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Anti-*Trichomonas vaginalis* activities and apoptotic effects of some Iranian medicinal plants

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Abstract. *Trichomonas (T.) vaginalis* a protozoan parasite that causes trichomoniasis is one of the most widespread sexually transmitted diseases. Aim of the present study is to evaluate the *in vitro* antitrichomonal properties of plant extracts of *Quercus (Q.) infectoria*, *Pistacia (P.) khinjuk*, and *Satureja (S.) khouzestanica* that are ethno-medicinally used in Iran against *T. vaginalis* trophozoites. In this study, the *in vitro* anti-*T. vaginalis* activities of the *Q. infectoria*, *P. khinjuk*, and *S. khouzestanica* extracts against *T. vaginalis* clinical isolates were assessed by Trypan Blue exclusion assay. The effect of the extracts on induced apoptosis in *T. vaginalis* trophozoites was evaluated using the fluorescein isothiocyanate (FITC) Annexin V staining kit. The *Q. infectoria* methanolic extract was significantly ($P<0.001$) more effective than the other tested extracts. It demonstrated lower IC_{50} values for trophozoite of *T. vaginalis*. *Q. infectoria* methanolic extract exhibited significantly ($P<0.001$) a higher rate of apoptosis on *T. vaginalis* trophozoite than other tested extracts and control group. Results of the study revealed that *Q. infectoria* extract can be considered as a suitable choice for medical studies to treat trichomoniasis. However, additional clinical studies are necessary to evaluate accurate biological effects of this plant on volunteer human subjects.

INTRODUCTION

Trichomonas vaginalis causing trichomoniasis is one of the most widespread sexually transmitted diseases (Van der Pol, 2007). Nearly 250 million people are infected by *T. vaginalis* worldwide (WHO, 2011). Thrichomoniasis considered the most prevalent sexually transmitted infection after viral ones; that causes urogenital track infection both in male and female (Satterwhite *et al.*, 2013). In the infected women, trichomoniasis causes genital pain, itching, acute vaginitis, and preterm delivery (Wolner-Hanssen *et al.*, 1989). Men are normally asymptomatic, however may hurt from urethral discharge, dysuria, urethritis, epididymitis and prostatitis (Wolner-Hanssen *et al.*, 1989). In recent years, serious complications including vulnerability to HIV infection, cervical cancer, or prostate cancer in men are correlated to trichomoniasis (Moodley *et al.*, 2002; McClelland *et al.*, 2007).

Currently, metronidazole considered the preferred medication for the treatment of trichomoniasis; however, it has resulted in some complications (Swygard *et al.*, 2003). Today, it has been shown that herbal supplies and their derivatives play a important role in exploration of new drugs in order to treat or prevent a broad spectrum of diseases such as infectious diseases (Cosa *et al.*, 2006; Mahmoudvand *et al.*, 2015).

Quercus (Q.) infectoria Olivier. (Fagaceae family) is commonly grown in Zagros Mountains, Western Iran (Jamzad *et al.*, 2012). Many parts of this plant are used as traditional and current medicine as an analgesic, anti-parkinsonian, anti-diabetic,

anti-inflammatory and anti-microbial drug (Iminjan *et al.*, 2014). *Pistacia (P.) khinjuk* Stocks (Anacardiaceae) frequently cultivated in different parts of the world including Iran (Mozaffarian, 2005). A number of pharmacological properties such as anti-inflammatory, anti-oxidant, anti-tumor, anti-asthmatic and anti-microbial properties have been related to this plant (Bozorgi *et al.*, 2013; Ezatpour *et al.*, 2015; Mahmoudvand *et al.*, 2016a). *Satureja (S.) khuzestanica* Jamzad belonging to the family Lamiaceae, extensively grows in different parts of Iran (Zargari 1990). Reviewers have reported some therapeutic properties of this plant such as anti-viral, anti-nociceptive, anti-inflammatory, antibacterial, anti-fungal, anti-spasmodic and anti-diarrhea properties (Zargari 1990; Hajhashemi *et al.*, 2002).

The present study aims to determine the *in vitro* anti-trichomonal properties of extracts from the plants *Q. infectoria*, *P. khinjuk*, and *S. khouzestanica* that are ethno-medicinally used in Iran against *T. vaginalis* trophozoites.

MATERIALS AND METHODS

Plant material

Plant materials from *Q. infectoria* (bark), *P. khinjuk* (fruit), and *S. khouzestanica* (leaf) were collected from rural regions of Khorramabad City (Lorestan Province, Iran) in June 2014. The plant materials were identified by a botanist and voucher specimens were deposited at the Herbarium of the Herbal Medicines Research Center (Khorramabad, Iran).

One hundred grams of plant materials (aerial parts) were dried and extracted by the means of the method of percolation via methanol (80.0%) for three days at 21°C. The extracts were then moved across a filter paper (Whatman No. 3, Sigma, Germany) to remove excess particles. Eventually, the extracts were evaporated by the means of a rotary evaporator in vacuum at 50°C and deposited at -20°C up to examinations (Tavakoli Kareshk *et al.*, 2015; Ezatpour *et al.*, 2016; Mahmoudvand *et al.*, 2016b).

Parasite

Isolates of T. vaginalis were prepared from vaginal discharges of female patients attending the Obstetric and Gynecology Clinic in Khorramabad City, Lorestan Province, Iran. The parasite was cultured at 37°C in the TYIS33 medium. The parasites in the log phase were diluted with the TYIS33 medium to reach 10^4 cell/ml.

Susceptibility assay

In vitro susceptibility of *T. vaginalis* isolates were tested against the extracts of the plants according to the method described by Upcroft & Upcroft, (2001). Primarily, *Trichomonas* trophozoites (5×10^3/ml) were put in 24-well culture plates and then were treated with metronidazole at the dose of 64 µg/ml as well as the extracts at the concentrations of 50, 100, 200, 400, and 500 µg/ml (the selection of the concentrations was based on the primary experiments) in the TYI-S33 medium at 37°C. On the first and second days, the viability of trophozoites was assessed using Trypan Blue exclusion assay by means of a haemocytometer under the microscope at 20× magnification. Moreover, 50.0% inhibitory concentrations (IC_{50} values) were calculated using Graph pad Prism5 software. All tests were repeated three times to calculate the IC_{50}.

Flowcytometry assay

Since flowcytometry has been employed for evaluating the effect of different antimicrobial agents on various microbial pathogens, we aim to evaluate the use of flowcytometry to study effects of these three extracts on apoptotic and necrotic cells of *T. vaginalis* (Nordin *et al.*, 2016). The effect of the extracts on induced apoptosis in *T. vaginalis* trophozoites was evaluated using the fluorescein isothiocyanate (FITC) Annexin V staining kit (Biovision, Palo Alto, California, USA). *T. vaginalis* trophozoites were cultured in 24-well culture plates with the different concentrations of the extracts (50, 100, 200, 400, and 500 µg/ml) in the TYI-S33 medium at 37°C for one and two days. After this time, trophozoites (5 × 104 cells/mL) were obtained after centrifuging at 1600 rpm for 5 min. In the next step, 0.5 ml of binding

buffer, 0.005 ml of annexin-V, and 0.005 ml of propidium iodide were poured into each well and were incubated at room temperature in darkness (Doroodgar *et al.*, 2016). The rate of apoptosis was determined by means of a flow cytometer (BD FACSCanto II, BD Bioscience, San Jose, California, USA); whereas the obtained results were analysis using the FlowJo software (Tree Star, San Carlos, California, USA).

Statistical analyses

Here, SPSS software, ver. 22, (SPSS Inc., Chicago) was applied for statistical analysis; variations among the tested groups were measured by one-way analysis of variance (ANOVA) test. Besides, *t*-test was used to compare IC50 values of the in the tested drugs. Finally, 0.05 was considered statistically significant.

RESULTS

Anti-T. vaginalis effects

In this study, the *in vitro* anti-*T. vaginalis* activities of the *Q. infectoria*, *P. khinjuk*, and *S. khouzestanica* extracts against *T. vaginalis* trophozoites were assessed by Trypan Blue exclusion assay. The results showed that all the tested extracts were effective in inhibiting the growth of *T. vaginalis* trophozoites based on a dose dependent manner after 24 and 48 h incubation (Figs. 1 and 2). Moreover, the *Q. infectoria* methanolic extract was significantly ($P<0.001$) more effective than extracts of the other tested plants and control group since it demonstrated lower IC_{50} values for trophozoites of *T. vaginalis*. The measured IC_{50} values for the three selected medicinal plants and metronidazole

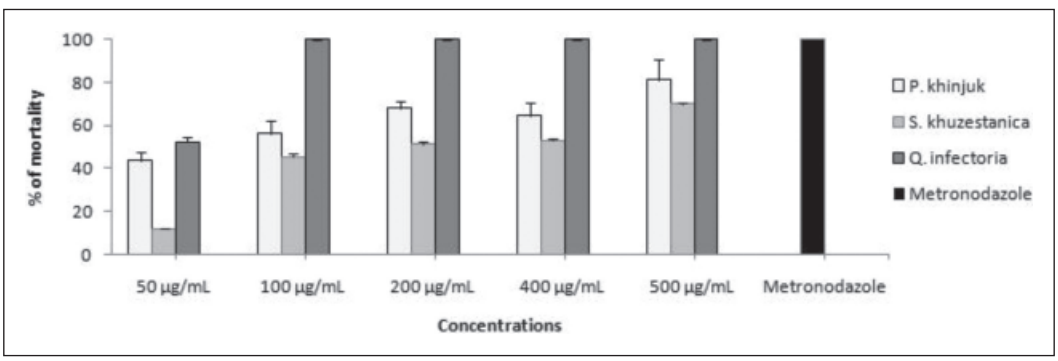

Figure 1. The mean of mortality rate of *T. vaginalis* trophozoites after 24 h treatment with *Q. infectoria*, *P. khinjuk*, *S. khouzestanica* extracts, and metronidazole (64 µg/ml) by Trypan Blue exclusion assay.

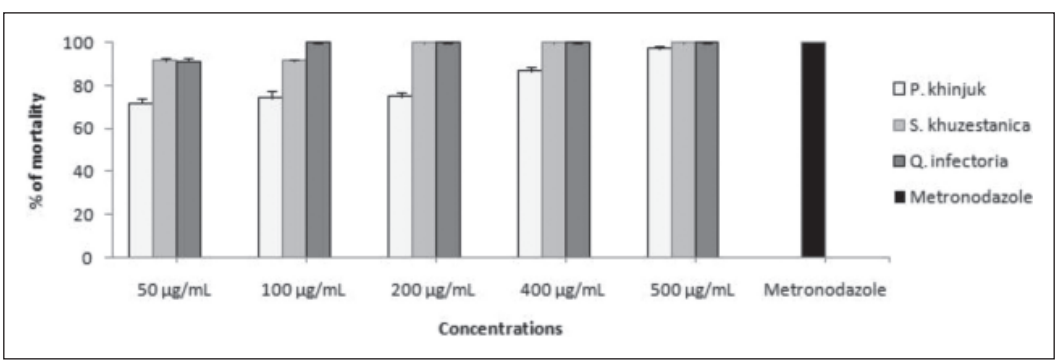

Figure 2. The mean of mortality rate of *T. vaginalis* trophozoites after 48 h treatment with *Q. infectoria*, *P. khinjuk*, *S. khouzestanica*, and metronidazole (64 µg/ml) by Trypan Blue exclusion assay.

Table 1. The IC_{50} values determined for some Iranian medicinal plants and control drug (metronidazole) against *T. vaginalis* trophozoites

Materials	IC_{50} (µg/ml)	
	24 h	48 h
Q. infectoria	21.3	3.4
P. khinjuk	93.6	26.6
S. khouzestanica	205.8	5.1
Metronidazole	0.5	0.09

as control drug against trophozoites of *T. vaginalis* are presented in Table 1.

Flowcytometry assay

The apoptotic effect of the *Q. infectoria*, *P. khinjuk*, and *S. khouzestanica* extracts against *T. vaginalis* trophozoites was evaluated by Annexin-V-FITC assay using a flowcytometer. The percentages of early apoptotic cells (annexin positive), late apoptotic cells (annexin and propidiumidoide positive), necrotic cells (propidium iodide positive) and living cells (annexin and propidium iodide negative) at various concentrations of the three tested plants were determined at 24 h after culture. The obtained findings revealed that all the tested extracts exhibited both the initial and late stages of apoptosis on *T. vaginalis* trophozoites after 24 h incubation. The *Q. infectoria* methanolic extract exhibited significantly ($P<0.001$) higher rate of apoptosis on *T. vaginalis* trophozoites in comparison with the other tested extracts and control group. Whereas, at the concentration of 50 µg/ml demonstrated 17.5% apoptosis in *T. vaginalis* trophozoites. In contrast, the control group revealed only 0.75% apoptosis on *T. vaginalis* trophozoites (Table 2).

DISCUSSION

Historical evidence have shown plants and spices to exhibit considerable therapeutic effects as well as a range of biological activities (Rocha *et al.*, 2005; Mahmoudvand *et al.*, 2014). In recent decades, chemical and synthetic antimicrobial drugs has decreased the interest in herbs as a natural resource for antimicrobial activities (Cowan 1999). However, there are a number of limitations in the use of synthetic drugs which has resulted in greater consideration for herbal drugs (McCutcheon *et al.*, 1992; Saedi Dezaki *et al.*, 2016). Both extracts tested in the current investigation showed remarkable anti-*Trichomonas* activity against trophozoites of *T. vaginalis*. However, the *Q. infectoria* methanolic extract was significantly ($p<0.05$) more effective than extracts of the other tested plants and control group as it demonstrated a lower IC_{50} values against trophozoite of *T. vaginalis*. According to the previous investigations, a range of therapeutic features such as anti-diabetic, anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral, and anti-fungal effects have been linked to *Q. infectoria* (Iminjan *et al.*, 2012; Baharuddin

Table 2. The apoptotic effect of *Q. infectoria*, *P. khinjuk*, and *S. khouzestanica* extracts against *T. vaginalis* trophozoites by Annexin-V-FITC assay using a flowcytometer

Materials	Concentrations (µg/ml)	% of Apoptosis	% of Late apoptosis	% of necrotic cells	% of living cells
Q. infectoria	12.5	7.8	1	0.15	90.4
	25	10.9	1.6	0.00	88.1
	50	17.5	2.6	0.25	79.6
P. khinjuk	25	4.1	0.05	0.00	95.8
	50	6.2	1.3	0.09	92.3
	100	7.9	0.7	0.04	91.4
S. khouzestanica	50	3.7	0.16	0.01	96.5
	100	3.7	0.15	0.07	96.1
	200	6.4	0.68	0.07	92.8

et al., 2015; Hussein *et al.*, 2000; Wan Nor Amilah *et al.*, 2014). Previous studies confirmed the existence of polyphenolic fractions including "tannins", "gallic acid" and flavonoids constituents such as quercetin in *Q. infectoria* (Hashim *et al.*, 2013). (Cowan 1999). The antimicrobial mechanisms of these phytoconstituents are still vague; However, it has been proven that phenolic structures appear to generate antimicrobial activity through destruction of the membrane and damaging cell peptidoglycans (Bisignano *et al.*, 1999; Kheirandish *et al.*, 2016). Moreover, recent studies confirmed that these components might disrupt in the synthesis of amino acids for the growth of microbes (Saija & Uccella, 2001).

Results showed that all the tested extracts had both the initial and late stages of apoptosis on *T. vaginalis* trophozoites after 24 h incubation. The *Q. infectoria* methanolic extract exhibited significantly ($P<0.001$) a higher rate of apoptosis on *T. vaginalis* trophozoites than other tested extracts; which indicated the anti-*T. vaginalis* effect of *Q. infectoria* extracts can closely related to induction of programmed death in *T. vaginalis* trophozoites.

CONCLUSION

The results showed that *Q. infectoria* extract can be used as a suitable alternative for the reatment of trichomoniasis after conucting complementary tests on laboratory animals and human cells.

Conflict of Interest

The authors declare that there is no conflict of interest in this study.

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