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In-vitro and in-vivo antitumour activity of physalins B and D from *Physalis angulata*

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Abstract

We have evaluated the in-vitro and in-vivo antitumour activity of physalin B and physalin D isolated from the aerial parts of *Physalis angulata*. In-vitro, both compounds displayed considerable cytotoxicity against several cancer cell lines, showing IC50 values in the range of 0.58 to 15.18 μg mL^{-1} for physalin B, and 0.28 to 2.43 μg mL^{-1} for physalin D. The antitumour activity of both compounds was confirmed in-vivo using mice bearing sarcoma 180 tumour cells. The in-vivo antitumour activity was related to the inhibition of tumour proliferation, as observed by the reduction of Ki67 staining in tumours of treated animals. Histopathological examination of the kidney and liver showed that both organs were affected by physalin treatment, but in a reversible manner. These compounds were probably responsible for the previously described antitumour activity of ethanol extracts of *P. angulata*, and their identification and characterization presented here could explain the ethnopharmacological use of this species in the treatment of cancer.

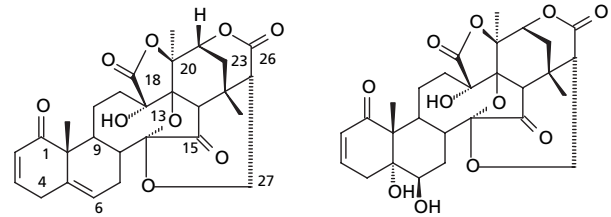
Introduction

Physalis angulata L. (Solanaceae), popularly known as 'camapum', is an annual herb that is distributed widely in the north and northeast regions of Brazil (Lorenzi & Matos 2002). Its known ethnopharmacological applications are anti-cancer, diuretic, anti-inflammatory, sedative, depurative and anti-septic (Lorenzi & Matos 2002). Crude extracts of this plant and several isolated secondary metabolites have been evaluated for therapeutic potential during the past decade and have been found to possess antibiotic, anti-inflammatory, immunomodulatory, antitumour, trypanocidal, molluscicidal and antiviral properties (Chiang et al 1992a, b; Lin et al 1992; Choi & Hwang 2003; Santos et al 2003; Soares et al 2003; Nagafuji et al 2004).

Concerning the anti-cancer potential of *P. angulata*, several studies have been performed showing that organic extracts and some purified compounds possess cytotoxic activity against several tumour cell lines (Chiang et al 1992a, b; Tomassini et al 2000; Soares et al 2003; Wu et al 2004). The cytotoxicity of this plant has been attributed to the physalins, compounds characterized as withasteroids, a series of C_{28}-steroidal lactones structurally based on the ergostane skeleton which are commonly produced by Solanaceae plants (Glotter 1991; Cárdenas et al 1994). Chiang et al (1992a) showed that physalins B and F, both isolated from *P. angulata*, inhibited the growth of several human leukaemia cells (K562, APM1840, HL-60, KG-1 and CTV1) and B cells, where physalin F was found to be more potent. Chiang et al (1992b) examined the cytotoxicity of physalins F and D in various cancer cell lines: KB (nasopharynx), HeLa (cervix uteri), Calu-1 (lung), HA22T (hepatoma), Colo205 (colon), Hep-2 (laryngeal), H1477 (melanoma) and 8401 (glioma), where physalin D was found to be inactive. Fang et al (2003) demonstrated the cytotoxicity of physalins B, H and F against a panel of human and murine cancer cell lines, and lately, Lee & Houghton (2005) showed that this toxicity was non-selective between normal and cancer cell lines. The in-vivo antitumour activity of physalins B and F in mice inoculated with leukemia cells has also been reported (Antoun et al 1981; Chiang et al 1992b). Recently, Wu et al (2004)

demonstrated the in-vitro anti-hepatoma activity of *P. angulata* and *P. peruviana* extracts.

In this study, the in-vitro and in-vivo antitumour activity of physalin B and D, both isolated from *P. angulata*, were demonstrated against a panel of tumour cell lines and sarcoma 180 tumour transplanted in mice. The histopathological and morphological examination of the tumour and the animal organs, including liver, spleen and kidney, were performed to determine toxicological aspects of physalin treatment.



1 **2**

Figure 1 Structures of physalin B (**1**) and physalin D (**2**) isolated from *Physalis angulata*.

Materials and Methods

Plant material

Specimens of the same population of *P. angulata* were collected during the flowering stage in June 2003 at Pentecoste County, State of Ceará, Brazil, and identified by Professor Edson P. Nunes, Departamento de Biologia, Universidade Federal do Ceará. A voucher specimen (number 33.576) is preserved at the Herbário Prisco Bezerra (EAC), Universidade Federal do Ceará.

Extraction and isolation

Aerial parts of *P. angulata* (4.3 kg) were ground to a coarse powder and percolated with hexane (2×10 L) followed by EtOH (2×10 L) at room temperature. The hexane and EtOH extracts were filtered and concentrated under reduced pressure to yield 1.5 and 70.0 g of crude extracts, respectively. The EtOH extract was fractioned over Si gel using hexane, CH_2Cl_2, EtOAc and MeOH as eluents. An amorphous precipitate was formed in the CH_2Cl_2 fraction, which after filtration yielded 2.0 g of white powder. This material was passed through a Si gel column and elution was initiated with hexane followed by solvents with progressively increasing polarity: hexane–CH_2Cl_2 (8:2, 6:4, 4:6, 2:8), CH_2Cl_2, CH_2Cl_2–EtOAc (9:1, 8:2, 6:4, 1:1), AcOEt and finally MeOH. Physalin D (1.1 g) was isolated from the fractions obtained by elution with CH_2Cl_2–EtOAc (9:1). The CH_2Cl_2 solution was evaporated to dryness in-vacuo to give a brown residue (7.0 g) which was further chromatographed on Si gel, eluting with hexane, hexane–EtOAc (9:1 to 2:8), EtOAc, EtOAc–MeOH (8:2, 6:4 and 4:6) and finally MeOH to give 14 subfractions. Physalin B (167 mg) was obtained from subfraction 5 (eluted with hexane–EtOAc 6:4) after recrystallization from MeOH.

The structures of physalins B and D (Figure 1) have been determined by spectroscopic means, including 1D (^{1}H and ^{13}C NMR, DEPT 135) and 2D NMR (^{1}H, ^{1}H COSY, HMQC, HMBC), physical properties and comparison with published data (Chiang et al 1992a, b).

Animals and tumour cells

In-vitro cytotoxicity was determined against nine tumour cell lines (National Cancer Institute, Bethesda, MD, USA): B-16 (murine skin), HCT-8 (human colon), PC3 (human prostate) MDA-MB-435, MDA-MB-231 and MCF-7 (human breast), K562, CEM and HL-60 (human leukaemia). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin at 37°C with 5% CO_2.

A total number of 53 Swiss mice (male, 20–30 g) obtained from the central animal house of the Federal University of Ceará, Brazil were used. Animals were housed in cages with free access to food and water. All animals were kept under a 12-h light–dark cycle (lights on at 0600 h). Animals were treated according to the ethic principles of animal experimentation of COBEA (Colégio Brasileiro de Experimentação Animal), Brazil. The Animal Studies Committee of Federal University of Ceará approved the experimental protocol.

Sarcoma 180 tumour cells were maintained in the peritoneal cavity of Swiss mice obtained from the central animal house of the Federal University of Ceará.

Cell viability

Tumour cell growth was quantified by the increase in cells capable of reducing the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a purple formazan product (Mosmann 1983). For the experiments, cells were plated in 96-well plates (10^5 cells/well for adherent cells or 3×10^5 cells/well for suspended cells in 100 μL medium). After 24 h, physalins B and D (0.039 to 25 μg mL^{-1}) dissolved in dimethyl sulfoxide (DMSO; 1%) were added to each well and the cells incubated for three days (72 h). Control groups received the same amount of DMSO. Doxorubicin was used as the positive control. Thereafter, the plates were centrifuged, and the medium was then replaced by fresh medium (200 μL) containing 0.5 mg mL^{-1} MTT. Three hours later, the MTT formazan product was dissolved in 150 μL DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). Drug effect was quantified as the percentage of control absorbance of reduced dye at 550 nm.

Assay of antitumour activity

Six-day-old sarcoma 180 ascites tumour cells (4×10^6 cell/500 μL) were implanted subcutaneously into the right hind groin of the mice. One day after inoculation, physalins B or D (10 and 25 mg kg^{-1}) or fluorouracil (25 mg kg^{-1}) were dissolved in 10% DMSO and administered intraperitoneally for seven days. The negative control was injected with saline plus 10% DMSO. On day 10, the mice were killed by decapitation. The tumour, liver, spleen and kidney were extirpated, weighed and fixed in 10% formaldehyde. Inhibition ratio (%) was calculated by the following formula:

inhibition ratio (%) = [(A − B)/A] × 100, where A is the tumour weight average of the negative control, and B is that of the treated group.

Histopathology and morphological observations

After being fixed with 10% formaldehyde, the tumour and organs were grossly examined for size or colour changes and haemorrhage. A portion of the tumour, liver, spleen and kidney were cut into small pieces, and histological sections were prepared and stained with hematoxylin and eosin. Histological analyses were performed by light microscopy. The presence and the extension of liver lesions attributed to drugs were considered suspect liver damage.

Ki67 immunohistochemical detection

Tumour sections were de-paraffinized with xylene and dehydrated with ethanol. The slides were then immersed in water for 10 min. For antigen retrieval, the slides were boiled in citrate buffer (pH 6.0) for 15 min in a microwave and subsequently cooled for 20 min. The slides were washed in phosphate-buffered saline (PBS), and endogenous peroxidase was then blocked by 0.3% hydrogen peroxide for 15 min. After washing with PBS, the sections were incubated overnight at 4°C with a mouse antibody to Ki67 at a 1:60 dilution. After 24 h, the slides were washed and incubated with a multilink antibody for 20 min, washed in PBS and incubated for 20 min with avidin–biotin–peroxidase complex. After washing with PBS, the slides were incubated for 3 min with diaminobenzidine, and finally counter-stained with hematoxylin before mounting. The percentage of proliferating neoplastic cells was determined directly by light microscopy. Cell proliferation rate was based on the number of Ki67-positive cells counted in four random fields per slide.

Statistical analysis

Data are presented as means ± s.e.m. The IC50 values and their 95% confidence intervals (CI 95%) were obtained by nonlinear regression using the GRAPHPAD program (Intuitive Software for Science, San Diego, CA, USA). The differences between experimental groups were compared by analysis of variance followed by Student Newman Keuls test ($P < 0.05$).

Results

In-vitro antitumour activity of physalin B and D were determined against several animal tumour cell lines: CEM, HL-60, K562, HCT-8, MCF-7, MDA-MB-435, MDA-MB-231, PC3 and B-16. Table 1 shows the IC50 values obtained. A significant suppression of cell growth was detected in the presence of both tested compounds with physalin D showing greater activity. The IC50 ratio for physalin B to physalin D varied from 1.58 on HL-60 cells to 18.86 on MDA-MB-435 cells.

Effects of physalins B and D on mice transplanted with sarcoma 180 tumours are shown in Table 2. Both compounds inhibited tumour growth. On day 10, the tumour weight average of control mice was 3.42 ± 0.24 g, while in the presence of physalin B it was 1.90 ± 0.40 and 1.68 ± 0.24 g at the doses of 10 and 25 mg kg^{-1}/day, respectively, and in the presence of physalin D was 1.86 ± 0.47 and 1.89 ± 0.29 g at the same doses. The tumour growth inhibition ratios were 44.44–50.87% and 45.61–44.73% for physalins B and D, respectively. There were no statistically significant differences among different compounds or different doses for the same compounds ($P > 0.05$). Fluorouracil reduced tumour weight by 71.05%.

After treatment with physalins B and D (25 mg kg^{-1}/day), the kidney weight was significantly reduced ($P < 0.05$), while there were no differences for liver and spleen (Table 2). Histopathological analyses of the kidneys from treated animals showed a discrete hydropic change in the proximal tubular epithelium and glomerular and tubular haemorrhage, but the structure of the glomeruli was essentially preserved. Besides the toxic effects observed in the kidney, histopathological analyses indicated that the liver was also a target organ for physalin toxicity, where the effects observed in the presence of physalin B (Figure 2C) were more intense than with physalin D

Table 1 Cytotoxicity of physalins B and D in tumour cell lines. Doxorubicin was used as the positive control

Cell line	Doxorubicin IC50 μg mL^{-1} (μM)	Physalin B IC50 μg mL^{-1} (μM)	Physalin D IC50 μg mL^{-1} (μM)
CEM	0.02 (0.04) 0.02–0.03	0.63 (1.23) 0.54–0.73	0.28 (0.51) 0.25–0.31
HL-60	0.02 (0.03) 0.01–0.02	0.87 (1.71) 0.59–1.29	0.55 (1.01) 0.42–0.74
K562	0.14 (0.24) 0.09–0.23	4.70 (9.21) 2.88–7.67	0.75 (1.38) 0.67–0.85
HCT-8	0.01 (0.02) 0.01–0.02	0.58 (1.14) 0.39–0.86	0.34 (0.62) 0.30–0.39
MCF-7	0.20 (0.34) 0.17–0.24	1.03 (2.02) 0.98–1.08	0.43 (0.79) 0.35–0.53
MDA-MB-435	0.48 (0.83) 0.34–0.66	7.92 (15.53) 6.13–10.22	0.42 (0.77) 0.28–0.63
MDA-MB-231	0.22 (0.38) 0.18–0.26	7.05 (13.82) 5.23–9.49	0.59 (1.08) 0.47–0.75
PC3	0.24 (0.41) 0.21–0.27	15.18 (29.76) 12.46–18.50	2.43 (4.47) 2.17–2.68
B-16	0.03 (0.06) 0.02–0.04	0.61 (1.20) 0.54–0.70	0.31 (0.57) 0.25–0.37

Data are presented as IC50 values with 95% confidence interval for leukemia (HL-60, CEM and K562) and colon (HCT-8), breast (MCF-7; MDA-MB-435 and MBA-MD-231), prostate (PC3) and skin (B-16) cancer cells. Experiments were performed in triplicate.

Table 2 Tumour growth inhibition rate in mice transplanted with Sarcoma 180 tumour and treated with physalins B and D

Drug	Dose (mg kg^{-1}/day)	Liver (g)	Spleen (g)	Kidney (g)	Tumour (g)	Inhibition (%)	n
Control	-	2.00 ± 0.13	0.29 ± 0.02	0.42 ± 0.02	3.42 ± 0.24	-	10
Fluorouracil	25	1.53 ± 0.10[a]	0.16 ± 0.02[a]	0.32 ± 0.01[a]	0.99 ± 0.25[a]	71.05	8
Physalin B	10	1.87 ± 0.08	0.28 ± 0.03	0.40 ± 0.01	1.90 ± 0.40[a]	44.44	11
	25	1.81 ± 0.09	0.25 ± 0.03	0.31 ± 0.01[a]	1.68 ± 0.24[a]	50.87	9
Physalin D	10	1.89 ± 0.09	0.32 ± 0.04	0.40 ± 0.02	1.86 ± 0.47[a]	45.61	11
	25	1.76 ± 0.12	0.29 ± 0.02	0.35 ± 0.02[a]	1.89 ± 0.29[a]	44.73	8

Data are presented as means ± s.e.m. for n experiments. Significant differences from control group were determined by analysis of variance followed Student Newman Keuls: [a]$P < 0.05$.

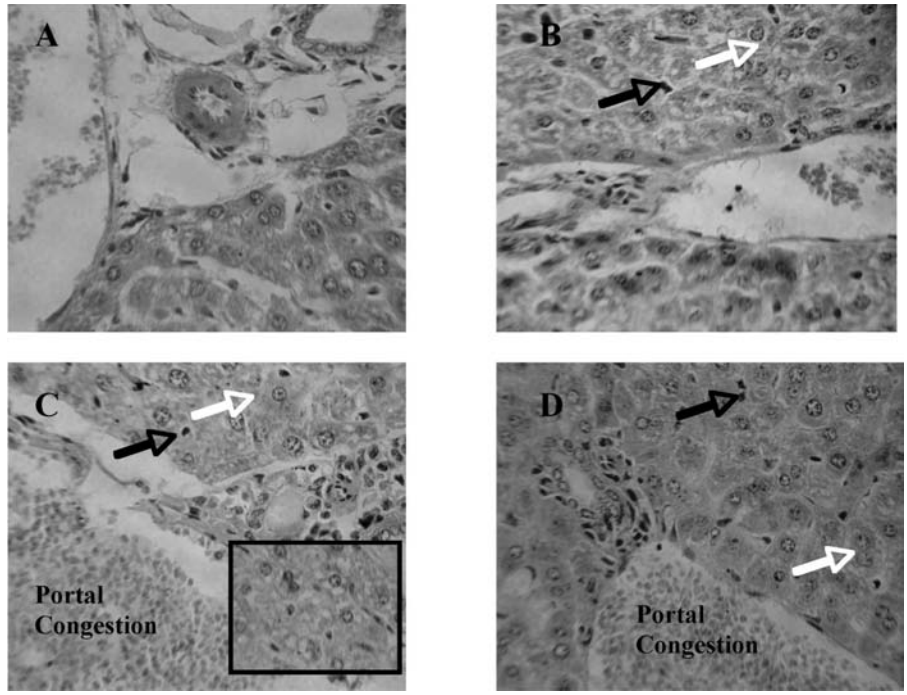


Figure 2 Histopathology of the liver from mice transplanted sarcoma 180 tumour. A. Control group. B. Fluorouracil (25 mg kg^{-1})-treated group. C. Physalin B (25 mg kg^{-1})-treated group. D. Physalin D (25 mg kg^{-1})-treated group. Microvesicular steatosis induced by physalin B treatment is shown on the detail of part C. Black arrows indicate Kupffer cells hyperplasia and white arrows indicate ballooning degeneration of hepatocytes.

(Figure 2D). These effects included Kupffer cell hyperplasia, portal tracts and centriolobular venous congestion, focal infiltrate of chronic inflammatory cells, intense ballooning degeneration of hepatocytes, microvesicular steatosis and sinusoidal haemorrhage, although rare areas of steatosis were observed with physalin D treatment (Figure 2D). As expected for a chemotherapeutic drug, fluorouracil produced a decrease in liver, spleen and kidney weights ($P < 0.05$, Table 2).

Histopathological analyses of the tumours extirpated from control mice showed groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei and binucleation (Figure 3A). Several degrees of cellular and nuclear pleomorphism were seen. Mitosis, muscle invasion and coagulative necrosis were also noticed. In the tumours extirpated from animals treated with fluorouracil and physalins B and D, there were extensive areas of coagulative necrosis (Figure 3B, C and D).

Ki67 staining for cell proliferation was performed in the tumours removed on day 10 from untreated animals and those treated with fluorouracil (25 mg kg^{-1}/day), physalin B (25 mg kg^{-1}/day) or physalin D (25 mg kg^{-1}/day). Nuclear staining and good preservation of morphological details were observed in all tumour sections immunostained with Ki67 antibody. In some of the samples examined, cells in mitosis showed a strong cytoplasmic positivity besides the expected Ki67 chromatin labelling. Background staining was seen in all cases. Figure 4 shows the number of Ki67 positive cells in

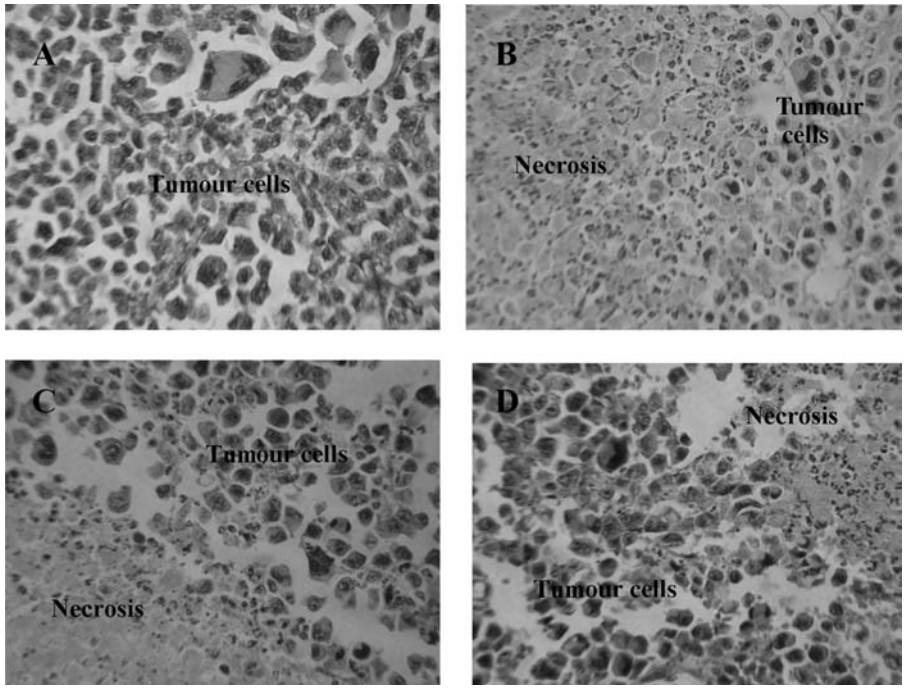


Figure 3 Histopathology of the sarcoma 180 tumour removed on day 10 from untreated (A), fluorouracil-treated (25 mg kg^{-1}/day, B), physalin B-treated (25 mg kg^{-1}/day, C) and physalin D-treated mice (25 mg kg^{-1}/day, D).

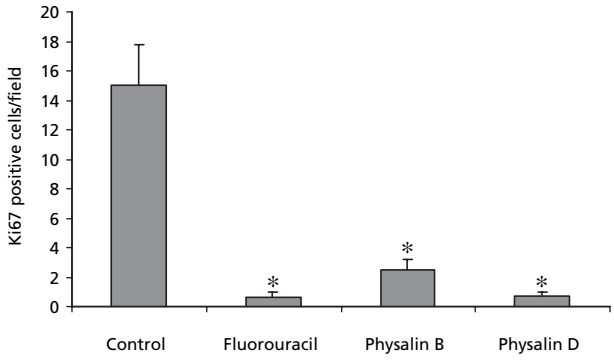


Figure 4 Effect of fluorouracil, physalin B and physalin D (25 mg kg^{-1}/day) on sarcoma 180 cell proliferation using Ki67 antibody. Ki67-positive cells from four fields/tumour were counted, and the mean ± s.e.m. of positive cells was calculated. $*P < 0.05$, analysis of variance followed by Student Newman Keuls.

the slides analysed. The results from this analysis showed that the relative number of Ki67-positive tumour cells was substantially less in tumours from mice treated with fluorouracil, physalin B or physalin D when compared with control tumours ($P < 0.05$).

Discussion

P. angulata is a herb widely distributed in Brazil, possessing great popular interest due to its reported medicinal properties, including anti-cancer activity (Chiang et al 1992a, b; Tomassini et al 2000; Lorenzi & Matos 2002; Soares et al 2003; Wu et al 2004). The aim of this study was to determine the in-vitro and in-vivo antitumour activity of two physalins, B and D, isolated from the organic solvent extracts of *P. angulata*.

Previous studies had already demonstrated that physalin B was cytotoxic to cultured tumour cell lines (Chiang et al 1992a, b; Fang et al 2003; Lee & Houghton 2005). Herein, the cytotoxicity of physalin B was shown in several different cell lines, more than previously reported (e.g. CEM, HCT-8, MDA-MB-231, MDA-MB-435, PC3 and B-16), reinforcing the findings that this compound exhibits a non-selective toxicity against various cell lines. On the other hand, previous data suggested that physalin D was inactive against cancer cell lines (Chiang et al 1992b). However, as can be seen from our findings, physalin D was even more cytotoxic than physalin B. Thus this is the first report demonstrating the cytotoxicity of physalin D.

The cytotoxic and antitumour properties of the physalins and withanolides have been investigated intensively (Kupchan et al 1969; Tomassini et al 2000; Minguzzi et al 2002; Veras et al 2004). Several studies demonstrated that the presence of the double bond between C-2 and C-3 (ring A) of these compounds was fundamental for those activities (Tomassini et al 2000; Veras et al 2004). In addition, Antoun et al (1981) demonstrated that the 5,6-epoxide ring of physalins was responsible for enhanced antitumour activity, and Lee & Houghton (2005) reinforced the importance of the functional groups located at C-5 and C-6. Our data corroborated this hypothesis and led us to propose that hydroxyl groups at the C-5 and C-6 positions of these compounds may

be relevant to activity, since an additional increment in the cytotoxic effect was observed for physalin D.

Although many reports have discussed the anti-cancer potential of *P. angulata* and its constituents, there are no data for studies using solid tumours. Herein, we reported the antitumour activity of physalins B and D in mice bearing sarcoma 180 tumours. Sarcoma 180 is a mouse-derived tumour and one of the most frequently used cell lines in antitumour related research in-vivo (Ito et al 1997; Lee et al 2003). Both compounds presented equal activity in this model. In-vivo antitumour activity for physalin B was demonstrated previously in leukemia models (Antoun et al 1981; Chiang et al 1992b), but this is also the first report on the antitumour activity of physalin D.

Immunohistochemical staining of cells for proliferation-associated proteins offers information about the tumour proliferation rate. The monoclonal antibody Ki67, described by Gerdes et al (1983), is a mouse monoclonal antibody that identifies a nuclear antigen associated with G1, S, G2 and M phases. This molecule is expressed all along the cell cycle, except in G0 and early G1 (Gerdes et al 1984). Thus, results obtained with Ki67 staining showed that the antitumour activity of physalins B and D was associated with a reduction in the tumour proliferation rate.

The histopathological analyses of organs removed from treated animals suggested that the kidney and liver could be considered as potential targets of physalin toxicity. The drugs should be considered as a possible cause of any liver lesion found in a biopsy. A large number of drugs of different chemical structures and with widely differing pharmacological actions occasionally give rise to substantial liver lesions (Scheuer & Lefkowitch 2000). Incriminated substances include antituberculosis drugs, non-steroidal anti-inflammatory drugs, anaesthetics, herbal remedies, methotrexate, chlorinated hydrocarbons and many others (Rang & Dale 1991; Scheuer & Lefkowitch 2000; Kummar et al 2004).

The liver shows great adaptive and regeneration ability. For example, the increase in endoplasmic reticulum produced by long-term treatment with anticonvulsant drugs is commonly regarded as an adaptive phenomenon. On the other hand, regeneration of hepatic tissues occurs in many diseases, except in the most deleterious ones. Even when hepatocellular necrosis is present but the conjunctive tissue is preserved, the regeneration proceeds almost to completion (Scheuer & Lefkowitch 2000; Kummar et al 2004).

The hepatic alterations observed after physalin treatment could be considered reversible (McGee et al 1992; Scheuer & Lefkowitch 2000; Kummar et al 2004), where physalin B was found to be more toxic. Ballooning degeneration of hepatocytes and portal tract and centriolobular venous congestion were also visualized in the control group, suggesting that these effects were related to hepatocyte metabolism (Curran 1985; Kummar et al 2004). Fluorouracil treatment resulted in Kupffer cell hyperplasia, which indicated the presence of a toxic agent (Kummar et al 2004). Microvesicular steatosis accompanied by ballooning degeneration of hepatocytes was observed in the group treated with physalin B, suggesting intrinsic hepatotoxicity, and physalin is known to produce liver damage when taken in sufficient quantities. Removal of drugs or dose adjustment usually leads to rapid improvement (Scheuer & Lefkowitch 2000).

Conclusion

Physalins B and D showed in-vitro and in-vivo antitumour activity, where the in-vivo activity was found to be associated with reversible toxic effects to the liver and kidney. These compounds were probably responsible for the previously described antitumour activity of ethanol extracts of *Physalis angulata*. Their identification could explain the ethnopharmacological use of this plant species in the treatment of cancer.

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