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In vitro antiprotozoal effect of alcoholic extract of hemolymph of *Galleria mellonella* larva against *Trichomonas gallinae*

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ABSTRACT

Trichomonas gallinae is a protozoan parasite that lives in the upper gastrointestinal tract of birds. Recently, the hemolymph of *Galleria mellonella* has been widely used as an anti-parasitic drug. The present work aimed to investigate the inhibitory activity of an alcoholic extract of *G. mellonella* hemolymph (AEGmH) on *T. gallinae in vitro* compared with that of metronidazole using wet smear and vital stain acridine orange (AO). The results showed that the degree of growth inhibition of *Trichomonas* was based on the AEGmH concentration. Furthermore, the highest reduction of parasitic growth (100%) was observed in cultures treated with 25, 12.5, and 6.25mg/mL AEGmH before 24h. Moreover, the same results were detected in cultures treated with 3.1 and 1.6mg/mL AEGmH, but after 48h, and doses of AEGmH (0.8 and 0.4mg/mL) after 96h. In comparison, complete inhibition of parasite growth (100%) was obtained by metronidazole at 25mg/mL after 72h. Besides, light microscopy observations revealed changes in trophozoites' morphology in culture treated with AEGmH. We concluded that the alcoholic extract of hemolymph of *G. mellonella* is suitable to counter trichomoniasis *in vitro* and showed antiprotozoal potential.

Key words: Antitrichomonal, *Galleria mellonella* larva, Hemolymph, Metronidazole, *Trichomonas gallinae.*

INTRODUCTION

Trichomonas gallinae is a flagellated protozoan parasite belonging to the family Trichomonadidae, a group of anaerobic protozoa causing disease in domestic animals and humans (Arfin et al. 2019). It generally influences the respiratory tract and upper digestive system, especially the crop and esophagus, of different avian species, causing avian trichomoniasis (Park 2011). The life cycle of *T. gallinae* includes two stages: motile trophozoite and non-motile pseudocyst (Marchiondo et al. 2019). *T. gallinae* is considered one of the most serious pathogens in pigeons globally, causing high mortality in young pigeons within 10 days of infection (Santos et al. 2020). It can cause granulomatous lesions within the digestive tract of affected birds. The lesions range from mild to severe inflammation, eventually leading to death by starvation (Amin et al. 2014). Through phylogenetic studies, Maritz et al. (2014) proved the possibility of zoonotic transmission of trichomonad parasites from humans to birds and vice versa.

Trichomoniasis diagnosis depends on microscopic evaluation of motile stages. Furthermore, other Romanowsky and Giemsa stains are commonly used to diagnose direct smears of *T. vaginalis*. However, the fast loss of the characteristic motility of the *Trichomonas* and scanty or rounded forms of trichomonads can be confused with polymorphonuclear leukocytes or dysplastic cells. As a result, wet smear evaluation is considered an insensitive technique for diagnosing infection (Khatoon et al. 2014). Recent reports recommended the usage of acridine orange dye to determine the viability of *Trichomonas* trophozoites, where it improved the ability to diagnose *Trichomonas* infection (Doshi 2017). Acridine orange is a non-specific nucleic acid stain that can be applied for fluorescence-based detection of *Trichomonas* infection. It is more sensitive than the wet mount, and it has been introduced as the standard method for the diagnosis of infection (Harrington and Gaydos 1984).

Metronidazole, or Flagyl, is a 5-nitroimidazole compound utilized to treat various pathogenic protozoa-induced infections, such as *Trichomonas*, *Entamoeba,* and *Giardia* (Löfmark et al. 2010). Nitroimidazole products such as tinidazole and metronidazole are the typical choices for treating avian trichomoniasis (Seddiek et al. 2014). These drugs have the possible disadvantages of

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carcinogenicity due to damaging DNA, proteins, and cell membranes (Baccega et al. 2021), as well as the development of drug resistance in some strains (Dingsdag and Hunter 2018). In addition, the long-term administration of these drugs can lead to toxicity and carcinogenicity (Baccega et al. 2021).

Recently, insects and their products have become essential ingredients in drug preparation across the globe. The therapeutic efficacy of insects results from a massive contribution to the nature conservation controversy and the opening of perspectives on the economic and cultural value of animals that have historically been overlooked (Ratcliffe et al. 2011). As many insects can survive harsh conditions such as microorganism infestation, the gastrointestinal tract, and dead flesh, it is not surprising that they have good immune defenses to combat infection. Specifically, insects have humoral and cellular innate immune defenses (Xiao et al. 2013). Humoral anti-microbial peptides (AMPs) are essential for developing new antibiotic drugs (Ratcliffe et al. 2014).

Shittu et al. (2013) reported that the methanol extract of *Musca domestica* maggots had anti-plasmodial activity. In addition, Lacerda et al. (2016) mentioned that the anti-parasitic activity of insect peptides was an interesting tool for treating neglected disorders, including leishmaniasis, malaria, and Chagas disease. Farghaly and Sadek (2020) investigated the trypanocidal efficacy of methanol extracts of the hemolymph of *Sarcophaga argyrostoma* larvae against *Trypanosoma evansi*-infected mice. Recently, several research groups have focused on using the hemolymph of the greater wax moth *Galleria mellonella* because it is a source of AMPs, or host defense peptides, acting as part of the innate immune response (Andrejko et al. 2014). Such peptides are active antibiotics acting against various microbes that can be novel therapeutic agents (Ageitos et al. 2017). Anti-microbial peptides also appear to destabilize biological membranes, may form transmembrane channels, and may even have the potential to boost immunity by acting as immunomodulators, unlike most traditional antibiotics (Reddy et al. 2004). *G. mellonella* larva has many advantages, including being widely available, procurable at a low cost, having no costs associated with breeding, no need for cultures or colonies to be preserved, and no need for other specialized equipment to house the larvae (Desbois and Coote 2012).

Patiño-Márquez et al. (2018) noted for the first time the anti-parasitic potential of peptides derived from the hemolymph of *G. mellonella*, such as anionic peptide 2 and cecropin-D peptides. El-Dirany et al. (2021) identified the major families of anti-leishmanial AMPs (cecropin, melittin, defensin, cathelicidin, temporin, magainin, eumenitin, dermaseptin, and histatin), as well as their potency against other pathogens. Against this background, we aimed to explore the efficacy of different concentrations of alcoholic extract of hemolymph of *G. mellonella* larva as an antitrichomonal drug *in vitro*.

MATERIALS AND METHODS

Ethical approval

Birds were collected and sampled as per the instructions of the Institutional Animal Care and Use Committee, National Research Centre Animal Care Unit in Egypt, which are compliant with the International Animal Ethics Committee (8th Edition 2011) guidelines by the Institute for Laboratory Animal Research (ILAR); Division of Earth and Life Studies (DELS); National Research Council (Record number: 13799, legacy ID: 8247), and the regulations and local laws.

Collection of *Galleria mellonella* larvae

Newly emerged larvae of *G. mellonella* were collected from the Apis Research Department, Research Institute of Plant Protection, Center of Agriculture Research, and reared on an artificial diet, as reported by Kulkarni et al. (2012). Hemolymph was collected from the last proleg using a syringe in an Eppendorf tube and kept at 0°C for a few days, and after that lyophilized to powder according to LaTorre-Snyder (2017).

Preparation of alcoholic extract of hemolymph

Three grams of the powder were percolated in 24mL of absolute ethanol and stored in the shade for 48h, and then it was filtered. The filtrate was gathered in a beaker, exposed to air, and permitted to evaporate at room temperature to yield an extract concentrate (Shittu et al. 2013).

Sample preparation and analysis using gas chromatography-mass spectrometry (GC- MS)

For the chemical composition analysis of the alcoholic extract of hemolymph, sterile water was used to dilute hemolymph samples 1:1000, and 10µL of this was transferred to a 1.5mL microcentrifuge tube. The aliquots were lyophilized overnight in a Lyophilizer Freeze Dryer (Labconco, Kansas City, MO, USA) to desiccate the samples. After that, samples were stored at -80°C for up to three days (Mayack et al. 2020). The analysis was performed according to Mayack et al. (2019). Samples standards were analyzed using an Agilent 7890 Gas Chromatograph (GC)/5975C Mass Spectrometer (MS).

Parasite collection and culture

This study was conducted at the Animal Health Research Institute, Department of Parasitology, Assiut, Egypt. *T. gallinae* parasites were collected from apparently healthy squabs. A total of 50 squabs aged between 30 and 90 days old were purchased from local markets in different parts of Assiut city. Samples were collected from the upper digestive tract (mouth and crop) using moistened microbiology swabs, in accordance with the procedure reported by Samour and Naldo (2003).

Wet specimens were made to ensure that the trichomonads were still active (flagellar motion). The swabs were spread over microscopy slides and examined microscopically at ×100 and ×400 magnifications. To identify the trichomonads' morphological characteristics, permanent slides were prepared and stained with Giemsa, in accordance with the work of Soulsby (1986).

The positive samples were cultured *in vitro* by immersing the swabs in a sterilized falcon tube containing beef extract–glucose peptone medium (growth medium-ATTC) at pH 7.4–7.6 (El-Sayed 2005) and incubated at 38°C until the number of parasites reached 10^4 ml. The tubes were examined daily for the presence of parasites by

microscopic examination using one drop from the bottom of the tube, per the work of Abd-El-Motelib and Galal (1994).

***In vitro* studies**

The minimum inhibitory concentration (MIC) was calculated as the lowest concentration of treatment that noticeably restricts the growth of *Trichomonas,* as previously reported by Meingassner and Thurner (1979). This step was crucial for *in vitro* assay.

To evaluate the effect of AEGmH on the growth of *T. gallinae*, 10^4 trophozoites were incubated in a glucose-serum broth medium containing AEGmH in different concentrations (25, 12.5, 6.25, 3.1, 1.6, 0.8, and 0.4mg/mL). Moreover, controls included cultures involving only the parasites and cultures treated with metronidazole (25mg/mL). All drugs were tested three times, and the growth of *T. gallinae* was examined by taking 10µl of the sample every 24, 48, 72, 96h, and 120h at 38°C post-inoculation.

Data analysis

The impacts of AEGmH and MTZ on the growth inhibition of *T. gallinae* were evaluated by comparing the trophozoites numbers in treated cultures with the numbers in untreated cultures using a hemocytometer (Neubauer Improved, bright line; Germany).

The percentage inhibition of multiplication was determined using the following equation:

Percent of growth inhibition $=\frac{a-b}{a}\times 100$

Where, a = the average number of trophozoites found in control tubes

b= the average number of trophozoites found in the tested tubes (Palmas et al. 1984).

Processing of smears

A drop of the freshly prepared sample was obtained on a clean slide and allowed to dry in the air. Air-dried film was fixed in absolute methanol by briefly immersing the film (two dips) in a Petri dish containing absolute methanol, after which it was left to dry. Two smears were made from each swab: one was used for Giemsa staining and the other for acridine orange (vital stain AO) staining.

Giemsa staining

Fixed smears were stained with diluted Giemsa stain (2 drops of stock solution+1mL of distilled water) for 20min. The film was then washed by briefly immersing the slide in a jar containing buffered water (one or two dips), after which it was left to dry in an inclined position (Soulsby 1986). It was assessed under the oil immersion lens of the microscope to detect any morphological changes in the treated samples.

Acridine orange staining

For buffered, low pH acridine orange (AO) staining, the stain from a commercial kit (Biodiagnostic Co., Dokki, Giza, Egypt) was used, as described in the manufacturer's guidelines. Fixed smears were stained with AO for 2 min, washed off with water, and then covered with a cover slide (Harrington and Gaydos 1984). Stained smears were evaluated under a fluorescence microscope (with a 470 - 490nm filter) at a magnification of 40× (Olympus Corporation of America, New Hyde Park, NY).

Interpretation of the microscopic findings

Green: living *T. gallinae*
Green/red: damaged *T. gallinae*
Red: dead *T. gallinae*

Statical analysis

The study used a factorial design with three replications and followed a randomized design. To establish the significance of differences between treatment means, the analysis of variance (ANOVA) approach was used in conjunction with SPSS 16 (SPSS Inc, USA) software.

RESULTS

The major chemical compounds of the GC-MS analysis for the alcoholic extract of *G. mellonella* hemolymph are presented in Table 1 and Fig. 1. Moreover, we assessed the potential impact of AEGmH on the survival of trophozoites of *T. gallinae in vitro* compared to metronidazole (MTZ). The *in vitro* study results revealed the high sensitivity of *T. gallinae* to AEGmH; so that with high concentrations of extract at 25, 12.5, and 6.25mg/mL at 24h (minimum time), showed a 100% mortality rate. Meanwhile, lower concentrations of AEGmH extract (3.1 and 1.6mg/mL) also caused 100% inhibition of growth of *T. gallinae* trophozoites at 48h post treatment. The lowest doses of AEGmH extract (0.8 and0.4 mg/ml) showed complete inhibition after 96h. Cultures treated with MTZ (25mg/mL) also showed a complete reduction of parasite growth after 72h (Table 2, Figs. 2 and 3).

Furthermore, the morphological features of fresh isolates from both the crop of naturally infected squabs and untreated culture revealed the presence of flagellated, actively motile trophozoites. In stained smears, the trophozoites were pear-shaped with four free flagella on their anterior aspect, an axostyle on the posterior end, and their nucleus located at the anterior third (Fig. 4a and b). The trophozoites measured 10.5–13.5×3–7.5µm. Trophozoites with low concentrations of AEGmH (5–7) were mostly spherical in shape (Fig. 4d) and sluggishly motile, while dead trophozoites were distorted (Fig. 4c).

Besides, the fluorescence microscopy examination of the slides stained with acridine orange revealed that the

Table 1: List of chemical compounds in alcoholic extract of *Galleria mellonella* hemolymph from GC-MS analysis.

Compound	%	Compound	%
Gm-prolinerich peptide 1	4.2	a-Glycerophosphate	2.1
Gm-defensin-like peptide	2.9	Citric acid	2.95
Gm-anionic peptide 1	0.9	Cobatoxin	12.9
Gm-anionic peptide 2	1.4	Fructose	0.40
Malic acid	0.92	Glucose	0.42
L-Aspartic acid	0.13	Glucitol	0.13
Pyroglutamic acid	3.35	Glucaric acid	0.1
2-Ketoglutaric acid	0.60	Sucrose	0.19
L-Glutamic acid	0.8	Moricin-B	22.15
Cecropin A	19.3	Moricin - C4	10.96
Cecropin D	13.2		
Total 100.00			

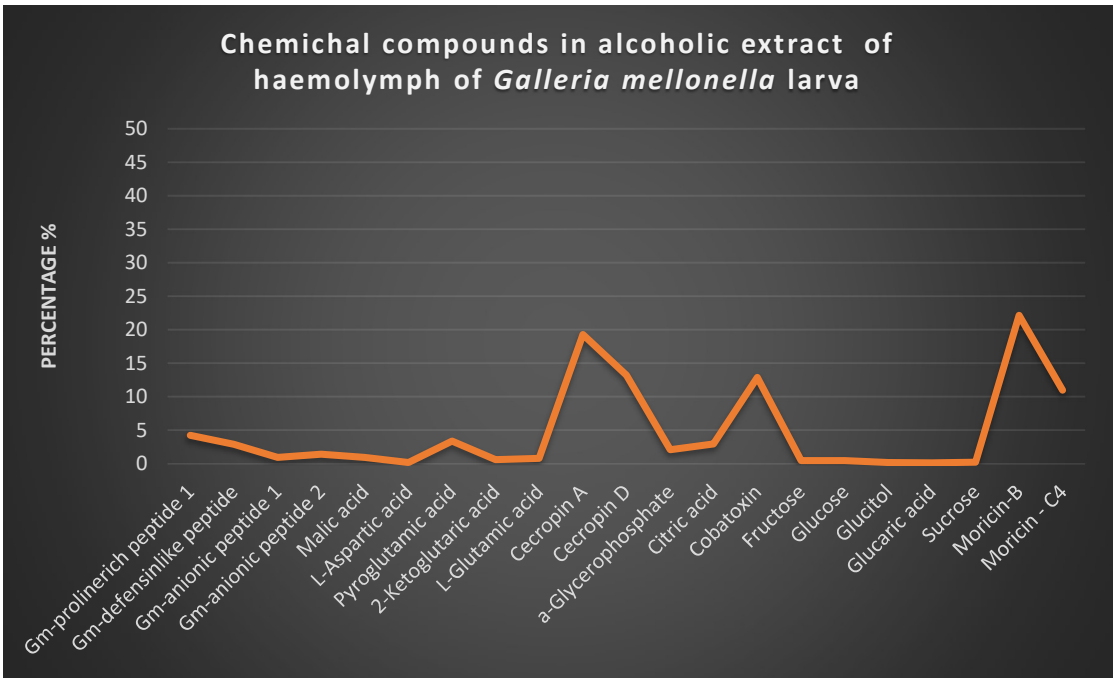

Fig. 1: Histogram showing different chemical compound concentrations in alcoholic extracts of hemolymph of *Galleria mellonella* larva (AEGmH) identified by GC-MS.

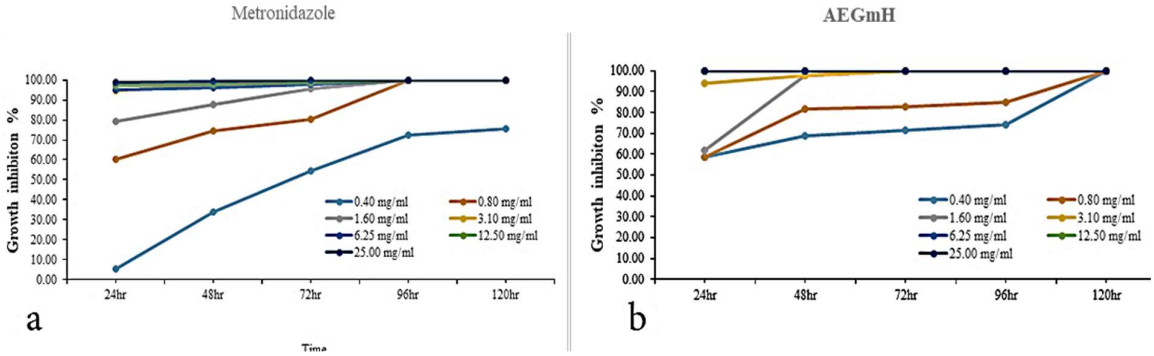

Fig. 2: Histogram showing a comparison between the effect of different concentrations of metronidazole (a) and alcoholic extract of hemolymph of *Galleria mellonella* larva (AEGmH) (b) on growth inhibition rate (%) of trophozoites.

Table 2: The antitrichomonal efficacy of alcoholic extract of hemolymph of *Galleria mellonella* larva and metronidazole against *Trichomonas gallinae* trophozoites (10^4) in comparison to normal control

Treatments	Conc. mg.mL^{-1}	No. of trophozoites				
		24hr	48hr	72hr	96hr	120hr
Control	-	56.40±0.37a	66.90±0.40a	66.90±0.27a	73.20±0.18a	73.20±0.20a
	0.40	53.40±0.33b	44.30±0.30b	30.60±0.17b	20.18±0.16b	17.80±0.18b
	0.80	22.40±0.18d	17.20±0.34d	13.20±0.08d	0.00e	0.00c
	1.60	11.60±0.21f	8.30±0.17f	2.90±0.03f	0.00e	0.00c
Metronidazole	3.10	3.20±0.14g	1.30±0.07h	1.30±014g	0.00e	0.00c
	6.25	2.90±0.20g	2.70±0.11g	1.50±0.20g	0.00e	0.00c
	12.50	1.70±0.10h	1.70±0.13h	0.93±0.7h	0.00e	0.00c
	25.00	0.63±0.15i	0.32±0.10i	0.10±0.01i	0.00e	0.00c
	0.40	23.40±0.24c	20.80±0.31c	19.20±0.07c	18.90±0.23c	0.00c
	0.80	23.40±0.22c	12.50±0.13e	11.70±0.08e	11.10±0.57d	0.00c
	1.60	21.70±0.2e	1.70±0.28h	0.00i	0.00e	0.00c
AEGmH	3.10	3.30±0.17g	1.70±0.11h	0.00i	0.00e	0.00c
	6.25	0.00j	0.00i	0.00i	0.00e	0.00c
	12.50	0.00j	0.00i	0.00i	0.00e	0.00c
	25.00	0.00j	0.00i	0.00i	0.00e	0.00c

Different superscript letters in a column indicate significant differences (P<0.05). Data are presented as mean±SD; AEGmH; Alcoholic extract of hemolymph of *Galleria mellonella* larva.

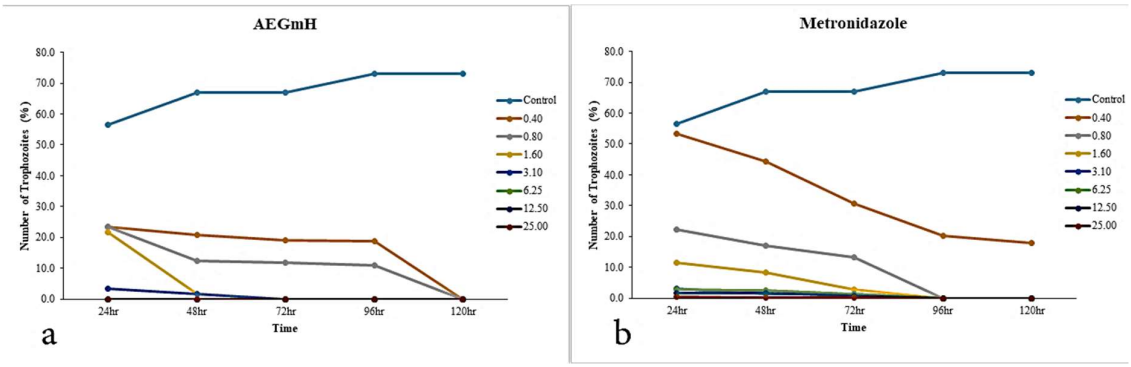

Fig. 3: Histogram showing a comparison between the effect of different concentrations of alcoholic extract of hemolymph of *Galleria mellonella* larva (AEGmH) (a) and metronidazole (b) on the *Trichomonas gallinae* trophozoites numbers (10^4).

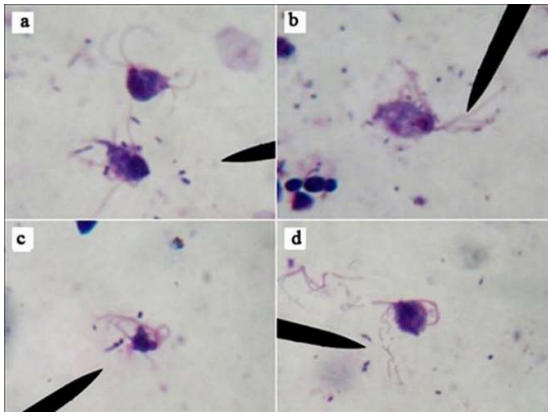

Fig. 4: Giemsa-stained smears of untreated and treated cultures of *T. gallinae*: (a, b) showing ovoid trophozoite with the anterior nucleus and posterior axostyle of trophozoite in untreated culture. (c) Deformity of trophozoite in treated culture. (d) Spherical trophozoite of *T. gallinae* in treated culture (×1000).

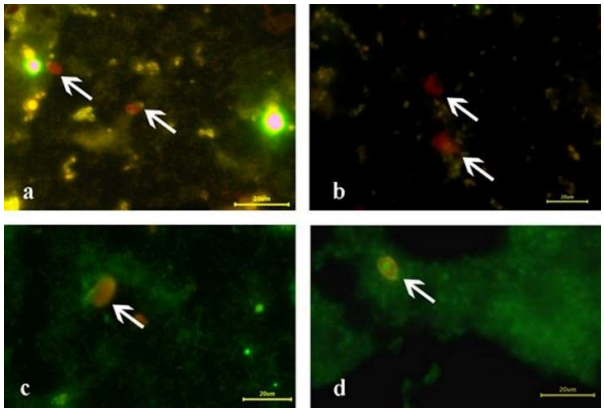

Fig. 5: Acridine orange, fluorescent staining showing brick red dead *T. gallinae* trophozoites after treatment (white arrows, a, b), brownish inactive trophozoites (white arrow, c), and greenish active untreated trophozoites with a round nucleus (white arrow, d; ×400).

dead trophozoites were characterized by a brick red color, potentially containing a yellowish nucleus (Fig. 5a and b). Inactive trophozoites were light brownish (Fig. 5c). Meanwhile, active trophozoites were characterized by yellowish cytoplasm with a bright green nucleus (Fig. 5d).

DISCUSSION

Trichomoniasis is a protozoan disease common in many bird species. The infectious agent mainly inhabits the anterior digestive tract of affected birds. It causes granulomatous lesions that occlude the esophageal lumen, leading to severe starvation and eventually death (Mehmood et al. 2019). Insects and their products are important for preparing drugs in traditional medicine (Sun-Waterhouse et al. 2016). When considering animal-derived therapeutic materials, the chemical research of arthropods, particularly insects, is most compelling for drug discovery. This is because they are more likely to be available in large quantities without possessing a significant negative impact on their native populations.

In addition, there are fewer ethical concerns about treating insects than with vertebrates, and insects are broadly reported to produce a diverse range of chemical compounds (da Silva et al. 2021). This, combined with the fact that insects have historically received less attention than plants, makes insects a suitable taxonomic group for chemical research (Seabrooks and Hu 2017). Anti-microbial peptides are often extracted from living organisms. These peptides are usually short in length (fewer than 50 amino acids), and cell membrane attack is the most common mechanism behind their anti-microbial activity (Wang and Wang 2015). In our study, we used the hemolymph of larvae because AMPs are produced in insects' hemocytes, epithelia, and fat bodies (Bulet et al. 1999).

Through this study, the chromatographic profile showed the high concentration of cecropin A and D. Cecropins anti-microbial peptides were discovered in the hemolymph of *Hyalophora cecropia* and constitute an important aspect of insects' innate immunity (Brady et al. 2019). After bacterial infections, other insects produce them spontaneously (tsetse flies, *Galleria mellonella*, and *Drosophila*) (Patiño-Márquez et al. 2018). Cecropin A and D in several studies showed a reduction in the growth of promasigotes and amastigotes of *Leishmania panamensis* (Patiño-Márquez et al. 2018) and *Leishmania aethiopica* (Pérez-Cordero et al. 2011). Our discussion was restricted to cecropin A and D from all families of AMPs because there is a limited publication on the general characterization of *G. mellonella* hemolymph polar metabolites, which would assist in characterizing its

potential as anti-parasitic, and our opinion was parallel to Killiny (2018).

Moreover, in this study, the high concentrations of alcoholic extract of hemolymph obtained from *G. mellonella* had as great an inhibitory effect on *T. gallinae* as metronidazole (MTZ). Concentrations of hemolymph extracts of 25, 12.5, and 6.2mg/mL eliminated 100% of *T. gallinae* within 24h *in vitro*. The same effect was obtained with MTZ at a 25mg/mL concentration. Meanwhile, both 3.1 and 1.6mg/mL hemolymph extracts eliminated 100% of *T. gallinae* trophozoites after 72h. Therefore, the minimal lethal concentration of hemolymph extract of *G. mellonella* was 1.6mg/mL.

Morphologically, *T. gallinae* trophozoites isolated in the present study from naturally infected squabs were like those described by Abd El-Rahman et al. (2008). Meanwhile, a distorted appearance of *T. gallinae* trophozoites was noted in some treatment media; these trophozoites were small and rounded or irregular in shape. Several research groups have suggested that small spherical forms appear when conditions for parasite survival become unfavorable. For example, Torrent et al. (2012) mentioned that there are two main mechanisms by which peptides perturb protozoan parasites: (1) disrupting the cellular membrane and (2) interfering with key processes in parasite metabolism. Pan et al. (2009) reported the effects of three peptides on *T. vaginalis* and confirmed their results by scanning electron microscopy. They found that the treatment of *T. vaginalis* with these peptides led to cell death, which was preceded by severe swelling and breakage of the outer membrane, in addition to efflux of the intracellular contents.

Furthermore, in the present work, both Giemsa and acridine orange techniques, in addition to the wet mount examination, were used to distinguish living and dead *T. gallinae* trophozoites in treated cultures. Both Giemsa and acridine orange staining techniques produced well-contrasted images of living, damaged, and dead trophozoites. Ferreira et al. (2006) mentioned that acridine orange is an organic compound, cell-permeable, and can interact with DNA by intercalation or RNA via electrostatic attractions. For this, the viability of protozoa was confirmed by fluorescent vital stain acridine orange, and it is widely accepted as an image-enhancing technique. According to the literature, the present work is considered preliminary on the impact of the alcoholic extract of hemolymph obtained from *G. mellonella* on protozoal parasites, particularly *T. gallinae.*

Conclusion

This research concluded that the alcoholic extract of hemolymph obtained from *G. mellonella* is suitable to counter trichomoniasis *in vitro*. It can be used as a new anti-trichomonas drug after further investigation to determine the appropriate therapeutic dose for treatment.

Author's Contribution

Responsible for paper idea and sample recruitment; Tasneme Ghazy, Gehan Sayed, Doaa Farghaly, and Mohsen Arafa. Writing, development, data analysis, biological tests, and revision; Al-Shaimaa Sadek, Doaa Farghaly and Basma Abou- El-Nour. Language editing and formatting the manuscript; Al-Shaimaa Sadek and Doaa Farghaly.

REFERENCES

Abd-El-Motelib TY and Galal B El-G, 1994. Some studies on *Trichomonas gallinae* infection in pigeons. Assiut Veterinary Medical Journal 30: 277-288.

Abd El-Rahman MM, Seddiek SA and Soliman AS, 2008. Some studies on trichomoniasis of pigeons at Qualiobia governorate. Egyptian Journal of Comparative Pathology and Clinical Pathology 21(2): 123-141.

Ageitos JM, Sánchez-Pérez A, Calo-Mata P and Villa PG, 2017. Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. Biochemical Pharmacology 133: 117-138. https://doi.org/10.1016/j.bcp.2016.09.018

Amin A, Bilic I, Liebhart D and Hess M, 2014. Trichomonads in birds–a review. Parasitology 141: 733-747. https://doi.org/10.1017/S0031182013002096

Andrejko M, Zdybicka-Barabas A and Cytryńska M, 2014. Diverse effects of *Galleria mellonella* infection with entomopathogenic and clinical strains of *Pseudomonas aeruginosa*. Journal of Invertebrate Pathology 115: 14-25. https://doi.org/10.1016/j.jip.2013.10.006

Arfin S, Sayeed MA, Sultana S, Dash AK and Hossen ML, 2019. Prevalence of *Trichomonas gallinae* infection in Pigeon of Jessore District, Bangladesh. Journal of Advanced Veterinary and Animal Research 6(4): 549.

Baccega B, Islabão YW, de Mello AB, Martins FO, Dos Santos CC, Ourique AF and Oliveira CB, 2021. *In vitro* and in vivo activity of the essential oil and nanoemulsion of *Cymbopogon flexuosus* against *Trichomonas gallinae*. Avicenna Journal of Phytomedicine 11(1): 32.

Brady D, Grapputo A, Romoli O, and Sandrelli F, 2019. Insect cecropins, antimicrobial peptides with potential therapeutic applications. International Journal of Molecular Sciences, 20(23): 5862.

Bulet P, Hetru C, Dimarcq JL and Hoffmann D, 1999. Antimicrobial peptides in insects; structure and function. Developmental & Comparative Immunology 23(4-5): 329-344.

da Silva MXG, Braga-Pereira F, da Silva MC, de Oliveira J, Lopes SD and Alves RRN, 2021. What are the factors influencing the aversion of students towards reptiles? Journal of Ethnobiology and Ethnomedicine 17: 1-10. https://doi.org/10.1186/s13002-021-00444-1.

Desbois AP and Coote PJ, 2012. Utility of greater wax moth larva (*Galleria mellonella*) for evaluating the toxicity and efficacy of new antimicrobial agents. Advances in Applied Microbiology 78: 25-53.

Dingsdag SA and Hunter N, 2018. Metronidazole: an update on metabolism, structure–cytotoxicity and resistance mechanisms. Journal of Antimicrobial Chemotherapy 73: 265-279. https://doi.org/10.1093/jac/dkx351.

Doshi NP, 2017. Comparison of Papanicolaou and Acridine Orange stains in the Diagnosis of *Trichomonas Vaginalis* Infection in Vaginal Discharge. Annals of Pathology and Laboratory Medicine 4: A750-A754

El-Dirany R, Shahrour H, Dirany Z, Abdel-Sater F, Gonzalez-Gaitano G, Brandenburg K, de Tejada GM and Nguewa PA, 2021. Activity of anti-microbial peptides (amps) against *leishmania* and other parasites: An overview. Biomolecules 11: 984. https://doi.org/10.3390/biom11070984

El-Sayed EMA, 2005. Some studies on trichomoniasis in pigeons in Sharkia province. MV Sc. Thesis, Zagazig Univ, Dept. of Avian and Rabbit Diseases, Egypt.

Farghaly DS and Sadek AM, 2020. Trypanocidal activity of methanol extracts of the hemolymph of *Sarcophaga argyrostoma* larva against *Trypanosoma evansi* infected mice. Veterinary World 13: 1599-1604. https://doi.org/10.14202/vetworld.2020.1599-1604

Ferreira CS, Bezerra RC and Pinheiro AA, 2006. Coloração vital com azul de metileno aplicada a tripomastigotas e epimastigotas de *Trypanosoma cruzi*. Revista do Instituto de Medicina Tropical de São Paulo 48(6): 347-349.

Harrington BJ and Gaydos JM, 1984. Low pH acridine orange stain for trichomonads. Laboratory Medicine 15: 180-182. https://doi.org/10.1093/labmed/15.3.180.

Khatoon R, Jahan N, Khan HM, Rabbani M and Ahmad S, 2014. Evaluation of different staining techniques in the diagnosis of *Trichomonas vaginalis* infection in females of reproductive age group. Journal of Clinical and Diagnostic Research 8: DC05.

Killiny N, 2018. Generous hosts: Why the larvae of greater wax moth, *Galleria mellonella* is a perfect infectious host model?. Virulence 9: 860-865. https://doi.org/10.1080/21505594.2018.1454172.

Kulkarni N, Kushwaha DK, Mishra VK and Paunikar S, 2012. Effect of economical modification in artificial diet of greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae). Indian Journal of Entomology 74: 369-374.

Lacerda AF, Pelegrini PB, de Oliveira DM, Vasconcelos EA and Grossi-de-Sa MF, 2016. Anti-parasitic peptides from arthropods and their application in drug therapy. Frontiers in Microbiology 7: 91.

LaTorre-Snyder M, 2017. Lyophilization: The basics. An overview of the lyophilization process as well as the advantages and disadvantages. Pharmaceutical Processing 32: 1-2.

Löfmark S, Edlund C and Nord CE, 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. Clinical Infectious Diseases, 50(Supplement_1): S16-S23. https://doi.org/10.1086/647939

Maritz JM, Land KM, Carlton JM and Hirt RP, 2014. What is the importance of zoonotic trichomonads for human health? Trends in Parasitology 30: 333-341.

Marchiondo AA, Cruthers LR and Fourie JJ, (Eds.) 2019. Parasiticide Screening: Volume 1: In Vitro and In Vivo Tests with Relevant Parasite Rearing and Host Infection/Infestation Methods. Academic Press.

Mayack C, Carmichael K, Phalen N, Khan Z, Hirche F, Stangl GI and White HK, 2020. Gas chromatography–Mass spectrometry as a preferred method for quantification of insect hemolymph sugars. Journal of Insect Physiology 127: 104115.

Mayack C, Phalen N, Carmichael K, White HK, Hirche F, Wang Y, Stangl GI and Amdam GV, 2019. Appetite is correlated with octopamine and hemolymph sugar levels in forager honeybees. Journal of Comparative Physiology A 205: 609-617. https://doi.org/10.1007/s00359-019-1352-2

Mehmood S, Nashiruddullah N, Ahmed JA and Borkataki S, 2019. Parasitic affections of domesticated pigeons (*Columba livia*) in Jammu, India. Annals of Parasitology 65: 53-64. https://doi.org/10.1016/j.pt.2014.05.005

Meingassner JG and Thurner J, 1979. Strain of *Trichomonas vaginalis* resistant to metronidazole and other 5-nitroimidazoles. Antimicrobial Agents and Chemotherapy 15: 254-57.

Palmas C, Wakelin D and Gabriele F, 1984. Transfer of immunity against *Hymenolepis nana* in mice with lymphoid cells or serum from infected donors. Parasitology 89(2): 287-294.

Pan CY, Chen JY, Lin TL and Lin CH. 2009, In vitro activities of three synthetic peptides derived from epinecidin-1 and an anti-lipopolysaccharide factor against *Propionibacterium acnes*, *Candida albicans*, and *Trichomonas vaginalis*. Peptides 30: 1058-1068.

Park FJ, 2011. Avian trichomoniasis: a study of lesions and relative prevalence in a variety of captive and free-living bird species as seen in an Australian avian practice. Australian Veterinary Journal 89: 82-88. https://doi.org/10.1111/j.751-0813.2010.00681.x

Patiño-Márquez IA, Patiño-González E, Hernández-Villa L, Ortíz-Reyes B and Manrique-Moreno M, 2018. Identification and evaluation of *Galleria mellonella* peptides with antileishmanial activity. Analytical Biochemistry 546: 35-42. https://doi.org/10.1016/j.ab.2018.01.029

Pérez-Cordero JJ, Lozano JM, Cortés J and Delgado G, 2011. Leishmanicidal activity of synthetic antimicrobial peptides in an infection model with human dendritic cells. Peptides 32: 683-690.

Ratcliffe NA, Mello P and Brasileiro C, 2014. Recent advances in developing insect natural products as potential modern day medicines. Evidence-based Complementary and Alternative Medicine 2014: 1- 21. https://doi.org/10.1155/2014/904958

Ratcliffe NA, Mello CB, Garcia ES, Butt TM and Azambuja P, 2011. Insect natural products and processes: new treatments for human disease. Insect Biochemistry and Molecular Biology 41: 747-769.

Reddy KV, Yedery RD and Aranha C, 2004. Antimicrobial peptides: Premises and promises. International Journal of Antimicrobial Agents 24: 536-547.

Samour JH and Naldo JL, 2003. Diagnosis and therapeutic management of trichomoniasis in falcons in Saudi Arabia. Journal of Avian Medicine and Surgery 17: 136-143.

Santos HM, Tsai CY, Catulin GEM, Trangia KCG, Tayo LL, Liu HJ and Chuang KP, 2020. Common bacterial, viral, and parasitic diseases in pigeons (*Columba livia*): A review of diagnostic and treatment strategies. Veterinary Microbiology 247: 108779. https://doi.org/10.1016/j.vetmic.2020.79

Seabrooks L and Longqin HU, 2017. Insects: an underrepresented resource for the discovery of biologically active natural products. Acta Pharmaceutica Sinica B 7: 409-426.

Seddiek ShA, El-Shorbagy MM, Khater HF and Ali AM. 2014. The antitrichomonal efficacy of garlic and metronidazole against *Trichomonas gallinae* infecting domestic pigeons. Parasitology Research 113: 1319-1329.

Shittu OK, Olayemi IK and Omalu ICJ, 2013. Anti-plasmodial properties of methanolic extract of *Musca domestica* maggot on P. berghei–infected mice. International Journal of Biology 2(5): 1064-1070.

Soulsby EJL. 1986. Helminths, Protozoa and Arthropods of Domesticated Animal. 7th. Edition. Bailliere Tindall, London.

Sun-Waterhouse D, Waterhouse GI, You L, Zhang J, Liu Y, Ma L, Gao J and Dong Y, 2016. Transforming insect biomass into consumer wellness foods: A review. Food Research International 89: 129-151. https://doi.org/10.1016/j.foodres.2016.10.001

Torrent M, Pulido D, Rivas L and Andreu D, 2012. Antimicrobial peptide action on parasites. Current Drug Targets 13: 1138-1147.

Xiao X, Wang P, Lin W, Jia J and Chou K, 2013. iAMP-2L: a two-level multi-label classifier for identifying antimicrobial peptides and their functional types. Analytical Biochemistry 436: 168-177. https://doi.org/10.1016/j.ab.2013.01.019

Wang G, Li X and Wang Z, 2015. The anti-microbial peptide database as a tool for research and education. Nuclic Acids Research 44(D1): D1087–D1093.