Journal of Shahrekord University of Medical Sciences
doi:10.34172/jsums.2023.627 2023;25(1):1-6 http://j.skums.ac.ir

Original Article

A survey on the anti-*Trichomonas vaginalis* effect of the hydroalcoholic extract of various medicinal plants *in vitro*

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Abstract

Background and aims: Trichomoniasis is the most common non-viral sexually transmitted infection worldwide; although it is treated by a 5-nitroimidazole drug family such as metronidazole (MTZ) with numerous side effects, and in this regard, alternative new drugs are required. Therefore, this study examined the anti-Trichomoniasis effect of the hydroalcoholic extract of some traditional medicinal plants of Iran *in vitro*.

Methods: In this experimental study, the hydroalcoholic extracts of medicinal plants were prepared by maceration at a stock concentration of 20 mg/mL in the saline solution and then used for *in vitro* anti-trichomonas experiments. *Trichomonas vaginalis* trophozoites were isolated from the patient and cultured in a Trypticase Yeast extract Iron-Serum-33 medium. In addition, 200 µL of the culture medium containing 5×10^4 trophozoites was diluted in plate wells, and 10 doses were separately added on trophozoites for each extract serially diluted between 0.12 and 16 mg/mL in triplicate. The plates were incubated for 48 hours at 37 °C with 5% CO_2. The number of trophozoites was counted with a hemocytometer and Trypan blue staining. Finally, the half maximal inhibitory concentration (IC_{50}) was calculated by probit analysis.

Results: Among the tested plants, *Eugenia caryophyllata*, *Camellia sinensis,* and *Terminalia chebula* Retz showed the best anti-trichomonal activity with IC50 values of 1.21, 1.62, and 1.66 mg/mL, respectively. All tested extracts had more IC50 than MTZ (IC_{50} 100 mg/mL), an antiprotozoal drug used as a positive control.

Conclusion: According to the results of this study, *E. caryophylata*, *C. sinensis* and *T. chebula* Retz affected the growth of *T. vaginalis*. Thus, it is recommended that other studies use this plant for the treatment of trichomoniasis infection.

Keywords: Medicinal plants, Hydroalcoholic extracts, *Trichomonas vaginalis*, Trophozoites, *In vitro*

Received: January 26, 2022, Accepted: February 26, 2022, ePublished: November 10, 2022

Introduction

Parasitic diseases are nowadays considered one of the health problems worldwide, especially in tropical and subtropical developing countries; they cause extensive damage at the social-psychological dimension annually. Trichomoniasis is caused by an anaerobic flagellated protozoan called *Trichomonas vaginalis* that inflicts urogenital infections and is one of the most common non-viral sexually transmitted diseases that affects the health and lives of people worldwide (1). Human is the only natural host for this parasite, and according to the information and statistical reports of some studies, about one hundred and forty-two million people in the world are infected with this parasite annually (2,3). The prevalence of trichomoniasis in the world's human population varies in different countries and is affected by geographical location, age, gender, health care, and socioeconomic and cultural status (4). Trichomoniasis may persist for a long time (months or even years) in females and generally persists for less than 10 days in men (5). Although Trichomoniasis, in most infected humans, is asymptomatic or slightly symptomatic, it is usually more severe in women than men. *T. vaginalis* in women causes obvious vaginitis, urethritis, and dysuria, as well as swelling of the cervix as a pelvic inflammatory disease, foamy discharge, and cervical cancer (6). In addition, in pregnant women, it can cause low birth weight, premature birth, ectopic pregnancy, spontaneous abortion, infertility, and premature rupture of the amniotic sac (7). Furthermore, it can be responsible for some non-genital urethritis, prostatitis, epididymitis, and infertility in men (5). Trichomoniasis is treated by the 5-nitroimidazole drug family such as metronidazole (MTZ), tinidazole, and secnidazole with a 95% success rate. However, the most common useful medical treatment for trichomoniasis is MNZ, which is approved by the United States Food and Drug Administration. Numerous studies have been reported about the prevalence of the resistance of *T. vaginalis* to MTZ; this is because higher doses used steadily (e.g., clinical isolates) are associated with

cross-resistance to other 5-nitroimidazoles (8). Further, MTZ has numerous side effects such as gastrointestinal disorders, hypersensitivity reactions, and dermatological symptoms (9). Moreover, MTZ has teratogenic and carcinogenic effects on fetuses. Therefore, these facts have prompted researchers to look for alternative drugs with slight or complementary side effects for the safe effective treatment of trichomoniasis (8). In this regard, the use of medicinal plants to treat various diseases has continued since ancient times. The benefits of medicinal plants with fewer side effects have often been considered available resources. Improper use of synthetic drugs has also raised the issue of resistance against microorganisms, necessitating the identification and research of medicinal plants (10). Thus, different parts of plants such as *Achillea wilhelmsii, Terminalia chebula* Retz*, Salvia hydrangea, Camellia sinensis, Alhagi maurorum, Teucrium polium, Malva sylvestris, Stachys inflate, Salvia officinalis, Eugenia caryophylata*, and *Ferulago angulata* have anti-parasitic and anti-microbial effects. The above-mentioned plants have been traditionally used in some areas of Iran and the world for the treatment of some disorders and diseases; they also have flavonoids, phenol contents, and antioxidant activities (11,12). Accordingly, the current study aimed to evaluate the effects of these extracts on *T. vaginalis in vitro*.

Materials and Methods

Collecting plant samples and extraction

To prepare the extracts for the screening of anti-trichomonas activity, different parts of the plants (*Terminalia chebula* Retz, *E. caryophylata*, *C. sinensis*, *A. maurorum*, *F. angulate*, *M. sylvestris*, *T. polium*, *S. hydrangea*, *S. inflate*, *S. officinalis,* and *A. wilhelmsii*) were purchased from the medicinal plant stores of Shahrekord and identified as the plants of interest by botanists, and voucher specimens at the Herbarium of Shahrekord University of Medical Sciences Chaharmahal va Bakhtiari province. Different parts of plants such as aerial parts, seeds, leaves, and flowers were thoroughly washed with water and dried at room temperature and shadow conditions for 3-5 days until they became fully dried. The dried parts were ground to a fine powder using a stainless steel blender, and the maceration was performed in 70% ethanol to prepare the extracts. Hence, the mixture of powder plants and methanol was continuously swirled at 150 rpm in a shaker incubator for 72 hours at room temperature, and the mixture was refined using a Buchner funnel and Whatman number 1 paper. The collected extracts were concentrated under a vacuum using a Rotary evaporator at 35˚C. Then, the extracts were incubated at 40˚C to dry, and ultimately, the dried extracts were stored at -20˚C until they were used for *in vitro* assay (13).

Parasites

First, a positive urine sample of *T. vaginalis* was taken from a female patient suspected of trichomoniasis in the Hajar hospital of Shahrekord and cultured in modified Trypticase Yeast extract Iron-Serum-33 (TYI-S-33) Diamond's medium supplemented with 10% fetal calf serum (FCS Gibco) to isolate the Trophozoites of *T. vaginalis* which are used in all experiments. Then, these Trophozoites were axenically grown and maintained in the TYI-S-33 medium by subculturing. The trophozoites were axenically cultured and maintained in Diamond's medium, then evaluated by an inverted microscope to ensure the viability and in the log phase of the growth of *T. vaginalis* trophozoites for investigating the anti-parasite of plant extracts *in vitro* assays (14).

In vivo experiments

After the mass cultivating of *T. vaginalis* trophozoites by using the TYI-S-33 medium, a dilution of trophozoites was prepared including 25×10^4 trophozoites per mL (stock), followed by adding 200 µL of stock (5×10^4 trophozoites) to each well of plates. The extracts were dissolved in the saline solution at a stock concentration of 20 mg/mL (DMSO, if necessary) and filtered by a filter Syringe of 0.2 microns. Additionally, 10 doses for each extract were prepared with serially diluted (0.12-16 mg/mL) and MTZ (1.62-250 µg/mL) and then added to the wells containing trophozoites in triplicate and incubated for 48 hours at 37˚C with 5% CO_2. Finally, the number of parasites was calculated with a hemocytometer and Trypan blue staining, and the 50% inhibitory concentration (IC_{50}) was calculated by probit analysis. MTZ was added as the positive control, and the negative control was *T. vaginalis* trophozoites in the TYI-S-33 medium (15).

Data analysis

IC_{50} was determined using probit regression, and analysis was performed by SPSS, version 23.

Results

The IC_{50} of the hydroalcoholic extract of various parts of different plants was determined on *T. vaginalis* trophozoites using the counting method. The plants included *Terminalia chebula* Retz, *E. caryophylata*, *C. sinensis*, *A. maurorum*, *F. angulate, M. sylvestris*, *T. polium*, *S. hydrangea*, *S. inflate*, *S. officinalis, A. wilhelmsii*, and MTZ. Next, their values were calculated by dose-response curve and regression analysis. IC_{50} values of plant extracts are provided in Table 1. Based on the results of this study, the IC_{50} values of MTZ were 100 µg/mL. Among the tested plants, *T. chebula Retz, E. caryophylata,* and *C. sinensis* showed the best anti-trichomonal activity with IC_{50} values of 1.667, 1.215, and 1.622 mg/mL, respectively. Moderate activity was observed with the *A. maurorum, F. angulate,* and *T. polium* with IC_{50} values 2.764, 2.924, and 3.766 mg/mL. However, *M. sylvestris, S. hydrangea, S. inflate, S. officinalis,* and *A. wilhelmsii* demonstrated poor activity with IC_{50} values of 4.931, 4.670, 6.955, 6.987, and 7.389 mg/mL, respectively. None of the extracts assayed were more active than MTZ (IC_{50} 100 µg/mL), which is used as

Table 1. Effect of selected plants on *Trichomonas vaginalis*

Plant name and family	Plant family	Voucher specimen	Plant part extracted	Traditional Use	Application Method	*T. vaginalis* IC_{50} (mg/mL)
Terminalia chebula Retz	Combretaceae	27	S	Asthma, sore throat, vomiting, diarrhea, bleeding piles, heart, and bladder diseases (12)	OD	1.667 (1.107-2.272)
Eugenia caryophylata	Myrtaceae	1002	S	Dental care as disinfectant, analgesic, and anti-inflammatory (12)	OD	1.215 (.792-1.825)
Camellia sinensis	Theaceae	349	L	Stimulant, diuretic , astringent, and to improve heart health (12)	OD	1.622 (1.215-2.087)
Alhagi maurorum	Papilionaceae	472	A	Gastrointestinal disorders, especially gastric ulcers and rheumatism (12)	OD	2.764 (2.053-3.654)
Ferulago angulata	Apiaceae	324	A	Lowering blood sugar and as a food seasoning (12)	OD	2.924 (2.482-3.426)
Malva sylvestris	Malvaceae	107	F	Cough, inflammatory diseases, and some skin diseases (12)	OD	4.931 (4.232-5.662)
Teucrium polium	Lamiaceae	552	A	Metabolic syndrome (12)	OD, VW	3.766 (2.547-5.417)
Salvia hydrangea	Lamiaceae	28	A	Carminative, and rheumatoid pain (12)	OD	4.670 (3.683-5.831)
Stachys inflate	Lamiaceae	1024	L	Infectious diseases, asthma, and rheumatism (12)	OD, VW	6.955 (5.352-8.927)
Salvia officinalis	Lamiaceae	663	A	Anti-inflammatory and analgesic (12)	OD	6.987 (5.492-8.965)
Achillea wilhelmsii	Asteraceae	207	A	Metabolic syndrome (12)	OD	7.389 (5.984-8.951)

Note. IC_{50}: Half maximal inhibitory concentration; The results are expressed as the mean (n=3), and 95% confidence intervals. The application method: VW: Vaginal wash; OD: Oral administration; Extracted parts of the plant: A (aerial parts), S (seeds), L (leaf), and F (flowers).

the antiprotozoal drug as the positive control.

Discussion

Trichomoniasis is the most common non-viral sexually transmitted disease, which is caused by the *T. vaginalis*, causing injuries, and complications such as preterm labor, low birth, weight, and miscarriage (1). A common treatment for this disease is MTZ, which has side effects for undertreatment patients. There are also many reports from many countries about the prevalence of MTZ resistance and its carcinogenic effects. The mechanism of action of MTZ as a synthetic drug used against *Amoebae*, *Giardia lamblia*, and *T. vaginalis* is the breaking of DNA strands that can occasionally cause dangerous side effects on the hosts (16). Therefore, the side effects of nitroimidazole compounds and increased resistance of *T. vaginalis* to treatment increase the importance of the need for new drugs such as those obtained from natural sources. Medicinal plants have long been widely used in the treatment of diseases. This widespread use can be due to various reasons such as the advantage of fewer side effects, better patient acceptance due to the recommendation of traditional medicine, and the use of previous generations, or the lower price of medicinal plants (17). Many of the known drugs are of plant origin; for example, quinine, which is used to treat malaria and has many synthetic derivatives such as Chloroquine, Amodiaquine, and Primaquine (18). Therefore, considering these risks and the antimicrobial effects of some medicinal plants, this study was conducted to investigate the anti-trichomoniasis activity of *A. wilhelmsii*, *T. chebula Retz*, *S. hydrangea*, *C. sinensis*, *A. maurorum*, *T. polium*, *M. sylvestris*, *S. inflate*, *S. officinalis*, *E. caryophylata*, and *F. angulate* on *T. vaginalis* Trophozoites compared with MTZ *in vitro*. In this study, the IC_{50} of the eleven plant extracts was determined on the growth inhibition of *T. vaginalis* trophozoite. The findings of this study showed that the inhibitory effect of *E. caryophylata*, *C. sinensis*, and *T. chebula* Retz was higher than those of the other plants, while less than that of MTZ with IC_{50}=100 µg/mL. *E. caryophylata* belongs to the *Myrtaceae* family and has compounds such as B-caryophyllene, acetyleugenol, alpha-humulene, thymol, eugenol, cinnamaldehyde, and methyl salicylate (19). The effects of this plant (e.g., anti-inflammatory, anti-epileptic, antibacterial, insecticide, antiviral, antioxidant, and anti-inflammatory activities, as well as cytotoxicity, and anesthesia) have been mentioned in some studies (20). Ibikunle et al investigated the anti-trichomonal activities of the methanol extract leaf of *Eugenia uniflora*, and its fractions of the *Myrtaceae* family. The results indicated that its subfractions E2-5 had LC50 and LC90 values of 4.77-5.28, 18.49-25.00, and 4.53-5.18, 18.32-19.07 µg/mL at 24 and 48 hours, respectively, which were better than those of MTZ. In addition, one study (21) reported the effective components of *E. uniflora*, including Mono- and sesqui-terpenoids, triterpenoids and their acetates, tannins, macrocyclic hydrolysable tannin dimers, and xanthine oxidase of its different parts, that can have anti-parasitic activities (e.g., anti-trypanocidal, anti-malarial, and anti-trichomonas activities). *C. sinensis* belongs to the *Theaceae* family and has high antioxidant power, as well as anti-cancer,

antimutagenic, anti-artherosclerotic, antibacterial, and antifungal activities (22). Its leaves contain polyphenols that are oxidized during the conversion of white tea to green tea and eventually to black tea. The main compounds of *C. sinensis* are epicatechin gallate (ECG), epigallocatechin gallate (EGCG), epigallocatechin (EGC), and eEpicatechin (EC). EGCG is the main ingredient in white and green teas. White tea contains relatively more caffeine, gallic acid, theobromine EGC, and ECG, which may be related to its greater antioxidant activity (23). *T. chebula* Retz belongs to the *Combretaceae* family. Dried and fresh fruit has been reported as a powerful antioxidant and rich in phenolic compounds. Important compounds of *T. chebula* Retz are chebulic, chebulinic, gallic, corilagin, and ellagic acids. This plant has antibacterial and antifungal, anti-amoebic, anti-malarial, antiviral, and antioxidant activities (24). A limited number of studies have reported the mechanism actions of these plants against *T. vaginalis*. Most of the properties of plants are attributed to their antioxidant activities, which are mostly related to the presence of phenolic components. These phenolic compounds usually have antimicrobial activities (25). However, the other components of the plants should be involved in the activities of these plants. Various studies have proven the effect of plants on *T. vaginalis*. Calzada et al found that among twenty-two medicinal plants investigated against *T. vaginalis*, the methanolic extracts of *Carica papaya* and *Cocos nucifera* represented the most antiparasitic properties with the IC_{50} values of 5.6 and 5.8 µg/mL, respectively (26). Likewise, Muelas-Serrano et al studied the anti-trichomoniasis activity of the nine plants of American plants and concluded that *Mikania cordifolia*, *Scutia buxifolia* Reiss (Asteraceae), and *Neurolaena lobata* (Rhamnaceae) have the most active extracts in this model against the *T. vaginalis* (27). Frasson et al evaluated the anti-trichomoniasis activity of forty-four aqueous extracts of plants. After screening these aqueous extracts, only the *Polygala decumbens* root extract was significantly effective in reducing trophozoite viability. The minimum inhibitory concentration value was 1.56 mg/mL, which is consistent with the results of this study. *A. maurorum* belongs to the *Papilionaceae* family with an IC_{50} of 2.764 mg/mL and shows anti-Trichomonas activity (28). Chemical studies on this plant have demonstrated the presence of unsaturated sterols, triterpenes, tannins, flavonoids, and flavanone glycosides such as alhagidin, alhagitin, proanthocyanidins, and resin. Numerous studies have indicated that the flavonoids in Quercetin and Catechin have antioxidant activities (29). In another study, Yousefi et al examined the effect of *S. officinalis* from the *Lamiaceae* family on the *T. vaginalis in vitro* and reported that different concentrations (2, 2.5,4, 5, 8, and 10 mg/mL) of the hydroalcoholic extracts of *S. officinalis* caused the inhibitor of the growing of trophozoites and was similar to the effect of MTZ on the parasites (30). Moreover, Rabbani et al investigated the effects of the *Stachys lavandulifolia* of the *Lamiaceae* family on the *T. vaginalis in vitro* and found that the concentrations of 500 µg/mL and 50 µg/mL of the aqueous extract and methanolic extract of *S. lavandulifolia* caused the death of *T. vaginalis* (31). Other studies evaluated the effects of the ethanolic and aqueous extracts of the aerial parts of *S. Lavandulifolia* and *S. sylvatica* on the *T. vaginalis in vitro* and reported that they are associated with effective components such as α-pinene, β-pinene, germacrene-D, and flavonoids. (32). *F. angulate* from the *Apiaceae* family with an IC_{50} value of 2.924 mg/mL showed good anti-trichomoniasis properties. It is considered to be a natural source of monoterpenes and sesquiterpenes, which have antimicrobial properties (33). The Oleogum resin extracted from *Ferula assa-foetida* had a strong effect on anti-trichomoniasis compared with MTZ (34). The essential oil of *Artemisia aucheri* has been found to be effective against *T. vaginalis* immediately after inoculation *in vitro* (35). *M. sylvestris* (the *Malvaceae* family) with an IC_{50} value of 4.931 mg/mL indicated moderate activity in comparison with an extract with an IC_{50} value of < 3 mg/mL. The plant has anti-inflammatory and antioxidant properties. The most important active ingredients in *M. sylvestris* are mucilage, flavonoids, tannins, phenolic compounds, terpenoid derivatives, and volatile oils (36). In this study, four genera of the *Lamiaceae* family were tested, including *T. polium, S. hydrangea, S. inflate*, and *S. officinalis*, which represented anti-trichomonas activities with IC_{50} values of 3.766, 4.670, 6.955, and 6.987 mg/mL, respectively. The Lamiaceae or Labiatae is a large family of flowering plants with more than two hundred and thirty genera which often include aromatic components with anti-microbial activated features. Different reports have been published about the anti-trichomonas activity of plants from this family. This family has different components such as phenylethanoid glycosides, triterpenoids, steroids, flavonoids, phenolic acids, phenylpropanoid glycosides, quinoidal, linalool borneol, alpha and beta caryophyllene, Tannins, Saponins, Sterols, and Volucoanthocyanins; they have a wide range of biological properties such as anti-inflammatory, antibacterial, antioxidant, and anti-cancer activities (37). Due to the composition and properties of the *Lamiaceae* family, it is expected that the species studied in this study demonstrate anti-trichomoniasis properties. Further, the unique compounds that are present in each plant cause different activities of inhibiting the growth of *T. vaginalis*. Thus, *T. polium* and *S. officinalis* are the strongest and weakest plants of this family, respectively. Ezz Eldin and Badawy concluded that the *Ocimum basilicum* essential oil inhibited the growth of *T. vaginalis* trophozoites in a culture medium. Their results revealed that increasing the concentration and time of exposure reduces the survival rate and mobility of *T. vaginalis*. Moreover, significant membrane damage, abnormally large cytoplasmic vacuolization, and extensive cytoplasmic damage have been found in trophozoites treated with this essential oil

(38). Furthermore, Zheng et al indicated that the aqueous extract of *Mosla chinensis* filled the cytoplasm of *T. vaginalis* with granules and vacuoles; some of them blew, and their cellular contents overflowed in the parasite, and finally its growth and proliferation was inhibited (39). The last plant evaluated in this study was *A. wilhelmsii* (the Asteraceae family) with an IC_{50} value of 7.389 mg/mL, which showed the weakest anti-*Trichomonas* property. The most important components produced by this family are isochlorogenic acid, sesquiterpene lactones, various alkaloids, terpenoid essential oils, pentacyclic triterpene alcohols, acetylenes, and tannins, which have anti-inflammatory, antibacterial, anti-cancer, anti-viral, and antioxidant effects (40). Probably due to having a lower percentage of phenolic compounds, it has lower antioxidant activities and less antiparasitic properties. In another study, the activity of different components of aqueous, methanolic, and chloroform extracts of *Xanthium brasilicum* and *Argemone mexicana* were investigated against a clinical strain freshly isolated from patients. All extracts represented weak anti-trichomonas activity; however, methanolic extracts demonstrated the strongest activity (41). In our study, the range of IC_{50} of the plant extracts was from 1.21 to 7.389 mg/mL; some cases had weaker activity than some plants and their IC_{50} values were much stronger than those of many plants against *T. vaginalis* compared with other studies. Research on different plants has led to the production of various anti-trichomoniasis drugs in the world. A few plants, which are sold in India, include *Emblica officinalis*, *Terminalia bellirica*, *Elettaria cardamomum*, *Rosa centifolia*, *Boerhaavia diffusa*, *Curcuma longa*, and *Vitex negundo* in PH5 as a vaginal gel (42).

Conclusion

The results of the present study indicated that the inhibitory effects of *E. caryophylata*, *C. sinensis*, and *T. chebula Retz* were higher than those of other plants. Thus, it is recommended future researchers prepare various extracts, other than the hydroalcoholic extract, including diethyl ether, ethyl acetate, and methanolic extracts. Each of these substances has a different activity in extracting plant compounds. According to evidence, most studies about the anti-trichomoniasis activity of medicinal plants were performed *in vitro*, and human studies were limited to a small number of works. Therefore, more human studies are needed to use these herbal compounds after animal trials in the treatment of Trichomoniasis in the form of cream or orally.

Authors' Contribution

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Funding/Support

This study was funded by Shahrekord University of Medical Sciences (Grant No. 5587).

Conflict of Interests

The authors declare that there is no conflict of interests.

Ethical Approval

The study protocol was approved by the Research Ethics Committee of Shahrekord University of Medical Sciences (IR.SKUMS.REC.1399.243).

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