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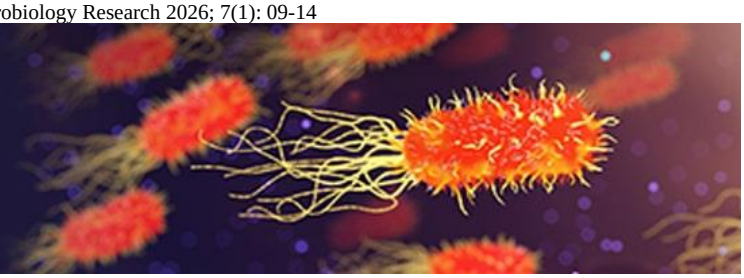

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In vitro anti-parasitic activity of *Enteromorpha intestinalis* methanolic extract against *Trichomonas gallinae*

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Abstract
The *T. gallinae* parasite, which inhabits the upper digestive and respiratory tracts of birds, causes avian trichomoniasis, a disease that poses a significant global threat to human health and livestock. Due to the negative effects of traditional treatments for the disease, such as nitroimidazole or its derivatives, researchers have been exploring more effective alternative therapies, including plant and algae extracts. Therefore, this study aimed to evaluate the biological efficacy of the methanolic extract of the algae *Enteromorpha intestinalis* against *T. gallinae in vitro*. Trophozoites of *T. gallinae* samples were collected from domestic pigeons and cultured on TYM (Tryptone/Yeast extract/Maltose medium) supplemented with 10% fetal calf serum. The pH was adjusted to 6.5-7, and the cultures were incubated at 37 °C. Meanwhile, samples of *Enteromorpha intestinalis* were collected in February 2025 from the Al-Hartha area in Basrah Governorate, Iraq. A methanolic extract was prepared, and five concentrations were made: 5000, 2500, 1250, 625, and 312.5 µg/ml. The chemical compounds of the extract were identified using gas chromatography-mass spectrometry (GC-MS). The median lethal concentration (LC_{50}) was determined via Probit analysis. The effectiveness of methanolic extract concentrations was compared with Ranidazole at 75 µg/ml against the Trophozoites of *T. gallinae*. Results showed that the highest inhibitory activity was observed within 24 hours at 5000 µg/ml, achieving 97.83% parasite killing, while the lowest was at 312.5 µg/ml, with 18.2% killing and significant differences (P = 0.000). The highest killing percentage after 48 hours was at 5000 µg/ml, reaching 98.54%, and the lowest was at 312.5 µg/ml, with 45.00%, and significant differences (P = 0.000). After 72 hours, 5000 µg/ml again achieved 98.62% killing, whereas 312.5 µg/ml resulted in only 75.86%, also significantly different (P = 0.000). The LC_{50} after 24 hours was 891.25 µg/ml, lowest to 512.87 µg/ml after 48 hours. Based on these findings, the methanolic extract of *Enteromorpha intestinalis* shows promise as a natural source of compounds capable of inhibiting *T. gallinae* and as a potential treatment for avian trichomoniasis.

Keywords: *Enteromorpha intestinalis*, *Trichomonas gallinae*, avian trichomoniasis, methanolic extract, *in vitro* antiparasitic activity

Introduction
T. gallinae, a protozoan parasite belonging to the Trichomonadidae family, is a single-celled eukaryote that causes avian trichomoniasis, also known as canker, a disease that poses a global threat to human health and animal wealth. This parasite infects the upper gastrointestinal and respiratory tracts of both wild and domestic birds (Quillfeldt *et al*., 2018; Farooq *et al*., 2018) [28, 16]. The most prominent symptoms in infected birds include weight loss, vomiting, increased thirst, and difficulty breathing (Amin *et al*., 2012; Stockdale *et al*., 2015) [11, 29]. Additionally, cheesy material may cover the mucous membrane of the mouth and partially obstruct the laryngeal inlet (Elbahy *et al*., 2023) [14]. Nitroimidazole and its derivatives, such as metronidazole and tinidazole, have been used since their introduction in the 1960s (Freeman *et al*., 1997) [17]. These compounds have revolutionized infection treatment; however, many human Trichomonas isolates have developed resistance (Kirkaldy *et al*., 2012) [24]. Bradshaw *et al*. (2006) [12] highlighted the toxicity of nitroimidazole compounds and their effects on human and animal health, especially with repeated or long-term use. Various issues have been noted following treatment with chemical compounds, including resistance among some biological pathogens and side effects on the body systems and tissues of patients. This has led to interest in using plants, algae, and their products natural compounds for treating many diseases caused by viruses, bacteria, fungi, or parasites.

These alternatives are also used in combating tumors and limiting their spread. *Enteromorpha intestinalis* is a type of green algae commonly known as 'Sea Lettuce' or 'Gutweed,' is a macroalga characterized by its bright green color." and widespread presence in freshwater environments. These algae are rich in biologically active compounds, making them of interest for potential pharmaceutical applications. Some of these compounds exhibit activity against viruses, bacteria, and fungi, and possess antioxidant, antitumor, and anti-inflammatory properties. Research has consistently highlighted the therapeutic potential of *Enteromorpha intestinalis*. Following the initial findings of Helio *et al*. (2000) [21], Zhang *et al*. (2013) [30] demonstrated the significant antifungal efficacy of aqueous and ethanolic extracts from algae against Candida species. These results corroborate the assertions made by Chiheb *et al*. (2009) [13] regarding the importance of *Enteromorpha intestinalis* as a rich source of bioactive compounds with clinical relevance. Its role as an antiparasitic agent: Bhakuni and Rawat (2005) [40] confirmed that *E. intestinalis* algae is effective against amoebic infections Spavieri *et al*. (2010) [31] demonstrated the efficacy of *Leishmania donovani* and *Trypanosoma cruzi* algae extracts against rhodesiense parasites. Its aqueous and methanolic extracts also had an antiparasitic effect against the malignant malaria parasite Al-Jaber *et al*., 2016 [32] *in vitro*. A study of *Plasmodium falciparum* showed the importance of the methanolic extract of this algae against the hydatid parasite *Echinococcus granuloses in vitro* and *in vivo*. In another study, the methanolic extract of the algae showed biological activity against the scabies mite Sarcoptic mange *in vitro* and *in vivo*
(Al-Mousawi *et al*. 2020) [10] Ivermectin compared to a drug. this study aimed to evaluate the biological activity of the methanolic extract of *Enteromorpha intestinalis* against *T. gallinae*, the parasite responsible for avian trichomoniasis. *T. gallinae* was chosen as a model organism representing other trichomonads because of its economic significance.

Material and Methods

Parasite Sample Collection

Samples of *T. gallinae* were collected from domestic pigeons of the Columba livia breed. The pigeons were brought from local markets in Basrah Governorate; their ages ranged between 2 and 6 months, and their weights ranged between 900 and 1100 grams. Initial identification of the infection was performed by a specialized veterinarian. The infected birds were then transferred to the Parasitology Laboratory in the Department of Biology at the College of Education for Pure Sciences for microscopic examination, confirmation of infection, and diagnosis of the parasite. The microscopic examination involved taking samples of secretions from the oropharyngeal area of the infected pigeons using sterile, moist swabs. These samples were placed in 0.5 ml of 0.9% warm sterile saline solution and stored warm at a temperature of 20-30 °C. The storage period should not exceed 30 minutes before examination. The samples were then examined directly to confirm the presence of the parasite that causes trichomoniasis. During the use of the wet mount method and observation of trophozoites of the *T. gallinae* parasite (2003, Samour and Naldo). The examination involved staining the samples transferred onto glass slides, after drying them at room temperature for 15-20 minutes, then staining them using Gram stain and leaving them at room temperature for 15 to 20 minutes, and then fixing them with methanol for 30 seconds. After that, the slides were washed with distilled water and left to dry at room temperature until microscopic examination (Tasca and DeCarli, 2003).

Parasite Culture and Maintenance

Positive *T. gallinae* samples were cultured in TYM (Tryptone/Yeast extract/Maltose medium) supplemented with 10% fetal calf serum. The pH was adjusted to 6.5-7 (Kharofa, 1999) [23]. The culture medium was distributed into sterile test tubes, and 100 µL of chloramphenicol was added to ensure contamination-free conditions. The tubes were then inoculated with the parasite, sealed, and incubated at 37±1°C. The tubes were examined after 48 hours to ensure parasite viability. Subsequent cultures were performed every 48 hours from the parent culture to obtain a pure culture. Only motile *T. gallinae* trophozoites were counted (Hamad & Hassan, 2017) [20].

Algal Collection and Extraction

Algal samples were collected during February 2025 from the Al-Hartha area in Basrah Governorate, Iraq, using clean and sterile containers designated for sample collection. The samples were transported directly to the laboratory in the Biology Department at the College of Education for Pure Sciences, where they were diagnosed by an algal expert. After the diagnosis process, the samples were rinsed with distilled water and dried away from direct sunlight at room temperature, then ground and stored for use at 18 °C (Weidman *et al*.). According to Rios *et al*.), the methanolic extract was prepared by dissolving 50 g of dried algae powder in 250 ml of absolute methanol. The mixture was then left to stand, stirring for 24 hours in a glass flask. The mixture was then filtered using Whatman No. 1 filter paper, and the filtrate was concentrated using a rotary evaporator at 40 °C. It was then allowed to dry. The extract was stored at 4 °C in sterile bottles until use.

Identification of the Chemical Components of the Methanolic Extract

The chemical components of the extract were identified using gas chromatography-mass spectroscopy (GC-MS). Tests were conducted at the Research and Quality Control Department of the Basra Oil Company, Nahran Omar.

The Effectiveness of Algal Extract Concentrations against the *T. Gallinae* parasite

Five concentrations of *C. glomerata* alcoholic extract (1600, 800, 400, 200, and 100) µg/ml were prepared. The effectiveness of these concentrations was tested in comparison with Ranidazole 75 µg/ml against the trophozoites of *T. gallinae*. Multi-well plates were used, with the wells in the plate divided into three groups: the first group included the five concentrations of the algal extract, the second group included the Ranidazole group, and the third group included the control group, which was untreated, with only the culture medium used to grow the parasite added. The method involves taking 100 µl of culture medium containing 1×10^4 parasites/ml using a micropipette and placing it in each well. The treatment, whether an algae extract concentrate or a conventional treatment, is then added, with five replicates for each treatment, in addition to a control group. The plate is then incubated under anaerobic conditions for 72 hours at 37 °C.

The improved Neubauer hemocytometer slide was used to count the parasites, after mixing the sample containing the parasite with an equal amount of 0.4% Trypan blue dye to distinguish between live and dead parasites. According to Palmas *et al*. (1984) [26], the results were recorded after every 24, 48, and 72 hours of incubation, using the following equation

Growth inhibition% = (A-B)/A×100

- **A:** The average number of trophozoites in the control group.
- **B:** The average number of trophozoites in the treatment group.

Determination of the median lethal concentration (LC_{50}) of the algal extract

The LC_{50} of the algal extract was calculated for all time periods of the experiment using a probit analysis, based on the method of Miller and Tainter (1944) [25].

Statistical analysis

Data were analyzed statistically using the chi-square test using the Statistical Package for the Social Sciences (SPSS) version 19, and significant differences were tested at a probability level of $p \leq 0.05$.

Results

Identification of the active compounds of the extract of *E.intestinalis*

Figure 1 shows the mass spectrum of the extract of *Enteromorpha intestinalis*, which shows a chemical compound represented by a group of peaks. The current study focused on three of these compounds based on their importance as active compounds according to previous scientific studies. These compounds are Loliolide, Palmitoleic acid, Phytol.

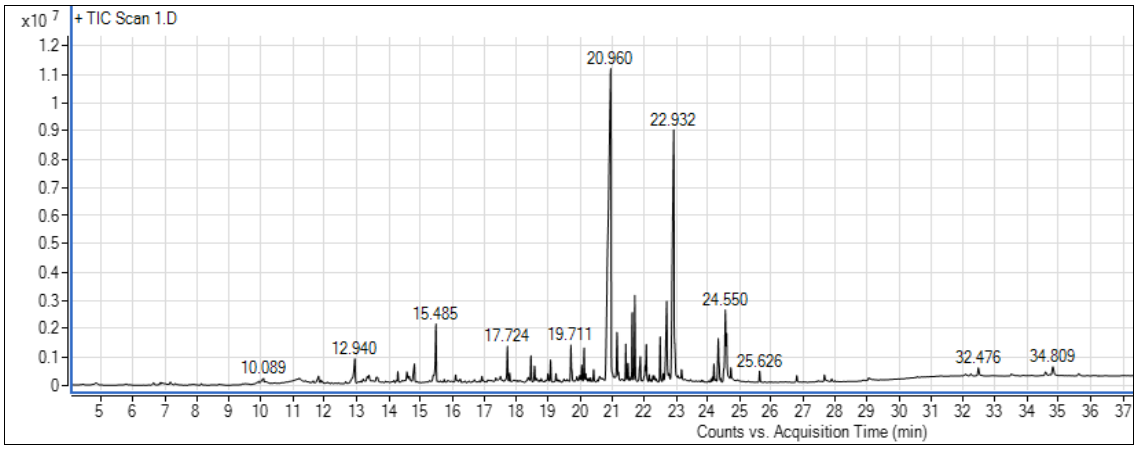

Fig 1: Mass spectrum of the alcoholic extract of *E. intestinali*

The effectiveness of the extract of *E. intestinalis* against the *T. gallinae*

Table 1 shows the percentages of growth inhibition of *T. gallinae* for the five concentrations of the extract of *E. intestinalis* compared to the Ranidazole over three time periods (72, 48, and 24 hours). The highest percentage of growth inhibition was achieved after 24 hours of the experiment for the concentration of 5000 µg/ml, at 97.83%, while the lowest percentage of growth inhibition was recorded at 18.2% for the concentration of 312.5 µg/ml. Significant differences (P-value = 0.000) were recorded for all the inhibition percentages for the five concentrations of the extract, as well as for the Ranidazole. As for the percentages of growth inhibition for the concentrations after 48 hours, the highest percentage was also achieved for the concentration of 5000 µg/ml, at 98.54%, while the lowest percentage of growth inhibition was recorded at 45.00% for the concentration of 312.5 µg/ml. There were significant differences (P-value = 0.000) for all inhibitory ratios of the five concentrations of the extract and the Ranidazole. The results of the percentages of inhibitory activity for the concentrations after 72 hours showed that the concentration of 5000 µg/ml recorded the highest percentage at 98.62%, while the lowest inhibition percentage was at 312.5 µg/ml, at 75.86%. There were significant differences (P-value = 0.000) for all inhibitory ratios of the five concentrations of the extract and the Ranidazole. It is worth noting that the inhibitory activity of the five concentrations of the extract of *E. intestinalis* decreased significantly with age, which may indicate the presence of chemical changes in the extract composition as the experiment progressed.

Table 1: Inhibitory activity of the extract of *E.intestinalis* against the *T. gallinae*

Inhibitory activity %			
Time (h) / Con. µg/mL)	24	48	72
Ranidazole (75)	100	100	100
5000	97.83	98.54	98.62
2500	73.91	79.2	86.21
1250	67.39	68.78	85.52
625	32.74	53.97	79.31
312.5	18.2	45.00	75.86
Chi-Square	86.65	34.86	5.67
P-Value	0.	0	0.399

Determination of the LC_{50} values of the extract of *E. intestinalis* against the *T. gallinae*

The LC_{50} values of the extract of *E. intestinalis* against the *T. gallinae* were determined during the different time periods of the experiment. After 24 hours of the experiment, due to the high inhibitory activity values for all concentrations exceeding 50%, it was not possible to determine the LC_{50} value. Whereas the LC_{50} value of the extract after 24 hours was 891.25 µg/ml (Figure 2), while the LC_{50} value after 48 hours was 512.87 µg/ml (Figure 3)

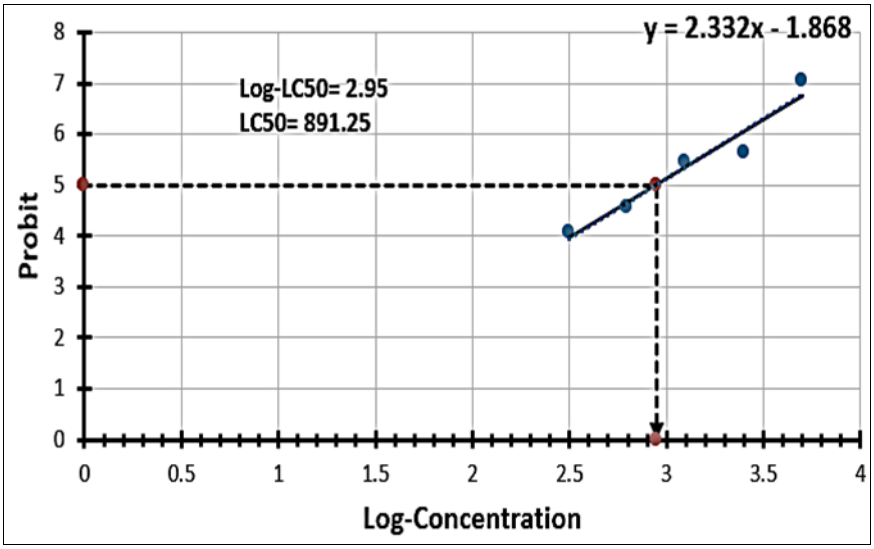

Fig 2: The LC_{50} value of the extract of *E. intestinalis* against the *T. gallinae* after 24 h

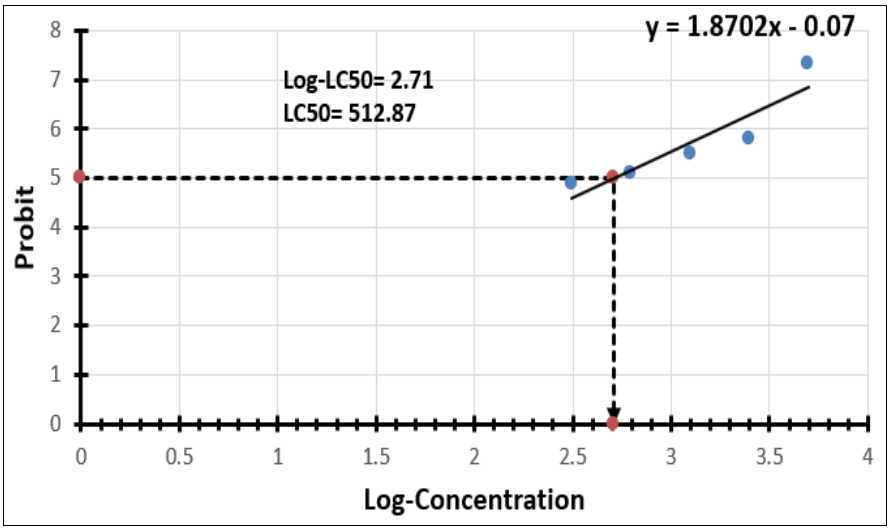

Fig 3: The LC_{50} value of the extract of *E .intestinalis* against the *T. gallinae* after 72 h

Discussion

he results of the test on the effectiveness of the methanolic extract of the algae *E. Intestinalis* in the vitality of the *T. gallinae* parasite have shown varying proportions compared to the traditional treatment Ronidazole during the time periods (24, 48, 72) hours, and the effect was progressive with increasing concentration. The effectiveness of extracts from this algae as an antiparasitic agent has been previously tested by Spavieri *et al*. (2010) [31], who found it effective against Trypanosoma cruzi, T. brucei, and *Leishmanai donovani*; and Ravikumar *et al.* (2011) [33], who recorded the effect of an effective methanolic extract of this algae against the parasite *Plasmodium falciparum in vitro*. Some local studies, such as Al-Jaber's (2016) [32] study, have also recorded the effectiveness of extracts from the same algae against the protozoa of *Echinococcus granulosus in vitro* and *in vivo*. Finally, Al-Mousawi *et al*. (2021) [34] demonstrated that the methanolic extract of *E. intestinalis* showed high efficacy against the rabbit mange mite Sarcopetes scabiei var. cuniculi *in vitro* and *in vivo*. Previous studies by Benjama and Masniyom (2011) [35] and Al-Jaber have also indicated this. (2016) It was noted that the green alga *E. intestinalis* is a source containing bioactive compounds such as proteins, phenols, sterols, alkaloids, polysaccharides, lipids, terpenes, amino acids, and saponins. Despite the differences in the parasitic groups used in previous studies compared to the parasite in the current study, and the similarity in the results obtained regarding the inhibitory activity of extracts from this alga, it can be concluded that the reason for this inhibitory activity of the methanolic extract of *E. intestinalis* is due to The chemical content of the active ingredients, confirmed by previous studies of the algal extract, includes carbohydrates, alkaloids, phenols, saponins, triterpenes, and sterols. Some of these were identified during the current study according to the results of chemical analysis using gas chromatography-mass spectrometry. The analysis confirmed the presence of several fatty acids and terpenes such as loliolide and phytol. Terpenes are considered biologically active compounds as antiparasitic agents, and their mechanism of action depends on the reaction with iron groups (Fe1, FeΠ), causing the release of free radicals in the cell, leading to damage and then the death of the parasite. Loliolide is a monoterpene that was previously isolated from *E. intestinalis* in a study by Güven *et al*. (2015) [36]. It has also been isolated from marine algae, plants, and animals (especially insects). It exhibits antioxidant and cytotoxic activity against tumors, as well as antifungal and antibacterial activity Loliolide's toxicity has been investigated against nasopharyngeal carcinoma and lymphocytic leukemia. Another study showed that loliolide in the marine brown alga Sargassum ringgoldianum exhibits

antioxidant activity (Yang *et al*., 2011) [37].

The current results also show that the methanolic extract of *E. intestinalis* contains palmitoleic acid, a saturated fatty acid found in plants, animals, and microorganisms. This acid is characterized by various properties, most notably its antitumor effect. Palmitoleic acid was isolated from red marine algae by Harada *et al*. (2001) [38]. Its cytotoxic activity against leukemia was tested, revealing high activity against cancer cells and no toxicity to normal cells (human dermal fibroblasts).

The methanolic extract of *E. intestinalis* also contains phytol, a diterpene with various antitumor and antioxidant activities (Pejin *et al*. 2014) [27]. Santos *et al*. (2013) [39] investigated the cytotoxic activity of phytol against seven types of tumors *in vitro* and also confirmed the importance of phytol as an antioxidant compound. It is known that the methanolic extract of algae contains alkaloids and phenols, which have an inhibitory effect on many pathogens. Phillipson and O'Nill (1987) stated that alkaloids significantly affect the DNA of parasite cells, while phenols play a role in altering cell membrane permeability and impairing its selectivity, leading to a loss of control over the entry of substances into the parasite cell and ultimately its death.

Extracts of the alga *E. intestinalis* have been tested for their antiparasitic properties by Bhakuni and Rawat (2005) [40], who demonstrated their efficacy against the parasite *Entamoeba histolytica*. Ravikumar *et al*. (2011) [33] also tested the efficacy of both methanolic and aqueous extracts of the same alga against the malaria parasite (Plasmodium falciparum) *in vitro* and observed high efficacy in both extracts. Spavieri *et al*. (2010) [31] noted the high efficacy of extracts of this alga against *T. rhodesiense*, *Trypanosoma cruzi*, and *Leishmania donovani*. Furthermore, Al-Jaber (2016) [32] demonstrated efficacy against hydatid cysts both *in vitro* and *in vivo*.

In another local study, Al-Mousawi *et al*. (2021) [34] reported high efficacy of *E. intestinalis* extracts against scabies mites both *in vitro* and *in vivo*. The results showed that the methanolic extract of *E. intestinalis* a potential natural source, at different concentrations achieved high rates of killing the *T. gallinae* parasite compared to the traditional treatment with Ronidazole.

Conclusion

The results of the current study, which demonstrated the effect of the extract of *E. intestinalis* against the *T. gallinae*, given its active compounds, could lead to the possibility of considering it a natural source of new compounds capable of inhibiting the *T. gallinae* and a promising treatment for avian trichomoniasis.

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Author's Contribution

Not available

Conflict of Interest

Not available

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