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Preliminary phytochemical screening and 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 to investigate the activity of *Ferula pseudalliacea* (*F. pseudalliacea*) against *T. vaginalis* and to perform a 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 performed on five *T. vaginalis* isolates using the microtiter plate method. Minimum lethal concentration (MLC) and growth inhibitory percent (GI%) of sub-MLC concentrations were reported after 24- and 48-hour exposures. Phytochemical screening of the extracts was carried out using a standard procedure.

Results: The antitrichomonal effect of the plant products depended on time and concentration, with the greatest effect observed after 48 hours of exposure. The essential oil and n-hexane extract of *F. pseudalliacea* demonstrated remarkable activity with MLC of 250 µg/ml, followed 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 antitrichomonal activity of its bioactive constituents.

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Introduction

Trichomoniasis is a common cause of human vaginitis caused by the 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 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 cases. 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 the 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 the Apiaceae family because of its pharmaceutical and industrial importance. In the Iranian flora, the genus *Ferula* contains 32 species, including 15 endemic plants, and this genus is typically called koma or kema. Several species of *Ferula* have been used in folk remedies as treatment of stomachache, hysteria, arthritis, rheumatoid disorders, 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 relieving 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 was 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 using the shade-drying method at room temperature (20±5 °C), and the dried plant materials were crushed into powder. The powder (100 g) was used to prepare the essential oil using a Clevenger-type apparatus. The obtained essential oil was kept in an airtight container in a refrigerator (4 °C) until use. Extraction of the dried plant

was performed using the maceration method. Briefly, the powdered plant (100 g) was macerated separately in n-hexane, ethyl acetate, and methanol solvents (3 × 2 L, room temperature for 72 h, 25 °C). Extraction was performed using a rotary evaporator below 40 °C. The obtained extracts were kept in dark containers in a refrigerator (4 °C) until use. Phytochemical analysis of the extracts was done using standard methods, and their constituent compounds were identified according to 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, pure trophozoites in the log phase of growth were used for the 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 the culture medium was considered as the criterion for determining plant product concentrations, and susceptibility testing was started with the highest concentration. Solutions were prepared in 2-fold dilutions in culture medium for susceptibility assays 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 kills all trophozoites after exposure. Growth inhibition due to sub-MLC and lower concentrations of the agents was considered as the percentage of growth inhibition (GI% = a − b / a × 100; a = mean number of viable trophozoites in the 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 96-well microtiter plates. First, the required serial dilutions of the agents were prepared, and 100 µL of the prepared solutions was dispensed into the wells. Then, the parasites in the 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 well. Finally, the number of *Trichomonas* cells 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 the MLC. The lowest concentration of the essential oil and extracts in the test well where no motile parasites were observed was considered the MLC concentration.

To evaluate the GI% rate of the agents, the number of parasites in the test wells was counted and compared with the number of parasites in the negative control well according to the equation mentioned above. The experiments were repeated in pairs and twice separately under sterile conditions. In each run, control wells (Negative control and metronidazole control) were used to check the experimental conditions. At the end, exposed parasites were cultured in fresh medium for MLC confirmation.

Statistical analysis

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

Results

Susceptibility testing revealed that the extracts and essential oil 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 Tables 1 and 2. At MLC concentrations, the tested agents were able to kill all trophozoites, which was confirmed by culturing treated trophozoites in fresh medium. The essential oil of *F. pseudalliacea* exhibited the highest anti-*Trichomonas* potential with an MLC of 250 µg/ml, followed by the ethyl acetate and n-hexane extracts with MLC of 500 µg/ml, after 24 hours of incubation (P=0.002). After 48 hours, the antitrichomonal activity of the n-hexane extract increased to an MLC of 250 µg/ml (Table 2). At the sub-MLC concentrations, growth inhibition of the trichomonads was observed, and the number of live trophozoites was remarkably reduced compared to the control (Tables 1 and 2). GI% of the agents at the sub-MLC concentrations ranged from 35.2% to 87.0% after 24 hours, and from 42.8% to 92.8% after 48 hours. Drug susceptibility testing demonstrated that the *Trichomonas* isolates were susceptible to metronidazole, with MLCs ranging from 6.2 to 12.5 µg/ml (Table 3).

Preliminary phytochemical tests for extracts were studied by standard methods. The results showed *F. pseudalliacea* extracts contain bioactive constituents including coumarins, terpenoids, steroids, phenolics, tannins, and glycosides (Table 4). The major constituents were coumarins, terpenoids, and steroids.

Table 1. 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	59.6±7.3 [b]	100.0±0.0 [a]	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	74.2±2.6 [b]	100.0±0.0 [a]	100.0±0.0	100.0±0.0	100.0±0.0
Ethyl acetate extract	0.0±0.0	0.0±0.0	35.2±2.4 [b]	100.0 [a] ±0.0 [a]	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	87.0±1.6 [b]	100.0±0.0 [a]	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 2. 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	92.8±1.9 [b]	100.0±0.0 [a]	100.0±0.0	100.0±0.0	100.0±0.0	100.0±0.0
N-hexane extract	0.0±0.0	50.6±2.1 [b]	100.0[a]±0.0 [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	85.2±2.2 [b]	100.0±0.0 [a]	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	42.8±1.9 [b]	100.0±0.0 [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

Table 3. 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	95.4±0.5 [b]	100.0±0.0 [a]	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	98.6±0.5 [b]	100.0±0.0 [a]	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

Discussion

In this research, the antitrichomonal efficacy of *F. pseudalliacea* was evaluated in comparison with metronidazole, the first-line treatment for trichomoniasis. The results showed that *F. pseudalliacea* was potentially effective against *T. vaginalis*. The essential oil and n-hexane extract of *F. pseudalliacea* were the most potent antitrichomonal agents. After 48 hours of exposure, the MLC of these two potent products was 250 µg/ml. At sub-MLC concentrations, the GI% of the oil and the n-hexane extract was 92.8% and 50.6%, respectively.

To our knowledge, the antimicrobial activity of essential oil and crude extracts of *F. pseudalliacea* has not been previously investigated. However, the in vitro antiplasmodial and antibacterial activities of coumarin derivatives from *F. pseudalliacea* have been demonstrated. Anti-*Plasmodium falciparum* activity of sanandajin, methyl galbanate, and kamolonol acetate was reported with IC50 values of 2.6, 7.1, and 16.1 µM, respectively (9). Sanandajin and ethyl galbanate were effective against *Staphylococcus aureus* and *Helicobacter pylori* (MIC=64 µg/ml), and methyl galbanate was effective against a vancomycin-resistant strain of *Enterococcus faecium* (MIC=64 µg/ml) (12).

To date, many studies have explored the antimicrobial effects of medicinal plants, some specifically targeting the *Trichomonas* parasite. Twenty-six Iranian medicinal plants with antitrichomonal activity were 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 strong antitrichomonal activity include *Foeniculum vulgare*, *Marrubium vulgare*, *Pistacia atlantica* subsp. *kurdica, Plantago lanceolata* L., and *Ferula* gummosa.

The antitrichomonal activity of *F. vulgare* was investigated by Karami et al. In their study, the methanolic and hexanic extracts of the medicinal herb showed the greatest potency with an MLC of 360 µg/ml. The essential oil demonstrated lower activity (MLC=1600 µg/ml). Chemical analysis of the essential oil revealed that E-anethole was the major component (19). In another study by Akbari et al., the essential oil of *M. vulgare* displayed the highest activity against *T. vaginalis* (MLC=291 µg/ml), while the n-hexane extract showed the lowest activity (MLC=1500 µg/ml) (20).

Matini and colleagues evaluated the efficacy of *P. atlantica* subsp. *kurdica* and *P. lanceolata L.* extracts against *T. vaginalis*. The ethyl acetate extract of these plants was considered the most potent antitrichomonal product, with MLC values of 337 and 1525 µg/ml, respectively (21,22). Anti-*Trichomonas* properties of various extracts of *F. gummosa*-including ethyl acetate, n-hexane, methanol, and its essential oil-were examined by Akbari et al. Their results showed that the extracts were more effective (MLC=125 µg/ml) than the essential oil (MLC=500 µg/ml). α- and β-Pinene, and β-eudesmol were identified as the major components of *F. gummosa* essential oil (23).

In another study, Mahmoudvand and colleagues examined the apoptotic effects of three Iranian herbs on *T. vaginalis*. They found that *Quercus infectoria* (IC50=3.4 µg/ml) was significantly more effective against the parasite than *Satureja khuzestanica* (IC50=5.1 µg/ml) and *Pistacia khinjuk* (IC50=26.6 µg/ml) (24).

Across studies reviewing the antitrichomonal potential of medicinal plants, the most active species typically belong to three families: Asteraceae, Lamiaceae, and Myrtaceae. Furthermore, terpenes, β-glycosides, saponins, essential oils, and alkaloids are among the major phytochemical compounds responsible for antitrichomonal activity (25). Berberine, an isoquinoline alkaloid used traditionally as a natural antibiotic, is one of the main components of Argemone mexicana methanolic extract. The IC50 antitrichomonal activity of *A. mexicana* extract was 70.8 and 67.2 µg/ml for the stem and leaf, respectively (26). Other plants with strong antitrichomonal effects include *Persea americana*, *Verbascum thapsus*, and *Ocimum basilicum* (27). The IC50 values of *P. americana* seed extracts against *T. vaginalis* were 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 attributed to the presence of bioactive components. Preliminary analysis demonstrated that coumarins, terpenoids, and steroids were the major bioactive constituents in the extracts. Phytochemical screening analyses are helpful for identifying bioactive compounds and supporting the discovery and development of medicinal agents. These studies also enable quantitative and qualitative evaluation of active pharmaceutical compounds in crude extracts.

One of the advantages of this study was the use of several *Trichomonas* isolates, which increases the reliability of the results. Additionally, essential oil and extracts were examined simultaneously, allowing a more accurate comparison of their effects. However, one of the main limitations of this study was the lack of access to metronidazole-resistant *Trichomonas* isolates, preventing evaluation of the compounds' effectiveness on resistant strains.

Conclusion

Despite limitations related to access to metronidazole-resistant isolates, *F. pseudalliacea* demonstrated potential anti-*Trichomonas* activity. The findings support the possibility of using components of *F. pseudalliacea* in the treatment of trichomoniasis. Therefore, further investigation into the bioactive components of *F. pseudalliacea* is recommended.

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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).

Conflicts of interest

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

Author 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 the manuscript. All authors 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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