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Cytotoxicity of aqueous extracts of *Rosmarinus officinalis* L. (Labiatae) in plant test system

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Abstract

This study investigated the cytotoxic activity of *Rosmarinus officinalis* L. (rosemary) aqueous extract on the cell cycle of *Allium cepa*. To this end, crude aqueous leaf extracts at four concentrations, 0.02, 0.04, 0.06 and 0.08 mg/mL, were tested on *A. cepa* meristematic root cells, at exposure times of 24 and 48h. Slides were prepared by the crushing technique, and cells analyzed throughout the cell cycle, totaling 5,000 for each control group and concentration. The four concentrations tested, including the lowest and considered ideal for use, at all exposure times, showed a significant antiproliferative effect on the cell cycle of this test system and presented a high number of cells in prophase. Our results evidenced the cytotoxicity of rosemary extracts, under the studied conditions.

Keywords: medicinal plant, rosemary, antiproliferative effect, *Allium cepa*.

Citotoxicidade de extratos aquosos de *Rosmarinus officinalis* L. (Labiatae) em sistema-teste vegetal

Resumo

Neste estudo investigou-se a ação citotóxica do extrato aquoso de *Rosmarinus officinalis* L. (alecrim) sobre o ciclo celular de *Allium cepa*. Para isso obteve-se extratos aquosos brutos de folhas secas desta planta em quatro concentrações, 0,02; 0,04; 0,06 e 0,08mg/mL, que foram testadas em células meristemáticas de raízes de *A. cepa*, nos tempos de exposição 24 e 48h. As lâminas foram feitas pela técnica de esmagamento, e analisaram-se células em todo ciclo celular, totalizando 5.000 para cada grupo controle e concentração. A partir dos resultados verificou-se que as quatro concentrações testadas, inclusive a menor e considerada ideal para consumo, em todos os tempos de exposição tiveram ação antiproliferativa significativa sobre o ciclo celular deste sistema teste, e apresentaram um grande número de células em prófase. Dessa forma, o alecrim, nas condições analisadas, mostrou-se citotóxico.

Palavras-chave: planta medicinal, alecrim, efeito antiproliferativo, *Allium cepa*.

1. Introduction

Worldwide, several plant species are used for the treatment and prevention of diseases, but the majority have not yet been satisfactorily evaluated as to their toxic potential, which is essential for the safe and effective use of these herbal medicines (Iganci et al., 2006).

Rosmarinus officinalis L. (Labiatae) is popularly known as rosemary and used in the form of tea, is originated from the Mediterranean region of Europe and grown in almost all countries with temperate and tropical climates. This species has a woody shrub size, is erect, little branched, with small linear, leathery-coriaceous leaves, and strong scent, with small blue flowers (Blanco et al., 2002; Silva et al., 2008).

The flavonoids diosmetin, genkwanin, luteolin, hispidulin and apigenin; caffeic, chlorogenic and rosmarinic acids; and terpenes - carnosol, rosmanol, epirosmanol, isorosmanol, rosmarini difenol, rosmariquinone and rosmadiol have been registered as chemical components of rosemary leaves (Ramalho and Jorge, 2006). Experimental studies have demonstrated that the essential oil has antimicrobial, anti-inflammatory, antiseptic, diuretic, anti-spamodic (Silva et al., 2008), chemopreventive, antitumor and antioxidant activities (Erkan et al., 2008). However, the toxic effect of *R. officinalis* needs to be further investigated (Ferreira et al., 2013).

Meristematic root cells of *Allium cepa* L. is an important test-system for the evaluation of the cytotoxicity of medicinal plant aqueous extracts (Stange et al., 2009; Delarmina et al., 2012) for their kinetic properties of proliferation and for having large and few chromosomes (2n = 16) (Leme and Marin-Morales, 2009; Herrero et al., 2012). It also presents

a satisfactory similarity to the results obtained in other bioassays, as those with animals and cell cultures, and is frequently used to alert the population about the use of herbal medicines (Belcavello et al., 2012).

Thus, since the population consumes tea from *R. officinalis* leaves for the treatment and prevention of diseases, and given the need for further studies in relation to the toxic potential of this plant, and considering the suitability of the *A. cepa* system in the evaluation of the toxicity on the cellular level of medicinal plants, this study aimed to assess the cytotoxic effects of crude aqueous extracts of rosemary leaves on the cell cycle of *A. cepa* roots at different concentrations and exposure times.

2. Material and Methods

This work was conducted in the Plant and Animal Cytogenetics Laboratory at the Campus Senador Helvídio Nunes de Barros of the Federal University of Piauí (UFPI), Picos, Piauí State, Brazil.

2.1. Plant collection

Samples of *R. officinalis* were collected from a medicinal garden located in the city of Teresina, Piauí State, in May 2012. They were identified by the specialist M.S. Maria do Socorro Meireles de Deus, Professor of Botany at UFPI. Afterwards, these samples were stored under controlled temperature and humidity for 6 months, for natural drying of leaves. Voucher specimens were deposited in the Herbarium Graziela Barroso (UFPI, Teresina, Piauí, Brazil).

2.2. Infusion preparation

Dried leaves were boiled in distilled water and infused for 10 minutes. Subsequently, aqueous extracts were filtered and cooled at room temperature, 25°C. Four concentrations were established for evaluation (0.02, 0.04, 0.06 and 0.08mg/mL); the concentration of 0.02mg/mL is considered usual and recommended by the Resolução de Diretoria Colegiada - RDC/48 of the Agência Nacional de Vigilância Sanitária - ANVISA (Brasil, 2004).

2.3. Obtaining meristematic cells for cytogenetic analysis

Onions (*A. cepa*) were allowed to root in flasks with aerated distilled water, at room temperature, approximately 25°C, until obtaining roots with about 1.0 cm length. For analysis of each concentration, an experimental group was set containing five onion bulbs.

Before testing each extract concentration, six roots, on average, of each bulb were collected and fixed to serve as control (CO) of the bulb itself. The remaining roots were placed on their respective concentrations, for 24 hours, a procedure called exposure time of 24 hours (ET 24h). After this, some roots were removed and fixed. The remaining roots of each bulb were once again placed on their respective concentrations for more 24 hours, this time called exposure time of 48 hours (ET 48 h). Again, six roots on average of each bulb were collected and fixed.

Roots were fixed in Carnoy 3:1 (ethanol: acetic acid), for approximately 6 hours. After this, roots were hydrolyzed in HCL for 8 minutes and then stained with 2% Orcein-Acetic. Next, cytology slides were prepared according to Guerra and Souza (2002) protocol. For each bulb, at least four slides were mounted (two roots per slide) and the analysis was conducted under a 40X ZEISS 2000 optical microscope to observe the number of dividing cells and check for the presence of cellular aberrations.

One-thousand-cells of each bulb were analyzed, totaling 5,000 cells per experimental group. The mitotic index was calculated through the number of dividing cells divided by the total cells analyzed. The statistical analysis of the data was carried out by the Chi-square test at 5% significance using the software BioEstat (Ayres, 2007).

3. Results and Discussion

Table 1 lists the number of cells in interphase and in different phases of cell division, as well as the values of mitotic index obtained from *A. cepa* root meristem cells treated with water (CO) and with *R. officinalis* extracts for 24 and 48 hours (ET 24h and ET 48h).

The four concentrations of rosemary extracts significantly reduced the mitotic index of *A. cepa* meristematic root cells compared with MI obtained for their respective controls ($p < 0.05$), proving to be cytotoxic (Table 1). The MI values obtained for each ET of each concentration were not significantly different to each other.

The antiproliferative role of this plant is attributed to the activity of rosmarinidifenol, rosmariquinone and rosmanol (Yesil-Celiktas et al., 2010). According to Visanji et al. (2006), high concentrations of these terpenes disrupt the cell cycle in the G_2 phase of the interphase, by interrupting the cytoplasm replication and the onset of chromosome condensation. This explains the antiproliferative effect in *A. cepa* in this study. However, this effect has already been observed at concentrations usually employed (0.02mg/ml) and recommended to the population by ANVISA. In all treatments, the inhibition of cell division occurred immediately at the 24h ET.

Visanji et al. (2006) reported that super-doses of diterpene carnosol affect cells under division, by acting on the B1cyclins during the process, disabling the proper formation of the mitotic spindle. This finding confirms results obtained herein, with a large number of cells in prophase in all TR, in the two ET (Table 1).

Several researches on the antiproliferative effect of rosemary were developed in recent years, such as Yesil-Celiktas et al. (2010) who evaluated the activity of aqueous and alcoholic extracts of *R. officinalis* leaf, at concentrations from 12.50 to 47.55 mg/mL on human cell lines NCI-H82 (lung carcinoma) DU-145 (prostate carcinoma), Hep-3B (hepatocellular carcinoma), K-562 (chronic myelogenous leukemia) and MCF-7 (breast adenocarcinoma) and verified that rosemary significantly inhibited cell division in all these cells. Similarly, Tai et al.

Table 1. Total number of cells analyzed and cell cycle phases of *Allium cepa* roots treated with water (control) and with infusion of *R. officinalis* leaf concentrations (treatment - TR) of 0.02, 0.04, 0.06, 0.08 mg/ml at ET 24 and 48 h. 5,000 cells were analyzed for each control group and concentration.

TR	ET	Undifferentiated Cells and Interphase	P	M	A	T	Total Cells in Cell Division	MI (%)
	CO	4432	195	174	58	71	498	9,9[a]
0,02mg/mL	ET 24h	4757	182	29	14	18	243	4,8[b]
	ET 48h	4749	194	16	20	11	251	5,2[b]
	CO	4548	112	121	108	111	452	9,0[a]
0,04mg/mL	ET 24h	4763	183	24	20	10	237	4,7[b]
	ET 48h	4757	191	22	20	10	243	4,8[b]
	CO	4414	178	155	40	113	586	11.7[a]
0,06mg/mL	ET 24h	4739	190	23	25	23	261	5,2[b]
	ET 48h	4770	194	14	11	11	230	4,6[b]
	CO	4485	224	66	77	58	515	10.3[a]
0,08g/mL	ET 24h	4757	184	23	24	12	243	4,8[b]
	ET 48h	4772	194	20	4	10	228	4,5[b]

CO - Control; ET - Exposure time; h - hour; TR - Treatment; P - prophase; M - metaphase; A - Anaphase; T - Telophase; MI - Mitotic Index. Means followed by the same letter do not differ significantly at the 5% level by the c^2 test.

(2012) and Cheng et al. (2011) observed a significant reduction in the mitotic index of the cell line A2780 (human ovarian cancer), and various cell lines of colon carcinomas from rodents treated with high concentrations of carnosol, respectively.

However, all studies reported on *R. officinalis* antiproliferative activity have had the cells of their test-systems treated with some clastogenic drug (and so some damage to the genetic material). However, in the present work, the meristematic cells of *A. cepa* roots were treated with water or with one concentration of rosemary extract, simulating the manner at which people use the plant, and showed that this plant also had antiproliferative activity on the cells without any pretreatment, demonstrating the toxic potential.

Furthermore, considering that common sense frequently considers medicinal plants free from adverse bodily reactions, which leads to their indiscriminate use, and that the *R. officinalis* plant is easily found in medicinal gardens, herbalists, natural food stores and markets, it is of utmost importance to perform further studies with *A. cepa* and other test-systems, applying different exposure times and treatments to thereby establish the optimal and safe concentration for the use of this plant.

In summary, under the present conditions, aqueous extracts of *R. officinalis* were cytotoxic to the test system employed, including the lowest concentration recommended for consumption. All concentrations at the two exposure times showed a high number of cells in prophase. Also importantly, the results obtained in this study also evidenced the importance of the *Allium cepa* test system, with results similar to those obtained in other bioassays.

References

AYRES, M., 2007. *Software BioEstat 5.0: aplicações estatísticas nas áreas das ciências biológicas e médicas*. Brasília: Sociedade Civil Mamirauá. 200 p.

BELCAVELLO, L., CUNHA, MR., ANDRADE, M. and BATITUCCI, MC., 2012. Citotoxicidade e danos induzidos pelo extrato de Zornia diphylla, uma planta medicinal. *Natureza on line*, v. 10, no. 3, p. 140-145.

BLANCO, MCSG., MING, LC., MARQUES, MOM. and BOVI, OA., 2002. Drying temperature effects in rosemary essential oil content and composition. *Acta Horticulturae*, vol. 569, p. 99-103.

BRASIL. Agência Nacional de Vigilância Sanitária – ANVISA, 2004. *Resolução RDC nº 48, de 16 de março de 2004. Dispõe sobre o registro de medicamentos fitoterápicos*. Diário Oficial da União. Poder Executivo, Brasília, 18 mar.

CHENG, AC., LEE, MF., TSAI, ML., LAI, CS., LEE, JH., HO, CT. and PAN, MH., 2011. Rosmanol potently induces apoptosis through both the mitochondrial apoptotic pathway and death receptor pathway in human colon adenocarcinoma COLO 205 cells. *Food Chemical Toxicology: toxicology : an international journal published for the British Industrial Biological Research Association*, vol. 49, no. 2, p. 485-493. http://dx.doi.org/10.1016/j.fct.2010.11.030. PMid:21112365

DELARMINA, JM., BATITUCCI, MCD. and GONÇALVES, JLO., 2012. Efeitos citotóxicos, genotóxicos e mutagênico da tintura de Matriarca chamomilla L. in vivo. *Revista Cubana de Plantas Medicinales*, vol. 17, no. 2, p. 149-159.

ERKAN, N., AYRANCI, G. and AYRANCI, E., 2008. Antioxidant activities of rosemary *Rosmarinus Officinalis* extract, blackseed *Nigella sativa* essential oil, carnosic acid, rosmarinic acid and sesamol. *Food Chemistry*, vol. 110, no. 1, p. 76-82. http://dx.doi.org/10.1016/j.foodchem.2008.01.058.

FERREIRA, LG., CELOTTO, AC., CAPELLINI, VK., ALBUQUERQUE, AAS., NADAI, TRD., CARVALHO, MTMD. and EVORA, PRB., 2013. Is rosmarinic acid underestimated as

an experimental cardiovascular drug? *Acta Cirurgica Brasileira*, vol. 28, suplemento 1, p. 83-87. http://dx.doi.org/10.1590/S0102-86502013001300016. PMid:23381830

GUERRA, M. and SOUZA, M., 2002. *Como observar os cromossomos: um guia de técnicas em citogenética vegetal, animal e humana*. Ribeirão Preto: FUNPEC. 191 p.

HERRERO, O., PÉREZ MARTÍN, JM., FERNÁNDEZ FREIRE, P., CARVAJAL LÓPEZ, L., PEROPADRE, A. and HAZEN, MJ., 2012. Toxicological evaluation of three contaminants of emerging concern by use of the Allium cepa test. *Mutation Research*, vol. 743, no. 1-2, p. 20-24. http://dx.doi.org/10.1016/j.mrgentox.2011.12.028. PMid:22249112

IGANCI, JRV., BROBOWSKI, G., HEIDEN, GVC., STEIN, L. and ROCHA, BHG., 2006. Efeito do extrato aquoso de diferentes espécies de boldo sobre a germinação índice mitótico de *Allium cepa* L. *Arquivos do Instituto Biologico*, vol. 73, no. 1, p. 79-82.

LEME, DM. and MARIN-MORALES, MA., 2009. Allium cepa test in environmental monitoring: a review on its application. *Mutation Research*, vol. 682, no. 1, p. 71-81. http://dx.doi.org/10.1016/j.mrrev.2009.06.002. PMid:19577002

RAMALHO, VC. and JORGE, N., 2006. Antioxidantes utilizados em óleos, gorduras e alimentos gordurosos. *Quimica Nova*, vol. 29, no. 4, p. 755. http://dx.doi.org/10.1590/S0100-40422006000400023.

SILVA, MDSA., SILVA, MAR., HIGINO, JS., PEREIRA, MSV. and CARVALHO, ADA., 2008. Atividade antimicrobiana e antiaderente in vitro do extrato de *Rosmarinus officinalis* Linn. sobre bactérias orais planctônicas. *Revista Brasileira de Farmacognosia*, vol. 18, no. 2, p. 236-240. http://dx.doi.org/10.1590/S0102-695X2008000200017.

STANGE, VS., GOMES, T., ANDRADE, MA. and BATITUCCI, MDCP., 2009. Avaliação do efeito mutagênico do extrato hidroalcóolico bruto, por meio de bioensaios in vivo e prospecção fitoquímica de Cecropia glaziovii Sneth (embaúba), Cecropiaceae. *Revista Brasileira de Farnacognosia*, vol. 19, no. 2b, p. 637-642. http://dx.doi.org/10.1590/S0102-695X2009000400023.

TAI, J., CHEUNG, S., WU, M. and HASMAN, D., 2012. Antiproliferation effect of Rosemary (Rosmarinus officinalis) on human ovarian cancer cells in vitro. *Phytomedicine*, vol. 19, no. 5, p. 436-443. http://dx.doi.org/10.1016/j.phymed.2011.12.012. PMid:22325591

VISANJI, JM., THOMPSON, DG. and PADFIELD, PJ., 2006. Induction of G2/M phase cell cycle arrest by carnosol and carnosic acid is associated with alteration of cyclin A and cyclin B1 levels. *Cancer Letters*, vol. 237, no. 1, p. 130-136. http://dx.doi.org/10.1016/j.canlet.2005.05.045. PMid:16019137

YESIL-CELIKTAS, O., SEVIMLI, C., BEDIR, E. and VARDAR-SUKAN, F., 2010. Inhibitory effects of rosemary extracts, carnosic acid and rosmarinic acid on the growth of various human cancer cell lines. *Plant Foods for Human Nutrition*, vol. 65, no. 2, p. 158-163. http://dx.doi.org/10.1007/s11130-010-0166-4. PMid:20449663