Efficacy of Paromomycin-Conjugated Nano Chitosan in Treating *Entamoeba histolytica* Infection in Mice

Hajar Falih Hishan Aldali [1*], Shatha Khudiar Abbas [1], Ahmed Naji Abd [1]

Abstract

Entamoeba histolytica is an intestinal parasite that causes amebiasis, a disease widespread in developing countries. This parasite primarily attacks the colon, producing ulceration of the colon's intestinal epithelial cells; nevertheless, the infection can spread to the liver, resulting in liver abscesses. The present study aimed to investigate treatment solutions for amebiasis. The chitosan nanoparticles were synthesized using the Ultrasonic method, and the antibiotic paromomycin was conjugated with the nanoparticles. seventy Swiss albino male mice infected with *E. histolytica* were treated with antibiotic-loaded nanoparticles, and the response to treatment was evaluated using liver enzymes (Alkaline phosphatase (ALP), Alanine transaminase (ALT), and Aspartate transaminase (AST). The study's findings revealed that The AST, ALT, and ALP activities increased significantly in the infected untreated group as they were 102.84±8.89, 104.06 ±7.50, and 533.41±21.18 (U/L) for AST, ALT, and ALP respectively; as for the groups that were infected and treated, they showed an improvement and a significant decrease in the concentration of liver enzymes for all treatments. The treatment of infected mice with 75% nano chitosan + 25% paromomycin showed the best therapeutic effect concerning AST and ALP as it minimized the levels of these enzymes as (62.12± 9.46) and (439.4527.43) (U/L) respectively, while for ALT enzyme, the highest significant therapeutic effect was observed in the group that treated with Nano-chitosan only (63.46±8.06) (U/L). The present study represented the efficiency of the prepared nanomaterial in conjugating with the antibiotic in treating mice infected with the parasite, as this was observed through a decrease in the concentrations of the three liver enzymes.

Keywords: Alanine transaminase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Chitosan, E. histolytica, Nanomaterials, paromomycin

Significance | *Entamoeba histolytica* causes widespread amebiasis. Chitosan-paromomycin nanoconjugates effectively treated infected mice, reducing liver enzyme levels, showing promising therapeutic potential.

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Introduction

Intestinal infections with parasites are among the most common infections in developing countries (Duguma and Tekalign, 2023). The parasite *Entamoeba histolytica* is a unicellular protozoon that causes Intestinal amebiasis with an estimated infection of 40,000 - 100,000 deaths worldwide annually and spreads up to 10% of the world's population (Sabeeh *et al.*, 2022; Jasni *et al.*, 2022). It has a global impact, especially in the tropics and subtropics (Shirley *et al.*, 2018)

The life cycle of *E. histolytica* is simple and depends on trophozoite (vegetative stage) and cyst (infective stage), Amebiasis infections are typically transmitted by fecal-oral transmission as the infection occurs after consuming food and drinks contaminated with cysts, particularly in areas with inadequate hand hygiene standards (Sharif and Mohammed, 2022). After the ingestion of the parasitic cyst, the excystation occurs in the small intestine, motile

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trophozoites are released and travel to the colon, and invade intestinal epithelial cells; also, the parasite infection may have concentrated in other and organs like the liver, lungs, and brain causing extra-intestinal amoebiasis (Kantor *et al*., 2018).

The main symptoms of the infection are dysentery, acute necrotizing colitis, toxic megacolon, chronic non-dysenteric colitis, ameboma, and perianal ulceration, with most patients experiencing stomach discomfort, tenderness, and diarrhea (Hamad, 2021; Houpt *et al*., 2016).

Metronidazole has wide lethal action against protozoa and the majority of anaerobic bacteria (Azad *et al*., 2023) and is the initial treatment of choice for invasive amebiasis despite having unwanted side effects (nausea, neurotoxicity, headache, and other unpleasant symptoms) (Sannella et al., 2003; Hillaker *et al*., 2023; Shrivastav *et al.,* 2021; Cuellar-Guevara *et al*., 2019). However, resistance and side effects have led to the need for novel medications. Nanoparticles have gained interest due to their smaller size, biocompatibility, and tissue penetration effectiveness. These materials offer distinct targets and mechanisms of action, making them a promising alternative to metronidazole (Zahra'a *et al*., 2017).

Aminoglycoside antibiotic paromomycin acts against a wide variety of protozoan species Although it is included in the treatment of HIV-infected patients and pregnant women in the first trimester because it is deemed safe in such circumstances, it is typically advised for the treatment of amoebiasis asymptomatic and slightly symptomatic individuals (Kappagoda *et al*.,2011). Chitosan nanoparticles are favoured due to their large surface area, enabling effective and high adsorption capacity for the pollutant in suspension (Sivakami *et al*., 2013; Li *et al*., 2021). Chitosan amine groups efficiently form complexes with anionic polymers and conjugate chemicals due to their positive charge, allowing for good target selectivity and immunological action. Furthermore, chitosan's mucoadhesive properties allow for the absorption of chitosan nanoparticles to particular locations. As a result, chitosan nanoparticles (NPs) are often used to carry medications, DNA, and vaccines in medicine and pharmaceutics. The safe encapsulation of the target molecule by NPs, its transport (with enzyme protection), and proper release at the target site are all crucial phases in the delivery process. The size, stability, binding affinity, and absorption of NPs increase (Lee *et al*., 2023). The aim of the present study was to assess the efficacy of paromomycin-conjugated nano chitosan as a therapeutic agent against *E. histolytica*

Materials and methods

Chitosan powder provided by Avonchem from the United Kingdom (U.K.) was used to prepare Nano chitosan by dissolving 1g of chitosan in 100 mL of deionized distilled water and heating to a temperature of 51 for 60 minutes after the samples were transferred to a sonicator for 30 minutes; Paromomycin (Humatin) capsules manufactured by (Pfizer Pharma PFE GmbH 250 mg, Germany) were diluted in Distilled water (1gm/ 100ml) then put in the ultrasonic bath for 30 minutes, The solution of chitosan nanoparticles was added to Paromomycin solution. It was then incubated in an ultrasonic bath for 15 min. Five solutions were prepared shown in Box 1.

X-ray diffraction (XRD) and Field Emission Scanning Electron Microscopy (FESEM) tests were used to characterize the solution. Five drops from each solution were added to a heated cover slide and imaged.

Parasite Collection

Parasites were collected from stool samples of patients with diarrohea who visited Emam Alli (A) hospital in Iraq from June to July 2022. The direct saline mount and Lugol's iodine wet mount for each stool sample were used to diagnose *E. histolytica* trophozoites and cysts microscopically (40X) (Jassim, 2014).

Animal model

Seventy Swiss albino male mice were used, their age was 6 to 14 weeks, and weighed 25 to 30 g. All mice were housed in hygienic plastic cages that included special mice diets and sterile water. Mice faeces were checked to make sure they did not have intestinal parasites before the experiment, and all groups except the control negative got daily oral cyclosporine (Sandimmune) (0.2 ml/mouse) to suppress their immune systems for 2 days. Then, all groups except the normal control were infected with *E. histolytica* cysts (10^3 cell/ mL).

Study design

The experiment design involved 7 groups each group having 10 mice and all groups (except negative control) were infected with *E. histolytica* trophozoites (1×103 cell/mL)

Control negatives: orally treated with 0.2 ml normal saline only.

Control positive: orally infected with the parasite and did not receive any treatment.

G I: infected with the parasite and treated with 0.2ml of 100% chitosan

G II: infected with the parasite and treated with 0.2ml of 100% paromomycin

G III: infected with parasite and treated with 0.2ml of mixed solution 50% chitosan + 50% paromomycin

G IV: infected with parasite and treated with 0.2ml of mixed solution 57% chitosan + 25% paromomycin

G V: infected with the parasite and treated with 0.2ml of mixed solution 25% chitosan + 75% paromomycin.

Measurement of liver enzymes

The toxicity of the prepared nanomaterials conjugated with the antibiotic was measured by determining the concentrations of liver

enzymes Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), and Alkaline phosphatase in serum using a competitive *in vitro* Enzyme-linked immunosorbent assay (ELISA) kit obtained from AGAPE, India.

Statistical analysis

The data of the present study were expressed as mean value ±SD and the differences between the groups were statistically analyzed by ANOVA. A P value ≥ 0.0001 was regarded as statistically significant.

Results and Discussion

Chitosan Nanoparticle characterization

X-ray Diffraction (XRD) assay

The XRD patterns of chitosan and chitosan-paromomycin (Fig. 1) showed a distinct peak at around 2θ≈20° corresponding to the formation of chitosan crystal structure. Further, adding paromomycin to the chitosan resulted in a broader peak at a slightly similar position (red curve). The latter suggests that the added paromomycin to the chitosan matrix was intercalated within the chitosan matrix, which in turn led to a broader XRD peak at the position mentioned above. This result is in agreement with Shujahadeen et al. (2019), who reported the XRD pattern of Cs and Cs: dextran nanoparticles, the CS has crystalline peaks at the 2θ value. while the peaks were more spacious in the case of Cs: dextran.

Field Emission Scanning Electron Microscopy (FESEM) observations

Fig. 2 shows SEM images of chitosan nanoparticles prepared with a hydrothermal method and deposited on a glass substrate at 40 C. SEM images confirm the different morphology of these NPS. The morphology of these NPs was not uniform and consisted of many small irregular nanoparticles with average sizes ranging from 10 to 60 nm. It was noticed that the particles are spherical or semi-spherical and unite with each other to form particles resembling clusters or cauliflower so that their dimensions reached 500 nm because of the superposition phenomenon, which is a natural phenomenon associated with nanoparticles. In the case of a paromomycin solution, it was noticed that there was a lot of variation in the shape and nanoscale assembly, so that the average particle size did not exceed 25 nm within a Gaussian distribution, as shown in Fig. 2,b; 4.6,c,d,f. A homogeneous mixing of the two materials was noticed indicating the particles' cohesions and adhesion to each other.

Enzymatic changes

The results in Table 1 reveal significant differences in liver enzymes (AST, ALT, and ALP) compared with the control group (P ≥ 0.0001). The AST, ALT, and ALP activities increased significantly in the positive control group (infected, un-treated group) as they were 102.84±8.89, 104.06 ±7.50, and 533.41±21.18 for AST, ALT, and ALP respectively (when P ≥ 0.0001) and for the negative control, which represents the normal (uninfected) group, the enzyme concentrations were the lowest significant compared to the rest study groups (GI, GII, GIII, GIV, and GV) for all the enzymes (55.84 ±9.83, 57.35 ±7.01 and 408.56±19.43 (U/L) for AST, ALT, and ALP respectively (P ≥ 0.0001), This was in the same line with Abas and Elagib (2021), Kurdi (2022) who studied the effect of *E. histolytica* on liver enzymes and found that the concentrations of AST, ALT, and ALP increase significantly with the infection of *E. histolytica*. This increase was due to *E. histolytica* leading to histological lesions in the liver as apoptosis death of cells and changes in biochemical parameters (ALP, AST, ALT) (Zangana *et al*., 2020).

The high levels of these enzymes in the serum enhanced the number of damaged cells caused by apoptosis, most likely because these enzymes are predominantly found in liver cells. However, when the liver is damaged for whatever cause, these enzymes are released into the bloodstream increasing the levels of (AST) and (ALT) enzymes in the blood and signaling liver disease, whereas ALP is a substance found in the bile ducts of the liver, intestine, and bone, Bile duct damage or blockage may occur the concentration of this enzyme increase in the bloodstream (Al-Hamairy *et al*., 2013).

The treatment of infected mice with 75% nano chitosan + 25% paromomycin showed the best therapeutic effect concerning AST and ALP as it minimizes the levels of these enzymes (62.12± 9.46) and (439.4527.43) respectively), while ALT enzyme the highest significant difference was observed in the GI group (63.46±8.06) (U/L) that treated with Nano-chitosan only. The results of the AST enzyme in Table 1 show that the largest significant difference appeared in the GV (62.12± 9.46), which was treated with 75% nano-chitosan+ 25% paromomycin compared to the positive control. This group showed no significant difference from GI (71.08± 11.46) (U/L). The least significant AST level was observed in the GII (87.31±9.44) treated with paromomycin only, while GIII and GV were 71.08± 11.46 and 82.95± 10.15 (U/L) respectively with a significant difference between them and the rest of the study groups.

The ALT enzyme results revealed a significant difference between the study groups. The highest significant difference was observed in the GI group (63.468.06) treated with Nano-chitosan only when compared to the positive control, and the least significant difference was observed in the GII group (93.079.14) that was treated with paromomycin only when compared to the positive control. There was also no significant difference between GI and GIV (68.758.64).

The ALP enzyme results showed a significant difference between the G4 group (439.4527.43) and the other study groups, with this group having the highest significant difference compared to the

Box 1. The formulation of Five solutions

Solutions	Ratio
Nano-chitosan	100%
Paromomycin	100%
Nano-chitosan+ paromomycin	50%+50%
Nano-chitosan+ paromomycin	75%+25%
Nano-chitosan+ paromomycin	25%+75%

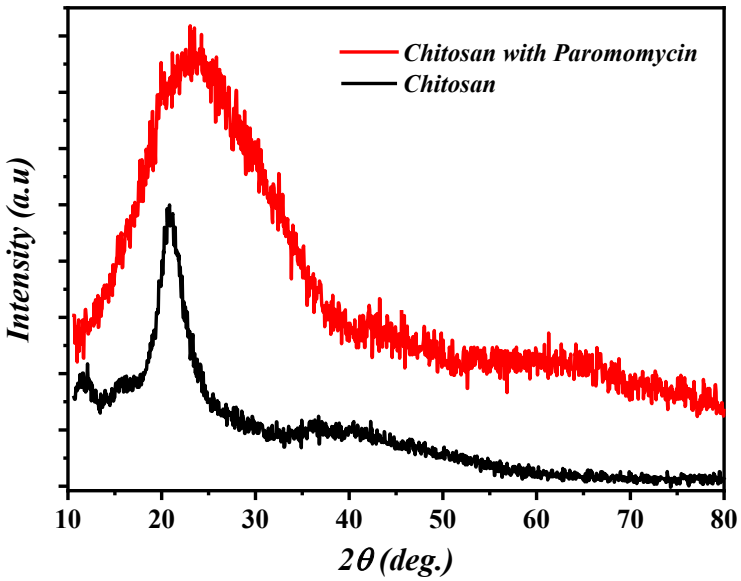

Figure 1. XRD patterns of chitosan and chitosan-Paromomycin.

Table 1. Mean concentration AST, ALT, ALP (U/L) in all studied groups

Groups	AST (U/L) Mean ± StDev.	ALT (U/L) Mean ± StDev.	ALP (U/L) Mean ± StDev
Control negative	f 55.84 ±9.83	e 57.35 ±7.01	d 408.56±19.43
Control positive	a 102.84±8.89	a 104.06 ±7.50	a 533.41±21.18
GI A 100%	e 71.08± 11.46	de 63.46±8.06	b 471.07±24.65
GII B 100%	b 87.31±9.44	b 93.07±9.14	b 476.58±24.48
GIII A50% + B 50%	d 75.73±11.64	c 7.16±78.99	b 483.54±25.35
GIV A 75% + B 25%	ef 62.12± 9.46	d 68.75±8.64	c 439.45±27.43
GV A 25% + B &5%	c 82.95± 10.15	c 84.17±8.89	b 478.78±22.65
(P ≥ 0.0001) Values with a different letter indicate a significant difference			

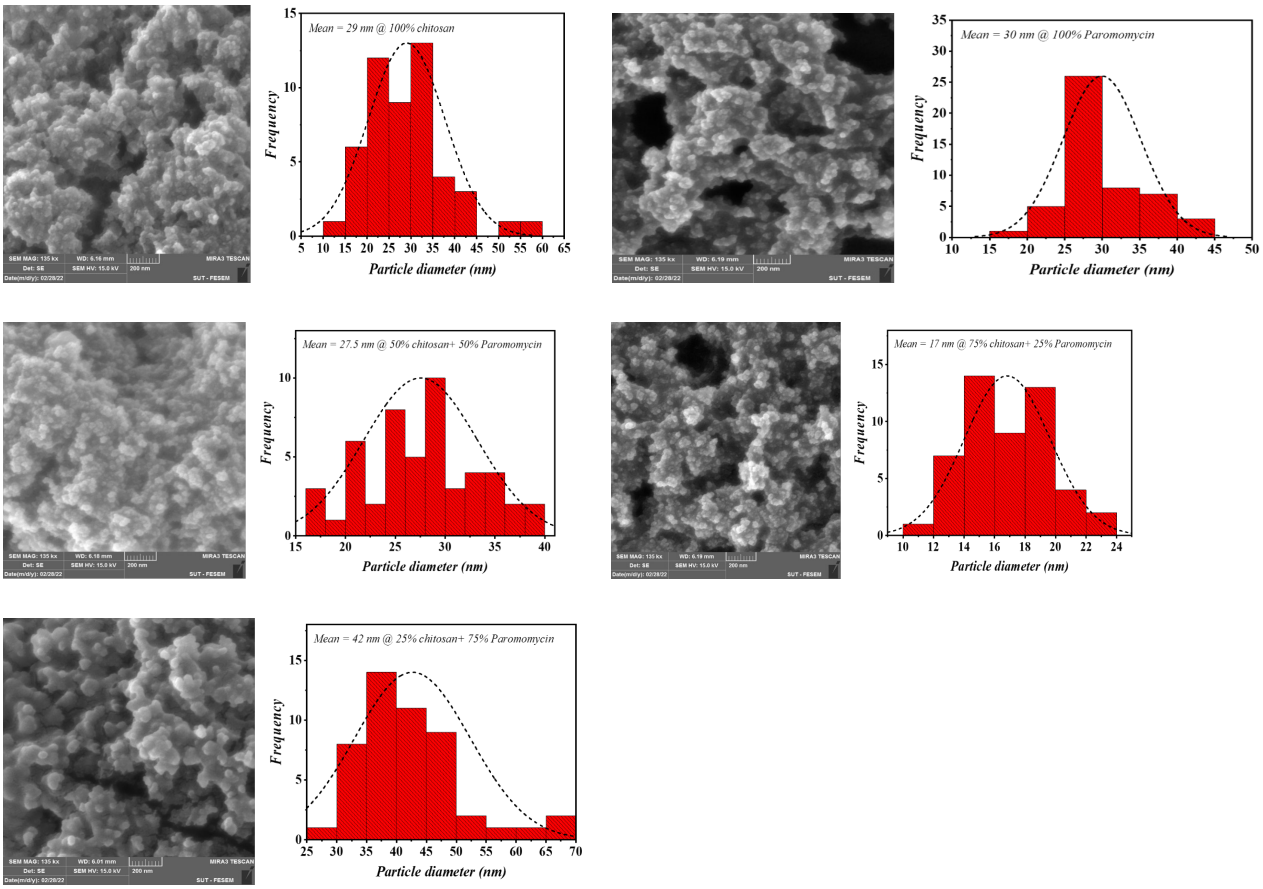

Figure 2. FE-SEM images of CNs conjugated with paromomycin (A: 100% chitosan, B: 100% paromomycin, C: 50% chitosan+ 50% paromomycin, D: 75% chitosan+ 25% paromomycin, E: 25% chitosan + 75% paromomycin).

positive control (104.067.50). There were no significant differences across the remaining research groups, which were as follows: 471.0724.65, 476.5824.48, 483.5425.35, and 478.7822.65 for GI, GII, GIII, and GV.

Many studies that looked for alternative therapeutic agents for E. histolytica represented in herbal extracts, Derivative of Benzothiazole (Sohni et al., 1995; Cimanga et al., 2006; Velásquez-Torres et al., 2023; Ranasinghe et al., 2023; Nsangou et al., 2023). Several nanomaterials-based delivery methods have previously been proven to be useful in the treatment of different intracellular parasite diseases in animal models (Alsharedeh et al., 2023) as in a study conducted by Zahra'a et al. (2017) Which indicated the effectiveness of silver nanoparticles in killing and reducing the vitality of *E. histolytica* trophozoites.

The study's results indicated a significant decrease in the value of liver enzymes ALP, AST and ALT in mice infected with E. histolytica correlated with the use of nanomaterial conjugated with paromomycin against *E. histolytica*. This is consistent with many studies that looked at providing therapeutic alternatives for E. histolytica, which indicated a decrease in liver enzyme concentrations after treatment (Zangana et al., 2020; Al-Kubaissi, 2002).

The therapeutic effect in the treatment groups may be because paromomycin has an activity and may be used in the treatment of asymptomatic infection with *Entamoeba* (Blessmann and Tannich, 2002); also to the capacity of Chitosan (CS) nanoparticles in facilitating the elimination of amoebiasis and CS is widely used for nanoparticles because of its recognized mucosa adhesivity and ability to enhance the penetration of huge molecules across the surface of mucosal membranes CS nanoparticles have been proved to possess great capacity for the association of proteins. Moreover, Chitosan nanoparticles are extensively investigated for the delivery of polypeptides such as snake venom, diphtheria toxoid, and tetanus toxoid (Saber et al., 2017).

From the results, the activity of nanomaterial conjugated with the paromomycin CS nanoparticle was better than the use of paromomycin. This agrees with a study done by Afzal et al. (2019) which concluded that loading paromomycin on nanocomposites increases its therapeutic efficiency as an oral therapy of visceral leishmaniasis also agrees with Jasim (2021) that the use of CS nanoparticles conjugated with paromomycin and estimated that The combined therapy gave best results than single.

Conclusion

In conclusion, paromomycin conjugated with nano chitosan was potentially effective as it significantly reduced the concentrations of liver enzymes concentrations (AST, ALP, and ALT) in Swiss albino mice infected with *E. histolytica*.

Author contribution

H.F.H.A., S.K.A., A.N.A. analyzed data, interpreted results, prepared manuscript, conceptualized and collected data.

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Competing financial interests

The authors have no conflict of interest.

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