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Influence of coagulant *Moringa oleifera* seed extract on reduction of *Toxoplasma gondii* oocysts and viability of *Toxocara* spp. eggs in water

Lucimara Fátima Beletini[a], Letícia Nishi[a], Priscilla de Laet Sant'Ana[b], Luis Henrique Garcia Muniz[c], Fernanda Ferreira Evangelista[a], Sérgio Cardim[d], João Luís Garcia[d], Francini Martini Mantelo[b], Ana Lúcia Falavigna-Guilherme[b,*]

[a]*State University of Maringá (UEM), Postgraduate Program in Health Sciences, Colombo Avenue 5790, CEP 87020-900, Maringá, Paraná, Brazil, Tel. +55-44-3011-4924; emails: lubeletini@hotmail.com (L.F. Beletini), leticianishi@hotmail.com (L. Nishi), fer.evangelista@hotmail.com (F.F. Evangelista)*
[b]*State University of Maringá (UEM), Department of Basic Health Sciences (UEM), Colombo Avenue 5790, CEP 87020-900, Maringá, Paraná, Brazil, Tel. +55-44-3011-4924; emails: alfguilherme@uem.br (A.L. Falavigna-Guilherme), priscillalsmariano@gmail.com (P. de Laet Sant'Ana), francinimartini@hotmail.com (F.M. Mantelo)*
[c]*University Hospital of Maringá, Division of Clinical Analyses and Pharmacy, Mandacaru Avenue, 1590, CEP 87083-240, Maringá, Paraná, Brazil, Tel. +55-44-3011-4924; email: lhgmuniz@gmail.com*
[d]*State University of Londrina (UEL), Department of Veterinary Medicine, Rodovia Celso Garcia Cid, PR 445 Km 380, Londrina, Paraná, Brazil, Tel. +55-43-999892069; emails: stcardim@hotmail.com (S. Cardim), jlgarcia@uel.br (J.L. Garcia)*

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ABSTRACT

This work provides insights of coagulant *Moringa oleifera* (MO) and aluminum sulfate (AS) on reduction of *Toxoplasma gondii* oocysts and viability of *Toxocara* spp. eggs in water. Raw (RW) and distilled water (DW) samples were added with 10^6 oocysts and 800 eggs L^{-1}, before coagulation/flocculation/sedimentation process, the samples were analyzed for oocysts/eggs removal, and eggs viability. The qPCR demonstrated that MO reduced 87.9% and AS, 97.8% of oocysts. Due to their high density, the reduction of eggs was 99%–100%. The eggs viability demonstrates that MO was able to reduce the infective eggs and increase the number of free larvae (non-infecting). In electronic microscopy, a thinner MO layer was observed on the eggs. MO was able to reduce 72.2% of turbidity in RW, while AS reduced 95.8%. MO reduced the quantity of *T. gondii* oocysts and viability of *Toxocara* eggs, consequently, the transmission, being useful, low cost, and biodegradable coagulant.

Keywords: Coagulation; Drinking water treatment; Pathogen removal; *Toxoplasma gondii*; *Toxocara* spp.; qPCR

1. Introduction

Waterborne parasite infections represent a public health risk in both developed and developing countries and the use of groundwater for drinking or cooking without adequate water treatment or sanitation can increase the risk of infection in individuals who consume it [1].

The purpose of drinking purified water is to reduce or eliminate numerous impurities, including pathogens, organic matter and chemical elements. One of the stages consists of coagulation and flocculation which facilitate the removal by conventional physical treatment such as sedimentation or filtration of particles that are colloidal [2]. Inorganic coagulants and polymers are typically used during

* Corresponding author.

the water treatment process to remove suspended solids, bacteria and viruses. Iron and aluminum salts are widely used for this purpose in coagulation/flocculation process, since they are low cost products and effective in removing impurities of water [3,4]. However, they present some drawbacks such as a residual trace of metals in the treated water, which can cause undesirable damages to the individuals, mainly to the central nervous system, as well as the environment, to accumulate high sludge production in the water treatment plant (WTP) [5–7]. Alternative treatments with natural coagulants can bring some advantages such as being biodegradable, non-toxic, and with less sludge production [8]. One of the natural coagulants used is the *Moringa oleifera* (MO) Lam seed extract [7,9]. Laboratory studies have demonstrated success of these seed extracts in the removal of bacteria, algae and intestinal protozoa *Cryptosporidium* spp. and *Giardia* spp. in the aquatic environment [10–12]. However, there are no experiments with *Toxoplasma gondii*, as well as evolutionary parasite forms such as helminth eggs, which have higher mass and higher sedimentation capacity.

The protozoan *T. gondii* and the nematode *Toxocara* spp. are zoonosis of worldwide distribution and the infection may be common in humans by the ingestion of *T. gondii* oocysts and embryonated eggs of *Toxocara* spp. in contaminated water, fruit, vegetables and soil [13]. An important clinical manifestation for both is eye disease [14,15] and severe cases of toxoplasmosis may occur in fetal infection resulting in severe neurological and ocular disorders [16]. Therefore, this study aimed to observe the influence of coagulant *M. oleifera* (MO) seed extract on reduction of *T. gondii* oocysts and viability of *Toxocara* spp. eggs in water.

2. Materials and methods

The experiments were performed according to the flowchart presented in Fig. 1.

In order to verify the efficacy of coagulants in the removal of *T. gondii* oocysts, 1,000 mL samples of distilled water (DW) and raw water (RW) were added with 10^6 oocysts L^{-1} and submitted to coagulation/flocculation/sedimentation process (CFS) using 50 mg L^{-1} of MO and aluminum sulfate (AS), separately. After CFS process, the supernatants were submitted to concentration and membrane filtration, followed by mechanical extraction and elution. The oocysts present were quantified by quantitative polymerase chain reaction (qPCR) [17,18].

To verify the efficacy of coagulants in the removal of *Toxocara* spp., 800 eggs L^{-1} were added to DW and RW samples. The CFS process was performed with 10, 30 e 50 mg L^{-1} of coagulants separately. After the process, the supernatants of the samples were submitted to the concentration by formalin-ethyl acetate sedimentation technique, followed by microscopy to count the eggs in the supernatant. It was also observed the viability of the eggs in the sediment after the CFS process, with cultured based in Harada and Mori [19] technique followed by microscopy observation.

The analysis and process are described below.

2.1. Water samples

Two different types of water were used in the experiment: distilled water (DW) and raw water (RW). The RW samples were obtained from treatment plant located in the City of Maringá, Paraná State, southern Brazil, that is supplied from Pirapó River, with turbidity of 50 NTU. A volume of 1,000 mL of water samples were analyzed for the presence of *T. gondii* through the PCR technique, primers B1 and Toxo4–5, and *Toxocara* spp. eggs by the formalin-ethyl acetate sedimentation technique, followed by microscopy, as described later, to assure that the samples were free of the parasites.

A volume of 1,000 mL of each water sample was used for CFS process.

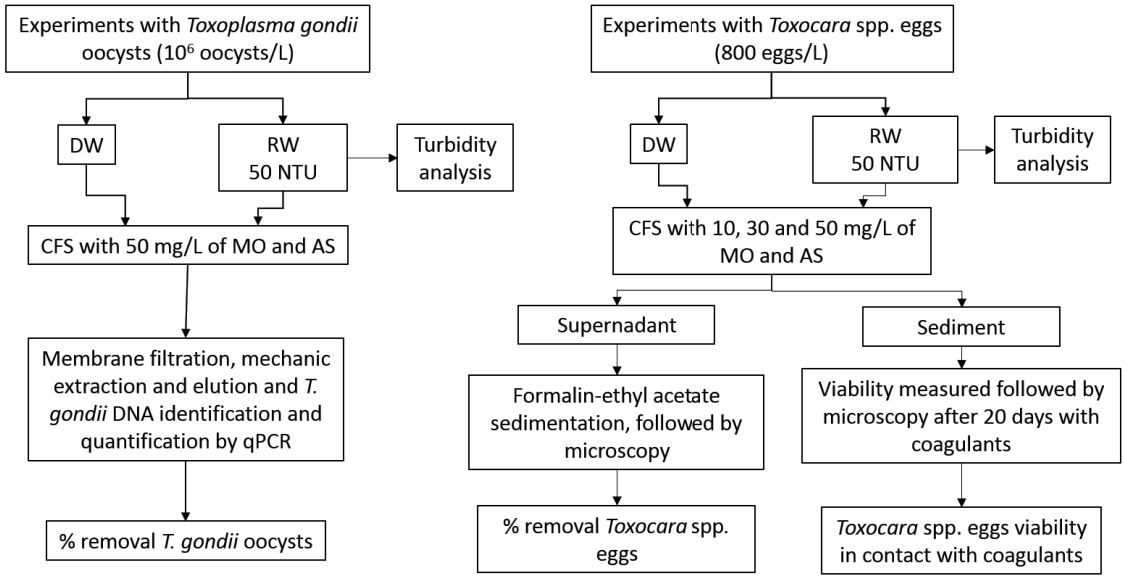

Fig. 1. Flowchart of coagulation/flocculation/sedimentation (CFS) experiments carried out in with samples of distilled water (DW) and raw water (RW) with coagulants MO and aluminum sulfate (AS).

2.2. Oocysts of T. gondii

Sporulated oocysts of the ME-49 strain of *T. gondii*, obtained from Medical Veterinary Department of State University of Londrina, Paraná State, Brazil, were used. The samples of RW and DW were contaminated with 10^6 oocysts in 1,000 mL, before the coagulant addition.

2.3. Eggs of Toxocara spp.

Fresh feces samples of dog infected with *Toxocara* spp. were prepared and the eggs obtained were counted in optical microscopy. An average of 800 eggs was added on each water sample (1,000 mL). About 95% of the eggs were non-embryonated.

2.4. Preparation of coagulants

Mature seeds of *M. oleifera* were previously removed from the pod and air dried. On the day of the experiments, the seed pulp was crushed, and a concentration of 1% weight/volume (w/v) was prepared to add 1 g of the powder in 100 mL of distillated water, agitated for 30 min and vacuum filtered in qualitative paper [20]. The 1% standard solution of the chemical coagulant was prepared by dissolving 10.3 g of aluminum sulfate powder (97% purity) in distilled water, and the volume was completed to 1,000 mL [21].

2.5. Coagulation/flocculation process

For each type of water (DW and RW), the tests were performed with 10^6 L^{-1} oocysts of *T. gondii*, at the concentration of 50 mg L^{-1} of *M. oleifera* and aluminum sulfate [11,21].

For the experiments with *Toxocara* spp., 800 eggs L^{-1} were added in two types of water (DW and RW) and the CFS process was performed with 10, 30, and 50 mg L^{-1} of each coagulant.

Experimental tests were conducted in jar test equipment Nova Ética-Model 218 LDB with six samples running simultaneously in beakers of 2,000 mL capacity. Water temperature was maintained at 25.0°C ± 3.0°C in all experiments [11].

The experimental conditions for the CFS process to MO were rapid mixing velocity (100 rpm), rapid mixing time (3 min), slow mixing velocity (15 rpm), slow mixing time (15 min) and settling time (60 min) [20]. To aluminum sulfate (AS), the experimental conditions for the CFS process were rapid mixing velocity (100 rpm), rapid mixing time (2 min and 30 s), slow mixing velocity (15 rpm), slow mixing time (20 min) and settling time (20 min) [21].

For the process control, water samples were submitted to the same conditions of the processes without addition of any coagulant solution.

The supernatant and sediment (for *Toxocara* eggs viability) obtained after the CFS process were collected and submitted to the following analysis:

2.6. RW samples turbidity

The nephelometric method is based on light intensity comparison by reference standard suspension. The turbidity measure was determined in Policontrol turbidimeter, AP2000 model, using formazin standard solutions with results expressed in NTU, according to Standard Methods [22].

2.7. T. gondii quantification in the supernatant

2.7.1. Membrane filtration, mechanic extraction and elution

After settling time, the supernatant from each water sample was gently aspirated until a volume of approximately 150 mL of sediment was left. Then, it was filtered in cellulose ester membrane (47 mm diameter, 0.3 μm pore size, Millipore®, Brazil) using a negative pressure vacuum pump (10–15 mm Hg). The membranes were then manually scraped as described by Cantusio Neto e Franco [23]. The material obtained was centrifugated at 2,500 rpm for 10 min in order to concentrate the material to a final volume of 1 ml and was stored in microtubes at 4°C.

2.7.2. T. gondii DNA identification and quantification by qPCR

A 150 μL sample was taken from the final volume of each sample and frozen in liquid nitrogen (–196°C) for 5 min, followed by thawing in a dry bath at 650°C for 5 min. This cycle was repeated five times [24]. Extraction was performed using a commercial kit ReliaPrep™ gDNA Tissue Miniprep System (Promega, USA), following the manufacturer's recommendations. The samples were analyzed by PCR using the primers B1 (B22–B23) to amplify 115 base pairs (bp) and Toxo4–5 to amplify a fragment of 529 bp [25]. In each reaction, a negative control (mixture without DNA) and a positive control (DNA extracted from ME-49 strain) were processed. The amplified products were observed on 4.5% polyacrylamide gel revealed by silver, and digitally recorded.

The qPCR amplification was performed following the methodology described by Opsteegh et al. [24]. The parasite load of each sample was quantified using the StepOne™Plus Real Time PCR System (Life Technologies, USA) and the Taqman System (Life Technologies, USA). Specific primers Tox9F and Tox11R were used to target a product of 162 bp. For each sample, PCR mixture contained 12.5 μL TaqMan®Universal PCR MasterMix (Life Technologies, USA), 0.7 μM of each primer, 0.05 μM of Probe, BSA (10 μg mL^{-1}), 2 μL of template and ultrapure water in a final solution of 25 μL. All PCR reactions were performed in duplicate and carried out as follows: initial denaturation at 95°C for 10 min, 45 cycles at 95°C for 10 s, 58°C for 20 s and 72°C for 20 s. Fluorescence was measured at the end of each cycle for quantification of the amount of *T. gondii* DNA in the samples, and a standard curve was constructed based on DNA concentration. Considering that this methodology was developed for tachyzoites genotype B1 with 35 genome copies [25], each result obtained in the amplification of the samples was multiplied by eight, and thus given in number of sporulated oocysts (eight sporozoites) [26,27].

The removal of *T. gondii* oocysts was calculated considering the initial concentration of oocysts added in the water before the CFS process and the final concentration of the oocysts in the supernatant samples after CFS process.

The percentage of oocysts removed in the process was calculated according to Eq. (1):

$$\%\text{removal} = \frac{\left(C_i - C_f\right)}{C_i} \times 100 \quad (1)$$

where %removal is the percentage of *T. gondii* oocysts removed in the process; C_i is the initial concentration of the oocysts added in the water, counted by qPCR, before the treatment process; C_f is the final concentration of the oocysts counted by qPCR in the supernatant samples.

2.8. Toxocara spp. eggs quantification in supernatant and eggs viability in the sediment

2.8.1. Quantification

After the CFS process of each sample, the supernatant was centrifuged at 1,500 rpm for 10 min and reduced to 1 mL volume and were counted by optical microscopy. The percentage of eggs removed in the process was calculated similarly to Eq. (1), where, in this case, % removal is percentage of *Toxocara* spp. eggs removed in the process, C_i is the initial concentration of the eggs added in the water, counted by microscopy, before the CFS process; C_f is the final concentration of the eggs counted by microscopy in the supernatant samples, after the CFS process.

2.8.2. Eggs viability after coagulants treatment

The sediment obtained after CFS process (150 mL) was removed from the jar and kept at temperature 28°C–30°C, for 20 d to check the viability of *Toxocara* eggs. These samples were maintained in distilled water according to the technique of Harada and Mori [19]. For each sample, 200 eggs were counted and their viability measured in percentage, observing the embryonation by using an optical microscopy. In the control, the eggs were left in water, without addition of coagulants. The experiment was performed in triplicate and the arithmetic mean of the count was obtained.

2.8.3. Scanning electron microscopy of Toxocara spp. eggs

A Shimadzu SS 550 Superscan scanning electron microscope (Shimadzu, Japan) was additionally used to examine the morphological characteristics of the *Toxocara* spp. eggs. The samples of *Toxocara* spp. were mounted and coated with metal [28] at a voltage of 20 kV after the water samples were treated with MO and AS. This experiment was performed with 50 mg L^{-1} of each coagulant in DW.

2.9. Data analysis

The obtained data were entered in a spreadsheet of the program Microsoft Excel 2010 and analyzed by the results means, standard deviation, maximum, minimum of the evaluated variables calculated.

3. Results

Sixty-six experiments involving *T. gondii* oocysts, *Toxocara* eggs, MO and AS were performed.

T. gondii DNA was detected in RW and DW after treatment with natural or chemical coagulants (Table 1). After CFS process, it was observed that 87.9% of *T. gondii* oocysts removal in RW using MO and 97.8% using AS. Water samples (DW and RW) that were not added with coagulants (controls) show 60% of oocysts reduction. Similarly, DW treated with both coagulants, MO and AS, also shows 60% of oocysts reduction (Table 1).

Few eggs of *Toxocara* spp. were found in supernatant either in samples submitted to CFS process using MO or AS or without the use of these coagulants, in both types of water. The egg reduction ranged from 98% to 100% (Table 2). Regarding its viability in the sediment, when using the process of CFS with *M. oleifera* in the dosages of

Table 1
Water experimentally contaminated with 10^6 *Toxoplasma gondii* oocysts and submitted to CFS process using 50 mg L^{-1} of MO and 50 mg L^{-1} of AS

Water type	Total of samples	Variables					
		PCR (+/–)	Turbidity (NTU)	qPCR (*n* oocysts L^{-1}) Mean ± standard deviation			% Reduction
Moringa oleifera							
DW	30	+	1.3	473,128.0	±	224,608.7	52.7
DW (control)	30	+	1.2	408,654.0	±	185,990.2	59.1
RW	30	+	13.9	120,626.9	±	40,277.0	87.9
RW (Control)	30	+	18.2	396,624.0	±	430,251.1	60.3
Aluminum sulfate							
DW	30	+	1.1	406,816.2	±	297,286.7	59.3
DW (control)	30	+	1.2	408,654	±	185,990.2	59.1
RW	30	+	2.1	21,211.2	±	42,867.8	97.8
RW (control)	30	+	24.1	396,624	±	430,251.1	60.3

DW = distilled water; RW = raw water.

Table 2
Results obtained from samples of water contaminated with *Toxocara* spp. after CFS with MO and AS

M. oleifera	Type of water	Eggs L^{-1} after CF	Count of 200 eggs after being kept in DW and CF for 20 d, 25°C–30°C			Turbidity (NTU)[a]
			Embryonated eggs	Non-embryonated eggs	Larvae	
10 mg L^{-1}	DW	1.2	190 (95%)	4	6	17.0 (66%)
	RW	0				
30 mg L^{-1}	DW	2.8	157 (78.5%)	32	11	15.4 (69.2%)
	RW	0				
50 mg L^{-1}	DW	0	161 (80.5%)	27	12	13.9 (72.2%)
	RW	0				
Aluminium sulfate						
10 mg L^{-1}	DW	2.8	184 (92%)	12	4	14.5 (71%)
	RW	4.0				
30 mg L^{-1}	DW	13.2	177 (88.5%)	23	0	3.2 (93.6%)
	RW	4.0				
50 mg L^{-1}	DW	4.0	179 (89.5%)	21	0	2.1 (95.8%)
	RW	0				
Control						
	DW	5.2	183	17	0	18.2 (44%)
	RW	4.0				

[a]Samples of RW with initial turbidity of 50 NTU.

30 and 50 mg L^{-1}, there was a lower percentage of viable eggs (78%–80%) and a higher number of free larvae when compared with sediments containing aluminum sulfate or control sediment (Table 2).

MO removed 66% to 72.2% of turbidity in RW and AS 71% to 95.8%. Control samples, without the addition of coagulants, showed 44% of turbidity removal.

In scanning electron microscopy, it was noted that the eggs in the sediment were coated with the coagulants, but greater adhesion and the thicker layer was observed with the aluminum sulfate (Fig. 2).

4. Discussion

The use of natural or chemical coagulants was important to promote the reduction of the rate of RW contamination with *T. gondii* oocysts, and even both treatments did not prevent the detection of the DNA of these protozoa in the water samples tested by molecular techniques. The coagulation with MO extract was able to remove 89.7% of the oocysts in the raw water. Since it is a natural and biodegradable coagulant, with no impact in the environment, it may be an alternative to reduce the oocysts in the aquatic environment in various circumstances and purposes, even if the infecting dose for most warm-blooded animals is unknown [29]. Nishi et al. [11] and Petersen et al. [30] observed oocyst reduction of *Cryptosporidium parvum* and *Giardia* spp. cysts, using high concentrations (150 mg L^{-1}) of MO. High concentrations did not simulate what normally occurs at the WTP. However, they may be useful for improving water quality, such as for irrigation [30]. Although AS, normally used in WTPs, in RW was able to remove larger amounts of oocysts (97.8%), the risk to human health should be considered. Keegan et al. [31] also obtained, in the same type of water, reduction of 95.9%–97.2% of oocysts of *C. parvum* using 40 mg L^{-1} of AS. These coagulants are capable of forming heavier and larger flocks with organic matter present in RW if compared with control samples and DW, which contributes to better sedimentation of the parasite structure [32]. Also, the reduction of the turbidity of the RW was accompanied by the decrease of the number of oocysts in these samples, in agreement with studies that have tested oocysts of other protozoans such as *Cryptosporidium* spp. also, cysts of *Giardia* spp. [11,33].

Toxocara spp. eggs presented high sedimentation because they are dense structures, as found by Sengupta et al. [34] with the *Ascaris suum* eggs, other Ascarididae family member. However, the use of MO showed a lower rate of viable eggs compared with control or AS, which is an interesting fact.

About the *Toxocara* spp. eggs viability, it is observed that the higher the concentration of the coagulant, the lower the proportion of viable eggs. Maybe there is a particular influence of the action of the coagulants concerning the viability of *Toxocara* spp. eggs. Besides, some free larvae (non-infective) were observed in the sediment of the samples treated with *M. oleifera*. The thinner layer of this coagulant on the eggs may have to cause larvae to hatch earlier, which can contribute to a reduction of environmental contamination since the infecting form to humans and animals are embryonated eggs. The residues generated by different water treatment processes can represent a font of

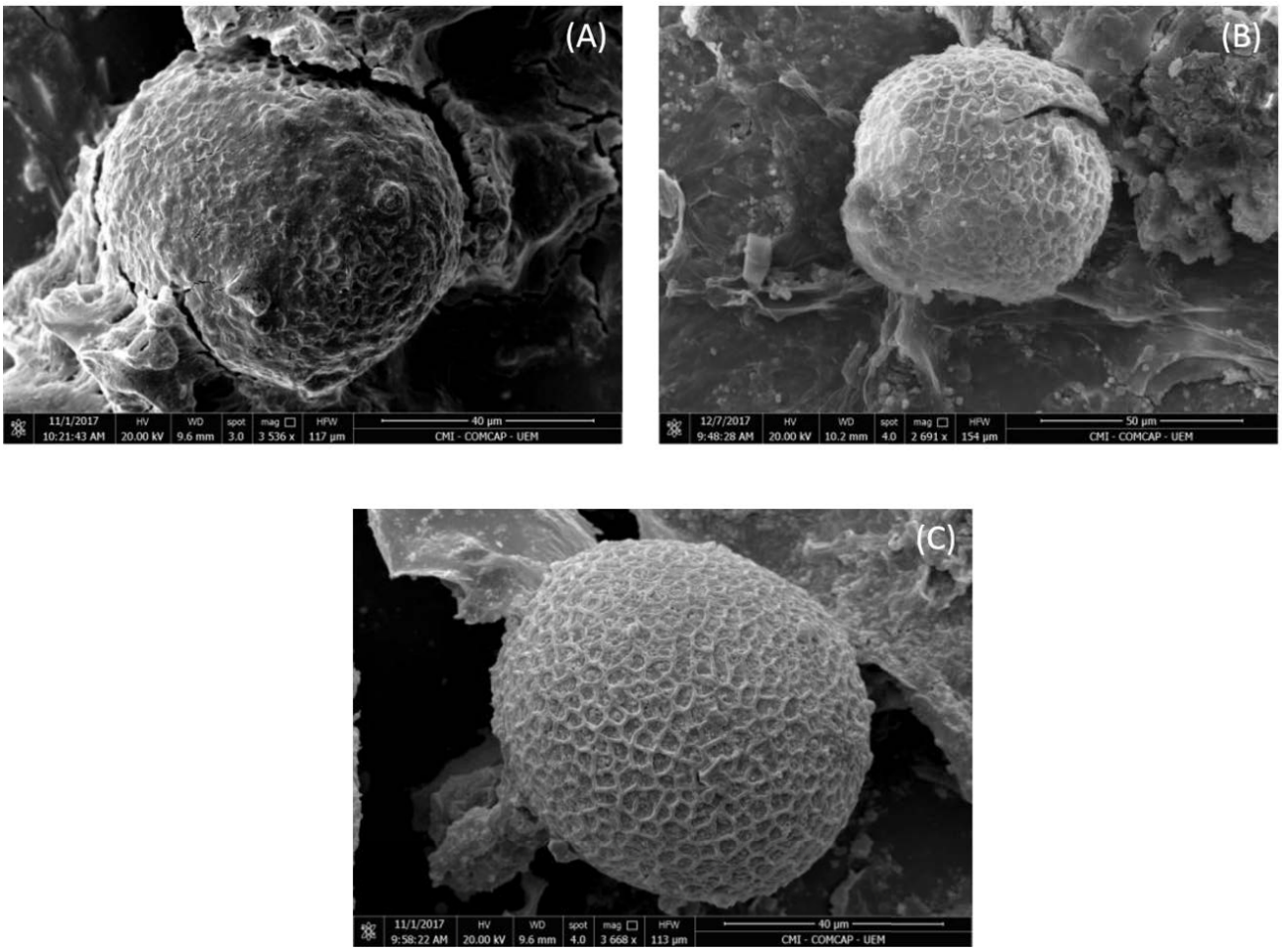

Fig. 2. Scanning electron microscopy of *Toxocara* spp. eggs after coagulation and flocculation process with 50 mg L^{-1} of coagulant in DW. *Toxocara* spp. – (a) AS (3.536×), (b) MO (2.691×) and (c) control (3.668×).[TS: Please change upper case part labels to lowercase in the artwork of Fig. 2.]

contamination to the environment leading to a higher risk of infection [35].

5. Conclusion

The coagulants were able to reduce most of the *T. gondii* oocysts present in RW. The addition of coagulants increases the removal of oocysts when compared with RW controls, as well as evidence that the presence of organic matter may aid in the formation of larger flakes, increasing the removal of oocysts when compared with clean water (DW). The use of the coagulants in clean water (DW), did not provide oocyst reduction with results similar to the control samples (DW and RW without the addition of coagulant). The aluminum sulfate was able to reduce 97.8% of *T. gondii* oocysts, while *M. oleifera* reduced 87.9% of them.

The sedimentation and *Toxocara* spp. eggs removal ranged from 99% to 100%, with or without coagulants addition, due to the high density of the egg. However, concentrations of 50 and 30 mg L^{-1} of *M. oleifera* and aluminum sulfate reduced the proportion of viable eggs in the sediment. Also, the sediment obtained after treatment with *M. oleifera* showed a higher amount of free larvae, and this makes it less infective since the larva in the environment is not able to infect humans and other animals. The sediment from *M. oleifera* may be a possibility to reduce the infecting potential of *Toxocara* spp. eggs in the environment, but further studies are needed to verify this effect.

This study points out the importance of these coagulants in the control of *T. gondii* in water treatment. Although some evolutive forms of *Toxocara* spp. became non-infecting, the majority of the eggs remain infective after the CFS processes with aluminum sulfate or *M. oleifera*.

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Conflict of interest

No potential conflict of interest was reported by the authors.

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