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Evaluation of the cytotoxic and mutagenic potential of *Rosmarinus officinalis* L. essential oil through the *Allium cepa* Leach bioassay

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ABSTRACT. The species *Rosmarinus officinalis* L. (rosemary) is an herb from the Lamiaceae family, widely used in cooking as a food preservative, seasoning or condiment. It also stands out for its therapeutic properties, mainly presenting antioxidant, antibacterial and antitumor activity. The aim of this study was to evaluate the cytotoxic and mutagenic activity of *R. officinalis* L. essential oil through the *Allium cepa* bioassay. This test constitutes an excellent plant model routinely used due to its sensitivity, low cost and good correlation with test systems in mammals. The defined concentrations for carrying out the test were 750, 243, 81 and 27 µg mL^{-1}. Five bulbs were used, 4 roots of each, measuring approximately 2 cm, and they were analyzed on two slides. All assays were performed at least in triplicate and compared to the negative control. The statistical test of analysis of variance (ANOVA with a fixed factor) was used, followed by Tukey's multiple comparisons test, for $p < 0.05$. For this purpose, the GraphPad Prism program (version 6.0) was used. The results showed a cytotoxic and mutagenic effect for all concentrations used of the essential oil of *R. officinalis* L. However, it is important to conduct further research using other genotoxicological tests with different endpoints and at different concentrations, in order to clarify the interaction of the essential oil of the species *R. officinalis* L. with the genetic material of the cell and its possible mechanism of action.

Keywords: *Allium cepa*; cytotoxicity; genotoxicity; mutagenesis.

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Introduction

The use of plants by humanity, from ancient times to the present day, for therapeutic purposes, has been a frequent and culturally stimulated practice. Due to the easy access, the use of medicinal plants has been revealed in different populations around the world, with the aim of improving people's quality of life, as well as maintaining health (Castro et al., 2020). However, plants produce chemical substances that can be beneficial or toxic (Frota, Amorim, Carneiro, & Oliveira, 2019). Its effectiveness and safety are validated through ethnopharmacological surveys, scientific documentation or clinical evidence. However, of the 200,000 species of native plants in Brazil, it is estimated that half have some therapeutic purpose and that only 1% of species with such potential have been studied (Vieira, Andrade, Seixas, Medeiros, & Carneiro, 2016). Most of them lack scientific studies regarding their compounds with cytotoxic, genotoxic or mutagenic effects, which can cause harm to human health (Frota et al., 2019).

The species *Rosmarinus officinalis* L., commonly known as rosemary and belonging to the Lamiaceae family, is considered a perennial and popular herb, originated in Mediterranean regions (Zaouali, Messouad, & Boussaid, 2003; Zegura, Dobnik, Niderl, & Filipič, 2011; Machado et al., 2013). This species is a plant cultivated worldwide, known for its nutritional value and pharmacological properties (Borges, Ortiz, Pereira, Keita, & Carvalho, 2018). In cooking, the oil of this species is used as a food preservative and as a seasoning or condiment (Zegura et al., 2011; Wang, Li, Luo, Zu, & Efferth, 2012). In medicine, *R. officinalis* L. is highly appreciated for its therapeutic properties, highlighting its antioxidant (Ozarowski et al., 2013), antibacterial (Rakover, Ben-Arye, & Goldstein, 2008) and antitumor (Ngo, Williams, & Head, 2011) activity. Its action has

also been reported as analgesic, antirheumatic, diuretic, antiepileptic (Nogueira de Melo et al., 2011) and expectorant (Takaki et al., 2008).

Rosmarinus officinalis L. essential oil is used as a biological preservative in food products (Ojeda-Sana, Van Baren, Elechosa, Juarez, & Moreno, 2013) due to its antioxidant and antimicrobial properties (Bozin, Mimica-Dukic, Samojlik, & Jovin, 2007). Essential oils represent a 'green' alternative for use in cosmetics, pharmaceuticals, agriculture and food, replacing chemical treatments (Jardak, Elloumi-Mseddi, Aifa, & Mnif, 2017). Despite the numerous therapeutic benefits reported for the species *R. officinalis* L., the cytotoxic and mutagenic effects of the total essential oil of this species are little known.

Cytogenotoxic effects produced by substances in natural products, especially plant extracts and their derivatives, can be evaluated through bioassays (Verri, Moura, & Moura, 2017), which are considered appropriately sensitive and simple in monitoring the toxic effects at the cellular level of chemical compounds (Lima, Guedes, Abreu, & Peron, 2018). Among them, *Allium cepa* L. root meristems are considered, in scientific circles, an efficient test for the initial screening of the genetic toxicity of chemical compounds (Parvan et al., 2020), due to their reduced chromosome number, which favors the detection of aneugenic or clastogenic alterations, and disturbances in the cell proliferation index (Lima et al., 2018). The *A. cepa* bioassay is a test system internationally accepted by research agencies as an accurate sensitivity assessment instrument for the analysis of cytogenotoxicity of substances of economic and/or social interest, such as, plant extracts (Terceiro & Oliveira, 2020). The results obtained through it show good correlations with other tests (Parvan et al., 2020), including in mammals (Fiskesjö, 1994). Therefore, this work aimed to evaluate the cytotoxic and mutagenic potential of *R. officinalis* L. essential oil through the *A. cepa* Leach bioassay.

Material and methods

Obtaining botanical material and concentrations used

The essential oil of *R. officinalis* L. (Sigma-Aldrich Brasil Ltda, CAS No: 8000-25-7) was made available by the Nucleus for Research in Medicinal Plants (NPPM) of Health Sciences Center at the *Universidade Federal do Piauí.*

The concentrations of 750, 243, 81 and 27 μg mL^{-1}, used in this research, were established through a previous study, where the DL50 acute toxicity test was performed (unpublished data).

Allium cepa bioassay

The *A. cepa* test was performed according to the protocol described by Guerra and Sousa (2002), with some modifications. Initially, the bulbs were grown in distilled water for 48 hours, at 25°C and constant aeration. Then, in order to analyze each concentration, five bulbs were used, 4 roots of each, measuring approximately 2 cm, and they were analyzed on two slides. After 24 hours of treatment, the roots were collected and fixed in *Carnoy* solution also for 24 hours. Subsequently, the roots were washed, followed by hydrolysis in 1N HCL for 5 min. to prepare the slides, which were then stained with 1% acetic orcein and analyzed under an optical microscope at a magnification of 400x, for the detection of chromosomal alterations and in the cell cycle. A total of 1,000 cells were counted in each of the 5 treated bulbs for each concentration tested to analyze the cytotoxic effects, at microscopic level. The cytotoxicity of *R. officinalis* L. essential oil was evaluated by determining the mitotic index (MI), which is used as a cell proliferation biomarker by measuring the proportion of cells dividing in the different phases of the cycle (Mercado & Caleño, 2020). The mitotic index (MI) was obtained using the following formula: MI = [(number of dividing cells/total analyzed cells) x 100]. For the counting of chromosomal aberrations (CA), chromosomal damage was quantified. The main chromosomal aberrations to be considered for analysis were nuclear sprouts, delays, fragments and bridges. Micronuclei (MN) were quantified in order to analyze the mutagenic effect. MN analysis makes it possible to identify any increase in the frequency of mutations in cells exposed to genotoxic agents (Mororo, Melo, Salazar, Leal, & Costa, 2019). As MN, the analysis of chromosomal alterations serves as a mutagenicity test (Bagatini, Silva, & Tedesco, 2007) and allows the evaluation of clastogenic and aneugenic actions (Leme & Marin-Morales, 2009). All assays were performed at least in triplicate and compared to the negative control.

Statistical analysis

The statistical test of analysis of variance (ANOVA with a fixed factor) was used, followed by Tukey's multiple comparisons test, for $p < 0.05$. For this purpose, the GraphPad Prism program (version 6.0) was used.

Results

The number of interphase cells for all tested concentrations of *R. officinalis* L. essential oil was greater than the number observed in the negative control (distilled water), with a consequent decrease in the number of dividing cells (Table 1). The concentration of 750 µg mL-1 showed the highest number of interphase cells, therefore, the lowest number of dividing cells. However, the concentrations of 243, 81 and 27 µg mL-1, despite also showing significant differences in relation to the negative control and the highest concentration tested, did not show statistically significant differences between them. Thus, it is not possible to establish a dose-response curve (dose-dependent effect) of *R. officinalis* L. essential oil for this parameter.

Regarding the mitotic index values, it is also observed a notable reduction of this parameter in the order of approximately 1000x for all tested concentrations of *R. officinalis* L. oil in relation to the negative control, thus presenting a strong cytotoxic activity of the oil essential species of *R. officinalis* L (Table 1). There was no significant difference between the concentrations tested, so that a dose-dependent response could not be established in this case as well.

Table 1. Cytogenetic effects of *Rosmarinus officinalis* L. essential oil infusion in *Allium cepa* cells.

Concentrations µg mL^{-1}	Total analyzed cells	Interphase	Dividing cells	IM (%)	AC	MN
Control	5,000	2,280	2,720	54.4	1	0
750	5,000	4,746*	254*	5.08*	136*	0
243	5,000	4,613$^{*\alpha}$	387$^{*\alpha}$	7.74*	233$^{\alpha *}$	0
81	5,000	4,610$^{*\alpha}$	390$^{*\alpha}$	7.80*	155$^{*\beta\delta}$	0
27	5,000	4,593$^{*\alpha}$	407$^{*\alpha}$	8.14*	151$^{*\delta}$	0

$^{*}p < 0.05$ for concentrations of 750, 243, 81 and 27 µg mL^{-1} in relation to the negative control. $^{\alpha}p \leq 0.01$ and $^{\beta}0.01 < p < 0.05$: significant when the concentrations are 243 for 750 µg mL^{-1}, 81 for 750 µg mL^{-1}, 27 to 750 µg mL^{-1} with $p < 1\%$ at these concentrations. However, for chromosomal aberrations, with the change in concentration from 243 to 750 µg mL^{-1} there was a significant difference at a level lower than 1%, whereas at the concentration from 81 to 750 µg mL^{-1} the changes were significant between 1 and 5%, and from the concentration of 27 to 750 µg mL^{-1} did not present significance. $^{\delta}p \leq 0.01$: was not significant when the concentrations are 243 for 81 µg mL^{-1} and 243 for 27 µg mL^{-1}, as well as cells in division and mitotic index in those concentrations. However, for chromosomal aberrations, with the change in concentrations, there was a significant change at a level lower than 1%. MI – mitotic index; AC – Chromosomal aberrations; MN – micronucleus.

Positive results for genotoxicity parameters are demonstrated by the significant increase in the number of chromosomal aberrations, in relation to the negative control, obtained for all concentrations of *R. officinalis* L. oil used (Table 1). Values increased from 1 (negative control) to 136 chromosomal aberrations at a concentration of 750 µg mL^{-1} and from 1 (negative control) to 233 chromosomal aberrations at a concentration of 243 µg mL^{-1}. For the concentrations of 81 and 27 µg mL^{-1}, although the number of chromosomal aberrations were similar to each other, they were still approximately 150 times greater than the negative control (Table 1). The presence of micronuclei was not verified.

In microscopic analysis, a wide spectrum of chromosomal aberrations was observed for all concentrations of *R. officinalis* L. oil used. It was identified from chromosomal delays and chromosome fragments, to loose chromosomes and chromosome bridges, as shown in the figures for concentrations of 750 µg mL^{-1} (Figure 1), 243 µg mL^{-1} (Figure 2), 81 µg mL^{-1} (Figure 3) and 27 µg mL^{-1} (Figure 4).

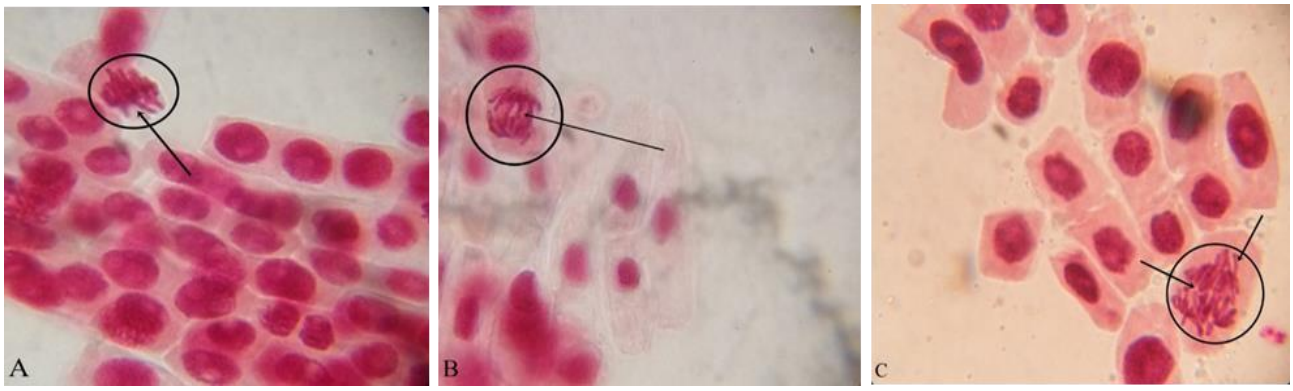

Figure 1. Chromosomal aberrations found after treatment of *Allium cepa* roots with a concentration of 750 µg mL^{-1} of *Rosmarinus officinalis* essential oil. A: Chromosomal fragment; B: Chromosomal bridge; C: Chromosomal delay and loose chromosomes.

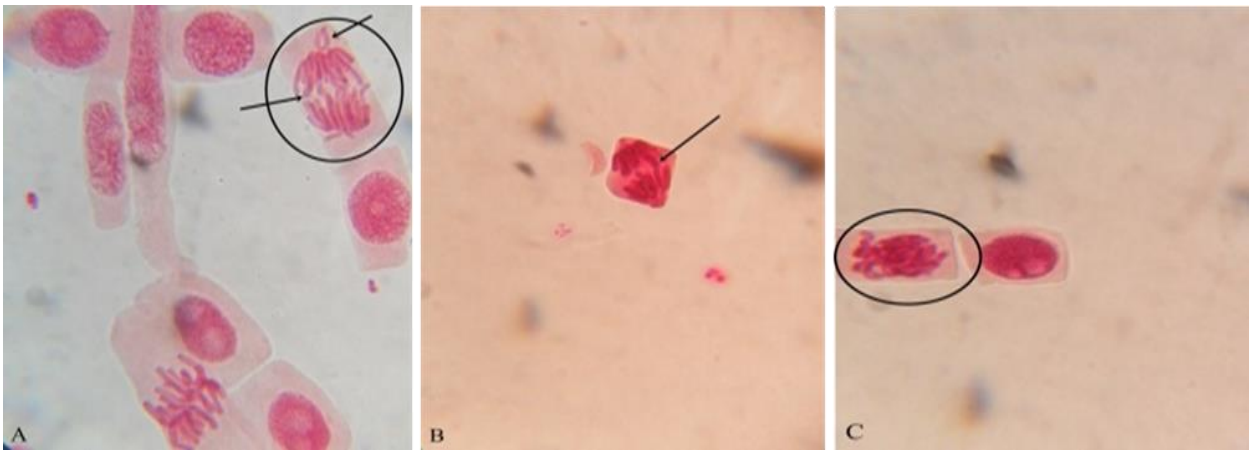

Figure 2. Chromosomal aberrations found after treatment of *Allium cepa* root cells with a concentration of 243 µg mL^{-1} of *Rosmarinus officinalis* essential oil. A: Chromosomal delay, loose chromosomes and a normal metaphase; B: Chromosomal bridge; C: Chromosomal fragment.

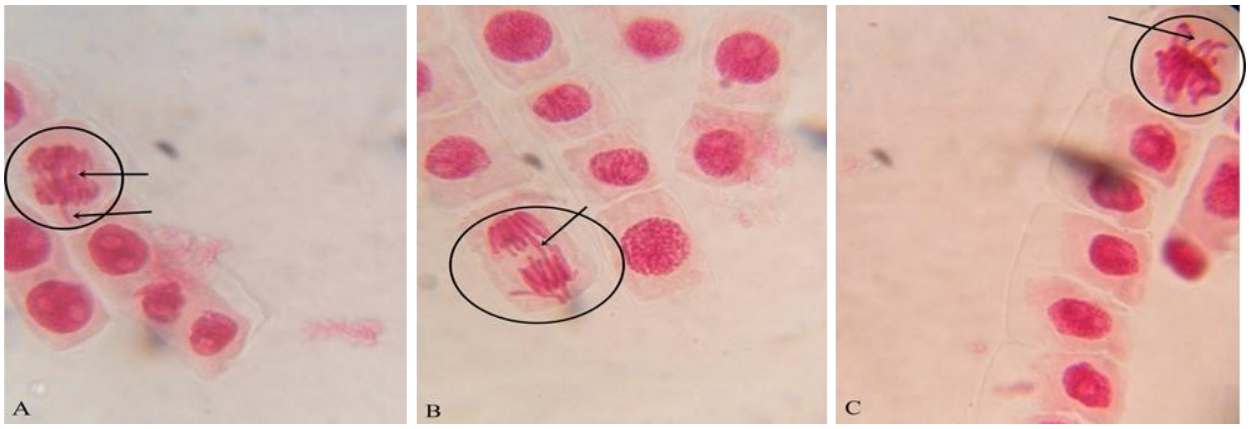

Figure 3. Chromosomal aberrations found after treatment of *Allium cepa* root cells with the concentration for the concentration of 81 µg mL^{-1} of *Rosmarinus officinalis* essential oil. A: Lag and loose chromosomes; B: Chromosomal bridge and loose chromosomes; C: Chromosomal fragment.

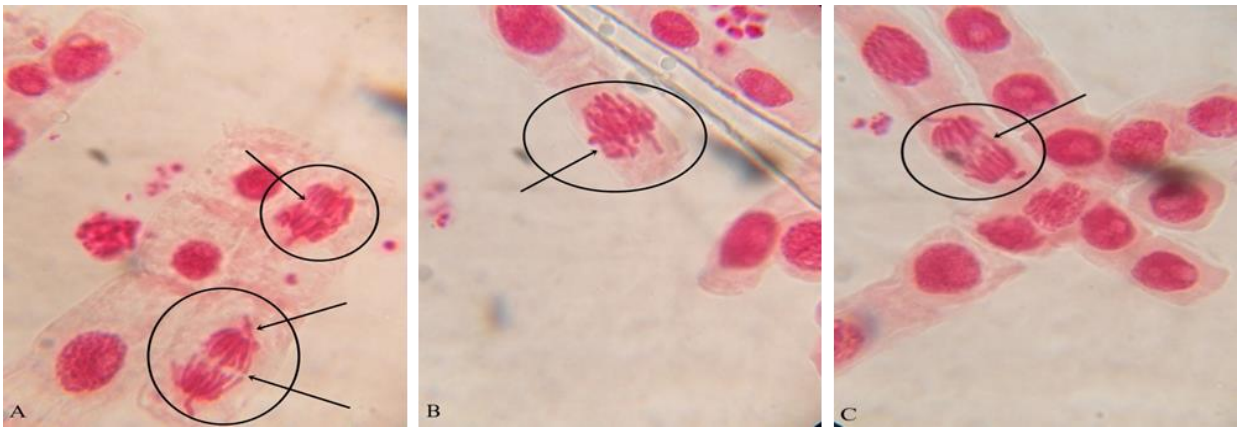

Figure 4. Chromosomal aberrations found after treatment of *Allium cepa* root cells with a concentration of 27 µg mL^{-1} of *Rosmarinus officinalis* essential oil. A: Chromosomal delay and loose chromosomes; B: Chromosomal fragment; C: Chromosomal bridge.

Discussion

Rosmarinus officinalis L. is a shrub that is widely scattered throughout the world. In recent decades, experimental research has confirmed the pharmacological potential of the species and of some of its primary compounds, also expanding the range of its possible therapeutic applications (Pérez-Sánchez et al., 2018). However, in this work it was observed that the oil of *R. officinalis* L. had a strong cytotoxic and mutagenic effect, since it remarkably decreased the mitotic index values and significantly increased the number of chromosomal aberrations, respectively, at all concentrations tested through the *A. cepa* bioassay.

The cytotoxic and mutagenic effects produced by substances present in natural products, mainly plant extracts and their derivatives, can be evaluated through different bioassays due to the considerable sensitivity and reliability of the results (Verri et al., 2017). The *A. cepa* bioassay used is the oldest test reported in the literature for the detection of cytotoxicity, genotoxicity (El-Shahaby, Abdel Migid, Soliman, & Mashaly, 2003) and mutagenicity (Seth, Misra, Chauhan, & Singh, 2008). This test is capable of simultaneously evaluate several parameters, such as the number of interphase and dividing cells, the mitotic index, the presence of chromosomal aberrations and micronuclei.

As the results obtained in this work show a reduction in MI for all concentrations of *R. officinalis* L. essential oil used, when compared to the negative control, it is suggested that there may be an inhibition of the cell cycle, thus preventing the entry of these cells into mitosis, with a possible arrest in interphase. This mechanism is proposed by Gömurgen (2005) and Sudhakar, Gowda and Venu (2001) who state that the possible cause of MI reduction indicates inhibition of DNA synthesis or blockage in the G2 phase of the cell cycle, preventing the cell from entering into mitosis.

The cytotoxicity found here for *R. officinalis* L. essential oil in the *A. cepa* test, however, was not dose-dependent. Wang et al. (2012), when evaluating three human tumor cell lines (SK-OV-3, HO-8910 and Bel-7402) exposed to increasing concentrations (0.0625% v v^{-1}, 1% v v^{-1}, 50 % v v^{-1}) of *R. officinalis* L. essential oil and three of its main isolated components (1.8 - cineole, α - pinene and β - pinene) through the MTT assay, showed dose-dependent cytotoxic activity. However, the essential oil of *R. officinalis* L. total had greater cytotoxic activity than its isolated components (Wang et al., 2012).

Regarding the cytotoxicity parameter, the different types of chromosomal aberrations (delays, fragments and bridges) found after the treatment of *A. cepa* meristematic cells with the concentrations of *R. officinalis* L. essential oil showed the direct interference of this substance with the molecule of DNA and its strong damaging power. Once adhered, the chromatids remain united and when separated, they can lead to chromosome breakage, thus promoting chromosomal fragmentation or breakage (Marcano, Carruyo, Campo, & Montiel, 2004). The presence of chromosomal delays may be related to the failure of the damaged chromosomes to move to one of the cell's poles, with the chromosomal bridges being the result of breakage and subsequent fusion of chromosomes and chromatids.

Micronuclei are small nuclear corpuscles representing the genetic material lost from the main nucleus during the separation of chromatids, in the mitotic process, resulting in the reconstitution of two nuclei, where each of the chromosomal sets are surrounded by a nuclear membrane. Although the presence of chromosomal breaks or fragments that could lead to the appearance of micronuclei. The results obtained here show that the essential oil of *R. officinalis* L. did not lead to the formation of micronuclei. but evidence the manifestation of disturbances in the mitotic process of cells exposed to it.

The absence of MN induced by *R. officinalis* L. oil in this test does not mean that it is devoid of any mutagenic risk, since numerous chromosomal aberrations are being identified. Leme and Marin-Morales (2009) claim that these reflect the mutagenic potential of the samples and that, therefore, the absence of micronuclei by itself would not nullify their ability to induce DNA mutations.

Although some reports are found in the literature that point to the protective, antioxidant and antimutagenic activity of phenolic compounds present in *R. officinalis* L. oil (Del Baño et al., 2006), a study that investigated the genotoxic and mutagenic potential of the total essential oil of *R. officinalis* L. in liver cells and peripheral blood of rats, using the comet test, showed positive results for genotoxicity (Maistro, Mota, Lima, Bernardes, & Goulart, 2010). Although the tests performed by Maistro et al. (2010) are different from the test used here, the results obtained by him corroborate the results found in the present study.

Although the phenolic compounds isolated from *R. officinalis* L. exhibit some important activities, mentioned above (Del Baño et al., 2006), it is considered a huge difficulty to attribute biological activity to a total essential oil, as it always contains a mixture of different chemical compounds, in addition to smaller compounds that can significantly contribute to the oil's activity (Wang, Wu, Zu, & Fu, 2008; Wang et al., 2012). The complexity of essential oils is due to the presence of about 20 to 60 constituents in different concentrations, having two to three main components in high concentrations (20 to 70%) and others in smaller amounts (Bakkali, Averbeck, Averbeck, & Idaomar, 2007). Thus, it can be inferred through this study that the genotoxic effect of *R. officinalis* L. essential oil is a result of the joint cooperation of its components, which is different from the effects obtained when its components are presented in isolation.

Regarding the chromosomal aberrations found after the treatment of *R. officinalis* L. on the meristematic cells of *A. cepa* for the concentration of 750 μg mL^{-1}, being reduced in relation to the other concentrations, it

is suggested that the strong toxicity attributed to this concentration is decreasing the number of cells able to be visualized for detection of chromosomal aberrations. It is also possible that this concentration stimulates DNA repair mechanisms, in face of the high and harmful genetic alterations. Increased DNA protection would reduce the molecule's primary damage more effectively, minimizing the observed changes. This mechanism is also proposed by (Kienzler, Bony, & Devaux, 2013). Another justification for this question may be related to the action of phenolic compounds present in *R. officinalis* L. oil, which at this high concentration (750 µg mL^{-1}) can actually show its antioxidant effect and act in the possible scavenging of free radicals formed in the cell. These compounds, at this concentration, could also stimulate the cell's enzymatic machinery, which would act directly against reactive oxygen species (ROS), repairing damage caused to the organism by these species (Barreiros & David, 2006).

At the other investigated concentrations (243, 81 and 27 µg mL^{-1}) the *R. officinalis* L. essential oil itself may be responsible for the release of excess free radicals, resulting in a high number of chromosomal aberrations when compared with the negative control, and with a concentration of 750 µg mL^{-1} and acting, instead of antioxidant, as a generator of ROS, free radicals that involve oxygen and that are preferentially generated by exogenous and environmental genotoxins (Scott, Rangaswamy, Wicker, & Izumi, 2014).

As the use of medicinal plants as part of human culture has exposed man to countless substances potentially hazardous to health, it is urgently necessary to increase scientific research in the area of Toxicological Genetics to analyze the cytotoxic, genotoxic and mutagenic potential of natural products, validating its use.

Conclusion

Taken together, the results obtained for the analysis of *R. officinalis* L. essential oil on the *A. cepa* system indicate a strong cytotoxic potential, at least in the assay and concentrations used here.

However, it is important to conduct further research, using other genotoxicological tests with different endpoints such as Ames test, alkaline Comet and CBMN (Cytokinesis-Block Micronucleus Test) and at different concentrations. Thus, the interaction of the essential oil of the species *R. officinalis* L. with the genetic material of the cell will be clarified, as well as its possible mechanism of action.

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