Original Article

In Vitro Assessment of the Anti-Proliferative and Anti-Viability Effects of Salivary Gland Extracts from *Hyalomma* ticks (Acari: Ixodidae) on Human Colorectal Cancer Cells

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Abstract

Background: The saliva and salivary glands of ticks possess a wide range of immuno-pharmacologically active molecules that effectively modulate the activity of enzymes, antibodies, and amines that have a role in different biological processes. Derived components from saliva and salivary glands of hard ticks Ixodidae have been characterized as potential natural sources for discovering promising anti-cancer drug candidates.

Methods: The anti-cancer activity of salivary gland extracts (SGEs) from *Hyalomma anatolicum*, *Hyalomma dromedarii*, *Hyalomma marginatum*, and *Hyalomma schulzei* was assessed. MTT assays and flow cytometry were done on the HT-29 colorectal cancer cell line to evaluate the anti-viability and proliferative inhibition.

Results: Based on the MTT assay results, the SGEs from *Hy. dromedarii* had the highest and lowest substantial anti-viability effects on the HT-29 cancer cell and human foreskin fibroblast (HFF) normal cell, respectively. The cytometric assessment revealed a significant increase in the apoptosis and necrosis ratio of the HT-29 cancer cells after treatment with *Hy. dromedarii* SGEs.

Conclusion: The results demonstrated that *Hy. dromedarii* SGEs have significant anti-proliferative, anti-viability, and apoptotic potential. The result of this study suggests that *Hy. dromedarii* SGEs is an appropriate candidate for further investigations to identify and purify the mechanisms and molecules involved in the anti-cancer activity of the SGEs.

Keywords: Tick; *Hyalomma*; Salivary gland extract; Anti-cancer; HT-29 cell line

Introduction

Cancer is the main cause of death and an important obstacle to increasing life expectancy in any country (1). Based on the World Health Organization (WHO) estimation for 2019, cancer rates as the first or second cause of mortality before the age of 70 in 112 out of 183 countries and also the third or fourth in another 23 countries (2). More than 1.9 million new cases of colorectal cancer and 935,000 deaths are estimated for 2020, which is about one in 10 cancer cases and deaths. Colorectal cancer ranks third in incidence and second in mortality (3). Conventional cancer treatments include surgery, radiation therapy, and chemotherapy.

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Despite technological advances, most cancers are still incurable, and many patients who undergo cancer treatment experience side effects and relapse after a period of remission. Therefore, any new effective and safe therapeutic agent will be in high demand (4).

Natural products can help pharmacists and clinicians develop novel medications and treat chronic and degenerative illnesses. These compounds can be found in plants and other living organisms. Recent advances in the development of arthropods' natural products as potential novel medicines indicate that arthropods and their compounds are an excellent option to achieve this goal (5–11). Among arthropods, ticks have received attention in biomedicine and treating complicated diseases (12–14). The ticks' saliva contains rich and potential chemical sources of neurotoxins, terpenoids, saponins, serpins, and carbohydrates (15–19). Moreover, it also provides a broad range of proteins and peptides to facilitate the production of the first line of defense against pathogens. It exhibits cytolysis, vasodilator, anticoagulant, anti-inflammatory, and immunosuppressive activities (20–23). The levels of non-protein substances and secreted proteins in tick saliva are effective in controlling host response mechanisms. These compounds activate the immune system with varying levels of regulatory activity of chemicals that affect cell signaling, such as enzymes, antibodies, vascular amines, molecular adhesion, cytokines, and chemokines, or directly affect target cells. Some peptides have been shown to suppress gene and protein expression, while others cause membrane lysis, apoptosis, or cell cycle arrest (24–29). This study aims to investigate the inhibitory effects of tick salivary gland extracts (SGEs) on cancer cell viability and proliferation.

Materials and Methods

Preparation of samples

Tick specimens were collected from different provinces of Iran, including East Azerbaijan, West Azerbaijan, Gilan, Isfahan, Qom, and Tehran, in favorable seasons from May to December 2021. Adult ticks were collected by fine-tipped angled forceps from the ear, mammary glands, under the tail, and the rest of the body of the appropriate host, including sheep and camels, following the standard method (30–31). The engorged and semi-engorged male and female ticks were used in this work. The specimens were preserved in the tubes, and relative information, including the code, date, gender, place of collection, and host type, was recorded. All tubes were surrounded with a wet towel, placed in a cold box containing an ice pack, and then transferred to the Vector Biology Laboratory at the School of Public Health, Tehran University of Medical Sciences. Ticks were identified using the identification keys of Hoogstraal (32) and Walker (33).

Preparation of the salivary gland extracts (SGEs)

In total, 700 males and females of the genus *Hyalomma*, including *Hy. anatolicum*, *Hy. dromedarii*, *Hy. marginatum*, and *Hy. schulzei*, were dissected under cold, sterile Dulbecco's PBS with a pH of 7.2. The SGEs of each species were divided into males and females, pooled, and frozen at −80 °C. Before the assays, pools of SGEs were homogenized and centrifuged at 18,000g at 4 °C for 30 min. Supernatants were pooled, and the soluble protein concentration of SGEs was determined using the bicinchoninic acid assay (34). The SGEs were lyophilized for further study.

Cell culture

The present study was conducted on the HT-29, a human colorectal adenocarcinoma cell line. The cell number IBRC C10097 was provided by the Iranian Biological Resource Center. The HT-29 cells were seeded for primary cultures at a density of 80,000 cells/cm^2 (T-25 cm^2 flask). The cells were grown in DMEM medium supplemented with 10% FBS, four mM L-glutamine, and 100 units/ml penicillin and strep-

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tomycin. It was incubated in a humidified atmosphere at 37 °C with 5% CO_2. The cells were detached with 0.25% trypsin and 0.02% EDTA for 3 min at 37 °C, spun at 300–1,000 g for 5–10 min, and resuspended in a complete medium. In this method, the cells were passaged three times per week. Also, human foreskin fibroblast (HFF) was used as a normal cell; the cell number RSCB0564 was provided by the Stem Cell Bank of Royan ATMP-TDC and maintained in DMEM (high glucose with 10% FBS under culture conditions similar to HT-29).

Cell viability assay

The effects of *Hy. anatolicum*, *Hy. marginatum*, *Hy. dromedarii*, and *Hy. schulzei* SGEs on the viability of HT-29 and HFF cells were assessed using the MTT assay (35). In this assay, mitochondrial succinate dehydrogenase catalyzes the enzymatic conversion of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to purple formazan dye. The dye produced is proportional to the number of metabolically active cells. In this study, the cells were seeded into a 96-well microplate at 10^4 cells per well density. Each well was filled with 100 µL of medium and cultured at 37 °C for 24 hours before being replaced with fresh media containing SGEs at various concentrations (60, 30, 15, and 7.5 µg/mL). Each experiment was repeated at least three times. Morphological changes were checked using an optical microscope. After the indicated time, 12 µL MTT (5 mg/mL) was added, and the cells were incubated for 4 h. After that time, the wells were removed entirely, and 100 µL of DMSO was added to solubilize the formazan crystals. The absorbance was determined spectrophotometrically at 570 nm on an Elx800 UV universal microplate reader (BioTek Co., Winooski, VT, U.S.). Control cells were those that had only been incubated with the medium. The half-maximal inhibitory concentrations (IC_{50} values) were calculated by fitting the survival curve from dose-dependent response data.

Evaluation of cell death by flow cytometry

The HT-29 cells were treated with each species' SGEs at the IC_{50} concentrations in a 12-well microplate at a density of 3 x 10^4 cells per well for 24 h and then washed with phosphate-buffered saline (PBS; 500 µL/well). To evaluate apoptosis and necrosis, at least 10^5 cells must be harvested and incubated with 2 mM Annexin-V and 10 mM of PI in PBS for 30 min at room temperature in the dark. The untreated cells served as a negative control. A flow cytometer immediately analyzed the cells (FACS Calibur, Becton Dickinson Co., San Jose, CA, U.S.). Data from three independent experiments were analyzed using FlowJo software (36).

Statistical methods

The data were described using the mean ± SD, or frequency (%). The chi-squared, or Fisher's exact, test was used to evaluate the relationship between categorical data. A two-way analysis of variance (ANOVA) was performed to compare genders and treatments, considering the interaction between gender and treatments. For pairwise comparisons (post hoc analysis), Tukey was used to improve the power of the comparisons. P-values less than 0.05 were considered significant. All of the analysis was done with SPSS version 26 software.

Results

The frequency of *Hyalomma* species in the study areas

A total of 3,299 hard ticks of the genus *Hyalomma* in four species, including *Hy. anatolicum*, *Hy. dromedarii*, *Hy. schulzei*, and *Hy. marginatum* were collected (Table 1). *Hyalomma anatolicum* was discovered in all sampling areas, including Tehran, Qom, Isfahan, Gilan, East Azerbaijan, and West Azerbaijan. *Hyalomma dromedarii* and *Hy. schulzei* were found at the highest rates in Tehran and Qom, while *Hy. marginatum* was collected in these two provinces, followed by Isfahan and Gilan. *Hyalomma anatolicum*, *Hy. marginatum*, and *Hy.*

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dromedarii were the dominant species during the sampling, while *Hy. schulzei* was collected late in the summer and during the autumn.

The protein concentration of salivary gland extracts (SGEs)

A standard curve was obtained to determine protein concentration. Table 2 displays the protein concentration of male and female *Hyalomma* species' SGEs. Generally, the protein concentration of female ticks' SGEs was lower than that of males. The highest and lowest protein concentrations were calculated for *Hy. dromedarii* and *Hy. schulzei* SGEs, respectively.

The effect of salivary gland extracts (SGEs) on cell viability

The mean of HT-29 cancer cell viability was compared to four SGE concentrations: 60, 30, 15, and 7.5 µg/mL. In all doses, the mean cell viability in *Hy. schulzei* was significantly higher than in all other treatments ($P< 0.001$). The cells with the lowest mean cell viability were referred to as *Hy. dromedarii* ($P< 0.001$). Mean cell viability at dose 7.5 was high for all treatments, and dose 60 was lower than the other doses (Fig. 1). Males had significantly lower mean cell viability than females across all doses ($P< 0.001$). In all doses, the IC_{50} values (Table 2) in females were higher than in males. The lowest IC_{50} value was referred to as *Hy. dromedarii*. There was a dramatic increase in the IC_{50} value in *Hy. marginatum* and *Hy. schulzei*. The cells exhibited more sensitivity after exposure to male SGEs; therefore, male ticks' SGEs were selected for further investigations. In the case of the HFF as a normal cell, SGEs of *Hy. dromedarii* and *Hy. schulzei* had the highest and lowest means of cell viability among male *Hyalomma* species' SGEs in doses of 30 and 15 µg/mL, respectively ($P= 0.004$).

Cell death, necrosis, and apoptosis

A flow cytometric analysis using the IC_{50} values revealed HT-29 cells' apoptosis and necrosis after 24h of treatment with male *Hyalomma* species' SGEs compared to untreated control cells (Fig. 2). The highest and lowest rates of late apoptosis were 32.2% and 19.9%, respectively, in *Hy. dromedarii* and *Hy. anatolicum. Hyalomma marginatum* and *Hy. anatolicum* had the highest and lowest percentages of early apoptosis, at 11.9% and 1.93%, respectively. Moreover, following an exposure of *Hy. anatolicum* and *Hy. schulzei* SGEs, the cells presented necrosis, with the highest value being 18.9% and the lowest value being 3.55%, respectively.

Table 1. Data on *Hyalomma* ticks sampling and collection sites established in Iranian provinces using a collection technique derived from captured hosts from May 1 to December 30, 2021

Species	Ticks (No.)		Location (Province)	Coordinates	Months
	Male	Female			
Hy. dromedarii	40	25	Tehran	35.7117°N 51.4070°E	July to December
Hy. schulzei	148	55			
Hy. marginatum	185	60			
Hy. anatolicum	50	25			
Hy. dromedarii	490	320	Qom	34.6456°N 50.8798°E	July to December
Hy. schulzei	220	60			
Hy. marginatum	266	85			
Hy. anatolicum	272	78			
Hy. anatolicum	60	95	Isfahan	32.6577°N 51.6692°E	June and July
Hy. marginatum	100	40			
Hy. anatolicum	30	140	Gilan	37°26′N 49°33′E	May, June, July, and August
Hy. marginatum	120	40			
Hy. dromedarii	20	130			

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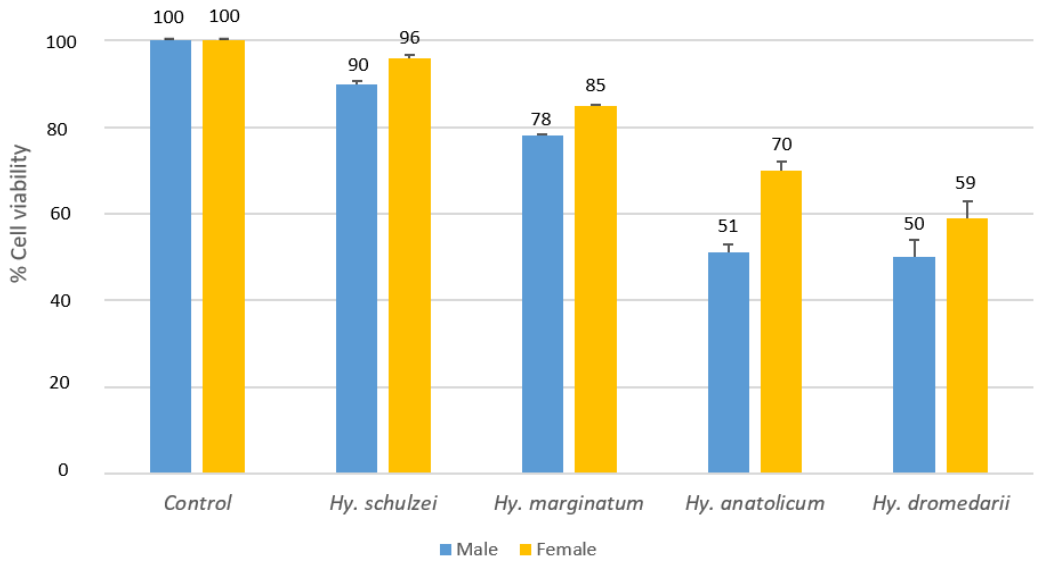
Table 1. Continued ...

Hy. anatolicum	85	20	East Azerbaijan	38.0766°N 46.2800°E	July and August
Hy. anatolicum	30	10	West Azerbaijan	37.5528°N 45.0759°E	June
Total	2116	1183			

Table 2. Comparison of the protein concentrations of salivary gland extracts (SGEs) and the IC_{50} (inhibitory concentrations) values of SGEs from *Hyalomma* species on the viability of human colorectal adenocarcinoma cell line (HT-29)

Species	Protein Concentration (mg/mL)		IC_{50} value (µg/mL)	
	Male	Female	Male	Female
Hy. dromedarii	11.48	8.36	7.70	9.89
Hy. anatolicum	10.38	8.04	7.84	14.13
Hy. marginatum	8.93	5.93	11.03	60.20
Hy. schulzei	6.75	5.81	13.44	67.07

A



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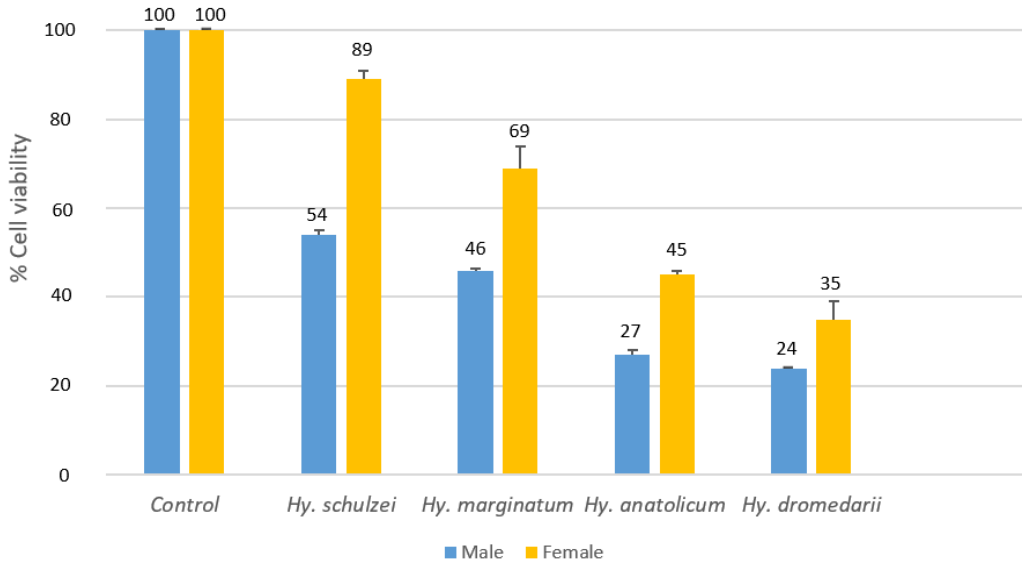

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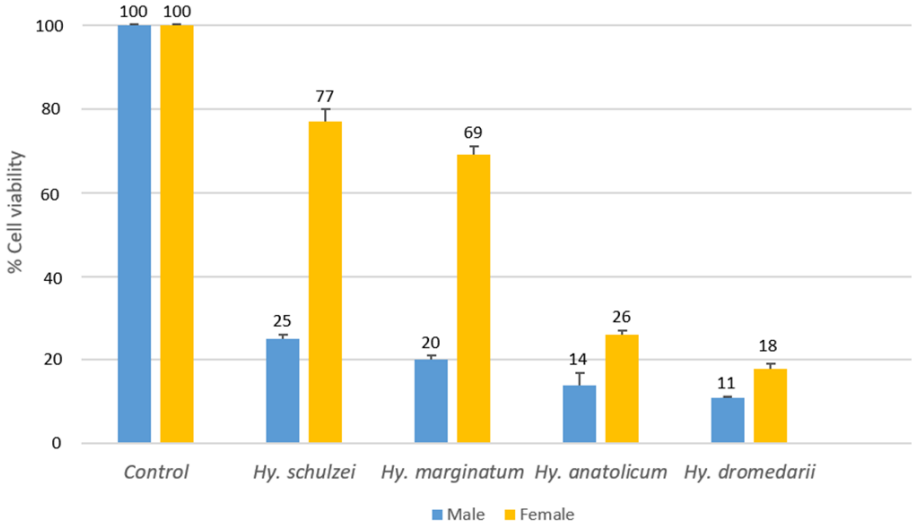
Fig. 1. Continued ...

C



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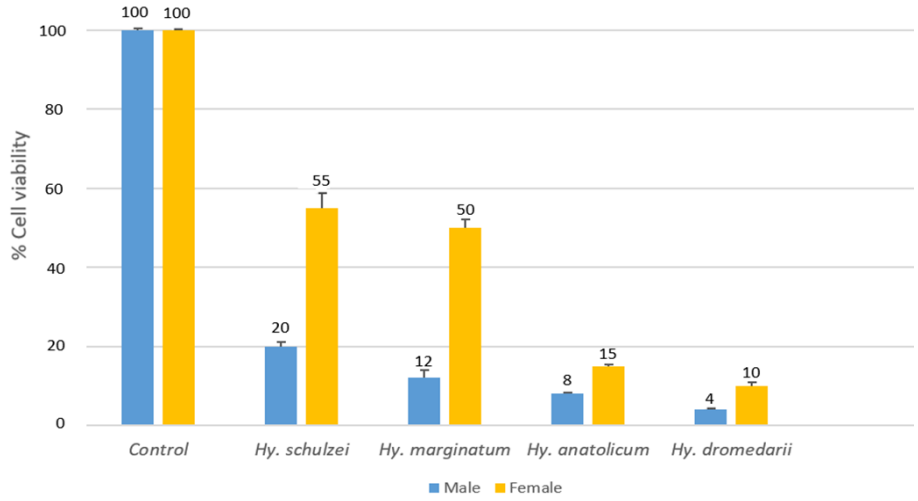

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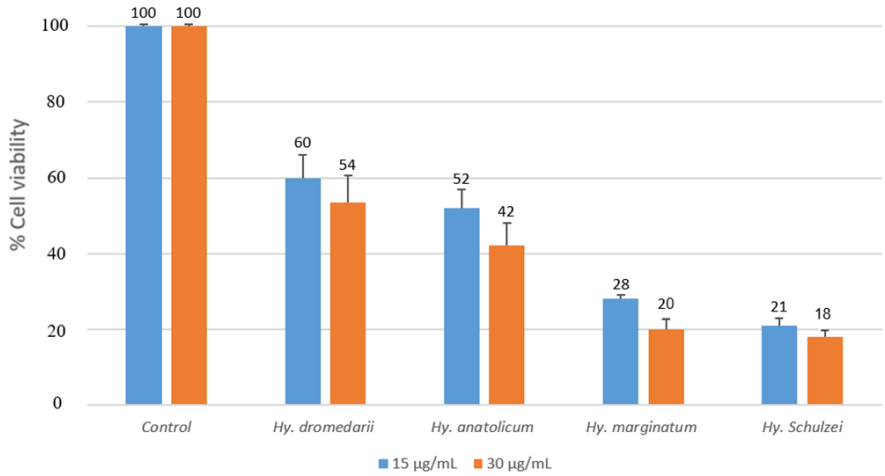

Fig. 1. The viability (mean ± SD) of human colorectal adenocarcinoma cell line (HT-29) after 24 h of treatment with salivary gland extracts (SGEs) from male and female *Hyalomma* species at various concentrations, A: 7.5, B: 15, C: 30, D: 60 (µg/mL), and (E) normal human foreskin fibroblast (HFF) cells after 24 h of treatment with 15 and 30 µg/mL of male *Hyalomma* species' salivary gland extracts. In each graph, the negative control refers to untreated cells.

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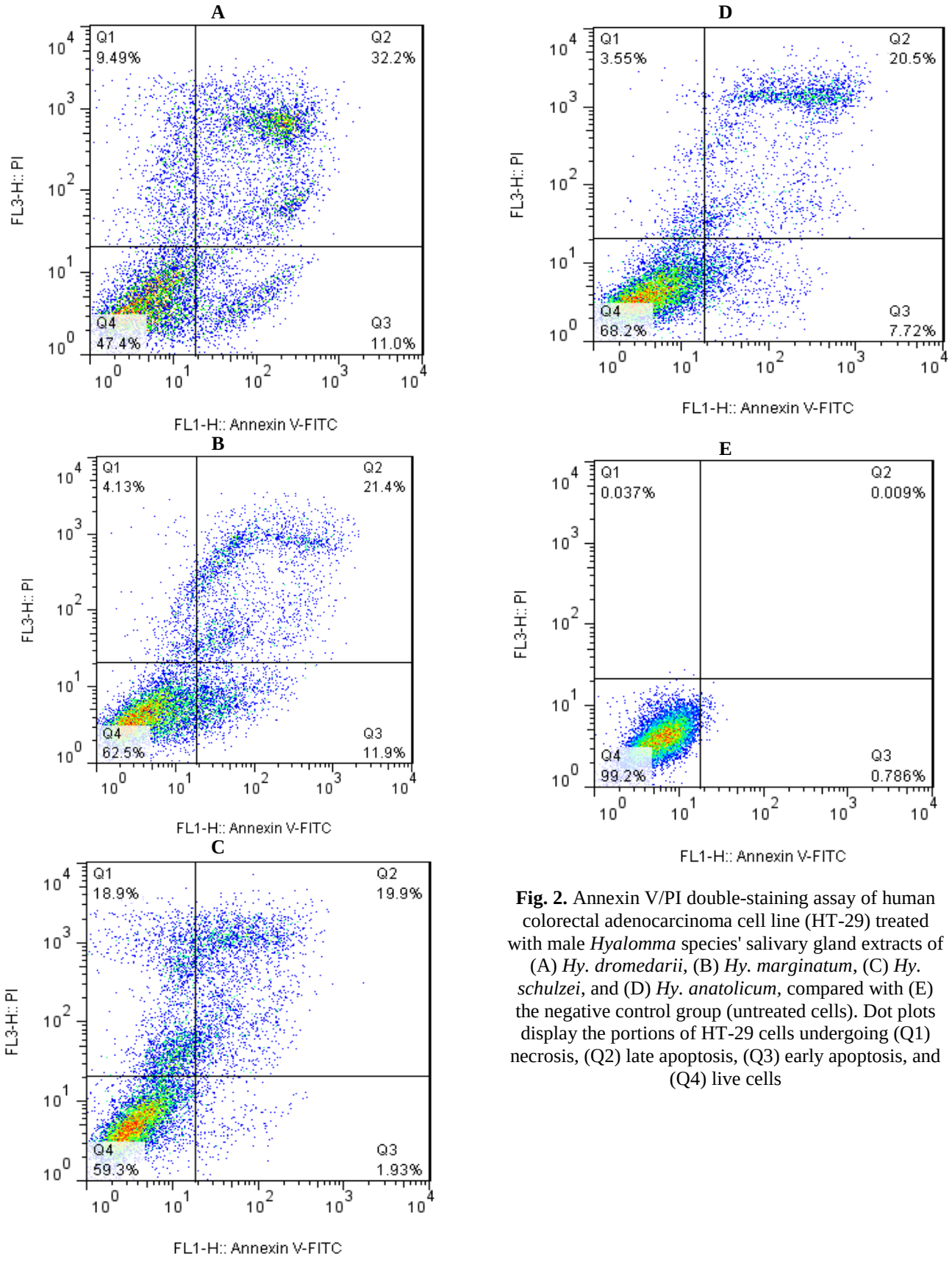

Fig. 2. Annexin V/PI double-staining assay of human colorectal adenocarcinoma cell line (HT-29) treated with male *Hyalomma* species' salivary gland extracts of (A) *Hy. dromedarii*, (B) *Hy. marginatum*, (C) *Hy. schulzei*, and (D) *Hy. anatolicum*, compared with (E) the negative control group (untreated cells). Dot plots display the portions of HT-29 cells undergoing (Q1) necrosis, (Q2) late apoptosis, (Q3) early apoptosis, and (Q4) live cells

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Discussion

Saliva is an essential biofluid secreted by the tick salivary glands, which modulates host defenses and facilitates the flow of blood to assure adequate feeding. Saliva and salivary gland extracts from Ixodidae were characterized as potential natural sources for the discovery of promising anti-cancer drug candidates (37). We investigated the effects of SGEs from *Hy. anatolicum*, *Hy. marginatum*, and *Hy. schulzei* on a cancer cell for the first time. Other researchers have done extra studies on *Hy. dromedarii* SGEs. Interestingly, our data demonstrate that *Hy. dromedarii* SGEs exhibit a higher anti-viability, anti-proliferative, and apoptotic potential on colorectal cancer cells among all of the *Hyalomma* species' SGEs. Sousa et al. (38) found that the saliva of Ixodidae contains proteinaceous molecules and potential anti-cancer properties. In the present research, according to the calculated concentration of protein, the protein concentration of *Hy. dromedarii* SGEs was higher than that of SGEs from other species. Our study is consistent with the suggestion that cancer cell cytotoxicity is influenced by the protein level (39–41). The anti-cancer effect of *Hy. dromedarii* SGEs on U87 glioblastoma cells was investigated earlier (42). *Hyalomma dromedarii* SGEs exerted an anti-viability and anti-proliferative effect on the glioblastoma cell. These findings make possible the characterization and development of novel molecules involved in the critical stages of tumor progression. Surprisingly, the viability of cancer cells largely decreased in a dose-dependent manner after seventy-two hours of treatment with *Hy. dromedarii* SGEs, with IC_{50} values of 98.68 and 84.76 µg /mL, respectively. The mean of HT-29 colorectal cancer cell viability in the current study was 0.04 at a concentration of 60 µg /mL after 24h of treatment with *Hy. dromedarii* SGEs and the estimated IC_{50} value was 7.7 µg /mL. This outcome is consistent with the earlier research (42). Though, our results go beyond the previous. Similar to Bensaoud's study (42), *Hy. dromedarii* was collected from camels in our study. Also, we collected *Hy. schulzei* from camels. The anti-viability and apoptotic effects of *Hy. schulzei* SGEs on HT-29 cancer cells were significantly lower than those of *Hy. dromedarii* SGEs. *Hyalomma anatolicum* and *Hy. marginatum* were collected from sheep, and the anti-viability and apoptotic effects of SGEs derived from these species were significantly different. The results revealed that the effects of SGEs from *Hy. anatolicum* are approximately similar to those of *Hy. dromedarii*. Our investigation demonstrated that the anti-cancer effects of SGEs were not related to the host's type. The means of cancer cell viability after treatment with *Hy. dromedarii* and *Hy. anatolicum* SGEs were similar to each other, but *Hy. anatolicum* SGEs had the lowest apoptotic effect among all of the species' SGEs. *Hyalomma schulzei* and *Hy. marginatum* SGEs had the lowest anti-cancer effects. Surprisingly, their anti-viability effects on normal cells were higher than all of the species' SGEs. The findings of *Hy. schulzei* and *Hy. marginatum* were opposite to those of *Hy. dromedarii* SGEs. It is noteworthy that in our study, a higher inhibitory effect on cancer cells was achieved after exposure to *Hy. dromedarii* SGEs compared to SGEs from the other species. Also, that was higher than the inhibitory effect of saliva from the other genera of Ixodidae in previous studies (43). According to our findings, after being exposed to *Hy. dromedarii* SGEs, 32.2 percent of HT-29 cancer cells induced late apoptosis. Previous studies have been conducted by utilizing laboratory ticks. Our results show that ticks from the field have a significant inhibitory effect on cancer cells. Previous studies have confirmed that salivary and salivary gland extracts from various genera and species of Ixodidae exhibit cytotoxicity against cancer cells, but not against non-cancer cells (44). In our study, SGEs derived from *Hy. dromedarii* yield-

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ed similar patterns of results. It supports the idea that the cytotoxic activity of the saliva and salivary gland extracts from some tick species is limited to cancer cells. Further studies are required to determine the relationship between molecular and functional properties of protein fractions and significant differences in the anti-viability of *Hy. dromedarii*, *Hy. marginatum*, and *Hy. schulzei* SGEs on normal and cancer cells. Our data illustrate that cancer cells exhibited significantly less sensitivity to female ticks' SGEs. Comparing our findings to previous research (45, 46), we suggest that male ticks are promising candidates for investigating the anti-cancer effect of saliva and salivary gland extracts in future studies. *Hyalomma* species were collected from different regions of Iran. The species' SGE qualities and quantities along with their anti-cancer properties, were not related to the collection sites, and they were strikingly similar. These findings suggest new avenues for investigating and developing the anti-cancer effect of SGEs and the molecules involved in the key steps of cancer progression from ticks in Iran.

Conclusion

The findings suggest that *Hy. dromedarii* SGEs are acceptable towards the recognition of new therapeutic compounds and effective molecules which could be further used in the development and management of colorectal cancer. However, thorough research is required to identify and purify the mechanisms and molecules involved in the inhibitory effect of *Hy. dromedarii* SGEs.

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Ethical consideration

The university research review committee revised the research proposal according to the rule and regulations. Accordingly, the study was approved by the Ethics Committee of Tehran University of Medical Sciences (ID: IR.TUMS. VCR.REC. 805).

Conflict of interest statement

The authors declare there is no conflict of interests.

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