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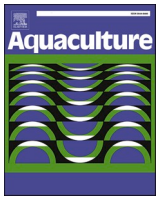

Efficacy of *Terminalia catappa-AgNP* nanocomposite towards *Saprolegnia parasitica* infection in angelfish (*Pterophyllum scalare)* eggs

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ABSTRACT

A nanocomposite based on silver nanoparticles (AgNPs) and extract of *Terminalia catappa* was developed, characterized, and evaluated in *in vitro* and *in vivo* conditions against *Saprolegnia parasitica* infection. The nanocomposite contained spheroidal silver-nanoparticles (1–55 nm) and presented active compounds as gallic acid, ellagic acid, and α and β punicalagin. The nanoparticles remained stable for one year after its production. A synergistic effect was observed between AgNPs and extract under *in vitro* and *in vivo* conditions against different stages of fungal development. In an *in vitro* assay, the nanocomposite showed fungistatic and fungicide effects to the fungal mycelium in solid and liquid media, respectively, through an increase in the contact surface. In an *in vivo* bioassay, the lowest concentration of nanocomposite (T1 = 45.75 $\mu g.L^{-1}$ AgNPs +62.5 $\mu g.L^{-1}$ *T. catappa* extract) demonstrated similar efficiency as the positive control (methylene blue) in preventing zoospore infectivity in eggs of angelfish (*Pterophyllum scalare*). The fungal zoospores were more sensitive to the nanocomposite than fungal mycelia. Our results exhibited the use of a nanocomposite containing AgNPs and *T. catappa* aqueous extract could reduce the required effective concentrations of AgNPs against saprolegniosis in fish eggs, thus, it may as an alternative to improve fish larval survival at the hatchery stage.

1. Introduction

Saprolegnia parasitica is one of the most significant fish pathogens from the genus oomycete, causing high rates of mortality for freshwater fish regardless of whether they live in natural environments or are reared in captivity (Van Den Berg et al., 2013; Lone and Manohar, 2018). Freshwater angelfish (*Pterophyllum scalare)* is an economically important ornamental fish species with easy reproduction, but its eggs frequently suffer fungal infections (Chapman et al., 1997; Forneris et al., 2003; Chambel et al., 2014).

The fungus breaks up the chorionic membrane of the fish eggs, especially the non-fertilized and dead eggs. As the infection increases, the fungus infects the viable embryos consequently causing their mortality (Liu et al., 2014; Songe et al., 2016). Without any chemical treatment, this mortality is common in the *P. scalare* eggs (see Eissa et al., 2013).

Chemical compounds including formalin, malachite green, and methylene blue have been widely used as anti-saprolegniosis in fish

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hatcheries until recently (Bassleer, 2011; Fuangsawat et al., 2011; Huang et al., 2015). Although the use of these compound is prohibited in the United States, Europe, and many other regions, their use is still permitted in the aquaculture sector in some countries, including Brazil (UEMS, 2016; Tancredo et al., 2019).

The Malachite green is carcinogenic and induces oxidative stress on fish when it reaches the water (Majeed et al., 2014). The use of malachite green is not approved by the U.S. Food and Drug Administration (Kwan et al., 2019) and is prohibited in aquaculture in the European Union due to its harmful effects on human health and the environment (EU regulation - EG1272, 2008).

Methylene blue also provokes problems in humans, such as tachycardia, vomiting, shock, cyanosis, and tissue necrosis (Razmara et al., 2011; Salazar-Rabago et al., 2017; Dinh et al., 2019). Japan, United States, and Europe have forbidden its use in aquaculture (Li et al., 2016; Lieke et al., 2020); however, in Brazil methylene blue is still in use due to lack of legislation and clear protocols regarding its use.

The use of metallic nanoparticles with antibacterial and anthelmintic may provide an alternative method to control fungal infections in fish (Swain et al., 2016; Vijayakumar et al., 2017). Studies about metal nanoparticles have proven them to be effective as an antifungal agent.

Silver nanoparticles (AgNPs) stand out for their antimicrobial activity, adhering to the cellular membrane, causing lipopolysaccharide degradation, increasing the permeability of membranes, and damaging the DNA as a result (Durán et al., 2016). A study found that silver nanoparticles show fungicidal effects against *Saprolegnia* spp. at 1800 and 2000 $mg.L^{-1}$ (Johari et al., 2015a). However, their use can have toxic effects in aquatic environments (Hosseini et al., 2014; Johari et al., 2015b). In response, a combination of silver nanoparticles and plant extracts (as nanocomposites) represent an eco-friendly alternative, since it reduces the concentration of AgNPs, replacing it with the plant extract action. Furthermore, the plant extract is quickly degraded in aquatic environments (Valladão et al., 2015). Thus, a more effective synergic effect is possible due the combination of nanoparticles and the plant extracts.

The leaf of the almond tree *Terminalia catappa* is a viable option to produce a nanocomposite due its chemical profile, with flavonoids, terpenoids, and tannins that present antimicrobial and antifungal activity in aqueous extracts (Chitmanat et al., 2005; Chansue and Assawawongkasem, 2008; Claudiano et al., 2009). In addition, the production of this nanocomposite using fallen leaves, avoids the death of plants and reduces production costs (Coccaro et al., 2013). For these reasons, this study produced, characterized, and evaluated a nanocomposite constituted by AgNPs and an aqueous extract of *T. catappa* against *S. parasitica in vitro* and *in vivo* assays.

2. Material and methods

2.1. Synthesis of AgNP-PVA

The silver nanoparticles were synthetized according to methodology adapted from Pencheva et al. (2012). Two formulations ($AgNPs_{30}$ and $AgNPs_{300}$) were synthesized using different concentrations of silver nitrate – $AgNO_3$ (Labsinth® – N1011.01.AD;P.A.-A.C·S; 30 and 300 mg. L^{-1}). For the nanoparticle synthesis, 5 g of polyvinyl alcohol (PVA) was dissolved in 95 mL of deionized water under agitation at 80 °C. In parallel, 30 or 300 mg of silver nitrate was dissolved in 5 mL of water and then added dropwise, under agitation, into the solution of 95 mL of PVA (5%). Subsequently, the prepared solution was heated at 100 °C for 1 h to form AgNP stabilized with PVA.

2.2. Production of Terminalia catappa aqueous extract

The *T. catappa* aqueous extract was produced and characterized according to Meneses et al. (2020). *Terminalia catappa* dried leaf powder (25 g) was soaked in 500 mL of distilled water, and heated at 80 ± 2 °C, for 1 h. The extracted material was filtered and submitted to a re-extraction process using 500 mL more of distilled water. The extract was lyophilized using a Labconco Freezone® 4.5 and maintained in a desiccator. For the experiment, the lyophilized extract was re-suspended in water at a concentration of 5 $g.L^{-1}$.

2.3. Synthesis of nanocomposite of silver nanoparticles and aqueous extract

This study produced two formulations of the nanocomposite labeled F30 and F300 constituted of synthetized silver nanoparticles at different concentrations ($AgNPs_{30}$ and $AgNPs_{300}$) (Pencheva et al., 2012; Meneses, 2017) and aqueous extract of *T. catappa* (Meneses et al., 2020).

To produce the nanocomposite, the colloidal dispersion of AgNPs ($AgNPs_{30}$ or $AgNPs_{300}$) were added dropwise, at room temperature, into the re-suspended aqueous extract of *T. catappa* (1:1 v:v). The nanocomposite was storage at 4 °C in the dark.

2.4. Ultraviolet visible spectroscopy

The morphology and the stability of nanoparticles within the nanocomposite were evaluated with an ultraviolet-visible (UV–Vis) spectrophotometer (FEMTO 800xi) at a wavelength of 400 to 800 nm one week and one year after the synthesis (adapted from Ghozali et al., 2015). The aqueous extract and the AgNP dispersion were subjected to wavelengths between 200 and 800 nm. Subsequently, all data were plotted in the software Origin 6.0 Professional.

2.5. Transmission electronic microscopy (TEM)

A transmission electronic microscope TEM-MSC JEOL 2100 (200 kV acceleration) was used to measure nanoparticle size. The nanoparticles were directly dripped onto a copper grid and measured using the software Image tool 3.0. All histograms were developed using the software Origin 6.0.

2.6. Dynamic light scattering and zeta potential

The nanoparticles within nanocomposites (F30 and F300) were characterized with dynamic light scattering (DLS) and zeta potential using ZetaSizer (Malvern®) at room temperature (25 °C), determining the diameter, the polydispersity index, and the stability of the colloidal dispersion (Nasiriboroumand et al., 2018). All samples underwent dilution in deionized water at a ratio of 1:100 (sample:water) to avoid any retro-mirroring effect (Bourezg et al., 2012; Namvar et al., 2015). The analysis was conducted at 0 h (synthesis moment), one week, two weeks, one month, and three months after the synthesis.

2.7. Inductively coupled plasma optical emission spectrometry (ICP-OES)

ICP-OES Varian 720-OES was used to measure and determine the silver ion concentrations in the nanocomposite with a standard solution of $AgNO_3$ Merck® ($AgNO_3$ at HNO_3 0.5 $mol.L^{-1}$). The samples were diluted with nitric acid solution (3%) and the concentrations were obtained at $mg.L^{-1}$. The concentration of AgNPs was calculated in terms of the difference between total silver and silver ions (Sun and Yang, 2014; Meneses, 2017).

2.8. High performance liquid chromatography (HPLC)

The chemical composition of aqueous *T. catappa* extract was determinate by HPLC. The extract was diluted with methanol/m-q water 50:50 v:v (4 $mg.L^{-1}$), submitted to an ultrasound sonicator (60 min) and then filtered at polytetrafluoroethylene membrane (0.45 μm). This study used chromatograph Shimadzu® containing one analytic column Phenomenex Luna® (4.6 × 250 mm – 5 μm particle size). The injection

Table 1
Volume and the different concentrations for both formulations of nanocomposite evaluated *in vitro* against *S. parasitica*.

Treatments	Volume of Nanocomposite		F30 Proportion between extract and nanoparticles		F300 Proportion between extract and nanoparticles	
	Solid medium	Liquid medium	AgNP (mg. L^{-1})	Extract (mg. L^{-1})	AgNP (mg. L^{-1})	Extract (mg. L^{-1})
Control	0	0	0	0.0	0.00	0.0
T_1	0.25 mL	1.25 mL	1.61	62.5	45.75	62.5
T_2	0.50 mL	2.50 mL	3.23	125.0	91.50	125.0
T_3	1.00 mL	5.00 mL	6.46	250.0	183.00	250.0

AgNPs – silver-nanoparticles.

volume was 20 μL, and the mobile phase flow rate was 1.0 mL.min^{-1} with gradient **A**: 0.1% acetic acid in ultrapure water (milli-q system) and **B**: methanol (HPLC degree) (adapted from Bensaad et al., 2017). The chemical compounds were quantified according to calibration curves of standard solutions (gallic acid, ellagic acid, α and β punicalagin) (Meneses et al., 2020). Because the nanocomposite could not be directly evaluated in the chromatograph, the active compounds were determined indirectly through the pure aqueous extract concentrations.

2.9. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) assay

Two *in vitro* assays were performed with different culture media (*i.e.*, solid and liquid). The first *in vitro* assay (solid medium) had a completely randomized design with three treatments – one control and three replicates for both formulations (F30 and F300) (Table 1). Petri dishes (80 × 10 mm and 10 mL volume) received potato dextrose agar (PDA) culture medium plus concentrations of the nanocomposite.

PDA medium discs of 9 mm diameter containing the mycelium of *S. parasitica* (GenBank accession no. KY418035) were inoculated in Petri dishes containing PDA and concentrations of the nanocomposite. Mycelial growth was evaluated every 24 h throughout the 96 h with the aid of digital pachymeter inox 150 mm LEE tools (adapted from Corrêa et al., 2013) (Fig. 1). At the end of the exposure time (96 h), new Petri dishes containing only PDA received the discs without any mycelial growth (more 96 h) to confirm a fungistatic or fungicidal effect (Fig. 1).

The second *in vitro* assay (liquid medium) had the same experimental design. Potato dextrose broth (50 mL) containing concentrations of the nanocomposite received four discs with *S. parasitica* mycelium in Erlenmeyers (125 mL). The four discs remained in constant agitation (Cientec® CT-712RN) with 125 rpm rotation at room temperature (25 °C) (Fig. 1).

Every day (until 96 h), one disc of each treatment was transferred into Petri dishes containing PDA without concentrations of the nanocomposite, to determine the fungistatic or fungicidal effect through mycelial growth, and observed for 96 h more (Fig. 1).

2.10. Prophylactic treatment against zoospore of S. parasitica (infecting phase) on eggs of angelfish Pterophyllum scalare

This study obtained the approval of the Brazilian Agriculture Research Corporation ethical committee for animals (CEUA/00262019).

The experiment used 15 adult Angelfish couples, which were kept in glass aquariums (75 L) plugged into a water recirculation system. Every glass aquarium had one ceramic piece as substrate for spawning, and the fish were fed twice a day with commercial ration (Poytara® with crude protein at 32%).

After spawning, the substrate was carefully removed and placed in a polyethylene incubator with a useful volume of 2 L. The incubator was placed into the parent's aquarium containing water from the recirculation system. The natural occurrence of fungal infections in eggs was recorded and photographed before and after experimental treatments. The experiment utilized a completely randomized design with a negative control (only the water of the system), a positive control (methylene blue – 1 mg.L^{-1}, Yeasmin et al., 2015; Rahman et al., 2017), and three concentrations of the nanocomposite F300 (based on the best results of the *in vitro* assays) with 6 repetitions (Table 2). The evaluation lasted 72

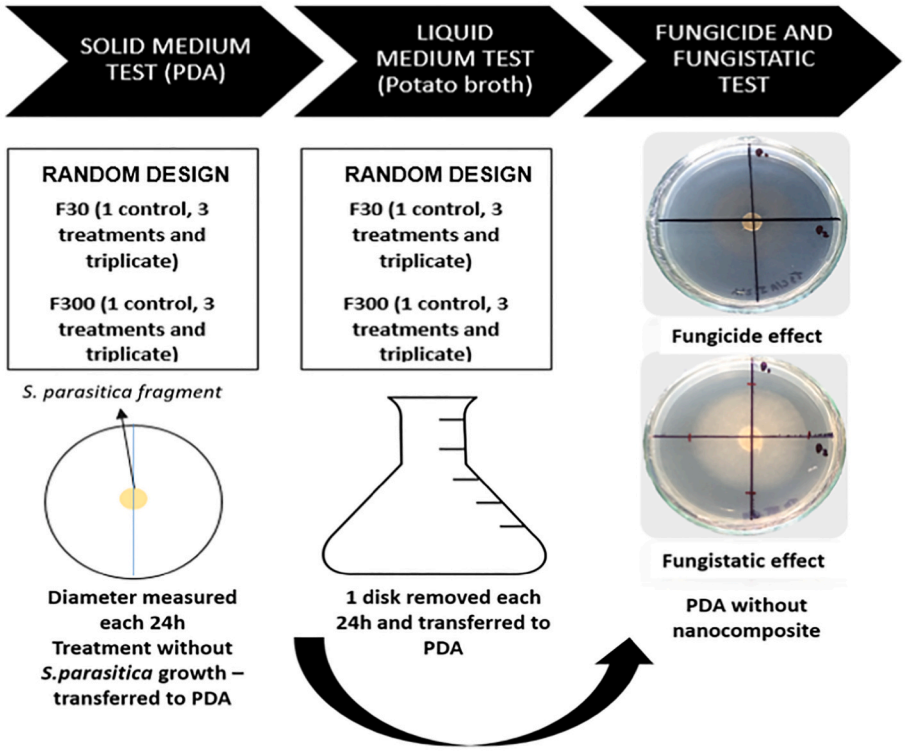

Fig. 1. Experimental design for both *in vitro* tests (solid and liquid medium).

Table 2
Different concentrations of nanocomposite F300 tested *in vivo* for eggs of Angelfish *Pterophyllum scalare*.

Negative control	Water of the system				
Positive control	Methylene blue (1 mg.L^{-1})				
Treatment with nanocomposite	F300				
	Concentration of nanocomposite (μL.L^{-1}) v:v[a]	Concentration of AgNPs within nanocomposite (μL.L^{-1}) v:v	Concentration of extract on nanocomposite (μL.L^{-1}) v:v	Concentration of AgNPs (μg.L^{-1}) in each nanocomposite concentration tested w:v[a]	Concentration of extract (μg.L^{-1}) in each nanocomposite concentration tested w:v
T_1	25	12.5	12.5	45.75	62.5
T_2	50	25	25	91.5	125
T_3	100	50	50	183	250

[a] v:volume; w: weight.

h, A total of 30 spawning with 503.77 ± 197.85 eggs was used in the experiment with each spawning considered a replicate.

Photographs were taken every 24 h to count viable, nonviable (opaque) (Oberlercher and Wanzenböck, 2016), and infected eggs. Counting was carried out with the aid of the software image J®. In addition, live and dead larvae were counted at the end of the experiment.

The water quality parameters such as temperature (YSI 55-12FT®), dissolved oxygen (YSI 55-12FT®), pH (AKROM KR20®), and electric conductivity (YSI 30-10FT®) were measured every day. Furthermore, total ammonia was determined at the end of the experiment using a colorimetric test.

2.11. Statistical analysis

In vitro data of solid and liquid media, after verification of normality (Shapiro Wilk Test) and homoscedasticity (Levene Test) assumptions were conducted of one-way analysis of variance (ANOVA) with post-hoc Tukey test for mean comparison. *In vivo* data about the angelfish eggs were submitted to non-parametric Kruskal Wallis test with post-hoc Mann Whitney for comparison of posts. All statistical analysis were performed on SISVAR 5.6, BioEstat 5.3, and Past considering 5% ($p < 0.05$) for any significant difference (Zar, 2009).

3. Results and discussion

3.1. Characterization of the nanocomposite

The analysis in the UV–Vis region confirmed the presence of the nanoparticles in the nanocomposite (Fig. 2D and E). The AgNPs (Fig. 2B and C), and the extract showed interaction. The presence of aqueous extract of *T. catappa* (Fig. 2A) turned the nanocomposite solution darker, displacing the absorption peak (F300) to the red region of the wavelength. The change of colour from yellow to brown in the nanocomposite was attributed to the collective oscillation of free conduction electrons, resulting in surface plasmon resonance by the electromagnetic field, as is common for AgNPs (Peng et al., 2010; Ajitha et al., 2015; Chandraker et al., 2019).

As expected, nanocomposite F30 showed a lower concentration of AgNPs (64.60 mg.L^{-1}) with greater variation at maximum absorbance (Fig. 2D) compared to F300 at a higher concentration (1830 mg.L^{-1}) (Fig. 2E).

For both formulations (F30 and F300), the nanoparticles demonstrated a spheroidal morphology (Fig. 3A and C), which is the most common shape for colloidal dispersion owing to thermodynamic stability (Elaissari, 2008). F30 ranged from 1 to 45 nm, with a higher frequency of 10–20 nm and a mean size of 19.93 ± 0.54 nm. F300 ranged from 1 to 55 nm, with a higher frequency of 5–10 nm and a mean size of 12.97 ± 10.90 nm (Fig. 3B and D).

The nanocomposite F30 presented reduced intensity of the plasmon band after one year, probably owing to the lack of silver ions and/or deposition of nanoparticles on the glass walls of the vessel (Pastoriza-Santos et al., 2000; Da Costa et al., 2011). In addition, the increased size of nanoparticle could be related to the excessive amount of aqueous extract without interaction with the silver ions (Bhainsa and D'Souza, 2006), making it more unstable and uneven (Shipway et al., 2000; He et al., 2002) (Fig. 2A and B).

The inverse occurred with the nanocomposite F300, as the amount (number) of nanoparticles increased, verified by the plasmon band after one year (Fig. 2E). This increase could be due to by the reduction of ions in the aqueous extract solution (Bhainsa and D'Souza, 2006; Da Costa, 2011). The higher concentration of silver ions used to synthetize AgNPs (300 mg) probably had a greater interaction with the extract, increasing the number of nanoparticles (Fig. 3C and D). Thus, this formulation showed smaller and more uniform nanoparticles than F30 (Fig. 3A and B).

Similar results were observed using aqueous extract of Pomegranate bark (*Punica granatum*) (contains the same majority compound as *T. catappa* extract – punicalagin), which effects the intensity of the plasmon band due to the extract concentration, reducing agglomeration and increasing the nucleation of nanoparticles (Nasiriboroumand et al., 2018). In addition, the uniform distribution of free silver atoms with extract molecules results in the homogeneity of the nanoparticles and a low polydispersity index (PDI).

All values of the PDI for both formulations remained below 0.7 throughout the study period, indicating the homogeneity of the particles (Barani et al., 2010; Barani et al., 2014; Nasiriboroumand et al., 2018). However, despite the small variations, F30 showed the greatest alterations from one month to three months (Table 3).

In both DLS and TEM analysis, F30 displayed greater particle sizes; however, the former recorded larger nanoparticles than the latter, as also reported by Erjaee et al. (2017). The differences between the analyses (DLS and TEM) occurred because the method used in the latter measures size based on metallic nanoparticles without organic cover agent (Baalousha and Lead, 2007; Diegoli et al., 2008; Cumberland and Lead, 2009). DLS measures particle diameter considering the ions or molecules that are linked to the nanoparticle surface (Sapsford et al., 2011). Thus, such ions or molecules associated to AgNPs appear larger on the Zetasizer than the transmission electronic microscopy. For this reason, the hydrodynamic size is always larger (Huang et al., 2007), rendering it an appropriate methodology to represent the nanocomposite.

Both formulations remained stable with adequate zeta potential, <−30 Mv. Its negative values confirmed the strong repulsion among the particles, which increase the stability of the colloidal dispersion (Rao et al., 2013; Anandalakshmi et al., 2016). Nanocomposite F30 exhibited greater variation for this index over time than F300 (Table 3).

The aqueous extract of *T. catappa* showed the following concentrations: ellagic acid 9.54 μg.mg^{-1}, gallic acid 7.36 μg.mg^{-1}, α punicalagin 13.32 μg.mg^{-1}, and β punicalagin 22.87 μg.mg^{-1}. These compounds

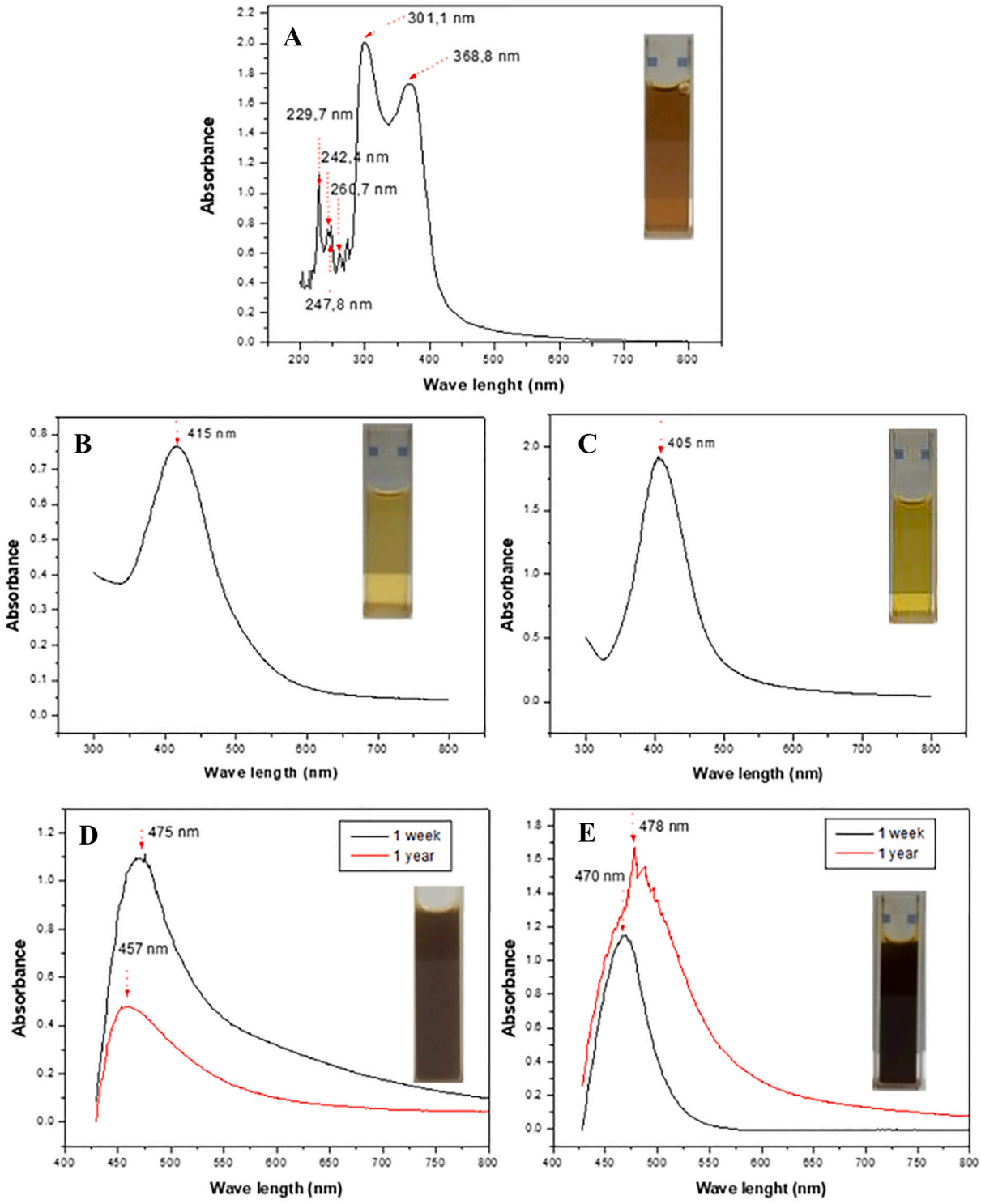

Fig. 2. Spectrum at ultraviolet-visible for aqueous extract of *T. catappa* (A); $AgNPs_{30}$ (B); $AgNPs_{300}$; (C); Nanocomposite F30 (D); Nanocomposite F300 (E).

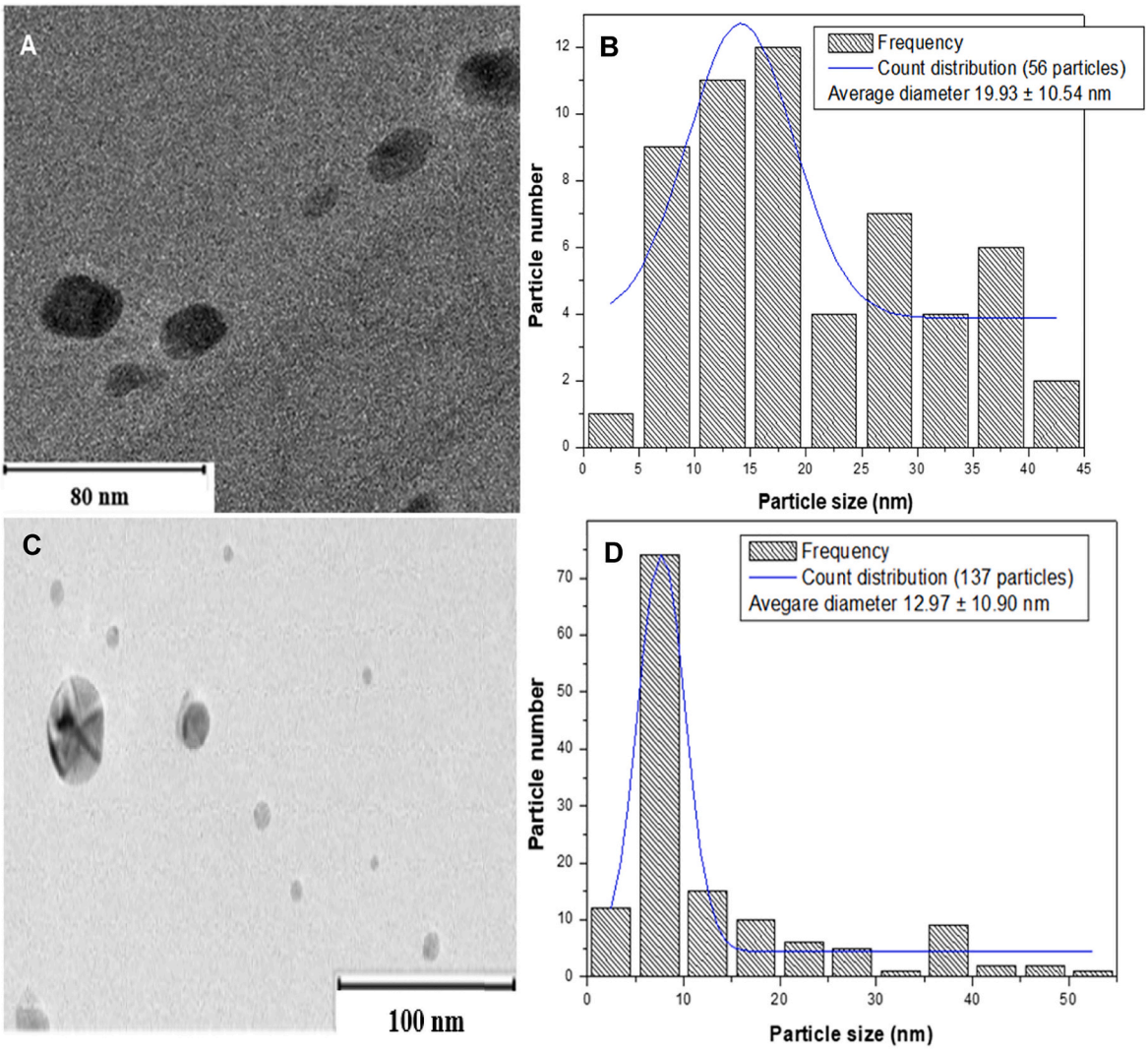

Fig. 3. Transmission electronic microscopy (TEM) of AgNPs in nanocomposite F30 (A), histogram with size distribution (B), TEM of AgNPs in nanocomposite F300 (C), histogram with size distribution.

Table 3
Mean size of nanoparticle, polydispersity index, and zeta potential of nanoparticles into both nanocomposite formulations (F30 and F300) over time.

Trial time	F30			F300		
	Mean size with DLS (d.nm)	PDI	Zeta (mV)	Mean size with DLS (d.nm)	PDI	Zeta (mV)
0 h	123.2	0.182	−13.85 ± 3.6	54.76	0.299	−14.46 ± 0.25
1 week	107.7	0.212	−11.76 ± 1.55	61.91	0.193	−18.53 ± 1.96
2 weeks	119.8	0.224	−24.03 ± 0.11	75.72	0.359	−14.53 ± 1.50
1 month	92.7	0.261	−18.56 ± 1.71	62.79	0.223	−13.53 ± 0.46
3 months	96.37	0.393	−13.43 ± 5.51	61.28	0.232	−14.93 ± 3.27

also occurred in the chemical profile of an aqueous extract of pomegranate (Bensaad et al., 2017; Nasiriboroumand et al., 2018). In another study about the aqueous extract of pomegranate (produced with ethanol + ether + water) and its characterization, the authors found similar compounds but at different concentrations: ellagic acid 34.5 ± 1.63 μg.mg^{-1}, gallic acid 3.37 ± 0.07 μg.mg^{-1}, α punicalagin 1.06 ± 0.02 μg.mg^{-1}, and β punicalagin 2.07 ± 0.03 μg.mg^{-1} (Singh et al., 2014). The present study obtained lower concentrations only for ellagic acid. The chemical compounds and concentrations from the aqueous extract of *T. catappa* were calculated for the nanocomposite F300 (Table 4).

3.2. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) assay – solid medium

For the *in vitro* test (solid medium), increases in nanocomposite concentration reduced the mycelial growth in any formulation at 24 h of exposure. Nonetheless, F300, showed the greatest inhibition value of 2.81 ± 0.45 mm, highlighted as the most effective treatment in the fungus control.

The lower concentrations of F30 promoted similar mycelial growth to the control group, but different from higher concentration of

Table 4
Concentrations of nanocomposite tested *in vitro* and *in vivo* and weight of active compounds present in each tested concentration (according to HPLC quantification of crude extract).

Concentrations of nanocomposite tested *in vitro* and *in vivo* containing *T catappa* extract and AgNPs[a]	Weight of active compounds present in each nanocomposite concentration tested *in vitro* (I·V) and *in vivo* (I.Vo)							
	Gallic acid (μg)		Ellagic acid (μg)		α-Punicalagin (μg)		β-punicalagin (μg)	
	I·V	I.Vo	I·V	I.Vo	I·V	I.Vo	I·V	I.Vo
T_1 of nanocomposite	460	0.46	600	0.60	830	0.83	1440	1.44
T_2 of nanocomposite	920	0.92	1190	1.19	1660	1.66	2870	2.87
T_3 of nanocomposite	1840	1.84	2390	2.39	3330	3.33	5740	5.74

[a] *in vitro* (I·V) concentrations: T_1: 45.75 mg.L^{-1} AgNPs +62.5 mg.L^{-1} *T. catappa* extract; T_2: 91.50 mg.L^{-1} AgNPs+125 mg.L^{-1} *T. catappa* extract; T_3: 183 mg.L^{-1} AgNPs +250 mg.L^{-1} *T. catappa* extract; *in vivo* (I.Vo) concentrations: T_1: 45.75 μg.L^{-1} AgNPs +62.5 μg.L^{-1} *T. catappa* extract; T_2: 91.50 μg.L^{-1} AgNPs+125 μg.L^{-1} *T. catappa* extract; T_3: 183 μg.L^{-1} AgNPs +250 μg.L^{-1} *T. catappa* extract.

Table 5
Mycelial growth of *S. parasitica* on solid medium in the *in vitro* assay.

Treatment	24 h		96 h	
	F30 Diameter (mm)	F300 Diameter (mm)	F30 Diameter (mm)	F300 Diameter (mm)
C*	51.11 ± 0.77d	51.11 ± 0.77 d	73.31 ± 0.13 b	73.31 ± 0.13 d
T_1	34.41 ± 1.27 c	10.18 ± 0.39 c	73.42 ± 0.02 b	56.96 ± 0.69 c
T_2	22.38 ± 0.30 b	5.45 ± 0.48 b	73.26 ± 0.04 b	29.02 ± 0.35 b
T_3	10.07 ± 0.25 a	2.81 ± 0.45 a	48.92 ± 0.47 a	16.12 ± 0.43 a

* C: control; T_1: 45.75 mg.L^{-1} AgNPs +62.5 mg.L^{-1} *T. catappa* extract; T_2: 91.50 mg.L^{-1} AgNPs+125 mg.L^{-1} *T. catappa* extract; T_3: 183 mg.L^{-1} AgNPs +250 mg.L^{-1} *T. catappa* extract; Different letters in the column means statistical difference by Tukey test ($p < 0.05$).

Table 6
Mycelial growth of *S. parasitica* exposed to nanocomposite (both formulations *in vitro*) into liquid medium for 24 h.

Treatments	Mycelial growth after 24 h of exposition to nanocomposite and 24 h without nanocomposite (PDA)		Mycelial growth after 24 h of exposition to nanocomposite and 96 h without nanocomposite (PDA)	
	F30 Diameter (mm)	F300 Diameter (mm)	F30 Diameter (mm)	F300 Diameter (mm)
C*	65.56 ± 0.90 a	65.56 ± 0.90 c	73.48 ± 0.10 a	73.48 ± 0.10 c
T_1	57.37 ± 2.04 a	51.86 ± 1.27 c	73.39 ± 0.10 a	73.24 ± 0.04 b
T_2	52.39 ± 3.33 a	36.08 ± 16.95 b	73.33 ± 0.05 a	73.34 ± 0.19 bc
T_3	51.73 ± 1.69 a	0.00 ± 0.00 a	73.30 ± 0.03 a	0.00 ± 0.00 a

* C: control; T_1: 45.75 mg.L^{-1} AgNPs +62.5 mg.L^{-1} *T. catappa* extract; T_2: 91.50 mg.L^{-1} AgNPs+125 mg.L^{-1} *T. catappa* extract; T_3: 183 mg.L^{-1} AgNPs +250 mg.L^{-1} *T. catappa* extract; Different letters in the column means statistical difference by Tukey test ($p < 0.05$).

nanocomposite (48.92 ± 0.47 nm) at 96 h of exposure. The higher concentration of F300 promoted higher reduction of mycelial growth compared to control group, achieving a greater inhibition of growth (16.12 ± 0.43 nm) at the same exposure time. Nevertheless, the higher concentrations of both formulations did not demonstrate fungicidal effect, only fungistatic (Table 5).

Table 7
Mycelial growth of *S. parasitica* exposed to nanocomposite (both formulations *in vitro*) in liquid medium for 96 h.

Treatments	Mycelial growth after 96 h of exposition to nanocomposite and 24 h without nanocomposite (PDA)		Mycelial growth after 96 h of exposition to nanocomposite and 96 h without nanocomposite (PDA)	
	F30 Diameter (mm)	F300 Diameter (mm)	F30 Diameter (mm)	F300 Diameter (mm)
C*	56.45 ± 3.33 b	56.45 ± 3.33 c	73.38 ± 0.13 a	73.38 ± 0.13 b
T_1	55.78 ± 0.56 b	42.61 ± 4.91 b	73.37 ± 0.05 a	73.40 ± 0.04 b
T_2	60.29 ± 1.63 b	0.00 ± 0.00 a	73.24 ± 0.05 a	0.00 ± 0.00 a
T_3	39.53 ± 1.13 a	0.00 ± 0.00 a	73.32 ± 0.09 a	0.00 ± 0.00 a

* C: control; T_1: 45.75 mg.L^{-1} AgNPs +62.5 mg.L^{-1} *T. catappa* extract; T_2: 91.50 mg.L^{-1} AgNPs+125 mg.L^{-1} *T. catappa* extract; T_3: 183 mg.L^{-1} AgNPs +250 mg.L^{-1} *T. catappa* extract; Different letters in the column means statistical difference by Tukey test ($p < 0.05$).

Another study using the ethanolic extract of snake jasmine (*Rhinacanthus nasutus*) and galanga (*Kaempferia galangal)* showed fungistatic effects, but at higher concentrations (2500 and 5000 mg.L^{-1}, respectively) (Udomkusonsri et al., 2007) compared to the present study at 24 h of exposure. Fungistatic effects were observed in 48 h of exposure in other studies using the aqueous extract of *Cnidium monnieri*, *Magnolia officinalis,* and *Aucklandia lappa* (Xue-Gang et al., 2013), and ethanolic extract of *Mentha longifolia*, *Satureja bachtiarica*, *Hyssopus officinalis*, *Tanacetum partheniu,* and *Myrtus communis* (Pirbalouti et al., 2009) at a concentration of 500 mg.L^{-1}, which is a higher concentration than used in the present study.

In a study using the same exposure method, Johari et al. (2015a) found a fungistatic effect at AgNP concentrations of 1000, 1200, 1400, and 1600 mg.L^{-1} against *S. parasitica*, as well as a fungicide effect at concentrations of 1800 and 2000 mg.L^{-1}. In our present study, a lower concentration was used to promote the same fungicide effect, probably indicating a synergistic effect between the nanoparticles and the aqueous extract (AgNPs 183 mg.L^{-1} + extract 250 mg.L^{-1}).

3.3. Minimum inhibitory concentration (MIC) and Minimum fungicidal concentration (MFC) assay – liquid medium

In the liquid medium assay, the greater surface contact between the fungus and the nanocomposite promoted increased efficacy in

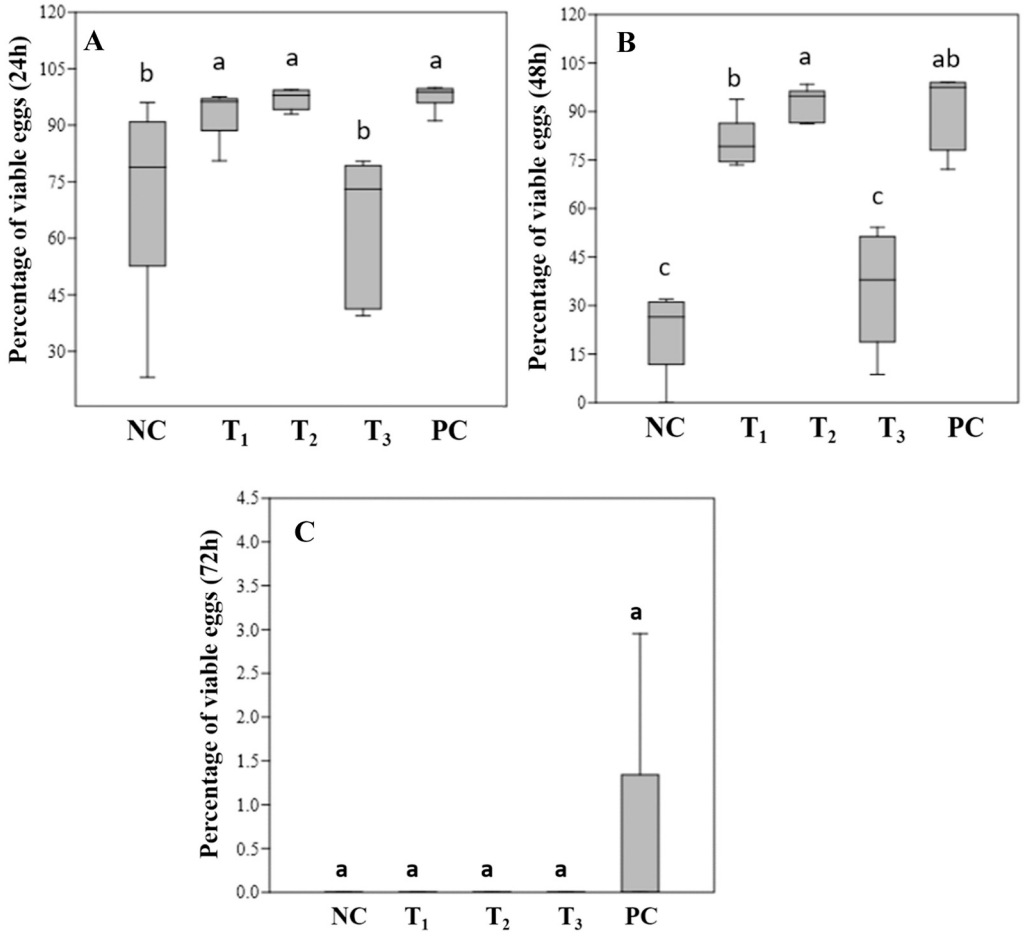

Fig. 4. Viable eggs rate in the different treatments at 24(A), 48(B), and 72 h(C). NC: negative control (water); PC: positive control (methylene blue); T_1: 45.75 μg.L^{-1} AgNPs +62.5 μg.L^{-1} *T. catappa* extract; T_2: 91.5 μg.L^{-1} AgNPs +125 μg.L^{-1} *T. catappa* extract; and T_3: 183 μg.L^{-1} AgNPs +250 μg.L^{-1} *T. catappa* extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

controlling the mycelial growth, different from that observed in solid medium assay. The F300 demonstrated a fungicide effect at concentration T_3 (AgNPs 183 mg.L^{-1} + aqueous extract 250 mg.L^{-1}) in the first 24 h of exposure, confirmed by the absence of mycelial growth in medium without the nanocomposite up to 96 h (Table 6).

Nonetheless, after 96 h of exposure, the second concentration of F300 (T_2 = AgNPs 91.5 mg.L^{-1} + aqueous extract 125 mg.L^{-1}) achieved a fungicide effect without any mycelial growth in the pure medium culture (Table 7).

Few studies have been conducted about the use of other nanocomposites against *Saprolegnia* sp. Johari et al. (2014) found a fungicide effect using a nanocomposite containing silver zeolite at concentrations of 1000 and 2000 mg.L^{-1}, 10 and 20 times greater, respectively, than the concentrations used in the present study with AgNPs.

For this reason, the nanocomposite used here should be regarded as an appropriate and eco-friendly product for *S. parasitica* control because of the use of lower concentrations, due to a synergistic effect between nanoparticles and *T. catappa* extract. This is an important factor due to the harmful conditions cause by extended permanence of silver ions in the environment (Fisher and Wang, 1998; Bianchini et al., 2002) and their toxic effects on aquatic life, mainly when used directly in water (Asghari et al., 2012; Mathivanan et al., 2012; Johari et al., 2013; Asztemborska et al., 2014; Hosseini et al., 2014; Tavana et al., 2014; Johari et al., 2015b).

3.4. Prophylactic treatment against zoospore of S. parasitica (infecting phase) in vivo for eggs of angelfish Pterophyllum scalare

In addition to the effects of the nanocomposite *in vitro*, this study evaluated its potential to control fungus in angelfish eggs, which are naturally susceptible to the zoospores (infectious phase of the fungus). According to one previous study, *in vitro* concentrations had toxic effects on eggs, requiring a readjustment of the concentrations. The new concentrations were defined by a sensibility test according to Yang et al. (2018).

The water quality parameters used were temperature (27.74 ± 1.47 °C), pH (7.01 ± 0.82), electric conductivity (0.16 ± 0.03 μS.cm^{-1}), dissolved oxygen (6.06 ± 0.88 mg.L^{-1}), and toxic ammonia with zero values. All of these remained within the required range for angelfish *Pterophyllum scalare* rearing (Pereira et al., 2016; Da Costa Sousa et al., 2019).

The percentage of viable eggs at 24 and 48 h showed statistical differences ($p < 0.05$) among the treatments (Fig. 4A and B). The control group had the lowest number of viable eggs at 48 h, demonstrating the high pathogenicity of the fungus in naturally infected eggs. The two lower concentrations of the nanocomposite at 24 and 48 h yielded the highest viable egg rates, statistically similar to the methylene blue (positive control) (Fig. 4A and B). After 48 h of exposure, the highest concentration of the nanocomposite (AgNPs 183 μg.L^{-1} + aqueous

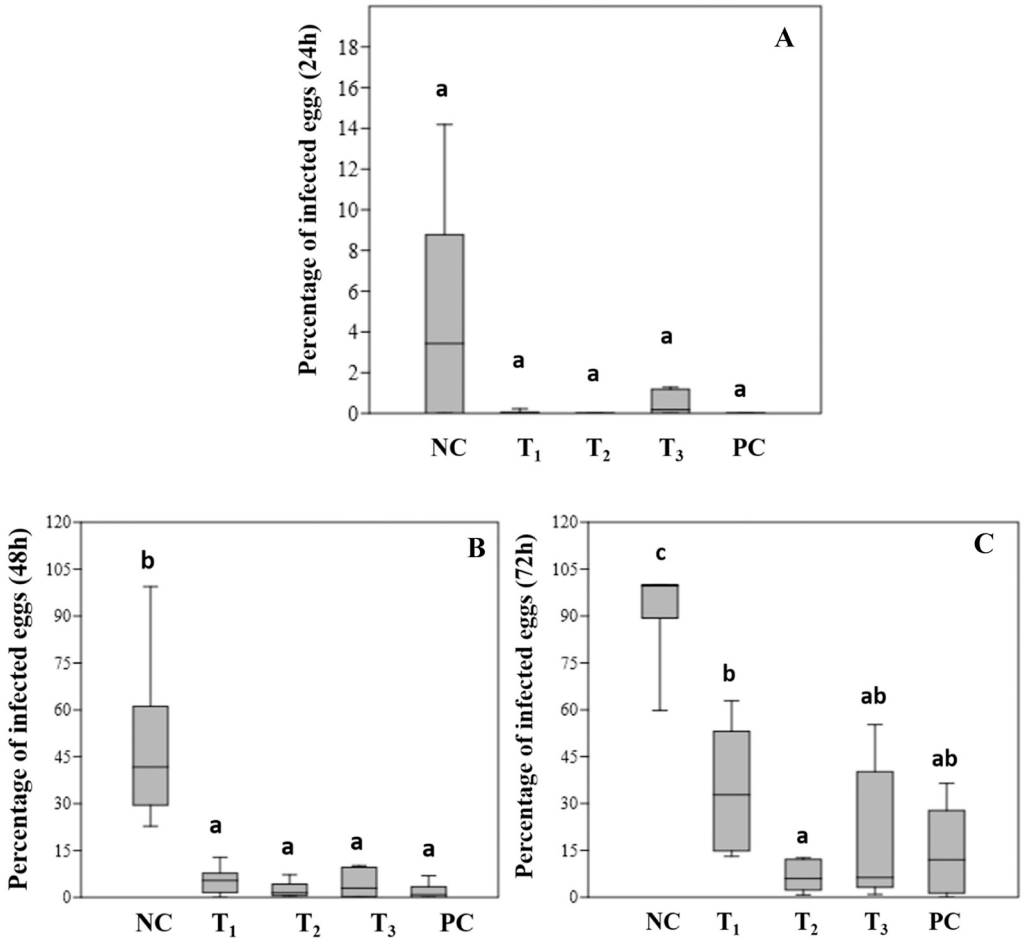

Fig. 5. Infected eggs rate with *S. parasitica* in the different treatments at 24(A), 48(B), and 72 h(C). NC: negative control (water); PC: positive control (methylene blue); T_1: 45.75 μg.L^{-1} AgNPs +62.5 μg.L^{-1} *T. catappa* extract; T_2: 91.5 μg.L^{-1} AgNPs +125 μg.L^{-1} *T. catappa* extract; and T_3: 183 μg.L^{-1} AgNPs +250 μg.L^{-1} *T. catappa* extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extract 250 μg.L^{-1}) produced a lower viable egg rate than the control group (only water), demonstrating the toxic effect in this concentration (Fig. 4B).

The eggs hatched into the treatments with the nanocomposite after 72 h (Fig. 4C), which is a normal time for this fish species. However, in methylene blue treatment for the same observation time (72 h), viable eggs were still founded, probably due to its retardant effect on embryo formation (Sanabria et al., 2009). Shapiro (1948) tested several chemicals for echinoderms in the initial stage of cleavage and observed that methylene blue retarded the first cleavage of sea urchin eggs. The toxic effect of methylene blue was reported in catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*) (Willford, 1966). In *Cyprinus carpio* eggs, increasing methylene blue concentration from 1 to 5 mg.L^{-1} reduced hatching (Yeasmin et al., 2015).

The treatments did not differ statistically for the infected eggs rate at 24 h, but after 48 h the negative control showed a higher infection rate (Fig. 5A and B), and the treatments (concentrations of nanocomposite and methylene blue) were statistically similar.

After 72 h (Fig. 5C), the nanocomposite at a concentration of AgNPs 91.5 μg.L^{-1} + aqueous extract 125 μg.L^{-1} achieved a similar effect to methylene blue, although its concentration was 10 times less than methylene blue 1000 μg.L^{-1}.

The second treatment (T_2) and the positive control (methylene blue) yielded more viable larvae (Fig. 6). The highest concentration (T_3) obtained less viable larvae probably due to a toxic effect. At the other end of the scale, the negative control (only water) also presented no viable larvae (Fig. 6).

The freshwater angelfish achieve greater hatching rate in wild environments or in captivity when the eggs remain with the parents, due the parental care. The parents remove non-fertilized and non-viable eggs and constantly agitate the water to avoid proliferation of the pathogen (Degani and Yehuda, 1996; Farahi et al., 2011). However, in intensive production, the spawns that are away from their parents have a hatching rate of zero according to Chambel et al. (2014), mainly due the *Saprolegnia* infection (Ahmed et al., 1990).

The utilization of prophylactic methods throughout egg development is crucial to avoiding Saprolegniosis. The fungus adheres and penetrates into the chorion, weakening eggs; absorbing nutrients, such as glycoprotein or lipoprotein; and thereby preventing embryo growth, which ultimately leads to death (Almufrodi et al., 2013; Van Den Berg et al., 2013; Humsari, 2017).

The mycelium and zoospore were differently affected by nanocomposite, mainly to applied concentrations. For the *in vitro* assay, this study evaluated the effect of the nanocomposite against mycelium growth. However, for the *in vivo* assay, the target was the zoospore. This difference was reported by Sun and Yang (2014) with copper sulfate, using a concentration of 5 mg.L^{-1} to control mycelium growth, 50 times greater (0.1 mg.L^{-1}) than that used to obtain a fungicide effect in zoospores.

Another study (Xue-Gang et al., 2013) described the same difference using *Cnidium monnieri* plus petroleum ether (mycelium 62.5 mg.L^{-1} and zoospore 25 mg.L^{-1}), *Magnolia officinalis* (mycelium 62.5 mg.L^{-1}

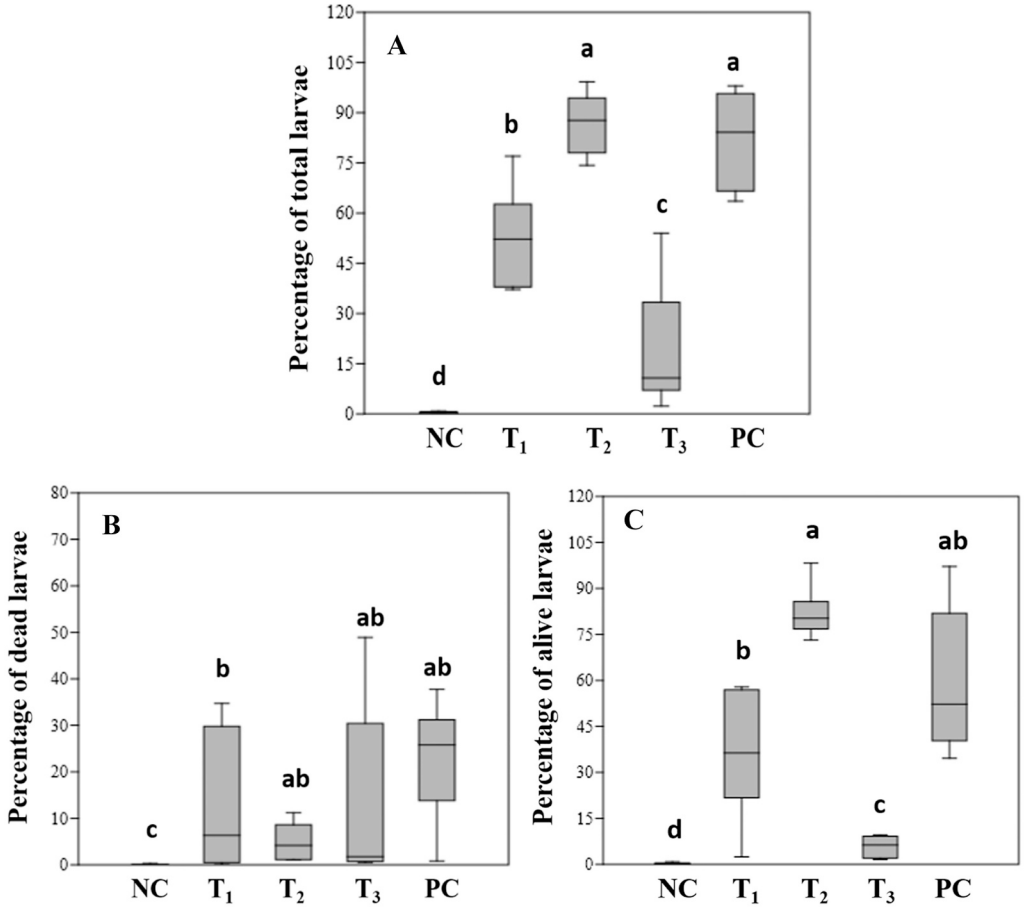

Fig. 6. Total viable larvae (A), dead larvae (B), and live larvae (C) among the different treatments at the end of experiment (72 h). NC: negative control (water); PC: positive control (methylene blue); T_1: 45.75 μg.L^{-1} AgNPs +62.5 μg.L^{-1} *T. catappa* extract; T_2: 91.5 μg.L^{-1} AgNPs +125 μg.L^{-1} *T. catappa* extract; and T_3: 183 μg.L^{-1} AgNPs +250 μg.L^{-1} *T. catappa* extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and zoospore 12.5 mg.L^{-1}), and *Aucklandia lappa* (mycelium 62.5 mg.L^{-1} and zoospore 25 mg.L^{-1}) extracts against *Saprolegnia*, all of which reported higher concentration for the same effects in the mycelium stage.

Thus, future studies using a nanocomposite to control mycelium at different life stages of fish must be conducted to determine toxic effects and ensure the fish's safety.

The use of aqueous extract of *T. catappa* improved the effect of the nanocomposite because its chemical profile with polyphenols (flavonoids) and tannins (gallic acid, ellagic acid, α and β punicalagin) has a fungicide effect, protecting eggs and avoiding fungal proliferation. Flavonoids form complex compounds with proteins, which penetrate cellular membrane, lysing it, avoiding fungal growth (Sulistyawati and Sri, 2009). In the present study, these effects allowed the use of lower concentrations of nanocomposites than other studies using isolated compounds (extracts or metallic nanoparticles) (Table 8).

The nanoparticle and the aqueous extract demonstrated a remarkable synergistic effect, reducing the amount of product to control *S. parasitica*. This is an important factor because less the metallic nanoparticles reach the egg (Böhme et al., 2017).

According to literature, silver ion is usually more toxic than nanoparticle due to different toxicity mechanisms for embryos (Zhao and Wang, 2011; Bilberg et al., 2012; Ribeiro et al., 2014). The chorion protected the eggs from external influences (Kimmel et al., 1995; Lee et al., 2007), it has pores (200 nm) (Hart and Donovan, 1983) that permit permeable gas and some nutrients (Rawson et al., 2000). However, the structure of chorion has negative charge, attracting and binding to positively charged metal ions (Hart and Donovan, 1983; Henn, 2011).

The toxicity of Ag + causes ionoregulatory disorders by sodium blockade (Potassium ATPase), triggering disturbances in fluid volume and circulatory collapse, leading to death (Mosselhy et al., 2016). Mortality of rainbow trout eggs by silver nitrate was associated to ionoregulatory impairment, in which 60% of Na + and Cl- was lost from the egg (Guadagnolo et al., 2000).

The transport of any particle across the chorion depends on size and particle properties, such as surface coating or stabilizers (Böhme et al., 2017). Some studies found that a smaller particle creates a higher specific surface area, increasing bioavailability or surface activity, which increases the particles toxicity (Park et al., 2010; Lin et al., 2013). The nanoparticles coated by the plant extract can avoid the internalization of the nanoparticles by increasing the total size of nanocomposite and decreasing the toxicity. Sarkar et al. (2014) reported higher toxicity of nanoparticles synthetized by chemical method in comparison to nanoparticles obtained by biological method (reduction through vegetal material) in zebrafish *Danio rerio*.

Silver nanoparticle accumulation in egg can also cause inadequate larval heart and caudal formation, as well as yolk sac edema at a concentration of 10 mg.L^{-1} AgNPs, as reported in *Danio rerio* (Orbea et al., 2017). Deformation and reduced physiological development of the embryo was observed at concentrations above 25 mg.L^{-1}, as well as the opacity of the chorion at 100 mg.L^{-1} (AgNPs + polyvinylpyrrolidone) (Caloudova et al., 2018). However, none of these alterations were observed after treatment with the nanocomposite (silver-nanoparticle + aqueous extract of *T. catappa*). Furthermore, the larvae development time and the number of viable eggs was not affected by exposure to our

Table 8
Different products used to control of *S. parasitica* with hatching rate and larval viability.

Treatment	Species	Concentration (mg. L^{-1})	Inhibition rate of *S. parasitica* (%)	Hatching rate (%)	Larval viability (%)	Reference
Aqueous extract with leaf of *Alpinia galanga*	Eggs of Catfish	600	Not available	64.78 ± 2.75	Not available	Saptiani et al., 2016
		800		68.86 ± 3.55	72.00 ± 2.65	
		1000		75.84 ± 0.77	74.33 ± 3.79	
Aqueous extract with rhizome of *Alpinia galanga*	Eggs of Catfish	600	Not available	75.91 ± 2.25	Not available	Saptiani et al., 2016
		800		80.73 ± 4.66	80.45 ± 1.72	
		1000		88.80 ± 1.37	83.42 ± 3.18	
Aqueous extract with leaf of *Alpinia galanga*	Eggs of Catfish	600	Not available	79.20 ± 3.87	Not available	Saptiani et al., 2016
		800		88.34 ± 3.01	73.67 ± 3.06	
		1000		88.48 ± 3.85	78.33 ± 1.53	
Aqueous extract with rhizome of *Alpinia galanga*	Eggs of Catfish	600	Not available	86.18 ± 4.59	Not available	Saptiani et al., 2016
		800		97.16 ± 0.96	89.79 ± 2.32	
		1000		96.21 ± 1.93	90.73 ± 2.20	
Ethanol extract of *Opuntia stricta*	Eggs of *Sander lucioperca*	163	32.8 ± 2.4	70	Not available	Khemis et al., 2015
		325	43.1 ± 1.0	>80		
		750	47.7 ± 1.4	>80		
		1500	50.2 ± 1.6	>80		
		3000	57.8 ± 0.8	>80		
Ethanol extract with rhizome of *Kaempferia galanga*	Eggs of catfish africano (*Clarias gariepinus*)	20	56.5 ± 0.090	56.50 ± 0.090	Not available	Humsari, 2017
		40	72.50 ± 0.136	69.00 ± 0.132		
		60	80.5 ± 0.139	75.50 ± 0.128		
		80	81.5 ± 0.073	66.5 ± 0.073		
Oxide Copper Nanoparticle	Eggs and larvae of *Labio rohita*	100	Not available	55.0 ± 2.2	45.8 ± 3.1	Swain et al., 2014
		250		54.0 ± 3.2	44.1 ± 3.2	
		500		77.0 ± 5.2	46.3 ± 5.2	
		1000		78.0 ± 3.1	47.85 ± 1.9	
		2500		72.1 ± 4.1	46.5 ± 2.5	
		5000		70.0 ± 1.7	46.1 ± 2.2	
Oxide Zinc Nanoparticle	Eggs and larvae of *Labio rohita*	100	Not available	63.0 ± 5.0	45.1 ± 2.4	Swain et al., 2014
		250		66.9 ± 3.1	45.2 ± 1.7	
		500		69.0 ± 4.1	44.7 ± 1.9	
		1000		78.05 ± 4.2	44.6 ± 4.0	
		2500		76.0 ± 3.4	42.5 ± 3.6	
		5000		69.3 ± 4.2	42.3 ± 2.8	
Nanoparticle of dioxide titanium and copper	Eggs and larvae of *Labio rohita*	100	Not available	30.0 ± 5.1	24.3 ± 2.8	Swain et al., 2014
		250		27.1 ± 2.8	24.6 ± 3.6	
		500		27.5 ± 4.3	23.5 ± 4.2	
		1000		24.8 ± 2.8	22.7 ± 3.1	
		2500		16.7 ± 3.1	0	
		5000		4.0 ± 0.3	0	

nanocomposite.

Thus, despite the possible metal internalization (Böhme et al., 2017), it was not harmful to the fish in the present nanocomposite formulation, because the nanoparticles may not have reached the embryo or the concentration was not sufficient to block the oxygen transportation.

Thus, the use of a nanocomposite allying AgNPs with the aqueous extract of *T. catappa* can reduce AgNPs concentration and hence its toxic effects for fish, the environment, or fish farmers, making it an eco-friendly alternative to improve larval survival in captivity conditions.

4. Conclusion

The nanocomposite (F300) showed silver-nanoparticles with small size and more stability, reflected in greater antifungal activity for both *in vitro* and *in vivo* tests. For prophylactic tests with eggs of angelfish *Pterophyllum scalare*, the concentration AgNPs 91.5 $\mu g.L^{-1}$ + aqueous extract 125 $\mu g.L^{-1}$ showed the best efficacy, providing a high rate of viable eggs, low rate of fungal infections, and greater larval survival.

Data availability statement

The data that support the findings of this study are available from the author upon reasonable request.

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This study has no funding to declare.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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