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Antiproliferative and genotoxic potential from extracts and fractions of *Richardia brasiliensis* Gomes (*Rubiaceae*) by the *Allium cepa* L. test system

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ABSTRACT - The medicinal species *Richardia brasiliensis* Gomes has secondary metabolites with considerable pharmacological activities. This study aims to determine the antiproliferative and genotoxic capacity of extracts and fractions obtained from the aerial parts of *R. brasiliensis,* in the *Allium cepa* test, on which one can detect the damages on chromosomes and the influence on cell proliferation during the mitotic cycle. The antiproliferative and genotoxic effect from extracts and fractions were evaluated at concentrations of 10 and 500 µg/mL and the mitotic index (MI) and the percentage of chromosomal alterations (CA) were calculated and used on the χ^2 test. It was observed antiproliferative capacity, the best results were from the autumn crude extract, and the hexane and butanol fractions with lower MI than the negative control. All treatments were genotoxic with CA, but with lower values than the ones found in the positive control.

Keywords: cell cycle, chromosomal alterations, mitotic index

RESUMO - Potencial antiproliferativo e genotóxico dos extratos e frações de *Richardia brasiliensis* Gomes (Rubiaceae) pelo sistema teste de *Allium cepa* L. A espécie medicinal *Richardia brasiliensis* Gomes possui metabólitos secundários com atividades farmacológicas consideráveis. Neste estudo foi determinada a capacidade antiproliferativa e genotóxica de extratos e frações obtidos a partir de partes aéreas de *R. brasiliensis*, no teste em *Allium cepa*, pelo qual se pode detectar danos nos cromossomos e a influência na proliferação celular durante o ciclo mitótico. O efeito antiproliferativo e genotóxico dos extratos e frações foram avaliados nas concentrações de 10 e 500 µg/mL e os índices mitóticos (IM) e a porcentagem de células com alterações (AC) foram calculados e utilizado o teste do χ^2. Foi observada capacidade antiproliferativa, destacando o extrato bruto de outono e as frações hexânica e butanólica, com IM inferiores ao controle negativo. Todos os tratamentos foram genotóxicos com AC, porém com valores inferiores aos encontrados no controle positivo.

Palavras-chave: alterações cromossômicas, ciclo celular, índice mitótico

INTRODUCTION

The use of plants for therapeutic purposes is one of the oldest forms of medical practice of mankind, and its use has been reported even before the written story (Delmondes *et al*. 2013, Rocha *et al*. 2015). In addition to the vast employment in various communities, the acceptance of plants as an alternative medicine is influenced by the easy access to plant resources and the high cost of synthetic drugs, linked to the socio-economic situation of the population (Argenta *et al.* 2011).

Natural products have played an important role in drug discovery over the years, contributing to their use *in natura* or even as a starting point for obtaining new molecules, (Niero 2010). Many vegetable species have their use disclosed, and sometimes they are used as the main alternative for the treatment of a specific disease.

Richardia brasiliensis Gomes (Rubiaceae) is a species of herbaceous habits and prostrated development, popularly known as "poaia-branca", "ervaço" or "ipeca" (Delprete *et al.* 2005, Lorenzi & Matos 2008). It is a native plant from South America, found in tropical and subtropical regions and in Brazil, it has wide distribution, especially in areas with intense agricultural activity, such as the Midwest, Southeast and South regions of the country (Figueiredo *et al.* 2009).

In folk medicine it is used as an antiemetic, anti-diabetic, anti-hemorrhoidal and vermicide (Pinto *et al.* 2008, Souza *et al.* 2009) and the decoction of the roots is used as an expectorant and diaphoretic (Grandi *et al.* 1989). The antibacterial activity of extracts and fractions was confirmed, with satisfactory results against Gram-positive and Gram-negative bacteria (Figueiredo *et al.* 2009).

Popularly, *R. brasiliensis* extracts are also used to treat skin diseases such as eczema, burns (Pinto 2008, Souza *et al.* 2009, Poonkodi & Ravi 2016) and even cancer, and this pharmacological effect can not be ruled out, since polyphenols and alkaloids with antitumor action were identified in the species (Unnati *et al.* 2013).

The therapeutic action of the species may be associated with the presence of active compounds such as flavonoids, alkaloids, steroids, triterpenes, resins, organic acids and phenolic compounds found in the extracts of *R. brasiliensis* (Figueiredo *et al.* 2009, Souza 2009) and isolated compounds such as scopoletin, isorhamnetin-3-O-rutinoside, oleanolic acid, p-hydroxy-benzoic acid and m-methoxy-p-hydroxy-benzoic acid (Pinto *et al.* 2008).

To use a plant as a medicine or for the production of a herbal medicine, it is necessary to elucidate its active compounds and investigate the mechanism of action by bioassays, (Maciel *et al.* 2002), also, to determine the degree of toxicity in doses compatible with its medicinal employment (Lorenzi & Matos 2008).

Large parts of the substances derived from the plant metabolism appear to have therapeutic effects and may act as antitumors, affecting different targets and transductions signals that modulate the gene expression and thus, the cell cycle progression, proliferation and death (Hemalswarya & Doble 2006). Therefore, studies of toxicity and mutagenicity are necessary for a safe and effective use of the therapeutic agent (Marsiglia *et al.* 2011).

The assay with the *Allium cepa* is among the available methods for assessing cellular changes, and it provides satisfactory results for the evaluation of antiproliferative, cytotoxic, genotoxic and mutagenic effects from various substances. It is considered as a standard assay for quick tests, also showing a positive correlation with other test systems (Chauan *et al.* 1999, Matsumoto & Marin-Morales 2006).

The antiproliferative and consequent chromosomal alterations can be viewed during the cell cycle of *A. cepa* when its roots are exposed to the action of extracts or other chemical compounds, highlighting the mutagenicity of a substance. The major chromosomal abnormalities found are irregular anaphases and metaphase, binucleate cells and adherent cells, breaks, formation of bridges and chromosome latecomers (Tedesco & Laughinghouse 2012). Furthermore, it is possible to determine the mitotic index (MI) by monitoring meristematic cells, used as an indicator of cell proliferation which is obtained by the number of cells in prophase, metaphase, anaphase and telophase, divided by the total number of observed cells (Vieira *et al.* 2009).

To assess the antiproliferative and genotoxic potential of extracts and fractions of *R. brasiliensis,* at different concentrations, on the cell cycle of *A. cepa* were the aims of this study.

MATERIAL AND METHODS

Botanical Material

The plant material used in the analysis was composed of the aerial parts of *Richardia brasiliensis* collected on the campus of the Federal University of Santa Maria (UFSM), RS, Brazil (S29°43.277' W053°42.844'). The witness material was deposited in the herbarium Santa Maria Department of Biology (SMDB) UFSM under registration 13.966. Samples were collected in January, during summer; in April, during fall; in August, during winter; and in October, during spring, in the year of 2013.

Acquisition of crude extracts and fractions

After each gathering, the botanical material was dried in a circulating air oven at 45°C ± 2 for a period of seven days and then reduced to powder using cutting mills. The hydroethanolic (70%) crude extract (CE) was obtained through the maceration of the powdered drug (300 g) with the renewal of the solvent for 30 days, concentrated, taken to a dry residue through lyophilization and stored in an amber bottle under refrigeration until its use.

According to Falkenberg *et al*. (2007), the crude extract was fractionated through the partitioning with organic solvents of increasing polarity, starting with n-hexane (HEX), chloroform (CLO), ethyl acetate (AcOEt) and butanol (BUT), thereby yielding the semi-purified fractions.

Pre-treatment

Sixteen treatments were prepared at concentrations of 10 and 500 µg/mL, using the crude extract of the four seasons and four fractions solubilized with 0.4% dimethyl sulfoxide (DMSO) in water. The controls were composed by positive control (Glyphosate® 1%), negative control (distilled water) and the diluent control (distilled water + 0.4% DMSO).

The *Allium cepa* bulbs were placed in 50 ml bottles with distilled water for rooting. After the rooting, the bulbs remained in contact with the solutions to be analyzed for a period of 24 hours. Subsequently, the radicles with a lenght of approximately 5 - 10 mm were collected and fixed in ethanol: acetic acid (3:1) for 24 hours, being kept in 70% ethanol and refrigerated.

Preparation of the slides

For the preparation of slides, the rootlets were hydrolyzed in hydrochloric acid (HCl) 1 N for 5 min, washed in distilled water and stained with acetic orcein 2%. The meristematic region was crushed with the aid of a glass rod and a cover slip placed on the material. The slides were observed and analyzed under an optical microscope with magnification of 40 X. Four bulbs were used and one thousand cells were counted, totalling 4.000 cells in each treatment.

The mitotic index (MI) was obtained from the dividing cells (prophase, metaphase, anaphase and telophase) by the total number of cells observed (Gadano *et al.* 2002, Vieira *et al.* 2009).

The percentage of chromosomal alterations (CA) were determined considering the total cells analyzed (interphase and division), comparing normal cells and altered cells.

Statistical analysis

The statistical analysis was performed by χ^2 with an error probability level of <0.05 through the 5.3 BioEstat program (Ayres 2007).

RESULTS AND DISCUSSION

The *Richardia brasiliensis* extracts and fractions were evaluated for their antiproliferative capacity and genotoxic effect through the *Allium cepa* test. The mitotic index (MI) was obtained from the observed cells in division (prophase, metaphase, anaphase and telophase) by the total number of cells observed including the interphase (Tab. 1).

The MI's positive control (7.7%) when compared to the negative control (10.7%) and the diluent control (10.4%) was significantly lower, demonstrating that the 1% Glyphosate® inhibits the cell division of rootlets of *A. cepa*. Souza *et al*. (2010), reported that Glyphosate® inhibited the cell division at a concentration of 15% in the study of the aqueous extract of *Artemisia verlotorum* using the same test system, demonstrating that this control is antiproliferative even at low concentrations, as it can be observed in the study with *R. brasiliensis*.

The negative control and the diluent control showed no significant difference in the MI (10.7% and 10.4%, respectively), demonstrating that the concentration of DMSO used to solubilize the samples had no effect on the cell proliferation. The same was observed in the treatment of leukemia cells, in which the vehicle DMSO solution did not affected the cell viability (Maioral 2013).

The autumn crude extract (T3) at a concentration of 500 µg/mL was statistically different from the other extracts or fractions, showing the lowest MI value (5.4%). The T3 was also significantly lower than the positive control (7.7%) and the negative control (10.7%), demonstrating the best antiproliferative action. The hexane fraction (T9) and the butanolic fraction (T15) at a concentration of 500 µg/mL, showed MI's of 7.0% and 6.7%, respectively, suggesting that the presence of polar compounds in these fractions, such as tannins, inhibited the cell division of the roots of *A. cepa,* which was also observed in infusion of *Psidium guajava* and *Achillea millefolium* (Teixeira *et al.* 2003).

The chlorogenic acid and the flavonoid rutin, identified in the ACE (Dornelles 2015) justify the best antiproliferative activity for this extract, as the effects induced by phenolic compounds have been explained by these being active on chemo-prevention of cancer, inhibition of cell cycle phases, cell proliferation, oxidative stress and induction of apoptosis (Pedriali 2005). Effects similar to those produced by the chemotherapic Neotaxel®, used in the treatment of Ehrlich tumor cells, have been obtained with the use of rutin (Machado 2006).

Similar results were observed in aqueous extracts of *Baccharis trimera* and *B. articulata* which contain tannins and flavonoids and showed antiproliferative activity in *A. cepa* test system, demonstrating that the ability to induce

Table 1. Mitotic index (MI) determination on *A. cepa* test, of extracts and fractions of *Richardia brasiliensis* at two concentrations, positive control = Glyphosate® 1%; negative control = distilled water; diluent control = distilled water + 0.4% DMSO and total number of cells observed in different cell cycle phases. Means followed by the same letter do not differ by the χ^2 test at 5% level of error probability.

Treatments (µg/mL)	Total number of cells observed	Interphase	Prophase	Metaphase	Anaphase	Telophase	Mitotic Index (%)
T1: CE Summer – 500	4.000	3.653	198	53	39	57	8.7f
T2: CE Summer – 10	4.000	3.504	261	73	63	99	12.4b
T3: CE Autumn – 500	4.000	3.783	100	42	29	46	5.4i
T4: CE Autumn – 10	4.000	3.592	175	88	62	83	10.2e
T5: CE Winter – 500	4.000	3.602	127	109	44	118	9.9e
T6: CE Winter – 10	4.000	3.597	129	104	69	101	10.0e
T7: CE Spring – 500	4.000	3.643	112	94	62	89	8.9f
T8: CE Spring – 10	4.000	3.551	175	105	60	109	11.2c
T9: Fraction Hex – 500	4.000	3.717	111	68	34	70	7.0h
T10: Fraction Hex – 10	4.000	3.594	141	100	64	101	10.1e
T11: Fraction Clo - 500	4.000	3.442	193	135	82	148	13.9a
T12: Fraction Clo – 10	4.000	3.443	174	153	110	120	13.9a
T13: Fraction AcOET - 500	4.000	3.688	132	72	42	66	7.8g
T14: Fraction AcOET - 10	4.000	3.602	127	129	55	87	9.9e
T15: Fraction But - 500	4.000	3.730	129	59	38	44	6.7h
T16: Fraction But - 10	4.000	3.684	119	93	61	43	7.9g
T17: Positive Control	4.000	3.693	160	63	16	68	7.7g
T18: Negative Control	4.000	3.570	227	67	60	76	10.7cd
T19: Diluent Control	4.000	3.583	184	110	41	82	10.4de

inhibition of cell division can be attributed to the presence of polyphenols (Fachinetto & Tedesco 2009).

The MI of the samples in concentrations of 10 µg/mL and 500 µg/mL were significantly different, with the highest concentration they showed the lowest MI, demonstrating antiproliferative activity, with the exception of the winter crude extract (WCE) and the chloroform fraction (CLO), in which there was no significant difference between the concentrations.

In infusion of *Psychotria brachypoda* at concentrations of 5 and 20 g/L there was a difference in the MI according to the concentration (Frescura 2012), the same happened with the methanol extract of *Euphorbia hirta* (Ping *et al.* 2012) and in different populations of *Citrus sinensis* (Tedesco *et al.* 2015) with a significant decrease of the MI according to the concentration, suggesting a dose dependency.

In relation to the cellular proliferation capacity, it was observed an increase in MI in relation to the negative control in treatments T2, T8, T11, T12. The treatments T2 and T3 are extracts of *R. brasiliensis* in the concentration of 10 µg/mL obtained in the summer and spring and the increase of the MI can be related to the low concentration tested, as well as the differences in the constitution and the concentration of the active substances in these extracts (Gobbo-Neto & Lopes 2007).

The treatments T11 and T12, in addition to presenting the highest values of MI, showed no significant difference between them. According to Macedo (2014) phytochemical analyzes of *Selaginella convoluta*, anthracene derivatives, flavonoids, naphthoquinones and the intense presence of triterpenes and/or steroids, substances commonly found in this fraction, have been identified. Be related to increased cell proliferation.

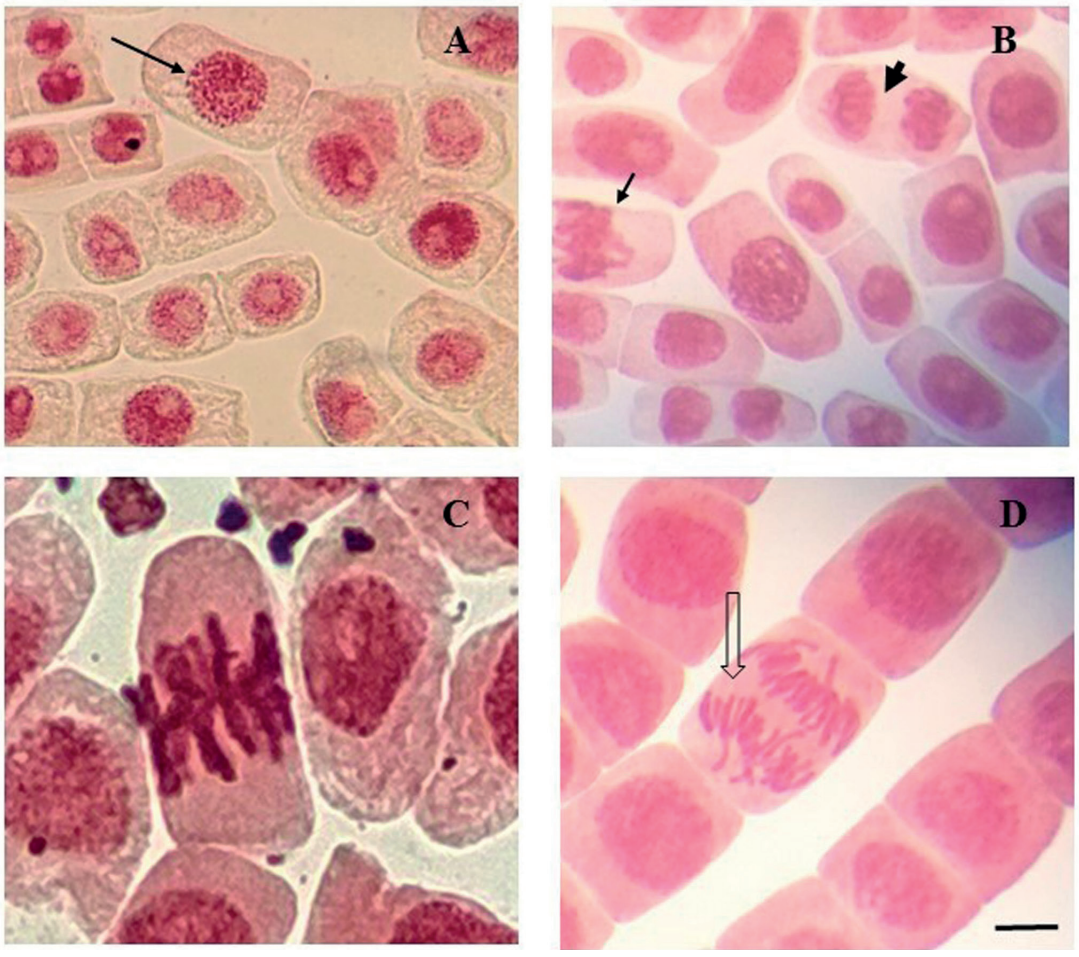
The extracts and fractions of *R. brasiliensis* showed antiproliferative capacity, but low genotoxicity when compared to the negative control (0.07%), which can be seen in the autumn crude extract (T3) and the butanolic fraction (T15), that presented 4 and 5 chromosomal alterations (CA) respectively. CA in cells of rootlets of *Allium cepa* treated with extracts and fractions of *R. brasiliensis,* controls, the total number of dividing cells, cells observed with alterations and the percentage of cell aberrations during the cell cycle are reported in Table 2. CA allow us to assess the genotoxicity of extracts and fractions during the phases of the cell cycle. Genotoxic substances have chemical and physical properties that interact with the nucleic acids leading to defects in germinal and somatic cells (Varanda 2006).

Table 2. Total of cells observed in the different cell cycle phases with chromosomal alterations (CA). Means followed by the same letter do not differ by the χ^2 test at 5% level of error probability. Positive control = Glyphosate® 1%; negative control = distilled water; diluent control = distilled water + 0.4% DMSO. B = binucleated; D = disorganized; BE = chromosomal breaks; BI = chromosomal bridge; M = micronucleus.

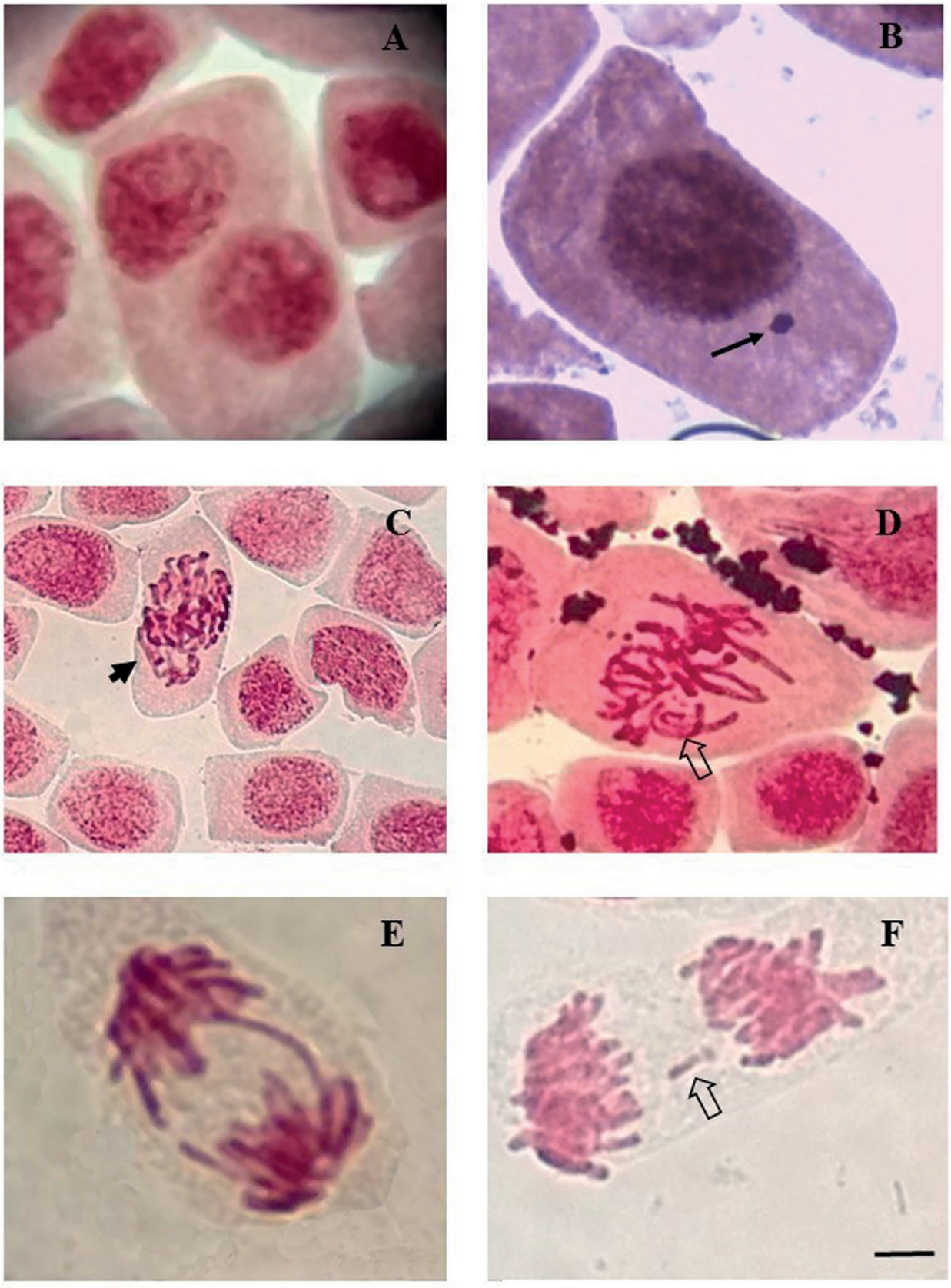
Treatments (µg/mL)	Interphase	Prophase	Metaphase	Anaphase	Telophase	Total number of cells observed with alterations	Total number of dividing cells	Total number of cells observed	Cells with CA (%)
T1: CE Summer – 500	1B	24D	1BE	3BE,1BI	0	30	347	4.000	0.7c
T2: CE Summer – 10	2M	0	5BE	4BE,5BI	0	16	496	4.000	0.4j
T3: CE Autumn – 500	2B	1D	0	1BI	0	4	217	4.000	0.1n
T4: CE Autumn – 10	0	14D	2BE	3BI	0	19	408	4.000	0.5i
T5: CE Winter – 500	1B	24D	0	1BE	0	26	398	4.000	0.6f
T6: CE Winter – 10	1B	16D	1BE	3BE,9BI	0	30	403	4.000	0.7b
T7: CE Spring – 500	1B	18D	0	10BI	0	29	357	4.000	0.7c
T8: CE Spring – 10	1B	13D	0	11BI	1BI	26	449	4.000	0.6e
T9: Fraction Hex– 500	0	9D	0	4BI	0	13	283	4.000	0.3k
T10: Fraction Hex– 10	0	12D	0	12BI	0	24	406	4.000	0.6f
T11: Fraction Clo – 500	0	10D	0	11BI	0	21	558	4.000	0.5g

Table 2. Continuação

Treatments (µg/mL)	Interphase	Prophase	Metaphase	Anaphase	Telophase	Total number of cells observed with alterations	Total number of dividing cells	Total number of cells observed	Cells with CA (%)
T12: Fraction Clo – 10	0	12D	0	10BI	0	22	557	4.000	0.5g
T13: Fraction AcOET – 500	0	13D	0	7BI	0	20	312	4.000	0.5h
T14: Fraction AcOET – 10	0	7D	0	15BI	0	22	398	4.000	0.5f
T15: Fraction But – 500	0	2D	0	3BI	0	5	270	4.000	0.1m
T16: Fraction But – 10	0	16D	0	12BI	0	28	316	4.000	0.7d
T17: Positive Control	0	84D	0	1BE,2BI	0	87	307	4.000	2.2a
T18: Negative Control	1D	0	0	2BE	0	3	430	4.000	0.07o
T19: Diluent Control	1B	9D	0	1BI	0	11	417	4.000	0.3



Figs. 1A-D. Meristem cells of *Allium cepa* root strain submitted to treatments with extracts and fractions of *Richardia brasiliensis*. **A.** Normal cells in interphase and prophase (long black arrow); **B.** Normal cell in metaphase (short black arrow) and telophase (short black arrow with filling); **C**. Normal cell in metaphase; **D.** Normal cell in anaphase (long arrow unfilled). Bars = 10 µm.



Figs 2A-F. Meristem cells of *Allium cepa* root strain submitted to treatments with extracts and fractions of *Richardia brasiliensis*. **A.** Cell binucleada; **B.** cell with micronucleus (long black arrow); **C.** Disorganized prophase (short black arrow); **D.** Break chromosome (short arrow unfilled) in metaphase; **E.** Anaphase bridge; **F.** Break chromosome in telophase. Bars = 10 µm.

The changes observed in the negative control (distilled water) were attributed to changes in the physiology of the plant and they were disregarded because of the low percentage of CA (0.07%).

The control of the T19 diluent (water + 0.4% DMSO) showed 0.3% of cell changes, demonstrating a large number of altered cells compared to water. Even in low concentration, the dimethylsulfoxide (DMSO) was capable of inducing cell damage, but without affecting the cell viability (10.4%).

The T17 positive control showed the highest percentage of cells with CA (2.2%), mainly cells disorganized in prophase. The Glyphosate® induces chromosomal alterations (Souza *et al.* 2010) due to the action in the biosynthesis of aromatic amino acids and the formation of proteins, reducing the levels of essential tubulin in the formation of microtubules (Yamada & Castro 2007) affecting the stages of the division and leading to aberrant divisions.

In most treatments the most observed CA were disorganized chromosomes (D) during prophase, followed by anaphase bridges (BI). All treatments showed low genotoxic effect when compared to the positive control with significantly different values of CA from the Glyphosate® (2.2%) observing binucleate cells, disorganized, micronucleus, breaks and chromosomal bridges (Figs. 1 and 2).

Following the results obtained in this study, it is necessary to conduct further investigations *in vitro* and *in vivo* with extracts and fractions of *R. brasiliensis* and compare the results between these test systems, in order to evaluate the possible therapeutic action of the specie.

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