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In vitro anti-parasitic activities of *Pulicaria dysenterica* and *Lycopus europaeus* methanolic extracts against *Trichomonas gallinae*

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ABSTRACT

Introduction: *Trichomonas gallinae* is the causative agent of trichomoniasis in birds. Although metronidazole is now the drug of choice for treatment of this infection, several studies reported metronidazole-resistant strains of *T. gallinae*. So it is important to explore for effective alternative compounds such as herbal extracts for treatment of avian trichomoniasis. This study was carried out to investigate the effects of methanolic extracts of *Pulicaria dysenteric* and *Lycopus europaeus* on the growth of *T. gallinae* trophozoites.

Methods: The methanolic extracts were obtained from aerial parts of plants. The anti-trichomonas activities of *P. dysenterica* at the concentrations of 200, 100, 50, 25, 12.5 and 6.25 mg/mL and *L. europaeus* at the concentrations of 227, 113.5, 56.75, 28.37, 14.1 and 7.09 mg/mL after 0, 1, 3, and 6 hours exposure time were evaluated.

Results: The results showed that both extracts decreased the viability of *T. gallinae*. The methanolic extract of *P. dysenteric* and *L. europaeus* showed 10% and 60% growth inhibition (GI %) at the highest concentration immediately after exposure. *P. dysenteric* methanolic extract at a concentration of 6.25 mg/mL completely inhibited the growth of parasite after 6 hours which was the minimum inhibitory concentration, while the lowest concentration of *L. europaeus* extract that showed 100% GI was 28.37 mg/mL that affected trophozoites after 6 hours.

Conclusion: Based on the results, both extracts revealed significant growth inhibitory effect on *T. gallinae*, suggesting the potential use of these plants in preparation of new anti-trichomonas compounds.

Implication for health policy/practice/research/medical education:
Methanolic extract of *Pulicaria dysenterica* and *Lycopus europaeus* showed remarkable growth inhibitory effects on *Trichomonas gallinae* trophozoites. Therefore, these extracts might be used as anti-trichomonas agents to treat avian trichomoniasis in future.

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Introduction

Avian trichomoniasis is a parasitic disease caused by the protozoan parasite *Trichomonas gallinae*. Parasites live mainly in the upper area of the digestive tract anterior to the gizzard (1). Depending on the strain virulence, they can live in the tissue of the head, thorax, or abdomen of various groups of birds, counting Columbiformes, Passeriformes, Psittaciformes and Falconiformes (2-4). Among these groups, the family Columbidae is known as the parasite's main host, particularly the domestic pigeon (*Columba livia domestica*) has been considered responsible for the worldwide spread of *T. gallinae* (1).

For more than 40 years, the 5-nitroimidazole drugs family, specifically metronidazole and tinidazole, have been used to treat the infections caused by certain gram-negative bacilli and anaerobic parasitic protozoa, especially trichomoniasis (5,6). Currently, metronidazole is the only drug approved for the treatment of *T. gallinae*. However,

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the existence of resistant strains of *T. gallinae* to these medications has been observed since the 1990s (6-8). The first therapeutic failures have been described for treatment of avian trichomoniasis. Besides, some metronidazole-resistant strains have been found frequently (5-7,9). Therefore, alternative curative therapies are necessary to provide a substitute treatment for avian trichomoniasis. Recently, the applications of many plants extract instead of chemicals have been recommended as remedies against parasites (10-14). Substances of herbal origin may have anti-trichomonas activities and offer new hope in the treatment of trichomoniasis. Two of the promising anti-protozoa herbal sources are *Pulicaria dysenterica* and *Lycopus europaeus*.

The genus *Pulicaria*, belonging to Asteraceae (Compositae) family, includes 100 species with a distribution from Europe to North Africa and Asia, particularly around the Mediterranean regions (15). Only five species of the genus grow in Iran, which includes *P. dysenterica, P. arabica, P. salvifolia, P. vulgaris,* and *P. gnaphalodes* (16,17).

The chemical literature survey shows the presence of flavonoids, sesquiterpenoids, polyacetylenes, polyacetylenes, sesquiterpenoid lactones, diterpenoids, thymol derivatives, and caryophyllene derivatives in the members of the genus *Pulicaria* (18-20).

Pulicaria dysenterica (*Inula dysenterica*), commonly called "Flea-bane" and "Meadow false fleabane", is a persistent plant with 10–30 cm high, with gold-yellow flowers. It is hermaphrodite and grows on sandy, stony places in Saudi Arabia, Iran, Afghanistan, and western Tibet (16). New compounds including methoxy-12-acetoxycaryophylla, methoxycaryophylla-2, dimethoxy-12-acetoxycaryophyll-2, hydroxycaryophyll-2, and dihydroxycaryophyll-2 have been isolated from this plant (18,19). In Iran, the original source of this plant is Razavi Khorasan province. *P. dysenterica* commonly is used as a medicinal plant to treat severe heatstroke, diarrhoea, inflammatory, and leishmaniosis diseases (14). Moreover, it has several biological activities such as antioxidant and insecticide properties (21,22).

Lycopus europaeus L. (Labiatae) also known as "gypsywort", is a genus of flowering plants of the Lamiaceae family, which is distributed in Europe, Asia, and the United States. Although active components of *L. europaeus* are not identified completely, some phytochemical studies on *Lycopus* spp. show that it contains phenolic compounds, cinnamic acid, rosmarinic acid, caffeic acid, gallic acid, luteolin-7-O-glucuronide, flavonoids, tannins, saponins, lithospermic acid, coumarins, saponins, alkaloids, sterols, caryophyllene, α-pinene, terpinene and terpenoids. Some studies on the extract of *L. europaeus* proved its analgesic and anti-inflammatory activities. *L. europaeus* is a perennial plant, which is conventionally suggested for treating mild hyperthyroidism. The anti-thyrotropic, anti-gonadotropic, antioxidant, antimicrobial and cardiotonic effects of this plant are attributed to phenolic compounds, mainly to derivatives of hydroxycinnamic and flavonoids (23-26).

This in vitro research was aimed to study the anti-trichomonal activities of methanolic extracts of *P. dysenterica* and *L. europaeus* in the treatment of *T. gallinae* and compare the efficacy of these natural products to metronidazole as a standard anti-trichomonal drug. According to available information, this study is the first research demonstrating the potential anti-trichomonal activities of these plants.

Materials and Methods

In vitro cultivation of parasite

Trichomonas gallinae trophozoites were taken by sterile swabs from the oral cavity, oesophagus and the crop of diseased domestic pigeon (*C. livia domestica*) with clinical features of trichomonosis and the oral cavity inoculated on Hollander's modification of trypticase yeast extract maltose (TYM) complete medium (pH 6.5). The TYM medium contained 10 g of trypticase peptone, 5 g of yeast extract, 2.5 g of maltose, 0.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.5 g of potassium chloride (KCL), 0.5 g of L-ascorbic acid, 0.5 g of potassium bicarbonate ($KHCO_3$), 0.05 g of ferrous sulfate ($FeSO_4$), and 0.02 g of agar (Merck, Germany) per 300 mL of distilled water. After sterilization of the medium for 15 minutes at 121°C, 30 mL foetal calf serum (Hyclone, USA) and antibiotic/antimycotic solution containing 0.15 µg/mL streptomycin, 0.15 µg/mL penicillin, and 0.15 µg/mL amphotericin B, (Sigma–Aldrich, Vienna, Austria) were added to the medium (27,28). Following incubation at 38°C, motile trichomonads were monitored daily by light microscopy. Isolates were sub-cultured every 48 hours by transferring 500 µL of cultured medium into a new sterile 15 mL Falcon, containing 5 mL of fresh medium. *T. gallinae* cells were counted using a haemocytometer (Neubauer Improved, bright line; Germany) and adjusted to 2×10^7/mL in the working suspension.

Preparation of methanolic extract

Aerial parts of *P. dysenterica* and *L. europaeus* were collected from rural areas of Mazandaran province. The plants were identified by experienced botanists at the faculty of medicinal plants, Amol University of special modern technologies.

The collected plants were washed thoroughly with distilled water and crushed into small pieces to facilitate drying. The pieces of plants were dried at room temperature under shade for three weeks. Finally, the dried plants were powdered using an electric blender and a fine powder was obtained.

Plant extracts were obtained by the following procedures: Three grams of each plant was suspended in 80 ml of 80% methanol (v/v). Suspensions were homogenized by vortex for 5 minutes, and the mixtures were placed in

an ultrasonic bath (Elmasonic S40H, 340 W, 37 kHz) at 30ºC and sonicated for 1 hour. Finally, the extracts were dried using a vacuum rotary evaporator in a water bath at 40°C and dried samples were weighed and transferred into the microtubes and stored at 4°C until the time of the experiments (29,30).

In vitro anti-trichomonas assay

To explore anti-trichomonas effects of *P. dysenterica* (200, 100, 50, 25, 12.5 and 6.25 mg/mL) and *L. europaeus* (227, 113.5, 56.75, 28.37. 14.1 and 7.09 mg/mL) different extract concentrations were diluted in phosphate buffer saline (PBS) and added to the sterile Eppendorf tubes. Approximately 1×10^7 trophozoites/mL *T. gallinae* was added to prepare concentrations of each extract. Metronidazole (100 µg/mL) was utilized as a positive control and PBS was utilized as a negative control. The growth of *T. gallinae* was observed 0, 1, 3, and 6 hours after treatment at 38°C. Each concentration was replicated three times.

Data analysis

In every sample, in each time live cells of *T. gallinae* were counted using hemocytometer slide. The active parasites and those with moving flagellum were considered as live cells. Percentage of growth inhibition (GI %) was calculated and reported using the Eq. 1 where a and b are a; mean numbers of viable parasites in control tube and mean b number of viable parasites in a test tube, respectively (31).

Eq. 1: GI% = a–b/a ×100.

Statistical analysis

The study was conducted as factorial based on a randomized design with three replications. Analysis of variance (ANOVA) procedure followed by Duncan's test using SPSS 16 (SPSS Inc., USA) software was applied to determine the significant differences between treatment means. *P* values of ≤0.01 were considered significant.

Results

The results showed that both methanolic extracts of *P. dysenterica* and *L. europaeus* were able to reduce the viability of *T. gallinae* trophozoites by causing cell death at each of the six concentrations. The alive trophozoites were decreased remarkably with enhancing the concentrations of extracts and exposure time.

In general, there was a little difference between the effectiveness of the higher concentrations of methanolic extract of *P. dysenterica* (200, 100 and 50 mg/mL) and *L. europaeus* (227, 113.5 and 56.57 mg/mL) with these concentrations at 1 hour after exposure (Figures 1 and 2, respectively).

In the highest concentrations of *P. dysenterica* (200 mg/mL) immediately after adding trophozoites to the dilutions, the motile of trophozoites were decreased and growth inhibitory effect was 10%. Exposure to the methanolic extract of *P. dysenterica* at 200, 100, and 50 mg/mL concentrations resulted in 100% cell dead within 1 hour after treatment (Figure 1). Also, 100% GI was detected with concentrations of 25, 12.5, and 6.25 mg/mL after 3 and 6 hours after exposure to the methanolic extract of *P. dysenterica* (Table 1).

Moreover, in a dose-dependent and time-dependent manner, the *L. europaeus* methanolic extract at all different concentrations had significant anti-trichomonal effects ($P \leq 0.01$) even immediately after exposure to trophozoites of *T. gallinae* (Table 2). According to Table 2, *L. europaeus* treated culture showed 60% inhibition of the growth of parasite with 227 mg/mL concentration in incubation at 0 hours. The growth inhibitory effect of *L. europaeus* methanolic extract at 227, 113.5, and 56.57 mg/mL concentrations was 100% at 1 hour after treatment. The *L. europaeus* methanolic extract in lower concentrations

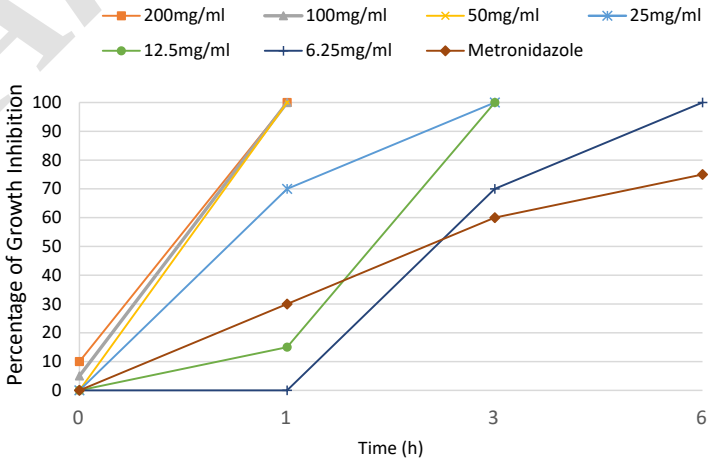

Figure 1. Effect of methanolic extract of *Pulicaria dysenterica* on *Trichomonas gallinae* trophozoites in TYM medium following 0, 1, 3 and 6 hours after treatment.

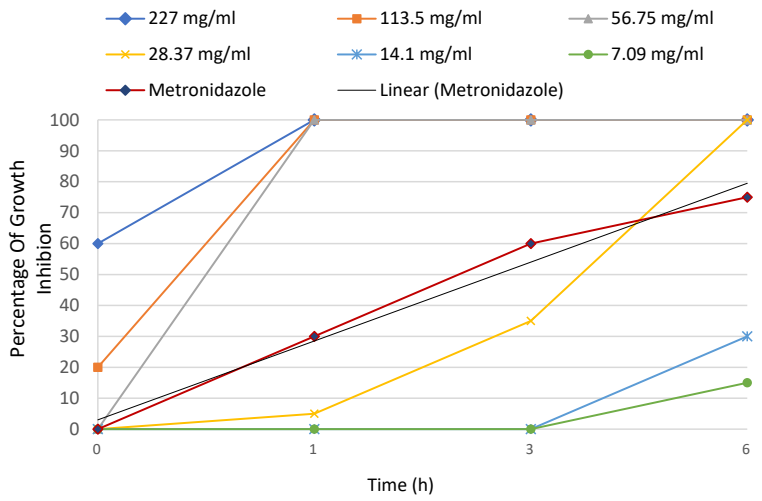

Figure 2. Effect of methanolic extract of *Lycopus europaeus* on *Trichomonas gallinae* trophozoites in TYM medium following 0, 1, 3 and 6 hours after treatment.

Table 1. Effect of *Pulicaria dysenterica* methanolic extract on the growth of *Trichomonas gallinae* trophozoites at different incubation periods

Concentration (mg/mL)		0	After 1 h	After 3 h	After 6 h
200	Mean ± SD	$10.1^{g} \pm 2.01^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	10.00	100	100	100
100	Mean ± SD	$5.02^{h} \pm 0.30^{++}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	5.00	100	100	100
50	Mean ± SD	$1.66^{i} \pm 0.30$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	100	100	100
25	Mean ± SD	$1.66^{i} \pm 0.41$	$70.3^{c} \pm 5.01^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	70.0	100	100
12.5	Mean ± SD	$1.66^{i} \pm 0.29$	$16.66^{f} \pm 2.50^{++}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	15.00	100	100
6.25	Mean ± SD	$1.66^{i} \pm 0.30$	$1.66^{i} \pm 0.40$	$70.3^{c} \pm 3.40^{++}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	0.00	70.0	100
Parasite Control	Mean ± SD	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$
	GI %	0.00	0.00	0.00	0.00
MTZ (100 µg/mL)	Mean ± SD	$0.00^{j} \pm 0.00$	$30.33^{e} \pm 2.51^{++}$	$59.01^{d} \pm 3.60^{++}$	$76.33^{b} \pm 3.21^{++}$
	GI %	0.00	30.00	60.00	75.00

SD, standard deviation.
++ $P<0.01$, statistically significant difference in comparison to parasite control in the same time interval.
** $P<0.01$, statistically significant difference in comparison to metronidazole 100 µg/mL in the same time interval.
Values having no common superscript are significantly different ($P < 0.01$).

showed different results. With a concentration of 28.37 mg/mL, 1.66, 5.01 and 35.01% prevention of the parasitic growth was found after 0, 1 and, 3 h, respectively and complete inhibition of growth (100%) 6 hours after exposure. Besides, with both concentrations of 14.1 and 7.09 mg/mL, 1.66% inhibition of the parasitic growth was observed after 0, 1, and 3 hours of treatment. At the concentration of 14.1 mg/mL, deterrence of growth was 30% after 6 hours. Moreover, at the lowest concentration (7.09 mg/mL), 15% inhibition of growth was found after 6 hours (Table 2).

No remarkable reductions in the number of trophozoites were observed in the control samples. Each concentration showed the statistically significant difference ($P \leq 0.01$) compared to parasite control and metronidazole groups.

Discussion

Trichomonas gallinae causes severe tissue lesions in the crop and oesophagus of birds. The only drugs recommended for treating trichomoniasis are metronidazole and a related 5-nitroimidazole, tinidazole, which have been utilized over 40 years. Many studies on clinical resistance

Table 2. Effect of methanolic extract of *Lycopus europaeus* on the in vitro growth of *Trichomonas gallinae* trophozoites after different exposure time

Concentration (mg/mL)		0	After 1 h	After 3 h	After 6 h
227	Mean ± SD	$60^{c} \pm 4.01^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	60.00	100	100	100
113.5	Mean ± SD	$20.01^{f} \pm 2.01^{++**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	20.0	100	100	100
56.75	Mean ± SD	$1.66^{i} \pm 0.30$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	100	100	100
28.37	Mean ± SD	$1.66^{i} \pm 0.30$	$5.01^{h} \pm 1.01^{++}$	$35.01^{d} \pm 3.01^{++}$	$100^{a} \pm 0.00^{++,**}$
	GI %	0.00	5.01	35.00	100
14.1	Mean ± SD	$1.66^{i} \pm .041$	$1.66^{i} \pm 0.40$	$1.66^{i} \pm 0.40$	$30.1^{e} \pm 2.01^{++}$
	GI %	0.00	0.00	0.00	30.00
7.09	Mean ± SD	$1.66^{i} \pm 0.39$	$1.66^{i} \pm 0.31$	$1.66^{i} \pm 0.30$	$15.01^{g} \pm 2.01^{++}$
	GI %	0.00	0.00	0.00	15.00
Parasite control	Mean ± SD	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$	$0.00^{j} \pm 0.00$
	GI %	0.00	0.00	0.00	0.00
MTZ (100 µg/mL)	Mean ± SD	$0.00^{j} \pm 0.00$	$30.33^{e} \pm 2.51^{++}$	$59.01^{d} \pm 3.60^{++}$	$76.33^{b} \pm 3.21^{++}$
	GI %	0.00	30.00	60.00	75.00

SD, standard deviation.
++ P<0.01, statistically significant difference in comparison to parasite control in the same time interval.
** P<0.01, statistically significant difference in comparison to metronidazole 100 µg/mL in the same time interval.
Values having no common superscript are significantly different (P < 0.01).

of these drugs have been well documented; for example, nitroimidazole resistant of isolates of *T. gallinae* was found in Belgium, Spain, and the United States (32,33). Nowadays, nitroimidazole resistant strains of *T. gallinae* are prevalent and only a few research have been published on substitutes of anti-trichomonal resources efficient against *T. gallinae*.

The application of medicinal plants and plant-based drugs has been increased to treat trichomoniasis, especially *Trichomonas vaginalis*. The effective native medicinal plants of Iran on trichomoniasis include *Zataria multiflora, Artemisia absinthium, Taxus baccata, Lavandula intermedia, Achillea millefolium, Pelargonium roseum, Juglans regia, Eucalyptus camaldulensis* Dehnh, *Stachys lavandulifolia, Artemisia aucheri, Myrtus communis, Freula assafoetida, Tanacetum parthenium, Mentha piperita, Allium sativum,* and *Salvia officinalis* (13).

Pulicaria is used as a medicinal herb, which has been traditionally utilized by humans as an anti-inflammatory remedy to treat severe heatstroke and diarrhoea. Another medicinal plant is *Lycopus* spp. which has antibacterial and antioxidant effects.

In this study for the first time, the effects of different concentrations of methanolic extracts of *P. dysenterica* and *L. europaeus* on the axenic culture of *T. gallinae* trophozoites comparing with metronidazole and negative control were evaluated.

The outcomes revealed that the minimal inhibitory concentrations at 1 hour interval of *P. dysenterica* were 200, 100 and 50 mg/mL, and for *L. europaeus* were 227, 113.5 and 56.75 mg/mL. The results showed a statistically significant difference compared to metronidazole 100 µg/mL after 1 hour incubation. The present study demonstrated that even low concentrations of methanolic extract of *P. dysenterica* and *L. europaeus* could eliminate *T. gallinae* in medium culture. Data obtained from high concentrations of *P. dysenterica* (200, 100 and 50 mg/mL) and *L. europaeus* (227, 113.5 and 56.75 mg/mL) exhibited strong and rapid anti-trichomonal activity. Also, a 10 % inhibition of growth with 200 mg/mL concentration of *P. dysenterica* and 60% GI with a 227 mg/mL concentration of *L. europaeus* was observed instantly following therapy (0 hours). In lower concentrations of methanolic extract of *P. dysenterica* (25, 12.5 and 6.25 mg/mL) and *L. europaeus* (28.37, 14.1 mg/mL) longer incubation period (3 and 6 hours) was required to obtain complete loss of viability and motility, while the 7.09 mg/mL concentration of *L. europaeus* needed 6 hours to completely inhibit the trophozoites growth. The effect of *P. dysenter*ica and *L. europaeus* on the inhibition growth of *T. gallinae* showed a statistically significant difference compared to parasite control and metronidazole. The results of all extracts revealed that the inhibition of trophozoites growth and trophozoites motility depended on the concentration and incubation time.

These findings are in agreement with the results of Youssefi et al who reported similar inhibitory effect using *Artemisia sieberi*, and findings of Seddiek et al using garlic against *T. gallinae* trophozoites in vitro and in vivo assays (12,34).

Phytochemical studies of *Pulicaria* spp. and *L. europaeus* indicated that there were various bioactive components in these plants, which might be responsible for their biological activities. Asghari et al and Zahabi et al reported that *P. gnaphalodes* extract had a good leishmanicidal effects and it seems that the leishmanicidal activity is mostly associated with the existence of components such as alpha-pinene, borneol, 1,8-cineole, p-cymene, geraniol, and thymol in the essential oils, which have already exhibited leishmanicidal activity (14,20).

Several studies presented an outline of chemical constituents of different *Lycopus* species. Peng et al isolated various compounds from *L. lucidus* Turcz and *L. europaeus*, including terpenoids, flavonoids, phenolic acids, and steroids (35). Fialova et al demonstrated the anti-staphylococcal activity of *L. europaeus* leaves water extract on clinical Staphylococcus aureus strains. Data obtained in their study showed that rosmarinic acid and luteolin-7-O-glucuronide were considered involved in biological activities (26).

According to other studies, biologically active compounds from plant material are mostly affected by dose and incubation time. Under the empirical conditions, increasing the concentrations of extracts from lowest to the highest dose increased the mortality rate of trophozoites of *T. gallinae*. On the other hands, inhibition of trophozoites growth and trophozoites motility in lower concentrations increased with a constant incubation time. Consequently, the higher the concentration of the extract and the longer the application time, the more significantly lethal effects of the extract.

In conclusion, the present study is the first in vitro investigation evaluating the efficacy of the methanolic extract of *P. dysenterica* and *L. europaeus* against *T. gallinae* trophozoites. Results support our perspective for the possibility of using the methanolic extract of *P. dysenterica* and *L. europaeus* as anti-trichomonas agents at several concentrations and can suggest the potential use of these plants for treating metronidazole-resistant isolates of *T. gallinae*. However, more comprehensive studies are needed to survey antitrichomonal activities of methanolic extracts of *P. dysenterica* and *L. europaeus* in vitro and in vivo conditions.

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Conflict of interests

There is no conflict of interest in this study.

Ethical considerations

This study protocol was approved by the ethics committee of Amol University of Special Modern Technologies (Ethical code: ir.ausmt.rec.961110). The ethical issues have been observed by the authors.

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