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Protective activity of *Cynara scolymus* L. leaf extract against chemically induced complex genomic alterations in CHO cells

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ABSTRACT

Cynara scolymus L., popularly known as artichoke, has been widely used in traditional medicine as an herbal medicament for therapeutic purposes. The study aimed at assessing the protective activity of *Cynara scolymus* leaf extract (LE) against DNA lesions induced by the alkylating agent ethylmethnesulphonate (EMS) in Chinese hamster ovary cells (CHO). The ability of *C. scolymus* L. LE to modulate the mutagenicity of EMS was examined using the cytokinesis block micronucleus (CBMN) cytome assay in three antigenotoxic protocols, pre- post- and simultaneous treatments. In the pre-treatment, *C. scolymus* L. LE reduced the frequencies of MNi and NBUDs induced by EMS in the lower concentration. In contrast, at the highest concentration (5 mg/ml) artichoke enhanced the frequency of MNi, potentiating EMS genotoxicity. In the simultaneous treatment only the induction of MNi was repressed by the exposure of cells to *C. scolymus* L. LE. No modification in genotoxicity was observed in LE post-treatment. The results obtained in this study suggest that lower concentrations of artichoke prevent chemically induced genomic damage in mammalian cells. In this context, the protective activity of *C. scolymus* L. could be associated to its constitutive antioxidants compounds.

Introduction

In the past 20 years, the interest in medicinal plants has increased together with the number of investigations into their biological effects on humans and animals (Newman & Gragg, 2007). *Cynara scolymus* L., popularly known as artichoke, belongs to the *Asteraceae* family, and is used in herbal medicine for therapeutic purposes. Artichoke is an edible vegetable from the Mediterranean, but it is easily found throughout Brazil, being more abundant in mountain regions. It grows to 1.4 – 2.0 meters tall, with arching, deeply lobed, silvery, glaucous-green leaves 50 – 82 centimeters long (Gebhardt, 1997).

In Brazilian herbal medicine systems, *Cynara scolymus* L. leaf preparations are used for hepato-biliary diseases, high cholesterol, hypertension and as diuretic (Gebhardt, 1997; Adzet, Camarasa & Laguna, 1987). It also has antioxidative and protective properties against hydroperoxide-induced oxidative stress in cultured rat hepatocytes (Gebhardt, 1997; Kraft, 1997; Miccadei et al., 2004).

Considering that humans are exposed daily to environmental mutagenic and carcinogenic compounds, investigating the antimutagenic and anticarcinogenic properties of a substance is essential (De Flora &, Ramel, 1988). Fruits and vegetables have become the object of intensive research, due to their antioxidant properties and consequent antimutagenic action (Kaur & Kapoor, 2001). Many phytonutrients induce apoptosis (cell death) and inhibit cancer cell proliferation (Reddy, Odhav & Bhoola, 2003). In fact, current literature has demonstrated that plants and their active constituents present protective effects against human carcinogenesis and mutagenesis (Surh & Ferguson, 2003). The antimutagenic action of a plant extract can be a good indication of anticarcinogenic activity. Thus, medicinal plants are an important source concerning the discovery of components that may be useful as chemopreventives, which could act on different steps of carcinogenesis (Surh & Ferguson, 2003; Abdillahi et al., 2012).

In this study we applied the cytokinesis-block micronucleus (CBMN) cytome assay to investigate the antimutagenic properties of *Cynara scolymus* L. leaf extract (LE) against DNA alkylations induced by ethylmethanesulphonate (EMS) in Chinese hamster ovary (CHO) cells.

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The CBMN - cytome assay is a useful short-term assay to measure chromosome damage, cytostatic effects and cytotoxicity in mammalian cells. The induction of micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were assessed in binucleated cells (BNCs). The nuclear division cytotoxicity index (NDCI) was used as an indicator of cytotoxicity (Fenech, 2000, Thomas, Umegaki & Fenech, 2003).

Materials and Methods

Plant Extract and Phytochemical Screening

The leaves of *Cynara scolymus* L., *Asteraceae* family, were collected in Gramado (Rio Grande do Sul / Brazil) and the specimen was deposited at the Department of Botany of Lutheran University of Brazil under number HERULBRA 4288. After harvest, LE was prepared by infusion (1/10 plant/solvent) in distilled water at 80 °C for 30 min. The infusion was left to stand to cool down at room temperature. After cooling, the filtered extract was frozen and concentrated by lyophilization for five days overnight to obtain the crude aqueous extract from leaves (13.7 g, yield: 11.4%) of *C. scolymus* L. The phytochemical profile and HPLC analysis of the leaf extract of *C. scolymus* L. were previously performed by our research group (Zan et al., 2013). Phenolic acids as clorogenic acid (778.7 μg/g), caffeic acid (43.8 μg/g) and flavonoids as isoquercitrin (1388.2 μg/g), and rutin (309.9 μg/g) were identified. Compounds such as gallic acid, hyperoside, quercetin, sakuranetin and rosmarinic acid were not detected in the extract.

Cell line and Chemical

The Chinese hamster ovary (CHO) cells were kindly supplied by Professor Salvadori (UNESP-Botucatu, São Paulo, Brazil). The cells were maintained in plastic cultures flasks (75 cm^2), in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a incubator chamber with 5% CO_2.

The alkylating agent ethylmethanesulphonate (EMS) (CAS, 62-50-0) was purchased from Sigma Chemical Company (Saint Louis, MO, USA). EMS was dissolved in DMEM medium immediately before use and was used as the DNA damage inducing agent in a dose of 350 μM. DMEM medium was used as the negative control.

Cell Treatment and the CBMN – Cytome Assay

Cells ($1x10^5$) were grown in 24-well plates at 37 °C and 5% CO_2 atmosphere, in complete DMEM medium for 24 h, washed with Dulbecco's phosphate-buffered saline (DPBS) and then submitted to the following treatments with different concentrations of *C. scolymus*: 0.62; 1.25; 2.5; 5.0 mg/ml.

Pre-treatment: cells were treated with *C. scolymus* LE for 1 h, washed with DPBS and exposed to EMS for another 1 h.

Simultaneous treatment: CHO cells were simultaneously treated with the *C. scolymus* LE concentrations and EMS for 1 h.

Post-treatment: cells were treated with EMS, after 1 h cells were washed with DPBS and treated with *C. scolymus*, for 1 h.

Then, 44 h after the beginning of the cultures, fresh complete medium containing cytochalasin B (5 μg/ml final concentration) was added. After 28 h, cells were washed with DPBS at 37 °C and trypsinized with 350 μl trypsin. After 5 min, the cells were gently resuspended in 650 μl of complete medium and harvested by cytocentrifugation for 5 min at 700 RPM. Slides were removed, fixed and stained with Instant Prov (Newprov®). After staining, slides were air dried and examined under 1000x magnification using a light microscope.

Table 1
Cytokinesis-block micronucleus (CBMN) cytome assessment of antigenotoxicity – Pretreatment of different concentrations of *Cynara scolymus* L. leaf extract (mg/ml) in CHO cells with EMS.

Treatments	MNi[a]	NPBs[a]	NBUDs[a]	NDCI[a]
NC	9.0 ± 2.2	3.2 ± 1.9	1.0 ± 0.8	1.9 ± 0.1
PC (EMS)	49.7 ± 4.8###	10.7 ± 1.7#	10.7 ± 2.2###	1.8 ± 0.1
0.62 + EMS	25.5 ± 1.9***	6.7 ± 1.7	2.0 ± 1.4***	1.9 ± 0.1
1.25 + EMS	53.7 ± 2.2	10.5 ± 3.1	5.0 ± 3.5**	1.9 ± 0.0
2.5 + EMS	45.7 ± 11.1	11.5 ± 6.8	2.7 ± 2.7***	1.9 ± 0.1
5.0 + EMS	73.5 ± 12.0**	9.0 ± 4.2	5.5 ± 2.1	2.0 ± 0.0

[a] Values are the mean ± standard deviation.
Significantly different from the negative control group ($p < 0.05$)
Significantly different from the negative control group ($p < 0.001$)
** Significantly different from the positive control group ($p < 0.01$)
*** Significantly different from the positive control group ($p < 0.001$)
NC: negative control, PC: positive control (EMS 350 μl), MNi: micronuclei, NPBs: nucleoplasmic bridges, NBUDs: nuclear buds, NDCI: nuclear division cytotoxicity index.

Scoring Cells

The procedure for scoring micronuclei (MNi), nucleoplasmatic bridges (NPBs) and nuclear buds (NBUDs) were carried out according to Fenech (2000). At least 3,000 binucleated cells (BNCs) were scored per concentration, totalizing the results of three independent experiments. Three experiments were carried out in duplicate, with 2000 cells analyzed per concentration/experiment. In total, 6.000 binnucleated cells were analyzed, for each concentration. For the evaluation of cytotoxicity, the nuclear division cytotoxicity index (NDCI) was estimated by scoring mononucleate and multinucleate as well as apoptotic and necrotic cells in a total of 500 cells. NDCI was calculated using the formula [Ap + Nec + M1 + 2M2 + 3(M3) + 4(M4)]/500, where M1–M4 represent the number of cells with one to four nuclei, respectively, Ap = number of apoptotic cells, Nec = number of necrotic cells. Analysis of variance (one- way ANOVA) with Dunnett's *post hoc* test at $p < 0.05$ was used to compare the differences between cells exposed only to EMS and the untreated group (negative control), as well as *Cynara scolymus* L. LE plus EMS with cells treated only with EMS. The MNi, NBUDs and NPBs frequencies and NDCI score was used for comparisons.

Results

The antimutagenic activity of *Cynara scolymus* L. LE against lesions induced by EMS was assessed in four viable concentrations (0.62, 1.25, 2.5, 5.0 mg/ml) which were previously selected by means of the trypan blue dye exclusion assay (data not shown). Only the concentrations that presented at least 85% of viable cells were tested in the CBMN-cytome assay.

The results of the pretreatment with *C. scolymus* L. LE are shown in Table 1. A significant reduction in the frequencies of MNi in BNCs was observed in cells treated with 0.62 mg/ml dose, when compared to EMS induced MNi alone frequencies. Hence, a reduction in the induction of NBUDs in CHO cells treated with 0.62, 1.25 and 2.5 mg/ml exposure concentrations was also observed in relation to EMS. However, in the cells treated with 5.0 mg/ml, *C. scolymus* L. LE potentiates EMS induced MNi since significant differences were observed.

In the simultaneous treatment, a modulatory effect was restricted to the induction of MNi. In fact, the 0.62 and 1.25 mg/ml *C. scolymus* L. LE exposure concentrations decreased the frequencies of MNi induced by EMS (Table 2).