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ORIGINAL RESEARCH

Protective Effect of Natural and Processed Coconut Water by Non-thermal Technologies Against Oxidative Stress in Brine Shrimp (*Artemia salina*)

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Abstract

Coconut water is widely consumed and appreciated due its sensory, nutritional, and functional characteristics. Despite being widely consumed, this beverage has a short shelf life that can be improved through processing technologies including non-thermal technologies. Although this processing is promising, it also can generate toxic bioactive compounds of natural and synthetic origin. Their safety has been long discussed, and concern for human food security is now clearly manifested by warnings added on products labels. The aim of this work was to evaluate the toxic and the protective effect of natural and processed coconut water by non-thermal technologies against oxidative stress in brine shrimp (*Artemia salina*). For acute toxicity test, *A. salina* nauplii instar II were exposed to different concentrations and ozone-processed (OTCW), plasma-processed (PTCW), and ultrasound-processed (UTCW) coconut water. The non-processed sample was the negative control. By the end of experiment (48 h), dead nauplii were counted and investigated under optical and electron microscopy. The protective effect was evaluated against H_2O_2 and morphological changes were also investigated. Coconut water treated with plasma and ultrasound was not toxic to *Artemia salina* nauplii at 10, 100, or 1000 μg mL^{-1}; however, ozone-treated artificial seawater caused a mild toxicity to nauplii exposed to 1000 μg mL^{-1}. All coconut water samples, included untreated samples, presented protective effect against oxidative stress caused by H_2O_2 reaching levels of 87.5% protection compared to control (24 h of experiment).

Keywords *Artemia salina* · Coconut water · Non-thermal processing · Protective effect · Toxicity

Introduction

Coconut (*Coccos nucifera* L.) water is a widely consumed beverage attractive for its sensory, nutritional (Augusto et al. 2015), and functional characteristics (Porto et al. 2020). Despite all these qualities, coconut water has a short shelf life, which can be improved by thermal processing. Thermally processed coconut water still needs preservative such as metabisulfite to extend its shelf life (Sucupira et al. 2017). For this reason, non-thermal processing can represent a significant change in this scenario because it does not require additional preservatives (Porto et al. 2020).

Non-thermal processing includes various technologies (e.g., pulsed electric fields, irradiation, intense pulsed lights, high hydrostatic pressure, membrane filtration, and hurdle technology) (Zhang et al. 2019). In food processing, non-thermal emerging might be an alternative to conventional thermal processing that can decrease the nutritional food values (Vollmer et al. 2020; Hernández-Hernández et al. 2019). For potato, non-thermal technology maintained the chemical composition and nutritional quality (Dourado et al. 2019). These processes are also important in inhibiting pathogenic microorganisms and promoting deteriorative enzyme inactivation. In a study carried out with 15 *Salmonella* strains, high hydrostatic pressure (HHP), pulsed electric fields (PEF), and ultraviolet radiation (UV) was effective in preventing these

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microorganisms growing (Guillén et al. 2020). Non-thermal plasma inactivated the microorganism *Bacillus subtilis* present in black pepper grains and still preserved the quality parameters of the grain (Charoux et al. 2020).

Non-thermal technologies are also used in the extraction of bioactive compounds such as phenolic acids and flavonoids. In a study using PEF, it was possible to increase enhance polyphenol extraction from fresh tea leaves (Liu et al. 2019). Moreira et al. (2019) using HHP, PEF, and UAE, extracted bioactive compound with improved biological activities. In another study, PEF altered the protein structure in rice and pea, enhancing their technological functionality (Melchior et al. 2020). The preservation of metabolites in different samples by this technique makes it the main tool for processing the most varied compounds (Li et al. 2017; Mieszczakowska-Frąc et al. 2016). However, due to the generation of free radicals, non-thermal technologies can also generate toxic or potentially toxic compounds as reported by (Alves Filho et al. 2020) in ultrasound potato peel extraction.

The protective effect of fruits and vegetables are related to their nutritional value (Bianchi and Antunes 1999) and the presence of bioactive with antioxidant properties. Compounds as vitamins, chlorophyllin, flavonoids, carotenoids, and curcumin are capable of restricting the spread of chain reactions and lesions induced by free radicals (Fotsis et al. 1997; Pool-Zobel et al. 1997). The nutritional value (amount of vitamins and antioxidant compounds) of food helps antioxidant enzymes (SOD and CAT) to combat lipid peroxidation and protect the DNA (Del Ré and Jorge 2012). Furthermore, the use of non-thermal technologies can contribute to the improvement the protective effect, because it can increase in the antioxidant activity of plant foods, due to greater availability of bioactive compounds after processing.

Even though non-thermal technologies are extensively studied in food science, studies regarding the toxicity of food exposed to non-thermal processing are scarce. Despite promising results on food properties and food preservation, using non-thermal technologies, little is known about their toxicity in biological systems. In this sense, for acute toxicity tests, biological systems must be used as an initial toxicity study. Currently, a model that stands out in the toxicity study is *A. salina*.

Artemia spp. is an aquatic invertebrate widely used as a source of live food to feed larvae of aquatic organisms in aquaculture (Sarkheil et al. 2018). *Artemia salina* makes up zooplankton being found in saline environments, distributed worldwide (Levaens and Sorgeloos 1996) and widely used in ecotoxicological studies (Khoshnood et al. 2017; Nunes et al. 2006).

The high adaptability to different testing conditions, the adaptation to laboratory conditions, low cost of maintenance, short life cycle, and high reproduction rate makes these species a good model for toxicity tests (Manfra et al. 2014). The reliability and validity of ecotoxicological tests using *Artemia* spp. has been confirmed by several trials using different stressors, including nanoparticles (An et al. 2019; Khoshnood et al. 2017; Sarkheil et al. 2018), pesticides (Cruzeiro et al. 2017; Garaventa et al. 2010), chemical compound (DEG) (Manfra et al. 2014), and pharmaceuticals (Nunes et al. 2006). In addition, *A. salina* has been used as study model for oxidative stress, as observed by Muthukrishnan et al. (2017) and Jiang et al. (2019). The H_2O_2 increase in the cell due to stress can inhibit antioxidant enzymes such as SOD and CAT (Gottschalk et al. 2013) that can result in cell death by apoptosis or necrosis (Sharma et al. 2012). In addition to NPs, oxidative stress caused by *Aloe vera* juice (*Aloe barbadensis* Miller) was also described (Sirdaarta and Cock 2010).

Different foods sources have shown a protective effect against oxidative stress on *A. salina* as pomegranate juice (Les et al. 2015). In addition to juice, the use of biosurfactants produced by bacteria had a protective effect on *A. salina* against pathogens (Hamza et al. 2018a, b). Another study evaluated the protective effect of 2-pyridine aldoxime methiodide (2-PAM) against insecticides (Victoria Barahona and Sánchez-Fortún 2007).

Although there are studies on the protective effect against oxidative stress of different compounds in *A. salina*, none described the protective effect of the coconut water. Despite non-thermal technologies generates free radicals and reactive species, toxicological studies regarding non-thermal processed foods are scarce. In this sense, the aim of this work was to evaluate the toxic and the protective effect of natural and non-thermally processed coconut water against oxidative stress in brine shrimp (*A. salina*).

Material and Methods

Coconut Water

Green coconuts (*Cocus nucifera L.*) in the 7th month of maturation were obtained from Paraipaba City, Ceará, Brazil. Coconuts were sanitized by immersion in water containing 200 ppm of active chlorine for 20 min. The coconut water was extracted manually with the aid of a sterile stainless-steel knife, homogenized, and filtered on standard filter paper. Then, the content was packaged in nylon-polyethylene bags (150-mL portions), vacuum-sealed, and stayed stored at − 18 °C.

For the toxicity and protective effect evaluation, unprocessed and processed samples were prepared in accordance with the bioassay protocol, whereby the substances are tested at concentrations 10, 100, and 1000 μg mL^{-1} (Les et al. 2015; Meyer et al. 1982b). Therefore, dilution of the samples was performed based on total soluble solids content. The total soluble solids values were 5.6° Brix at 25 °C for unprocessed

samples and 5.7, 5.9, and 6.3 Brix for ozone, plasma, and ultrasound samples, respectively.

Non-thermal Processing of Coconut Water

For ozone processing, 100-mL portions of coconut water were processed in a glass column reactor with a 4.5 cm of diameter and 30 cm high containing a distribution porous plate made of sintered glass (pore size of 40–50 μm), coupled to a portable ozone generator (Model O & L15, OzoneLife, Sao José dos Campos, Sao Paulo, Brazil), which was fed by an oxygen tank with a flow rate of 125 mL/min. The ozone dosage provided by the generator was set at 29.5 mg/L of inlet flow. Three different ozone charges (0.074, 0.222, and 0.370 mg/mL of liquid sample) were tested, according to a previous study (Porto et al. 2020). The treatments were performed at room temperature (25 ± 2 °C).

For plasma processing, aliquots of 20 mL of coconut water were placed in a petri dish and exposed directly to atmospheric cold plasma. Plasma was generated through dielectric barrier discharge (DBD) in a system consisted of plasma generator (Model PLS0130, Inergiae, Florianópolis, SC, Brazil) coupled to a chamber containing two aluminum discs (8-cm diameter) separated at a distance of 15 mm. Two acrylic sheets (5-mm thick) were used as a dielectric barrier. The frequency and voltage applied were 730 Hz and 20 kV, respectively and the processing time was 15 min, according to previous study (Porto et al. 2020).

The ultrasound processing was carried out in a continuous flow system composed of an Ultrasonic Disruptor (maximum power: 500 W; model QSONICA SONICATIORS, USA) with a titanium macro tip with 9 mm of diameter coupled to a metal reactor (50 mL). The time required for a 300-mL sample of coconut water to pass through the entire system was 102 s and this was defined as one cycle. Three ultrasonic powers were tested (50, 75, and 100% of the total power of the equipment). For a better use of ultrasonic energy, the sample passed four cycles through the system at each tested power.

Test Organism

The life cycle of *Artemia salina* begins with the breaking of dormancy cyst and small spherical-like structures of high physical and chemical resistance. Dormancy breaks when the spheres meet saline water (SW) (Morgana et al. 2018). Hatched cysts originate Instar I nauplii that became instar II nauplii. High-hatching cysts were purchased in Fortaleza city. Dehydrated cysts were kept at 17 °C and sheltered from light until use. Instar II stage nauplii (48 h post hatching) were obtained as described by Meyer et al. (1982a, b). Briefly, cysts, (0.2 g) in 200 mL of artificial seawater, were incubated for 48 h at 24 °C ± 2 under 16 h of light, 8-h dark conditions, and continuous aeration of the cyst suspension in artificial sea water. The hatched nauplii were separated from non-hatched cysts based on their positive phototaxis and then transferred to 24-well plates containing artificial seawater.

Toxicity Assays

Toxicity assays were carried out with the lethality test of *Artemia salina* 24-h exposure according to Johari et al. (2019). Briefly, three different test concentrations (10, 100, and 1000 $\mu g.mL^{-1}$) of unprocessed coconut water (UCW) and ozone (OTCW), plasma (PTCW), and ultrasound (UTCW) non-thermal processed coconut water were administered in nauplii within 24 h. Negative control group was exposed only to artificial seawater (SW) and positive control was exposed to potassium dichromate ($K_2Cr_2O_7$) 0.5 M. The experiment was carried out on 24-well plates with final volume of 2 mL. Each concentration carried out by quintuplicate and each replication contained ten newly hatched nauplii (Instar II) (Johari et al. 2019). The experiment was carried out at 24 °C ± 2 with a photoperiod of 8-h dark/16-h light. After that, the dead larvae were counted under a stereomicroscope (Zeiss Stem 508). The test is valid only when survival rate in the control group was ≥ 90% (Johari et al. 2019).

Protective Effect of Coconut Water Against Hydrogen Peroxide (H_2O_2)–induced Toxicity in *Artemia salina*

After the acute toxicity test, only in cases where no deaths were observed, an experiment was carried out to evaluate the protective effect of coconut water against hydrogen peroxide (H_2O_2)–induced toxicity in *A. salina*. Therefore, the nauplii (Instar II) were exposed to their H_2O_2 LC_{50}. To determine the LC_{50}, nauplii were exposed to different H_2O_2 concentrations (50 mM, 100 mM, 150 mM, and 200 mM). The percentage of deaths were determined and converted into probity values by a computer program (Microsoft Excel) to determine the lethal concentration for 24-h exposure (Ates et al. 2020).

Cysts were hatched as described above and instar II nauplii were placed in 24-well microplates containing artificial seawater. Then, H_2O_2 was added, final concentration 127.45 mM (LC_{50}). After 2 h of nauplii interaction of H_2O_2, unprocessed coconut water (UCW) and non-thermal processed coconut water—ozone (OTCW), plasma (PTCW), and ultrasound (UTCW)—was added to wells at final concentration of 10, 100, and 1000 μg mL^{-1}. The counting of the individuals as well as the investigation of morphological changes were carried out in 24, 48, and 72 h of experiment, according to Les et al. 2015.

Control group was exposed only to artificial seawater. Positive control was exposed to hydrogen peroxide (127.45 mM) more yeast suspension. The experiment was carried out on the conditions of cytotoxicity test. The nauplii were feed with a yeast suspension (3 mg/5 mL artificial seawater).

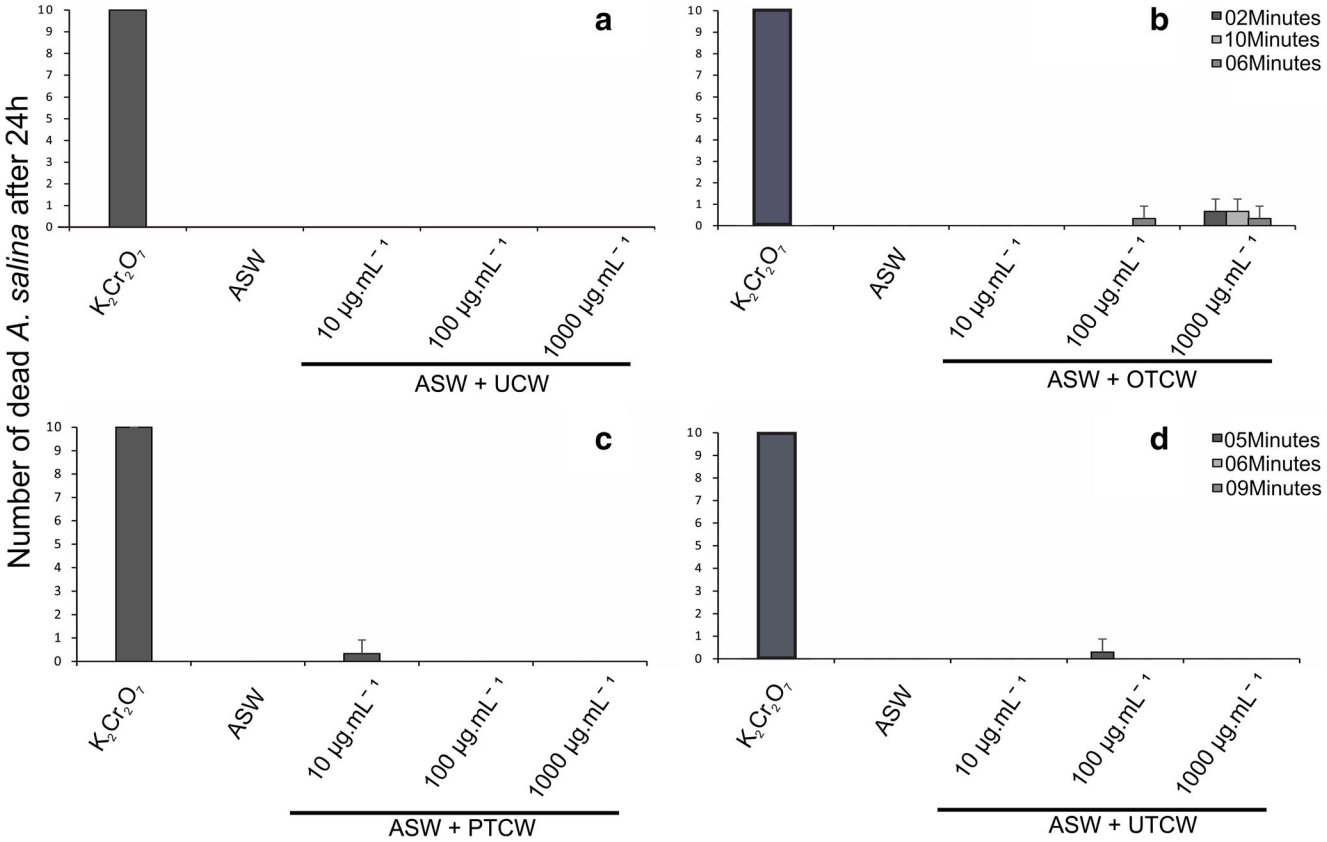

Fig. 1 Acute toxicity of unprocessed coconut water (UCW) and processed coconut water (10, 100, and 1000 μg ml^{-1}) in *Artemia salina* instar II nauplii at 24 h of exposure. **A** No acute toxicity of negative control—artificial seawater (ASW) and UCW was observed in *A. salina*. The positive control ($K_2Cr_2O_7$) was lethal for 100% of individuals. **B** OTCW did not present acute toxicity in any of the time intervals (2, 6, and 10 min). **C**, **D** Same result presented by PTCW and UTCW, with low acute toxicity for *A. salina*. SW, seawater; UCW, unprocessed coconut water; OTCW, coconut water processed with ozone; PTCW, coconut water processed with plasm; UTCW, coconut water processed with ultrasound

Morphological analysis

Light microscopy

For investigation of morphological changes, nauplii were collected at 24 h for toxicity assay, and 24, 48, and 72 h for protective assay washed in seawater, mounted on a glass slide, and investigated under light microscope (Primo Star-Zeiss) equipped with a with Zen Light software.

Scanning electron microcopy

For SEM investigation, samples of *A. salina* nauplii instar II were collected as described for light microscopy and fixed in solution of glutaraldehyde 2.5%, formaldehyde 4.0% in cacodylate buffer 0.1 mol L^{-1}, pH 7.2, at room temperature for 24 h. Subsequently, the material was rinsed in sodium cacodylate buffer 0.1 mol L^{-1}, pH 7.2, three times for 45 min each and post-fixed for 1 h at room temperature with 1.0% osmium tetroxide in cacodylate buffer 0.1 mol L^{-1}, pH 7.2. After the washes, the samples were increasing series dehydrated with acetone for 45 min each step. After dehydration, the material was critical point dried (Q150T ES). Dried samples were placed in stubs sputtered with a 20-nm gold. Observation and documentation were performed in scanning electron microscope (Quanta FEG 450 FEI).

Statistical analysis

For the statistical analyses, the data were collected daily. The results were expressed as multiple mean comparison for each sample with different concentrations. To find differences between treatments by one-way ANOVA and Student *t* test were used. The level of statistical significance was established in $p < 0.05$.

Results and discussion

Toxicity assays

After 24 h of interaction, no toxic effect of unprocessed coconut water (UCW) was observed in *A. salina* nauplii, even in high concentrations (1000 μg mL^{-1}). The positive control

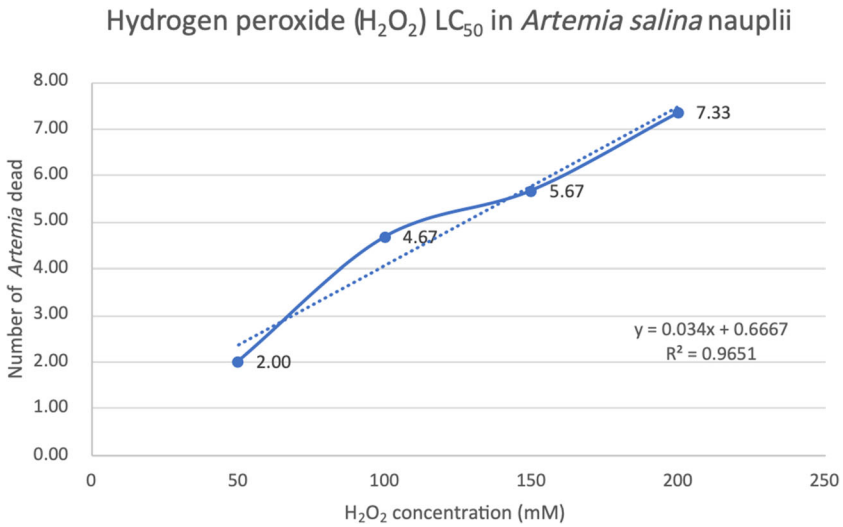

Fig. 2 Lethal concentration that kills 50% of individuals (LC_{50}). *A. salina* instar II nauplii submitted to different concentrations of hydrogen peroxide (H_2O_2). Mortality increase is directly proportional to the concentration of H_2O_2. The concentration of 127.45 mM killed 50% of the tested individuals. Experiments were conduct on triplicates. $y = 0.034x + 0.6667$; $R^2 = 0.9651$. $LC_{50} = 127.45$ mM)

($K_2Cr_2O_7$ 0.5 M) was lethal in 100% of the individuals in contrast to the negative control which did not present dead individuals (Fig. 1a). The controls exhibited the same results in all experiments.

Ozone processed coconut water (OTCW) did not cause toxicity to nauplii in the concentration of 10 μg mL^{-1} and 100 μg mL^{-1}; however, 10-min-processed samples at 100 μg mL^{-1} presented low toxicity, lethal for only 3.3% of the individuals (Fig. 1b). At the maximum tested concentration (1000 μg mL^{-1}), lethality was observed for 6.5% of the individuals in the processing times of 2 (0.074 mgO_3 mL^{-1}) and 6 min (0.370 mgO_3 mL^{-1}) (Fig. 1b). The low percentage of dead individuals did not represent a significant difference in relation to the negative control with a value of 0% *A. saline* dead ($P > 0.05$) (Table 1 - Supplementary data).

Plasma processed coconut water did not cause toxicity at any of the concentrations tested. Only the concentration of 10 μg mL^{-1} caused lethality in 3% of the individuals, with no significant difference in relation to the control (Fig. 1c). Ultrasound-processed coconut water did not cause statistically significant deaths ($P > 0.05$) in individuals in the three concentrations and processing tested. However, at a concentration of 100 μg mL^{-1}, 3.3% of individuals died (Fig. 1d). All the results are summarized in Table 1 (Supplementary data). In addition, in all tested concentrations (10, 100, and 1000 μg ml^{-1}), the percentage of dead nauplii was below 10% indicating no toxicity of unprocessed and non-thermal technologies processed coconut water. Amado et al. (2019) studied the antioxidant and toxicity of avocado varieties confirming that these plants are non-toxic to *A. salina.*

Bevilacqua et al. (2018) revised a series of non-thermal technologies applications for fruit and vegetable juices and beverages including high-pressure, ultrasound, radiation, inert gas, cold plasma, and membrane processing. Little is known about the toxicity of products that can be generated along these processing. Pérez-Andrés et al. (2018) demonstrated that non-thermal food processing technologies can modify the structure of lipids and proteins. On the other hand, cold

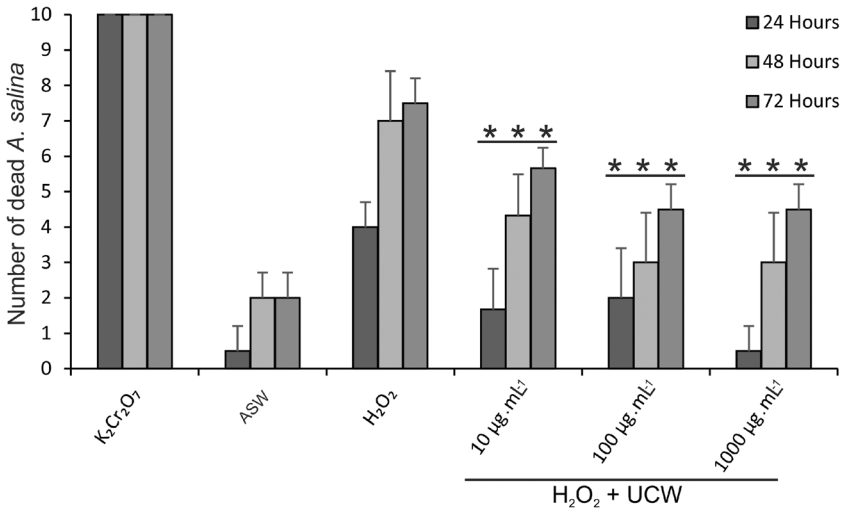

Fig. 3 Protective effects of unprocessed coconut water (UCW) coconut water (10, 100, and 1000 μg mL^{-1}) on H_2O_2 in *A. salina* at 24, 48, and 72 h of exposure. Note that in all time intervals (24, 48, 72 h) samples treated with H_2O_2 + UCW showed a significant difference in relation to H_2O_2. However, the protector was more effective within 24 h of exposure. * Significance differences ($P < 0.05$) were observed between the H_2O_2 and H_2O_2 + UCW samples at 24, 48, and 72 h in all tested concentrations

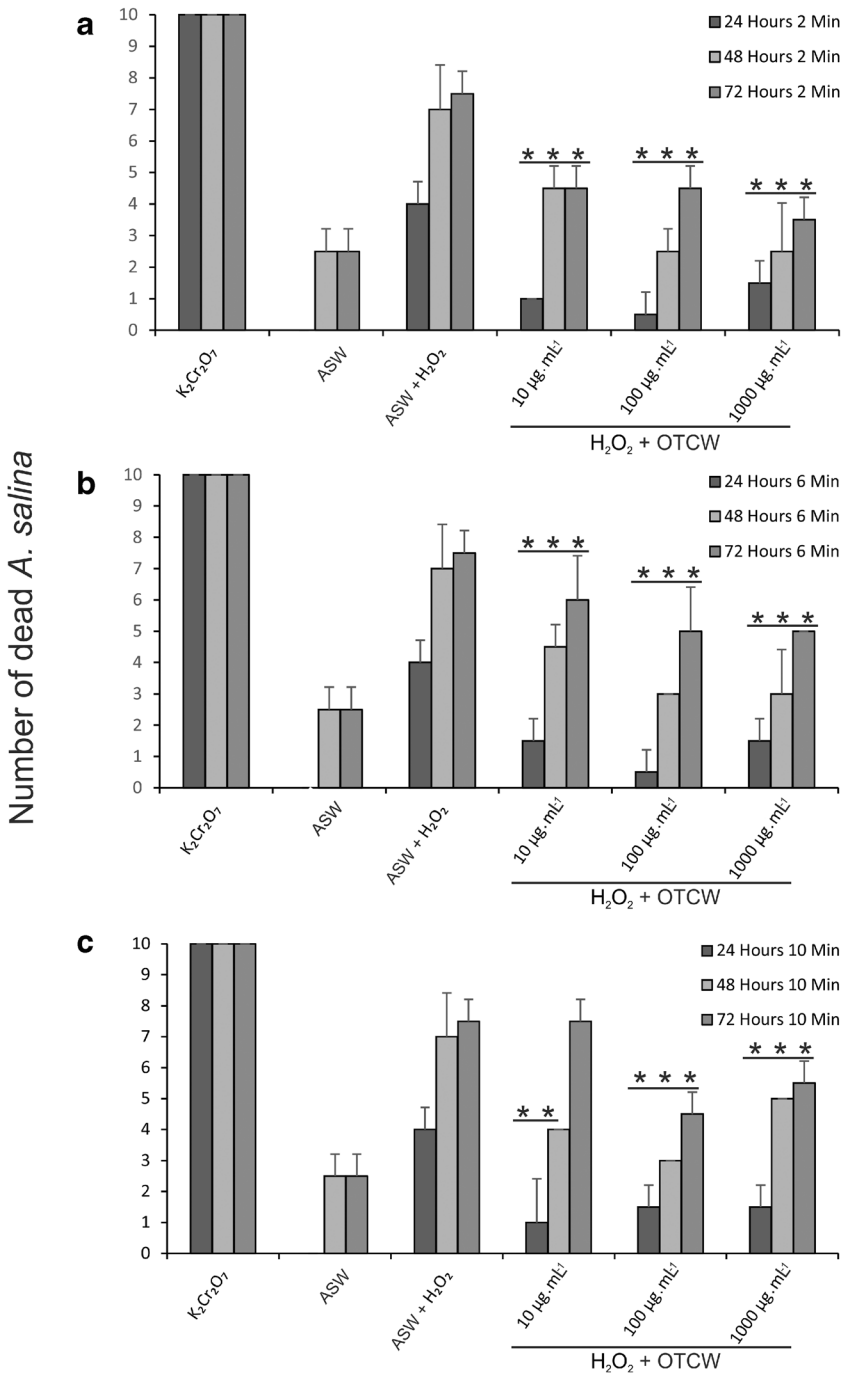

Fig. 4 Protective effects of ozone treated coconut water (OTCW) (10, 100, and 1000 μg mL^{-1}) on hydrogen peroxide (H_2O_2) in *A. salina* at 24, 48, and 72 h of exposure. * Significance differences ($P < 0.05$) were observed between the H_2O_2 and H_2O_2 + OTCW samples at 24 and 48 h in all concentrations used. At 72 h, only 10 μm/ml within 10 min showed no statistical difference between the H_2O_2 and H_2O_2 + OTCW samples

plasma processing may not affect functional compounds of siriguela juice (Paixão et al. 2019).

Protective Effect of Coconut Water Against Hydrogen Peroxide (H_2O_2)–induced Toxicity in *A. salina*

To accurately assess the possible protective effect of unprocessed coconut water, and coconut water processed with ozone, plasma, and ultrasound in relation to oxidative stress caused by hydrogen peroxide (H_2O_2), an experiment was carried out to determine the concentration of the stressor that kills 50% of individuals (LC_{50}) in 24 h (Fig. 2). The concentration of 127.45 mM of H_2O_2 was lethal for 50% of individuals ($y = 0.034x + 0.6667$; $R^2 = 0.9651$).

Hydrogen peroxide 127.45-mM-induced significant toxicity at different experiments times; however, co-treatment of nauplii with 10, 100, and 1000 mg ml^{-1} of unprocessed coconut water decreased nauplii dead at 24, 48, and 72 h (Fig. 3). The protective effect aims to minimize the damage caused by stressors (H_2O_2) by the reduction of ROS content. In coconut

water compounds as methionine; L-arginine; cytokines; selenium; vitamin C; and Zn, Mn, and Cu can be responsible to the antioxidant proprieties (Zulaikhah 2019). Our results show a protective effect in all UCW concentrations tested, mainly in 24 h, especially in 1000 mg ml^{-1} (Fig. 3). The addition of UCW caused the lethality rate to drop to 16.7% (10 μg mL^{-1}), to 16% (100 μg mL^{-1}) and to 5% (1000 μg mL^{-1}). The increase in the exposure time to H_2O_2 caused increased nauplii dead. At 48 and 72 h, the mortality rate was 70% and 75% respectively (Fig. 3). Despite this, the mortality of nauplii was close to 50%, on UCW treat samples, indicating the protective effect with a p value < 0.05 (Table 2 - Supplementary data). The protective effect of processed and unprocessed coconut water was compared to H_2O_2. Asterisk shown in the graph indicates significant protective effect.

Ozone processed coconut water (OTCW) for 2 min (0.074 mgO_3 mL^{-1}), decreased the number of deaths nauplii in the 24-h treatment, at the three tested concentrations (Fig. 4a) with a variation of 5 to 10% of mortality. In study using blueberry fruit, it was observed that ozone treatment activated a defense mechanism against oxidative stress (Piechowiak et al. 2020). The exposure time increase (24–48 h) reduced the protective effect against H_2O_2, increasing the percentage of mortality. Similar results were found when the experiment carried out with OTCW treated by 6 min (0.222 mgO_3 mL^{-1}) (Fig. 4b), with best result in 24 h of exposure. Forty-eight and 72 h presented higher mortality rate. Same condition was also observed for OTCW treated for 10 min (0.370 mgO_3 mL^{-1}) at 24, 48, and 72 h (Fig. 4c). However, in 72 h with concentration of 10 μg mL^{-1} did not produce a protective effect, presenting 75% of dead nauplii with p-value > 0.05 (Fig. 4c).

Plasma-processed coconut water (PTCW) sample were able to decrease the number of dead nauplii at concentrations of 10, 100, and 1000 μg mL^{-1} in the times of 24 and 48 h (Fig. 5). Strong protective effect was observed in 24 h of exposure with 0% nauplii dead in the concentration of 100 and 1000 μg mL^{-1} (Fig. 5). Within 72 h of exposure, no protective effect of PTWC was observed in any of the concentrations tested.

Ultrasound-processed coconut water (UTCW) by 5 min was efficient in decreasing the number of dead nauplii at concentrations of 10, 100, and 1000 μg mL^{-1} in experiments of 24 and 48 h (Fig. 6a). In the 72-h experiment, on the other hand, toxicity appears to have increased at all concentrations (Fig. 6a). The sample processed with ultrasound for 6 min induced a decrease in the number of dead nauplii in all situations, except under the condition of 10 μg mL^{-1} in the experiment conducted for 72 h (Fig. 6b). The sample processed with ultrasound for 9 min also induced a decrease in the number of dead nauplii in all situations, except for 1000 μg mL^{-1} in the experiment conducted for 72 h (Fig. 6c).

Despite the protective effect of coconut water, naturally, all aerobic organisms produce ROS such as H_2O_2, O_2^-, and OH (Ahsan et al. 2003). These radicals can negatively affect organisms and chances their survival (Sirdaarta and Cock 2010). In vivo, H_2O_2 is formed by oxidizing enzymes by reducing the superoxide anion O_2 (Halliwell and Gutteridget 1984). The H_2O_2 does not show sufficient reactivity to cause damage to organic substrates; however, H_2O_2 can cross the membrane and generate highly reactive OH through interaction with ions such as Fe_2^+ e Cu^+ (Ahsan et al. 2003). In our study, all non-thermally processed coconut water samples presented a similar protective effect in *A. salina* along the experiment interval (72 h). PTCW was the most efficient sample at 24 h of exposure, once prevent more deaths than other treatments. The better efficiency of the protective can be attributed to non-thermal processing technology, which preserves the nutritional value of food (Dourado et al. 2019). The nutritional value of food helps antioxidant enzymes (CAT and SOD) to combat oxidative stress responsible for cell damage (Del Ré and Jorge 2012). The lower efficiency of the protector was observed in nauplii exposed to H_2O_2 within 72 h, a common result for all solutions used (UCW, OTCW, PTCW, and UTCW). In this

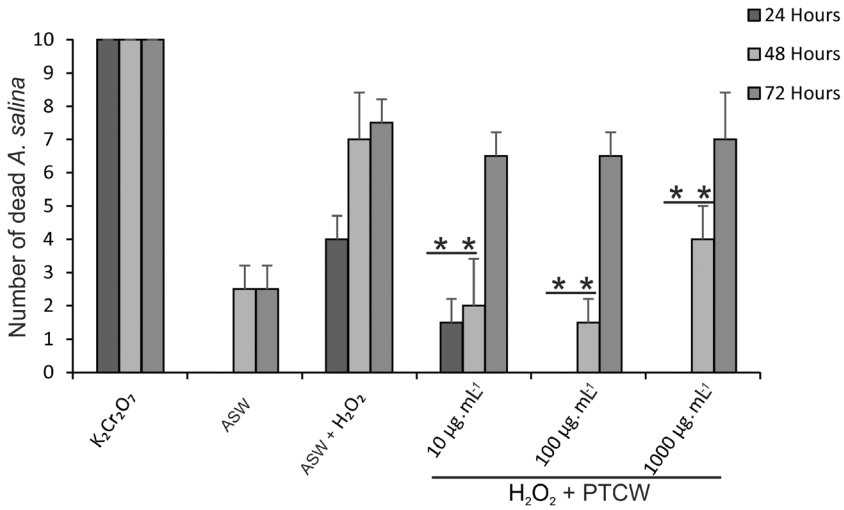

Fig. 5 Protective effects of plasm treated coconut water (PTCW) (10, 100, and 1000 μg mL^{-1}) on hydrogen peroxide (H_2O_2) in *A. salina* at 24, 48, and 72 h of exposure. * Significance differences (P < 0.05) were observed between the H_2O_2 and H_2O_2 + PTCW samples at 24 and 48 h in all concentrations used. At 72 h, all concentrations (10, 100, and 1000 μg ml^{-1}) showed no statistical difference between the H_2O_2 and H_2O_2 + PTCW samples

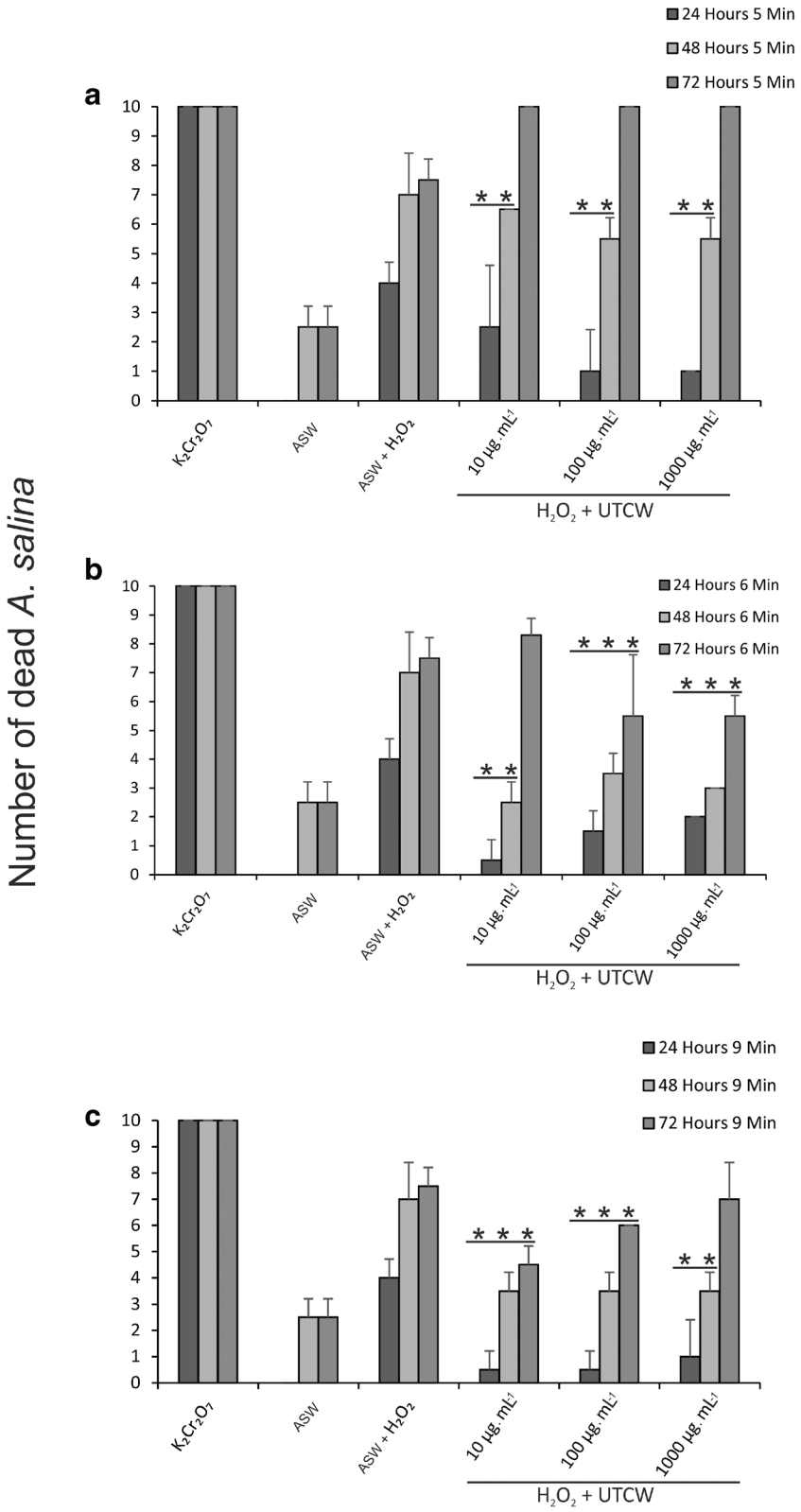

Fig. 6 Protective effects of ultrasound-processed coconut water (UTCW) on hydrogen peroxide (H_2O_2) in *A. salina*. * Significance differences ($P < 0.05$) were observed between the H_2O_2 and H_2O_2 + US samples at 24 and 48 h in all concentrations used (10, 100, and 1000 μg ml^{-1}) within 5 min. At 72 h, all concentrations of the protective (H_2O_2 + UTCW) within 5 min it was more toxic than the H_2O_2

interval, the protective effect of coconut water losses its effectiveness. The low effectiveness may be related to a reduction in the nutritional value of coconut water. Moreover plasma treated solution or ozonated treated solution contains high concentration of ROS such as OH^-, H_2O_2, O_3, NO^{3-} (Chen et al. 2019).

In addition, biological systems when exposed to stressors can undergo metabolic changes due to increased ROS in the

cell, including H_2O_2. This increase in ROS content is responsible for degrading biomolecules (AshaRani et al. 2009; Clément et al. 2013) and indirectly changes the morphology of species such as malformation of limbs, body size, and anomalies that affect the survival of species in the short and long term.

Morphological Analysis of Toxicity Assay

Nauplii exposed to ASW (negative control) did not present morphological damage. They presented a slightly wrinkled surface and preservation of all characteristic structures of the nauplii such as antenna, mandible, and endite (Fig. 7a and Fig. 7b). The abdomen, posterior region, and appendages were preserved (Figs. 7c and d). The $K_2Cr_2O_7$ caused many morphological changes in *A. salina* such as surface wrinkling, loss of body integrity, and severe damage to antenna, mandible, and endite (Fig. 7e and f). The posterior region and appendages presented deformations (Fig. 7g and h).

A. salina exposed to UCW 1000 μg ml^{-1} showed similar morphology compared with ASW without alteration and preservation of nauplii survival structures (Fig. 7i and j). However, the digestive tract was dense (Fig. 7i). The posterior region and appendages did not present structural changes (Fig. 7k and l). Nauplii exposed to OTCW 1000 μg ml^{-1} showed no morphological change in the body, compared to the control (Fig. 7m and n). The abdomen and posterior region exhibited slight deformation (Fig. 7o); however, the underdevelopment

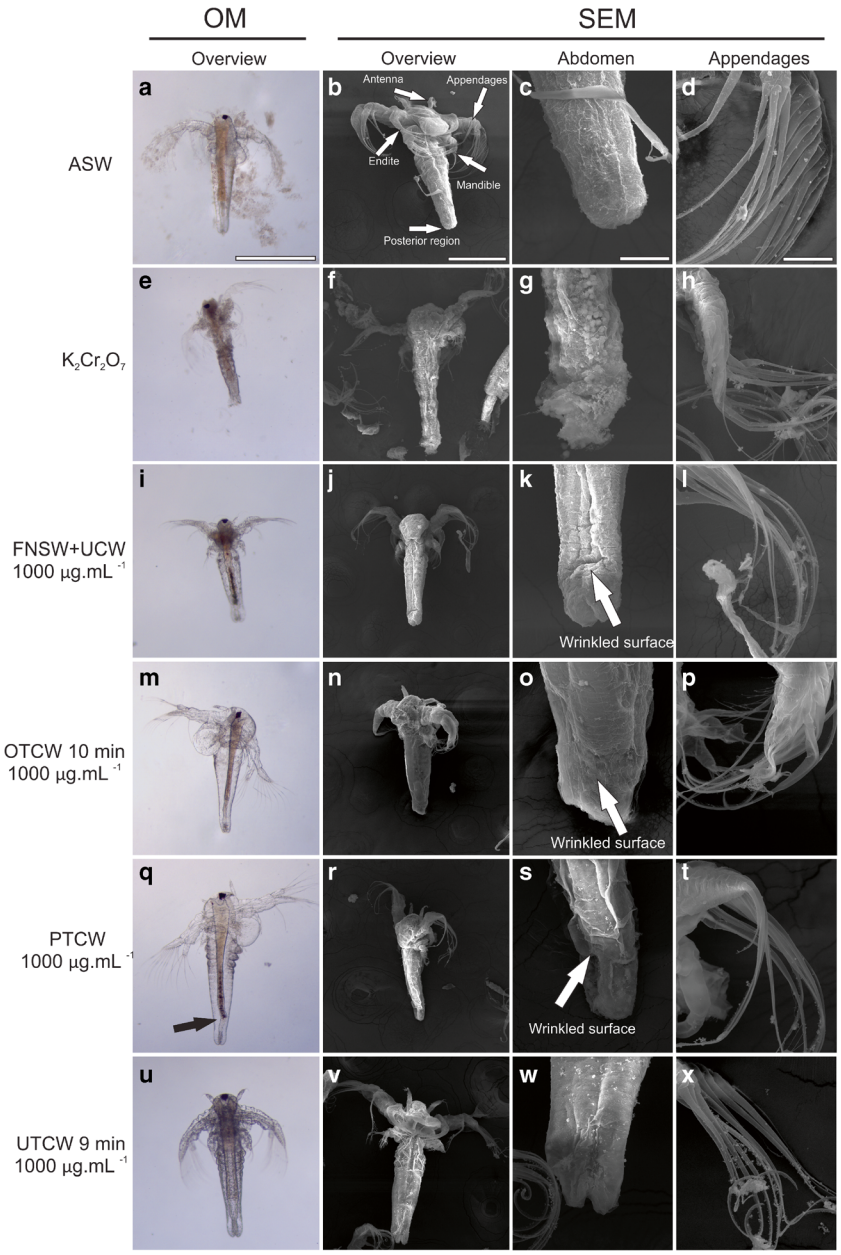

Fig. 7 Optical microscopy and scanning electron microscopy in *A. salina* within 24 h. **A**, **B**, **C**, **D** Negative control (ASW); **E**, **F**, **G**, **H** positive control ($K_2Cr_2O_7$); **I**, **J**, **K**, **L**, **M**, **N**, **O**, **P**, **Q**, **R**, **S**, **T**, **U**, **V**, **W**, **Y**, **Z** no significant morphological changes were observed in any of the treatments. Bars, A, B, E, F, I, J, M, N, Q, R, U, and V 500 μm; C, D, G, H, K, L, O, P, S, T, X, and Z—50 μm

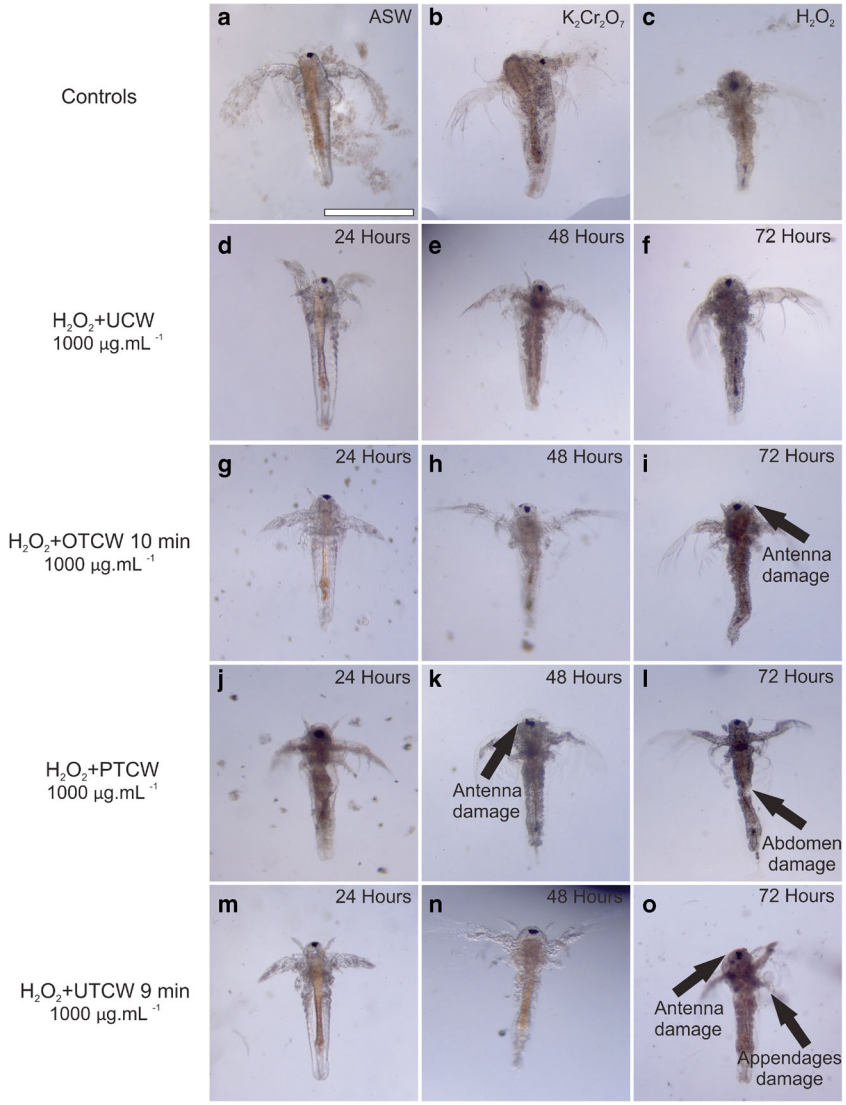

Fig. 8 Protective effect of unprocessed and processed coconut water against H_2O_2 using *A. salina*. **A**, **B**, **C** Controls: ASW, $K_2Cr_2O_7$ and H_2O_2; **D**, **E**, **F** Protective effect of H_2O_2 + UCW; **G**, **H**, **I**, **J**, **K**, **L**, **M**, **N**, **O** Morphological changes with damage to the animal's body were observed more frequently in the 72 h of exposure to H_2O_2. Bars, 500 µm

of the appendages was observed (Fig. 7p). The nauplii submitted to PTCW 1000 µg ml^{-1} solution presented a dense digestive tube (Fig. 7Q), without significant morphological changes in the body (Fig. 7r). The abdomen suffered deformation with wrinkling surface but showed normal development of the appendages (Fig. 7s and t). The UTCW solution 1000 µg ml^{-1} caused marked surface wrinkling without changing the morphology of the body of the nauplii (Fig. 7u and v). The abdomen and posterior region without morphological changes and normal development of appendages (Fig. 7x and z).

Choi (2017) studied the toxic effect of grapefruit seed extract on *A. salina*. Although no morphological changes were evaluated, the extracts presented strong larvicidal effect. According to Sarker Apu et al. (2013) different fractions of leaves, flowers and fruits of *Solanum sisymbriifolium* and *Jatropha gossipyfolia* were also toxic to *A. salina*. Most part of toxicity studies on *A. salina* model are conducted on instar II nauplii, although some studies investigates many life stages including adults (Madhav et al. 2017). This is justified by the fact that instar I nauplii are less sensitive compared to instar II nauplii (Ocaranza-Joya et al. 2019). This difference is due instar I nauplii cannot incorporate toxic through the digestive tract, once mouth and anus are still closed (Ocaranza-Joya et al. 2019). For this reason, the use of instar II nauplii can evaluated the toxicity more accurately.

In study using mitoxantrone (anti-tumor drug), high mortality rates of nauplii were observed which allowed determination of a LC_{50} value of 17.7 mL mL^{-1} (da Rosa et al. 2019). The same result was observed in a study using *Moringa oleifera* flower extract (Rocha-Filho et al. 2015). In addition, Yi et al. (2020) observed that gonyautoxins (GTXs) excreted by *Alexandrium minutum* inhibited the expression of genes related to chitin metabolism resulting in the interruption of the morphogenesis process in *A. salina*.

Morphological changes on *Artemia salina* are consistent to nauplii exposed to silver nanoparticles (Arulvasu et al. 2014), food dyes (Motta et al. 2019), copper oxide (Madhav et al. 2017), and microplastic particles (Batel et al. 2020).

Morphological Analysis of Protective Effect Assay of Coconut Water Against Hydrogen Peroxide–induced Toxicity

Nauplii exposed to ASW, exhibited typical morphology (Fig. 8a). $K_2Cr_2O_7$ generated deformations in the animal's body and loss of structures such as eye, antenna, and appendages (Fig. 8b). H_2O_2 is a strong oxidizer that degrades organic substrates, causing morphological changes in the *A. salina* entailing in loss of structural integrity of the body (Fig. 8c).

The protective effect of UCW, OTCW, PTCW, and UTCW at 1000 μg ml^{-1} against H_2O_2 LC_{50} was more effective to prevent nauplii morphological damage at 24 h of experiment (Fig. 8d, g, j and m). Despite the morphology of the nauplii has been, in general, preserved, the gut has a dense content, especially for PTCW (Fig. 8q). Within 48 h of experiment, it was possible to notice nauplii structural damage, such as damage to the antennas and appendages deformation, in all samples (Fig. 8e, h, k and m). These structural changes were accentuated in 72 h (Fig. 8f, i, l and o) and included abdomen damage on PTCW sample (Fig. 8l).

The observations under scanning electron microscopy confirm the damage caused by H_2O_2 at LC_{50} after 24 h of exposition (Fig. 9d, g, j and m). Within 72 h of exposure, the nauplii exposed to OTCW, PTCW, and UTCW showed antenna damage, abdomen wrinkling and underdeveloped appendages (Fig. 9i, l and o). In a toxicity study on *A. salina* submitted to TiO_2NPs and $AgTiO_2$, changes were observed on the ocular surface, malformations in the body, loss of the antenna, and enlargement of the intestine resulting from the increase in the ROS content in the cell (Ozkan et al. 2016).

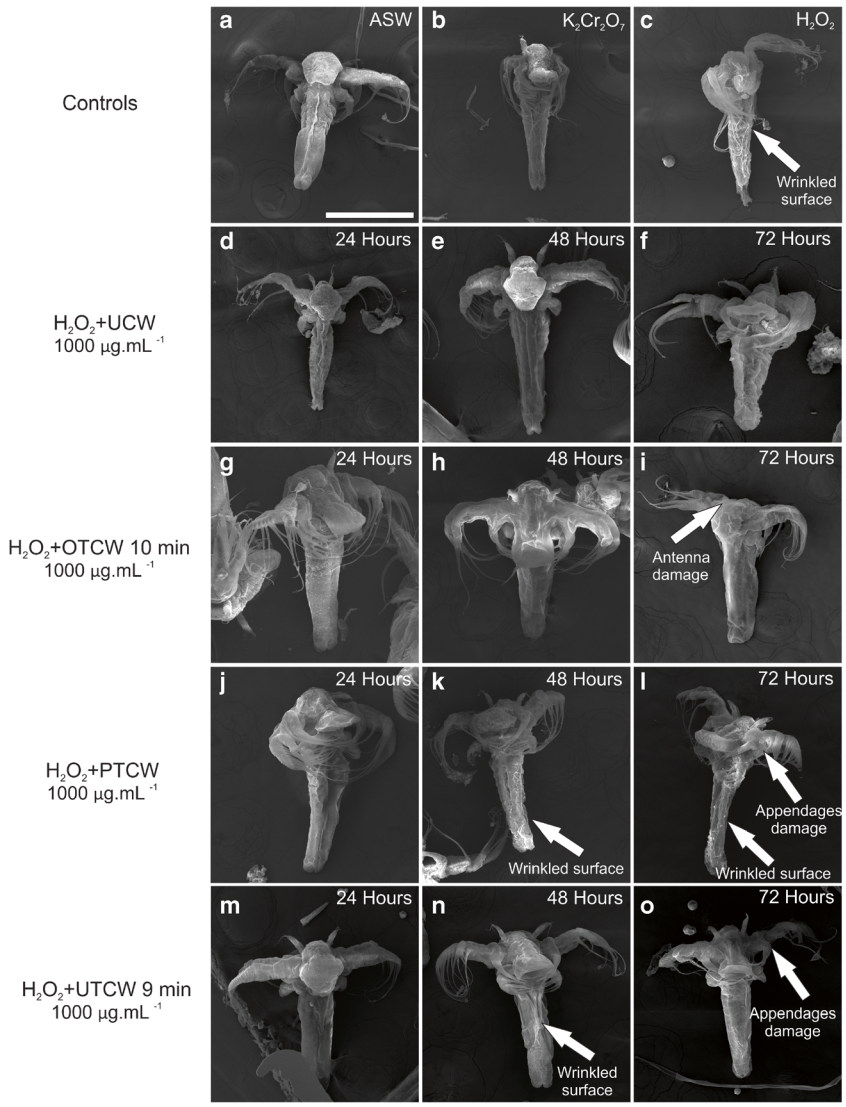

Fig. 9 **A**, **B**, **C**, **D**, **E**, **F**, **G**, **H** Protective effect of unprocessed and processed coconut water in the 24–72-h interval against hydrogen peroxide (H_2O_2) using *A. salina*. **I**, **J**, **K**, **L**, **M**, **N**, **O** Analysis by scanning electron microscopy showed changes in the animal's body in the 72 h of exposure. Bar, 500 μm

This result was similar to our study, in which *A. salina* presented morphological changes such as marked wrinkling of the body surface, loss of antenna, and malformation of the appendages in the 72 h of exposure to the stressor (H_2O_2). In another study conducted by Shaala et al. (2015), nauplii of *A. salina* submitted to the chemical compound tributyltin chloride (TBTCL) presented improper development of mandibles, underdeveloped end pod and endite, as well as swimming site in the second pair of antenna.

The damage caused in the animal's body is related to the increase of ROS. The means to reverse the increase in ROS in the cell would be to balance the amount of species reactive against the total of antioxidant enzymes (Bianchi and Antunes 1999). This result can be achieved by reducing the stress in *A. salina* by adding a compound with a protective effect as observed in our study with coconut water. Different compounds and extracts have been used to protect *A. salina* from stressors. In a study using the biosurfactant (BS-SLSZ2 with antibiofilm properties) produced by *Staphylococcus lentus* SZ2 protected *A. salina* against *Vibrio harveyi*. However, after 48 h of exposure to the pathogen, an increase in the mortality rate of *A. salina* was observed (Hamza et al. 2018b). Another study evaluated the protective effect of the supernatant (BLDZ1) produced by *Bacillus licheniformis* in *A. salina* against *Vibrio alginolyticus* and *Pseudomonas gessardii*. In this study, the survival rate in the 48-h interval was above 90%; after this incubation period (96 h), a decrease in the survival rate was observed (Hamza et al. 2018b). Result comparable to our study, in which the protective effect of coconut water was more effective in 24 h of exposure.

In addition to biosulfants, endotoxins such as lipopolysaccharides (LPS) can have protective activity, increasing the survival rate of individuals. The pre-treatment of *A. salina* with cyanobacterial lipopolysaccharide (LPS) decreased the mortality rate against the stressful microcystin-LR and cylindrospermopsin in the 24-h interval (Lindsay et al. 2006). As mentioned earlier, pesticides are also toxic to *A. salina*. In a study conducted by Victoria Barahona and Sánchez-Fortún (2007), the protective effect of atropine and 2-pyridine aldoxime methoiodide (2-PAM) against organophosphorus (OP) insecticides such as fonofos and phosphamidon was evaluated and the results indicate protection of *A. salina* in 24 h of exposure interval, the same experiment time demonstrated more effective on our experiments. Gerbino et al. (2020) evaluated protective effect of zinc, oligosaccharides, and its association on *A. salina*.

Studies that evaluate the protective effect of different compounds are unanimous regarding the effectiveness of the treatment in relation to *A. salina*; however, the effect of the protective is more effective within the 24-h interval. All of these results corroborate our study.

Conclusion

Unprocessed and non-thermal processed coconut water were non-toxic to *A. salina* presenting low mortality rate. In addition, the non-toxic coconut water samples presented protective effect against oxidative stress caused by H_2O_2, avoiding deaths and morphological changes. The protection was more significant within 24 h. With the increase in the exposure time, the protective effect decreases raising the number of dead nauplii, especially in 72 h.

Further studies are needed for detailed investigation of the products generated by the unprocessed and non-thermal technologies processed coconut water interaction with *A. salina* nauplii.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11947-021-02600-7.

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