor lines HUT-78 and SEAX and other tumor cell lines and normal cell lines. It will also be very interesting to test also the effect of *Helix aspersa* extracts on Human umbilical vein endothelial cells (HUVEC) and study the expression of VEGF gene in these cells.

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CONFLICT OF INTERESTS

All the authors declare they have no conflict of interest. This work has not published previously, and it is not under considerations for publication elsewhere.

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