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Original Article

An Electrolytically Prepared Colloidal Silver Preparation Protects against Citrate-induced Toxicity in *Artemia franciscana* Nauplii

Ian Edwin Cock[1, 2,*], Michael Whitehouse[2]

[1]Centre for Planetary Health and Food Security, Griffith University, Nathan, AUSTRALIA.
[2]School of Environment and Science, Griffith University, Nathan, AUSTRALIA.

ABSTRACT

Introduction: Nanotechnology is promising for the development of new effective medicines. Silver nanoparticle preparations have been particularly well studied and a range of beneficial effects have been identified. Despite this, the sale of Colloidal silver (CS) preparations for therapeutic purposes has been banned by multiple regulatory authorities (including the FDA and TGA) on the basis of their perceived toxicity. This study evaluates the toxicity of electrolytically produced (CS) preparation, as well as some compounds used to produce CS by chemical synthesis. **Materials and Methods:** Toxicity of the CS preparation and the chemical toxins were evaluated using the *Artemia* nauplii toxicity assay. Additionally, the CS was tested for its protective effects against citrate and tannic acid-induced toxicity by screening combinations of CS and the toxins. **Results:** The CS preparation was nontoxic in the *Artemia* nauplii bioassay at all concentrations tested and did not induce mortality substantially above the seawater control at all concentrations ≤50 µg/mL. In contrast, citrate and tannic acid were strongly toxic, with LC_{50} values of 11.6 and 26.8 µg/mL respectively. Interestingly, co-incubation of varying concentrations of the CS preparation with 30 µg/mL of citrate resulted in protection against toxicity, with 50 µg/mL CS inhibiting ~65% of citrate-induced toxicity. In contrast, the CS preparation had no apparent effects on tannic acid-induced toxicity. **Conclusion:** The CS preparation tested in our study was nontoxic at all concentrations tested. Furthermore, the CS mitigated the toxic effects of citrate, but had no apparent effect on tannic acid-induced toxicity. Further studies are required to verify these findings in other toxicity models and to study the protective molecular mechanisms.

Keywords: Silver nanoparticles, Colloidal silver, Citric acid toxicity, Tannic acid, Brine shrimp toxicity, Protection against toxicity.

Correspondence:
Dr. Ian Edwin Cock
[1]Centre for Planetary Health and Food Security, Griffith University, Nathan, AUSTRALIA.
[2]School of Environment and Science, Griffith University, Nathan, AUSTRALIA.
Email: i.cock@griffith.edu.au

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INTRODUCTION

Recent developments in nanotechnology have highlighted the industrial applications of nanoparticles,[1,2] and have stimulated considerable interest in the use of nanomaterials for medical applications.[1] The diagnostic applications, including the detection of biological molecules and disease states, have been particularly well reported in recent years.[3,4] Recent studies have also explored the therapeutic potential of some engineered nanomaterials for diverse applications, including cancer chemotherapy,[5] and for the treatment of bacterial infections.[6]

Silver nanoparticles have been amongst the most studied categories of nanomaterials, with an impressive range of pharmaceutical, cosmetic and industrial uses.[7] Indeed, numerous studies have reported noteworthy antimicrobial efficacy for silver nanoparticles, including against antibiotic resistant bacterial strains.[8-11] Silver nanomaterials are relatively simple to produce particles of different sizes, shapes and chemical complexities and therefore to modulate their bioactivities.[12,13] However, despite their medical potential, the uptake of silver nanoparticles for therapeutic applications has been slow, largely because of the reported toxicity of some silver nanomaterials.[14,15] Whilst the cytotoxicity may be beneficial in the treatment of some cancers,[16-18] the toxicity of silver preparations limits their uses for other therapeutic applications, including for the treatment of pathogen infections.

Notably, the majority of studies reporting toxicity for silver nanomaterials, focus on materials containing ionic (particularly, oxidized Ag+) forms of silver[7] and relatively few studies have investigated the toxicity and therapeutic potential of finely dispersed Colloidal silver (CS) nanoparticles containing nonionic/uncharged (Ag^o) silver. Furthermore, those studies

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that have examined the toxicity of zerovalent silver colloids generally focused on preparations produced by the addition of chemical reductants to silver salts. This approach complicates the evaluation of the CS toxicity due to the presence of other toxic compounds in the preparation. Substantially fewer studies have evaluated the toxicity of electrolytically produced zerovalent CS and substantially more study is required in this field. Additionally, the effects of CS on toxicity of other molecules remains largely unexplored.

This study evaluates the toxicity of CS prepared by electrolytic methods and compares it to two biological toxins (citrate and tannic acid) using *Artemia* nauplii Lethality assays (ALA). This assay was selected due to its sensitivity and reproducibility.[19] The ALA is also substantially less expensive than cell line toxicity assays and produces results much more rapidly. In addition, a nontoxic concentration of the CS preparation was tested in combination with the citrate and tannic acid toxins to evaluate the ability of CS to mitigate the toxicity of those compounds.

MATERIALS AND METHODS

Colloidal silver sample

A colloidal silver sample (Figure 1a) was kindly donated by Hans Laroo (Security Research, Ipswich Qld) for this study. The sample, which contained nanoparticles with a mean diameter of ~33 µm (Figure 1b; Table 1) was prepared electrolytically as previously described.[11] The physical parameters of the colloidal silver preparations were determined in the earlier study and are reproduced in Table 1.

Citrate and tannic acid solutions

ACS grade citric acid (≥99.5% purity; Figure 1c) and tannic acid (≥99.5% purity; Figure 1d) were purchased from Sigma Aldrich, Australia and were dissolved in sterile deionised water to produce 1 mg/mL stock solutions. The stocks were aliquoted and stored at -30°C until use. For toxicity screening, aliquots were thawed and diluted to the required concentrations in artificial seawater (Reef Salt, AZOO Co., USA).

Toxicity screening

Reference toxin

Potassium dichromate ($K_2Cr_2O_7$) (AR grade, Chem-Supply, Australia) was prepared as a 1.6 mg/ml solution in distilled water and was serially diluted in synthetic seawater for use in the toxicity assay. The stock was serially diluted in artificial seawater for use in the bioassay.

Artemia franciscana nauplii toxicity screening

Toxicity was tested using the *A. franciscana* nauplii Lethality assay (ALA) developed for the screening of active plant constituents with the following modifications.[19] *A. franciscana* cysts were obtained from North American Brine Shrimp, LLC, USA (harvested from the Great Salt Lake, Utah). Artificial seawater was prepared using Reef Salt, AZOO Co., USA. Seawater solutions at 34 g/L distilled water were prepared prior to use. A mass of 2 g of *A. franciscana* cysts were incubated in 1000 mL of synthetic seawater under artificial light at 25°C, 2000 Lux, with continuous aeration. Hatching commenced within 16-18 hr of incubation. Newly hatched *A. franciscana* (nauplii) were used within 10 hr of hatching. A 400 µL volume of seawater containing approximately 46 (mean 46.2, n=125, SD 12.8) nauplii were added to the wells of a 48 well plate and immediately used for bioassay. The colloidal silver preparations, as well as the citrate and tannic acid solutions, were diluted in seawater for testing in the bioassay. A volume of 400 µL of all tests or controls were transferred to individual wells and incubated at 25±1°C under artificial light (1000 Lux). A negative control (400 µL seawater) was run in triplicate on each plate. The wells were checked at regular intervals and the number of dead counted. The nauplii were considered dead if no movement of the appendages was observed within 10 sec. After 72 hr all nauplii were sacrificed by the addition of 50 µL of glacial acetic acid and counted to determine the total number per well. The LC_{50} within 95% confidence limits was calculated for each treatment using Probit analysis.[19]

Toxin/colloidal silver co-treatment

The ability of the colloidal silver preparation to block the toxic effects of citric and tannic acids was tested but screening these toxins in combination with the colloidal silver preparation against *Artemia nauplii* as described above, with modifications. A volume of 200 µL of colloidal silver dilutions prepared in artificial seawater were dispensed into individual wells to test across the concentration range 7.5-240 µg/mL in the assay. A volume of 200 µL of the citrate or tannic acid toxins was then added separately to individual wells to give a final concentrations of 30 µg/mL of the test toxin in the assay. The nauplii were exposed to the colloidal silver/toxin combinations for 24 hr at 25°C. The number of dead brine shrimp in each well was subsequently counted and expressed as the % mortality in the well. All tests were performed in three independent experiments, each with internal triplicates (n=9) and are expressed as the mean % mortality±SEM.

Statistical analysis

Data are expressed as the mean±SEM of at least three independent experiments. The paired t-test was used to calculate statistical significance between control and treated groups with a p value <0.01 considered to statistically significant.

RESULTS

Toxicity of colloidal silver, citrate and tannic acid

The CS preparation and the citrate and tannic acid toxins were serially diluted in artificial seawater for toxicity testing in the

ALA toxicity assay (Figure 2). The reference toxin potassium dichromate (1000 µg/mL) was tested in parallel as a comparison. Potassium dichromate rapidly induced toxicity, with 100% mortality noted within 3 hr of exposure (unreported results). Citrate and tannic acid also induced mortality with 30 min, with 100% of the nauplii dead within 3 h for concentrations ≥30 µg/mL or ≥60 µg/mL respectively. Furthermore, mortality induction by citrate and tannic acid was significantly greater than noted the negative seawater control at all concentrations tested ($p<0.05$). As solutions that induce >50% mortality are defined as toxic, citrate and tannic acid were deemed to be toxic at concentrations ≥15 µg/mL or ≥30 µg/mL respectively in our study. The toxicity of these compounds was further quantified by determination of their LC_{50} values using Probit analysis (Table 1). LC_{50} values of 11.6 µg/mL and 26.8 µg/mL following 24 hr exposure were calculated for citrate and tannic acid respectively. Therefore, 30 µg/mL was selected for further testing to evaluate the effects of CS-toxin combinations.

The 100 and 50 µg/mL concentrations of the CS preparation also induced mortality significantly above that of the seawater control within the first 24 hr of exposure, although the % mortality was substantially lower than noted for the potassium dichromate control and for the citrate and tannic acid test toxins. Indeed, substantially less than 25% *Artemia* mortality was evident at each of these concentrations. All other CS concentrations tested did not induce mortality above that of the negative seawater control. Therefore, the CS preparation was defined as nontoxic at all concentrations tested and it was not possible to calculate 24 h LC_{50} values for the CS preparation (Table 1). CS concentrations ≤50 µg/mL were selected to evaluate the effects of the CS preparation on mitigating toxicity induced by citrate and tannic acid.

A further trend was also evident for the ALA tests: The induction of mortality became substantially greater as the toxin exposure time increased (Figure 3). Previous studies have also reported that *Artemia* spp. toxin sensitivity increased substantially as the nauplii develop through the larval stage.[20] *Artemia* nauplii hatch with a yolk sac, which provides them with nourishment

Table 1: Physical parameters and toxicity evaluations of the colloidal silver preparation and the citrate and tannic acid toxins.

Measured parameter		Colloidal silver preparation	Citrate	Tannic acid
Physical parameters of colloidal preparation	Total silver content measured (µg/mL).	135$^{\Phi}$	0	0
	Conductivity (µSiemens).	18$^{\Phi}$	1.2	0.07
	pH	8.1$^{\Phi}$	2.58	5.83
	Light scattering index.	98$^{\Phi}$	-	-
	Median nanoparticle size (µm).	33±4.1$^{\Phi}$	-	-
Toxicity (LC_{50}) in the ALA (µg/mL)	24 hr exposure.	CND	11.6	26.8
	48 hr exposure.	44.8	<7.5	<7.5
	72 hr exposure.	3.9	<7.5	<7.5

$^{\Phi}$ denotes values reproduced from;[11] -=not values are available; CND=Could not determine as mortality was <50% at all concentrations tested. The physical parameters of citrate and tannic acid were determined using 1 mg/mL solutions.

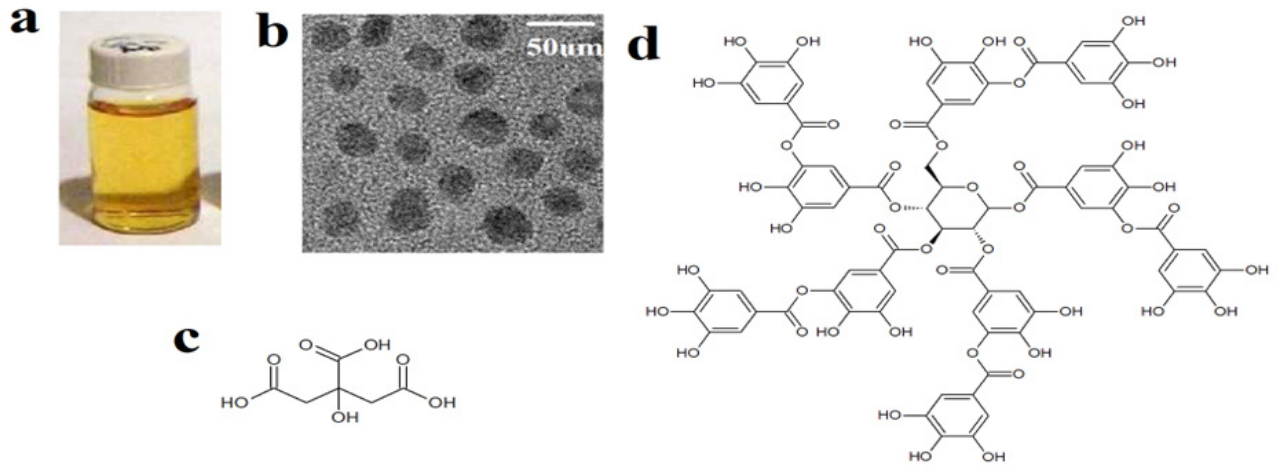

Figure 1: (a) The colloidal silver sample and (b) the individual particles visualised by SEM, as well toxins (c) citrate and (d) tannic acid.

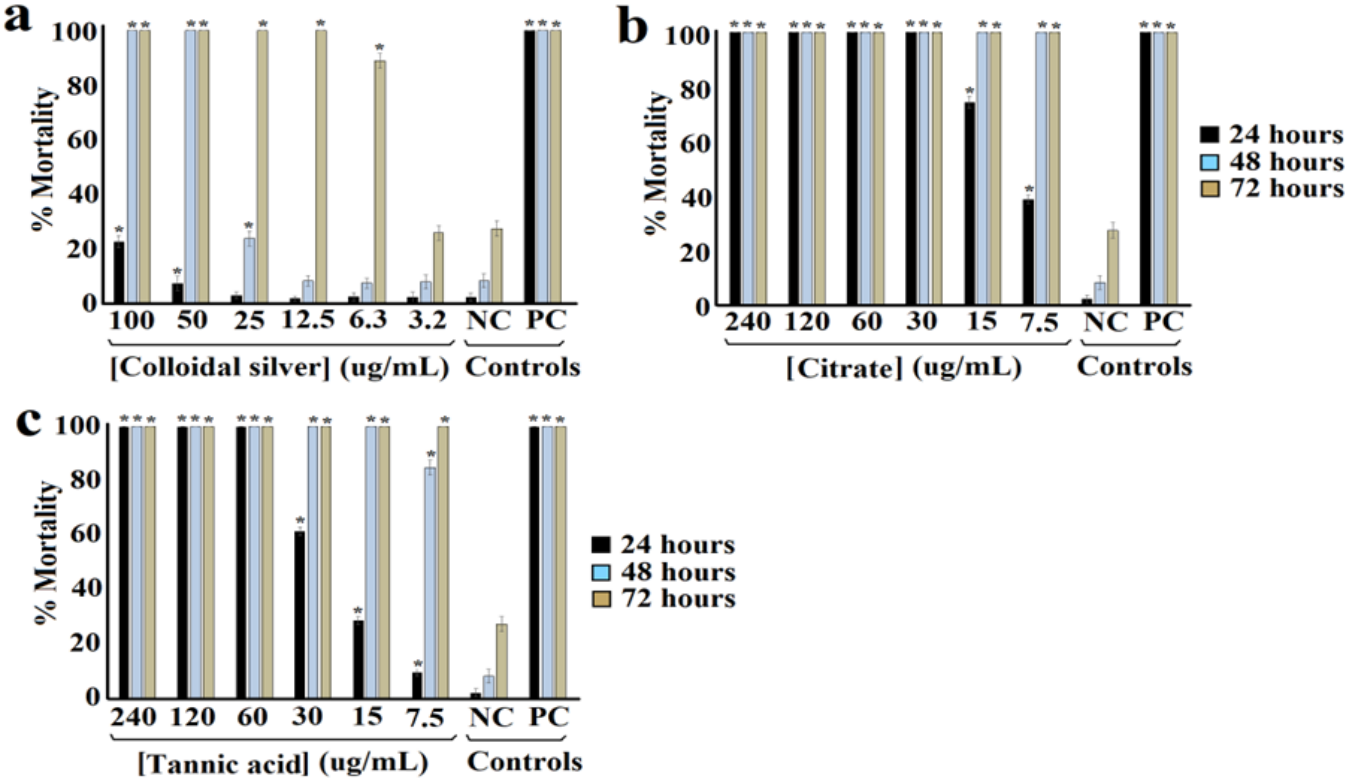

Figure 2: Evaluation of toxicity in the *Artemia* nauplii toxicity assay for various concentrations of (a) colloidal silver preparation, (b) citric acid, (c) tannic acid following 24, 48 and 72 hr exposure. NC=Negative (seawater) control; PC=Potassium dichromate control (1000 µg/mL); * indicates results that are significantly different to the negative control (p<0.01). All assays were performed in triplicate, each with three internal replicates (n=9).

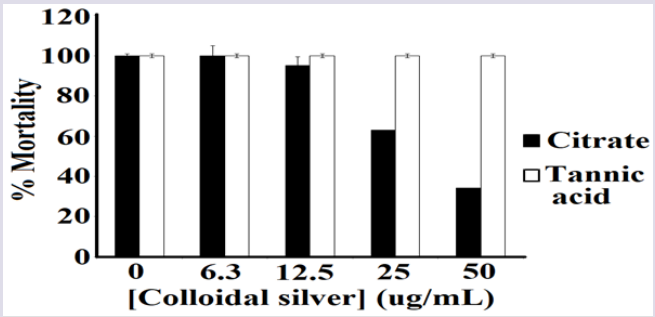

Figure 3: The effects of varying concentrations of the colloidal silver preparation against citrate and tannic acid induced toxicity following 24 hr exposure. The citrate and tannic acid toxins were each tested at constant 30 µg/mL concentrations in the assay. All assays were performed in triplicate, each with three internal replicates (n=9).

for several hours post hatching.[21] Therefore, environmental toxin uptake and distribution is minimal during that period. Mortality induction during this period is most frequently due to changes in physical conditions (e.g. pH changes), although other toxicity mechanisms may also occur. Once the yolk sac has depleted, the nauplii become increasing reliant on uptake of external substances for their nutrition and there is a substantial increase in uptake and distribution of external chemicals across the first 12-36 hr of their development. Indeed, a study that examined the toxicity of Fe_2O_3 nanoparticles reported that uptake and distribution was greatest during that period and that this uptake mirrored the Fe_2O_3 nanoparticles-induced mortality.[20] After the 36 hr period, if additional nutrition is not provided, the nauplii begin to die, even in the absence of toxin. Indeed, an increase in spontaneous mortality was evident in the negative (seawater) control, with mortality increasing from ~2% to ~28% across this period. Therefore, the 24 hr exposure period was selected for further studies to evaluate the effects of CS/toxin combinations.

Protective effects of colloidal silver against citrate and tannic acid toxicity

An interesting trend was evident when citrate was tested at toxic levels (30 µg/mL) in combination with the CS preparation. Whilst citrate induced 100% mortality in combinations containing ≤6.3 % CS, the levels of mortality decreased as the concentration of CS was increased. Indeed, the addition of 25 µg/mL CS, inhibited citrate-induced mortality by approximately 40%, whilst 50 µg/mL CS decreased mortality by more than 65% compared to citrate alone. This indicates that the CS preparation has a noteworthy protective effect against citrate-induced toxicity. Interestingly, this effect was not evident for tannic acid-induced toxicity, with no

changes in mortality evident at the CS and toxin concentrations tested.

DISCUSSION

Nanoparticle preparations (including CS preparations) have potential as therapeutics and have attracted substantial interest, particularly for their antimicrobial properties. Metallic silver has been used to sterilise and disinfect drinking water since ancient times.[22] Additionally, silver preparations have also been used as post-operative antiseptics and to prevent ophthalmia neonatorum in neonates. Furthermore, CS preparations were approved by the US Food and Drug Administration (FDA) for antiseptic wound treatment in the 1920s. More recently, the sterilisation properties of silver have been exploited by NASA on the Apollo spacecraft and the MIR space station.[23,24] Despite the initial approval of silver preparations by regulatory agencies and their recent and historic use as antiseptic agents, the US FDA and the Australian Therapeutics Goods Administration (TGA) reversed their approval of CS for therapeutic use in the 1980s and banned claims regarding their efficacy, citing concerns about their safety.[25,26] Whilst multiple previous studies have reported toxicity for silver preparations, the majority of those studies examined ionic silver preparations, or CS preparations prepared by the addition of reducing agents (including citrate and tannins), which may themselves be toxic, to silver salts.[7,27,28]

Our study examined the toxicity of an electrolytically produced CS preparation, which is devoid of the chemical agents (including citrate and tannins) that are included in many chemical CS preparation methods to produce Ag^0 and instigate colloid formation. Notably, the electrolytic CS preparation tested in our study was nontoxic in the *Artemia* model assay at all concentrations tested. This contrasts substantially with multiple other studies, which have reported substantial toxicity for silver nanoparticles. Notably, those studies generally tested ionic silver solutions, or products containing other chemicals used in their preparation. Therefore, our study also tested the toxicity of citrate and tannic acid (reducing agents commonly used in the formation of form CS preparations). Notably, both citrate and tannic acid induced substantial mortality in *Artemia* nauplii, verifying that they may contribute to the toxicity of some of the CS preparations previously examined. Whilst our study did not determine the toxic mechanisms of these compounds, pH changes may account for some of these effects. Whilst *Artemia* nauplii are generally considered a good model system for toxicity studies, they are sensitive to pH changes.[29] Indeed, decreasing the pH of the *Artemia* nauplii's environment from 7 to 5 decreases the number of viable *Artemia* nauplii by nearly 100%.[30] Citrate is a tri-basic acid that has pKa values of approximately 3.1, 4.8 and 6.4.[31] Therefore, it is almost completely deprotonated in aqueous solutions, resulting in low pH values. Indeed, the pH pf the citrate solution used in our study was measured to be ~2.6. It is likely that this decrease in pH may be responsible for the toxicity reported in our study.

Interestingly, exposure to the CS preparation in our study substantially reduced *Artemia* nauplii mortality when tested in combination with citrate. The effects of the CS were dose-dependent, with ~35% in mortality (~65% decreased toxicity) noted when the citrate was tested in combination with 50 µg/mL. The CS preparation may block the toxic effects of the citrate in several ways. The Ag units in the colloids may be directly removing protons from the solution, reducing the pH changes and thereby decreasing *Artemia* nauplii mortality. Notably, citrate is included in some CS preparation protocols to reduce ionic silver to Ag^0, thereby minimising ionic repulsion affects between charge Ag ions. The proton accepts an electron, forming water. Interestingly, previous studies have reported that lower pH's induce the formation of larger silver colloids due to increased aggregation as the levels of ionic silver in the preparations decrease.[32] It is possible that the CS preparation used in our study has also reduced the citrate-induced pH decreases in our study, although this remains to be verified.

In contrast, it is unlikely that pH effects alone are responsible for the toxicity of tannic acid reported herein. Tannic acid is a weak acid (pKa ~6)[31] and therefore does not fully deprotonate in aqueous solutions, resulting in higher pH values. Indeed, the pH of the tannic acid solution examined in our study was measured to be ~5.8. Whilst pH changes can induce *Artemia* nauplii mortality, a greater decrease in pH is needed for the toxicity to become apparent, with a previous study reporting >80% nauplii survival at pH 5.0.[30] It is therefore likely that pH changes are not solely responsible for the mortality of the tannic acid solution reported in our study and other factors are likely to contribute. More work is required to determine the toxicity mechanisms. However, many gallotannins (including tannic acid) are known to bind to cell surface proteins, phospholipids and sugars, thereby blocking their activities and/or regulating membrane signalling pathways.[33] It is possible that the toxicity of tannic acid towards *Artemia* nauplii is mitigated by these effects, although this remains to be verified.

In contrast to the effects of the CS preparation on citrate toxicity, there was no apparent effect for the CS on tannic acid-induced toxicity. This is an interesting result, which may relate to the concentrations of the citrate and tannic toxins tested in our study, or to differences in the toxicity mechanisms. Both toxins were tested at 30 µg/mL in the combination studies. However, tannic acid is a much larger molecule than citrate (molecular masses of ~1701 and 189 g/mole respectively). Therefore, the molar concentrations of these molecules tested in the combination

studies was approximately 17.6 µM and 158.7 µM respectively (i.e. approximately a nine-fold difference). Interestingly, the greatest protective effect was noted when the CS preparation was tested in combination with the higher molar concentration toxin (citrate). Tannic acid is a hydrolysable molecule that consists of ten gallic acid moieties esterified together and to a central glucose moiety. These ester bonds are readily hydrolysed in mild acidic conditions, releasing multiple gallic acid moieties.[34] Therefore, it is possible that the 17.6 µM tannic acid solution tested in our study hydrolyses to give up to 176 µM gallic acid (if hydrolysis is complete), which may account for the resistance of the tannic acid toxin solution to the protective effects of the CS preparation.

CONCLUSION

The results reported herein demonstrate that an electrolytically produced CS preparation was nontoxic towards *Artemia* nauplii. In contrast, citrate and tannic acid (both of which may be used as reducing agents for chemical CS synthesis) displayed substantial toxicity in the assay. Interestingly, the CS preparation reversed the toxicity of citrate, but had no apparent effect against tannic acid. These results indicate the safety and protective effects of electrolytic CS preparations. However, these effects were evaluated using an *Artemia* nauplii assay model. Whilst this assay is robust and generally correlates well to mammalian cell toxicity assays, additional studies are required to confirm these effects in mammalian cells. Furthermore, studies are required to determine the molecular mechanism(s) by which the CS preparation protects against the toxicity of citrate and tannic acid.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

- The toxicity of an electrolytically produced Colloidal Silver (CS) preparation was evaluated using *Artemia* nauplii toxicity assays.
- The CS preparation was nontoxic at all concentrations tested.
- Citrate and tannic acid were toxic in parallel experiments, with 24 hr LC_{50} values of 11.6 and 26.8 µg/mL respectively.
- CS mitigated the toxicity of citrate (but not tannic acid) when tested in combination.

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