UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
Faculdade De Farmácia
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Evaluation of the protective effect of α-lipoic acid, vitamin C, coenzyme Q10, curcumin and pomegranate extract against the harmful effects caused by docetaxel and carboplatin in the alternative model *Caenorhabditis elegans*

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ABSTRACT

Breast cancer affects a great part of the female population worldwide. Antioxidants are being tested to help to minimize the side effects caused by chemotherapy. This study aimed to evaluate if an antioxidants mixture (Mix) (α-lipoic acid, vitamin C, coenzyme Q10, curcumin) and pomegranate extract (POMx) can minimize some harmful effects caused by docetaxel and carboplatin, using alternative model *Caenorhabditis elegans*. The nematodes were treated with POMx (5, 10, 20, 40 and 50 mg/mL) and Mix (0.25, 0.5, 1, 2 and 3 doses) followed by docetaxel (0.2 and 0.1 mg/mL) and carboplatin (99.4 and 4.7 µg/mL). Mortality rate, body area and ROS production were evaluated. Paraquat (PQ) and Metil methanesulfonate (MMS) were used as positive damage controls as a prior assay. POMx did not help to reduce the mortality when comapared to PQ and MMS. Mix did not reduced mortality compared to PQ. Mix 2 doses helped to decrease mortality when compared to MMS, docetaxel and the lower dose of carboplatin tested. *C. elegans* body area was significantly reduced when treated with the harmful agents, Mix and POMx were not able to revert it. ROS production was noticed when added docetaxel and carboplatin and only Mix 2 reduced ROS in the higher dose of carboplatin tested. Worms that received Mix had a better outcome than the ones treated with POMx, probably because there was more than one antioxidant, since it lowered the mortality rate proving that the use of a combination of antioxidants is better than using only one.

Keywords: antioxidants; pomegranate extract (POMx); docetaxel; carboplatin; *Caenorhabditis elegans.*

1. Introduction

Breast cancer is known to be the most frequent type of cancer in women worldwide. It has a high mortality rate, being responsible for 15.403 deaths of women in 2015 (INCA, 2018). In 2018, it is estimated 59.700 new cases among Brazilian women. The etiology of breast cancer is multifactorial, including reproductive and endocrine factors, inherited mutations and environmental factors (WHO, 2014).

There are more than 20 subtypes of breast cancer. Approximately 20% of the cases of primary breast cancer have the amplification on the human epidermal growth factor receptor 2 (HER2) gene (WHO, 2014; Nabholtz et al., 2002). The overexpression of this gene is associated with a poor prognosis, since it is involved with regulation of normal and neoplastic cell growth and differentiation (Nabholtz et al., 2002).

The current treatment to HER2-positive breast cancer includes chemotherapy and the two most used regimens are doxorubicin and cyclophosphamide followed by docetaxel (AC-T) and a combination of docetaxel, carboplatin and trastuzumab (TCH). The use of the combination TCH is favorable when compared to the AC-T since it has shown less acute toxic effects, revealed by fewer neutropenia, leukopenia and a greater cardiological safety, since docetaxel and carboplatin are not cardiotoxic compounds (Bayo et al., 2017).

Docetaxel is an antimicrotubule agent from the taxoid antioneplasic class (Bayet-Robert et al., 2010). Carboplatin is a platinum derivate that binds to the DNA present in the nucleus, causing an interference in the DNA replication and normal transcription (Fuertes et al., 2003). Trastuzumab is a monoclonal humanized anti-HER2 antibody that inhibits the proliferation and survival of tumors that depends on HER2 (Hudis, 2007). These drugs are known to form reactive oxygen species (ROS) that leads to oxidative stress and can be related to some of the side effects of the treatment (Kabel et al., 2007; Mir et al., 2009; Block et al., 2007).

The cardiotoxicity caused by trastuzumab is related to its great affinity in binding to the HER2, making the receptor incapable to dimerize. The

cardiomyocytes are then left in stress, not being able to activate cell survival pathways that would deal with the excessive ROS production (Zeglinski et al., 2011). The ROS can be produced not only as a result of the side effect, but also as a mechanism of the chemotherapy. Carboplatin has ROS production as a primary mechanism against cancer cells (Block et al., 2008) and docetaxel is known to induce the apoptotic cell death by ROS production (Cao et al., 2005).

Antioxidants control oxidative stress, being the intake of these substances an option to patients with cancer as supplementation to chemotherapy. This complementary treatment has become popular, and it is estimated that 13% to 87% of the patients are taking supplements based on antioxidants (Block et al., 2007). However, the self-medicating by patients with antioxidants may be a problem in complex diseases, such as cancer, because these compounds may interfere in the chemotherapy protecting not only the normal, but also the cancer cells (Moss et al., 2006). Despite that, there are studies showing that antioxidants helped relieving symptoms and appeared to be a good alternative to help patients to stay under treatment (Moss et al., 2006). Previous studies have reported that patients with cancer who are undergoing treatment chemotherapy have ingested antioxidants such as α-lipoic acid, vitamin C, coenzyme Q10, curcumin and pomegranate extract (POMx) (Block et al., 2007; Kim et al., 2012).

Therefore, to investigate whether antioxidants could protect against the toxic effects of chemotherapies, the nematode *Caernohabditis elegans* was applied as an alternative model in this study. *C. elegans* has a short life cycle and represents a less expensive and time-consuming alternative of *in vivo* assays when compared to mammals (Hunt et al., 2016). An advantage of the nematode over *in vitro* models is that the whole organism response could be evaluated. Besides, the nematodes genome has the majority of genes and disease pathways similar to the humans (Kaletta et al., 2006).

Paraquat (PQ) and methyl methanesulfonate (MMS) are used as damage positive control on *C. elegans*. These harmful agents are known to cause alteration on the redox cycle and damage directly on the DNA,

respectively (Charão et al., 2015; Qureshi et al, 1989). These agents are used as a prior study, to analyze if the antioxidants are able to prevent toxic effects caused by PQ and MMS.

Thus, the main aim of this study was to evaluate whether the following antioxidants mixture (α-lipoic acid, vitamin C, coenzyme Q10, curcumin) and POMx could minimize the harmful effects caused by docetaxel and carboplatin, drugs used in the chemotherapy treatment of breast cancer, in *C. elegans.*

2. Materials and Methods

2.1. Reagents

The antioxidants curcumin and pomegranate extract (POMx) were obtained from Fagron®. Vitamin C and coenzyme Q10 were purchased from Purifarma® and α-lipoic acid was obtained from Infinity Pharma®. Docetaxel, carboplatin, 2,7-dichlorofluorescein diacetate (DCF-DA), methyl methanesulfonate (MMS) and paraquat were supplied by Sigma-Aldrich Co®. (St Louis, MO, USA). Bacto-agar and bacto-peptona were obtained from Becton Dickinson BD® (New Jersey, USA) and HiMedia Laboratories® (Mumbai, India), respectively. All other chemicals and solvents were from analytical grade. The nematode strains used in this work were N2 Bristol, obtained from the *Caenorhabditis Genetics Center* (CGC).

2.2. Antioxidants mixture and extract preparation

The antioxidants mixture (Mix) consisted in α-lipoic acid, curcumin, vitamin C and coenzyme Q10. The concentration of each antioxidant used in the mixture was according to previous studies that tested these antioxidants in *C. elegans*: 100 *μ*M α-lipoic acid, 200 *μ*M curcumin, 150 *μ*g/mL coenzyme Q10 and 140 *μ*M vitamin C (Brown et al., 2006; Liao et al., 2011; Ishii et al, 2014; Sonane et al., 2017). The stock solution was prepared by weighting the antioxidants and dissolving them in dimethylsulfoxide (DMSO). Working solutions were prepared by diluting the stock solutions in water.

Five solutions of antioxidants mixtures were prepared with different concentrations of the antioxidants and were called according to the dose: 0.25 (0.94 mg of coenzyme Q10, 0.37 mg of curcumin, 0.155 mg of vitamin C and 0.123 mg of α-lipoic acid), 0.5 (1.875 mg of coenzyme Q10, 0.74 mg of curcumin, 0.31 mg of vitamin C and 0.257 mg of α-lipoic acid), 1 (3.75 mg of coenzyme Q10, 1.48 mg of curcumin, 0.62 mg of vitamin C and 0.515 mg of α-lipoic acid), 2 (7.25 mg of coenzyme Q10, 2.96 mg of curcumin, 1.24 mg of vitamin C and 1.03 mg of α-lipoic acid) and 3 doses (11.25 mg of coenzyme Q10, 4.44 mg of curcumin, 1.86 mg of vitamin C and 1.545 mg of α-lipoic acid). Doses 2 and 3 were diluted in 25 mL of DMSO and the others in DMSO and water.

The stock solution of POMx was prepared by dissolving the extract in DMSO. The concentration used was 40 mg/mL, according to a previous *in vitro* study (Jeune et al., 2005), and the dosage was adjusted to *C. elegans*

Control solutions were prepared according to the diluents used: 5% DMSO, 0.05% Tween 80, and 5% DMSO with 0.05% Tween 80. A negative control of 0.5% saline was also used.

Paraquat (PQ) and methyl methanesulfonate (MMS) were prepared at 0.5 mM and 1 μM, respectively, in water (Charão et al., 2015). Docetaxel and carboplatin were prepared in distilled water and 0.05% tween 80 at the day of the experiment to avoid drug degradation.

2.3. *C. elegans* strain and synchronization

N2 *C. elegans* strain (wild-type) was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50, as source of food, at 20°C. For the synchronization, gravid *C. elegans* were washed off the plates into centrifuge tubes and then lysed with a bleaching mixture (1% NaOCl; 0.25 M NaOH), followed by flotation on a 30% sucrose solution (m/v) to separate eggs from dissolved worms and bacteria debris. The eggs were washed with M9 buffer (0.02 M KH_2PO_4, 0.04 M Na_2HPO_4, 0.08 M NaCl, and 0.001 M $MgSO_4$) and allowed to hatch overnight in NGM agar plates without bacteria.

2.4. Exposure to antioxidants and chemotherapeutics

After synchronization, 2.500 L1 larvae were treated with POMx from 5 to 50 mg/mL and antioxidants mixture (Mix) ranging from 0.25 to 3 doses namely (Mix 0.25, Mix 0.5, Mix 1, Mix 2 and Mix 3). The organism were incubated for 24h at 20°C, by constant agitation in a rotator in a 0.5% NaCl liquid media. Additionally, worms exposed to 5% DMSO were used as controls. After incubation, worms were washed three times with 0.5% NaCl to remove the antioxidants and then, transferred to NGM recovery plates inoculated with *Escherichia coli* - OP50 for posterior assays (controls) or it was added 50 μL of 0.5 mM PQ, 1μM MMS, 0.2 mg/mL (D1) and 0.1 mg/mL (D2) docetaxel or 99.4 μg/mL (C1) and 49.7 μg/mL (C2) carboplatin to the worms (Rantanen et al., 1994). After 24 h of incubation, the nematodes were washed three times with 0.5% NaCl to remove the drugs and the worms were placed on new NGM plates seeded with OP50.

2.5. Mortality evaluation

At the end of the 24 h incubation with the drugs, worms were washed three times with 0.5% NaCl and plated on NGM seeded with *E. coli OP50*. Mortality parameter was evaluated by counting after 24 h time recovery on plates. All experiments were performed in duplicate and repeated at least three independent times.

2.6. Body area

Body area was evaluated in 20 worms at L4 stage per treatment 48 h after drugs removal. The organisms were washed of the NGM plates with distilled water and transferred to a centrifuge tube, allowing the worms to settle and separated from the bacteria. The process is repeated until the solution is clear. Then, 15 μL of the solution were deposited on a blade covered with agarose and added 25 μL of 2.25% levamisole. The worms were photographed and the flat surface area was measured with AxioVision software LE version 4.8.2.0 for Windows.

2.7. ROS measurement

For ROS measurement, 1.500 N2 L1 worms were resuspended in 100 µL of 0.5% NaCl and transferred to 96-well plates with 100 μL of 0.05 mM 2,7-dichlorofluorescein diacetate (DCF-DA). Fluorescence was measured at 485 nm excitation and 535 nm emission, for 90 minutes using a microplate reader (Spectramax Me2; Molecular Devices LLC, Sunnyvale, CA, USA) at 20°C. The values were expressed as percentage of fluorescence intensity relative to control. At least three independent experiments were performed in duplicate.

2.8. Statistical analysis

Analyses were performed using GraphPad Prism version 7 (GraphPad Software). Statistical analysis of significance was performed by one-way ANOVA, or repeated measures ANOVA for ROS quantification, followed by Bonferroni post-test. Significance was accepted at $p<0.05$.

3. Results

3.1. Toxicity of pomegranate extract (POMx) and antioxidants mixture (Mix) after 24 h of exposure

Figure 1 shows the mortality of the worms after 24 h of incubation with POMx and Mix. Negative control when compared to DMSO 5% did not presented significant differences. In relation to POMx exposure, it was possible to observe a significant mortality at 20, 40 and 50 mg/mL ($p<0.001$) when compared to 5% DMSO (Figure 1A). For Mix, all tested concentrations presented significant mortality ($p<0.01$) compared to 5% DMSO (Figure 1B).

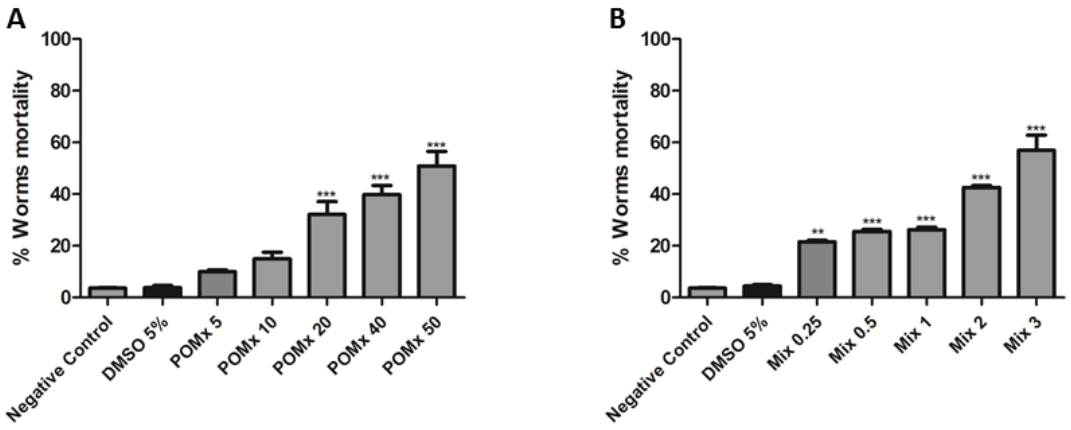

Figure 1. *C. elegans* mortality after 24 hours of exposure to different concentrations of pomegranate extract (POMx) and antioxidants mixtures (Mix). **(A)** POMx (5, 10, 20, 40 and 50 mg/mL). **(B)** Mix (0.25, 0.5, 1, 2 and 3 doses). Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using ANOVA/Bonferroni. (**p<0.01; ***p<0.001 vs. control group).

3.2. Antioxidants mixtures (Mix) protected nematodes against MMS but not PQ induced mortality

It was possible to observe in Figure 2 that PQ and MMS significantly increased worms mortality compared to negative control group (p<0.001). POMx pre-incubation did not show significant difference compared to PQ (Figure 2A) or MMS (Figure 2B), being not able to protect nematodes from toxicity.

In Figure 2C, the nematodes incubated with Mix did not present a significant reduction in PQ induced mortality. However, when mortality was induced by MMS, Mix 2 and 3, the worms mortality was significantly reduced (p<0.05), as observed in Figure 2D.

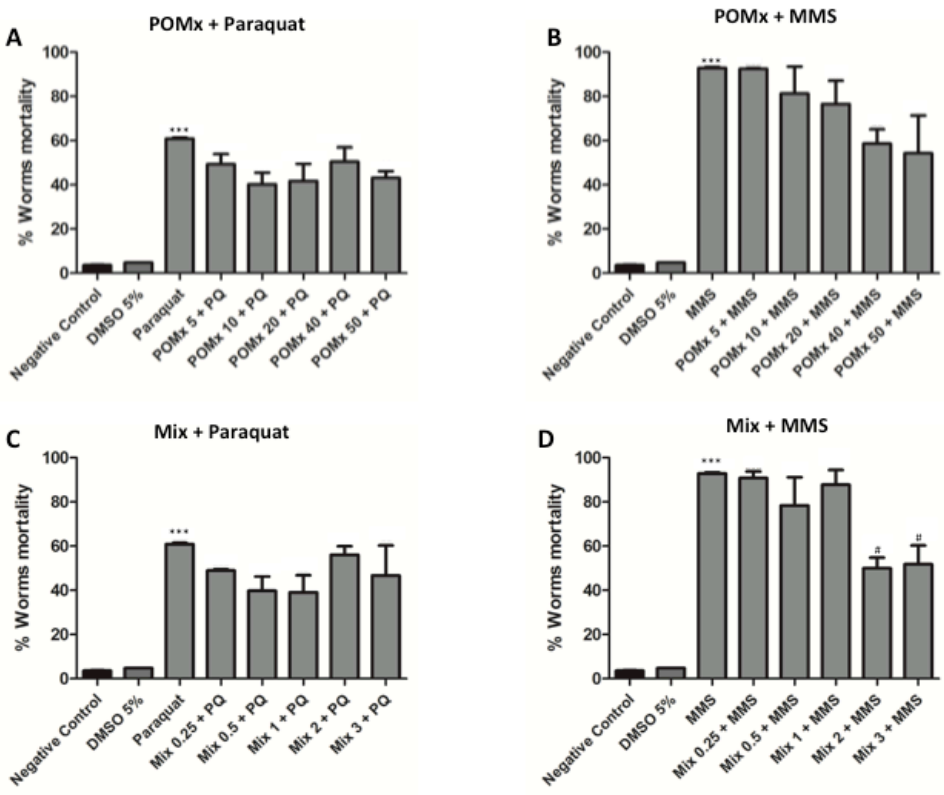

Figure 2. *C. elegans* mortality after 48 hours of exposure to pomegranate extract (POMx) and antioxidants mixture (Mix) followed by paraquat (PQ) and methyl methanesulfonate (MMS). Concentrations of groups: POMx (5, 10, 20, 40 and 50 mg/mL), Mix (0.25, 0.5, 1, 2 and 3 doses), PQ (0.5 mM) and MMS (1 μM). **(A)** POMx + Paraquat. **(B)** POMx + MMS. **(C)** Mix + Paraquat. **(D)** Mix + MMS. Values are expressed as mean ± SEM (n=3 independent experiments performed in duplicate). Statistical comparisons were made using ANOVA/Bonferroni post-hoc test (*p< 0.05, **p<0.01, ***p<0.001 vs. control group; ^{ø}p< 0.05, ^{øø}p<0.01, ^{øøø}p<0.001 vs. PQ; $^{\#}p$< 0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. MMS).

3.3. Antioxidants mixture, but not pomegranate extract (POMx), protects nematodes from docetaxel and carboplatin toxicity

As demonstrated in Figure 3, 0.05% Tween 80 or 5% DMSO did not affect nematodes mortality. Docetaxel, in both concentrations, leads to a significant increase in worms mortality compared to 0.05% Tween 80 (p<0.01; Figure 3AC). Pre-incubation with POMx 40 was not able to protect the worms

against mortality induced by docetaxel (Figure 3A), however pre-incubation with Mix 2 caused a significant decrease in mortality induced by both docetaxel concentrations (p<0.05; Figure 3C).

Carboplatin, in both concentrations, caused a significant increase in worms mortality compared to 0.05% Tween 80 (p<0.01; Figure 3BD). Pre-incubation with POMx 40 was not able to protect the worms against mortality induced by carboplatin (Figure 3B), however pre-incubation with Mix 2 showed a significant decrease in mortality induced by C2 (p<0.05), but not C1 (Figure 3D).

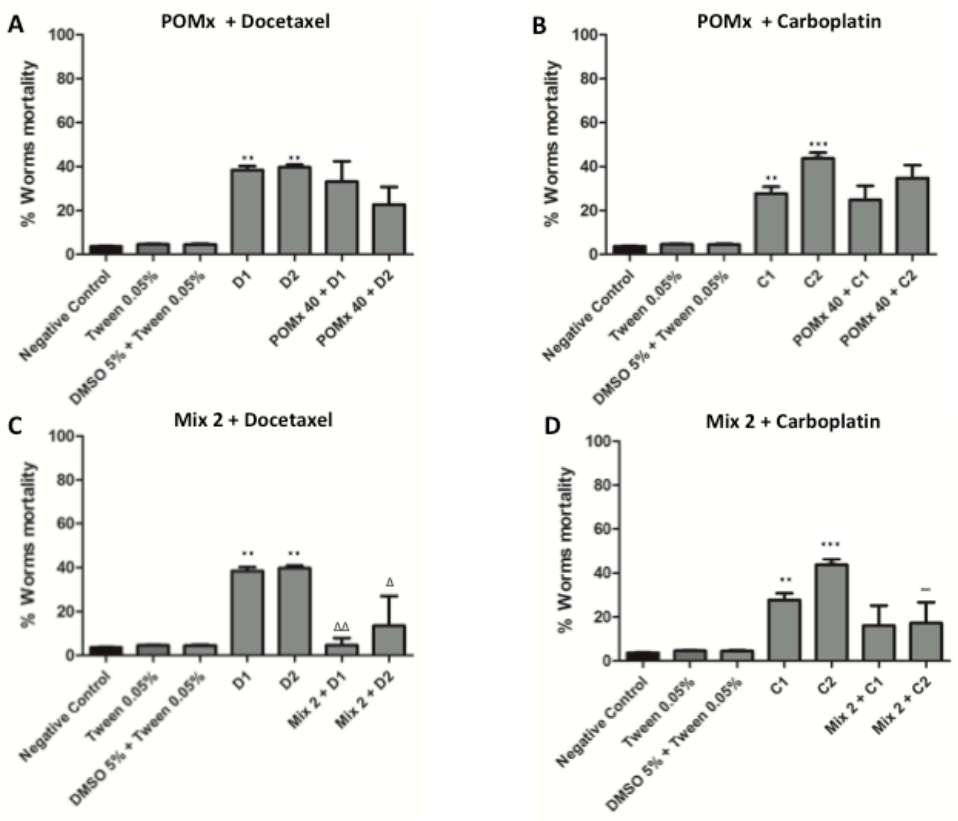

Figure 3. *C. elegans* mortality after 48 hours of exposure to pomegranate extract 40 mg/mL (POMx 40) and antioxidants mixture 2 doses (Mix 2) followed by docetaxel (D1 and D2) and carboplatin (C1 and C2). **(A)** POMx 40 mg/mL + Docetaxel; **(B)** POMx 40 mg/mL + Carboplatin; **(C)** Mix 2 + Docetaxel; **(D)** Mix 2 + Carboplatin. Concentrations of groups: D1 (0.2 mg/mL) and D2 (0.1 mg/mL); C1 (99.4 μg/mL) and C2 (49.7 μg/mL). Values are expressed as mean ± SEM (n=3 independent experiments performed in duplicate). Statistical comparisons were made using ANOVA/Bonferroni post-

hoc test (*p< 0.05, **p<0.01, ***p<0.001 vs. control group; $^{\Delta}p$< 0.05, $^{\Delta\Delta}p$<0.01, $^{\Delta\Delta\Delta}p$<0.001 vs. docetaxel; $^{\infty}p$< 0.05, $^{\infty\infty}p$<0.01, $^{\infty\infty\infty}p$<0.001 vs. carboplatin).

3.4. *Caenorhabditis elegans* body area is not affected by pomegranate extract (POMx) or antioxidants

Regarding to body area, 5% DMSO caused a significant decrease when compared to negative control group for both POMx and Mix (p<0.01), according to Figure 4. No significant differences were observed for tested concentrations of POMx (Figure 4A) and Mix (Figure 4B) when compared to 5% DMSO.

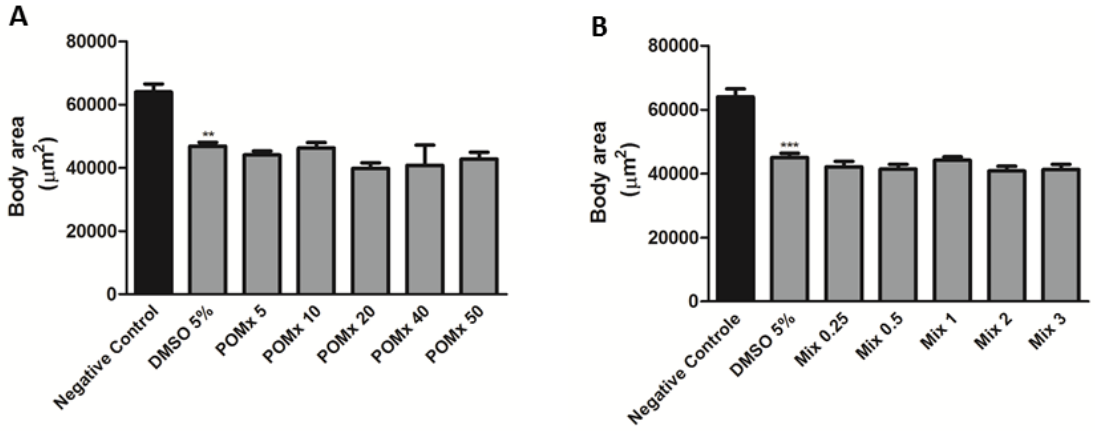

Figure 4. Body areas of *C. elegans* after 24 hours of exposure with pomegranate extract (POMx) **(A)** and antioxidants mixtures (Mix) **(B)** in different concentrations. Values are expressed as means ± SEM from three independent experiments (n=3). Statistical comparisons were made using ANOVA/Bonferroni post-hoc test (*p< 0.05, **p<0.01, ***p<0.001 vs. control group).

3.5. Pomegranate extract (POMx) or antioxidants were not able to revert PQ or MMS effect in body area

In Figure 5, it is possible to observe that DMSO 5% showed significant difference when compared to negative control (p<0.01). PQ (Figure 5AC) and MMS (Figure 5BD) reduced worms body area compared to 5% DMSO (p<0.001). Neither POMx nor Mix pre-incubations reverted the PQ or MMS decreased body area.

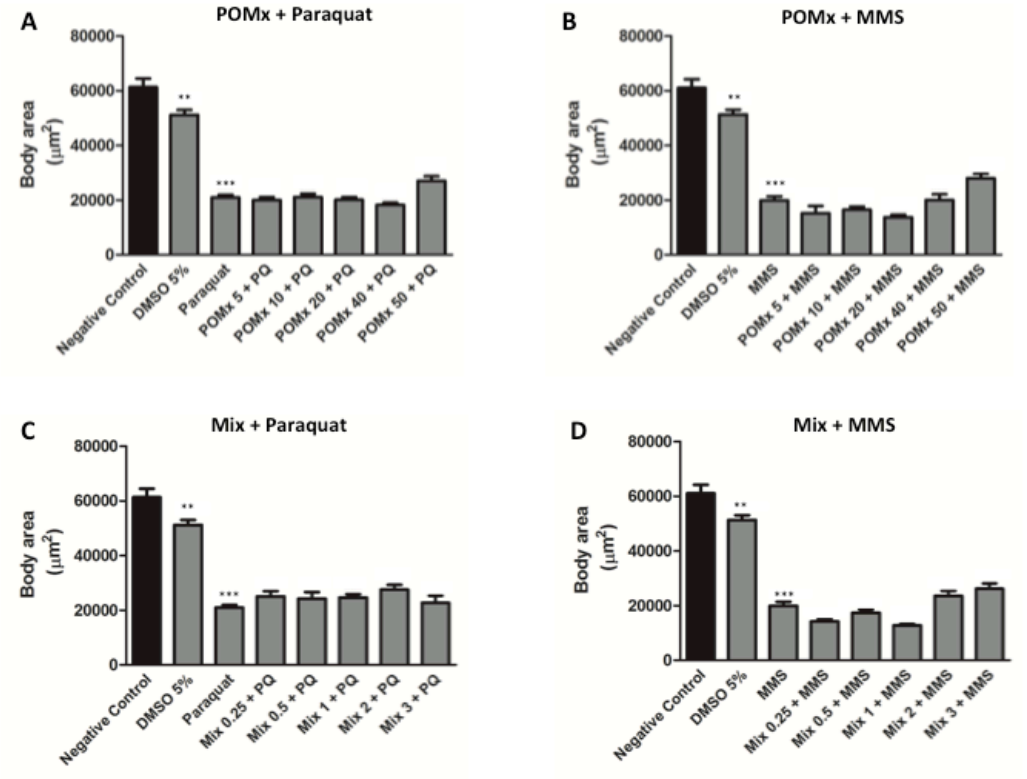

Figure 5. Body areas of *C. elegans* after 48 hours of exposure to pomegranate extract (POMx) and antioxidants mixture (Mix) followed by paraquat (PQ) and methyl methanesulfonate (MMS). Concentrations of groups: POMx (5, 10, 20, 40 and 50 mg/mL), Mix (0.25, 0.5, 1, 2 and 3 doses), PQ (0.5 mM) and MMS (1 μM). **(A)** POMx + Paraquat. **(B)** POMx + MMS. **(C)** Mix + Paraquat. **(D)** Mix + MMS. Values are expressed as mean ± SEM (n=3 independent experiments performed in duplicate). Statistical comparisons were made using ANOVA/Bonferroni post-hoc test (*p< 0.05, **p<0.01, ***p<0.001 vs. control group; ^{ø}p< 0.05, ^{øø}p<0.01, ^{øøø}p<0.001 vs. PQ; $^{\#}p$< 0.05, $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 vs. MMS).

3.6. Body area of *C. elegans* after 48 h exposure to pomegranate extract 40 mg/mL (POMx 40) and antioxidants mixture 2 doses (Mix 2) followed by docetaxel and carboplatin

Figure 6 shows that *C. elegans* body area was significantly reduced by 5% DMSO + 0.05% Tween 80 incubation when compared to negative control (p<0.05; ANOVA/Bonferroni). Both concentrations of docetaxel (Figure 6AC)

and carboplatin (Figure 6BD) significantly reduced worms area when compared to tween 0.05% group (p<0.001; ANOVA/Bonferroni). Neither POMx 40 nor Mix 2 were able to revert the developmental toxicity induced by the chemotherapeutics.

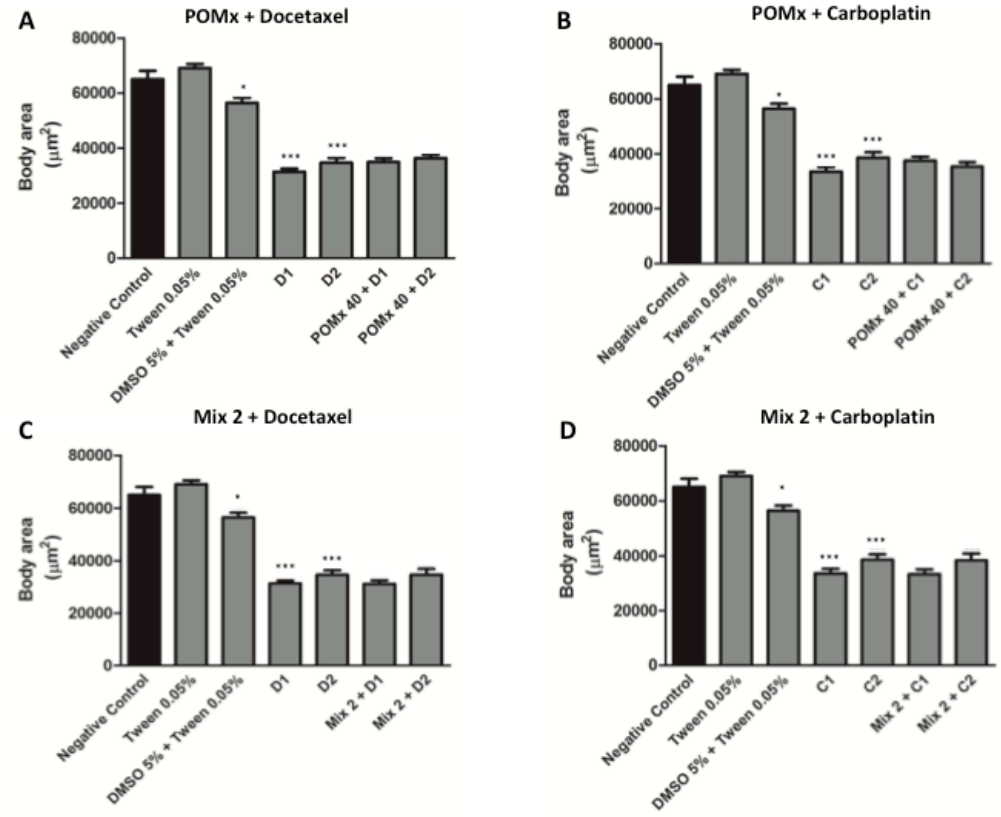

Figure 6. Body areas of *C. elegans* after 48 hours of exposure to pomegranate extract 40 mg/ mL (POMx 40) and antioxidants mixture 2 doses (Mix 2) followed by docetaxel (D1 and D2) and carboplatin (C1 and C2). **(A)** POMx 40 mg/mL + Docetaxel; **(B**) POMx 40 mg/mL + Carboplatin; **(C)** Mix 2 + Docetaxel; **(D)** Mix 2 + Carboplatin. Concentrations group: D1 (0.2 mg/mL) and D2 (0.1 mg/mL); C1 (99.4 μg/mL) and C2 (49.7 μg/mL). Values are expressed as mean ± SEM (n=3 independent experiments performed in duplicate). Statistical comparisons were made using ANOVA/Bonferroni post-hoc test (*p< 0,05; **p<0.01; ***p<0.001 vs. control group).

3.7. ROS production

With regard to ROS production evaluation, 0.05% tween 80 and 5% DMSO + 0.05% tween 80 caused a significant increase in ROS levels when

compared to control (p<0.05; Figure 7). In Figure 7A, it is possible to observe that D1 had a significant difference compared to Tween 0.05% (p<0.05) and POMx 40 + D2 to DMSO 5% + Tween 0.05% (p<0.05). Figure 7B, shows that C1 has a significant difference when compared to Tween 0.05% (p<0.05) and POMx 40 + C2 when compared to DMSO 5% + Tween 0.05% (p<0.05). POMx 40 + D1 when compared to D1 did not show any significant difference, for POMx 40 + D2 also did not compared to D2, as seen in Figure 7A. POMx + C1 and POMx + C2 compared to C1 and C2, respectively, did not present significant differences (Figure 7B).

For the Mix 2, D1 showed significant difference when compared to tween 0.05% and did when compared with C1 (p<0.05), as seen in Figures 7C and 7D, respectively. In addition, Figure 7C shows that Mix 2 + D1 and Mix 2 + D2 had a significant difference when compared to DMSO 5% + Tween 0.05% (p<0.05). Figure 7D, Mix 2 + C1 and Mix 2 + C2 showed significant difference when compared to DMSO 5% + Tween 0.05% l (p<0.05). Mix 2 + D1 and Mix 2 + D2 did not show significant differences compared to D1 and D2, respectively (Figure 7C). In figure 7D, Mix 2 + C1 compared to C1 had a significant difference (p<0.01;), however Mix 2 + C2 did not when significant difference when compared to C2.

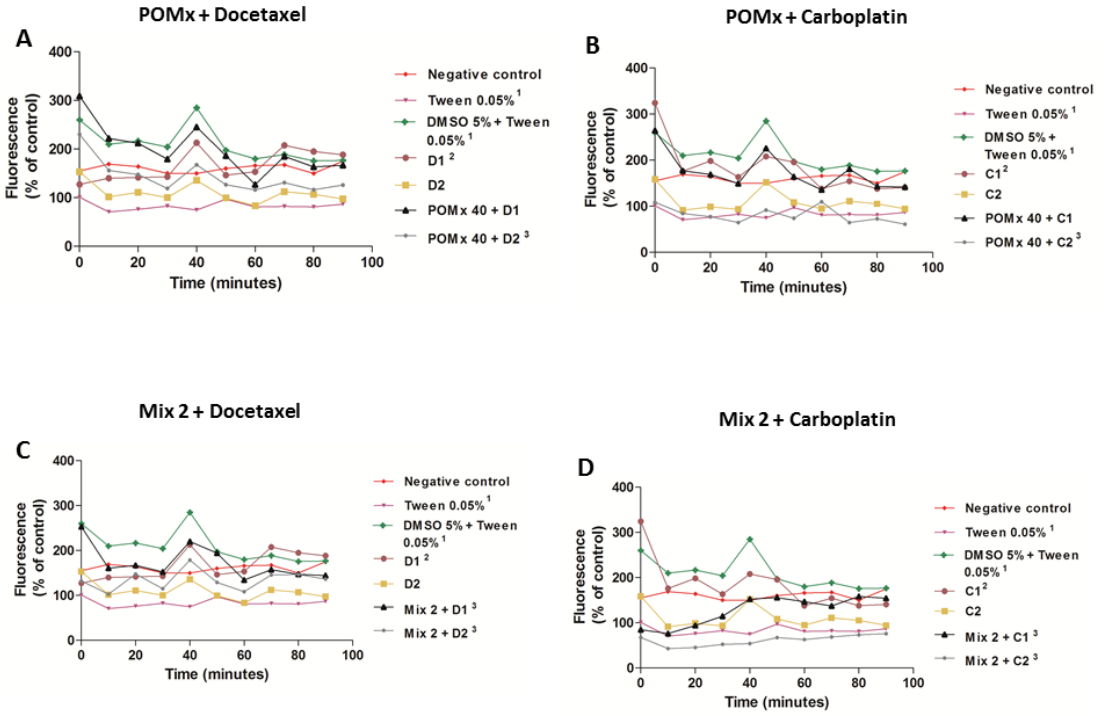

Figure 7. ROS levels measure by DCF-DA: **(A)** POMx 40 mg/mL + Docetaxel; **(B)** POMx 40 mg/mL + Carboplatin; **(C)** Mix 2 + Docetaxel; **(D)** Mix 2 + Carboplatin. D1 (0.2 mg/mL) and D2 (0.1 mg/mL); C1 (99.4 μg/mL) and C2 (49.7 μg/mL). Data are expressed as mean ± SEM (n=3 independent experiments performed in duplicate). Statistical comparisons were made using repeated measures ANOVA/Bonferroni post-hoc test ([1] $p<0.05$ vs. negative control; [2] $p<0.05$ vs. Tween 0.05%; [3] $p<0.05$ vs. DMSO 5% + Tween 0.05%).

4. Discussion

The intake of antioxidants including α-lipoic acid, curcumin, vitamin C and coenzyme Q10 by breast cancer patients to alleviate the symptoms of chemotherapy treatment is common. However, their benefic effects on harmful effects caused by chemotherapeutic agents is not known. The aim of this study was to evaluate whether the antioxidants mixture (α-lipoic acid, curcumin, vitamin C and coenzyme Q10) and pomegranate extract are able to minimize the toxicity of chemotherapeutic agents docetaxel and carboplatin, using the alternative *in vivo* model *C. elegans*.

The worms are a good option to *in vivo* assays, since its genome has been already mapped and its signaling pathways and genes are well conserved comparing to humans (Hunt et al, 2017). Although worms are used as a model for many antioxidants studies, for our knowledge, this was the first study to use the antioxidants in combination with docetaxel and carboplatin.

Pomegranate extract (POMx) has been used for a long time to treat some diseases, going from diabetes to cancer and the tanins and anthocyanines content is known by their anti-inflammatory action and antioxidant power (Sharma et al., 2017). Our findings showed that POMx concentrations increased *C. elegans* mortality. A recent study showed that *C. elegans* longevity was impaired when the worms are exposed to higher doses of POMx, but was improved when lower doses were used, showing that toxicity and benefics effects is dose dependent (Kiling et al., 2015). Furthermore, it is known that the IC_{50} of POMx in C4-2 cell line (prostate cancer cell) is 42 *μ*g/mL and our finding was approximately a thousand times higher, an expected comparison since the assays were made *in vivo*, in a much more complex system (Wang et al, 2014).

Similarly to POMx, the tested concentrations of antioxidants Mix also showed significant differences when compared to control groups. The lethal dose was found in approximately 3 doses. Curcumin concentration in the LD_{50} was higher than 200 *μ*M. A previous study showed that worms exposed to 200 *μ*M did not have an increased lifespan, fact that could be associated with the result found here, because if that concentration was lethal to 50% of the nematodes, it would not be able to prolong their lives (Liao et al, 2011).

According to Ishii et al. (2004), 150 *μ*g/mL of coenzyme Q10 prolonged *C. elegans* lifespan, therefore the concentrations used to treat the worms were between that. Lifespan in model of *Drosophila melanogaster* was 0.005% concentration of α-lipoic acid, causing a high rate mortality (Bauer et al, 2004). In relation to the lethal dose of 50% (LD_{50}), there was more than that of the antioxidant, contributing to our findings. Vitamin C has been used to help minimize the damage caused by TiO_2 and ZnO nanoparticles in *C. elegans*

and it lowered the toxicity of those compounds when used at 140 μM (Sonane et al, 2007). The dosage that represented the LD_{50} had a higher concentration.

Paraquat and MMS were used to test the protective effect of the antioxidants. The main toxicity mechanism of paraquat is by altering the redox cycle, causing a higher production of ROS (Charão et al, 2015). MMS is a cytotoxic agent that acts directly in the DNA (Qureshi et al, 1989). Both agents caused a significant mortality when compared to control group in the present study. The concentration of POMx tested did not reduce the mortality caused by paraquat. Mix 2 and 3 doses helped to reduce the mortality caused by MMS. Since the chemotherapeutics drugs used in this study acts on DNA level of the cancer cells, we decided to use the concentrations of Mix that had a better outcome in MMS, being that Mix 2.

Docetaxel and carboplatin alone caused a significant mortality when compared to control group. The worms that were pre-treated with POMx did not have a significant difference of mortality when compared the worms that only received chemotherapeutics agents. Previous study showed that POMx when combined with docetaxel can help to enhance the apoptosis of prostate cancer cell lines, C4-2, PC3 and $ARCaP_M$ (Wang et al, 2013). The concentration of POMx used on that study was similar to the one tested herein, but the docetaxel concentration was lower. Since it is an *in vivo* study, the chemotherapeutic agent dose is assumed to be lower when compared to an *in vitro* study.

However, Mix 2 showed a significant difference in both docetaxel concentrations and in the lower carboplatin one. It was presumed that the mixture would be better than the POMx, due to the fact that antioxidants mixtures when combined to apoptotic agents enhance apoptosis in cancer cells, without causing the death of normal cells (Pathak et al, 2013). Some antioxidants vitamins, when used together, have a better outcome than when used alone, being able to use a lower dose of those to affect the growth of cancer cell (Cole et al, 1997). There is evidence that mixture of vitamins used in human cancer lung H520 cells could cause a synergic effect with paclitaxel

and carboplatin, helping the treatment by raising cancer cell apoptosis (Pathak et al, 2013). Although that study used paclitaxel, it can be assumed that it would help when used docetaxel, since both drugs have similar mechanisms.

In the ROS production assay, carboplatin and docetaxel were oxidants, result that was expected since such chemotherapeutics agents are known to increase the ROS production (Conklin, 2004). Analyzing the results, Mix showed better outcome than POMx. Since it is a mixture, it probably works on more than only one mechanism in the worms, helping to minimize toxicity. This study showed that using antioxidants mixtures along cancer treatment seems to be a good option, but more studies are needed, being a great way to help breast cancer patients to stay on chemotherapy. Since there is no studies that used docetaxel and carboplatin in *C. elegans*, to further assays, it should be used lower concentrations of the drugs and also of the antioxidants, because maybe the concentrations tested in this study were high, being extremely toxic and the antioxidants were not able to help.

5. Conclusion

Considering that breast cancer is the main type of cancer among women worldwide and, because of this, it is of extreme importance to find ways to make the treatment easier for those patients. Our study showed that antioxidant mixture (Mix) had better results than pomegranate extract (POMx), since it decreased mortality of *C. elegans* caused by docetaxel and by the lower dose of carboplatin, proving that the use of a combination of antioxidants is better than using only one.

Funding

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Declaration of Interest

All authors declare that there are no conflicts of interest.

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Beers, S. R., & De Bellis, M. D. (2002). Neuropsychological function in children with maltreatment-related posttraumatic stress disorder. *The American Journal of Psychiatry, 159*, 483–486. doi: 10.1176/appi.ajp.159.3.483

Ramus, F., Rosen, S., Dakin, S. C., Day, B. L., Castellote, J. M., White, S., & Frith, U. (2003). Theories of developmental dyslexia: Insights from a multiple case study of dyslexic adults. *Brain, 126*(4), 841–865. doi:

10.1093/brain/awg076

Example of reference with more than 7 authors

Rutter, M., Caspi, A., Fergusson, D., Horwood, L. J., Goodman, R., Maughan, B., … Carroll, J. (2004). Sex differences in developmental reading disability: New findings from 4 epidemiological studies. *Journal of the American Medical Association, 291*(16), 2007–2012. doi: 10.1001/jama.291.16.2007

Example of other references

van Bergen, E., de Jong, P. F., Maassen, B., Krikhaar, E., Plakas, A., & van der Leij, A. (in press). IQ of four-year-olds who go on to develop dyslexia. *Journal of Learning Disabilities*. doi: 10.1177/0022219413479673

Howell, K. W., Fox, S. L., & Morehead, K. W. (1993). *Curriculum-based evaluation: Teaching and decision making* (2nd ed.). Pacific Grove, CA: Brooks/Cole Publishing Company.

Fan, K. Y. (1986, September). *Graphic symbol of the Chinese character*. Paper presented at the meeting of the Symposium of Chinese Character Modernization, Beijing, China.

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