Preliminary phytochemical screening and antitrichomonal activity of *Ferula pseudalliacea*

Running title: Antitrichomonal Activity of *Ferula pseudalliacea*

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Abstract

Background: *Trichomonas vaginalis* (*T. vaginalis*) causes human trichomoniasis, a common type of protozoan vaginitis. Due to the increasing incidence of drug-resistant trichomoniasis, new pharmacological research is needed. The aim was the investigation of the activity of *Ferula pseudalliacea (F. pseudalliacea)* against *T. vaginalis*, and preliminary phytochemical analysis of its extracts.

Methods: Essential oil and various extracts of *F. pseudalliacea* roots, including n-hexane, ethyl acetate and methanol, were obtained. Susceptibility testing of the plant products was done on five *T. vaginalis* isolates by using microtiter plate method. Minimum lethal concentration (MLC) and growth inhibitory percent (GI%) of sub-MLC concentration were reported, after 24 and 48 hours' exposures. Phytochemical screening of the extracts was done using standard procedure.

Results: The antitrichomonal effect of the plant products depended on time and concentration, with the greatest effect observed after in 48 hours of exposure. The essential oils and n-hexane extract of *F. pseudalliacea* demonstrated a remarkable activity with MLC of 250 µg/ml and following by the ethyl acetate (MLC=500 µg/ml) and methanol extract (MLC=1000 µg/ml), with GI% 92.8, 50.6, 85.2, and 42.8, respectively. The bioactive constituents of the extracts were coumarins, terpenoids, steroids, phenols, tannins and glycosides.

Conclusion: The results of this study demonstrated *in vitro* antitrichomonal properties of *F. pseudalliacea*. Therefore, further studies are needed to investigate the potential of the antitrichomonal activity of its bioactive constituents.

Keywords: Antitrichomonal agents; Essential oil; Extract; *Ferula pseudalliacea*; Phytochemicals; *Trichomonas vaginalis*

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Introduction

Trichomoniasis is a common human vaginitis caused by a protozoan parasite, *Trichomonas vaginalis*. The infection is one of the most prevalent STIs worldwide. According to the World Health Organization in 2016, there were 156 million new cases of *Trichomonas* infection among persons aged 15 to 49 years. Trichomoniasis in women may present with a wide variety of clinical features, from an asymptomatic infection to a severe vaginitis. Adverse pregnancy outcomes, infertility and cervical neoplasia may be seen as complications after trichomoniasis. Furthermore, the infection increases the risk of spreading HIV infection in the community (1-2).

Metronidazole therapy is commonly the standard treatment for trichomoniasis worldwide. Since 1961, when metronidazole was introduced to treat trichomoniasis, it has faced challenges. One of these is drug resistance. Drug-resistant *T. vaginalis* is involved in an increasing number of refractory trichomoniasis. The first metronidazole-resistant trichomoniasis was reported in 1962 and it has been on the rise. According to the Center for Disease Control and Prevention, 2 to 5% of clinical *T. vaginalis* isolates are metronidazole- resistant in the United States (3-4).

Due to the presence of bioactive compounds in plants, they are of particular interest in pharmaceutical research. Plant essential oils and extracts are known to be a rich source of natural ingredients for treatment of various diseases and are more compatible with biological systems. *Ferula* is a genus of plants with about 185 species. This genus has a special position in Apiaceae family because of pharmaceutical and industrial importance. In Iranian flora, the genus *Ferula* contains 32 species including 15 endemic plants which this genus is typically called koma or kema. Several species of *Ferula* have been used in folk remedy as treatment of stomachache, hysteria, arthritis, rheumatoid and etc. Recent studies have proven antibacterial, antileishmanial, antimalarial, antioxidant, anti-epileptic and anti-inflammatory effects of *Ferula* species (5-7). In previous studies, the methanol extract of *F. szowitsiana* was able to inhibit the growth of *T.vaginalis* cells (8).

F. pseudalliacea, is an indigenous species of Iran and grows in the Sanandaj Mountains (West of Iran) and their gum has been used in traditional medicine for healing wounds and relieves itching. Recently, different studies have been done on the antibacterial, antiplasmodial, phytotoxic and anticancer activity of *F. pseudalliacea* (9-12). But so far, a study to evaluate the antitrichomonas effect of this species has not been done.

In this study, we have examined the antitrichomonas effect of *F. pseudalliacea* essential oil and extracts against *T. vaginalis*. Also the preliminary phytochemical analysis of the extracts were performed by standard methods.

Methods

Plant material

The roots of *F. pseudalliacea* were obtained from natural habitats in western Iran. The plant was identified in the herbarium of the Department of Pharmacognosy of Hamadan University of Medical Sciences, and Voucher number 234 was allocated.

Preparation of the essential oil, extract and phytochemical screening

The plant was dried during the shade-drying process at room temperature (20±5 ° C) and the dried plant's materials were crushed into powder. The powder (100 g) was used to prepare essential oil using a Clevenger-type apparatus. The obtained essential oil was kept in airtight container in refrigerator (4 °C) until use. Extraction of the dried plant was performed using maceration method. Briefly, the powder plant (100g) was macerated separately in n-hexane, ethyl acetate and, methanol solvents (3 × 2 L, room temperature for 72 h, 25°C). Extraction was done using a rotary

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evaporator below 40 °C. The obtained extracts were kept in dark container in refrigerator (4 °C) until use.

Phytochemical analysis of the extracts was done using standard methods and their constituent compounds were identified using the method of Ugochukwu and Bargah (13,14).

Parasite culture and solutions

Five clinical *T. vaginalis* isolates were cultured in TYI-S-33 medium supplemented with 10% heat-inactivated adult bovine serum, and antibiotics (100 IU/ml penicillin, and 100 µg/ml streptomycin). After several 48-hour subcultures, the pure trophozoites in log phase of growth were used for susceptibility assay (15,16). Metronidazole (Sigma-Aldrich, St Louis, USA) was dissolved in distilled water. The plant products were dissolved in Dimethyl sulfoxide (D2650 SIGMA, BioReagent) or distilled water, according to solubility. Solubility of the essential oil and the extracts in culture medium was considered as criteria for the plant products concentration and susceptibility testing was started with the highest concentration. The solutions were prepared in 2-fold dilutions in medium culture for susceptibility assay at the following concentrations 200, 100, 50, 25, 12.5, 6.2, 3.1, 1.6, 0.8, 0.4, 0.2, 0.1 µg/ml for metronidazole and 4000, 2000, 1000, 500, 250, 125, 62.5 µg/ml for the plant products.

Susceptibility Assay

The minimum lethal concentration (MLC) corresponds to the lowest concentration of the antitrichomonal agents that kill all trophozoites after exposure. Growth inhibition due to sub-MLC and lower concentrations of the agents was considered as percentage of growth inhibition (GI% = a–b/a ×100; a= mean numbers of viable trophozoites in negative control well, b= number of viable trophozoites in the test well at ≤sub-MLC concentration). Susceptibility testing was assessed according to the method recommended by the CDC (17). The experiments were performed in the 96-well microtiter plates. First, required serial dilutions of the agents were prepared and 100 µL of the prepared solutions was dispensed in the wells. Then, the parasites in logarithmic growth phase were counted with a hemocytometer (Neubauer cell chamber) and 100 µL of parasite-containing medium (2×10^5 trophozoites/mL) was added to each test wells. Finally, the number of *Trichomonas* was set to 2×10^4 cells/well. The plates were aerobically incubated at 35.5°C. After 24 and 48 hours of exposure, the test plates were examined with an inverted microscope to determine MLC. The lowest concentration of the essential oil and the extract in the test well where no motile parasites were observed was considered as MLC concentration. To evaluate GI% rate of the agents, parasites number in the test wells were counted and compared with the number of parasites in the well negative control according to mentioned equation. The experiments were repeated in pairs and two times separately under sterile conditions. In each runs, control wells (negative control and metronidazole control) were used to check the experimental condition. At the end, exposed parasites were cultured in fresh medium for MLC confirmation.

Statistical analysis

Analysis was done using SPSS statistical software, version 16. The results were shown as MLC and mean values. Friedman test was used to compare the averages. A P value less than 0.05 was considered statistically different.

Results

Susceptibility testing revealed that the extracts and essential oils of *F. pseudalliacea* had a lethal effect on the *Trichomonas* parasite. The antitrichomonal activity of *F. pseudalliacea* depended on concentration and time of exposure as shown in Table 1 and 2. At MLC concentrations, the tested agents were able to kill all trophozoites, which was confirmed by culturing treated trophozoites in

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fresh medium. Essential oils of *F. pseudalliacea* exhibited the highest anti-*Trichomonas* potential with a MLC of 250 µg/ml and followed by ethyl acetate, and n-hexane extract with MLC of 500 µg/ml), after 24 hours' incubation (P=0.002). After 48 hours, antitrichomonal activity of the hexanic increased to MLC of 250 (Table 2). At the sub-MLC concentrations, growth inhibition of the trichomonads was observed and the number of live trophozoites were reduced remarkably compared to the control (Table 1 and 2). GI% of the agents at the sub-MLC concentrations were between 35.2% to 87.0%, after 24 hours, and between 42.8% to 92.8%, after 48 hours. Drug susceptibility testing demonstrated that the *Trichomonas* isolates were susceptible to metronidazole with MLCs ranged from 6.2 to 12.5 µg/ml (Table3).
Preliminary phytochemical tests for extracts was studied by standard method. Result showed *F. pseudalliacea* extracts contain bioactive constituents include: coumarins, terpenoids, steroids, pheolics, tannins and glycosides. (Table 4). The major constituents were coumarins, terpenoids and steroids.

Discussion

In this research, antitrichomonal efficacy of *F. pseudalliacea* was evaluated in comparison with metronidazole, the initial choice of therapy for trichomoniasis. The results showed that *F. pseudalliacea* was potentially effective against *T. vaginalis* parasite. The essential oil and n-hexane extract of *F. pseudalliacea* were the most potent antitrichomonal agents. After 48 hours' exposure, the MLC of the two potent products was 250 µg/ml. At sub-MLC concentration, GI% of the oil and the n-hexane extract were 92.8% and 50.6%, respectively.
To our knowledge, antimicrobial activity of essential oil and crude extracts of *F. pseudalliacea* has not previously investigated but, *in vitro* antiplasmodial and antibacterial activity of coumarin derivatives from *F. pseudalliacea* was previously demonstrated. Anti-*Plasmodium falciparum* activity of sanandajin, methyl galbanate and kamolonol acetate was exhibited with IC_{50} of 2.6, 7.1 and 16.1 µM, respectively (9). Sanandajin and ethyl galbanate were effective on *Staphylococcus aureus* and *Helicobacter pylori* (MIC=64 µg/ml) and also, methyl galbanate was effective on vancomycin resistant strain of *Enterococcus faecium* (MIC=64 µg/ml) (12).
To date, many studies have been performed to investigate the antimicrobial effect of medicinal plants, some of which have been conducted on *Trichomonas* parasite. Twenty six Iranian medicinal plants showing antitrichomonal activity has been reviewed by Ziaei Hezarjaribi et al (18). In this review, *Artemisia aucheri*, *Zataria multiflora*, and *Lavandula angustifolia* were highlighted as the most potent medicinal plants. Other Iranian medicinal plants with potent antitrichomonal activity include *Foeniculum vulgare, Marrubium vulgar*, *Pistacia atlantica* subsp. *Kurdica*, *Plantago lanceolata L.*, and*, Ferula gummosa*.
Antitrichomonal activity of *F. vulgare* was investigated by Karami et al. In this study, methanolic and hexanic extract of the medicinal herb with MLC of 360 µg/ml were the most potent extracts. *F. vulgare* essential oil assay showed less activity (MLC=1600 µg/ml) than the extracts. Chemical analysis of the essential oil revealed that E-Anethole is the major component of *F. vulgare* essential oil (19). In a study conducted by Akbari et al., essential oil of *M. vulgare* had the highest activity on *T. vaginalis* (MLC=291 µg/ml) among the tested natural products. The lowest activity related to the n-hexane extract of *M. vulgare* (MLC=1500 µg/ml) (20). Bakhtiarnejad and colleagues evaluated the efficacies of *P. atlantica* subsp. *Kurdica* and *P. lanceolata L.* extracts against *T. vaginalis*. The ethyl acetate extract of the plants were considered as more potent antitrichomonal products with MLC of 337 and 1525 µg/ml, respectively (21,22). Anti-*Trichomonas* properties of various extracts of *F. gummosa* including ethyl acetate, n-hexane and methanol, and its essential

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oil were tested by Akbari et al. The results of the study showed that the extracts are more effective on *Trichomonas* (MLC=125 µg/ml) than the essential oil (MLC=500 µg/ml). α- and β-Pinene, and β-Eudesmol were reported as the main ingredients of *F. gummosa* essential oil (23). In another study, Mahmoudvand and colleagues examined the apoptotic activities and effects of three Iranian herbs against *Trichomonas*. They showed that *Quercus infectoria* (IC_{50}=3.4 µg/ml) was significantly more effective on the parasite than *Satureja khuzestanica* (IC_{50}=5.1 µg/ml) and *Pistacia khinjuk* (IC_{50}=26.6 µg/ml) (24).

In a review of the antitrichomonal properties of medicinal plants, most plants with high antitrichomonal potential belong to three families including Asteracea, Lamiaceae, and Myrtaceae. Also, terpenes, β-glycosides, saponins, essential oils and alkaloids are phytochemical compounds that have antitrichomonal activity (25). Berberine, an alkaloid of isoquinoline, has been used as a natural antibiotic in traditional medicine. Berberine alkaloid is one of the main components of *Argemone mexicana* methanolic extract. The IC_{50} antitrchomonal activity of the *A. mexicana* extract was 70.8 and 67.2 µg/mL for the stem and leaf, respectively (26). Other plants with high antitrichomonal properties include *Persea americana*, Verbascum thapsus, and Ocimum basilicum (27). The IC50 activity of *P. americana* seeds against *T. vaginalis* was 0.524 and 0.533 µg/ml for the chloroform and ethanolic extracts, respectively (28). Alcoholic extract of *V. thapsus* and *O. basilicum* essential oil were effective against *T.vaginalis* at 30 and 39.17 µg/ml, respectively, after 24 hours of exposure (29,30).

In the present study, the observed antitrichomonal properties of *F. pseudalliacea* may be due to the presence of bioactive components. The preliminary analysis of the extracts dmonstrated that coumarins, terpenoids and steroids were the major bioactive components in the extracts of *F. pseudalliacea*. The phytochemical screening analysis are helpful in the identification of bioactive compounds and provides the possibility of discovery and development of medicinal compounds. These researches provide the possibility of quantitative and qualitative estimation of active pharmaceutical compounds in crude extracts. One of the advantages of this study is that several isolates of *Trichomonas* have been used to obtain more reliable results. Also, the essential oil and extracts have been used simultaneously to better compare the effects of the compounds. But one of the main limitations of this study was the lack of access to metronidazole resistant *Trichomonas* isolates and investigating the effect of the compounds on them.

Conclusion

Despite restrictions on access to metronidazole-resistant isolates, *F. pseudalliacea* has potential anti-*Trichomonas* property. The findings support our perspective for the possibility of using the components of *F. pseudalliacea* for treatment of trichomoniasis. Therefore, further investigation on bioactive components of *F. pseudalliacea* could be useful.

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Ethical statement

This study was approved by the Ethical Committee of Hamadan University of Medical Sciences, Hamadan, Iran (UMSHA.REC.1394.84 IR).

Conflict of interests

The Authors declare that they have no conflict of interests to disclose.

Authorship contributions

DD, MF, AHM and MM designed the study. ZA, DD and MM conducted the experiment and data collection. MM and DD analyzed and interpreted the data and prepared a manuscript. Everyone read and approved the final manuscript for publication.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table1. Efficacy of essential oil and extracts of *F. pseudalliacea* on *T. vaginalis* after 24 hours' exposure

Agents	Mean and standard deviation of growth inhibition percent (GI%) at different concentrations of plant products						
	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)	1000 (µg/ml)	2000 (µg/ml)	4000 (µg/ml)
Essential oil	47.6±5.6	[b]59.6±7.3	[a]100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
N-hexane extract	0.0±0.0	35.0±4.5	[b]74.2±2.6	[a]100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Ethyl acetate extract	0.0±0.0	0.0±0.0	[b]35.2±2.4	[a]100.0[a]±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Methanol extract	0.0±0.0	0.0±0.0	0.0±0.0	34.2±3.7	[b]87.0±1.6	[a]100.0±0.0	100.0±0.0

[a]Minimum Lethal Concentration (MLC) is related to the lowest concentration of the antitrichomonal agents that kill all trichomonads

[b]Sub-MLC concentration is related to inhibition of trichomonads growth

Table2. Efficacy of essential oil and extracts of *F. pseudalliacea* on *T. vaginalis* after 48 hours' exposure

Agents	Mean and standard deviation of growth inhibition percent (GI%) at different concentrations of plant products						
	62.5 (µg/ml)	125 (µg/ml)	250 (µg/ml)	500 (µg/ml)	1000 (µg/ml)	2000 (µg/ml)	4000 (µg/ml)
Essential oil	62.4±7.7	[b]92.8±1.9	[a]100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
N-hexane extract	0.0±0.0	[b]50.6±2.1	[a]100.0[a]±0.0	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Ethyl acetate extract	0.0±0.0	0.0±0.0	[b]85.2±2.2	[a]100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
Methanol extract	0.0±0.0	0.0±0.0	0.0±0.0	[b]42.8±1.9	[a]100.0±0.0	100.0±0.0	100.0±0.0

[a]Minimum Lethal Concentration (MLC) is related to the lowest concentration of the antitrichomonal agents that kill all trichomonads

[b]Sub-MLC concentration is related to inhibition of trichomonads growth

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Table3. Efficacy of metronidazole on *T. vaginalis*

Incubation time	Mean and standard deviation of growth inhibition percent (GI%) at different concentrations of metronidazole									
	0.4 (µg/ml)	0.8 (µg/ml)	1.6 (µg/ml)	3.1 (µg/ml)	6.2 (µg/ml)	12.5 (µg/ml)	25 (µg/ml)	50 (µg/ml)	100 (µg/ml)	200 (µg/ml)
24 hours	51.8 ±2.4	59.0 ±5.6	71.8 ±2.6	83.6± 2.5	[b]95.4± 0.5	[a]100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0
48 hours	63.0 ±2.9	82.4 ±2.1	93.5 ±1.3	[b]98.6 ±0.5	[a]100.0 ±0.0	100.0± 0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0	100.0 ±0.0

[a]Minimum Lethal Concentration (MLC) is related to the lowest concentration of the antitrichomonal agents that kill all trichomonads

[b]Sub-MLC concentration is related to inhibition of trichomonads growth

Table 4. Preliminary phytochemical screening of *F. pseudalliacea* extracts

Compounds	**Test methods**		**Result**	
		n-hexane	**ethyl acetate**	**methanol**
Carbohydrates	Fehling's solutions	-	-	-
Glycosides	Keller-kilani	-	-	+
Pheolics	Ferric chloride	+	+	2+
Tannins	Ferric chloride	-	-	+
Alkaloids	Dragendorff's	-	-	-
Proteins and amino acids	Ninhydrin test	-	-	-
Coumarins	UV test	3+	3+	3+
Saponins	Foam test	-	-	-
Flavonoids	Alkaline reagent	-	-	-
Phlobatannins	Precipitate test	-	-	+
Terpenoids	-	3+	3+	2+
Steroids	Salkowski,s test	2+	2+	2+

[+] Presence; [-] Absence

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