

Therapeutic Efficacy and *in vivo* *Giardia lamblia* Morphological Alterations Induced by Some Natural Medicinals

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Abstract: *Giardia lamblia*, the causative agent of giardiasis is a protozoan intestinal flagellate that infects humans, specially children all over the world. The current therapeutic agents have considerable adverse effects or be contraindicated in certain clinical situations or show failure due to drug resistance. Using a Mouse Model, the intent of the present study was to evaluate the *in vivo* therapeutic efficacy of garlic plant, wheat germ agglutinin WGA and selenium in comparison with metronidazole and to declare their impact on ultrastructure of luminal trophozoites. The patent period, intensity of infection, cyst shedding and cure rate in groups treated by the natural therapeutics 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 at least in clinical situations where current chemotherapy is contraindicated. All of the tested therapeutics proved to be injurious to the parasite with evident morphological changes. While garlic appeared to induce changes that interfere with the progress of trophozoite motility, selenium and WGA seemed to be evidently lethal to the parasite.

Key words: *Giardia lamblia*, garlic, wheat germ agglutinin, selenium, metronidazole, therapeutic efficacy, *Giardia lamblia* ultrastructure

INTRODUCTION

Giardiasis caused by the protozoan intestinal flagellate *Giardia lamblia* is a common diarrheic disease among humans worldwide (Escobedo *et al.*, 2010). Clinical presentation varies from severe disease to asymptomatic carrier state and the disease may resolve spontaneously but frequently lasts for several weeks or months if left untreated (Ali and Hill, 2003). Malabsorption of fat, lactose, vitamin A and vitamin B₁₂ may occur even when the infection is asymptomatic (Lengerich *et al.*, 1994; Faubert, 1996). The pathogenic stage, the trophozoite has an extensive cytoskeleton of microtubules and contractile proteins and includes a ventral adhesive disk and four pairs of flagella (Robert and Jr. Janovy, 1996). Direct damage to the intestinal mucosa by trophozoite attachment is one of the theories put forward to interpret the pathogenesis. The adhesive disk on the ventral surface and the anterior limitation of the ventrolateral flange to the adhesive disk and its flexibility are participated in the mechanism of attachment (Salem *et al.*, 1990). In spite of the recognition of the pathogenicity of *G. lamblia* >50 years, the current therapeutic agents

including metronidazole and other nitroimidazole derivatives mostly have considerable adverse effects or be contraindicated in certain clinical situations or show failure due to drug resistance (Busatti *et al.*, 2009; Escobedo *et al.*, 2010; Tian *et al.*, 2010). In recent years, several natural and phytotherapeutic agents have been proposed for treatment of giardiasis (Shukla *et al.*, 2008; Busatti *et al.*, 2009; Islamova *et al.*, 2010) but still not valid. Although, garlic plant (*Allium sativum*) has been proved to cause *in vitro* loss of flagella movement and cell motility (Harris *et al.*, 2000; Hawrelak, 2003) and Wheat Germ Agglutinin (WGA) has been found to interfere with *in vitro* excystation of *Giardia lamblia* (Meng *et al.*, 1996), nevertheless *in vivo* confirmation still indeterminate. Selenium was reported to have *in vitro* and *in vivo* inhibitory effect on some other protozoan parasites as *Cryptosporidium parvum* (Huang and Yang, 2002) and *Trypanosoma cruzi* (De Souza *et al.*, 2003).

Hoping for a new less problematic treatment for giardiasis particularly in infections out of tune or resistant to other medications, the present research was designed to evaluate the impact of some natural substances represented by garlic, WGA and selenium in comparison

with the chemotherapeutic metronidazole on the course of infection and on the ultrastructure of *Giardia lamblia* trophozoites in experimentally infected mice.

MATERIALS AND METHODS

Source of the parasite: *Giardia lamblia* cysts were isolated from fresh stool samples of an infected diarrheic child attending the Out-patient Clinic of the Pediatric Department, Riyadh Military Hospital, Riyadh city, Saudi Arabia. Isolation of cysts was done after concentration by repeated centrifugation and washing in saline.

Animal groups: A total of 75 weanling laboratory bred Swiss albino mice, 3-4 weeks old, weighed 20 g/mouse and free from parasitic infections were orally inoculated with 10^5 *Giardia lamblia* cysts/mouse (Hill *et al.*, 1983) and maintained as 5 groups, 15 mice each. Group I (control) kept untreated, groups II-V (experimental groups) treated with garlic, WGA, selenium sulfide and metronidazole, respectively.

All procedures including euthanasia procedure were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) and the Ethical Guidelines of the Experimental Animal Care Center (College of Pharmacy, King Saud, Saudi Arabia).

Therapeutic agents: Garlic cloves obtained from the local markets in Riyadh city were used as freshly chopped preparation suspended in tap water and inoculated in a dose of 13 mg/mouse 3 times a day (tds). Wheat Germ Agglutinin (WGA) and selenium sulfide (Sigma, UK) were used as aqueous preparations in a dose of 10 µg/mouse tds and 13 µg/mouse twice daily, respectively.

Metronidazole (Rhone-Poulenc, France) was given as aqueous preparation in a dose of 0.69 mg/mouse twice daily. All therapeutic agents were inoculated orally for 10 days starting from the 7th Post-infection (P.I) day (Hill *et al.*, 1983).

Parasitological study: All groups were subjected for determination of the patent period; intensity of infection, percentage of control in cyst shedding on day 10, 13 and 17 P.I and cure rate as described by Blagburn *et al.* (1998).

Transmission Electron Microscopy (TEM) study: The study was carried out in the Electron Microscopy Unit at King Saud University. Five animals from each of the control and experimental groups were sacrificed on the 10th post-infection day after being anesthetized with chloroform. The upper part of the small intestine was dissected, cut into 1 mm blocks fixed in 2.5% cold buffered glutaraldehyde solution then processed for electron microscopy. Specimens were washed twice in PBS and post-fixed in 1% osmium tetroxide (OsO₄) in phosphate buffer for 3 h at 4°C then washed twice in water and transferred to 1% uranyl acetate in 50% ethyl alcohol for 1 h. Specimens were then dehydrated in ascending grades of ethanol up to 100% and finally embedded in Epon 812 resin. Polymerization of the resin was achieved at 70°C over a period of 12 h. Sections for TEM were cut on the ultramicrotome using a diamond knife and were loaded onto 200 mesh copper grids. They were stained with uranyl acetate and lead citrate (Smith and Croft, 1991) and examined using a Zeiss EM 906 transmission electron microscope.

Statistical analysis: Results were analyzed using the one way ANOVA (SPSS, 1999).

RESULTS AND DISCUSSION

Parasitological study: These are shown in Table 1-4.

Table 1: The patent period of *G. lamblia* infection in different groups

Groups*	Patent period (days) (mean±SD)
I	29.40±3.64 ^a
II	13.80±1.64 ^b
III	13.80±1.92 ^b
IV	14±1.58 ^b
V	12.40±0.54 ^b
Statistical analysis	F = 56.6, p<0.0001

*I: Control non-treated; II-V: Treated with garlic; WGA: Selenium and metronidazole. ^{a, b}A highly significant difference is found between means with no common letter

Table 2: Giardia cyst shedding at different post-infection days in different groups

Groups*	No. of shed cysts (mean±SD**) (Post-infection days)			Statistical analysis
	10	13	17	
I	11.00±2.15 ^{aA}	8.46±2.10 ^{aA}	4.10±1.53 ^{bA}	F = 15.9, p<0.0001
II	6.86±0.37 ^{aB}	4.82±0.46 ^{bB}	1.18±0.37 ^{bB}	F = 249.8, p<0.0001
III	7.24±0.46 ^{aB}	5.66±0.71 ^{bB}	1.68±0.19 ^{bB}	F = 161.4, p<0.0001
IV	4.96±0.32 ^{aC}	3.18±0.25 ^{bC}	0.82±0.33 ^{bC}	F = 229.2, p<0.0001
V	3.70±0.49 ^{aC}	2.32±0.46 ^{bC}	0.27±0.06 ^{bC}	F = 95.5, p<0.0001
Statistical analysis	F = 35.9, p<0.0001	F = 26.2, p<0.0001	F = 20.7, p<0.0001	

*I: Control non-treated; II-V: Treated with garlic; WGA: Selenium and metronidazole. **Mean number in microscopic field ×400/mouse; ^{a, b, c}A significant difference is found between means with no common letter in each row. ^{A, B, C}Significant difference is found between means with no common letter in each column

Table 3: Percentage of control in cyst shedding among different treated groups

Groups*	No. of control in cyst shedding (mean±SD**) (Post-infection days)		
	10	17	13
II	34.96±17.06 ^b	39.21±20.37 ^b	70.14±5.51 ^b
III	32.43±11.46 ^b	30.79±13.37 ^b	53.60±18.80
IV	53.02±12.07 ^a	59.87±12.91 ^a	79.03±6.37 ^{ab}
V	65.89±3.70 ^a	71.93±5.58 ^a	92.40±3.26 ^a
Statistical analysis	F = 8.6, p<0.0001	F = 8.9, p<0.0001	F = 12.2, p<0.0001

*II-V: Treated with garlic; WGA: Selenium and metronidazole. **A significant difference is found between means with no common letter in each column

Table 4: Cure rate among different groups

Groups*	No. of examined on days 17-19 P.I. ^a	No. cured ^b	Cure rate (mean±SD)
I	14	0	0.00±0.00 ⁰
II	14	12	86.67±11.54 ^b
III	14	11	78.33±20.21 ^b
IV	11	8	72.23±25.45 ^b
V	14	13	93.33±11.55 ^b
Statistical analysis	F = 16.2, p<0.0001		

*I: Control, II-V: Treated for 10 days (7-16th P.I) with garlic; WGA: Selenium and metronidazole. ^aAll mice examined for Giardia cysts on days 17-19 P.I and 3 mice from each group were sacrificed on the 19th P.I day for luminal trophozoites in H and E stained jejunal sections. ^bCure determined by absence of cysts and trophozoites at the time of testing. **No significant difference between means having a common letter and highly significant difference between means having no common letter

TEM study: Examination of ultrathin sections (400-500 Å) obtained from control non-treated and treated groups revealed notable differences in ultrastructure of luminal trophozoites.

Control trophozoite (Gr. I): Luminal trophozoite (Fig. 1a-f) showed poorly differentiated cytoplasm with the outer ectoplasm containing a lot of vacuoles beneath the cell membrane mainly in the dorsal surface and also in the Naked area (NK). Such vacuoles were absent beneath the suction disk (VD) and the Ventrolateral Flange (VLF). The cytoplasm appeared dark due to its content of dark staining granules. The endoplasm contained in addition, finely granular free ribosomes, endoplasmic reticulum, randomly distributed microtubules and other cell organelles. The two nuclei appeared ovoid with no observed nucleoli. Four pairs of flagella were found, run their intra-cytoplasmic course as axonemes consisting of nine pairs of peripheral microtubules encircling one central pair and emerge as anterolateral (AF), posterolateral (PLF), Ventral (V) and caudal (CF) pairs, the latter being accompanied by special microtubules (the funis).

The part of the Ventrolateral Flange (VLF) surrounding the suction disk appeared supported by a striated Marginal Plate (MP) and a dorsal fibrous lamella. The suction disc appeared to be composed of a

layer of microtubules underlying the plasma membrane. The Naked area (NK) was seen devoid of disk structure.

Garlic treated trophozoite (Gr. II): Evident changes (Fig. 2a-f) in the overall morphology and cytoplasm were noticed. These included swelling of the cell and striking distortion in shape with roundish, ovoid and irregular appearance, disappearance of the peripheral vesicles beneath the dorsal plasma membrane, appearance of cytoplasmic protrusions on the surface of the plasma membrane, internalization of flagella, appearance of vacuoles in the cytoplasm, membranous and lamellar structures in the cytoplasm, grossing of the endoplasmic reticulum and disaggregation of the ribosomes. In few sections, misshaping of the nuclei and swelling of the axonemes with increased distance between the microtubules and the surrounding membrane were noticed. On the other hand, the structure of the adhesive disk and the flagella was rarely affected.

Wheat germ agglutinin treated trophozoite (Gr. III): A strong impairment of the cell membrane and cytoskeleton of trophozoites were evident (Fig. 3a-c). The changes included disruption of the plasma membrane, extensive outside shedding of vesicles, extensive vacuoles in the cytoplasm, loss of the normal integrity of cell structures including the cytoskeleton, electron-dense deposits on the cell surface, nuclei and other cytoplasmic structures. Death of trophozoites and its transformation to ghosts were mostly apparent.

Selenium treated trophozoite (Gr. IV): Massive changes in the morphology and cell structure (Fig. 4a, b) were detected as distortion in shape, disruption of the cell membrane and shedding of the peripheral vesicles to the outside, appearance of empty vacuoles and lamellar structures in the cytoplasm, fragmentation of adhesive disk and microtubules, appearance of electron-dense deposits in the cytoplasm and nuclei. Fragmentation of trophozoites, death and ghost's appearance were marked.

Metronidazole treated trophozoite (Gr. V): Prominent changes (Fig. 5a-c) were noticed. The cells were swollen with ovoid to roundish contour, cytoplasmic membranous and lamellar structures were abundant, cytoplasm showed depletion of the dark granules and extensive vacuoles, endoplasmic reticulum was distended, nuclei deformed with heavy electron-dense deposits on the nuclear membrane, the cell membrane was disrupted with appearance of cytoplasmic protrusions on the cell surface.

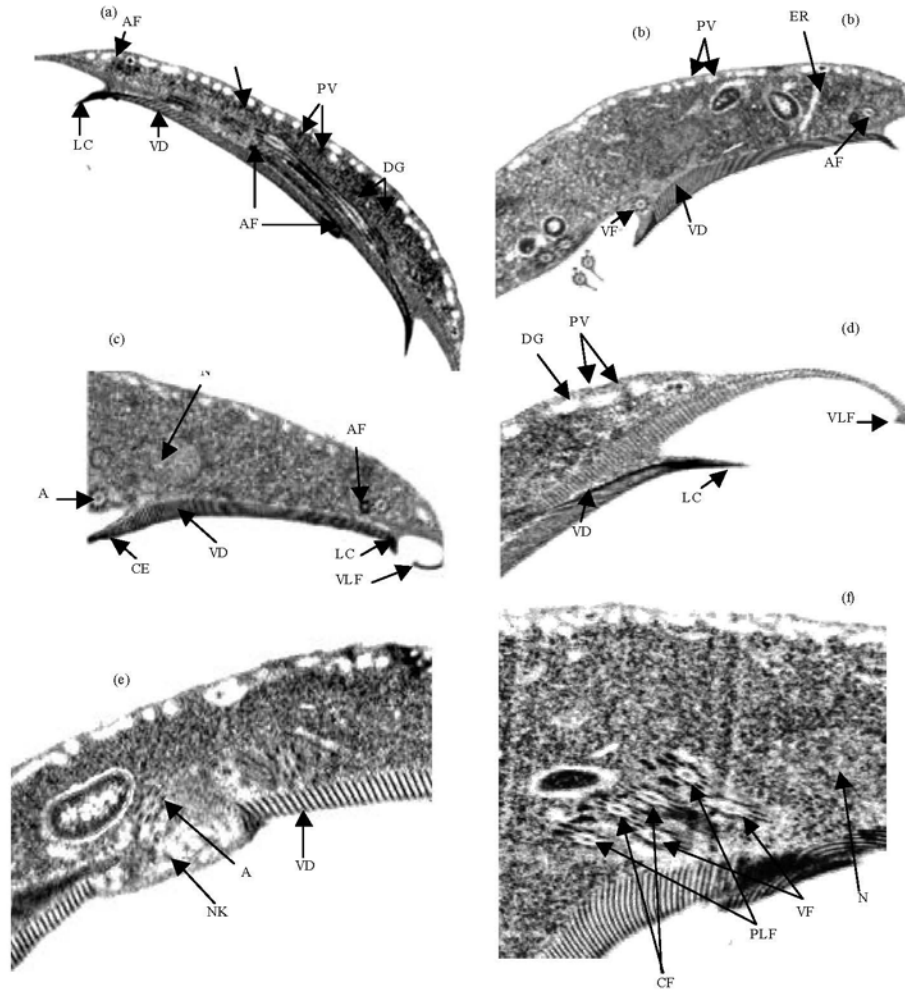


Fig. 1: a-f) TEM sections of luminal *G. lamblia* trophozoites of control non-treated group showed a convex dorsal surface covered from outside by the Plasma Membrane (PM). A lot of vacuoles (Peripheral Vesicles (PV)) found beneath the plasma membrane and in the Naked area (NK). The Cytoplasm (CY) appeared dark due to its content of Dark staining Granules (DG), finely granular free ribosomes, microtubules, Endoplasmic Reticulum (ER) and other cell organelles. The two Nuclei (N) appeared ovoid in the broad anterior half of the cell. Four pairs of flagella were found arising from the kinetosomal complex present between the two nuclei, run their intra-cytoplasmic course as Axonemes (A) consisting of nine pairs of peripheral microtubules encircling one central pair and emerge as AF: Anterior Flagella; VF: Ventral Flagella; CF: Caudal Flagella; PLF: Postero-lateral Flagella. The Ventral Disk (VD) appeared to be composed of a layer of microtubules underlying the plasma membrane with cytoplasmic fold known as the Ventro-lateral Flange (VLF) surrounding the anterior border and sides of the ventral disk, a striated Marginal Plate (MP), Lateral Crest (LC) and Caudal Edge (CE)

In search of new anti-giardial natural substances and focusing on their impact on trophozoite ultrastructure in experimentally infected mouse model and on the course of infection, garlic plant, WGA and selenium were evaluated for their activity in comparison with the chemotherapeutic metronidazole. In the present study, all treated groups showed significantly less patent period, significant decrease in intensity of infection and significant control

in cyst shedding at all of the 10, 13 and 17th P.I. days in comparison with the non-treated control. On the other hand, no significant difference found between results of the tested natural therapeutics in comparison with each other and in comparison with metronidazole. At the end of the treatment period although, none of the garlic, WGA and selenium achieved complete cure but the obtained high cure rates with insignificant difference

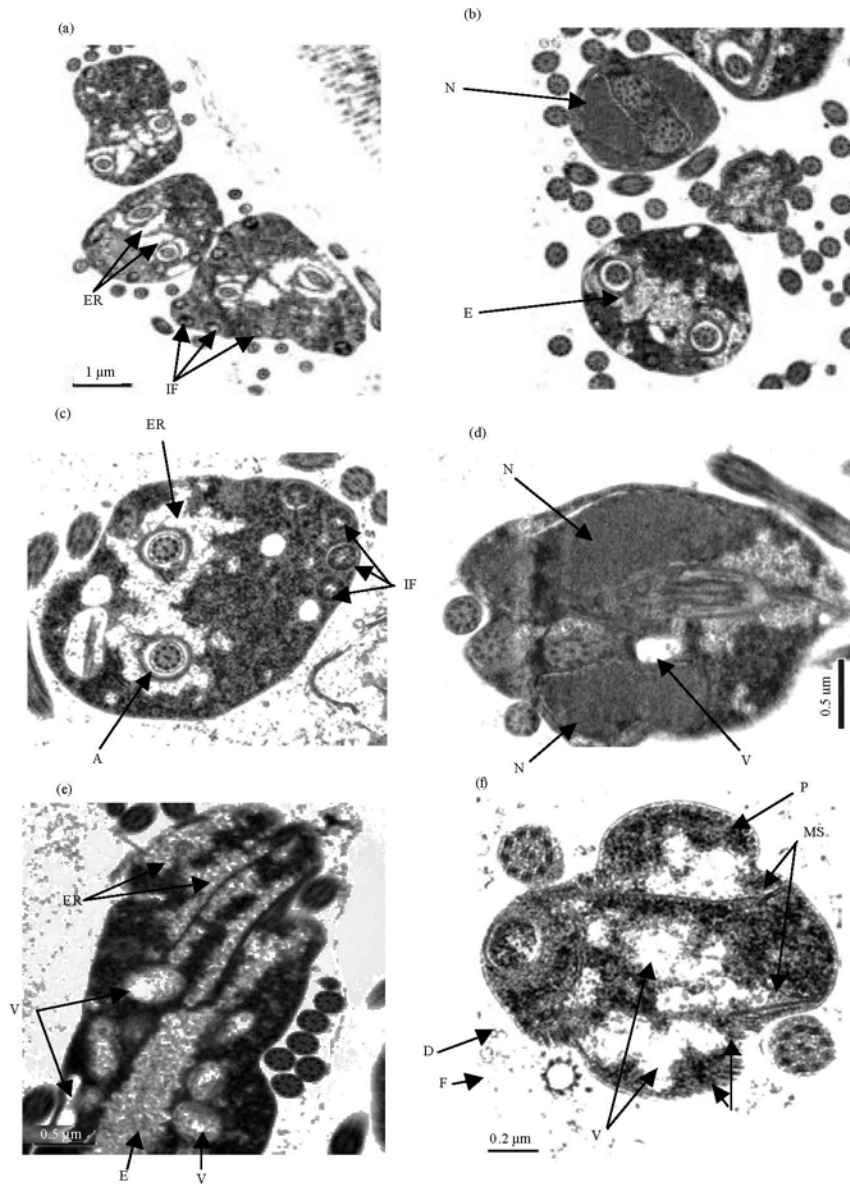


Fig. 2: a-f) TEM sections of luminal trophozoites from garlic treated group showing swelling of the cell with striking distortion in shape with roundish, ovoid or irregular appearance, Internalization of Flagella (IF) and its Crowding (CF) on the outer cell surface, grossing of the Endoplasmic Reticulum (ER), formation of intra-cytoplasmic Vacuoles (V), Membranous and lamellar Structures (MS) in the cytoplasm, appearance of cytoplasmic Protrusions (P) on the cell surface, swelling of the Axonemes (A) with increased distance between the microtubules and the surrounding membrane and misshaping of the Nuclei (N). Disintegration of the disk (VD) and Flagella (F) was rarely seen

in comparison with metronidazole could indicate their potential use as anti-giardial therapeutics that might be beneficial at least in clinical situations where current chemotherapy is contraindicated. The evident distortion in shape of trophozoites noticed in all treated groups indicates diffusion of the treating agents through cell surface and loss of the Osmoregulatory System through

the plasma membrane. While plasma membrane was seen intact in the garlic treated group, distinct disruption was observed in the WGA treated one. This may be due to the capability of WGA to specific binding with surface carbohydrate residues NAG of Giardia as mentioned by Ortega-Berria *et al.* (1994). In both the selenium and metronidazole treated groups,

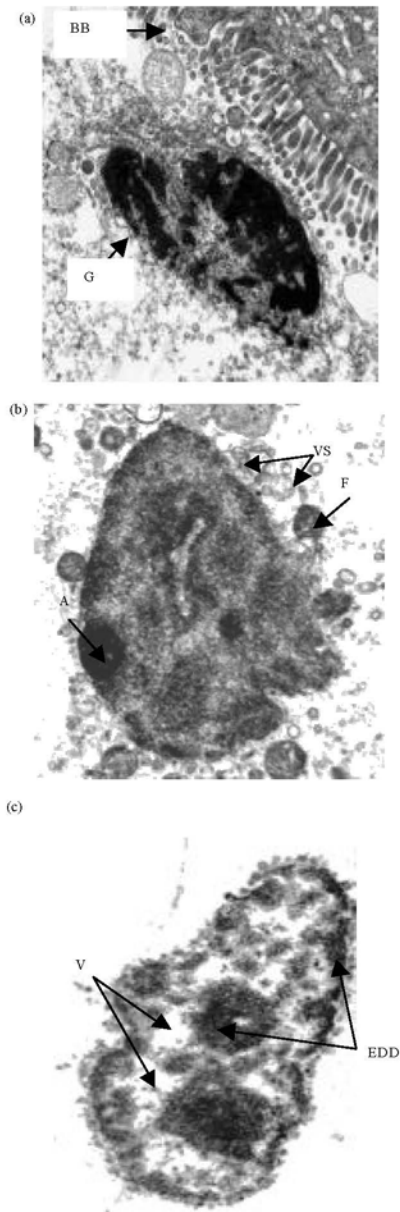


Fig. 3: a-c) TEM sections of luminal trophozoites from WGA treated group showing irregular shape, evident reaction on the cell surface, extracellular shedding of Vesicles (VS), extensive intra-cytoplasmic Vacuoles (V) Electron-dense Deposits (EDD), loss of normal integrity of cell structures and destruction of the Brush Border (BB) of intestinal epithelial cells. Replacement of cell structures by vacuoles and electron dense deposits was prominent (Ghost's appearance)

pronounced destruction of the cell membrane and most of cell components were evident. The selenium-induced changes are most probably due to its direct cytotoxicity

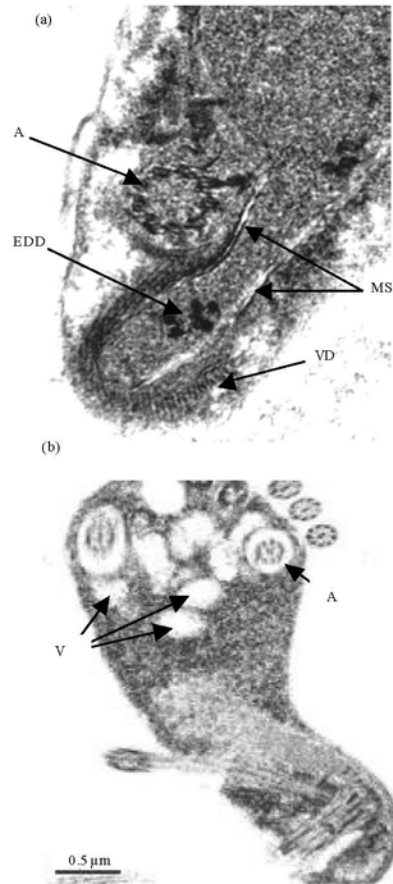


Fig. 4: a-b)TEM sections of luminal trophozoite from selenium treated group showing intra-cytoplasmic Vacuoles (V), loss of normal integrity of cell membrane; Axonemes (A) and microtubules of Ventral Disk (VD) and evident Lamellar Structures (LS) and Electron-dense Deposits (EDD) in the cytoplasm and nuclei. Ghost's appearance (G) and trophozoite fragmentation were prominent

which may be induced by a high inoculated dose. Death of trophozoites by metronidazole was explained by the reduction of the nitro group in the drug by electrons from the ferredoxins in the parasite with consequent drug activation and binding to the DNA molecules of the cell (Gillis and Wiseman, 1996; Uperoft and Uperoft, 1998; Samuelson, 1999). Disappearance of the peripheral vesicles beneath the dorsal plasma membrane in all treated groups may reflect severe inhibition of pinocytosis or complete digestion of its content. Owen (1980) mentioned that the size of these vesicles differs according to their activity for obtaining soluble substances in intestinal lumen. Cell membrane disruption may be the cause of outside shedding of vesicles as seen in WGA-treated

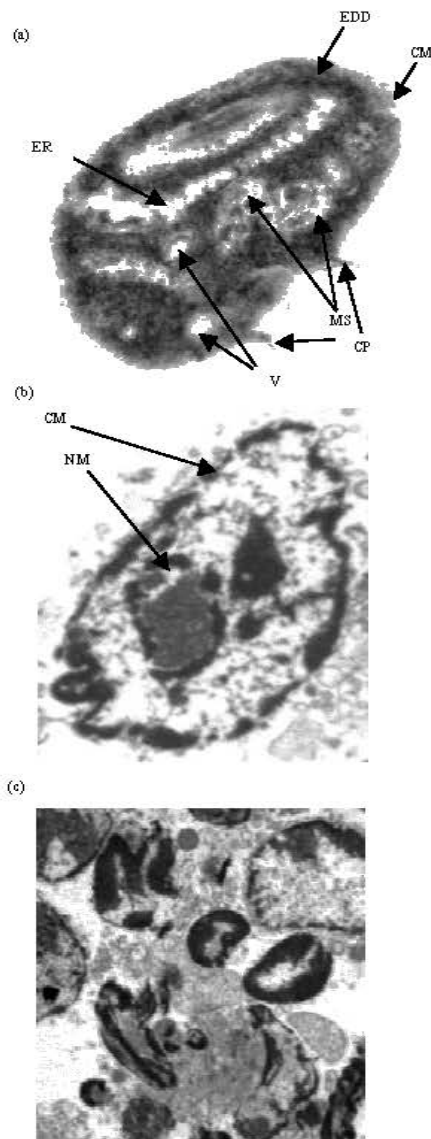


Fig. 5: a-c) TEM sections of luminal trophozoites from metronidazole treated group showing swelling of the cell with disruption in cell shape, appearance of Cytoplasmic Protrusions (CP) on the cell surface together with some destruction of the Cell Membrane (CM), appearance of intra-cytoplasmic Vacuoles (V), Lamellar Structures (LS) and Membranous Structures (MS), distention of Endoplasmic Reticulum (ER), Electron-dense Deposits (EDD) on the Cell surface (CM) and Nuclear Membrane (NM) and replacing cytoplasmic structures including Axonemes (A). Luminal ghosts were plentiful

group. Grossing of the endoplasmic reticulum seen in all treated groups might reflect its hyperactivity as a

response against exposure to the injurious toxic effects of treating agents. Owen (1980) mentioned that a tubular network extending from the endoplasmic reticulum to the peripheral vesicles, carries lytic enzymes to digest its contents. So, suggested increased secretion of lytic enzymes by the endoplasmic reticulum may be another explanation for disappearance of the peripheral vesicles. It has to be mentioned that such grossing of the endoplasmic reticulum was more distinct in the garlic treated group.

Vacuolization of the cytoplasm, a finding that reflects the parasite injury was detected in all treated groups but was more pronounced in WGA treated group. Depletion of the dark granules in the cytoplasm, noticed in all treated groups, reflects the parasite's behavior to obtain energy by consuming stored food due to loss of capability to attach to the intestinal mucosa. Solari *et al.* (2003) mentioned that the cytoplasmic dark granules are stored ferretin and glycogen. Membranous and lamellar structures apparent in groups treated with selenium and metronidazole might indicate the direct lethal effect of such therapeutic agents. The evident injury on the cytoskeleton in the group treated with selenium may be due to induced oxidative stress against the parasite and interaction of free radicals with the micro-tubules. This injury indicates inability of the parasite to attach to the intestinal mucosa with consequent inability to obtain nutrients and to reproduce. In the garlic treated group, swelling of the axonemes with increased distance between the microtubules and the surrounding membrane was detected.

Also, internalization of flagella, a finding that interferes with parasite motility and mucosal attachment was markedly noticed. It is known that the propulsive efforts of the ventral flagella generate a suction force beneath the ventral disk with subsequent attachment. The structure of the adhesive disk and the flagella was very rarely affected in the garlic-treated group and when present may represent a pre-lethal stage. Another finding in the garlic-treated group detected in few trophozoite sections was misshaping and faint staining of the nuclei which may point at the probability of DNA affection.

Electron dense deposits were noticed in the nuclei and cytoplasm of WGA and selenium treated groups. This may reflect their lethal anti-giardial effect. In the group treated with metronidazole, electron dense deposits were in addition seen on the nuclear membrane, a finding coinciding with Campanti and Monteiño-Leal (2002).

The anti-giardial efficacy of garlic in published literature has been reported to be due to anti-oxidant effect of its components (Prasad *et al.*, 1995), rapid reaction of the allicin with the thiol groups in some of the parasite proteins (Rabinkov *et al.*, 1998), a suppressive effect of

the allicin substance on the cystein proteinases of Giardia (Ankri and Mirelman, 1999); the metabolic biproducts of thiosulphinates immediately formed by crushing of fresh garlic gloves (Harris *et al.*, 2000) and direct action of allicin on the parasite's DNA (Miron *et al.*, 2000). Harris *et al.* (2000) found that *in vitro* treatment of *G. lamblia* trophozoites with garlic extract caused swelling of the cell and loss of flagella movement with some alterations in the adhesive disk. Harris *et al.* (2000) and Hawrelak (2003) reported that the strategy of garlic *in vivo* may be due to stimulation of production of nitric oxide synthase at the intestinal mucosa with consequent increase in production of nitric oxide suggested to have a toxic and lethal effect on Giardia. As regards WGA, Meng *et al.* (1996) suggested that its efficacy may be due to capability to agglutinate trophozoites with consequent prevention of reproduction.

They added that WGA is difficult to be digested and absorbed with more activity against luminal parasites. Grant *et al.* (2001) suggested a potential immunostimulant role of WGA and protection of parasite antigens from being digested with their increased rapid handling to the mast cells. The interpretation confirms that WGA induces severe injury on trophozoite surface *in vivo*. Also, the immunostimulant effect of WGA was markedly noticed by the researchers in intestinal sections.

The suggested parasite cytotoxicity in selenium treated group is in agreement with De Souza *et al.* (2003) and Rivera *et al.* (2003) who decided that the effect of selenium varies according to the inoculated dose and that high doses are cytotoxic. The role of selenium as an immunostimulant and in activation of the antioxidant glutathione peroxidase and other antioxidants as vitamin E were also reported (ATSDR, 2003; Rivera *et al.*, 2003).

CONCLUSION

The *in vivo* anti-giardial therapeutic efficacy of all of garlic, WGA and selenium has been confirmed in the present study. Garlic efficacy seems to be due to ready permeability through the cell membrane, interference with trophozoite motility and attachment to the intestinal mucosa and to a less extent its activity against the internal structures including the nucleus. Wheat germ agglutinin efficacy seems to be due to its lethal effect on trophozoites by agglutination in addition to its immunostimulant action. Selenium efficacy is most probably due to direct cytotoxicity and destructive effect on the trophozoite cytoskeleton. Continuation of research is recommended to analyze and quantify the safe therapeutic doses and to complete elucidation of their mode of action.

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