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Genotoxic effect of *Physalis angulata* L. (Solanaceae) extract on human lymphocytes treated *in vitro*

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ABSTRACT: *Physalis angulata* L (Solanaceae) is a medicinal plant from North of Brazil, whose different extracts and infusions are commonly used in the popular medicine for the treatment of malaria, asthma, hepatitis, dermatitis and rheumatism. However, the genotoxic effects of *P. angulata* on human cells is not well known. The main purpose of the present study was to evaluate the *in vitro* genotoxic effects of aqueous extract of *P. angulata* using the comet assay and the micronucleus assay in human lymphocytes provided from 6 healthy donors. Treatments with *P. angulata* extracts were performed *in vitro* in order to access the extent of DNA damage. The comet assay has shown that treatments with *P. angulata* at 0.5, 1.0, 2.0, 3.0 and 6.0 μg/mL in culture medium were genotoxic. Lymphocytes treated with *P. angulata* at the concentrations of 3.0 and 6.0 μg/mL in culture medium showed a statistically significant increase in the frequency of micronucleus ($p<0.05$), however, the cytokinesis blocked proliferation index (CBPI) was not decreased after *P. angulata* treatment. In conclusion, the present work demonstrated the genotoxic effects of *P. angulata* extract on human lymphocytes *in vitro.*

Introduction

The plant kingdom is vast and the antique use of plants to treat various diseases in human beings is not well known. There are a considerable number of natural products used in the traditional medical systems in many countries as alternative medicine for the treatment of various diseases (Choi and Hwang, 2003). Many of these medicinal plants provide relief of symptoms comparable to that obtained from allopathic medicines (Choi and Hwang, 2003). One of these plants, *Physalis angulata*, known in Brazil as "camapú" or "balãozinho", is a branched annual shrub that belongs to the Solan-

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aceae family (Januário *et al.,* 2002). This plant is widely distributed throughout tropical and subtropical regions of the world and its extracts or infusions have been used in many countries in popular medicine in the treatment of a variety of diseases such as malaria, asthma, hepatitis, dermatitis and rheumatism (Chiang *et al.*, 1992a; Lin *et al.*, 1992; Soares *et al.*, 2003). Some *in vitro* studies showed that purified compounds of *P. angulata* such as physalins (A, B, D and F) and glycosides (e.g. Myricetin-3-O-neohesperidoside) isolated form organic fractions of *P. angulata* exhibited antitumoral activities on HA22T (hepatoma), HeLa (cervix uteri), leukemia, lung adenocarcinoma and epidermoid carcinoma of the nasopharynx KB-16 cell lines (Chiang *et al.*, 1992a,b; Ismail and Alam, 2001; Soares *et al.*, 2003).

The number of evidences related to the biological effects of plant extracts is constantly increasing. The composition of these natural extracts that apparently exhibit only benefic properties may include chemical components with mutagenic, teratogenic and/or carcinogenic activities. If a genotoxic compound is present, it can interact with DNA molecule, leading to genetic damage in regions of fundamental importance to cycle control and apoptosis, giving rise to a neoplasic process. Thus, it is very important the inclusion of a genotoxic approach in toxicological evaluation of therapeutic compounds.

A great number of endpoints are commonly used in toxicological genetics. The alkaline version of "single cell gel electrophoresis", or comet assay, has been used by many investigators to evaluate *in vitro* and/or *in vivo* genotoxicity of several chemicals and provides a direct determination of the single and double-stranded DNA breaks in the response of individual cells (Singh *et al.*,1988). On the other hand, the cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring micronuclei (MN) in cultured human cells because scoring is specifically restricted to once-divided cells (Fenech and Crott, 2002). MNs are fragments originated from chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division (Fenech, 2000).

In the present report we examined the *in vitro* cytotoxic and genotoxic activities of aqueous extract from *Physalis angulata* using temporary cultures of human lymphocytes. The frequencies of MN in binucleated cells, the nuclear division index and the extension of DNA damages detected by comet assay were analyzed. As far as we know, to date there are no data in the literature about genotoxic or mutagenic activities of *P. angulata* on human cells.

Materials and Methods

Plant extract and chemicals

P. angulata specimens were collected in Belém, Pará State, Brazil, and identified (Herbarium, Neurochemistry Laboratory, Department of Physiology, University Federal of Pará, Voucher number 15). 150g from *P. angulata* roots were washed in water and boiled in 700 mL of ultrapure water (Milli-Q plus), remaining only 14% from the initial volume that was frozen (-20ºC), lyophilized and 2.712g of extract were obtained. This extract was dissolved in distilled water and filtrated in sterile 0.22 μm Milipore7 filter. This solution was kept at 4ºC and protected from light incidence until use.

Bleomycin (CAS 9041-93-4) was purchased from Biosintética (Brazil) and used in the micronuclei assay as a positive control, and Doxorubicin (CAS 25316-40-9) was purchased from Laboratórios Eurofarma (São Paulo, Brazil) and used as a positive control in comet assay. Cytochalasin B (CAS 14930-96-2) was purchased from Sigma (St. Louis, MO, USA).

Cell culture and treatments

Genetic study was approved by the Universidade Federal do Triângulo Mineiro´s Ethics Committee (Protocol no. 440) and Hospital Universitário João de Barros Barreto de Belém-PA Ethics Committee, Brazil. Human peripheral blood lymphocytes were used for *in vitro* comet assay and micronucleus assay. Lymphocytes were obtained from six healthy, non-smoking volunteers, three males and three females aged 18-30 years with their completed informed consent. Heparinized total blood (0.5 mL) was added to 4.5 mL medium, containing 78% RPMI 1640 (Sigma Chemical Co., USA), 20% inactivated fetal bovine serum (Gibco-Invitrogen, Denmark), antibiotics (penicillin and streptomycin), and stimulated with 2% of phytohemagglutinin (PHA; Gibco-Invitrogen, Denmark).

Preliminary tests were carried out in order to determine the concentrations of the extract to be used on cell cultures. Concentrations above 6.0 μg/mL culture medium inhibited cell viability detected by Trypan Blue Exclusion Dye (at least 70%) for comet assay. Therefore, the final concentrations of the extract in culture media were 0.1, 0.5, 1.0, 2.0, 3.0 and 6.0 μg/mL for the following experiments.

For comet assay, cells were treated with different concentrations of *P. angulata* (0.1, 0.5, 1.0, 2.0, 3.0 and 6.0 μg/mL of culture medium) 6 h after culture initia-

tion and treatment with doxorubicin in positive control (0.15 μg/mL) was performed after 24 h culture since this antitumoral agent is very cytotoxic and disturbs cell cycle (Islaih *et al*., 2005). In micronucleus assay, cells were exposed to different concentrations of the extract at 24 h culture. With the blood sample from each volunteer, seven cultures were prepared, and treated with the extract (0.5, 1.0, 2.0, 3.0, and 6.0 μg/mL in culture medium). The tested concentrations were established also in preliminary experiments. A negative control untreated culture and a positive control treated with 1.5 μg/mL bleomycin were also performed.

Comet assay

After 48 h at 37 ºC, the alkaline version of comet assay was performed as described by Singh *et al.* (1988). Slides were prepared in duplicate and 100 cells were screened per sample (50 cells from each slide) in a fluorescent microscope (Zeiss, Germany) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm using a 40X objective. The nucleus were classified visually according to fragments migration in: class 0 (no damage); class 1 (little damage with a short tail of length smaller than the diameter of the nucleus); class 2 (medium damage with a tail length one or two times the diameter of the nucleus); class 3 (large damage with a tail length between two times and half and three times the diameter of the nucleus); class 4 (great damage with a long tail of damage superior to three times the diameter of the nucleus) (Maistro *et al*., 2004).

Micronuclei assay

Cytochalasin B (6 μg/mL) was added at 44 h. After 72 h at 37ºC, cells were collected by centrifugation, rinsed and submitted to a mild hypotonic (1% sodium citrate) treatment and immediately fixed with methanol:acetic acid. Slides were prepared according to standard cytogenetic procedures and staining with 4% Giemsa.

Slides were coded and scored by light microscopy at 400X or 1000X magnification as necessary. For each experiment, 2,000 binucleated lymphocytes with well-preserved cytoplasm were scored. Micronuclei were identified according to the criteria of Fenech *et al*. (2003). As a measure of cytotoxicity, the cytokinesis-block proliferating index (CBPI) was calculated according to the following formula: CBPI = [MI+2MII+3(MIII+MIV)]/*N* as proposed by Surrallés *et al*. (1995), where MI-MIV represent the number of human lymphocytes with 1-4 nuclei determined on 500 cells.

TABLE 1.

DNA damage index, frequencies of cells with comets and distribution of comet classes in human lymphocytes treated *in vitro* with different concentrations of *Physalis angulata* L. extract. 600 nuclei/treatment were analyzed.

Treatments μg/mL	Comet classes (%) 0	1	2	3	4	DNA damage index ± SD	Cells with comet (%)
Negative control (n=6)	57.2	32.2	8.2	2	0.4	0.51 ± 0.19	42.8
Positive control* (n=6)	32	29.7	32	3	3.3	1.15[a] ± 0.19	73.8[a]
Extract							
0.1 (n=6)	50.2	34	2.2	3.6	0.2	0.75 ± 0.14	49.8
0.5 (n=6)	38.2	41	16.2	4.2	0.4	0.87[a] ± 0.28	61.8
1.0 (n=6)	32.6	45.2	16.8	4.2	0.4	0.93[a] ± 0.33	69.4
2.0 (n=6)	38.6	33.4	22.2	4.2	1.6	0.90[a] ± 0.26	61.4
3.0 (n=6)	40	33.8	19.4	5	1.8	0.95[a] ± 0.12	60.0
6.0 (n=6)	42.7	28	20	8.3	1	1.14[a] ± 0.65	57.3

[a] statistically different from control group ($p<0.05$)
SD: standard deviation
* DXR: 0.15μg/mL

Statistical analysis

For micronuclei assay the results were tabulated and experimental values were expressed as mean ± SD (standard deviation). The statistical significance comparing data between different treatment groups was assessed by one-way Anova and student t test. A P value of <.05 was considered significant.

The one-way Anova for repeated measures followed by Newman-Keuls post-test was applied for comet assay results comparing the different treatment groups in six independent repetitions. The level of significance set was α=0.05 (Bailar and Mosteller, 1992).

Results

Since differences between sexes were not observed, statistical analysis considered males and females together.

Table 1 presents data from comet assay analysis, i.e., distribution of different comet class, DNA damage index and frequency of cells with comet. Treatments with the four highest extract concentrations significantly increased the frequency of cells with comets and the DNA damage index when compared to the negative control ($p<0.05$). Distribution of comets among classes 1, 2, 3 and 4 was also increased by the increasing doses of the extract (0.5, 1.0, 2.0, 3.0 and 6.0 μg/mL) and class 1 and 2 was most frequently observed.

Data of micronucleus analysis in binucleated lymphocytes are presented in Table 2. Results of total binucleated cells with micronucleus and total micronucleus after treatment with the test compound in a large range of concentrations are presented in Table 3. Treatments with the four lowest concentrations did not show significant variations in the total of binucleated cells with micronucleus, when compared to negative control ($p>0.05$). Lymphocytes treated with *Physalis* extract at the concentrations of 3.0 and 6.0 μg/mL culture medium showed a statistically significant increase in total binucleated cells with micronucleus ($p<0.05$). In general, one micronucleus was found in each binucleated cell, but cells with two or three micronuclei were also observed (Table 2).

The percentage of binucleated cells was not altered by treatments, when compared to the negative control (Table 3). As regard to CBPI, the results indicate that *Physalis* extract was not cytotoxic, and no significant overall difference was found between treatments and negative control (Table 3).

TABLE 2.

Induction of micronuclei in human lymphocyte cultures treated with extract of *Physalis angulata* L. (PAE)

Treatments μg/mL	Total of BN with MN	Total of MN	Distribution of MN in BN			
			0MN	1MN	2MN	3MN
Negative control (n=6)	23	25	11977	21	2	0
Positive control* (n=6)	245	274	11755	219	23	3
PAE (n=6)						
0.1 (n=6)	25	26	11975	24	1	0
0.5 (n=6)	23	27	11977	19	4	0
1.0 (n=6)	26	26	11974	26	0	0
2.0 (n=6)	34	37	11966	32	1	1
3.0 (n=6)	38[a]	40	11962	36	2	0
6.0 (n=6)	37[a]	41	11963	34	2	1

12000 cells analyzed per treatment; BN: binucleated cells; MN: micronuclei

*Bleomycin: [BLM] = 1.5 μg/ml (10 μl/culture)

[a] $P<0.05$ treated vs. negative control

Discussion

Approximately 30% of drugs available worldwide are based on natural products (Canalle *et al*., 2001). The active principles in extracts from a variety of plant sources from tropical and sub-tropical regions of the world are studied for possible applications in human health (Aruoma, 2003). *Physalis angulata* is an important herb used in traditional medicine with biological effects against malaria, asthma, hepatitis, dermatitis and rheumatism. In the present work the genotoxic and cytotoxic effects of its extract was evaluated *in vitro* using human lymphocytes.

The *Physalis* extract was not cytotoxic in the present study. The distribution of cells according to the number of nuclei observed on CBMN in untreated control was similar to that observed in the treated cultures. Wu *et al*. (2004) had observed that the *Physalis* extract did not produce cytotoxic effect towards mouse BALB/C normal liver cells. Other studies suggested the cytotoxic effects of *Physalis* compounds. Physalins B, F, and G purified from *Physalis* extract have potent immunosuppressive activities in macrophages and in lypopolysaccharide-induced shock in mice and trypanocidal activity against both epimastigotes and trypomastigotes of *Trypanosoma cruzi in vitro* probably due to its cytotoxic activity (Soares *et al*., 2003; Nagafuji *et al*., 2004). Magalhaes *et al*. (2006) observed that physalins B and D displayed considerable citotoxicity against several cancer cell lines, but the tested concentrations were in the range of 0.58 to 15.18 μg/mL, much higher than those in the present study. In our study, concentrations above 6.0 μg/mL were cytotoxic, in agreement with the data of Kuo *et al*. (2006) reporting the citotoxicity of physalins U and V in concentrations nearby 4 μg/mL on tumor cell lines.

In the present work, the genotoxic effects of the *Physalis* extract, once it showed the ability to induce DNA damage, were shown. Treatments with 0.5, 1.0, 2.0, 3.0 and 6.0 μg/mL increased significantly DNA lesions detected with the comet assay. However, it was observed that only 3.0 and 6.0 μg/mL of the extract increased the frequency of binucleated cells with micronuclei. This difference in the response to the comet assay and in micronucleus assay is not surprising since the comet assay is a rapid, simple and highly sensitive method for the detection of single and double DNA strand breaks and alkali-labile sites. The comet assay is not used to detect mutations, but to detect genomic lesions that could lead to a mutation (Gontijo and Tice, 2003). On the other hand, micronuclei are more drastic lesions revealed as small extranuclear bodies originated in mitosis from acentric chromosome fragments or whole chromosomes that are not included in the main nuclei following DNA replication and nuclear division (Schmid, 1973; Fenech, 1997).

TABLE 3.

Induction of cytotoxicity in human lymphocyte cultures treated with extract of *Physalis angulata* L.

Treatments	Distribution of cells according to number of nuclei				%BN	CBPI ± SD
μg/mL	1	2	3	4		
Negative control (n=6)	1227	1438	191	144	47.93	1.742 ± 0.31
Positive control* (n=6)	1852	1019	71	58	33.96	1.426 ± 0.18
PAE (n=6)						
0.1 (n=6)	1157	1584	136	123	52.80	1.701 ± 0.28
0.5 (n=6)	1284	1442	138	136	48.06	1.661 ± 0.27
1.0 (n=6)	1291	1496	119	94	49.86	1.642 ± 0.24
2.0 (n=6)	1186	1514	151	149	50.46	1.705 ± 0.22
3.0 (n=6)	1289	1517	121	73	50.56	1.635 ± 0.27
6.0 (n=6)	1199	1500	163	138	50.00	1.817 ± 0.14

12000 cells analyzed per treatment; %BN: percentage of binucleated cells; SD: standard deviation. CBPI: cytokinesis blocked proliferation index {[M1 + 2(M2) + 3(M3 + M4)]/ N}
*Bleomycin: [BLM] = 1.5 μg/ml (10 μl/culture)

The fact that some plants may have genotoxic effects depends on various compounds present in the extracts. Some previous studies reported physalins B, F and D as bioactive compounds of the genus *Physalis* (Chiang *et al*., 1992a,b; Ismail and Alam, 1992). These compounds may influence mitochondria dysfunction producing large amount of hydrogen peroxide, an important reactive oxygen species that damages the DNA molecule (Wu *et al*., 2004). It was also observed that the *Physalis* extract presents some possible direct effect on the DNA molecule. The *Physalis* extract induced a cell cycle arrest in the G2/M phase in MDA-MB 231 cells inhibiting synthesis or mRNA stability and their downstream protein levels of cyclin A and cyclin B1, increasing $p21^{waf1/cip1}$ and $P27^{kip1}$ levels (Hsieh *et al*., 2006).

In conclusion, results of the present work clearly showed that the *Physalis* extract was not cytotoxic but exhibited important genotoxic effects under our experimental conditions, indicating that its use requires caution, since its genotoxic effects *in vivo* have not been evaluated yet.

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