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Original Article

Alpha-Pinene and Tannic Acid Inhibit *Trichomonas vaginalis* Protozoan Cells by Inducing Apoptosis

Masoumeh Moradi¹, Dara Dastan^{2,3}, Mohammad Fallah¹, Manizhe Kashi Nahanji¹,
*Mohammad Matini¹

1. Department of Medical Parasitology and Mycology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
2. Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
3. Department of Pharmacognosy and Pharmaceutical Biotechnology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran

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*Correspondence

Email:
matini@umsha.ac.ir

Abstract

Background: Trichomoniasis is one of the most common sexually transmitted infections worldwide. The growing concern of drug resistance of this infection has cautioned the need for new drug development. We evaluated the potential antiproliferative and apoptotic effect of α -pinene and tannic acid (TA) on *Trichomonas vaginalis* cells. In addition, the cytotoxicity of agents on Vero cells was investigated.

Methods: *Trichomonas* cells were axenically cultured in TYI-S-33 medium. In vitro antiproliferative activity of α -pinene, TA, and metronidazole was investigated against *Trichomonas* cells. The assays were carried out in triplicate using microtiter plate and trypan blue staining method. Annexin V/PI staining with flow cytometry was used to evaluate apoptosis induction. In addition, the cytotoxic effect was measured by MTT assay.

Results: α -Pinene and TA exhibited significant inhibition of the *Trichomonas* cells and the lowest IC₅₀ values were 22.9 μ g/ml and 140 μ g/ml at 48 hours' incubation, respectively. The CC₅₀ was found at 116 μ g/ml for α -pinene and 473 μ g/ml for TA, after 48 hours of treatment. The flow cytometry study demonstrated that the natural compounds induced apoptosis in *Trichomonas* cells. After 24 hours of treatment, the induction of apoptosis was 5.2% - 36.6% at concentrations of 3.9 - 62.5 μ g/ml for α -pinene and TA induced-apoptosis was 6.1% - 53.8% at concentrations of 125-2000 μ g/ml.

Conclusion: Although the results show the antiproliferative and apoptotic effect of α -pinene and TA on *Trichomonas* cells, in vivo studies are needed to further clarify the effects of these compounds.



Introduction

Trichomonas vaginalis is a flagellated protozoan responsible for human urogenital infection. Trichomoniasis is considered as the most prevalent non-viral sexually transmitted infection worldwide. According to WHO bulletin 2016, the prevalence of trichomoniasis is 5.3% and 0.6% in women and men, respectively. Its annual incidence of 156 million cases is more than that of syphilis, chlamydia and gonorrhea (1). *T. vaginalis* is a common causative agent of vaginitis in female. However, it can also cause urethritis, epididymitis, or prostatitis in men. This disease may range from asymptomatic to severe infection with serious consequences. Common symptoms in women include a malodorous and purulent discharge, which results in genital irritation and itching. In addition to increased risk of adverse pregnancy outcomes, pelvic inflammatory disease, and cervical cancer, trichomoniasis can increase predisposition to HIV infection in both sexes. More than 75% of cases of the infection in men are asymptomatic, so they go undiagnosed and untreated. Consequently, persistent and long-term trichomoniasis is likely to impair male fertility (2-5).

Since 1961, metronidazole remains the regimen of choice for the treatment of trichomoniasis. The first metronidazole treatment failure was reported in 1962. Drug resistance has been documented in *T. vaginalis*, occurring in up to 4–10% of clinical isolates in the United States (6-7). In addition to this important public health concern, side effects and low tolerance of metronidazole, especially in high doses, there is an increasing need to develop safe and effective antitrichomonal drugs. Generally, natural products have played an important role in the discovery and production of drugs, especially for infectious diseases; therefore, phytochemicals are of particular interest in antimicrobial research and development. α -Pinene is a member of the monoterpene class found in nature, like coniferous trees, particu-

larly the genus *Pinus*, which have several biological activities, e.g., antioxidant, anti-inflammatory, anticancer, and antimicrobial activity (8-9). Tannic acid (TA), a hydrolysable plant tannin, is classified as a phenolic compound that occurs naturally in almost all aerial plant tissues. Besides being a well-known food additive, TA has several biological properties. While it has properties to increase cell proliferation, tissue regeneration and wound healing processes, it also exhibits antimicrobial activity (10-12).

Until now, the biological effects of some medicinal plants or their natural derivatives on pathogenic protozoa have been evaluated. However, there is little evidence of the anti-protozoal potential of α -pinene and TA and their possible mechanisms. Therefore, we study aimed to evaluate apoptosis and growth inhibitory effect of α -pinene and TA separately on *Trichomonas* cells. Moreover, cytotoxicity effect of the compounds on Vero cell line was investigated.

Materials and Methods

Ethical considerations

This project was approved by the research ethics committee of the Hamadan University of Medical Sciences (Ethics code: IR.UMSHA.REC.1401.066).

Cell culture and Treatment

The *Trichomonas* cells (TVH1 strain, isolated in 2017 from 35-year-old female symptomatic patient in Hamadan) were axenically grown in TYI-S-33 medium supplemented with 10% bovine serum, 2 ml of vitamin mix #18, and 100 $\mu\text{g}/\text{ml}$ Pen/Strep (Sigma-Aldrich Chemical Co.) mixture at 35.5 °C (13). Subcultures were done every 48 hours to keep the trichomonads in the exponential growth phase. For experiments, *Trichomonas* cells ($1 \times 10^5/\text{ml}$) were seeded in 48-well culture plates and then were treated separately with different concentrations of TA (62.5-4000 $\mu\text{g}/\text{ml}$) and α -

pinene (3.9-250 µg/ml), Sigma-Aldrich Chemical Co., in TYI-S-33 medium. Cell viability was evaluated by trypan blue cell counting at different time intervals between 12-48 hr. Viable cells were counted, using trypan blue exclusion assay in a haemocytometer, to determine the percentage of cell growth inhibition (GI%), according to the following formula:

$GI\% = \frac{a-b}{a} \times 100$ a=the average number of cells in negative control wells and b=the average number of cells in test wells. Also, 50% inhibitory and cytotoxicity concentration (IC₅₀ & CC₅₀) and minimum lethal concentration (MLC) were determined by GraphPad prism 8 software and inverted microscope, respectively (14). The experiments were performed aerobically in three replicate series compared to the negative control (wells without any therapeutic agent), and positive control (wells containing metronidazole).

Annexin V/PI apoptosis assay

The treated *Trichomonas* cells with TA (125-2000 µg/ml) and α -pinene (3.9-62.5 µg/ml) were assayed for apoptosis using annexin V/PI staining, according to the manufacturer's kit (BioLegend, USA) instructions. Briefly, at the end of the 24 hours' incubation (15), the cells were harvested by centrifuged at 2000 rpm for 5 min. The pellet was washed twice with phosphate buffer saline (PBS) and then resuspended in binding buffer at a concentration of 1.0×10^6 cells/ml. The cell suspension (100 µl) was transferred to a 5 ml test tube and 5 µl FITC annexin V and 10 µl PI were added, then the cells were gently vortexed and incubated at room temperature in the dark. After 15 min, 400 µl of annexin V binding buffer was added to the test tube and finally, analyzed by FACScalibur flow cytometer.

MTT Cytotoxicity Assay

Cytotoxicity of various concentrations of TA (62.5-4000 µg/ml) and α -pinene (3.9-250 µg/ml) was evaluated on Vero cells by using MTT tetrazolium reduction assay (Cell Viabil-

ity Assay Kit from Kia Zist, Iran). The Vero cells maintained and subcultured in DMEM supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), Pen/Strep (100 mg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with 0.2% trypsin/EDTA in PBS solution. The Vero cells were seeded at a concentration of 1×10^5 cells per well in 96-well plates. They were treated with different concentrations of the agents for 12, 24 and 48 h at 37 °C. After incubation, 20 ml of MTT solution (5 mg/ml in PBS) were added to each well and incubated at 37 C for 4 h at 37 °C. Subsequently, the supernatant was removed from wells and 100 ml of Dimethyl sulfoxide (BioReagent, Sigma-Aldrich Chemical Co.), was added. The plates were shaken for 5 min and the optical density of the tests was measured against controls at 570 nm, using a microplate reader (16). The 50% cytotoxic concentration (CC₅₀) determined using GraphPad prism (version 8.0.2) software.

Statistical analysis

Data are shown as mean \pm standard deviation (SD) of three independent experiments. The effects of the agents were analysed using one-way ANOVA test (GraphPad prism, version 8.0.2). The statistical significance threshold was considered as $P < 0.05$.

Results

Cell viability inhibition effect of the agents was determined at different concentrations at 12, 24- and 48-hours' exposure. The results showed that α -pinene and TA suppressed the cell viability and induced apoptosis in *Trichomonas* cells, as compared to the control group ($P < 0.001$). The effect of the agents was time- and concentration-dependent. α -Pinene exhibited greater inhibitory and lethal effect against *Trichomonas* cells, with an IC₅₀ of 22.9-38.9 µg/ml and a MLC of 125-250 µg/ml. The IC₅₀ for TA was 140-1398 µg/ml

and the MLC was 2000-4000 $\mu\text{g/ml}$. In sublethal concentrations, the most growth inhibitory effect was observed after 48 hours' exposure as follows: 96.7% for α -pinene and 87.6% for TA (Table 1). Results of drug susceptibility test showed that the isolate was sensitive to metronidazole with MLCs of 25, 12.5 and 6.25 $\mu\text{g/ml}$ for 12, 24- and 48-hours' incubation, respectively (Table 1).

In flow cytometry analysis using annexin V and PI staining, cells are divided into four categories. The cells in the annexin V⁺/PI⁻ quadrant represent early apoptotic cells, and those in the annexin V⁺/PI⁺ quadrant represent late apoptotic cells. In addition, the cells located in the annexin V⁻/PI⁻ and annexin V⁻/PI⁺

quadrants indicate live cells and necrotic cells, respectively. α -Pinene induced apoptosis and necrosis in *Trichomonas* cells at lower concentrations than TA. After 24 hours' exposure, α -pinene induced apoptosis from 5.2% to 36.6% at concentrations of 3.9 to 62.5 $\mu\text{g/ml}$ (Fig. 1). TA-induced apoptosis was 6.1% to 53.8% at concentrations of 125 to 2000 $\mu\text{g/ml}$ (Fig. 2). Necrosis caused by α -pinene and TA was 13.8%-50.5% and 4.4%-31.9%, respectively.

The toxicity of α -pinene and TA on Vero cells was investigated at different concentrations and incubation times, and their selectivity index (SI = CC₅₀/IC₅₀) values were calculated. Results indicated that the SI of α -pinene was higher than that of TA (Table 2).

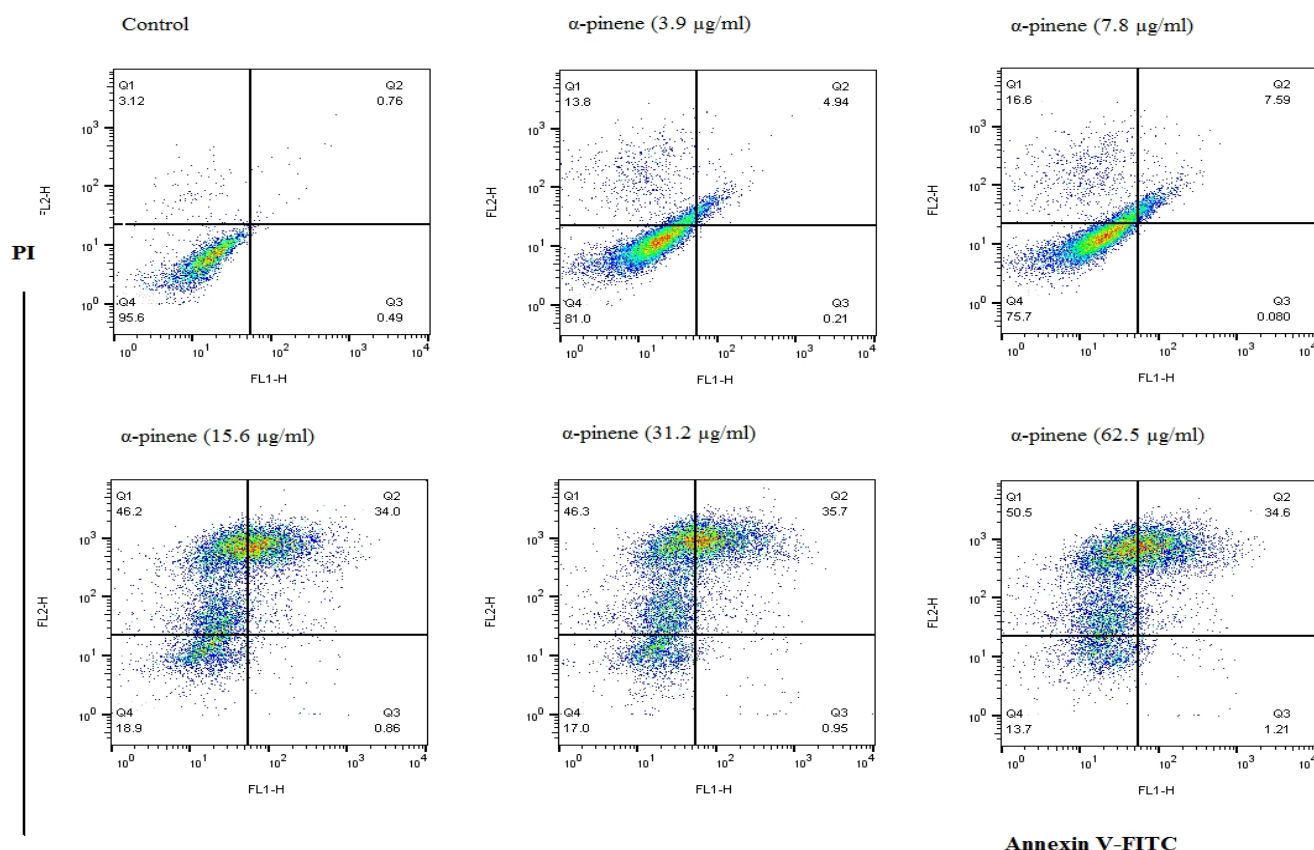


Fig. 1: Flow cytometry histogram of apoptosis assay by Annexin V/propidium iodide (PI) staining method corresponding to *Trichomonas vaginalis* cells, TVH1 strain, treated with different concentrations of α -pinene after 24 hours. Q1: necrotic cells; Q2: late apoptotic cells; Q3: early apoptotic cells; Q4: living cells

Table 1: Viability inhibition effect of α -pinene, tannic acid, and metronidazole on *Trichomonas vaginalis* cells, TVH1 strain.

Incubation time	Growth inhibition percent (Mean \pm SD) at different concentrations						
	α -Pinene						
	3.9 ($\mu\text{g/ml}$)	7.8 ($\mu\text{g/ml}$)	15.6 ($\mu\text{g/ml}$)	31.2 ($\mu\text{g/ml}$)	62.5 ($\mu\text{g/ml}$)	125 ($\mu\text{g/ml}$)	250 ($\mu\text{g/ml}$)
12 hours	21.4 \pm 7.6	26.3 \pm 5.5	40.2 \pm 6.3	48.5 \pm 4.1	54.5 \pm 3.3	88.6 \pm 4.2	100 \pm 0.0
24 hours	15.5 \pm 5.8	29.2 \pm 6.3	69.3 \pm 7.7	76.5 \pm 8.8	80.9 \pm 6.4	100 \pm 0.0	100 \pm 0.0
48 hours	10.2 \pm 6.5	45.3 \pm 4.7	74.3 \pm 5.2	84.1 \pm 4.3	96.7 \pm 6.2	100 \pm 0.0	100 \pm 0.0
	Tannic acid						
	62.5 ($\mu\text{g/ml}$)	125 ($\mu\text{g/ml}$)	250 ($\mu\text{g/ml}$)	500 ($\mu\text{g/ml}$)	1000 ($\mu\text{g/ml}$)	2000 ($\mu\text{g/ml}$)	4000 ($\mu\text{g/ml}$)
12 hours	1.4 \pm 5.2	6.3 \pm 6.6	13.4 \pm 8.3	16.9 \pm 5.2	19.8 \pm 6.7	78.8 \pm 5.1	100 \pm 0.0
24 hours	4.3 \pm 4.9	8.5 \pm 7.1	26.4 \pm 3.9	33.8 \pm 4.8	59.9 \pm 6.2	80.4 \pm 3.8	100 \pm 0.0
48 hours	21.1 \pm 6.2	39.4 \pm 3.2	71.5 \pm 6.8	74.9 \pm 5.1	87.6 \pm 4.4	100 \pm 0.0	100 \pm 0.0
	Metronidazole						
	0.2 ($\mu\text{g/ml}$)	0.4 ($\mu\text{g/ml}$)	0.8 ($\mu\text{g/ml}$)	1.6 ($\mu\text{g/ml}$)	3.1 ($\mu\text{g/ml}$)	6.2 ($\mu\text{g/ml}$)	12.5 ($\mu\text{g/ml}$)
12 hours	4.9 \pm 3.9	19.3 \pm 5.5	38.9 \pm 7.1	59.2 \pm 8.6	64.6 \pm 5.3	77.5 \pm 6.3	90.2 \pm 4.6
24 hours	10.3 \pm 6.7	32.8 \pm 2.6	55.0 \pm 4.6	72.6 \pm 2.3	84.6 \pm 2.7	93.4 \pm 0.6	100.0 \pm 0.0
48 hours	25.9 \pm 4.8	44.0 \pm 2.2	73.4 \pm 2.5	91.5 \pm 2.3	98.2 \pm 0.4	100.0 \pm 0.0	100.0 \pm 0.0

Data are representative of at least three independent experiments.
Statistical comparison groups, $p=0.001$

Table 2: Antitrichomonal activity and cytotoxic effect of α -pinene and tannic acid on Vero cells.

Incubation time	α -Pinene				Tannic acid			
	MLC ($\mu\text{g/ml}$)	IC ₅₀ ($\mu\text{g/ml}$)	CC ₅₀ ($\mu\text{g/ml}$)	SI ^a	MLC ($\mu\text{g/ml}$)	IC ₅₀ ($\mu\text{g/ml}$)	CC ₅₀ ($\mu\text{g/ml}$)	SI ^a
12 hours	250	38.9	226	5.8	4000	1398	747	0.5
24 hours	125	30.2	209	6.9	4000	689	677	0.98
48 hours	125	22.9	116	5	2000	140	473	3.4

^aSI: selectivity index.

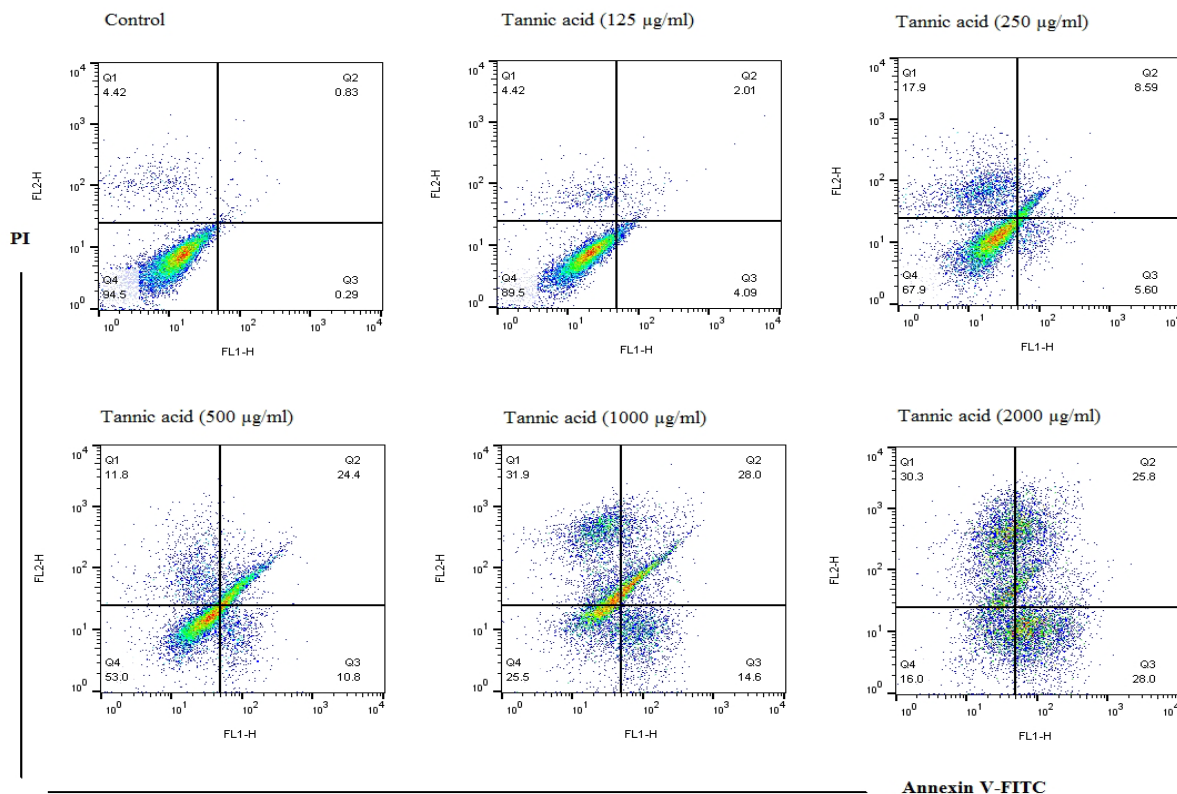


Fig. 2: Flow cytometry histogram of apoptosis assay by Annexin V/propidium iodide (PI) staining method corresponding to *Trichomonas vaginalis* cells, TVH1 strain, treated with different concentrations of tannic acid after 24 hours. Q1: necrotic cells; Q2: late apoptotic cells; Q3: early apoptotic cells; Q4: living cells

Discussion

Our study focused on the biological effects of natural products on *Trichomonas* cells and the possibility of inducing apoptosis in vitro. To evaluate the antitrichomonal activity of α -pinene and TA, we used GI%, IC₅₀ and as well as annexin V/PI staining to investigate the possible induction of cell death (necrosis and/or apoptosis). The results of this study show that α -pinene and TA have a statistically significant effect on the growth of *Trichomonas* and Vero cells compared to the control.

The potential of antimicrobial activity of natural compounds to differentiate between host cells and microorganisms is an important factor in the design and discovery of new antibiotics. According to this concept, antimicrobial susceptibility and cytotoxicity tests

confirmed selective antitrichomonal activities of α -pinene and TA. The average of IC₅₀ values in different treatment times (12, 24, and 48 hours) exhibited that α -pinene with mean IC₅₀ of 30.7 μ g/ml has more activity than TA with mean IC₅₀ of 742.3 μ g/ml on *Trichomonas* ($P < 0.001$). This finding is supported by the average selectivity index (SI) of α -pinene (5.9) and TA (1.6). Annexin V/PI flow cytometry methods were used to clarify further the pathways of antiprotozoal activity of the tested natural compounds. Annexin V/PI assay showed that the compounds, in addition to necrosis, are capable of inducing apoptosis in *Trichomonas* cells. Recently, the apoptotic effect of α -pinene and TA was shown on human ovarian and lung cancer cells, respectively (17-18).

So far, antibacterial and antiviral activities of α -pinene and TA have been reported (8-10). However, according to our knowledge, the antiprotozoal activity of TA has only been investigated on *Acanthamoeba* spp. (11-12). Padzik et al. demonstrated antiamebic effect of tannic acid-modified silver nanoparticles (AgTANPs) on clinical strains of *Acanthamoeba* spp. (T4 genotype). The IC₅₀ of AgTANPs against different strains of *Acanthamoeba* was reported 16 and 14 ppm after 72 h and 96 h, respectively (11). In another study, the synergistic effect of AgTANPs on the antiprotozoal potential of contact lens solutions against *Acanthamoeba* was evaluated. The results indicated an increase in the antiamebic activities of the contact lens solutions by AgTANPs (12).

Indirect evidence indicates the antitrichomonal potential of tannins. Studies conducted on *Manilkara Rufula* and *Poinciana (Caesalpinia) Microphylla* agree with this. Therefore, that tannins enriched-fractions of the extract of *M. rufula* and *P. microphylla* had antiproliferative properties on *Trichomonas* spp. The results of our study further confirmed this previous evidence (19-20).

In relation to the antiprotozoal properties of α -pinene, its antimalarial and antileishmanial activity have been reported. Among 20 nature identical essential oil constituents that investigated by Robyn et al., pulegone, nerolidol, linalyl acetate and α -pinene had the most potent antimalarial activity. In this study, α -pinene was found to inhibit the growth of *Plasmodium falciparum* with IC₅₀ value of $1.2 \pm 0.2 \mu\text{M}$ (21). In the other study conducted by Rodrigues et al., the effects of *Syzygium cumini* essential oil and its major component α -pinene were investigated on *Leishmania amazonensis*. α -Pinene exhibited antiproliferative effect on promastigotes of *L. amazonensis*, with an IC₅₀ of 19.7 $\mu\text{g/ml}$ and a SI of 21.5. The cytotoxic effect of α -pinene on intramacrophagic amastigotes was 15.6 $\mu\text{g/ml}$, with a SI of 27.2. The effect of *S. cumini* essential oil on *Leishmania* was lower in comparison with α -pinene.

The IC₅₀ and SI of the essential oil were 60 $\mu\text{g/ml}$ and 10.2; 38.1 $\mu\text{g/ml}$ and 16.1 on promastigotes and intramacrophagic amastigotes of *L. amazonensis*, respectively. The results of this study showed that the antileishmanial effects are carried out by immunomodulatory effect, so that increased phagocytic and lysosomal activity and high levels of nitric oxide were observed (22).

Ginger officinale and *Quercus infectoria* are plants that have shown the potential to cause apoptosis in *T. vaginalis*. Apoptosis effect of ethanolic extract of *G. officinale* was investigated by Arbabi et al. The tested extract caused the death of all *Trichomonas* cells at a concentration of 800 $\mu\text{g/ml}$ after 48 hours. In addition, the IC₅₀ of the ginger extract was 93.8 $\mu\text{g/ml}$ after 24 hours. In comparison with our results, the antiproliferative activity of *G. officinale* extract against *Trichomonas* is lower than α -pinene (IC₅₀= 30.2 $\mu\text{g/ml}$) and higher than TA (IC₅₀= 689 $\mu\text{g/ml}$). In this study, apoptosis was measured after 48 hours and in different concentration. At concentrations of 50-400 $\mu\text{g/ml}$, the early and late apoptosis were 0.70% - 1.53% and 16.3% - 74.7%, respectively (16). The results indicate that the rate of Ginger-induced apoptosis in *Trichomonas* cells is higher than that of α -pinene (5.2%-36.6%) and TA (6.1%-53.8%). This difference may be due to difference in exposure time or apoptotic potency of the compounds. Regardless of the difference of incubation time in this study, the rate of Ginger-induced apoptosis in *Trichomonas* cells is apparently higher than that of α -pinene and TA. While in sub-MLC concentration, the necrotic effect of α -pinene (50.5%) and TA (31.9%) was higher than *G. officinale* extract (~0.5%).

Mahmouvand and her colleagues evaluated the effect of apoptosis of some Iranian medicinal plants. Among *Quercus infectoria*, *Pistacia khinjuk*, and *Satureja Khuzestanica*, the extract of *Q. infectoria* had the most apoptotic effect on *T. vaginalis*. The 24-hour IC₅₀ values of the herbs were 21.3 $\mu\text{g/ml}$, 93.6 $\mu\text{g/ml}$, and 205.8 $\mu\text{g/ml}$ for *Q. infectoria*, *P. khinjuk*, and *S.*

Khuzestanica, respectively. At the same incubation time and at a concentration of 50 µg/ml, inducing apoptosis in *Trichomonas* cells by methanolic extracts of *Q. infectoria*, *P. khinjuk*, *S. Khuzestanica* was reported 20.1%, 7.5%, and 3.8%, respectively (15). The comparison of the results shows that although the *Q. infectoria* extract has more antitrichomonal potency, it induces less apoptosis in the parasite cells. Fakhrieh-Kashan et al. investigated the antitrichomonal and apoptotic effect of the combination of the alcoholic extract of *Verbascum thapsus* and *G. officinale* on *T. vaginalis*. IC₅₀ of the combination extract was 73.80 µg/ml after 24 hours' treatment. The apoptosis and necrosis rates of the extract were reported 8.97-77.19 and 1.35-4.09, respectively, at concentration of 25-400 µg/ml and 48 hours' incubation (23). The higher apoptosis induction of the mixed extract compared to α-pinene and TA may be due to its longer exposure time (48 hours) to the parasite cells.

Similar to α-pinene and TA, the investigated medicinal plants have antitrichomonal activity, but show significant differences in the induction of apoptosis and necrosis in *Trichomonas* cells. These dissimilarities probably indicate different mechanisms of antiprotozoal action of their bioactive compounds. However, the lack of a standard method to investigate the effects of medicinal plants on protozoa should not be ignored. On the other hand, plant extracts are a combination of different types of bioactive compounds or phytochemicals with different activity and potency. Synergistic or antagonistic interactions between the compounds of plant extracts are an important part of their nature, and the biological activity of medicinal plants is the result of the interaction between their components (24). So, the development of antiprotozoal drugs using medicinal plant derivatives requires identifying their effective compounds and understanding the nature of interactions between bioactive compounds.

Cell death can occur through apoptosis or necrosis. Apoptosis is an active and programmed process for cell death. While external factors play a role in necrosis process, which causes cell destruction and release of cell contents. In the present study, it was shown that α-pinene, as a monoterpene and TA, a polyphenolic compound, can lead to cell apoptosis. Monoterpenes can cause cell death by inhibiting the DiHydroFolate Reductase enzyme and impaired protozoan cell metabolism (25). According to our knowledge, there is no information regarding the mechanism of the antiprotozoal effect of TA. However, induction of TA-induced apoptosis through caspase-dependent and caspase-independent mechanisms has been reported in hepatocellular carcinoma (26). In addition to the apoptotic properties of α-pinene and TA, considerable cell necrosis was observed in exposed *Trichomonas* cells. It seems that the cell membrane is one of the important targets of the cell in antiprotozoal mechanism of α-pinene and TA. Changes in cell membrane permeability and cell wall lysis have been attributed to monoterpene substances (27). The antimicrobial effect of tannins is due to their ability to pass through the cell wall and interfere with the metabolism of microorganisms (10).

Conclusion

α-pinene and TA have antitrichomonal potency and induce programmed death in *Trichomonas* cells at non-toxic concentrations. However, these promising results require further research to evaluate in vivo efficacy and safety of the natural components. Furthermore, the precise identification of the apoptosis mechanism of α-pinene and TA in protozoan cells and the understanding of the nature of the interaction between bioactive phytochemicals can lead to the development of new antiprotozoal combination therapies.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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