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Original Article

The Effect of Fig Leaves on *Trichomonas vaginalis* and Macrophages *In-vitro*

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ABSTRACT

Trichomonas vaginalis, an anaerobic flagellated protozoan, can infect the cervix, vagina, pelvis, urethra, and epididymis. The development of microbial resistance and the occurrence of side effects with synthetic anti-trichomonas drugs, such as metronidazole, requires the need to expand research on the use of natural medicines in the control of this infection. The present study was conducted to investigate the effect of hydroalcoholic and aqueous extracts of fig leaves on the viability of *Trichomonas vaginalis* in vitro. Hydroalcoholic and aqueous extracts of fig leaves were obtained by percolation and perfusion, respectively. The effect of the hydroalcoholic extract (2000, 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 µg/ml) and the aqueous extract (3000, 1500, 750, 375, 187.5, 93.7, 46.8, 23.4, 11.7, 5.8 µg/ml) were evaluated on *T. vaginalis* cells in-vitro. Otherwise, the effect of the hydroalcoholic and aqueous extracts (3200, 1600, 800, 400, 200, 100, and 50 µg/ml) was studied on J774 macrophage cells. Using the hemocytometry method, the cell growth inhibition in different concentrations was calculated after three times incubation (24, 48, and 72 hrs). Fig leaves' hydroalcoholic and aqueous extracts could inhibit the growth of *T. vaginalis* and macrophage cells in-vitro. The cell growth inhibition in different concentrations and incubation times were significantly different. The anti-trichomonas activity of fig leaves' extracts allows their use in treating trichomoniasis.

Running title: Fig on Trichomonas vaginalis and Macrophages

INTRODUCTION

Trichomonas vaginalis, the protozoan causing trichomoniasis, causes disorders such as premature birth, low birth weight babies, miscarriage, ectopic pregnancy, endometritis, salpingitis, cervical cancer, as well as increasing the risk of contracting the human immunodeficiency virus (HIV), prostatitis, urethritis, vaginal secretions, urination, redness, itching, burning, or abdominal and pelvic pains [1-4]. According to the announcement of the World Health Organization (WHO), the number of people suffering from this disease is estimated to be around 250 million people in the world [5]. The prevalence of this disease in women is estimated at 5-74% and in men at 5-29%. Women in the age range from 16 to 53 years are more at risk of this infection [6]. The most common medications used in the treatment of this disease are the nitroimidazole family, including metronidazole and tinidazole. These drugs have side effects such as carcinogenicity, teratogenicity on the fetus, resistance to *T. vaginalis*, nausea, diarrhea, headache, skin itching, and leukopenia [7]. Due to the importance of the disease and the problems caused by it, the side effects of the drugs, and the drug resistance that has developed to metronidazole, the traditional use of medicinal plants has increased day by day due to the belief that their side effects are little and they are cheap [8, 9]. *Ficus carica* is one of the medicinal plants whose various parts are widely used in the control and treatment of diseases. This plant

has antioxidant, antibacterial, anti-parasitic, and anti-fungal effects due to its polyphenols such as gallic acid, chlorogenic acid, catechin, epicatechin, anthocyanin, flavonoid, and carotenoid [10-12]. Therefore, the present study was conducted to investigate the efficacy of the hydroalcoholic and the aqueous extracts of fig leaves on the viability of *T. vaginalis in vitro*.

MATERIALS AND METHODS

Plant Preparation

The leaves of the fig tree with the scientific name *F. carica* were purchased from an authentic medicinal plant center (Yazd, Iran) and the originality of the plant was confirmed by the Department of Pharmacognosy, School of Pharmacy, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Preparation of the Fig Leaf Hydroalcoholic Extract

The hydroalcoholic extract of fig leaf was extracted by percolation method. First, the fig leaves were placed in a dark and humid room for a week, and after they were dried, they were powdered using an electric mill, then 200 grams of dried fig leaf powder was mixed with ethanol (80%) in a beaker. The mixture was poured into the decanter funnel and the valve of the funnel was opened in such a way that 20 drops came out of it every minute. After the complete extraction of the solvent, the resulting solution was returned to itself again. After one week, the resulting solution was put under the hood.. After four weeks, the extracts were mixed and put under the hood to dry. Finally, the weight of the dry fig leaf extract was measured, which in this study was 25 grams.

Preparation of the Fig leaf Aqueous Extract

The fig leaf aqueous extract was extracted by perfusion method. Fifty grams of the dried fig leaf powder was mixed with 20 times distilled water (about 1000 ml) and placed on a magnetic hot plate device at a temperature of 80 °C for 20 minutes. Finally, the mixture was filtered with filter paper and kept under the hood to dry. The weight of the resulting dry extract was 7 grams.

Preparation of the Serial Concentrations of Fig Leaf Extracts

A concentration series from the hydroalcoholic extract (2000-3.9 µg/ml) of fig leaves, and a concentration series from the aqueous extract (3000-5.8 µg/ml) were prepared. To prepare the hydroalcoholic and aqueous extracts, 80% ethanol and 20% ethanol were used as solvents, respectively. To dissolve the aqueous extract, 490 mg of the aqueous extract were dissolved in 62.5 ml of 20% ethanol and was sonicated for 20 minutes; The concentration of this extract was 7.84 mg/ml. The first tube should have a concentration of 3000 µg/ml in a volume of 200 µl, so 10 tubes containing 100 µl of phosphate buffered saline (PBS) were prepared. In the first tube, 100 µl of a solution containing 76 µl of PBS and 24 µlof culture medium containing *T.* with an initial concentration of 7.84 mg/ml were added until its volume reached 200 µl. Then 100 µl of the solution from the first tube was added to the second tube until the dilution of the second tube was half of the first tube. This process was repeated up to the tenth tube until the concentration of each tube is halved compared to the previous tube (including dilutions of 3000, 1500, 750, 375, 187.5, 93.7, 46.8, 23.4, 11.7, and 5.8 mg/ml, respectively). Dilution of the hydroalcoholic extract was also prepared with the same process (including 2000, 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 mg/ml, respectively).

To investigate the cytotoxicity effect of the extracts on human cells, the hydroalcoholic and the aqueous extracts of fig leaves were prepared on J774 macrophage cells with a concentration series of 3200, 1600, 800, 400, 200, 100, 50 mg/ml.

Standardization

The Catechin method was used to measure flavonoid compounds [13]. Three concentrations of hydroalcoholic extract were prepared in distilled water. Sodium nitrite (300 _µL, 5%, Merck, Germany) was added into the blank and test tubes. After 5 minutes, aluminum chloride (300_µL , 10%, Merck, Germany) followed by LNaOH (2ml, 1M) was added and distilled to 100 mL. The optical absorbance of the solution was read at 510 nm by a spectrophotometer.

Cultivation of *T. vaginalis* Parasite and Macrophage

T. vaginalis strain was isolated from the vaginal secretions of women suffering from trichomoniasis at Isfahan University of Medical Sciences, Iran, and stored at -70 °C. Its authenticity was confirmed by the microbiology department of Shahid Sadoughi University of Medical Sciences, Yazd, Iran. To cultivate *Trichomonas vaginalis*, a TYIS-33 culture

medium was used at 37 ºC, Ph = 5-6, and for 24-48-72 hours.

Macrophages of the J774 cell line were obtained from Pasteur Institute Cell Bank, Tehran, Iran. They were kept diagonally in an incubator with a temperature of 37 ºC.

Evaluation of the Effect of Fig Leaf Extract on *T. vaginalis* Trophozoite

The cytotoxic effect of the extracts on J774 macrophage cells was evaluated. Using the hemocytometry method (performed with a proper cell counting using a Neubauer chamber) with trypan blue staining. The percentage of cell growth inhibition was studied after 24, 48, and 72 hours of incubation. Metronidazole with a concentration of 65 µg/ml and PBS was considered as a standard and control, respectively. All of the tests were repeated three times, so the average results were considered. The amount of growth inhibition for each of the various concentrations of fig leaf extract on *Trichomonas* and macrophages was calculated using the following formula: GI=(a-b/a); GI: Growth inhibition, a: The number of living cells in the control sample, b: The number of living cells in the extract (14).

Statistic Analysis

The data were evaluated using SPSS 21 software, ANOVA statistical test, and Tukey post hoc. $P \leq 0.05$ was considered as the level of significance.

RESULTS

Standardization Results

The total flavonoid content of the extract was calculated

by the following formula as 26.66 _µg/mL:

Y = 0.0002 X + 0.0129; Y = 0.0179, X = 25.12 _µg/mL, R2 = 0.9796

Growth Inhibition Rate of *Trichomonas Vaginalis* in the Presence of the Hydroalcoholic Extract of Fig Leaves

The growth inhibition percentage of *Trichomonas vaginalis* among the concentrations of 2000, 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 µg/ml of the alcoholic extract of fig leaves, throughout 24, 48, and 72 hours, three times repetition was compared and analyzed. The results showed that the inhibitory effect of the hydroalcoholic extract of fig leaves on *Trichomonas va*ginalis after 24 hours of incubation starts from a concentration of 125 µg/ml, and after 48 and 72 hours from a concentration of 62.5 µg/ml (Fig. 1).

Growth Inhibition Rate of *T. vaginalis* in the Presence of the Fig Leaf Aqueous Extract

The growth inhibition percentage of *Trichomonas vaginalis* in the concentrations of 3000, 1500, 750, 375, 187.5, 93.7, 46.8, 23.4, 11.7, 5.8 µg/ml of the fig leaf aqueous extract, throughout 24, 48 and 72 hours in three repetitions were compared. The results showed that the inhibitory effect of the fig leaf aqueous extract on *Trichomonas vaginalis* starts after 24 hours of incubation at a concentration of 375 µg/ml, after 48 hours of incubation at a concentration of 23.4 µg/ml, and after 72 hours of incubation at a concentration of 23.4 µg/ml, and with the increase of concentration, more inhibition growth happened, so that finally with all three types of incubation time, they lead to complete inhibition of protozoa. In the comparison between different assessment times, from the concentration of 23.4 µg/ml and above, increasing the incubation time significantly increased growth inhibition (P<0.5, Fig. 2).

The Effect of Metronidazole in Inhibiting the Growth of *T. vaginalis*

The results of our study showed that except for the concentration of 3000 µg/ml of the aqueous extract in 24 hours and the concentration of 2000 µg/ml of the alcoholic extract in 48 and 72 hours, the rest of the concentrations were significantly different in all the three times of incubation compared to metronidazole (65 microgram/ml) (P<0.05, Fig. 3).

Growth Inhibition Rate of J774 Macrophage Cells in the Presence of Fig Leaf Alcoholic Extract

The growth inhibition percentage of J774 macrophage cells in concentrations of 3200, 1600, 800, 400, 200, 100, and 50 µg/ml of fig leaf alcoholic extract, during 24, 48, and 72 hours, were compared in the three times incubation. The results showed that the inhibitory effect of fig leaf hydroalcoholic extract on macrophages starts after 24 and 48 hours of incubation from a concentration of 100 µg/ml and after 72 hours of incubation from a concentration of 50 µg/ml. With increasing incubation time, significantly more growth inhibition occurred (p<0.05, Fig. 4).

Growth Inhibition Rate of J774 Macrophage Cells in the Presence of Fig Leaf Aqueous Extract

The growth inhibition percentage of J774 macrophage cells in concentrations of 3200, 1600, 800, 400, 200, 100, and 50 µg/ml of the fig leaf aqueous extract, during 24, 48, and 72 hours of incubation, were compared. The results showed that the inhibitory effect of the fig leaf aqueous extract on macrophages was started after 24 hours of incubation from a concentration of 200 µg/ml, after 48 hours from a concentration of 100-400 µg/ml, and after 72 hours from a concentration of 50 µg/ml. With increasing concentration, more growth inhibition occurred. In the comparison between different incubation times, more growth occurred ($P<0.05$, Fig. 5).

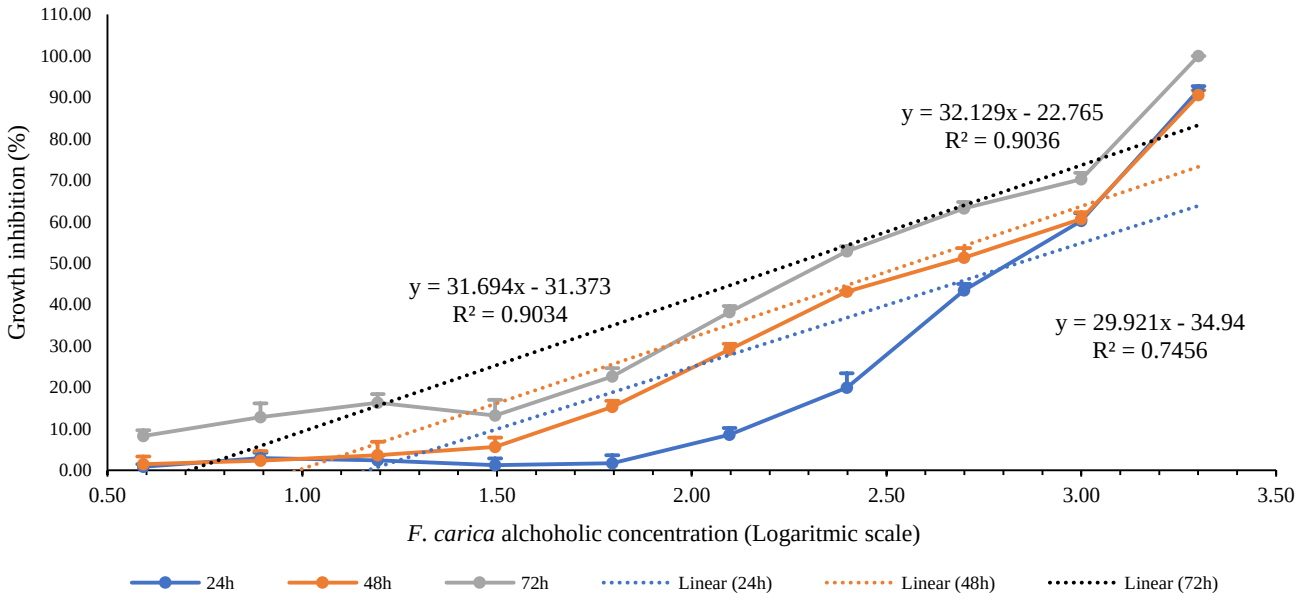

Fig. 1 Diagram of the effect of the hydroalcoholic extract of fig leaf on inhibiting the growth of *T. vaginalis* after 24, 48, and 72 hours of incubation.

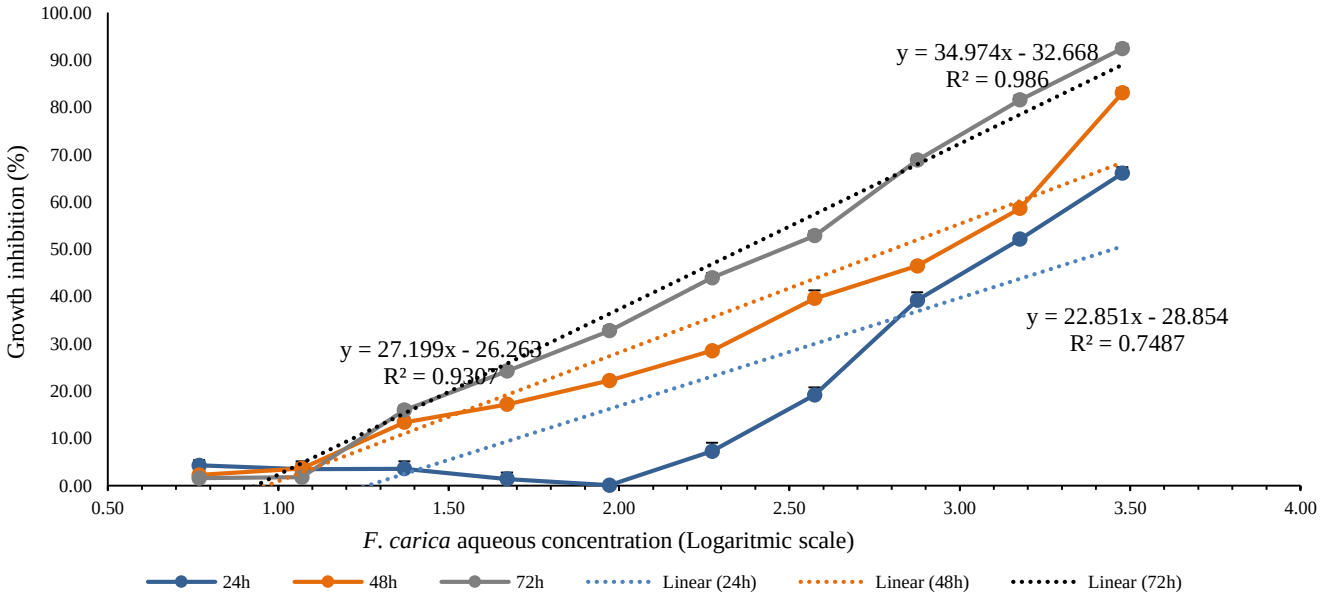

Fig. 1 Diagram of the effect of fig leaf extract on inhibiting the growth of *T. vaginalis* after 24, 48, and 72 hours of incubation. The data were replicated three times and included the standard error.

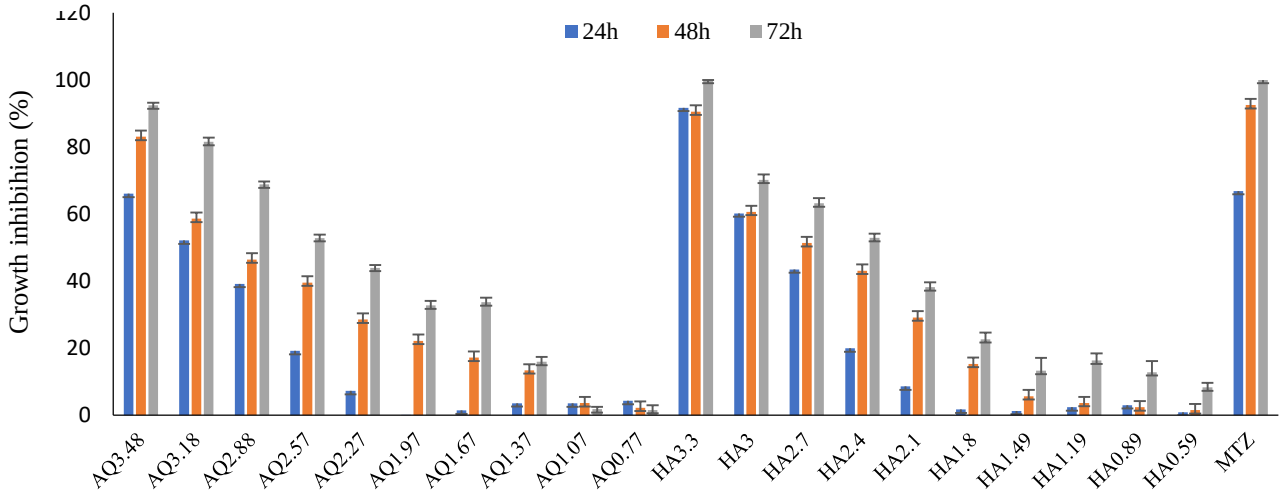

Fig. 2 The effect of metronidazole in inhibiting the growth of *T. vaginalis* in comparison with aqueous and hydroalcoholic extract of fig leaf in logarithmic scale. AQ, aqueous; HA, hudroalcoholic; MTZ, Metronidazole (65 microgram/ml)

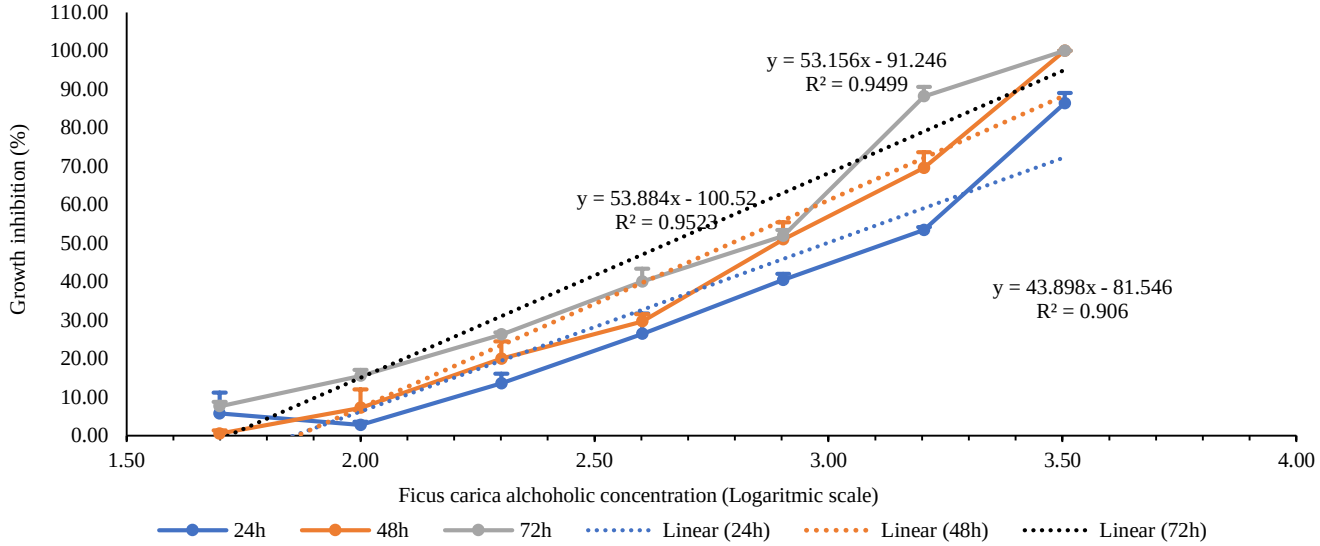

Fig. 3 Comparison of the average growth inhibition percentage of J774 macrophage cells in different concentrations of fig leaf alcoholic extract after 24, 48, and 72 hours Of incubation, The data were replicated three times and included the standard error.

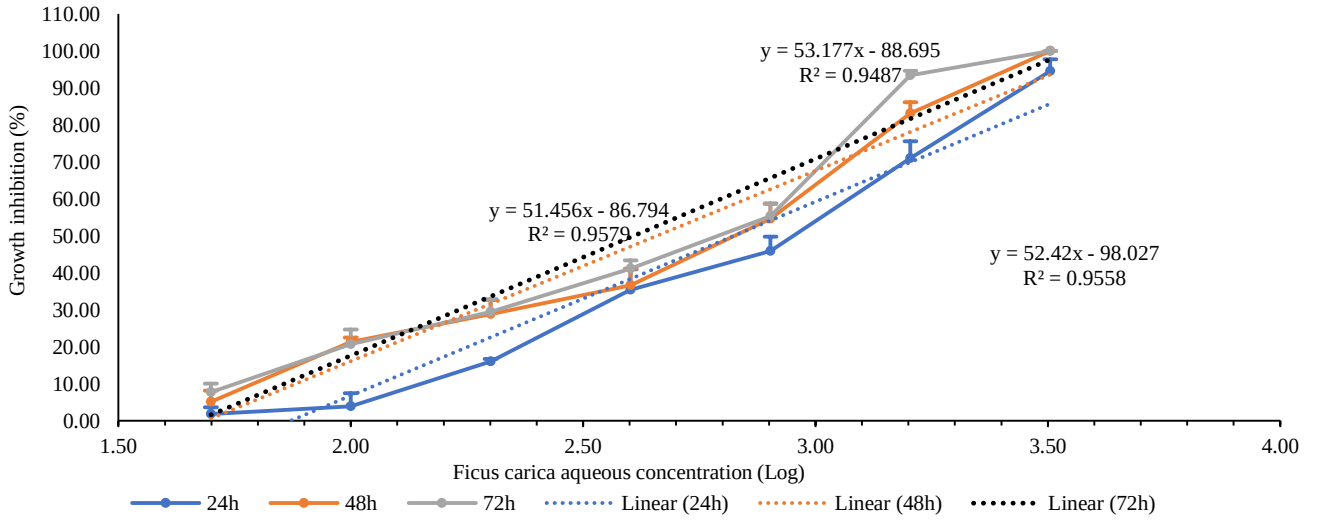

Fig. 4 Comparison of the average growth inhibition percentage of J774 macrophage cells in different concentrations of fig leaf aqueous extract for three times of 24, 48, and 72 hours of incubation. The data were replicated three times and included the standard error.

DISCUSSION

The results of this research showed that different concentrations of the hydroalcoholic and aqueous extracts of fig leaves and a concentration of 65 µg/ml of metronidazole inhibit the growth of *T. vaginalis* in the logarithmic scale, and with the increase in the proximity time of the extracts with this protozoan, more inhibition occurs. In addition, it was found that both hydroalcoholic and aqueous extracts of fig leave completely inhibit *T. vaginalis* at a specific concentration and in all three incubation times of 24, 48, and 72 hours, which are equivalent to 65 µg/ml metronidazole.

The percentage of inhibition of macrophage cells, like *T. vaginalis*, in different concentrations of the aqueous and alcoholic extract increases over time and increasing concentration, so that after 48 and 72 hours in the highest concentration, the concentration of 3200 µg/ml of aqueous and alcoholic extract no living cells remain.

Meanwhile, in the control group that included normal saline instead of plant extract, the growth of *T. vaginalis* was normal and no harm was done to macrophages in any of the incubation times of 24, 48, and 72 hours.

Metronidazole is the drug of choice for the treatment of trichomoniasis, but its toxicity and side effects, as well as drug resistance that has recently developed, are among the most important problems of its use, which have prompted researchers to search for alternative drugs [15,16]. Fig leaf with the scientific name *F. carica* is one of the medicinal plants that has been the focus of traditional medicine. Many pharmacological effects such as antibacterial, anti-parasitic, anti-cancer, anti-inflammatory, and anti-oxidant effects have been studied for fig leaves [17]. The results of this study also showed that both hydroalcoholic and aqueous extracts of fig leaves have anti-trichomonas activity comparable to metronidazole.

In a study by Rocha *et al*., the anti-leishmanial activity of Artemisa annua and Arrabideea Brachiopoda flavonoids were investigated *in vitro.* And the results showed that flavonoids are effective on Leishmania. In a study conducted by Abdelhakim Bouyahya *et al*., they showed that the *F. carica* plant has antibacterial, antioxidant, and antioxidant effects due to having several chemical substances, which are mainly polyphenols and flavonoids. It is anti-cancer and anti-inflammatory [10]. Therefore, it can be concluded that effective substances such as flavonoids and alkaloids can exert destructive effects on *Trichomonas* and be useful against this protozoan. Therefore, according to this study and other studies that have reported the anti-protozoal effects of flavonoids, it can be concluded that probably the flavonoids of fig leaf extract are responsible for its anti-trichomonas effect [18].

In a study conducted by Hussan Ara Begum *et al*., the antibacterial and antioxidant activity of the hydroalcoholic extract of fig leaves in the concentration range of 1000, 750, 500, 250, and 125 µg/ml for antioxidant effect and concentrations of 500 and 200 µg/ml was evaluated for antibacterial effect. The obtained result showed that the hydroalcoholic extract of fig leaves has an antioxidant and antibacterial effect. Therefore, this plant can be effective against trichomoniasis in the form of aqueous and alcoholic extracts [19]. In a study by Amol. p. p *et al*., to investigate the anti-helminthic activity of methanolic and aqueous extracts of fig leaves in comparison with the drug mebendazole as a positive control, it was shown that methanolic and aqueous extracts of fig leaves have anti-parasitic activity. Therefore, it can be concluded that the extract of fig tree derivatives can be used against pathogenic agents such as protozoa [20]. In a study conducted by Joel *et al*., it was shown that the methanol extract of the leaves and stems of Argemone mexicana (Mexican anemone) has an inhibitory effect on *Trichomonas vaginalis*, which is due to phenolic compounds including flavonoids, and its inhibitory effect is dependent on the concentration of the extract, and with the increase in concentration, the percentage of *Trichomonas* inhibition also increases, so that at a concentration of 1000 µg/ml, the inhibitory effect was 100% respectively [21]. Therefore, it can be concluded that fig leaf extracts have a good inhibitory effect on *T. vaginalis*. In this study, after 72 hours, the concentration of 2000 µg/ml of the alcoholic extract of fig leaves has a 100% inhibitory effect and the concentration of 3000 µg/ml of aqueous extract of fig leaves has a 92.39% inhibitory effect on Trichomonas.

Fakhri *et al*.'s (2014) investigated the effect of aqueous and alcoholic extracts of geranium on the growth of *Trichomonas vaginalis in-vitro* and showed an IC50 of 27.63, 54.67 µg/ml after 24 hours [22]. According to the IC50 value, 1.28 and 1.18 in the present study for alcoholic and aqueous extracts

after 24 hours, it can be said that the inhibitory power of fig leaf extract was higher than that of the geranium plant.

Negozi *et al*. (2015) investigated the trypanocidal activity of flavonoid compounds in the leaf extract of Vitex simplicifolia from the Shah Pandian family. The result of the study showed that the lowest IC50 of flavonoid derivatives of this plant on trypanosides and mouse myoblast cells is 4.7 and 1.58 µg/ml, respectively [23]. In the present study, the IC50 value of the hydroalcoholic extract of fig leaves after 72 hours on *Trichomonas* and mouse macrophages, is 0.90 and 1.72, respectively, and also the IC50 value of the aqueous extract of fig leaves after 72 hours on *Trichomonas* and macrophages, which it is equal to 0.72 and 1.67, respectively. It can be concluded that both the fig leaf extracts have a good inhibitory effect on *Trichomonas vaginalis* compared to Vitex simplicifolia, while they have little toxicity on human cells.

CONCLUSION

Results of this study showed that the fig leaf extract, like metronidazole, has significant therapeutic effects on *Trichomonas vaginalis* in such a way that it can completely inhibit this protozoan as well as macrophages, and the growth inhibition percentage of *Trichomonas vaginalis* by hydroalcoholic and aqueous extracts of fig leaves in the logarithmic phase depends on time and concentration. Therefore, after conducting more research and conducting human experiments, this plant can be considered an effective medicinal plant in the treatment of trichomoniasis.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Ethical Considerations

This study was approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences, Yazd, Iran. (Code: IR.SSU.MEDICINE.REC.1397.234).

Authors' Contribution

Conceptualization, Supervision, and Methodology: F.M and M.Z; Investigation: F.Z, M.Z, H.M; Writing – original draft: A.M, H.M; Review & editing: M.Z and F.M; Data collection: H.M and A.M; Data analysis: H.M; Funding Administration: M.Z.

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