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Evaluation of micronuclei frequency in *Tradescantia pallida* pollen mother cells treated with ethanolic extracts isolated from *Cryptocarya mandioccana, Cryptocarya moschata* and *Pterogyne nitens*

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Resumo

Apesar de extratos vegetais possuírem uma série de compostos com atividade farmacológica, eles podem também apresentar substâncias com potencial mutagênico. O objetivo do atual estudo é avaliar a mutagenicidade do extrato etanólico das plantas: *Cryptocarya mandioccana, Cryptocarya moschta* e *Pterogyne nitens* utilizando o ensaio do micronúcleo em células mãe de grão de pólen (tétrades) de *Tradescantia pallida* (Trad-MCN). Inflorescências de *T. pallida* foram tratadas com diferentes concentrações do extrato etanólico das plantas selecionadas. Para *C. mandioccana, C. moschata* e *P. nitens* o ensaio Trad-MCN foi executado os tratamentos, controle positivo (formaldeido 10000 ppm), controle negativo (solução de Hoagland), e controle de veículo (Tween 20% ou DMSO 3%). Os micronúcleos foram quantificados em 300 tétrades/inflorescência, a média e o erro padrão foram estabelecidos no mínimo para 10 inflorescências/tratamento. Os extratos avaliados demonstraram efeito clastogênico dose resposta, respectivamente: *C. mandioccana* (0.5, 1.0 e 2.0 mg/mL) e *P. nitens* (1.0 and 2.0 mg/mL). Entretanto, *C. moschata* não demonstrou atividade clastogênica/aneugênica nas concentrações avaliadas no presente estudo. A partir desses resultados foi possível concluir que os extratos de *C. mandioccana* and *P. nitens* apresentaram atividade clastogênica/aneugênica nas maiores concentrações enquanto o extrato *C. moschata* não apresentou o mesmo efeito.

Unitermos: clastogênico, aneugênico, *Cryptocarya mandioccana, Cryptocarya moschata, Pterogyne nitens*, Trad-MCN.

INTRODUCTION

Brazil is one of the twelve countries reported to have the highest biodiversity, presenting a variety of plant species with medicinal properties. Plant extracts are commonly used in Brazilian folk medicine, based on popular knowledge accumulated over centuries for treatment of many diseases (ALBUQUERQUE *et al*., 2007; WITAICENIS *et al*., 2007). Several species have been found in tropical forests located in the Brazilian Atlantic Rain Forest and the Cerrado, which are the two main biomes in Sao Paulo State. Among the plant species, which cover these areas, *Cryptocarya moschata, Cryptocarya mandiocana* and *Pterogyne nitens* have been studied in order to search for potential anti-cancer compounds.

Pterogyne nitens (Fabaceae) is the sole member of genus Pterogyne (Leguminosae) and this plant species has been reported to contain terpenoid guanidine alkaloids with anti-fungal and anticancer activity (GUNATILAKA *et al*., 1992, BOLZANI *et al*., 1995). Its alkaloids also presented moderate cytotoxicity against Chinese hamster ovary cell lines (BOLZANI *et al*., 1995). Cryptocarya is one of the largest pantropical genera in Lauraceae, which is present in South America, South Africa, Madagascar, Asia, Australia and Oceania (VAN DERR WERFF, 1991). The genus includes about 350 species, with approximately 18 neotropical species, mostly localized in South America, especially in the southern part of Brazil (VAN DERR WERFF, 1991; ROHWER, 1993; MORAES, 2005). Cryptocarya species possess antiinflammatory activity (DREWES *et al*., 1997; ZSCHOCKE e VAN STADEN, 2000). Phytochemical studies on steam bark and leaves of *C. moschata* from Atlantic Rain Forest show the occurrence of styrylpyrones and flavonoid

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glycosides on this species (CAVALHEIRO e YOSHIDA, 2000; NEHME *et al*., 2002). Some of the pyrones and styrylpyrones isolated from this genus have shown larvicidal and antifertility activities, as well as inhibition of breast cancer cell lines (HAWARIAH e STANSLAS, 1998; PIHIE *et al*., 1998; EE *et al*., 1999).

Although these studies have been conducted to search for pharmacological activity of herbal extracts, green plants may contain carcinogenic and mutagenic substances. Micronuclei (MCN) assays in mice and pollen mother cells have been proposed as a useful tool in order to characterize the mutagenic potential of complex matrices (MA *et al*., 1994, KLUMPP *et al*., 2004). Genetic damage that results in chromosome breaks, structurally abnormal chromosome, or spindle abnormalities leads to MCN formation in animal or plant cells (MA *et al*.,1983). Two major mechanisms are responsible for the formation of MCN: double strand DNA breaks (clastogenic events) and chromosome loss (aneugenic events) (MULLER *et al*., 2008), being MCN frequency considered a sensitive biomarker of mutagenicity (MA *et al.*, 1983).

Considering that few studies have been conducted to verify mutagenicity (clastogenic and/or aneugenic effects) of plant extracts, the aim of the present study was to screen the dose response micronuclei frequency of the extracts obtained from *C. moschata, C. mandiocanna* and *P. nitens*, using Trad-MCN bioassay.

MATERIAL AND METHODS

Plant Material

Cryptocarya mandioccana Meissner (Lauraceae) was collected at the Carlos Botelho State Park (São Paulo State, Brazil) in February, 2002. *Cryptocarya moschata* Nees (Lauraceae) was colleted at Mata da Mariana (Ibaté, São Paulo State, Brazil) in February, 2002. Leaves of Pterogyne nitens Tullasne (Fabaceae) were collected at Parque Estadual Fontes do Ipiranga (São Paulo, São Paulo State, Brazil) in March, 2003. Voucher specimens *C. mandioccana* and *P. nitens* (AGS13, Cavalheiro CB353, respectively) were deposited at the State Herbarium "Maria Eneida P. Kaufmann" of the Botanic Institute (SP) and *C. moschata* voucher specimen (Moraes 2347) was deposited in the Herbarium of ESALQ (ESA).

Preparation and chemical characterization of extracts

Shade-dried and powdered leaves of P. nitens (10.2 kg) were extracted by re-maceration with ethanol (3x) (Labsynth Ltda., São Paulo, SP, Brazil, 95%) and the combined extracts were concentrated under reduced pressure to yield 766.0 g of residue. The crude extract of *P. nitens* was analyzed by HPLC-DAD using a Phenomenex® ODS column (250 x 4.6 mm, 5 µ m) eluted with water (solvent A) and methanol (solvent B) in the gradient mode (5 to 100% B, in 60 min; flow rate of 1.0 mL/min). The chromatogram showed peaks with UV spectrum (λ_{max} = 237 nm) characteristic of terpenoid guanidine alkaloids (BOLZANI et al., 1995), typical for *P. nitens* leaves in addition to peaks with UV spectrum (λ_{max} = 257 and 349 nm) characteristic of flavonoid glucosides.

Dried and powdered leaves of *C. mandioccana* (3.6 kg) and *C. moschata* (2.3 kg) were extracted by sonication with ethanol (3x). After concentration under reduced pressure the combined ethanol extracts yielded 176.5 g for *C. mandioccana* and 73.8 g for *C. moschata*. The combined ethanol extracts yielded 176.5 g for *C. mandioccana* and 73.8 g for C. moschata (NEHME *et al.*, 2005). The crude extracts were analyzed by HPLC using a Supelcosil® LC18 coluMCN (250 x 4.6 mm, 5 µm) eluted with phosphoric acid-triethylamine (Labsynth Ltda., São Paulo, SP, Brazil, 95%) pH 2.0 (solvent A) and acetonitrile (Labsynth Ltda., São Paulo, SP, Brazil, 99.9%) (solvent B) in a gradient mode (15 to 60% B, in 60 min), followed by an isocratic step (60% B by 10 min) at a flow rate of 1.0 mL/min (NEHME *et al*., 2005). The chromatograms showed peaks with UV spectra characteristic of quercetin-glycosides (λ_{max} = 250 and 350 nm) and styrylpyrones (λ_{max} = 250 nm). All samples were dried under reduced pressure with silica before the experiment, reducing ethanol to non-significant levels. Therefore, the ethanol was removed into the extracts in levels incapable to interfere in Trad-MCN assay. This procedure is commonly used to obtain extracts from plants, which they will be submitted to biological assays (MESIA *et al*., 2008; OH *et al*., 2007).

Mutagenicity (Clastogenic and/or Aneugenic) assays

1. Extract Solubilization

Solutions of each plant extract were prepared in four different concentrations according to their solubility. The ethanol extract of *P. nitens* was solubilized in Tween 20 (20%) (Labsynth Ltda., São Paulo, SP, Brazil, 99.9% of purity) in four different concentrations (0.13, 0.5, 1.0, 2.0 mg/mL) whereas to solubilize the ethanol extract of *C. mandioccana*, a 5% ethanol solution (Labsynth Ltda., São Paulo, SP, Brazil, 95% of purity) and Tween 20 (20%) was prepared in four different concentrations (0.13, 0.5, 1.0, 2.0 mg/mL). The ethanol extract of *C. moschata* had lower solubility in Tween and needed to be solubilized in DMSO 3% (Labsynth Ltda., São Paulo, SP, Brazil, 99.9% of purity). Likewise, lower concentrations (0.03, 0.13, 0.25 and 0.5 mg/mL) of *C. moschata* extract were prepared. The results were normalized, subtracting the micronuclei frequency obtained in the vehicle controls from the frequency of test groups. Therefore, we excluded the potential inteference of vehicles into the evaluation of the mutagenic effect of the ethanolic extracts.

2. Tradescantia pallida Micronuclei Assay (Trad-MCN)

2.1. Sampling of Inflorescences and Treatment Procedures

Twenty young inflorescences of *T. pallida* were collected at random for each group (controls and test), immersed into Hoagland's solution, prepared as proposed by EPSTEIN (1975), and mantained in adaptation period for 24 hours under natural day light illumination. The pH of all

solutions was adjusted to a tolerable level to treat the inflorescences (5.5 to 8.5) and the inflorescences submitted to aeration during the experiment. Control groups were composed of (1) positive control (formaldehyde 10000 ppm), (2) negative control (Hoagland's solution), and (3) vehicle control (Tween 20 to 20% or DMSO 3%). For each test group, the inflorescences were treated with four diferent concentrations of *C. mandioccanna, C. moschata* and *P. nitens* for 8 hours. After the treatment, the cuttings were returned to clean Hoagland solution for a recovery period of 24 hours. At the end of recovery time, the inflorescences were fixed in ethanol-acetic acid (3:1 ratio, freshly prepared) for 48 hours and then transferred into 70% ethanol for storage in refrigerator for 2 weeks (MA, 1981).

2.2. Slide preparation and pollen mother cell counting

The slide preparation procedure was based on the acetocarmine squash method for plant chromosomes and the selection of appropriate buds in early tetrad stage was performed with a series of buds of an inflorescence as described by MA and colleagues (1983).

3. Statistical Analysis

The statistical analysis of the data was performed with the statistical program Bioestat 4.0 (Belem, PA, Brazil). Individual treatment groups were compared with the negative control group using Mann-Whitney U-test, and the results are indicated as means +/- S.E. Statistical significance was accepted as $p < 0,05$.

RESULTS

The analysis of the extracts of *Cryptocarya mandioccana, Cryptocarya moschata* and *Pterogyne nitens* by reverse phase HPLC-DAD indicated the major classes of secondary metabolites in the different samples. Retention times on the chromatograms (data not shown) and the UV profile of *Cryptocarya mandioccana and C. moschata* evidenced the presence of quercetin glycosides (λ_{max} at 250 and 350 nm) and styrylpyrones (λ_{max} = 250 nm). HPLC-DAD analyses of Pterogyne nitens extract analyses indicated presence of terpenoid guanidine alkaloids and

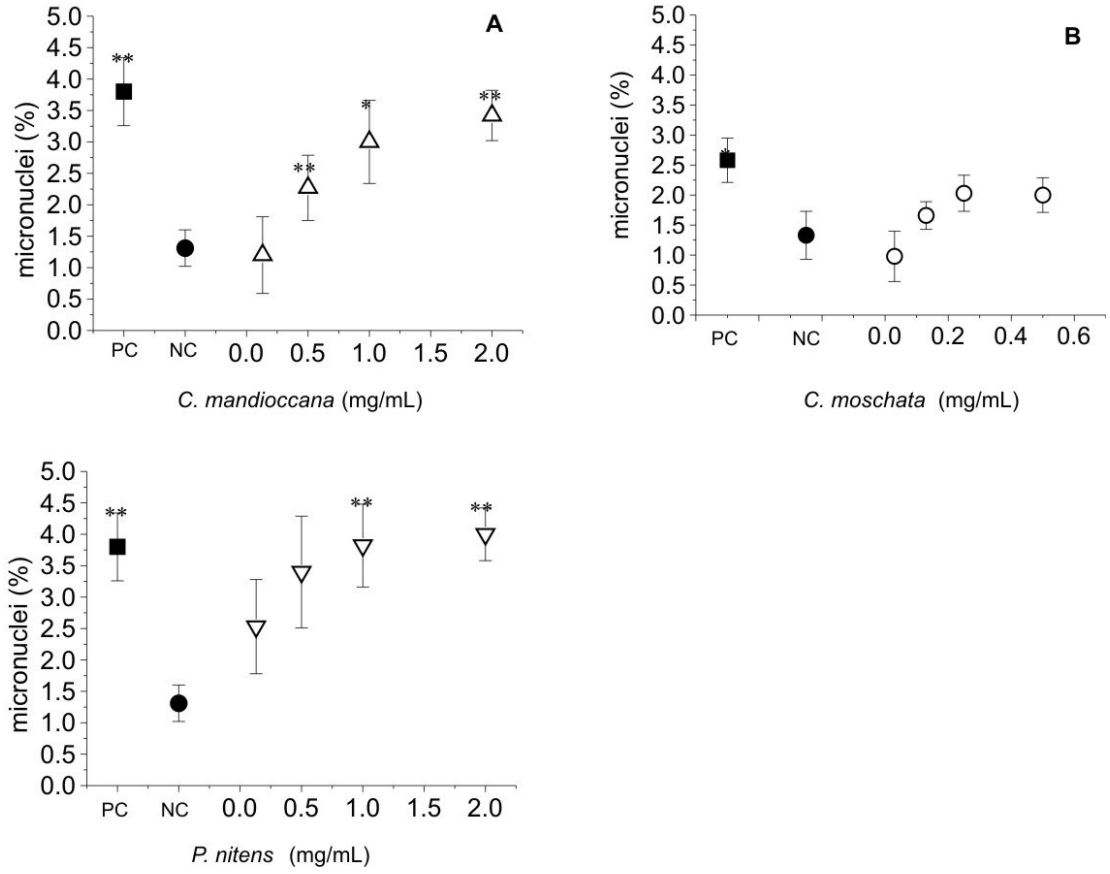

Figure 1. Mean values and standard error of the MCN in pollen mother cells of Tradescantia pallida treated with different concentrations of ethanol extracts followed by positive control (PC) (■) = formaldehyde 10000 ppm and negative control (NC) (●) = Hoagland solution. Mann Whitney for Independent Samples. A) Treatment with *C. mandioccana* (Δ) at 0.13, 0.5, 1.0, 2.0 mg/mL, ** p<0.01 (PC versus NC; NC versus 0.5, and 2.0 mg/mL) * p<0.05 (NC versus 1.0 mg/mL); B) *C. moschata* (○) at 0.03, 0.13, 0.25 and 0.5 mg/mL * p<0.05 (PC versus NC); C) Treatment with *P. nitens* (▽) at 0.13, 0.5, 1.0, 2.0 mg/mL, ** p<0.01 (PC versus NC; NC versus 1.0 and 2.0 mg/mL).

flavonoid glucosides, as major constituents, from their UV data with bands maxima at 237 nm and at 257 and 349 nm, respectively. *T. pallida* MCN Assay (Trad-MCN), showed mutagenicity (clastogenicity and/or aneugenicity) of extracts tested with dose-response effect (Figure 1). Figure 1A shows high frequency of micronuclei was observed to *C. mandioccana* treatment (0.5, 1.0 and 2.0 mg/mL), MCN +/- SE obtained in concentrations tested were (1.2+/-0.61, 2.27+/-0.52, 3.0+/-0.66, 3.42+/- 0.4). In contrast the micronuclei frequency to *C. moschata* treatment was no different to the negative control (non-treated pollen mother cells) and the number of MCN +/- SE in concentrations tested was (0.98+/-0.42, 1.66+/-0.23, 2.03+/-0.3, 2+/-0.29) (Figure 1B). Likewise to *C. mandioccana*, higher frequency of micronuclei was observed to *P. nitens* treatment at 1.0 and 2.0 mg/mL. For P. nitens the number of MCN +/- SE in four different concentrations evaluated was (2.53+/-0.75, 3.4+/-0.89, 3.82+/-0.66, 4.0+/-0.42) (Figure 1C).

DISCUSSION

To evaluate mutagenic effects of crude extracts isolated from Brazilian plant, we submitted the extracts to Trad-MCN assay. Although micronucleus mice assay is pretty standard for hazard analysis, the Trad-MCN assay has been considered a quick, simple and efficient method to evaluate clastogenic or mutagenic effects of many compounds including natural products (ZHANG *et al*., 1999). Moreover, the evaluation of MN in polen mother cells of Tradescantia pallida (Trad-MCN) has been described as an inexpensive bioassay for monitoring mutagenic effects (MA, 1981).

Our findings demonstrated that ethanolic extract of *C. moschata* did not present clastogenic effect in all concentrations. These data have important implications for analysis of risks associated with the popular use of *C. moschata*. Although the concentrations of the use of this plant by population are unknown, our data suggests that ethanolic extract of *C. moschata* in tested concentrations is not associated with enhanced risk of diseases associated with DNA damage like cancer.

In the present study, ethanolic extracts of *C. mandioccana* and *P. nitens* were clastogenic in higher concentrations, which might be associated with a potential anticancer activity. Clastogenic/aneugenic properties must be considered according the biological activity that you wish to target. When anticancer therapy is attempted, mutagenicity properties of commercial drugs and/or natural products are relevant. Several commercial anticancer drugs and natural products have been seen associated to DNA (adduct), which results in high cytotoxic and the tumor cells death (ISHIKAWA *et al*., 2003; SILVA *et al.*, 2005). Moreover, the DNA adducts of those compounds could trigger apoptosis signals in the tumor cells (KAWANISHI e HIRAKU 2004; HAVELKA *et al*., 2007; WHEATE *et al*., 2007). Conversely, clastogenicity can be harmful to the normal cells as well observed in several studies with compounds isolated from plants (JHOO *et al.*, 2007; SILVA *et al*., 2007). Then, the evaluation of mutagenicity of natural products should be seriously considered, once most of the natural products have popular use and, also, when their pharmacologic properties are investigated (SANNOMIYA *et al.*, 2007).

We can conclude that higher concentrations of the ethanolic extracts from *P. nitens* and *C. mandioccana* demonstrated clastogenic/aneugenic effect whereas ethanolic extract from *C. moschata* did not present these effects.

ABSTRACT

Although herbal extracts contain several classes of compounds with pharmacological activity, they also present toxic substances with mutagenic effects. The aim of the present study was to verify the mutagenicity of *Cryptocarya moschata, Cryptocarya mandioccana* and *Pterogyne nitens* using micronucleus assay in pollen mother cells (tetrads) in Tradescantia pallida (Trad-MCN). T. pallida inflorescences were treated with different concentrations of ethanolic extracts from the selected plant species. For *C. mandioccana C. moschata* and *P. nitens*, Trad-MCN assays were carried out simultaneoulsly, followed by positive control (formaldehyde 10000 ppm), negative control (Hoagland's solution), and vehicle control (Tween 20 20% or DMSO 3%). MCN present in tetrads were quantified in 300 tetrads/inflorescence and the mean (%) and standard error (SE) were established for at least 10 inflorescences per treatment. The extracts demonstrated dose response mutagenicity (clastogenic/aneugenic effects), respectively, *C. mandioccana* (0.5, 1.0 and 2.0 mg/mL) and *P. nitens* (1.0 and 2.0 mg/mL) However, no mutagenic effect was observed to *C. moschata* at the concentrations evaluated in the present study. We can conclude that the *C. mandioccana* and *P. nitens* extracts demonstrated clastogenic/aneugenic effects in highest concentrations whereas *C. moschata* extract did not demonstrate the same effect.

Keywords: Clastogenic, aneugenic, *Cryptocarya mandioccana, Cryptocarya moschata, Pterogyne nitens*, Trad-MCN.

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