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Evaluation of cytotoxicity, genotoxicity and ecotoxicity of nanoemulsions containing Mancozeb and Eugenol

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ABSTRACT

Mancozeb is a fungicide widely used in agriculture, mostly against the pathogen *Glomerella cingulata* responsible for the rot of ripe grape, but presents high toxicity. Strategies are sought to reduce the toxicity of this fungicide and alternative treatments are welcome. An alternative could be the use of clove oil, which has Eugenol as its major compound, and has antifungal potential against *G. cingulata*, however, Eugenol is susceptible to degradation processes which may compromise its efficacy. The nanoencapsulation of Mancozeb and Eugenol is a possible strategy to overcome the limitations of toxicity, solubility and instability of these compounds. Therefore, the objective of this study is to develop nanoemulsions containing Mancozeb (0.1 mg/mL) and Eugenol (33 mg/mL), isolated or associated, and evaluate the safety of these formulations through cytotoxicity, genotoxicity and ecotoxicity tests. Nanoemulsions were developed by the spontaneous emulsification method, cytotoxicity and genotoxicity were evaluated in healthy human cells through MTT, Dichlorofluorescein diacetate and Picogreen tests, and ecotoxicity assessment was carried out using the chronic toxicity test in springtails. After preparation, the physicochemical characterization of the nanoemulsions were performed which presented mean particle size between 200 and 300 nm, polydispersity index less than 0.3, negative zeta potential and acid pH. The nanoencapsulation was able to avoid the reduction of the cell viability caused by Mancozeb, while Eugenol was shown to be safe for cell use in both free and nanostructured forms, however the association of the two active compounds showed toxicity in the higher doses of Mancozeb. In the ecotoxicity tests, both free Mancozeb and Eugenol forms presented high toxic potential for soil, whereas the nanoencapsulation of these compounds did not cause a reduction in number of springtails. Therefore, from the tests performed, it was possible to observe that nanoencapsulation of Mancozeb and Eugenol is a safe alternative for the application of these compounds mainly in agriculture.

1. Introduction

The toxicity and carcinogenicity of fungicides stand out as one of the main public health problems (Belpoggi et al., 2002). The greatest concern is related to potentially exposed individuals, such as workers involved in the development and application of these products, residents of areas where products are sprayed, as well as contamination of food, soil and water with fungicide residues (Paro et al., 2012). About 90% of applied fungicides do not reach the target organism and end up contaminating soil and water (Soares et al., 2012). The accumulation of fungicides, metabolites and heavy metals in soil and water is one of the main problems of environmental contamination.

Mancozeb, a manganese and zinc ethylene-bis-dithiocarbamates, is one of the most frequently used commercial fungicides, but some studies have shown a variety of toxic effects that this fungicide can cause (Belpoggi et al., 2002; Calviello et al., 2006; Hoffman et al., 2016; Pavlovic et al., 2016). Mancozeb is classified by the National Health Surveillance Agency as a highly harmful to the environment, and is rapidly metabolized to Ethylenethiourea, which has a high carcinogenic, mutagenic and teratogenic potential (Pavlovic et al., 2016). This rapid decomposition can occur through processes such as photolysis, hydrolysis or biological decomposition, releasing its sub-compounds

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manganese and zinc in soil, besides its metabolite Ethylenethiourea that has high mobility in soil due to its high aqueous solubility (Geissen et al., 2010).

Mancozeb is the most widely used preventive treatment against the fungus *Glomerella cingulata*, which causes the ripe grape rot, but due its toxic effects mentioned above, worries the environment and human health. In this context, essential oils, which are compounds of natural origin, have been highlighted as promising alternatives due to their antimicrobial properties, antioxidants and may still present low toxicity (Bakkali et al., 2008). Among the essential oils that present antifungal activity against *G. cingulata*, is clove oil which major compound is Eugenol. Barrera-Necha et al. (2008) observed that clove essential oil inhibited the germination of the conidia *Colletotrichum gloeosporioides* of the fungus *G. cingulata* (Barrera-Necha et al., 2008). However, Eugenol is susceptible to degradation processes when exposed to light, oxygen, humidity and high temperatures which may compromise its stability and efficacy (Karkamar et al., 2012).

Considering the limitations of Mancozeb in relation to toxicity and those of Eugenol in relation to instability, solubility and volatilization, the nanoencapsulation of these compounds may be an alternative to solve these challenges and provide stable, effective and safe formulations. Among the nanostructured systems are nanoemulsions, used with the aim of increasing the bioavailability of drug, ensure a controlled release and protection against mechanisms of degradation (Finke et al., 2012).

Preliminary studies carried out by this research group demonstrated that isolated and associated nanoencapsulation of Mancozeb and Eugenol potentiated its antifungal activity against *Glomerella cingulata*. However, as important as effectiveness is ensuring the safety of the formulations. So, in this context, the objective of this study is to develop nanoemulsions containing Mancozeb and Eugenol, associated and isolated, and to evaluate the safety of these formulations through cytotoxicity, genotoxicity and ecotoxicity tests.

2. Materials and methods

2.1. Materials

Mancozeb, Eugenol, Sorbitan monooleate (Span 80), MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and DPPH (2,2-diphenyl-1-picril-hydrazila) were obtained from Sigma Aldrich (São Paulo, Brazil). Polysorbate 80 (Tween 80) was purchased from Synth (São Paulo, Brazil) and Crodamol from Alpha Chemistry (Porto Alegre, Brazil).

2.2. Development of nanoemulsions

Four formulations, described in Table 1, were developed by the spontaneous emulsification method, following the methodology described by Flores et al. (2011): Blank nanoemulsion (NE-B), Eugenol nanoemulsion (NE-E), Mancozeb nanoemulsion (NE-M) and Mancozeb + Eugenol nanoemulsion (NE-ME). After individual homogenization of each phase, the organic phase was poured into the aqueous phase under moderate agitation, and then the organic solvent was evaporated under reduced pressure with the aid of a rotary evaporator to desired volume of formulation (25 mL) with temperature control (35 °C) (Flores et al., 2011).

2.3. Physicochemical characterization of the nanoemulsions

2.3.1. Determination of mean droplet size, polydispersity index, zeta potential and pH

After preparation, the nanoemulsions were characterized according to the mean droplet size, polydispersity index and zeta potential by Zetasizer equipment (Zetasizer® nano-ZS model ZEN 3600, Malvern). To determine the particle size and polydispersity index of the nanoemulsions the dynamic light scattering technique was used after sample dilution (500 times) in ultrapure water. Zeta potential was determined by the electrophoretic mobility technique after sample dilution (500 times) in aqueous solution of sodium chloride (10 mM). The pH values were determined using a potentiometer (DM-22, Digimed®) previously calibrated with standard solution. All readings were performed in triplicate and results were expressed as mean ± SD (Gündel et al., 2018).

2.3.2. Determination of Eugenol content in nanoemulsions

The Eugenol content in NE-E and NE-ME was performed by High Performance Liquid Chromatography (HPLC) following the methodology described by Saran et al. (2013), with modifications. For the extraction of the Eugenol from the nanoemulsions, 45 µL of each formulation was withdrawn and added to 25 mL of methanol, then the samples were homogenized for 30 min (without heating), centrifuged for 30 min (2332*g* at 15 °C), filtered (0.45 µM) and injected into the chromatographic system. For these tests was used a Prominence® chromatograph (Shimadzu, Japan) C18 column (150 mm × 4.6 mm × 5 µm), mobile phase composed of water and methanol (40:60), flow 1 mL/min, detection at wavelength 270 nm, with an injection volume of 20 µL and temperature of 40 °C (Saran et al., 2013).

2.4. Evaluation of corona effect

This assay aims to evaluate the behavior of proteins present in RPMI 1640 culture medium when in contact with nanoemulsions following the methodology described by Konduru et al. (2017), with modifications. The formation of corona protein was evaluated by determining the mean particle size, polydispersity index, zeta potential and pH as mentioned above. It was evaluated the corona effect at the highest concentrations of each treatment, that is NE-M was evaluated at 5 µg/mL, NE-E and NE-B were evaluated at 500 µg/mL, whereas NE-ME was evaluated in the two concentrations mentioned. RPMI 1640 culture medium was prepared containing 10% fetal bovine serum and 1% antibiotics Streptomycin/Penicillin (Sigma-Aldrich). The nanoemulsions were incubated in contact with the culture medium for 24 h in a greenhouse with 5% CO_2 at 37 °C, simulating the same cell culture conditions (Konduru et al., 2017).

2.5. Evaluation of antioxidant activity

For the evaluation of the antioxidant activity, the treatments Mancozeb, Eugenol, Mancozeb + Eugenol and NE Mancozeb + Eugenol were diluted in ethanolic solution in different concentrations.

Table 1
Qualitative and quantitative composition of the formulations.

	Organic phase					Aqueous phase	
Formulation/Components	**Mancozeb**	**Eugenol**	**Crodamol**	**Span 80**	**Acetone**	**Tween 80**	**Ultrapure water**
NE-B	–	–	825 µL	0.2 g	67 mL	0.2 g	134 mL
NE-M	0.0025 g	–	825 µL	0.2 g	67 mL	0.2 g	134 mL
NE-E	–	825 µL	–	0.2 g	67 mL	0.2 g	134 mL
NE-ME	0.0025 g	825 µL	–	0.2 g	67 mL	0.2 g	134 mL

In test tubes, an aliquot of 400 µL of the sample was mixed with 2 mL of the DPPH solution (0.004% m/v). The control was prepared with 400 µL of ethanol and 2 mL of DPPH solution. All tubes were left at room temperature for 30 min in the dark. The results were read at 517 nm wavelength using the UV/VIS-UV-1650 PC spectrophotometer (Shimadzu®). The percent inhibition of the radical was calculated by Eq. (1) and the results were expressed in µL of the sample required to reduce the initial concentration of the radical by 50% (IC_{50}) (Brand-Williams et al., 1995; Roesler et al., 2007).

$$\% \text{ Inhibition} = [(\text{ADPPH–AExtr})/\text{ADPPH}] \times 100 \quad (1)$$

where ADPPH is the absorbance value of the control and AExtr is the absorbance value of the sample. AExtr was evaluated as the difference between the absorbance value of the sample and the absorbance value of the control.

2.6. Cell culture and treatments

In the cytotoxicity and genotoxicity experiments, were used mononuclear cells of peripheral blood obtained from discard samples which were obtained from the Laboratory of Clinical Analysis of the Universidade Franciscana, on the advice of the Ethics Committee of the institution and CAAE record: 31211214.4.0000.5306. The cells were density gradient separated in 15 mL tubes using Histopaque® reagent and centrifugation at 1000 rpm for 30 min. The concentration of 2×10^5 cells was obtained by counting in Neubauer's chamber with 0.4% Tripan blue dye. The cytotoxicity and genotoxicity of the following treatments were evaluated for 24 h: free Mancozeb (0.5, 1, 2.5 and 5 µg/mL), free Eugenol (0.5, 5, 50 and 500 µg/mL), Mancozeb (0.5, 1, 2.5 and 5 µg/mL) + Eugenol (0.5, 5, 50 and 500 µg/mL) association, blank nanoemulsion (0.5, 1, 2.5 and 5 µg/mL), Mancozeb nanoemulsion (0.5, 1, 2.5 and 5 µg/mL), Eugenol nanoemulsion (0.5, 5, 50 and 500 µg/mL) and Mancozeb (0.5, 1, 2.5 and 5 µg/mL) + Eugenol (0.5, 5, 50 and 500 µg/mL) nanoemulsion. Treatment concentrations were chosen according to the antifungal activity against *Glomerella cingulata* (data not shown).

2.6.1. Evaluation of cell viability

Through the MTT cell viability assay was assessed the toxicity of the formulations in peripheral blood mononuclear cells following the methodology described by Krishna et al. (2009) with modifications. For comparison purposes, the cytotoxicity of Mancozeb, Eugenol and Mancozeb + Eugenol in free form diluted in DMSO (0.5%) was also evaluated. The experiment was performed in 96-well plates containing the treatment, cells in the concentration 2×10^5 cells/mL and RPMI culture medium (supplemented with 10% fetal bovine serum and 1% antibiotic). After incubation for 24 h (37 °C and 5% CO_2), 20 µL of MTT salt was added to each well and stored in a greenhouse for an additional 4 h. After the incubation, 200 µL of the supernatant was removed and 200 µL of dimethylsulfoxide (DMSO) was added, then the results were read in ELISA reader (TP-READER, Thermo-Plate®) at 570 nm. The negative control of experiment was composed of cells and culture medium, and the positive control by cells, culture medium and hydrogen peroxide (10 µM). The experiment was performed in triplicate (Krishna et al., 2009).

2.6.2. Dichlorofluorescein diacetate (DCFH-DA) assay

In order to verify the total free radicals ratio, the reagent 2,7-dichlorofluorescein diacetate (DCFH-DA), according to Esposti (2002), was used which has the capacity to cross the cell membrane, being deacetylated by mitochondrial enzymes, producing 2,7-dichlorodihydrofluorescein which reacts with reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and produces 2,7-dichlorofluorescein that emits fluorescence. Therefore, the fluorescence in the spectrofluorimeter equipment (Shimadzu, Japan) was determined based on the wavelengths of 488 nm excitation and 525 nm emission (Esposti, 2002).

2.6.3. Fluorimetric DNA quantification assay

The fluorimetric DNA quantification assay was performed following the methodology described by Sagrillo et al. (2015), in order to complement the determination of cell viability. For the quantification of free DNA the DNA-PicoGreen® reagent was used, which has as a principle a fluorescent dye that binds to the double-stranded DNA formed when the treatment causes disruption and cell death. The experiment was performed in 96-well plates containing culture medium, cells and treatment. After 24 h of incubation (37 °C and 5% CO_2), the dye was added, then the experiment was incubated for a further 5 min under the same conditions and the results were read using the Spectrofluorimeter (Shimadzu, Japan) equipment, with a wavelength of 480 nm of excitation and 520 nm of emission (Sagrillo et al., 2015).

2.7. Evaluation of ecotoxicity

2.7.1. Soil

For the test, a soil was used as reference, which is recommended by ISO 112682 (ISO, 1998) the Tropical Artificial Soil (TAS). This soil is commonly used in ecotoxicological assessments in tropical climate regions and consists of a mixture of 75% industrial sand, 20% kaolinite clay, and 5% coconut husk powder.

2.7.2. Soil organisms to be tested

Ecotoxicity tests were carried out in accordance with ISO 11267 guideline (ISO, 1999), in order to evaluate the toxic potential of treatments on the reproduction of springtails species *Folsomia candida* by the chronic toxicity test. The organisms were obtained from laboratory cultures maintained at a temperature of 20 ± 2 °C and photoperiod of 12:12 h (light/dark). In plastic containers (3.5 cm diameter × 11.5 height) were added 30 g of soil, 10 springtails (10–12 days) and the treatments free Mancozeb (4.2, 2.1 and 1.05 µg/30 g soil), free Eugenol (230, 115, and 57.5 µg/30 g soil), Mancozeb + Eugenol association (1.5 + 230, 0.75 + 115 and 0.375 + 57.5 µg/30 g soil), NE-M (1.5, 0.75 and 0.375 µg/30 g soil), NE-E (230, 115, and 57.5 µg/30 g soil), NE-ME (1.5 + 230, 0.75 + 115 and 0.375 + 57.5 µg/30 g soil) and NE-B (230, 115, and 57.5 µg/30 g soil). After 28 days, water and black ink were added to the containers, so that juveniles generated remained on the surface of the water and could be counted (Zortéa et al., 2017).

2.8. Statistical analysis

The results were submitted to analysis of variance (ANOVA) and the means were analyzed using Dunnett's test, where the significance level was set at 5% ($p < 0.05$). The graphs were performed on Graphpad Prism 5.0 software (Graphpad Software, INC).

3. Results and discussion

3.1. Characterization of nanoemulsions

After preparation, the nanoemulsions were characterized according to the mean droplet size, polydispersity index, zeta potential, pH and Eugenol content, as shown in Table 2. The formulations had mean droplet size between 200 and 300 nm, which according to the literature, since nanoemulsions are nanostructured systems composed of two immiscible phases stabilized with the aid of surfactants, presenting a size of 20–500 nm (Soliman, 2017). The formulations had a polydispersity index of less than 0.3, indicating uniformity of particle size. The pH of the nanoemulsions presented acidic characteristics and the Eugenol content in NE-E and NE-ME was around 50%. The zeta potential is another important parameter in the characterization of nanoparticles, since values different from zero indicate a greater stability

Table 2
Physical-chemical characterization of the formulations. The results are expressed as mean ± SD (n = 3).

	Size (nm)	Polydispersity index	Zeta potential (mV)	pH	Eugenol content (%)
NE-B	194 ± 0.64	0.15 ± 0.01	−12.1 ± 1.20	5.45 ± 0.09	–
NE-M	198 ± 1,17	0.16 ± 0.02	−11.5 ± 0.75	5.67 ± 0.13	–
NE-E	306 ± 2.57	0.25 ± 0.04	−7.14 ± 0.31	4.12 ± 0.11	49.35%
NE-ME	243 ± 3.56	0.27 ± 0.01	−7.34 ± 1.04	4.41 ± 0.15	47.82%

of the system, and the use of Tween 80 surfactant allowed the development of formulations with zeta potential values ranging from − 7 to − 12 mV (Flores et al., 2011).

3.2. Evaluation of corona effect

Nanoparticles when in contact with biological medium can receive a protein coating, a process known as the corona effect, and consequently causes changes in the interaction of the nanoparticles with the cells, modifying their mechanism in biological medium and being able to generate toxicity (Docter et al., 2014; Lee et al., 2015; Rodriguez-Emmenegger et al., 2011). To evaluate the formation of corona protein, the formulations were maintained in contact with RPMI culture medium (supplemented with 10% fetal bovine serum and 1% antibiotic) for 24 h in an oven at 37 °C with 5% CO_2.

Contact with the RPMI medium and exposure to serum proteins resulted in a significant increase on the mean particle size due to formation of corona protein on surface of nanoparticles. This increase was observed in NE-E and NE-ME, probably due to the presence of Eugenol in these two formulations, and both formulations were evaluated in the highest concentration of this active substance (500 µg/mL). While NE-M showed no protein formation on its surface, the highest concentration of Mancozeb was 5 µg/mL. In this case, it is believed that the formation of corona protein with Eugenol was dose-dependent, the higher the concentration of the active, greater the affinity of the proteins for the nanoparticles.

NE-E and NE-ME showed an increase of 150 and 300 nm, respectively, compared to the initial characterization, whereas NE-M and NE-B maintained an average size around 200 nm. The NE-E and NE-ME formulations also showed an increase in the polydispersity index to values of 0.56 and 0.68, respectively, indicating the absence of particle size uniformity. The zeta potential of formulations did not show variations after contact with culture medium, in addition, the presence of nanoemulsions did not alter the pH of medium with values around 7 in all conditions.

Paula et al. (2013) observed a significant increase in size of silica nanoparticles after contact with the culture medium RPMI supplemented with fetal bovine serum. This increase is related to the presence of proteins strongly attached to surface of nanoparticles, called corona hard. In addition, after formation of corona protein, there were no changes in the zeta potential values (Paula et al., 2013).

3.3. Evaluation of antioxidant activity, cytotoxicity and genotoxicity

Mancozeb free presented IC_{50} of 91.16 µL, however, it was able to reduce the generation of reactive oxygen species (ROS) and did not cause DNA damage. Although, there was reduction of cell viability at all concentrations tested, as shown in Fig. 1. This reduction in the viability confirms the action mechanism of Mancozeb (Fig. 1A), which impairs the transport of electrons in the respiratory chain and reduces the production of adenosine triphosphate (ATP), causing mitochondrial dysfunction (Domico et al., 2007; Liu et al., 2017). On the other hand, the nanoencapsulation of Mancozeb caused increased ROS production and DNA damage, however, it was able to protect against the reduction of cellular viability (Fig. 1B), except in the higher concentration, demonstrating the potential of nanostructured systems to reduce the cytotoxic effect of this fungicide.

Pirozzi et al. (2016) also evaluated the toxicity of Mancozeb in HepG2 hepatocarcinoma cells through the MTT assay, and found that this active was highly cytotoxic because it reduced cell viability (below 50%) at all concentrations after 24 h of exposure (Pirozzi et al., 2016). Pavlovic et al. (2016) observed that prolonged exposure to Mancozeb can induce genotoxicity and apoptosis in cells through ROS formation (Pavlovic et al., 2016). Srivastava et al. (2012) also confirmed that the genotoxic and pro-oxidant potential in lymphocytes after exposure for 24 h in different doses of Mancozeb, in addition, the oxidative stress caused by the increase of ROS is related to the mechanism of apoptosis induced by Mancozeb (Srivastava et al., 2012). In the results obtained in this study, there were observed that Mancozeb in the free form caused reduction of viability due to a mitochondrial mechanism, whereas NE-M did not cause a reduction in viability, but it had an impact on ROS generation and DNA damage.

Eugenol is considered safe by Food and Drug Administration (FDA), also not presenting mutagenic and carcinogenic potential (Baskaran et al., 2010). Due to its lipophilic properties, Eugenol is rapidly incorporated into the lipid bilayer of the cell membrane (Atsumi et al., 2001). At low concentrations, Eugenol presents antioxidant potential while at high concentrations have pro-oxidant activity increasing the production of free radicals (Porto et al., 2014), and the apoptotic potential of Eugenol is related to high ROS production (Yoo et al., 2005).

The free Eugenol, through the analysis of antioxidant activity, presented an IC_{50} of 1.71 µL responsible for reducing ROS production in the cells, not causing damage to DNA (Fig. 2A), however, the highest concentration evaluated showed moderate cytotoxicity and caused a reduction of 30% of cell viability, as shown in Fig. 2. Only the highest NE-E concentration showed an increase in ROS production (123%) and greater damage in the genetic material (143%) (Fig. 2B), which consequently caused cell death due to oxidative stress. Eugenol after oxidation or alkalinization is converted to a phenoxyl radical which presents low toxicity, and the nanoencapsulation aims to protect the Eugenol from oxidation, delaying this conversion and may increase the toxicity of this active when nanoencapsulated, in addition, alkaline conditions of the medium of culture increase ROS production (Nagabu et al., 2010).

After the association of Mancozeb and Eugenol, there was an increase in antioxidant activity where the free association had IC_{50} of 1.71 µL, and this result was potentiated with nanoencapsulation of these active compounds with IC_{50} of 0.30 µL. In the free Mancozeb + Eugenol combination, only the higher concentration containing 5 µg of Mancozeb and 775 µg of Eugenol resulted in a reduction in cell viability, as shown in Fig. 3, verifying that the presence of Eugenol was able to reduce cytotoxicity of Mancozeb previously observed. In addition, the association was able to reduce generation of ROS and did not cause DNA damage (Fig. 3A).

However, NE-ME only at concentrations below 0.5 µg of Mancozeb did not result in reduced cell viability, while higher concentrations resulted in increased ROS levels and DNA damage, resulting in cell death (Fig. 3B).

Based on the results obtained, was observed that the isolated nanoencapsulation of Mancozeb (NE-M) and Eugenol (NE-E) would be the best alternative for the application of these compounds taking into consideration the safety in healthy human cells. In addition, the doses

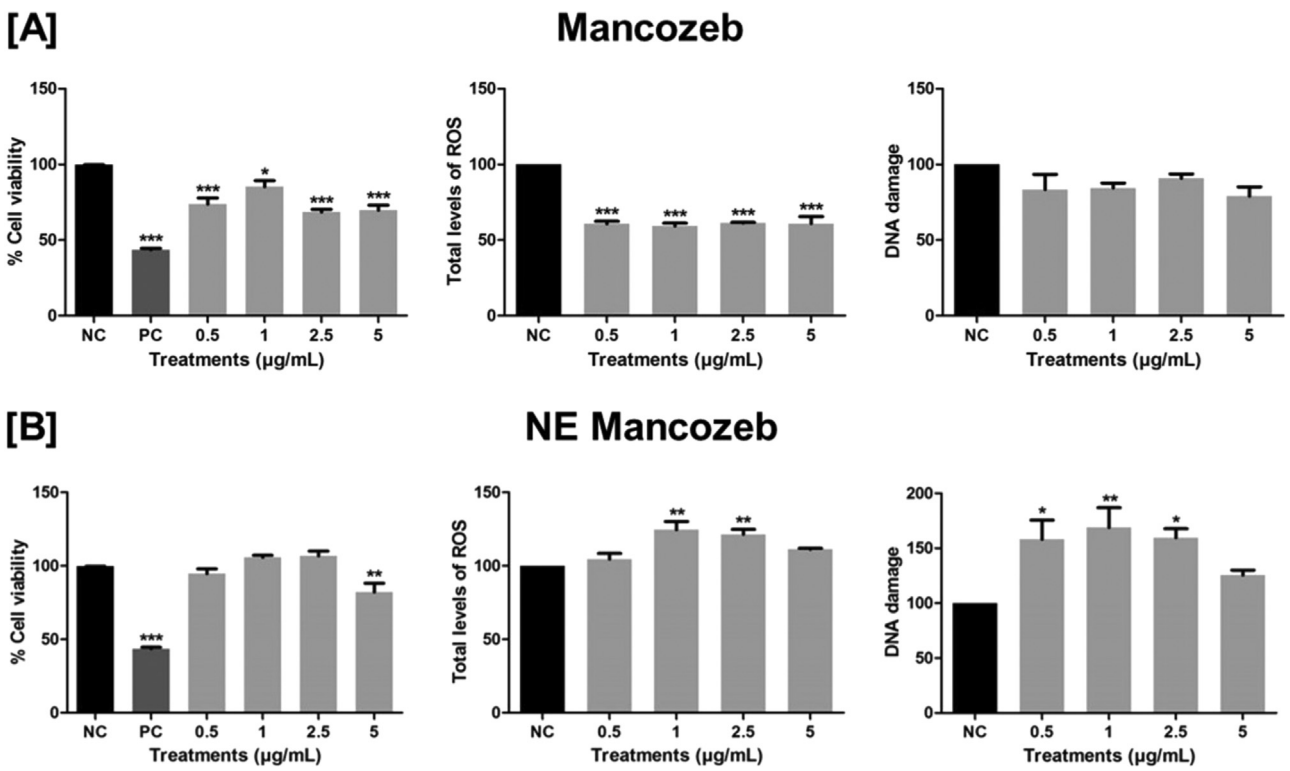

Fig. 1. Evaluation of cell viability, total levels of ROS and DNA damage in healthy human cells from the free Mancozeb [A] and NE-M [B] treatments. All the results were compared with the negative control (NC). The results followed by *** presented statistical difference ($p < 0.05$).

used were superlative because they are probably above the concentration that could be considered toxic for human consumption. Preliminary trials of antifungal activity against *G. cingulata* performed by the research group demonstrated potentiation of efficacy after nanoencapsulation of the compounds, where for treatment with NE-M a dose about 1000 times lower than the active in free form was required, while for NE-E a dose 3 times lower was required. The effective dose of NE-M was about 50 times lower than the lowest concentration (0.5 µg/

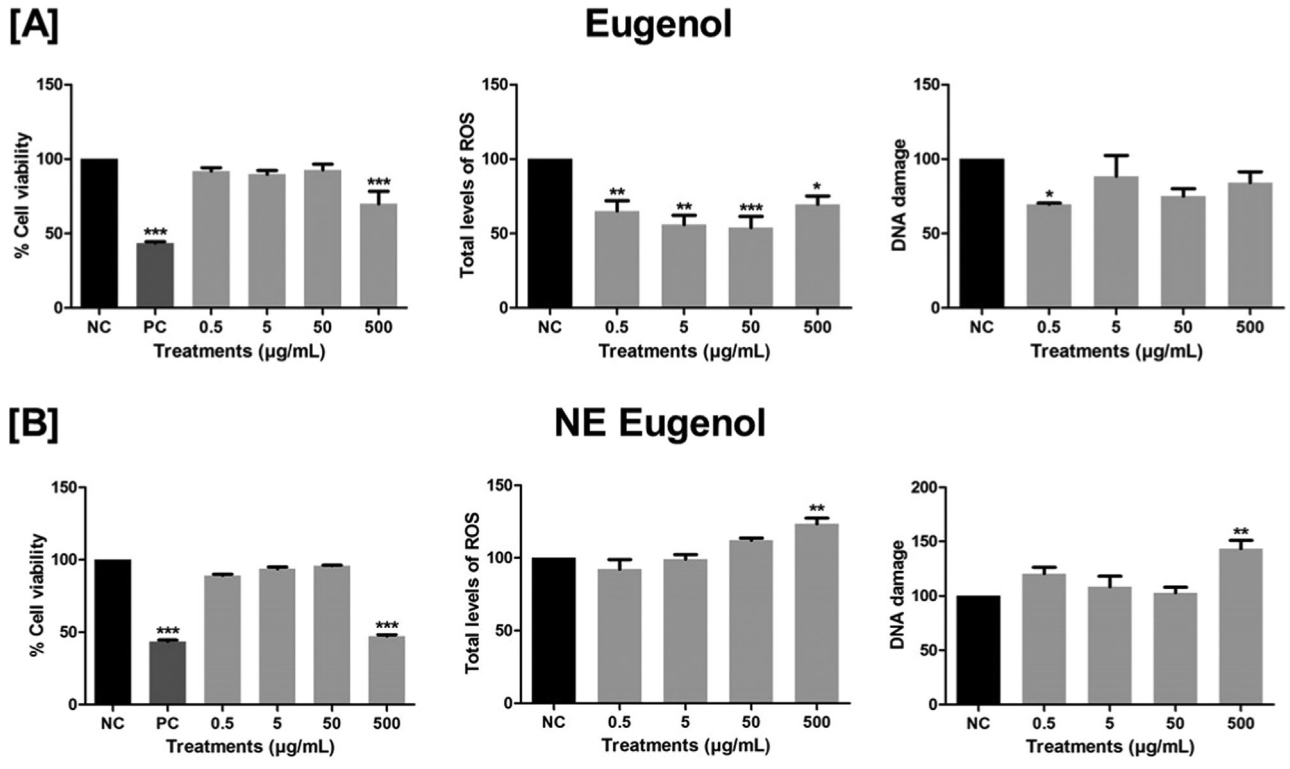

Fig. 2. Evaluation of cell viability, total levels of ROS and DNA damage in healthy human cells from the free Eugenol [A] and NE-E [B] treatments. All the results were compared with the negative control (NC). The results followed by *** presented statistical difference ($p < 0.05$).

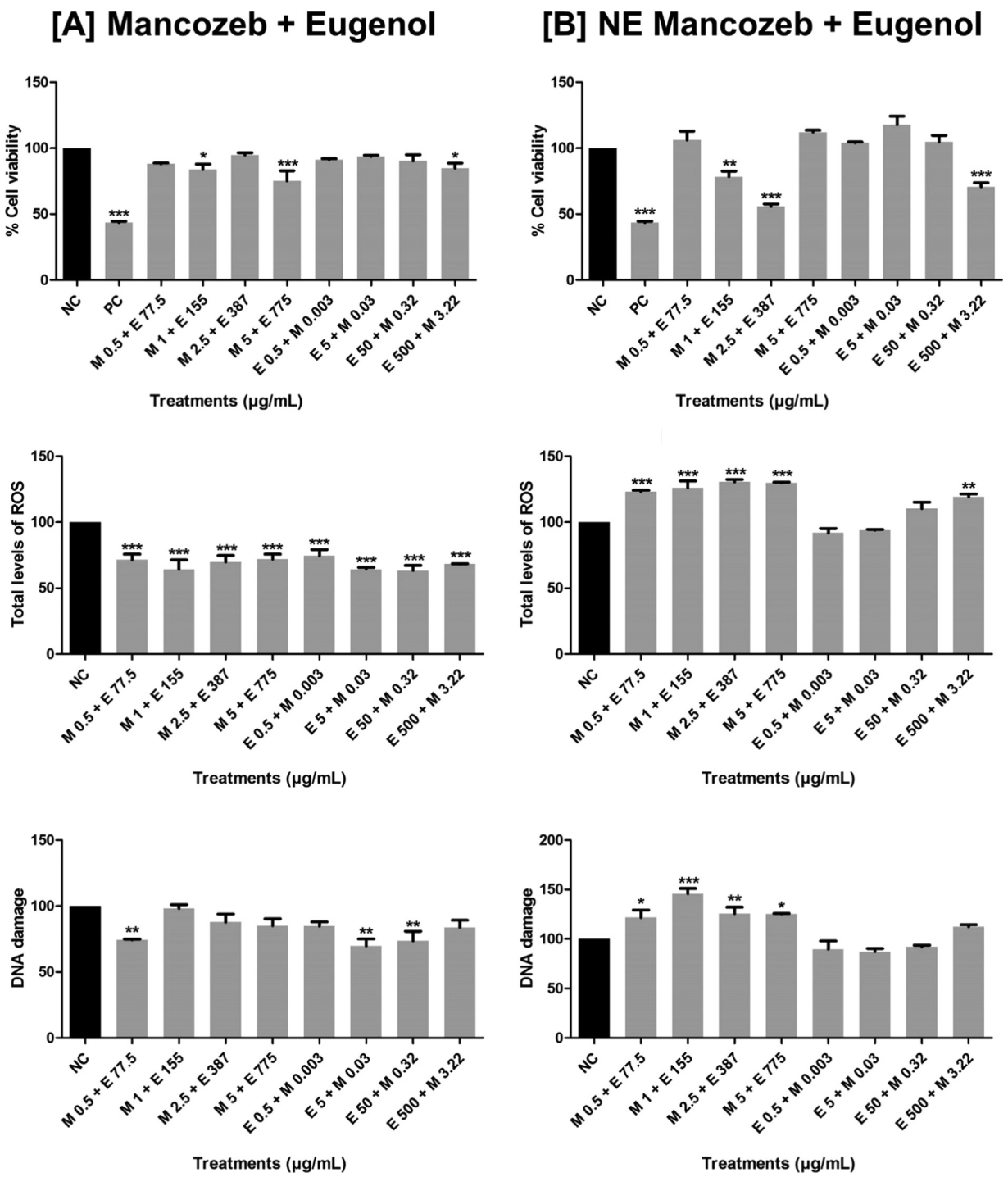

Fig. 3. Evaluation of cell viability, total levels of ROS and DNA damage in healthy human cells from the free Mancozeb + Eugenol [A] and NE-ME [B] treatments. All the results were compared with the negative control (NC). The results followed by *** presented statistical difference ($p < 0.05$).

mL) evaluated in the present study, since the effective dose of NE-E is within the range of safe concentrations determined in this study.

In order to verify the influence of other constituents of formulation, the activity of NE-B was evaluated, in which there is no presence of active Mancozeb and Eugenol. It was observed that NE-B did not present increased ROS production and generation of DNA damage, in addition, the formulation did not cause a reduction in cell viability, suggesting the safety of its constituents.

3.4. Evaluation of ecotoxicity

The reproduction test presented the criteria to be validated according to the guidelines of ISO 11267 (1999). It should contain more than 100 organisms per vessel and the coefficient of variation should be less than 30% for control. A few studies have been done on the impact of fungicide use on tropical ecosystems (Garcia et al., 2008), in addition, frequent use of fungicides, such as Mancozeb, results in high levels of environmental pollution as well as significant risks to human health. In this context, alternative treatments are needed in order to reduce the use of ethylene-bis-dithiocarbamates or approaches to reduce their

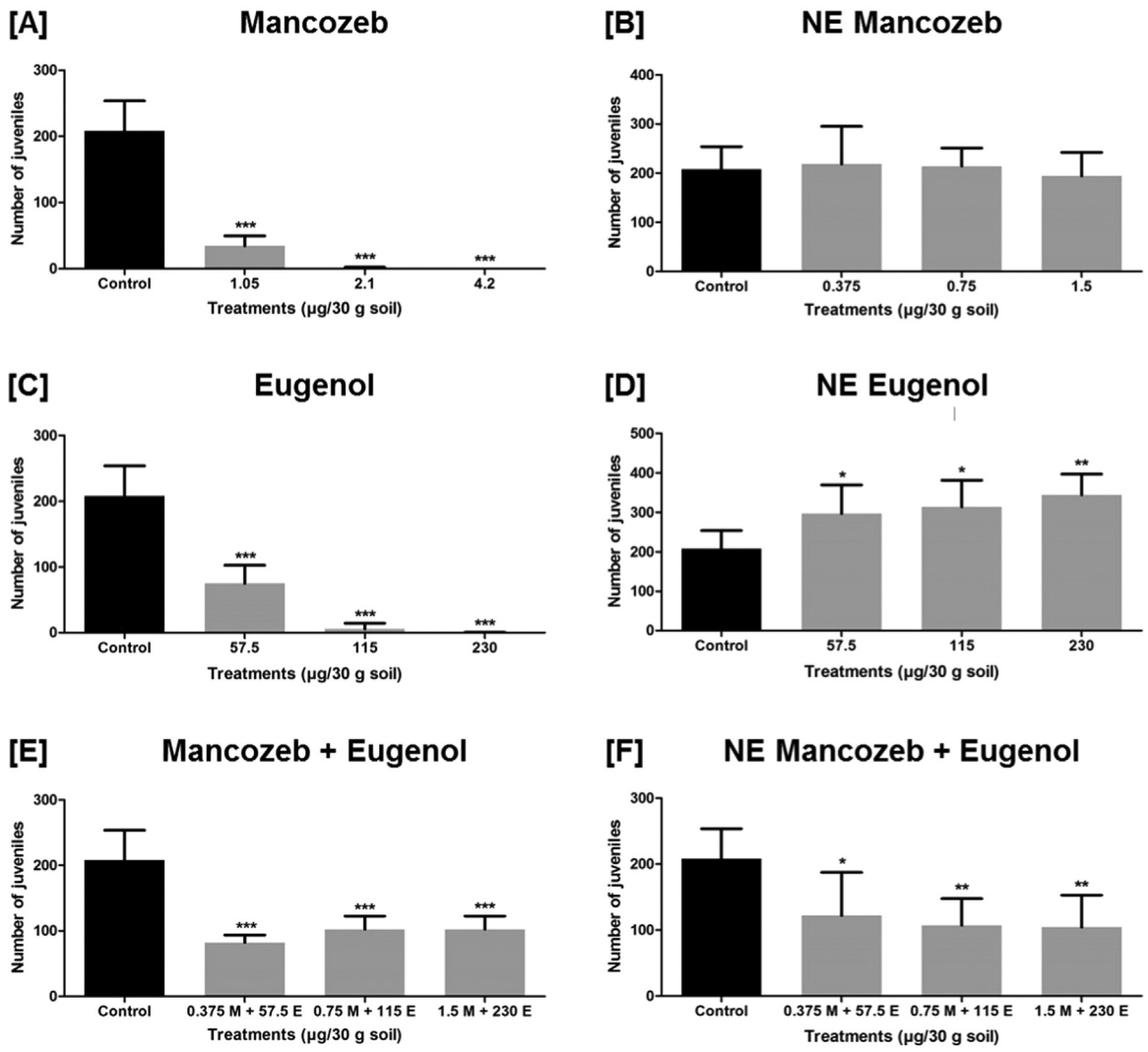

Fig. 4. Evaluation of chronic toxicity of treatments free Mancozeb [A], NE-M [B], free Eugenol [C], NE-E [D], free Mancozeb + Eugenol [E] and NE-ME [F] the reproduction of *Folsomia candida*. All results were compared with the control. The results followed by *** presented statistical difference ($p < 0.05$).

toxic effects (Geissen et al., 2010). The chronic toxicity test on the reproduction of springtails *Folsomia candida* was carried out in order to evaluate the ecotoxicity of the treatments, as shown in Fig. 4.

The free Mancozeb was tested at the reference concentration of 280 g/ha (4.2 µg/30 g soil) (Naves et al., 2006), used for the control of the fungus *Glomerella cingulata*, and also two smaller doses. The lowest dose of Mancozeb tested was 1.05 µg/30 g soil, equivalent to 70 g/ha, and showed a reduction of around 85% in the number of juveniles generated. While at two higher doses (2.1 and 4.2 µg/30 g soil) they reduced 100% the reproduction of springtails, confirming the toxic potential of Mancozeb to the soil (Fig. 4A). On the other hand, NE-M showed no toxicity to organisms in the soil in any of concentrations tested, while maintaining the number of juveniles about 200 individuals (Fig. 4B), this may be related to the ability of nanoencapsulation of this active to protect against decomposition processes, avoiding the accumulation of manganese and zinc in soil.

Geissen et al. (2010) evaluated the accumulation of Ethylenethiourea, manganese and zinc in soil and water after the decomposition of Mancozeb, and observed that the frequent application of this fungicide caused a high accumulation of manganese and zinc in the soil, in addition, surface and underground water presented high contamination of Ethylenethiourea (Geissen et al., 2010). The toxic effect of mancozeb on edaphic organisms (*Porcellionides pruinosus*) was evaluated by Morgado et al. (2016) where they found a reduction in the feed rate of the individuals in soils contaminated by the product, which resulted in greater loss of body weight. This condition can lead to the death of the organisms, not only of *Porcellionides pruinosus*, but also of springtails that present routes of exposure, to the products, very similar (Morgado et al., 2016).

The free Eugenol in the lowest concentration (57.5 µg/30 g soil) had a reduction of springtails around 65%, and in the two highest concentrations (115 and 230 µg/30 g soil) the reduction was 100% (Fig. 4C), however, NE-E maintained the number of juveniles generated above 200 individuals in all concentrations (Fig. 4D). The toxic potential of Eugenol was also observed by Gueretz et al. (2017), where Eugenol at different doses (50, 75 and 100 mg/L) presented acute toxicity for bacterium *Aliivibrio fischeri* and for microcrustacean *Daphnia magna* and chronic toxicity for alga *Desmodesmus subspicatus* (Gueretz et al., 2017). According to Plata-Rueda et al. (2018), the mechanisms of toxic action of terpenoids such as Eugenol, can show altered locomotor behavior, and muscle contractions (legs and abdomen) that can cause mortality, low respiration rate, and behavior repellency response in the edaphic organisms (Plata-Rueda et al., 2018).

Furthermore, Mancozeb and Eugenol have low solubility in water, which favors accumulation in the soil. When these are associated with nanoemulsions oil in water occurs an increase in solubility, possibly related to the reduction of the toxic effects of these when nanoencapsulated alone (Du et al., 2016). However, when the compounds

Mancozeb and Eugenol were associated in both free and nanostructured form (Figs. 4E and 4F), there was a reduction in the reproduction of the springtails, and there was no statistical difference between the free and nanostructured treatment ($p < 0.05$). Beyond that, NE-B did not cause any reduction of organisms, confirming that the other constituents of formulation do not present toxicity.

4. Conclusion

Nanotechnology in agriculture aims to develop formulations less harmful to environment and consequently to human health, besides reducing the decomposition of active in soil, increasing its bioavailability. In this study, nanoemulsions containing Mancozeb and Eugenol, isolated or associated were developed and the safety of these formulations was evaluated through cytotoxicity, genotoxicity and ecotoxicity assays. From the tests carried out, the ability of nanostructures to prevent toxic effects caused by a synthetic fungicide, Mancozeb, and a natural fungicide, Eugenol, was demonstrated. Therefore, on the basis of the tests carried out, it was possible to observe that the nanoemulsion containing Eugenol was the safest formulation for use in agriculture, as it did not cause reduction of cell viability, ROS production and DNA damage and did not present ecotoxicity to *Folsomia candida*.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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