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THE EFFECT OF MELITTIN FROM THE VENOM OF THE BEE APIS MELLEFERA ON PATHOGENIC PARASITES IN VITRO

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ABSTRACT

Melittin is the major bioactive peptide of honeybee venom, with membrane active biological properties which could lie the foundation for the anti-parasitic effect. The in vitro effect of melittin prepared from the Iraqi and Italian strains of *Apis mellifera* on Entamoeba histolytica cysts, *Giardia lamblia* cyst and Trichomonas vaginalis was evaluated. In addition, a reference preparation of commercial melittin was added. Standardized parasites $1x10^5$ viable trophozoites/mL) were cultured for 24 h in the presence of 0.6 or 0.8 micro mole of melittin. Unless treated, and vehicle, and metronidazole-treated teams were incorporated as controls. The viability of parasites was determined by dye exclusion, hemocytometer and microscopic analysis of motility and morphology. Three biological experiments were independent and each treatment was replicated three times technically. Overall death rates of 0.6 µmol/mL were 70.1% for E. histolytica, 60.7% for G. lamblia and 84.8% for T. vaginalis. At 0.8 µmol/mL, mortality increased to 90.4%, 80.2%, and 90.1%, respectively. The concentration had a significant influence on mortality for all three parasites ($p < 0.001$). The mean activity was the highest for the commercial one and the Iraqi-bee ones produced comparable effects. These results indicate in vitro anti-parasitic activity that is concentration dependent and this activity needs to be verified by mechanistic, cytotoxicity, and in vivo activity studies.

KEYWORDS: Melittin; Apis mellifera; Bee venom; Entamoeba histolytica; Giardia lamblia; Trichomonas vaginalis; Antiparasitic activity.

1. INTRODUCTION

Diseases caused by protozoa with *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis* are still significant and cause intestinal and urogenital disease. *E. histolytica* is the cause of amoebiasis, a disease that can be either entirely asymptomatic with intestinal colonization or one with severe colitis and extraintestinal disease. Drug-screening studies searching for new anti-amoebic agents have proven to be an indicator for the need of new drugs. Same applies for *G. lamblia* which is a major cause of diarrheal disease: experimental studies indicate that decreased susceptibility to metronidazole can be accompanied by phenotypic changes in parasitic attachment and fitness [2]. There are also some differences among *Giardia* strains and clones in their susceptibility to nitroimidazoles, as well as variations in the levels of susceptibility to treatment [3]. In the case of *T. vaginalis*, decreased susceptibility to the 5-nitroimidazole drugs has been reported through surveillance and molecular investigation of several cellular pathways potentially related to resistance have been reported [4, 5]. These observations highlight the need for further study of antiperoxidative drugs that don't work in the same way as traditional nitroimidazole-based drugs.

Bee venom is a complex biological secretion which includes peptides, enzymes and other bioactive molecules. One of the main peptide components of *Apis mellifera* venom, melittin is a 26-amino-acid cationic amphipathic peptide that can be isolated and quantified by chromatographic and mass-spectrometric methods [6]. The biological activity of it is mostly linked to its interaction with lipid membranes. Melittin attaches to phospholipid bilayers, extracts an integral membrane lipid, causes destabilization of the membrane, induces pore formation, disrupts the permeability control and ultimately leads to damage of the cell [7]. The biological activity, membrane directed in nature, encourages the testing of melittin against protozoan parasites which, like many organisms, depend on the integrity and ionic homeostasis of their plasma membranes. However, due to the possibility of melittin acting on mammal cells as well, appropriate concentrations and untreated and vehicle controls need to be used when determining its activity.

Melittin and melittin-derived peptides have already been shown to have antiparasitic activity via previous experimental studies. Melittin has been found to cause cellular changes and kill various life stages of *Trypanosoma cruzi* [8]. It also showed activity against *Leishmania infantum* with parasite damage and modulation of the host-cell response [9]. Furthermore, a cecropin–melittin hybrid peptide showed in-vitro anti-amoebic activity against *E. histolytica* trophozoites [10]. The results favor the antiparasitic activity of melittin; however, more studies are required for the activity of melittin against *E. histolytica, G. lamblia* and *T. vaginalis* both individually and in comparison, with each other.

The aforesaid results, prompted the current study to assess the in-vitro antiparasitic activity of melittin isolated from Iraqi and Italian A. mellifera venom in comparison with a commercial melittin preparation. The viability and mortality of parasites

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were assessed after their contact with melittin at concentrations of 0.6 or 0.8 µmol/mL, under standard incubation in vitro. The hypothesis to be tested was that melittin would result in a concentration-dependent decrease in protozoan viability and may be different depending on the type of parasite or the source of melittin.

2. MATERIALS & METHODS

2.1. STUDY DESIGN AND SETTING

The purpose of this experimental study was to evaluate the in vitro antiparasitic activity of melittin purified from the venom of *Apis mellifera* Synthesized from its Iraqi and Italian strains. A commercially available form of melittin was used as a reference product. The Venom collected were from the apiary at the Faculty of Agriculture at University of Baghdad/ Al-Jadriyah, Baghdad, Iraq and the in vitro assays and parasite culturing under aseptic conditions were performed at the Parasitology Laboratory at Mustansiriyah University/ Baghdad-Iraq.

2.2. CHEMICALS AND REAGENTS

Melittin featured in this research was purchased from Sigma–Aldrich (catalogue No. M2272, St. Louis, MO, USA) with a purity of ≥85%, where the purity was determined by high performance liquid chromatography (HPLC) and this commercial source of melittin was extracted from honeybee venom. Fetal bovine serum, heat in-activated, phosphate buffered saline (PBS) at pH 7.2, penicillin – streptomycin solution, metronidazole and several analytical reagent grade chemicals from Merck (Germany) and Sigma-Aldritch (USA). HPLC-grade acetonitrile (LiChrosolv®, Merck, (Germany) and trifluoroacetic acid were used in purification of melittin.

2.3. SOURCE OF BEES AND VENOM COLLECTION

The venom source was from healthy colonies of Iraqi strain and Italian strain *A. mellifera* kept in the apiary of the Faculty of Agriculture, University of Baghdad. Three colonies were sampled from each strain in an attempt to minimize the colony-specific variation. Bee venom was harvested in early morning hours (06:00–08:00) when collectors (type BV0508) that collect bee-venom electrically from the entrance of each hive were used.

The device stimulated the worker bee into stinging onto a sterile glass collection plate through mild electrical pulses without the loss of its stinger. Collection device was run for 30 minutes in each hive. The deposited venom was left to dry in the dust-free environment and with care, was scraped off from the glass plate by using a sterile stainless-steel blade after collection. Venom was collected from both Iraqi and Italian strains of bees and placed in sterile amber containers and stored at 4°C until it could be purified of melittin and stored at −20°C. Before purification, venom was pooled from different hives of bees of the same strain.

2.4. EXTRACTION AND PURIFICATION OF MELITTIN

Melittin was isolated from the venom of two strains of bees from Iraq and Italy. In brief 100mg of dried crude venom was dissolved in 4mL of sterile ultrapure water for 2 minutes. The solution was centrifuged at 10,000 ×g for 15 min at 4°C, to remove any insoluble material. Afterward, the supernatant was filtered through a 0.22-µm membrane filter.

Melittin was purified by reverse-phase HPLC on a C18 column. Solvents A (ultrapure water with 0.1% trifluoroacetic acid) and B (acetonitrile with 0.1% trifluoroacetic acid) were the mobile phase. The separation was carried out by running a gradient from 10% to 60% acetonitrile (acetonitrile/0.1% TFA) for 30 min at a flow rate of 1.0 mL/min and measuring the peptides at 214 nm.

The peak of melittin was identified by comparing the retention time and ultraviolet (UV) absorption spectra of the peak with that of the commercial reference standard of melittin. The fractions corresponding to this were collected and pooled and lyophilized. Purity was calculated by the integrated HPLC peak area and only the purity of the melittin fractions that achieved purity greater than or equal to 95% were used for the antiparasitic experiments. The purified melittin was kept in sterile, light-proofed containers in a refrigerator at −20°C until they were applied. The purification steps were performed according to the method by Zhou et al. [6] with slight modifications.

2.5. PREPARATION OF MELITTIN SOLUTIONS

Each of the melittin preparations was made to a 10 mmol/L stock concentration in sterile PBS. The solutions were filtered through sterile 0.22-µm syringe filters and aliquoted for single use and stored at −20°C to minimize the number of times they were frozen and thawed. Working solutions were freshly prepared in the respective culture medium used for the parasites to give a final concentration of 0.6 and 0.8 µmol/mL respectively.

Three melittin preparations were evaluated:

1. Melittin purified from Iraqi *A. mellifera* venom.
2. Melittin purified from Italian *A. mellifera* venom.
3. Commercial melittin obtained from Sigma–Aldrich.

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2.6. PARASITE ISOLATION AND IDENTIFICATION

The stools were collected from the patients and new clinical isolate of *E. histolytica* and *G. lamblia* were prepared from the stool samples in the Parasitology Laboratory at Al Kadhimiyah Teaching Hospital, Baghdad, Iraq. *Trichomonas vaginalis* isolates were collected from vaginal samples in which the parasite was detected in the regular examination of the parasites in the working parasitological laboratory.

The parasites were first recognized by using direct wet mount microscopy and their morphology and movement. In addition, those slides were stained with iodine and permanent stain and reexamined for intestinal parasite. Diagnosis of *E. histolytica* was made by using *E. histolytica*-specific antigen test to differentiate it from morphologically similar nonpathogenic *Entamoeba* species. Only that sample with live trophozoites was used for culture which was devoid of fungal and bacterial contamination visually.

2.7. PARASITE CULTURE AND MAINTENANCE

E. histolytica and *G. lamblia* trophozoites were cultured in TYI-S-33 medium containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. The cultures were placed in sterile culture tubes tightly capped and the incubation temperature was 37°C, cultures were subcultured at 72 h intervals.

The trophozoites of *Trichomonas vaginalis* were cultured in Diamond's TYM medium containing the same antibiotics, penicillin and streptomycin, as well as 10% heat inactivated fetal bovine serum. These cultures were cultivated at 37°C and sampled for subcultures every 48 h.

Logarithmic-growth-phase trophozoites were used in all experiments. The parasites were harvested prior to treatment with fresh culture medium after repeated centrifugation at 500 ×g for 5 min and washing twice with sterile PBS. The number of trophozoites was determined with the help of a Neubauer hemocytometer and the suspension adjusted to the concentration of 1×10^5 viable trophozoites/mL. The isolates were used in the experiment if over 95 % of them were viable initially.

2.8. IN VITRO ANTIPARASITIC ASSAY

The antiparasitic assay was carried out on sterile 96-well culture plates. A total volume of 200 µL was filled into each well with 180 µL of a suspension of the standardized trophozoite suspension, and 20 µL of the various melittin working solutions, which resulted in a final melittin concentration of 0.6 or 0.8 µmol/mL.

These experimental groups were used for each of the parasites:

- Control one with the trophozoite and culture medium and untreated methylene blue (inhibition caused by methylene blue itself).
- Vehicle control containing trophozoites and an equivalent volume of sterile PBS.
- Positive control treated with 20 µg/mL metronidazole.
- Iraqi-bee melittin at 0.6 and 0.8 µmol/mL.
- Italian-bee melittin at 0.6 and 0.8 µmol/mL.
- Commercial melittin at 0.6 and 0.8 µmol/mL.

Plates were kept at 37°C for 24 h under the above-described culture conditions for each parasite. Three separate biological experiments were conducted different days under normal conditions, with freshly made parasite cultures and melittin solutions. Three technical wells were used to test each treatment for each independent experiment. The mean of the three technical wells was regarded as one experimental replicate and three independent replicates per treatment group were used.

2.9. ASSESSMENT OF PARASITE VIABILITY AND MORTALITY

After incubation, parasite viability was determined by trypan-blue exclusion, and by direct counting using a Neubauer haemocytometer. Those parasites that did not take up dye and had normal morphology were said to be viable; stained parasites, immotile parasites or structurally disrupted parasites were said to be nonviable.

Motility of trophozoite form of *T. vaginalis* was further evaluated by light microscopy. The organisms with active flagellar movement were considered to be "viable" and the completely immotile organisms were recorded as "nonviable" organisms.

The percentage of parasite mortality was calculated as follows:

$$Parasite\ mortality\ (\%) = \frac{N_c - N_t}{N_c} \times 100$$

where $N_c$represents the mean number of viable trophozoites in the untreated control and $N_t$represents the mean number of viable trophozoites in the melittin-treated group.

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2.10. STATISTICAL ANALYSIS

IBM SPSS Statistics 26.0 was used for statistical analyses. The data presented were the mean ± SD of three independent experiments. Normality of data was tested using the Shapiro-Wilk test and homogeneity of variances was tested using Levene's test.

Two-way analysis of variance was used to test the effect of the different sources and concentrations of melittin on each parasite. When a statistically significant effect was found, Tukey's honestly significant difference post hoc test was employed to make pairwise comparisons. One-way analysis of variance was followed by Tukey test to compare untreated con, Vehicle con, Positive con and Melittin treated group. Where appropriate, percentage data were transformed to meet the criteria for parametric analysis. Statistically significant differences were determined at $P<0.05$ and exact P values were given if possible.

Mean values and the most important comparisons of the means were obtained with estimated 95% confidence intervals (CIs). 95% CIs for the main effects were obtained from the corresponding ANOVA models and 95% Tukey-adjusted pairwise CIs were calculated.

2.11. ETHICAL CONSIDERATIONS

Clinical parasites were isolates from de-identified residual specimens from routine clinical diagnosis. No identifiable patient data was used/accessed. Study was approved by the responsible institutional scientific and ethics committees and conducted according to institutional guidelines.

3. RESULTS

3.1. ASSAY PERFORMANCE AND CONTROL GROUPS

Before treatment, all of the parasite cultures had an initial viability of more than 95%, which was the inclusion criterion. There was no statistical difference between untreated and Vehicle-Control group for any of the three parasites (Tukey test, $p > 0.05$), which indicates that PBS alone had no effect on trophozoite viability.

After 24 h, viable counts in the untreated groups remained at $97.0 \pm 1.0 \times 10^3$ cells/mL for *E. histolytica*, $96.0 \pm 1.0 \times 10^3$ cells/mL for *G. lamblia*, and $96.5 \pm 1.0 \times 10^3$ cells/mL for *T. vaginalis*.

Metronidazole produced mortality rates of 93.3 ± 1.3%, 91.0 ± 1.5%, and 94.0 ± 1.0% against *E. histolytica*, *G. lamblia*, and *T. vaginalis*, respectively. One-way ANOVA demonstrated a significant overall treatment effect for *E. histolytica* [$F(8,18) = 2899.14$, $p < 0.001$], *G. lamblia* [$F(8,18) = 1224.63$, $p < 0.001$], and *T. vaginalis* [$F(8,18) = 1655.59$, $p < 0.001$].

Table 1: Viable trophozoite counts after 24 h of treatment

Parasite	Untreated control	Vehicle control	Metronidazole	Melittin 0.6 µmol/mL	Melittin 0.8 µmol/mL
E. histolytica	97.0 ± 1.0	97.0 ± 0.5	6.7 ± 1.3	29.9 ± 2.2	9.6 ± 1.7
G. lamblia	96.0 ± 1.0	96.0 ± 0.5	9.0 ± 1.5	39.3 ± 2.9	19.8 ± 2.5
T. vaginalis	96.5 ± 1.0	96.5 ± 0.5	6.0 ± 1.0	15.2 ± 2.4	9.9 ± 2.2

The mean numbers of viable $\times 10^3$ trophozoites/mL ± SD. The initial inoculum was 1×10^5 trophozoites/mL. The numbers of experiments used in control and metronidazole were three, but the numbers of melittin in pooled were three at each concentration (three melittin source concentrations).

3.2. CONCENTRATION-DEPENDENT ANTIPARASITIC ACTIVITY

In all three parasites, melittin activity was irrelevant with significance less than 0.001 for each melittin treated group, when compared with vehicle group. Combined results of all three melittin sources showed, treatment with 0.6 µmol/mL produced mortality rates of 70.1 ± 2.2% for *E. histolytica*, 60.7 ± 2.9% for *G. lamblia*, and 84.8 ± 2.4% for *T. vaginalis*. Increasing the concentration to 0.8 µmol/mL increased mortality to 90.4 ± 1.7%, 80.2 ± 2.5%, and 90.1 ± 2.2%, respectively.

The concentration effect was statistically significant for *E. histolytica* [$F(1,12) = 1182.40$, $p < 0.001$, partial $\eta^2 = 0.990$], *G. lamblia* [$F(1,12) = 522.43$, $p < 0.001$, partial $\eta^2 = 0.978$], and *T. vaginalis* [$F(1,12) = 36.79$, $p < 0.001$, partial $\eta^2 = 0.754$].

For every melittin source, 0.8 µmol/mL was more active than 0.6 µmol/mL. Tukey-adjusted comparisons were significant at $p < 0.001$ for *E. histolytica* and *G. lamblia* and at $p < 0.05$ for *T. vaginalis*.

T. vaginalis was the most susceptible parasite (0.6µmol/mL), while *G. lamblia* was least susceptible. Mortality was >88% for *E. histolytica* and *T. vaginalis* at 0.8 µmol/mL for all sources of melittin.

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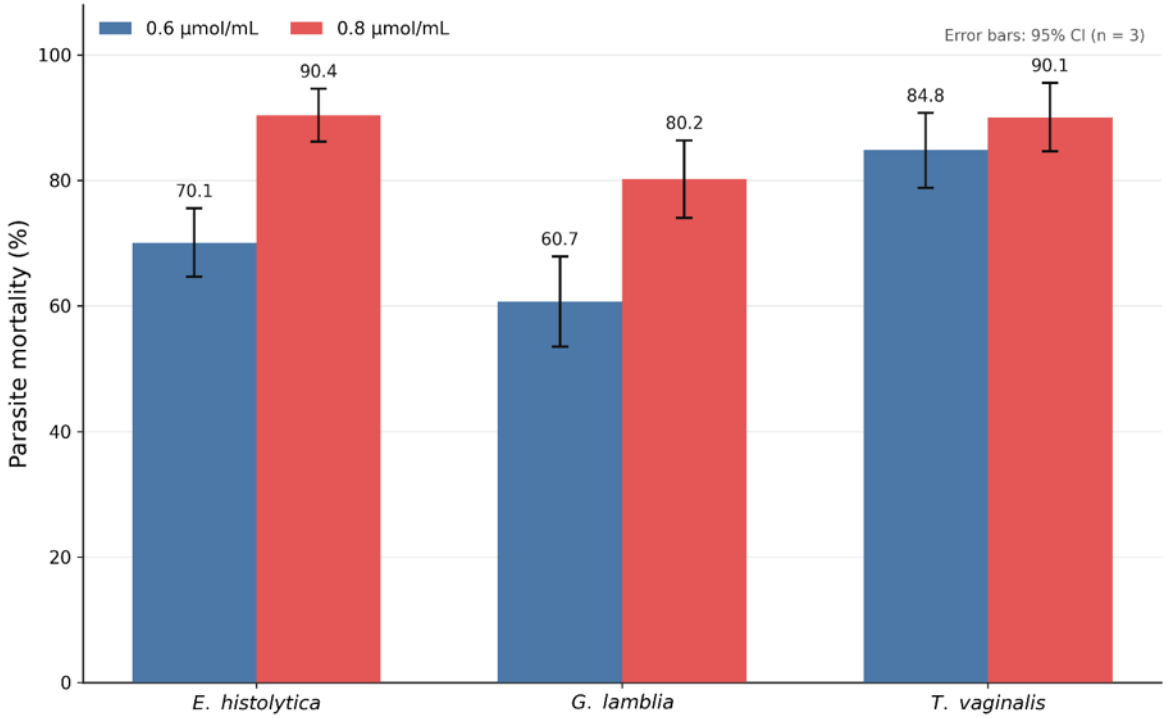

Figure 1: Concentration-dependent mortality of the three parasites following 24 h of melittin exposure. Bars represent estimated marginal means, and error bars represent 95% confidence intervals

Two higher melittin concentrations (0.6-0.8 µmol/mL) were able to increase mortality by 20.3, 19.5 and 5.3 percentage points for *E. histolytica, G. lamblia* and *T. vaginalis*, respectively. The three confidence intervals suggest that the relationship of concentration with the number of deaths was precisely estimated and that it was always positive (i.e., greater than 0) for the three parasites.

3.3. COMPARISON OF MELITTIN SOURCES

Both the concentrations of commercial melittin gave the highest mortality percentage followed by the melittin purified from Iraqi bee strain giving the second highest mortality percentage. The first hen strain provided, the Italian strain, gave a slightly lower mortality for melittin purified from it; but the direction of the concentration response was as in all other sources.

At 0.8 µmol/mL, commercial melittin caused 92.0 ± 1.0% mortality in *E. histolytica*, 82.7 ± 1.5% in *G. lamblia*, and 92.0 ± 1.5% in *T. vaginalis*. The corresponding values for Iraqi-bee melittin were 90.5 ± 1.0%, 80.0 ± 1.5%, and 90.0 ± 1.5%, respectively.

There were no significant differences between the two bee melittins (commercial and Iraqi-bee) in any of the three assay types based on Tukey test at $p > 0.05$. Commercial melittin was more active than Italian-bee melittin against *E. histolytica* at 0.6 and 0.8 µmol/mL ($p = 0.023$ and $p = 0.022$, respectively) and against *G. lamblia* ($p = 0.033$ and $p = 0.034$, respectively). For *T. vaginalis*, the individual pairwise differences among melittin sources did not reach statistical significance after Tukey adjustment ($p > 0.05$).

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Table 2: Parasite mortality according to melittin source and concentration

Parasite	Melittin source	0.6 µmol/mL	0.8 µmol/mL
E. histolytica	Iraqi bee strain	70.0 ± 1.5	90.5 ± 1.0
	Italian bee strain	68.0 ± 1.5	88.7 ± 1.2
	Commercial melittin	72.3 ± 1.2	92.0 ± 1.0
G. lamblia	Iraqi bee strain	60.0 ± 2.0	80.0 ± 1.5
	Italian bee strain	58.3 ± 2.2	78.0 ± 2.0
	Commercial melittin	63.7 ± 1.5	82.7 ± 1.5
T. vaginalis	Iraqi bee strain	85.0 ± 2.0	90.0 ± 1.5
	Italian bee strain	82.7 ± 2.3	88.3 ± 2.2
	Commercial melittin	86.7 ± 1.5	92.0 ± 1.5

Values are the mortality percentage means ± SD of three independent biological replicates. Three technical replications were performed for each biological replicate.

Commercial melittin at 0.8, µmol/mL did not show any significant difference with respect to metronidazole in the activity results against *E. histolytica* ($p = 0.855$) and *T. vaginalis* ($p = 0.809$). The activity of metronidazole was still significantly greater than that of commercial melittin against *G. lamblia* ($p < 0.001$).

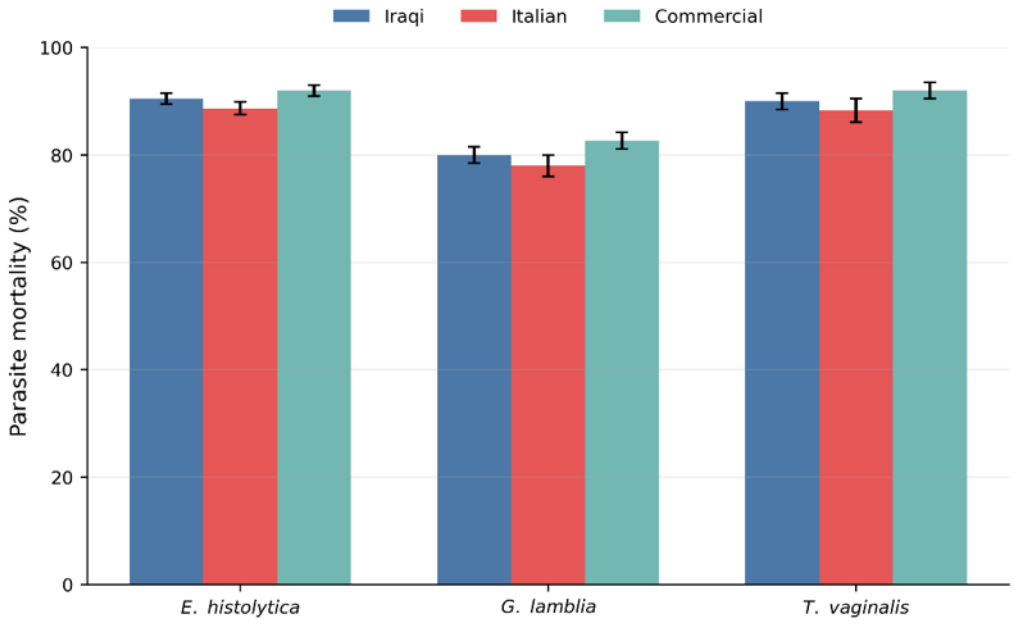

Figure 2: Comparison of melittin purified from Iraqi and Italian bee strains with commercial melittin at 0.8 µmol/mL

3.4. FACTORIAL ANALYSIS OF MELITTIN SOURCE AND CONCENTRATION

Results of the two-way ANOVA indicated that the main effects were significant for source of melittin and concentration for all three parasites. The factor that contributed the greatest variance across the observed data was concentration, especially in the data of *E. histolytica* and *G. lamblia*.

No significant source × concentration interaction was observed, which means as the concentration increased from 0.6 to 0.8 µmol/mL the increase of activities was in the same manner with both the Iraqi, Italian and commercial preparations.

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Table 3: Two-way ANOVA examining melittin source and concentration

Parasite	Effect	df	F	p-value	Partial η^2
E. histolytica	Melittin source	2, 12	13.81	<0.001	0.697
	Concentration	1, 12	1182.4	<0.001	0.99
	Source × concentration	2, 12	0.27	0.77	0.043
G. lamblia	Melittin source	2, 12	11.71	0.002	0.661
	Concentration	1, 12	522.43	<0.001	0.978
	Source × concentration	2, 12	0.12	0.888	0.02
T. vaginalis	Melittin source	2, 12	6.42	0.013	0.517
	Concentration	1, 12	36.79	<0.001	0.754
	Source × concentration	2, 12	0.04	0.958	0.007

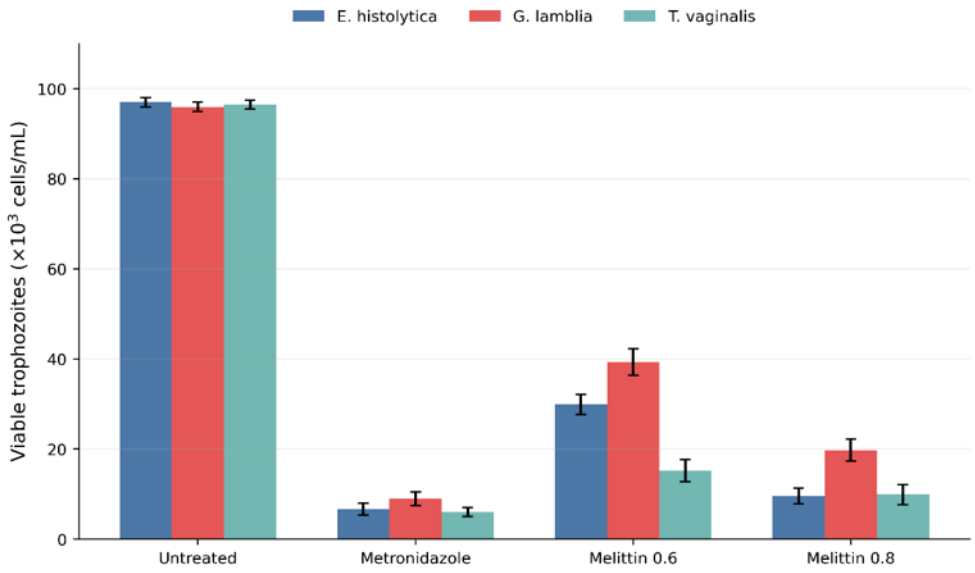

Figure 3: Viable trophozoite counts after 24 h in the untreated, metronidazole-treated, and melittin-treated groups. Melittin values are pooled across the three sources

3.5. MICROSCOPIC FINDINGS

The results of the quantitative viability assessment were supported by microscopic examination. The morphology and movement of trophozoites remained intact after no treatment. When cells were treated with melittin (0.6 µmol/mL), reduced motility, cell rounding, cytoplasmic granularity and appearance of intracellular vacuoles were observed.

At 0.8 µmol/mL these changes were more evident and most of the trophozoites were completely immotile showing loss of normal outline of the cell, membrane disruption and cellular debris.

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Table 4: Qualitative microscopic changes following melittin exposure

Parasite	Untreated control	0.6 µmol/mL melittin	0.8 µmol/mL melittin
E. histolytica	Active amoeboid movement, intact outline, and prominent pseudopodia	Reduced pseudopod formation, slower movement, rounding, and vacuolization	Complete immobility, marked rounding, membrane disruption, and cellular debris
G. lamblia	Preserved pear-shaped morphology, bilateral symmetry, and flagellar movement	Reduced flagellar motility, swelling, and irregular peripheral margins	Loss of symmetry, complete immobility, membrane damage, and fragmented cells
T. vaginalis	Vigorous jerky motility with intact flagella	Marked reduction in motility, rounding, and cytoplasmic granularity	Complete motility inhibition, membrane blebbing, loss of flagellar movement, and fragmentation

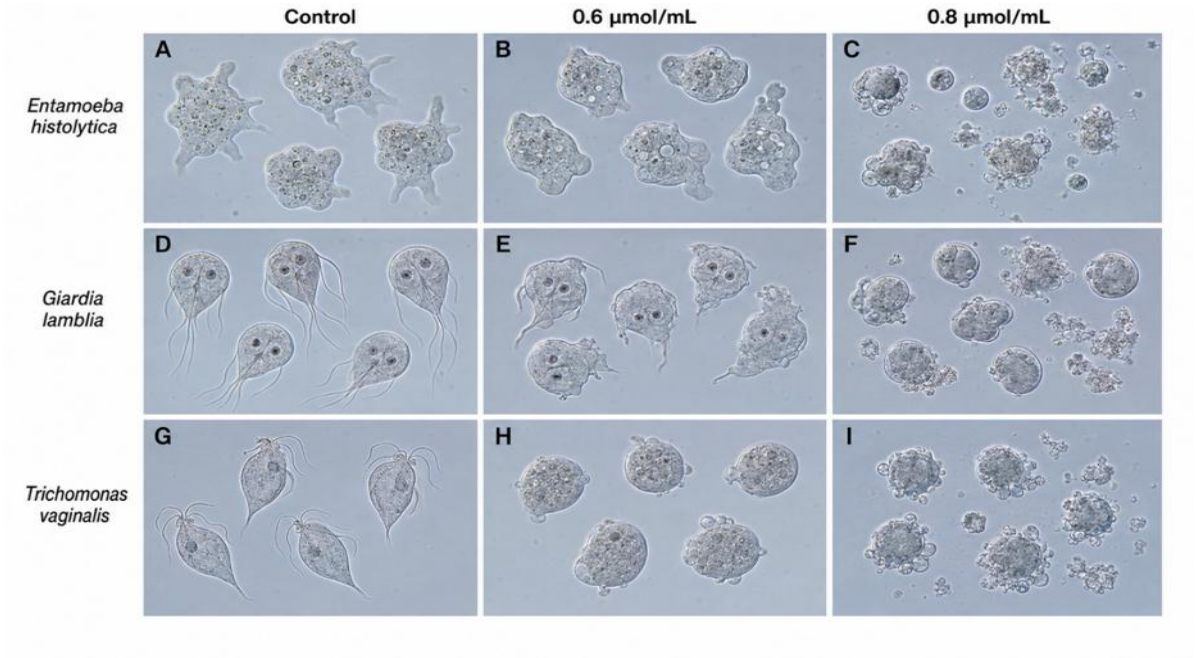

Figure 4: Representative light-microscopy images of the cultured *E. histolytica*, *G. lamblia*, and *T. vaginalis* trophozoites before treatment

4. DISCUSSION

In the present research, the in vitro parasiticidal effect of melittin was found to be concentration dependent on *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis*. As TEM concentrations were raised from 0.6 to 0.8 µmol/mL, death rates subsequently rose to 70.1% to 90.4% in *E. histolytica*, 60.7% to 80.2% in *G. lamblia* and 84.8% to 90.1% in *T. vaginalis*. Most variation was due to concentration; source x concentration interaction was not significant indicating a similar concentration response from three preparations (Iraqi, Italian & commercial) used.

Melittin's membrane-disruptive property could explain the activity observed. Experimental investigations have revealed that melittin is able to aggregate within phospholipid membranes, form transient pores and the detergent like permeabilization of membranes [11,12]. This is in agreement with the microscopic findings of reduced motility, cell rounding, vacuolization, disruption of the cell membrane and fragmentation of cells in treated trophozoites. However, these results are not indicative of apoptosis and necrosis as molecular parameters indicating cell death, leakage of cell membranes and alteration of mitochondrial functions were not taken into account.

Results are overall similar to other finished antiparasitic studies. Bee venom caused evident morphological changes in various developmental stages of *Trypanosoma cruzi* [13]. Moreover, Cecropin-melittin hybrid peptides have been found to disrupt the plasma membrane of *Leishmania donovani* promastigotes [14] and to decrease the viability of tachyzoites of

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Toxoplasma gondii [15]. However, direct comparisons are excluded due to the various species of parasites, types of peptides, concentration, and experimental conditions.

There was species variation in susceptibility. *T. vaginalis* had the lowest susceptibility with 0.6 µmol/mL, while *G. lamblia* had the lowest susceptibility with both concentrations. Possible differences between these reflect differences in membrane composition, surface proteins, glycocalyx structure or cellular stress reactions. Due to the investigation of these factors, their contribution in this system is still speculative and is needed further to be examined on a mechanistic level.

The highest mean mortality was caused by commercial melittin whereas Iraq bee strain-purified melittin closely followed. Preparation from Iraq and commercial preparations showed no significant differences, but the preparation from Italy had slightly less activity against and *G. lamblia*. The composition of bee-venom varies depending on the colony, season, geography and ecology of the area [16]. But only by performing further LC–MS/MS peptide identification analysis and peptide oxidation and purity analysis can it be concluded whether the differences between the preparations are due to bee strain or to the possible presence of melittin isoforms.

The viability of the parasites seemed unaffected by the vehicle control and strengthens the conclusion that the mortality was due to melittin exposure. However, if the concentration of commercial melittin were lowered to 0.8 µmol/mL, it was not possible to observe any statistical difference between this dose and metronidazole for *E. histolytica* and *T. vaginalis*, and metronidazole continues to be the most effective against *G. lamblia*. This is not a statement of therapeutic equivalence since the compounds have not been compared at the same molar concentration, and only three independent replicates were provided.

The drawbacks were small numbers of biological replicates, only 2 concentrations and 1 incubation period and the lack of IC_{50} determinations and/or quantitative mechanistic assays and mammalian-cell toxicity testing. The present in-vitro findings cannot be construed to imply clinical safety and efficacy and the hemolysis and mammalian-cell cytotoxicity caused by melittin can be substantial [17]. Further experiments should be carried out on broader concentrations and chronological scales, by determination of a parasite-selectivity index, in a quantitative study of membrane damage and by determination of efficacy and safety in proper in-vivo models.

5. CONCLUSION

In in vitro conditions, melittin was found to have strong, concentration dependent antiparasitic activity against *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis*. Significantly higher parasite mortality and a significantly decreased number of viable trophozoites were observed when the concentration of melittin was raised from 0.6 to 0.8 µmol/mL. Commercial melittin was more active than the other melittin Ence and had similar effects as melittin purified from the Iraqi *Apis mellifera* strain with respect to the mean. This was slightly weaker for the Italian bee strain preparation of melittin but was consistent across all three preparations and for both dose and concentration.

The greatest susceptibility was found for *T. vaginalis* (0.6 µmol/mL) and the least susceptibility was found for *G. lamblia* at both concentrations. The lack of a significant interaction between melittin source and concentration revealed that raising the concentration resulted in a similar increase in antiparasitic action, from both sources of melittin.

The results of this experiment are preliminary evidence for further research to consider melittin as a potential anti-parasitic peptide. But the findings can only be viewed in in vitro system and cannot be considered as evidence of clinical effectiveness. More studies are needed to identify MINIC, MLIC, mechanism of action of the parasite killing, cytotoxicity tests on mammalian cell models and efficacy/safety tests on suitable in vivo models.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest to disclose.

Declaration Regarding Generative AI and AI-Assisted Technologies in Writing Process

No AI tools were used by the authors during the preparation of this manuscript.

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