International Journal of Pharmacology

*Int. J. Pharmacol.* **2026**; 22(3): 50906
https://doi.org/10.31083/IJP50906

*Research Article*

# Effects of GGsTOP-Silver Nanoparticles on Nymph Viability and Nit Sheath Structure in Treated Head Louse Eggs

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Academic Editor: Mehmet Ozaslan
Submitted: 10 February 2026 Revised: 19 April 2026 Accepted: 15 May 2026 Published: 30 June 2026

**Abstract**

**Background**: The eggs of Pediculus humanus capitis are the most frequent cause of recurrence of lice infection. A few days post-drug treatment in a sub-lethal dose environment, the eggs hatch, and the nymphs are able to survive in this low-drug environment. Female lice lay eggs and cover them with a nit sheath, which is important for the viability of the eggs. The enzyme involved in nit sheath protein solidification is transglutaminase (TG). **Methods**: In this study, the effects of GGsTOP, an inhibitor of transglutaminase, and its silver nanoparticles (AgNPs) formulation on eggs of lice, were investigated *in vitro*. **Results**: The results showed that free GGsTOP and GGsTOP-silver nanoparticles had comparable effects on the eggs, with 20 and 10%, of hatched nymphs, respectively ($p = 0.470$). However, ELISA test showed that transglutaminase activity was lowest in eggs treated with GGsTOP-silver nanoparticle ($1.02 \pm 0.04$ and $0.80 \pm 0.02$), relative to other groups ($p = 0.0001$), indicating that GGsTOP-silver nanoparticle was effective in inhibiting TG, the enzyme involved in nit sheath solidification. This result may imply that even partial inhibition of TG, such as that shown by the effect of free GGsTOP, affects egg viability. **Conclusions**: This study provides valuable insights into a promising novel formulation with the potential to impair louse eggs and disrupt the life cycle of infestations, especially when used at higher doses.

**Keywords:** silver nanoparticles; GGsTOP; eggshell; transglutaminase; Pediculus humanus capitis

## 1. Introduction

Infestation with all species of human lice is a global public health problem. In recent studies, it was estimated that 19% of school children were infected with Pediculus humanus capitis worldwide [1,2] Pediculus humanus capitis is an obligate wingless blood-sucking insect. There are three stages of the life cycle of the head louse, the first of which is the egg stage, while the nymph and the adult form the second and third phases, respectively [3]. The eggs hatch in approximately 5–11 days into nymphs that leave behind an empty eggshell. The nymph resembles the adult louse, but it is smaller in size. Nymphs have three instars. The first stage spends more time feeding than moving around and are also much harder to detect among the hairs, while the second and third stages have the ability to move, but not as fast as the adult. The nymph becomes an adult 7–14 days post-hatching [3].

After fertilization, the adult female begins to lay eggs. The eggs are laid within 1 cm of the scalp surface [4].

The egg is divided into four parts: the nit body, where the embryo develops, the aeropyles, the operculum, and the cement-like substance (cementum). The body of the louse eggshell is fixed basally to the hair shaft with the cementum that surrounds the hair and partially covers the eggshell, referred to as the "nit sheath" [5]. The rigidity of the nit sheath is due to proteins, hyaluronic acid, and possibly lipophilic membranes. The nit sheath is essential for protecting the embryo and supporting its development [5,6].

The nit sheath comprises two large 50-kDa proteins called louse nit sheath proteins 1 and 2 (LNSP1 and LNSP2) [6]. Louse nit sheath protein 1 (LNSP1) comprises more than 85 residues of the amino acid glutamine (Gln), while LNSP2 contains more than 110 Gln residues. For the solidification process, Gln and Lys are linked by the enzyme transglutaminase (TG) in the reproductive system of the female. If this process does not occur, the egg may become suffocated due to the obstruction of its respiratory passages [5].

Head lice infestation is treated using insecticide and non-insecticide products or physical methods. A non-insecticidal product such as IPM/D5 modifies the lipid layer of the exoskeleton [7]. When this layer is disrupted, the louse dies due to loss of control of dehydration [8]. An example of a purely physical treatment is wet combing [9]. Examples of the insecticidal options are over-the-counter pyrethroids which act on voltage-gated sodium channels, thereby causing spastic paralysis in lice [10]. However, head lice have developed resistance to both pyrethrin and permethrin [10,11].

Several studies focused on finding alternative insecticides to overcome the growing problem of the diminished efficacy or complete loss of responsiveness due to resis-

**Table 1. Anti-louse nano drug formulation.**

| Size of AgNPs (nm) | Drug | Method of application | Targeted stages | Effective concentration (mg/L) | Reference |
|---|---|---|---|---|---|
| 50–80 | *Tinospora cordifolia* | Applied to the dorsal side | Adult lice | 25 | [17] |
| 59.52 | *Lawsonia inermis* | Applied to the dorsal side | Adult lice | 80 | [18] |
| 25–110 | *Ocimum canum* | Larval exposure (24 hours contact) | Tick nymphs | 13.85 to 48.86 (Depending on tick species) | [19] |
| 60–150 | *Musa paradisiaca* | Larval exposure (24 hours contact) | Tick nymphs | 50 | [20] |

AgNPs, silver nanoparticles; mg/L, milligram per liter; nm, nanometer.

tance to conventional drugs. In a study targeting lice with some chemical insecticides, Lotilaner achieved the highest rate of lice elimination at a concentration of 10 µM within 3 hours, while ivermectin eliminated 91% of lice within 24 h at a concentration of 100 µM. On the other hand, Pirotoxin did not record any significant result compared to the rest of the insecticides [12]. A study conducted on Varroa destructor mites showed that amitraz had a higher effectiveness of 81.3%, while Cinnamomum verum, Thyme oil, Melaleuca viridiflora, and Syzygium aromaticum showed effectiveness ranging from 67.4% to 73.5% [13]. An *in vitro* study evaluating the efficacy of eight essential oils at a 6% concentration against Dermanyssus gallinae revealed varying activity. Satureja montana oil achieved the highest killing efficacy of 100% upon direct oil application, while Thymus vulgaris oil showed the highest residual effect of 11% [14]. A previous study presented by Omar and Albarrak demonstrated the effects of several essential oils on different stages of tick development and recommended their use as a promising method for tick control, reducing reliance on chemical pesticides and potentially limiting the development of resistance [15].

GGsTOP is a phosphonate-based inhibitor that targets TG [16]. When this inhibitor was injected into gravid lice, it decreased egg-hatching, reduced the number of eggs laid, and caused desiccation of eggs when applied to hair. This implies that GGsTOP blocks the activity of TG and prevents protein cross-linking, resulting in inhibition of nit-sheath solidification. Thus, the eggs fail to adhere to the cementum and may suffocate [5].

Several previous studies employed silver nanoparticles to formulate anti-louse or acaricidal drugs as summarized in Table 1 (Ref. [17,18,19,20]).

As shown by the results of the previous studies, finding a novel drug which has a different target from the conventional drugs, is urgently needed. In addition, it is hypothesized that the Ag formulation of GGsTOP will improve its efficacy on larval viability and TG inhibition. The future implications of this work include the development of a nano-formulation of a non-conventional drug that has a new targeted site in head lice. This target-specific mechanism may reduce lice resistance.

The aim of this study was to determine the effect of GGsTOP silver nanoparticles formulation on egg viability and TG activity in nit sheath. This study hypothesizes that GGsTOP AgNPs will lead to decreased egg viability and reduced TG enzyme activity compared to the control group.

## 2. Materials and Methods

### *2.1 Materials*

GGsTOP (GC16612, molecular weight: 331.26 Da) was provided from Glpbio Technology (Montclair, CA, USA). Silver nitrate and chitosan were brought from Thermo Fisher Scientific (Waltham, MA, USA). Phosphate-buffered saline (PBS; BPBS74-01X) was purchased from UFC BIO, Amherst, NY, USA.

### *2.2 Louse Egg Samples*

Two hundred live head lice were obtained from randomly selected 35 infected school children using a fine-toothed metal comb. Pairs of male and female lice were kept for 48 h in *in vitro* feeding apparatus containing human hair tufts in the upper chamber, and human blood in the bottom chamber, with a nylon parafilm membrane separated the two chambers and a surrounding tube circulating continuous warm water [21]. The laid eggs (aged 0–2 day; not counted) were kept in the hair tufts after removing adult lice [22]. The presence of yolk granules under the stereomicroscope was considered as a sign of viability of the eggs. Nonviable and late-stage eggs were excluded.

This study was approved by the Committee of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University (approval reference no.: 130-22). The informed consent of participating in this study was obtained from the guardians of all children.

### *2.3 Drug-AgNPs Formulation*

Silver nitrate (1 mL, 0.11 M) and chitosan (28.7 mL, 2.5 mg/mL) were dissolved in 1% acetic acid solution and

mixed until a homogeneous solution was obtained. Subsequently, the mixture was transferred to an ice-cold bath, and 3 mL of freshly prepared 0.8 M sodium borohydride was added under vigorous stirring [23].

For the conjugation with GGsTOP, 10 mg of GGsTOP was dissolved in 5 mL of distilled water. Then, 4 µL of GGsTOP solution was added to the solution of AgNPs. The two solutions were mixed in a volume ratio of 4:1, stirred for 3 h, and sonicated for 10 min. The mixture was centrifuged to ensure that the drug particles were bound to the AgNPs, and the precipitate from the mixture was resuspended in water to obtain the final suspension [18].

### *2.4 Characterization of Silver Nanoparticles*

Transmission electron microscopy (TEM) (JEM-2100, JEOL, Tokyo, Japan) was used to determine the size and shape of the nanoparticles. The optical properties of the nanoparticles were analysed using UV-Vis spectroscopy with an Ocean Optics USB2000+VIS-NIRFIBER spectrophotometer (Ocean Optics, Dunedin, FL, USA), while Fourier-transform infrared (FTIR) (Vertex 70 RAM II Bruker spectrometer, Bruker Optics GmbH, Ettlingen, Germany) was used to obtain the FTIR spectra. The zeta potential of the nanoparticles was measured by suspending the nanoparticles in deionized water. The absorbance of the tested sample was recorded at a wavelength of 234 nm.

### *2.5 Drug Release Profile*

In the determination of drug release profile, 5 mL of GGsTOP-AgNPs was put in a dialysis bag with a 10–12 kDa MW cut-off, and the bag was immersed in 50 mL of PBS solution at 37 °C. The initial concentration of GGsTOP at each time point was recorded. Then, the cumulative percentage release was calculated.

### *2.6 Experimental Design*

A total of 150 louse eggs were divided into five groups and placed in 15 Petri dishes. The first group served as the negative control group, which received no treatment. The free GGsTOP group was treated with free GGsTOP dissolved in distilled water, while the free drug vehicle group was treated with distilled water alone. The GGsTOP-AgNPs group and the AgNPs vehicle group received GGsTOP-AgNPs and plain AgNPs, respectively. Each experiment was repeated three times with ten eggs per Petri dish. Each egg was immersed in 69 nL of the formulation for 15 consecutive days, during which it was incubated at 30 °C under 75–85% RH humidity [5]. All experiments were conducted simultaneously across all groups.

### *2.7 ELISA Technique*

Based on the TG inhibitor screening assay kit (catalogue number: MBS846796; MyBioSource, San Diego, CA, USA), the following test was performed: 50 treated eggs were initially divided into five groups and placed in Eppendorf tubes. Then, they were crushed using a fine-tipped drawing brush. To homogenize the eggs, 100 µL of PBS was added to each tube containing crushed eggs, and the tubes were centrifuged at 300 rpm for 15 min at room temperature. The next step involved inhibitor preparation. Then, enzyme and inhibitor solutions were added to a 96-well plate. The reaction components (background, enzyme, solvent, positive inhibitor and controls, and test compound) were added individually (75 µL each) to the wells. Additionally, 100 µL of the prepared egg samples from the first step was added to the wells. After the test, 100 µL of PBS was added to bring the final volume to 200 µL. The plates were incubated for 15 min at 37 °C. Finally, in the substrate preparation and assay procedure, 75 µL of the mixture containing TG buffer, donor substrate, acceptor substrate, 1 M DTT, and dH2O was added to the wells. Then, the plates were incubated for 2 h at 37 °C. The reaction was stopped using 75 µL of the stop solution which also served as the color developer. After completion of the kit steps, a microplate reader was used to measure the absorbance at 525 nm.

### *2.8 Statistical Analysis*

The activity of TG enzyme in the treated eggs was expressed as mean ± standard error of the means (S.E.M). The Chi-square ($\chi^2$) test was conducted to evaluate whether the differences in the number of viable eggs amongst the treated groups were statistically significant.

After checking the normality and variance homogeneity, one-way analysis of variance (ANOVA) test was used to determine statistically significant differences in the TG activities amongst the groups of treated eggs. Paired *t*-test was used to test the statistical significance of differences in the release profiles of GGsTOP and GGsTOP-AgNPs. All statistical analyses were conducted at a 95% confidence level.

## 3. Results

A silver nanoparticle GGsTOP formulation was prepared for the treatment of head lice, and its effect was compared to that produced by the free drug (GGsTOP).

### *3.1 Characterization of Silver Nanoparticles*

The prepared suspension of AgNPs had a deep yellow-to-brown color. The average size of the nanoparticles was 12 ± 2 nm. The zeta potential value of GGsTOP-AgNPs formulation was high (33.7 ± 2.34 mV), while the free GGsTOP formulation exhibited a lower zeta potential value of –10.7 ± 1.28 mV. Blank AgNPs has a zeta potential of 47.7 ± 2.15 mV.

As shown in Fig. 1, the peaks around 3200–3500 $cm^{-1}$ correspond to -OH stretching vibrations in the hydroxyl groups, while the sharp peaks near 1650 $cm^{-1}$ indicate C=O stretching vibrations in carbonyl groups.

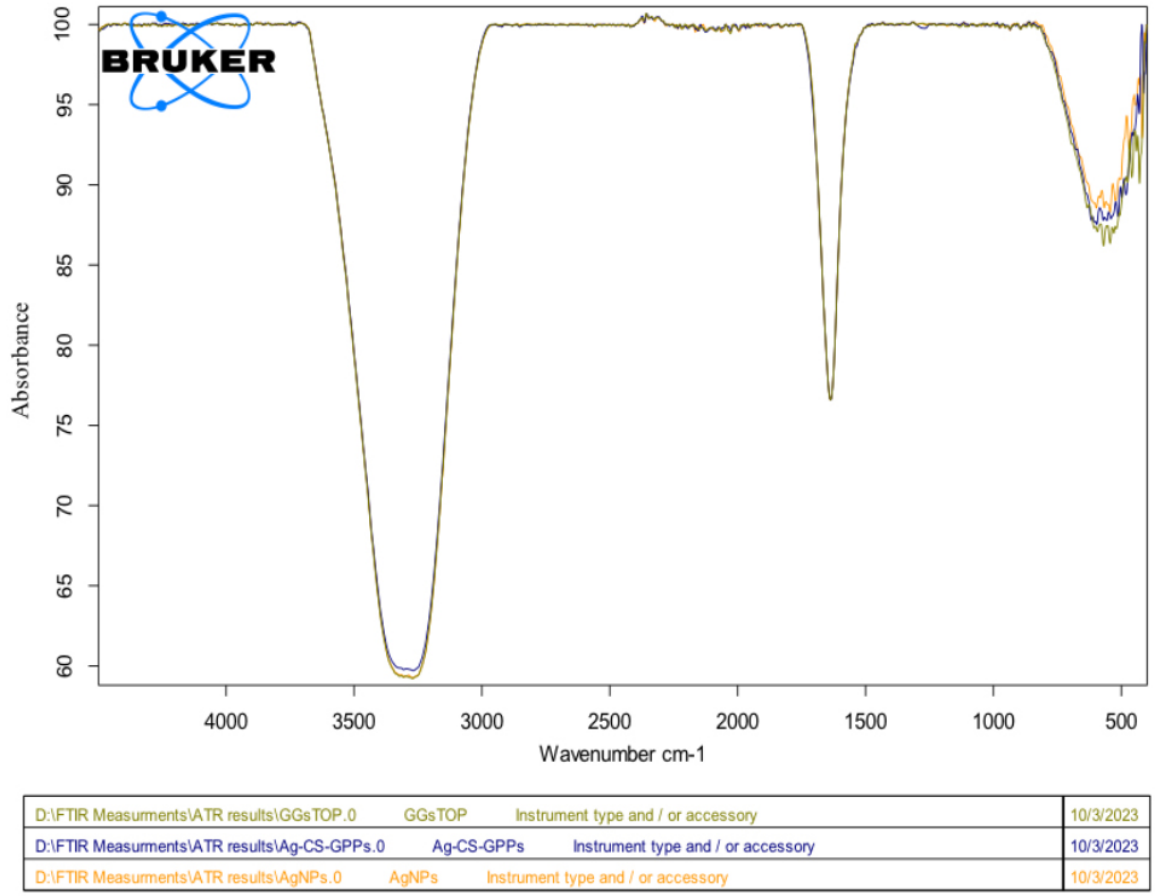


**Fig. 1. FTIR spectra of GGsTOP, AgNPs, and GGsTOP-AgNPs**. Using FTIR to determine the infrared absorption properties of the analyzed material, the broad peak around 3200 $cm^{-1}$ corresponds to -OH stretching vibrations, while sharp peaks near 1650 $cm^{-1}$ indicate C=O stretching vibrations. FTIR, Fourier-transform infrared; AgNPs, silver nanoparticles; $cm^{-1}$, inverse centimeters.

The standard curve showed strong linearity ($R^2$ = 0.999), thereby validating the use of intensity to determine GGsTOP concentration (y = 0.09011 + 1.1156 x; x = µg $mL^{-1}$).

*3.2 Release Profiles of GGsTOP and GGsTOP-AgNPs*

Free GGsTOP showed a rapid release initially, reaching 27% within the first 50 h, followed by a slight decrease, and then stabilization, suggesting that majority of the drug was released early in the process. In contrast, GGsTOP-AgNPs exhibited a slower release, reaching 20% at approximately 425 h (Fig. 2). The release profile of GGsTOP was significantly faster than that of GGsTOP-AgNPs ($p$ = 0.0056).

*3.3 Effect of GGsTOP Formulations on Head Lice*

The experiment lasted for 15 days. The embryo viability was assessed as the number of nymphs that hatched from eggs 20 days post-treatment (Table 2). Notably, no egg hatching was observed after 17 days. Each treatment group contained 30 eggs (10 eggs per dish), and there were 3 replicates per group.

The number of recorded eggs amongst the treated samples was recorded up to the 20th day. The results showed that most eggs hatched into viable nymphs in the and negative control (96.7%) and free vehicle (90%) groups ($p$ = 0.605). AgNPs vehicle group showed a fewer number of viable nymphs (23/30), although this number was not significant compared to negative control group ($p$ = 0.058). The lowest number of hatched nymphs was observed in the treated groups, but no statistically significant difference was observed between free GGsTOP (6/30) and GGsTOP-AgNPs (3/30; $p$ = 0.470).

*3.4 Evaluation of TG Enzyme Activity*

Transglutaminase (TG) is an important enzyme that solidifies the nit sheath. Thus, head louse eggs are destroyed by inhibiting TG, thereby breaking the life cycle of the parasite. Results from ELISA (Table 3) revealed significant reductions in TG activity in Pediculus eggs treated with 100 µL and 200 µL dilutions of free GGsTOP and GGsTOP-AgNPs.

The free GGsTOP and GGsTOP-AgNPs groups had significant reductions in TG enzyme activity at both di-

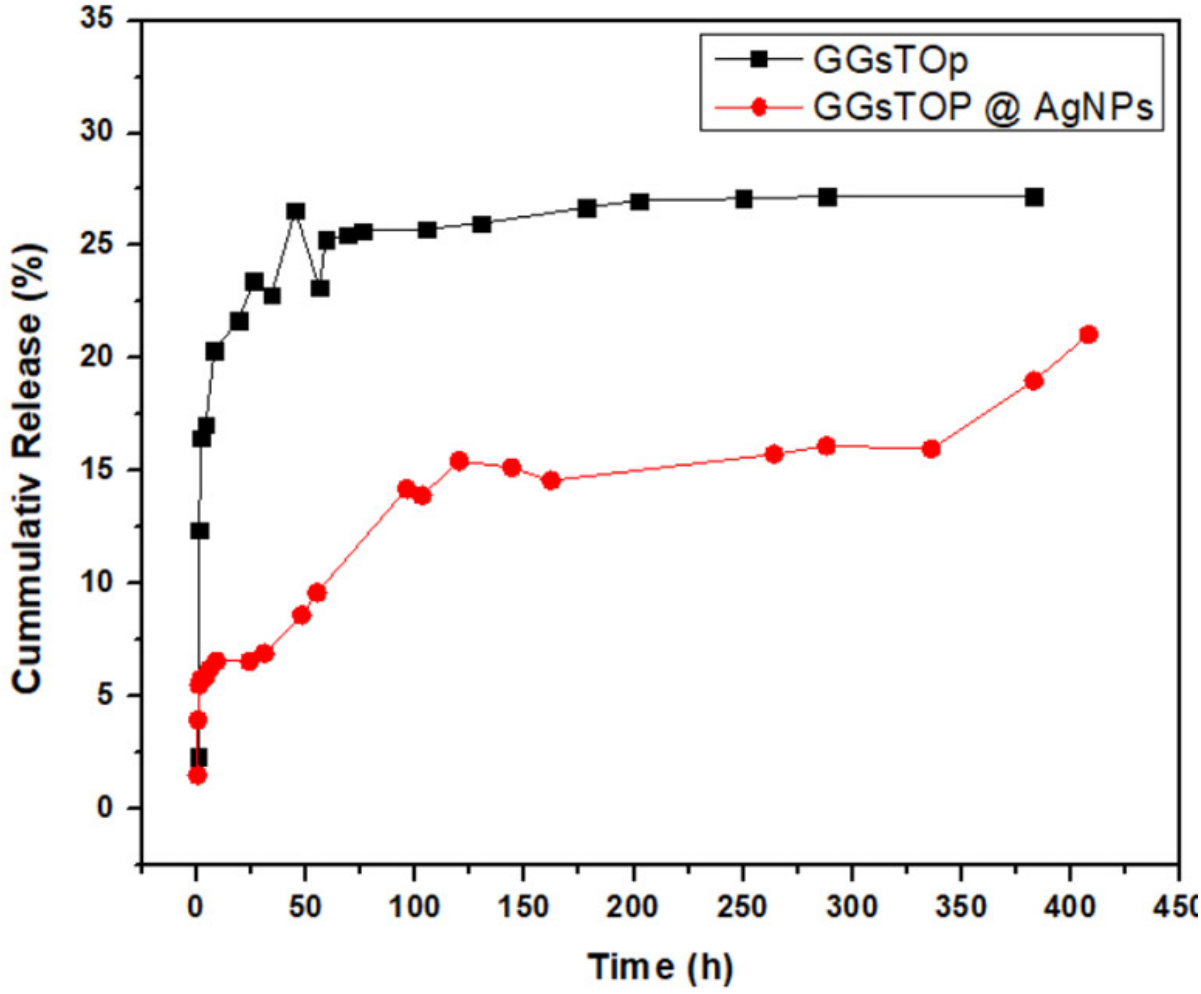


**Fig. 2. Cumulative release profiles of GGsTOP and GGsTOP-AgNPs in PBS (pH 7.4).** Five milliliters of GGsTOP silver nanoparticles were added to a dialysis bag, immersed in 50 mL of PBS solution at 37 °C. UV-visible absorbance was recorded over time, showing that the free GGsTOP exhibited the highest release initially before stabilizing, whereas GGsTOP AgNPs displayed a gradual increase in release throughout the observation period. AgNPs, silver nanoparticles; h, hours; PBS, phosphate-buffered saline.

**Table 2. The percentage of hatched nymphs post treatment (n = 30 for each group).**

| Negative control group | Free drug vehicle | AgNPs vehicle | Free GGsTOP | GGsTOP-AgNPs |
|---|---|---|---|---|
| 96.7% (29/30) | 90% (27/30) | 76.7% (23/30) | 20% (6/30) | 10% (3/30) |

**Table 3. Activities of TG in eggs in various treated groups (n = 10).**

| PBS volume added (µL) | The activity of TG (U/mL) | | | | |
|---|---|---|---|---|---|
| | Treated egg groups | | | | |
| | Negative control group | Free drug vehicle | AgNPs vehicle | Free GGsTOP | GGsTOP-AgNPs |
| 100 | 5.53 ± 0.20 | 4.00 ± 0.17 | 1.93 ± 0.03 | 2.56 ± 0.13 | 1.02 ± 0.04 |
| 200 | 4.60 ± 0.21 | 3.46 ± 0.33 | 1.64 ± 0.06 | 2.21 ± 0.13 | 0.80 ± 0.02 |

PBS, phosphate-buffered saline; AgNPs, silver nanoparticles; TG, transglutaminase.
µL, microliters; U/mL, units per milliliter.

lutions, when compared to the negative control group ($p$ = 0.0001). Additionally, the free GGsTOP group demonstrated a significant reduction in TG activity at 100 µL ($p$ = 0.0008), when compared to its vehicle, although the reduction was not significant at 200 µL ($p$ = 0.09). The GGsTOP AgNPs group showed marked decreases in TG activity at both dilutions, relative to its vehicle group ($p$ = 0.0191; $p$ = 0.0025). Moreover, the GGsTOP-AgNPs group had a sig-

nificant reduction in TG enzyme activity, when compared to the group treated with free GGsTOP at both concentrations ($p = 0.0001$).

## 4. Discussion

Head lice are one of the public ectoparasitic infections that affect humans globally. Their prevalence which affects primary school children, causes physical and psychological effects such as skin irritation and anxiety, respectively [24].

Within the framework of nanoparticle synthesis, previous pediculosis studies have reported the synthesis of larger nanoparticles (50–80 nm [17], 59.52 nm [18], 25–110 nm [19] and 60–150 nm [20]) than the present study (12 ± 2 nm). This may be attributed to the use of chitosan and acetic acid as stabilizer and solvent, respectively. The positively-charged chitosan molecules, along with their functional groups (-NH2, -OH, and -COCH3) enhance nanoparticle stabilization by effectively binding to silver ions, particularly in acetic acid solutions [25]. This results in the formation of more uniform and smaller nanoparticles due to minimization of aggregation.

When the release level was checked and measured for hours, the GGsTOP-AgNPs showed greater efficacy than free GGsTOP. Free GGsTOP reached its peak release (27%) after 2 days, while GGsTOP-AgNPs reached 20% after 17 days. This may be attributed to high stability of the formulation which led to a slower release.

Our study revealed that only 20% of eggs treated with free GGsTOP and 10% of those treated with GGsTOP-AgNPs hatched into viable nymphs, whereas most (90–97%) eggs hatched successfully in the negative control group. This effect may be attributed to the early developmental stage of the embryo at the time of inhibitor application. A previous study has shown that injection of the female lice with GGsTOP at the first stages of pregnancy produced more effective results than treating the eggs after the female lice had laid them [5]. Injection of 69 nL of GGsTOP into the female louse and coating the hair led to reduced egg-laying capacity, lower hatchability, and an increased number of desiccated eggs. Furthermore, eggs laid on GGsTOP-coated hair exhibited a 12.3% detachment rate, most likely due to impaired nit sheath formation. It is also preferable to use the drug in the early stages of egg laying on the hair to ensure its effect on the targeted enzyme, thus achieving greater effectiveness in the therapeutic intervention. This does not negate that more studies are needed to study the effect of GGsTOP formulations in different stages of embryonic development.

When head lice were treated with an aqueous extract of L. inermis leaves, 94% mortality was achieved at a concentration of 80%, whereas, in contrast, 100% mortality was observed with just 10% of the corresponding synthesized AgNPs formulation [18]. Similarly, treatment with T. cordifolia leaf extract at a concentration of 100 mg/L resulted in 80% mortality of lice, whereas AgNPs synthesized from these leaves resulted in 100% mortality at a much lower concentration (25 mg/L) [17]. In addition, AgNPs demonstrated significantly higher acaricidal efficacy compared to crude plant extracts, reducing LC50 and LC90 values by approximately 90–97%, indicating substantial enhancement potency [19,20]. All these studies suggested that the use of AgNPs improved the insecticidal efficacy of the formulation. However, in this study, the incorporation of AgNPs slightly enhanced ovicidal activity of GGsTOP The number of hatched nymphs in GGsTOP AgNPs and free GGsTOP treated group was 3 and 6, respectively out of a total of 30 eggs ($p = 0.470$). Therefore, neither formulation can be considered superior to the other based on the current data.

To gain a clearer understanding of the effect of the tested formulations, ELISA was used to measure TG enzyme activity in eggs post-treatment. The results showed that the highest TG enzyme activities were observed in the negative control group (5.53 ± 0.20 U/mL and 4.6 ± 0.21 U/mL for the two dilutions). In contrast, the lowest enzyme activity was detected in eggs treated with GGsTOP-AgNPs (1.02 ± 0.04 U/mL and 0.80 ± 0.02 U/mL at 100 and 200 µL dilutions, respectively. This indicates the efficacy of GGsTOP-loaded silver nanoparticles against TG. Eggs treated with free GGsTOP also exhibited significant reductions in TG enzyme activity (2.56 ± 0.13 U/mL and 2.21 ± 0.13 U/mL at the two dilutions), when compared to the free drug vehicle (4 ± 0.17 U/mL at 100 µL dilution). However, the difference was not significant at the second dilution, which may be attributed to the lower activity of TG at a higher dilution with PBS. These findings are in agreement with those reported in previous studies which demonstrated that nanomaterials enhance the biological activity of various compounds [26]. The GGsTOP formulation specifically targeted TG enzyme by binding to its active site and reducing its affinity for its natural substrates in the nit sheath, i.e., LNSP1 and LNSP2. This interaction disrupted the integrity of the nit sheath and prevented proper egg adhesion and development.

It is worthy of note that this study is the first to measure TG enzyme activity in treated head lice eggs. Unlike most previous studies that only focused on mortality of viable lice stages, the present study aims to interrupt the life cycle by targeting lice eggs through investigating TG activity as a biochemical marker within the eggs, offering a novel perspective on treatment efficacy. A previous study focused on the effect of GGsTOP on TG-mediated cross-linking of LNSP1 and LNSP2 in female head lice during oviposition [5]. Our findings further validate the potent inhibitory effect of GGsTOP-AgNPs, and they highlight their potential as a novel therapeutic strategy for preventing egg attachment to hair and disrupting embryonic development, thereby providing an effective approach to controlling infestation by head lice.

As previously mentioned, the ELISA results revealed distinct effects of different treatments on the targeted TG enzyme activity in eggs. This underscores the importance of employing multiple evaluation methods for assessment of the effectiveness of treatment.

## 5. Limitations

The experiments were conducted in small sample size under controlled laboratory conditions; *in vivo* settings may lead to some variations in results. This study lacks an examination of the histopathological changes of the treated eggs and a study of the effect of a positive control medication such as pyrethroid. These could provide more valuable and comprehensive information. In addition, the characterization of AgNP formulation lacks examination of particle size distribution and the polydispersity index which may give clearer view about the physicochemical properties of the formulation.

## 6. Conclusions

The results of this study revealed that GGsTOP-AgNP and free GGsTOP exhibited higher egg-killing efficacy compared to the control group as shown by the number of hatched eggs. These formulations reduced TG activity as shown by ELISA results. This indicates a clear anti-enzyme effect of the treatment groups and disruption of TG enzyme activity. This disruption affects LNSP1 and LNSP2 proteins, which play a crucial role in eggshell hardening and their close association with the hair. The study may contribute to further progress in lice control by incorporating *in vivo* evaluation, clinical applications, diverse laboratory techniques, testing higher doses and different stages of embryonic development. Further studies may yield more comprehensive and conclusive results.

## Abbreviations

AgNPs, silver nanoparticles; FTIR, Fourier-transform infrared; IVM, Ivermectin; LC, Lethal Concentration; LNSP, Louse nit sheath protein; TG, transglutaminase.

## Availability of Data and Materials

All data reported in this paper will be shared by the corresponding author contact upon request.

## Author Contributions

NZ and IA designed the research study. IA performed the research. NZ made the Critical revision of the article. NZ and IA provided help on Collection and/or assembly of data, Data analysis and interpretation, Writing the article. Bothauthors contributed to the writing and revision of the first draft of the manuscript and approved the final version. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

This study was approved by the Committee of Biomedical Ethics, Faculty of Medicine, King Abdulaziz University (approval reference no.: 130-22). The informed consent of participating in this study was obtained from the guardians of all children. The study was carried out in accordance with the guidelines of the Declaration of Helsinki.


## Acknowledgment

The authors would like to express their gratitude to KAU Endowment (WAQF) and the Deanship of Scientific Research (DSR) at King Abdulaziz University for supporting this work.

## Funding

This research received no external funding.


## Conflicts of Interest

The authors declare no conflicts of interest.

## Declaration of AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work the authors used ChatGPT-3.5 in order to check spell and grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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