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ORIGINAL ARTICLE

Therapeutic Efficacy of Tomex (Allicin) versus Metronidazole in Experimental Giardia lamblia infection: Ultrastructural and Histopathological studies

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ABSTRACT

Background: *Giardia lamblia*, a protozoan intestinal flagellate, is the causative agent of human giardiasis all over the world. The current therapeutic agents have considerable adverse effects or are contraindicated in certain clinical situations and may show failure due to drug resistance. **Aim of the study:** This study was conducted to evaluate the in vivo therapeutic efficacy of Tomex versus metronidazole against giardiasis and determine the histopathological changes of the intestinal mucosa and their impact on the ultrastructure of the pathogenic trophozoites. **Methods:** Fourty-seven Swiss albino mice were divided as following: Group I: 2 non-infected, non-treated mice. Group II: 15 mice, only infected (non-treated). Group III: 15 mice infected with *G. lamblia* cysts and treated orally with Tomex. Group IV: 15 mice infected with *G. lamblia* cysts and treated orally with metronidazole. These mice were subjected to parasitological, histopathological, and ultrastructural studies. **Results:** The patent period, intensity of infection, cyst shedding, and cure rate in groups treated by Tomex revealed a highly significant difference in comparison with the non-treated control and insignificant difference in comparison with metronidazole, a finding that might be beneficial in clinical situations, at least, where current chemotherapy is contraindicated. **Conclusions:** Tomex proved to be injurious to the parasite with evident morphological changes, causing an improvement in the histopathological changes of the intestinal mucosa, rapid clearance of the parasite, and control of giardiasis.

Keywords: Giardia; mice; Tomex; Histopathology; Ultrastructure.

1. INTRODUCTION

Giardiasis, an intestinal infection caused by *Giardia lamblia* represents one of the most common parasitic diseases worldwide [1]. The World Health Organization reports about 400 million new cases of *G. lamblia* infection per year [2]. Transmission of giardiasis usually occurs indirectly following the ingestion of food or water contaminated with cysts, or by person-to-person contact, particularly among individuals living in conditions of poor hygiene and sanitation [3]. *Giardia* trophozoite has an extensive cytoskeleton of microtubules and contractile proteins, including a ventral disc and four pairs of flagellae [4]. Direct damage to the intestinal mucosa by trophozoite attachment is one of the theories that interpret the pathogenesis. The adhesive disc on the ventral surface and its flexibility participate in the mechanism of attachment [5]. Clinical manifestations of giardiasis vary from severe to asymptomatic carrier state and the disease may resolve spontaneously in immuno-competent children, but frequently last for several weeks or months if left untreated [6]. The infection can cause diarrhea, weight loss, dehydration,

abdominal pain, malabsorption, maldigestion, and steatorrhea [7]. Typical treatment for giardiasis is chemotherapy using one or more drugs, predominantly 5-nitroimidazole derivatives such as metronidazole, as a first-line drug. Other nitroimidazoles (secnidazole, tinidazole, and ornidazole), benzimidazoles (albendazole, mebendazole), Furazolidone, Quinacrine and benzimidazole derivatives have been used for giardiasis treatment [8]. Because of the adverse reactions of the current conventional drugs, such as gastrointestinal disturbances, headache, leukopenia, and neurotoxic effects, or contraindication in some clinical situations, or failure due to drug resistance [9], discontinuation of the treatment may be a must, and new effective and safe natural agents [10] have been proposed for treatment of giardiasis. Allium sativum could be considered as an appropriate candidate therapy for the treatment of giardiasis [11]. The anti-giardial efficacy of the garlic has been reported due to the antioxidant effect of its components [12], and rapid reaction of the allicin against some of the parasite proteins [13].

2. METHODS

The experiment complied with the ARRIVE guidelines and was carried out in accordance with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

Source of the parasite: Fresh stool samples were obtained from a heavily infected patient attending the outpatient clinic of the Pediatric Department of Zagazig University Hospital.

Animal groups: Weanling laboratory-bred Swiss albino mice of either sex, aged 3-4 weeks old, weighting 15-20 gm each, free from intestinal parasites and protected against acquisition of any parasitic infection were infected by 100.000 cysts/mouse [14] and divided into the following groups: Group I: Two mice, non-infected, non-treated. Group II: 15 mice, only infected (non-treated). Group III: 15 mice infected with G. lamblia cysts and treated orally with Tomex for 10 days from the 7th day post-infection. Group IV: 15 mice infected with G. lamblia cysts and treated orally with metronidazole (flagyl) for 10 days from the 7th day post-infection.

Ethical consideration: Mice were maintained in accordance with the research protocols following the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals, Faculty of Medicine, Zagazig University. The experimental protocol was approved by the Parasitology department and by local medical ethics committee in faculty of medicine of Zagazig University (Institutional Review Board, IRB, ZU-IRB 1757- (10-4-2016).

Drugs: Tomex tablets 200 mg (Sekem, Egypt), containing (Allicin only) which is considered the active principle of *Allium sativum*, given orally in the form of aqueous suspension, in a single daily dose of 1.04 mg /mouse for 10 days starting from the 7th day post infection and calculated according to the table of Pagets and Barnes as follows (the daily human dose was 400 mg multiplied by 0.0026 = 1.04mg) [15]. Metronidazole (Flagyl) tablets 500mg (EPICO, Egypt) was given orally in aqueous suspension, a daily dose (1.37 mg/mouse divided into 2 doses) for 10 days starting from the 7th day post-infection according to the drug table of Pagets and Barnes [15].

All mice groups were subjected to:

1. Parasitological examination: for determination of patent period, intensity of infection, percentage of reduction at 10, 13 and 17th post infection days and cure rate as described by Blagburn [16].

2. *Ultra-strustural changes:* All infected groups were subjected to TEM study in the Electron Microscope unit at Al-Azhar University, Cairo, Egypt. One mouse from each infected treated, and non-treated group was sacrificed on the 10th day p.i. by cervical dislocation. Specimens from the duodenum and jejunum were rapidly dissected out and fixed in fixative in phosphate buffer for 3 hours. Specimens were then post-fixed in 25% osmium tetroxide in the same buffer at 4°C for two hours, then washed in the buffer. Subsequently, the fixed tissues were dehydrated

in ascending ethanol series and finally embedded in Epon-Araldite. Polymerized tissue blocks were sectioned on the ultra-microtome using diamond knives. Thick plastic sections were stained with toluidine blue in 1% borax and examined by light microscope to define the desired area for ultra-structural examination. Ultrathin sections were mounted on uncoated copper grids and double stained with aqueous uranyl acetate for 10 minutes and Reynolds lead nitrate for 2 minutes. Grids were examined with a JEOL-JEM 1010 Transmission Electron Microscope at an accelerating voltage of 80 KV as described by Smith and Croft [17].

3. Histopathological study: On the 10^{th} and 17^{th} post-infection days, the upper part of the jejunum of the sacrificed mice from non-infected control, infected treated and non-treated groups was removed aseptically, fixed in 10% formalin, processed, stained with hematoxylin and eosin, and examined under the light microscope.

3. STATISTICAL ANALYSIS

Data were presented as mean ±SD. Statistical significance was determined by the paired "t" test for differences within the same group. Differences between groups were determined by a one-way ANOVA and correlation coefficient (r). P >0.05 was considered not significant, P <0.05 was considered significant, and P<0.01 was considered highly statistically significant. SPSS version (14) program for Windows (SPSS Inc., Chicago, IL, USA) was used.

4. RESULTS

shedding (in x40 microscopic field) at the 10^{th}, 13^{th}, and 17^{th} post-infection days was (4.90±1.6), (2.90 ±1.77) and (1.17 ±0.40), respectively.

3. The percentage reduction and cure rate: The percentage of reduction of Tomex-treated group at the 10^{th}, 13^{th}, and 17^{th} post-infection days was (60.89±15.05), (70.9±10.4) and (87.70±3.65), respectively with a highly significant difference *(P<0.01)*. The percentage of reduction of the metronidazole-treated group was (68.95±8.8), (77.69±5.09) and (90.4

Inoculation of 45 mice with 100.000 living *G. lamblia* cysts/mouse resulted in 100% take-up of infection by recording the presence of cysts and/or trophozoites in the stool by direct iodine stained smears. Shedding of *Giardia* cysts started 4-6 days post-infection.

Parasitological results:

1. Patent period: mice belonging *to* the *Tomex-treated group* had a short duration of excretion of *Giardia* cysts (12.3±3.1) compared to the control infected group (30.1 ± 4.6) with a highly significant difference (P<0.01) in between. But with no significant difference (p>0.05) in comparison with metronidazole-treated group patent period (11.5 ± 3.4).

2. Intensity of infection: mice belonging to *control–infected (group II)* showed no significant (p > 0.05) reduction in the mean count of cyst shedding at 10^{th}, 13^{th}, and 17^{th} post-infection days. The mean count of cyst shedding (in an x40 microscopic field) of *control-infected groups* at 10^{th}, 13^{th}, and 17^{th} post-infection days was (15.60±1.92), (13.00±1.49) and (12.20±1.78), respectively. *Tomex-treated (group III)* showed a significant reduction in the mean count of cyst shedding at the 10^{th}, 13^{th}, and 17^{th} post-infection days. The mean count of cyst shedding (in x40 microscopic field) of *Tomex-treated group* at the 10^{th}, 13^{th}, and 17^{th} post-infection days was (6.1±1.46), (4.4±1.46) and (1.50±0.49), respectively with a highly significant difference (P<0.01). *Metronidazole-treated (group IV)* showed a significant reduction in the mean count of cyst shedding at 10^{th}, 13^{th}, and 17^{th} post-infection days. The mean count of cyst ±3.18), respectively with a highly significant difference *(P<0.01)*.The cure rate of the Tomex-treated group was (92.30 %) while the mice of metronidazole-treated group were completely cured (100 %). Cure rate was measured on 13 mice of each of the treated groups by the absence of cysts/trophozoites in the stool for 3 consecutive days after finishing treatment on days 17^{th}-19^{th} post-infection days and absence of luminal trophozoites in H&E stained jejunal sections of 3 mice/group sacrificed on the 19^{th} post-infection day.

Transmission Electron Microscopic (TEM):

Fig. 1&2 show the normal ultra-structure of the infected control luminal trophozoite. The trophozoite showed a lot of peripheral vesicles beneath the cell membrane, mainly in the dorsal surface and also in the naked area. Such vacuoles were absent beneath the ventral disc and the ventrolateral flange. The cytoplasm appeared dark due to its content of dark granules. The cytoplasm contained in addition, endoplasmic reticulum and randomly distributed microtubules. The two nuclei appeared ovoid with no observed nucleoli. Four pairs of flagellae were found, running in their intra-cytoplasmic course as axonemes consisting of nine pairs of peripheral microtubules encircling one central pair (9+2) and emerging as ventrolateral, posterolateral, ventral, and caudal pairs, the latter being accompanied by special microtubules (the funis). The part of the ventrolateral flange surrounding the ventral disc appeared supported by striated marginal plates. The ventral disc appeared to be composed of layer of microtubules underlying the plasma membrane. The naked area was seen devoid of disc structure.

Fig. 3, 4 & 5 reveal evident changes in the overall morphology and cytoplasm of the luminal trophozoite treated with Tomex at the 10th post-infection day. These included swelling of the cell and distortion in shape with roundish, ovoid, and irregular appearances, cell membrane defects, disappearance of the peripheral vesicles beneath the dorsal plasma membrane, appearance of cytoplasmic protrusions on the surface of the plasma membrane, appearance of vacuoles in the cytoplasm, membranous and lamellar structures in the cytoplasm, and grossing of the endoplasmic reticulum. Additionally, misshaping of the nuclei and swelling of the axonemes were noticed. The structure of the adhesive disc was seriously affected with destruction and internalization of some flagellae.

Fig. 6&7 demonstrate deformed shape with many vacuoles seen inside the cytoplasm of the luminal trophozoite treated with metronidazole at the 10th post-infection day, disappearance of the peripheral vesicles beneath the dorsal plasma membrane and the contents of the cytoplasm were depleted of the endoplasmic reticulum with the appearance of cytoplasmic protrusions on the cell surface. Deformed nuclei with heavy electron dense deposits on the cytoplasm were noticed. Microtubules associated with the ventral disc, the flagellae were notably altered (the parasite appeared as a luminal ghost).

Histopathological results:

Histopathological examination of the jejunal sections of the control non-infected group (I) (*Fig. S1*) showed healthy, normal mucosal epithelial lining and normal villous/crypt ratio 4:1 compared with shortening, broadening and fusion of villi, decrease in the villous/crypt ratio due to crypt hyperplasia along with the infiltration of inflammatory cells in the lamina propria in control-infected group (II) (*Fig. S2*). It was observed that the therapeutic supplementation of Tomex to mice infected with Giardia (group III) at the 10th post-infection day showed an abnormal villous architecture with lymphoid hyperplasia (*Fig. S3*). At the 17th post infection day, Tomex helped in restoring the normal mucosal architecture with increased villi and crypts ratio but had mild inflammation in the lamina propria (*Fig. S4*). Most notably, it was also observed that the therapeutic supplementation of metronidazole to mice infected with Giardia in group (IV) at the 10th post-infection day showed an abnormal villous architecture with increased cellular infiltration in the lamina propria (*Fig. S5*). At the 17th post-infection day, histopathological examination of (group IV) treated with metronidazole revealed almost normal gut morphology like that of normal control mice (*Fig. S6*).

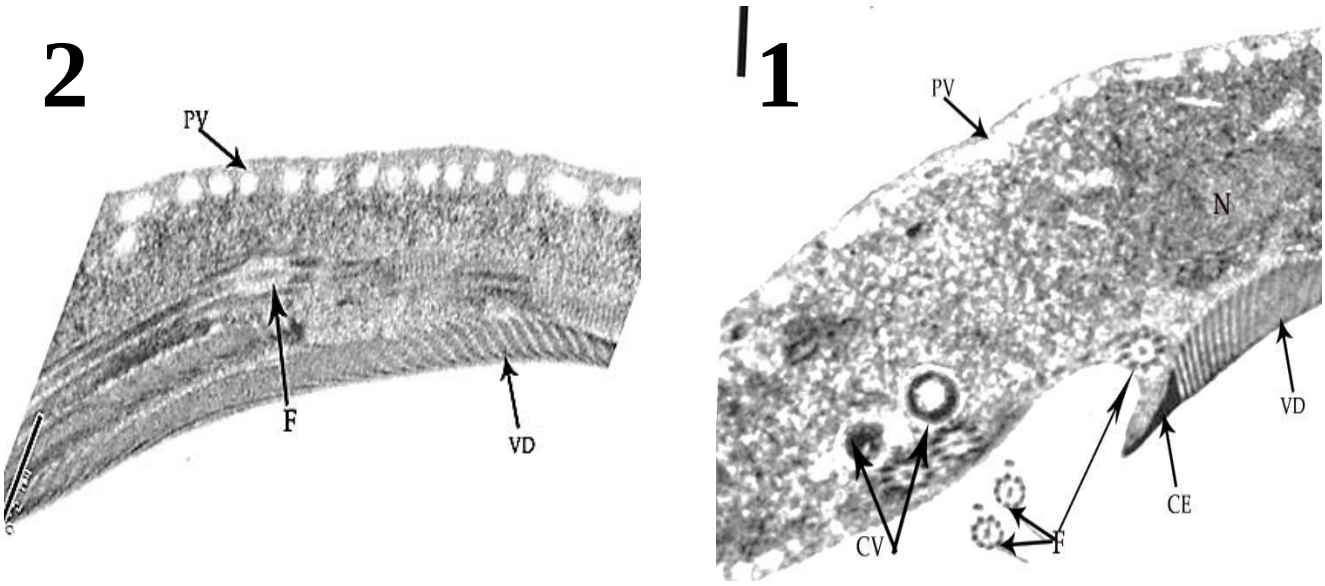
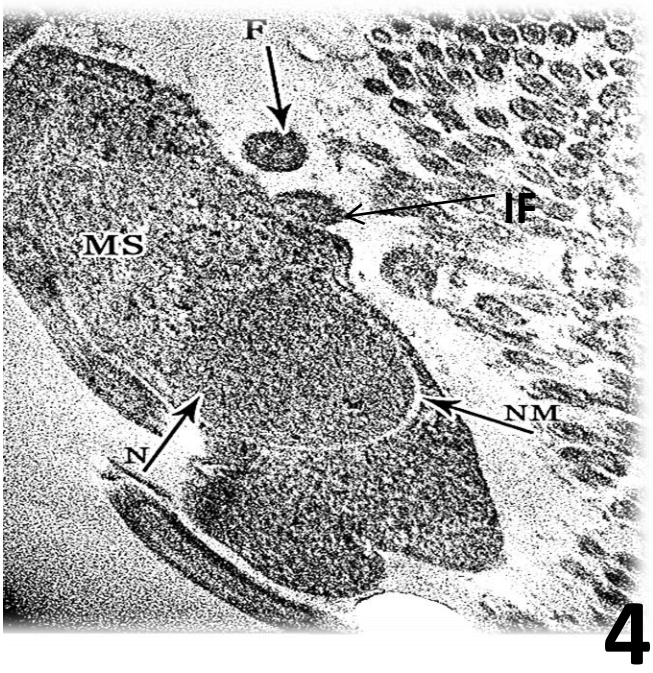
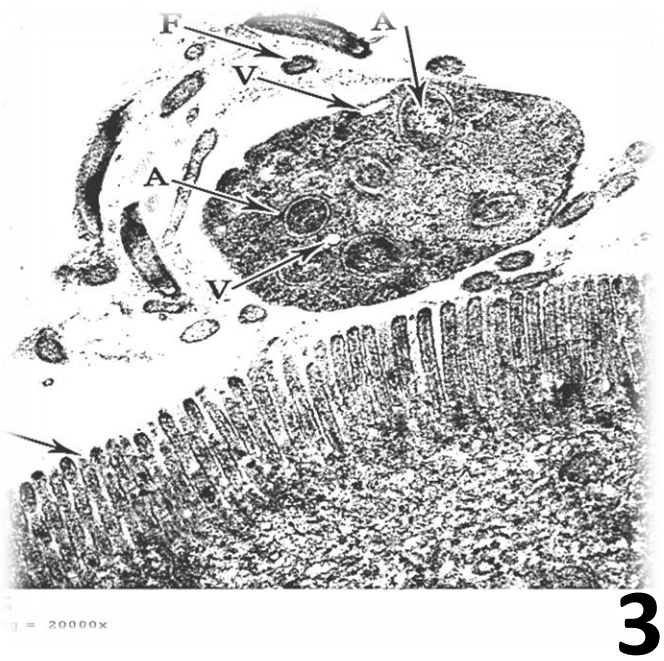


Fig.1&2: A TEM section of a luminal *G. lamblia* trophozoite from a control, infected non-treated mouse showing a normal convex dorsal surface with normally distributed peripheral vesicles (PV), normal appearance of cytoplasmic vacuoles (CV), normal shape of the nucleus (N), normal pattern of flagellar microtubules (9+2) and normal ventral disk microtubules (VD) with preserved caudal edge (CE).





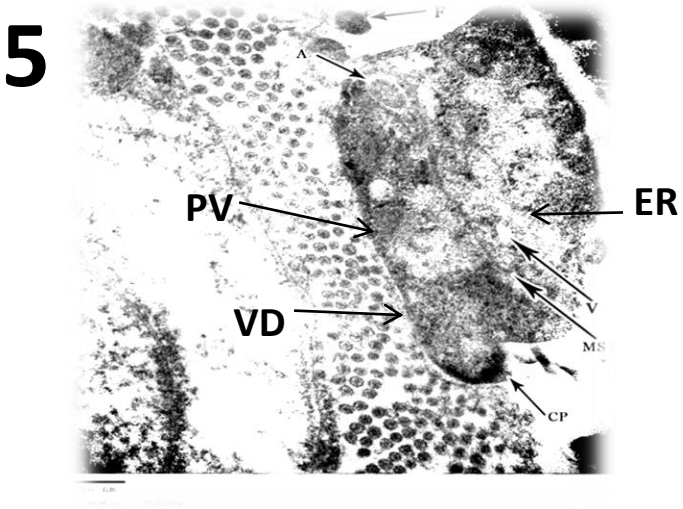
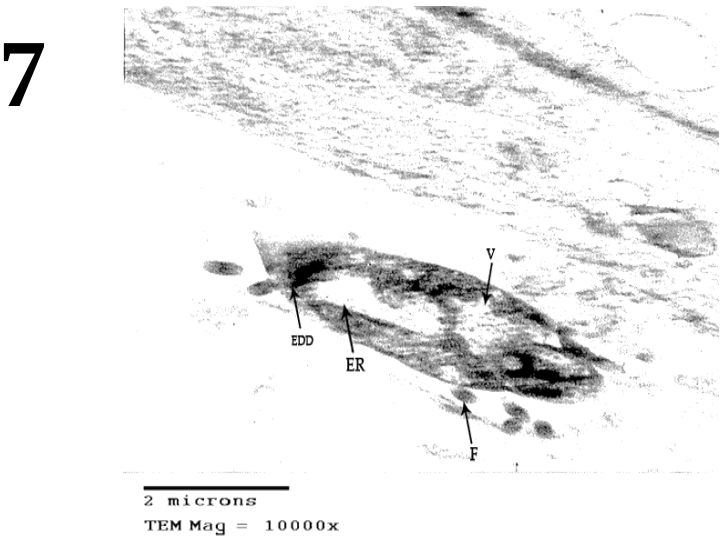


Fig. 3, 4 &5: ATEM section of luminal *G. lamblia* trophozoites from Tomex-treated group showing swelling of the cell with an irregular appearance and disruption in the cell membrane (CM). Swelling of the axonemes (A) with increased distance between the microtubules and the surrounding membrane was noticed and the flagellar microtubules (F) were destructed. Disappearance of the peripheral vesicles (PV) beneath the dorsal plasma membrane and appearance of intra-cytoplasmic vacuoles (V) were noticed. Appearance of the membranous structures (MS), misshaping of the nuclei (N), appearance of nuclear membrane (NM), internalization of some flagellae (IF) and grossing of endoplasmic reticulum (ER).



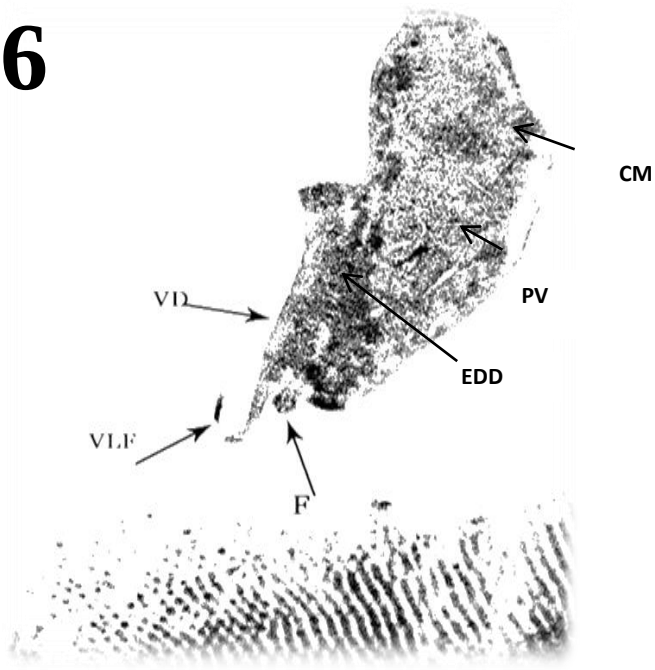


Fig. 6 & 7: A TEM section of luminal *G. lamblia* trophozoites from the metronidazole-treated group showing disruption in the cell shape, appearance of cytoplasmic protrusions (CP), appearance of intra-cytoplasmic vacuoles (V), electron dense deposits (EDD) on the cell surface, distension of the endoplasmic reticulum (ER), destruction of microtubules of flagellae (F) and the parasite appeared as a luminal ghost. Destruction of microtubules of the ventral disc (VD), destruction of the shape of ventro-lateral flange (VLF) and disappearance of peripheral vesicles (PV) were noticed.

5. DISCUSSION

Clinical failures and adverse effects of antibiotics have inspired scientists to look for alternative phytotherapeutic agents for giardiasis [18]. Garlic (Allium sativum) has been used by humans for many years due to its numerous health benefits. It is not only antibacterial, antiviral, antifungal, and anti-protozoan, but also it has beneficial effects on human immune systems [19]. Most of these properties of garlic are attributed to Allicin, its effective substance [20]. Therefore, the present study was designed to assess the efficacy of Tomex treatment on experimental giardiasis.

In the present study, the Tomex-treated group showed significantly less patent period, a decrease in the intensity of infection and a significant increase in the percentage of reduction of cyst shedding at all of the 10^{th}, 13^{th}, 17th post-infection days in comparison to the control-infected group ($P<0.01$). On the other hand, no significant difference was found between results of Tomex-treated group in comparison with metronidazole ($P>0.05$). At the end of treatment Tomex-treated group achieved 92.3 % cure rate, with no significant difference with metronidazole-treated group, which achieved complete cure (100%).

As regards Tomex, our parasitological results were in agreement with Sanad and Al-Ghabban [21], who reported that fresh chopped garlic caused a significant reduction in the intensity of infection and an increase in the percent reduction of cysts shedding in comparison to the infected-control group. Similarly, Abid AL-Khfaji [22] reported that the increasing doses of alcoholic extract of garlic cloves were more active in reducing the number of G. lamblia trophozoites in the stool of infected mice than watery extract. The mechanisms by which garlic inhibit G. lamblia is attributed to the sulphur-containing compounds, especially

allicin, that shows a variety of anti-microbial activities [21]. Also, Ibrahim [23] reported that commercially available garlic tablets (Tomex) was similar to metronidazole in inhibiting the motility of the Trichomonas vaginalis in vitro with increasing percentages of immotile trophozoites, with the added advantage of being a natural product containing allicin only which is considered the active substance responsible for garlic's anti-microbial activity [20].

As regards the ultra-structure of luminal trophozoites of infected-treated groups, the present study demonstrated that many ultra-structural changes of G. lamblia trophozoites occurred after treatment by Tomex and metronidazole at the 10th post-infection day.

The evident distortion in the shape of trophozoites noticed in the Tomex and metronidazole-treated groups indicated the diffusion of the treating agents through the cell surface. Distinct disruption was observed in the Tomex and metronidazole-treated groups. This may be due to the capability of Allium sativum organosulfur compounds to interact with the membrane proteins or aminoacids, forming thioallyl compounds, which are considered potentially life threatening for Giardia parasites [24]. Vannier-Santos and de Castro [25] mentioned that grossing of the endoplasmic reticulum of Tomex and metronidazole might reflect its hyperactivity as a response against the injurious toxic effect of treating agents (Tomex and metronidazole). Abodeely et al. [26] declared that a tubular network extending from the endoplasmic reticulum to the peripheral vesicles, carries lytic enzymes to digest its contents. So, increased lytic secretions by endoplasmic reticulum may be another explanation for the disappearance of the peripheral vesicles. Vacuolization of the cytoplasm, a finding that reflects the parasite injury, was detected in Tomex and metronidazole-treated mice. Membranous and lamellar structures apparent in groups treated with Tomex and metronidazole-treated groups indicate a direct parasite lethal effect of the treating agents. In Tomex-treated groups, swelling of the axonemes was observed. Also, flagellae and ventral disc were affected, indicating the lethal effect of Tomex. The misshaping of the nuclei reflects DNA affection. Electron dense deposits were noticed in the cytoplasm of Tomex and metronidazole-treated group, this indicates their lethal anti-giardial effect. Additionally, in the group treated with metronidazole, electron dense deposits were seen on the nuclear membrane, a finding coinciding with Busattia et al. [27].

The results of the present study were in agreement with Harris et al. [28], who studied the effect of in vitro treatment of G. lamblia trophozoites with freezed whole garlic, which resulted in a swelling of the cells, loss of flagellar movement, and fragmentation of the disc due to the direct action of allicin on parasite DNA [29].

Also, Sanad and Al-Ghabban [21] observed that the whole fresh chopped garlic in a dose of 13mg/mouse three times per day in vivo resulted in changes in the overall morphology, such as swelling of the parasite, disappearance of peripheral vesicles, vacuolization, depletion of dark granules in the cytoplasm and grossing of endoplasmic reticulum. Swelling of axonemes and internalization of the flagellae, a finding that interferes with the parasite motility and mucosal attachment, were markedly noticed. Misshaping and faint staining of the nuclei was also observed. The structure of the adhesive disc and the flagellae were rarely affected. Similarly, Argüello-García et al. [11] reported that in vitro incubation of Giardia trophozoites with several thioallyl compounds (TACs) from fresh crushed aqueous garlic extracts (AGEs) led to a complete loss of the integrity of plasma membrane and diffuse destruction of cytoplasmic contents. On the other hand, the authors observed that the cytoskeletal elements of the ventral disc and flagellae remained structurally unaltered.

Regarding metronidazole-treated group, complete distortion of the trophozoites and the appearance of ghosts in this group, were explained by the reduction of nitro group in the drug by electrons from the ferrodoxins in the parasite, with subsequent drug activation and

binding to the DNA molecules of the Giardia trophozoite [30]. The effect of metronidazole on Giardia trophozoite ultra-structure was studied by Campanti and Montero-Leal [8], who observed membranous structures in the cytoplasm and a more rounded appearance of the trophozoite when they were incubated in vitro with 1µ/ml of metronidazole for 6 hours.

As regards our histopathological results, the present study showed no improvement in the histopathological changes at the start of treatment by Tomex (3rd day of treatment), but by the end of treatment (10th day post treatment), either by metronidazole or even Tomex, there was a marked improvement in the histopathological changes. Capasso [31] suggested that the toxic anti-giardial efficacy of garlic in vivo was due to the stimulation of the production of nitric oxide synthase (NOS). NOS is thought to be the ultimate mediator of immune function within the cell and has been shown to be cytotoxic to Giardia [33]. Garlic has been shown to affect the physiology of the gastrointestinal tract. It decreases episodes of diarrhea by relaxing smooth muscles to decrease peristaltic action. This may also indicate the role of NOS, the effector of smooth muscle relaxation [37]. The restoration of villi and crypts to normal morphology in mice treated with Tomex in the present study suggests that this therapy abrogated the Giardia-induced mucosal damage.

6. CONCLUSION

The in vivo anti-giardial efficacy of Tomex has been confirmed in the present study. Tomex readily diffuses into the trophozoite cell membrane, with serious effects on the internal structures, including nucleus, ventral disc and, axonemes. It interferes with the adherence of the trophozoite to the intestinal mucosa, subsequently causing a rapid clearance of the parasite and control of giardiasis. This natural medicinal therapy can be considered as an important reliable line for the treatment of giardiasis, especially when the standard chemotherapeutic agents are contraindicated.

7. RECOMMENDATIONS

Continuation of research is recommended to complete the interpretation of the mode of action of Tomex and other garlic preparations and to quantify and specify the safe therapeutic doses in different clinical conditions.

Conflict of interest

The authors of this manuscript declare no conflicts of interest, and no relationships with any companies, whose products or services may be related to the subject matter of the article.

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Ethical approval

Institutional review boards' approval was obtained.

Statistics and biometry

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