JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

In vitro anti-*Trichomonas vaginalis* activity of *Haplophyllum myrtifolium*

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Abstract

Introduction: In the classic treatment of *Trichomonas vaginalis* infection, although metronidazole has been used since the 1960s, there has been an increase in MTZ-resistant *T. vaginalis* strains and failure in the treatment of trichomoniasis causes serious concerns. Therefore, the present study aimed to investigate the *in vitro* antitrichomonal activities of extracts (ethanol and total alkaloid) and pure compounds (chrysosplenetin, dictamnine, gamma-Fagarine, skimmianine) of *H. myrtifolium* against *T. vaginalis*.

Methodology: *H. myrtifolium* was collected from the town of Honaz in Denizli, located in the Aegean region of Turkey, and preparation of extracts and isolation and structure elucidation of pure compounds were performed. Later, different concentrations of extracts and pure compounds were incubated with *T. vaginalis* trophozoites isolated from Turkey, which are known to be sensitive to metronidazole.

Results: It was found that ethanol extract caused a more effective lysis on *T. vaginalis* trophozoites compared with total alkaloid extract ($P < 0.05$). No compounds except for furoquinoline alkaloid skimmianine prepared above 37.5 µg/mL were found to have any inhibitory effect on *T. vaginalis* trophozoites.

Conclusion: The ethanol extract of *H. myrtifolium* and skimmianine can be considered as potential candidates for antitrichomonal drug development.

Key words: *Trichomanas vaginalis*; *Haplophyllum myrtifolium*; antitrichomonal activity.

J Infect Dev Ctries 2019; 13(3):240-244. doi:10.3855/jidc.10854

(Received 12 September 2018 – Accepted 28 February 2019)

Introduction

Trichomoniasis, caused by the pathogenic trichomonad *Trichomonas vaginalis*, is the most common, non-viral sexually transmitted disease (STD) worldwide, with 276 million new cases each year [1].

The natural host of this flagellated protozoan is humans, and it can cause urogenital tract infections both in females and males [2]. In females, *T. vaginalis* leads to vaginitis, showing symptoms of vaginal itching, odor, and discharge, whereas in males, symptoms are not generally noticeable, although urethritis and swelling of the prostate gland can be observed [3]. In addition, trichomoniasis can give rise to serious health consequences in females such as adverse pregnancy outcomes resulting in low-birth-weight infants and premature rupture of placental membranes, pelvic inflammatory disease, cervical cancer, and infertility. It has also been reported in previous studies that trichomoniasis can increased risk of transmission of HIV [2,4-8].

The incidence rates for STDs vary according to age, sexual activity, number of sexual partners, menstrual cycle, methods of diagnosis, and socioeconomic status [9]. A limited number studies have investigated the prevalence of trichomoniasis among female and male patients living in Turkey [10]. According to results obtained from a few studies, the prevalence of *T. vaginalis* among female patients with vaginal discharge is 7-8.69%, whereas the prevalence in male patients with suspected urinary system infection is 6.5% [10-12].

In the classic treatment of *T. vaginalis* infection, metronidazole (MTZ), the only effective approved drug, has been used since the 1960s. On the other hand, there has been an increase in the number of MTZ-resistant *T. vaginalis* strains, and failure in the treatment of trichomoniasis causes serious concerns [2]. Erroneous cases are generally treated using increased doses of MTZ, which may cause an increase in the rate of adverse effects [2]. In addition to this, poor absorption and ineffective delivery of drug on the

infection site are accepted as additional negative factors in the failure of trichomoniasis treatment [13]. These reasons are sufficient to investigate new natural antitrichomonal therapeutic agents that have fewer adverse effects and are relatively inexpensive.

A study conducted in 2018 listed natural plants that showed anti-*Trichomonas vaginalis* activity [2]. According to the list, dried bean seed coats, lectins of *Phaseolus vulgaris*, Perla black bean, leaves of *Voacanga globose*, leaves of *Cussonia species L*., leaves of *Eucalyptus camaldulensis*, seeds and oil of *Nigella sativa*, roots of *Polygala decumbens*, leaves of *Campomanesia xanthocarpa*, and seeds of *Persa americana* and *Pistacia lentiscus* were shown to be effective against *T. vaginalis*. In addition, natural secondary metabolites such as flavonoids, alkaloids, cumarins, saponins, and glycosides were also reported to have anti-*Trichomonas vaginalis* activity [13].

To the best of our knowledge, there has been no previous work on the anti-*T. vaginalis* activity of *H. myrtifolium,* a medicinal plant endemic in Turkey. Therefore, the present study aimed to investigate the *in vitro* anti-*Trichomonas vaginalis* activities of extracts and pure compounds (chrysosplenetin, dictamnine, gamma-Fagarine, skimmianine) of *H. myrtifolium* against *T. vaginalis* in comparison with MTZ, which is the reference drug for the treatment of trichomoniasis.

Methodology

Plant material and preparation of extracts

H. myrtifolium was collected from the town of Honaz, Denizli, located in the Aegean region of Turkey. The plant was taxonomically identified and deposited in the Department of Pharmacognosy, Faculty of Pharmacy, Ege University, Izmir, Turkey.

The preparation of extracts and isolation and structure elucidation of pure compounds were performed as previously described [14-15]. Briefly, the aerial and underground parts of the plant were cut into small pieces, dried and powdered in a grinder. For ethanol extraction, the powdered material was first dissolved in ethanol at room temperature. Later, extraction solvent was filtered and evaporated under reduced pressure in a rotary evaporator (Buchi, Essen, Germany). Finally, a part of extract was lyophilized and used as ethanol extract. In the next step, for total alkaloid extraction, the remaining extract, a dark syrupy material, was acidified using 2% hydrochloric acid and filtered. Later, 25% aqueous ammonium hydroxide was added to the filtrate to adjust to pH 9-10 and the filtrate was extracted using chloroform. Finally, the chloroform was evaporated to yield the total alkaloid fraction. The extracts and pure compounds were kept below -80ºC until required for analysis.

Parasite cultivation

T. vaginalis trophozoites isolated from Turkey that are known to be sensitive to metronidazole were used in this study. *T. vaginalis* trophozoites were axenically grown at 37°C using Trypticase yeast extract-maltose medium (TYM, pH: 6) supplemented with 15% (v/v) fetal calf serum (FCS, Sigma-Aldrich, Taufkirchen, Germany) and penicillin/streptomycin at a dilution of 1:100, as previously described [3].

Antitrichomonal assay

For *in vitro* testing, 50, 100, 200, 400, and 800 µg/mL concentrations of the ethanol extract and total alkaloid fraction of *H. myrtifolium* were prepared. Also, 12.5, 37.5, 50, 75, 100, and 150 µg/mL concentrations of chrysosplenetin, dictamnine, gamma-Fagarine, and skimmianine were prepared. All extracts and pure compounds were dissolved in dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany) at a final concentration of 0.5% (v/v), which does not affect parasite growth rate, mobility or morphology, and diluted in TYM medium supplemented with 15% FCS [3].

T. vaginalis trophozoites in the logarithmic phase of growth were transferred to a 24-well plate (10^5 parasites/well) containing TYM medium supplemented with 15% (v/v) FCS and penicillin/streptomycin (Sigma–Aldrich, Buchs, Switzerland) at a dilution of 1:100. Thereafter, different concentrations of extracts and compounds were added to each well and incubated at 37°C. As a reference drug, metronidazole prepared in DMSO (25 µg/well) was used. For untreated groups, two wells were used; one well contained DMSO (25 µg/well) in TYM medium with 15% FCS and *T. vaginalis* trophozoites (10^5 parasites/well), and the other contained only TYM medium with 15% FCS and *T. vaginalis* trophozoites (10^5 parasites/well).

The number of parasites was counted using a hemocytometer under a light microscope (Olympus, CK 40, UK) at the 12, 24, and 72 hours. All in vitro experiments were run in triplicate and the results are expressed as mean percentages of the number of parasites.

Results

No immotile *T. vaginalis* trophozoites or active lysis was observed when ethanol and alkaloid extracts were applied at a concentration of 50 µg/mL and 100 µg/mL at 24, 48, and 72 hours of incubation. *T.*

Table 1. Effect of ethanol and alkaloid extracts of *H. myrtifolium* on *T. vaginalis* trophozoites.

Concentration (µg/mL)		Ethanol extract			Alkaloid extract		
		24th hour	48th hour	72th hour	24th hour	48th hour	72th hour
50	Movement	+	+	+	+	+	+
	Lysis	-	-	_	-	-	_
100	Movement	+	+	+	+	+	+
	Lysis	-	-	-	-	-	-
200	Movement	+	-	-	+	+	+
	Lysis	-	-	-	-	-	-
400	Movement	+	-	-	+	-	-
	Lysis	-	+	+	-	-	-
800	Movement	+	-	-	+	-	-
	Lysis	-	+	+	-	+	+

vaginalis trophozoite motility disappeared at 48 hours after exposure with 200 µg/mL of ethanol extract and 400 µg/mL of alkaloid extract. Trophozoite lysis was observed for the first time at 48 and 72 hours when 400 µg/mL of ethanol extract and the highest dose (800 µg/mL) of alkaloid extract were used, respectively (Table 1).

When the percent lysis values obtained from extracts or pure compounds were compared with the control groups at the first 24 hours, no statistically significant difference was found ($P > 0.05$). On the other hand, use of ethanol extract at a concentration of 400 µg/mL resulted in the lysis of 50% and 100% of *T. vaginalis* trophozoites at 48 and 72 hours of incubation, respectively. Also, it was found that the highest doses (800 µg/mL) of ethanol and alkaloid extracts led to the lysis of 100% of *T. vaginalis* trophozoites at 48 hours.

The minimum inhibitory concentration (MIC) for the ethanol extract and alkaloid extract at 48 hours was 200 µg/mL and 400 µg/mL, respectively. The minimal lethal concentration (MLC) was 400 µg/mL at 72 hours for the ethanol extract and 800 µg/mL for alkaloid extracts at 48 hours.

Among the pure alkaloid compounds, dictamnine and gamma-Fagarine showed no activity against *T. vaginalis* trophozoites. Similarly, skimmianine and the flavonoid compound chrysosplenetin had no anti-*T. vaginalis* activity at concentrations of 12.5 µg/mL-37.5 µg/mL. Interestingly, although no motile *T. vaginalis* trophozoites were observed at 48 and 72 hours when chrysosplenetin was applied over 37.5 µg/mL, lysis was not detected. Otherwise, skimmianine from 50 µg/mL to 150 µg/mL showed anti-*T. vaginalis* activity at 48 and 72 hours (Table 2).

At 48 hours of incubation, it was found that skimmianine at a concentration of 100 µg/mL caused the lysis of 50% of *T. vaginalis* trophozoites. Also, 100% of *T. vaginalis* trophozoites were observed to be lysed when skimmianine concentration was increased to 150 µg/mL.

The MIC values at 48 hours for skimmianine and chrysosplenetin were 50 µg/mL. The MLC value at 48 hours for skimmianine was 150 µg/mL. The MLC value was not estimated for chrysosplenetin because it had no lethal effect.

MTZ at concentration of 50 µg/mL caused the lysis of 100% of *T. vaginalis* trophozoite at 72 hours. DMSO

Table 2. Effect of skimmianine and chrysosplenetin pure compounds on *T. vaginalis* trophozoites.

Concentration (µg/mL)		Skimmianine			Chrysosplenetin		
		24th hour	48th hour	72th hour	24th hour	48th hour	72th hour
12.5	Movement	+	+	+	+	+	+
	Lysis	-	-	_	-	-	_
25	Movement	+	+	+	+	+	+
	Lysis	-	-	-	-	-	-
37.5	Movement	+	+	+	+	+	+
	Lysis	-	-	-	-	-	-
50	Movement	+	-	-	+	-	-
	Lysis	-	-	-	-	-	-
100	Movement	+	-	-	+	-	-
	Lysis	-	+	+	-	-	-
150	Movement	+	-	-	+	-	-
	Lysis	-	+	+	-	-	-

prepared in a final concentration of 0.5% (v/v) showed no statistically important anti-*T. vaginalis* activity when compared with the negative control groups ($P > 0.05$).

Discussion

MTZ has long been used in the classic treatment of *T. vaginalis* infections. However, new alternative drugs obtained from natural sources are being investigated for the treatment of trichomoniasis due to occurrence of MTZ-resistant *T. vaginalis* strains in approximately 9.6% of cases, in addition to the adverse effects of MTZ [16].

Plant materials are being used as an alternative approach for the treatment of various diseases, including parasitic diseases, because they have useful therapeutic activities. Accordingly, several studies have been conducted to develop alternative drugs for the treatment of trichomoniasis and it was found that flavonoids, alkaloids, cumarins, saponins, and glycosides obtained from plant materials showed anti-*T. vaginalis* activity [2,13].

H. myrtifolium, which was used in this study, is a member of the Rutaceae family. This family is represented by 161 genera and about 1900 species around the world. Its most characteristic features are secretory cavities that contain volatile oils, alkaloids, resin, hesperidin, and some other chemical compounds [17].

A study reported that ethanol and alkaloid extracts of *H. myrtifolium* showed anti-leishmanial activity against *Leishmania tropica*, which is a causative agent of cutaneous leishmaniasis [15]. To the best of our knowledge, the antitrichomonal effect of *H. myrtifolium*, which contains biologically active compounds such as alkaloids, lignans, and glycosides, has not been studied before. In the present study, the antitrichomonal effect of *H. myrtifolium* was investigated for the first time and ethanol extract was found to be more effective for the lysis of *T. vaginalis* trophozoite than the alkaloid extract. This result is not surprising because the ethanol extract contains therapeutics such lignans, coumarins and flavonoids, in addition to alkaloids.

In addition, the antitrichomonal impact of furoquinoline alkaloids such as dictamnine, gamma-Fagarine, and skimmianine was investigated in this study, and skimmianine over 37.5 µg/mL was found to have an inhibitory effect on *T. vaginalis* trophozoites. Also, it was observed that chrysosplenetin only prevented the movement of *T. vaginalis* trophozoites when used at its highest dose (150 µg/mL).

Similar to our study, the results of different studies indicated that alkaloids showed an anti-*T. vaginalis* effect. In a previous study, alkaloids obtained from seeds of *Crolatalaria pallida* were indicated to have an inhibitory effect on *T. vaginalis* trophozoites [18]. In another study, it was reported that alkaloids from *Hippeastrum breviflorum* showed an anti-*T. vaginalis* effect [19]. It was shown that furoquinoline alkaloids extracted from *Teclea afzelii* (*Rutaceae*) plants had anti-plasmodial activity [20]. Another study reported that some alkaloids (furoquinoline and acridone) obtained from plants form the Rutaceae family had anti-plasmodial activity against *Plasmodium falciparum* [21].

Conclusion

The results of this study indicated that the ethanol extract of *H. myrtifolium* and isolated compound skimmianine could serve as an efficacious alternative therapeutic agent for the treatment of *T. vaginalis* infection.

Acknowledgements

The authors would like to thank Prof. Dr. Ahmet Özbilgin from University of Manisa Celal Bayar, Turkey for providing *T. vaginalis* trophozoites. No financial support for the study has been received.

Authors' Contributions

This study was conceived and designed by A.A.G., H.C., H.K., and S.K. All experiments were performed by A.A.G., H.C., B.P., and H.K. The manuscript was written by A.A.G., H.C., and H.K. The manuscript was reviewed by H.C., H.K., and S.K.

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Conflict of interests: No conflict of interests is declared.