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Evaluation of genotoxic and antigenotoxic effects of hydroalcoholic extracts of *Zuccagnia punctata* Cav.

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Abstract

Zuccagnia punctata Cav. (Fabaceae), a widely used plant species in Argentine folk medicine, has been shown to have a broad spectrum of antibacterial, antifungal, antioxidant and cytoprotective activities. In this study, the hydroalcoholic extract of *Zuccagnia punctata* and 2′,4′-dihydroxychalcone isolated from it were investigated for genotoxicity/antigenotoxicity in the *in vitro* comet assay test on human hepatoma HepG2 cells. No acute toxicity of the extract could be determined. HepG2 cells were treated with three different concentrations (2.5, 5.0 and 10.0 μg/mL) or 2′,4′-dihydroxychalcone (0.01, 0.10 and 1.00 μg/mL). To explore the potential mechanisms of action, two approaches were followed: co-treatment with 4-nitroquinoline-*N*-oxyde (4-NQO), a direct genotoxic compound, and a pre-treatment protocol with benzo[*a*]pyrene (B[*a*]P), an indirect genotoxic compound. The natural products neither affected cell viability nor induced DNA damage in the concentration range tested. *Zuccagnia punctata* tinctures were able to diminish the DNA damage induced in HepG2 cells by 4-NQO and B[*a*]P in 31% and 10%, respectively at 10 μg/mL. Pre-treatment of HepG2 cells with 2′,4′-dihydroxychalcone was highly effective in decreasing B[*a*]P-induced DNA damage at a statistically significant level, with an almost clear dose–response relationship. The inhibition values were 28.2–43.9% for the tested concentrations of 0.01–1 μg/mL, respectively. The results clearly indicate that the phytoextract from *Zuccagnia punctata*, under the experimental conditions tested, is not genotoxic and that 2′,4′-dihydroxychalcone contributes to a high degree to the antigenotoxic effects of *Zuccagnia punctata* tincture.

Keywords: *Zuccagnia punctata*; 2′,4′-Dihydroxychalcone; HepG2 cells; Antigenotoxic activity; Comet assay

1. Introduction

Many people in developing countries use traditional drugs including medicinal herbs to meet their primary health care needs (WHO, 2002). Surveys in Argentina revealed that a large number of indigenous plants are still in use as a source of herbal therapies (Ratera and Ratera, 1980; Toursarkissian, 1980). *Zuccagnia punctata* Cav. (Fabaceae) is one of them. This South American plant species is widely distributed in arid and semiarid areas in western Argentina from Jujuy to Chubut (Burkart, 1952; Cabrera, 1971; Kiesling, 1994). This native shrub, known under the common names of jarilla pispito, puspus and jarilla macho, is used in folk medicine as foot antiseptic and against bacterial and fungal infections, asthma, arthritis and rheumatism (Ratera and Ratera, 1980; Toursarkissian, 1980).

Its favourable antimicrobial (Zampini et al., 2005), antifungal (Quiroga et al., 2001; Svetaz et al., 2004), gastroprotective of gastric mucosa against ethanol-induced damage (Ortega et al., 2003), and antioxidant (Zampini et al., 2004) properties are worth reporting. Regarding its chemical composition, flavanones, flavones, chalcones and caffeoyl ester derivates have been previously isolated from the aerial parts of *Zuccagnia punctata* (Pederiva et al., 1975; Pederiva and Giordano, 1984; Svetaz et al., 2004) and 2′,4′-dihydroxychalcone was reported as one of the major constituents isolated from the leaf resin of *Zuccagnia punctata* (Pederiva et al., 1975). This compound showed cytoprotective effects on the ethanol-induced gastroduodenal tract injury in rats (De la Rocha et al., 2003), antifungal

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properties (Svetaz et al., 2004), antimicrobial activity against multi-resistant Gram-negative bacteria (Zampini et al., 2005) and interesting properties as singlet molecular oxygen [O_2 ($^1\Delta_g$)] scavengers (Avila et al., 2001; Montaña et al., 2003).

Frequently used plants in traditional medicine are assumed to be safe, due to their long-term use (Elgorashi et al., 2002) and are considered to have no side effects because they are natural. This concept is largely circumstantial and the toxicology of plant extracts, especially those that are frequently used over long periods should be determined. In view of the fact that the aerial parts of *Zuccagnia punctata* are widely used and due to the lack of information about their genotoxicity, it is important to evaluate the effects of this complex mixture on genetic alterations. Among short-term toxicity assays, the comet assay, also known as the single-cell gel-electrophoresis (SCGE) assay, is a very sensitive test for the quantification of DNA damage and provides direct determination of DNA single- and double-strand breaks in individual cells. Many authors have used the comet assay to evaluate the *in vitro* and/or *in vivo* genotoxicity/antigenotoxicity of several chemicals with various cell lines (CHO, V79, HepG2) (Valentin-Severin et al., 2003). The HepG2 cells present endogenous bioactivation capacity, retain many of the morphological characteristics of liver parenchymal cells, and contain phase I and phase II drug metabolizing enzymes which play an essential role in the activation/detoxification of promutagens/procarcinogens, the latter being the major advantage of HepG2 cells for their use in mutagenicity/antimutagenicity studies (Knasmüller et al., 1998). The aim of the present work was to determine the genotoxicity and antigenotoxicity potential of *Zuccagnia punctata* extracts and 2′,4′-dihydroxychalcone.

2. Materials and methods

2.1. Reagents

The genotoxic compounds B[*a*]P and 4-NQO and general laboratory reagents were purchased from Sigma (Munich, Germany). The test compounds were dissolved in dimethylsulfoxide (DMSO; Merck, Darmstadt, Germany) for stock solutions and were stored at −20 °C.

2.2. Plant material

Zuccagnia punctata aerial parts (leaves and stems) were collected from January to February, 1998–2001 at 2000 m above sea level in Amaicha del Valle, Tucumán, Argentina. The samples were dried in a dark place at room temperature. Voucher specimens (IML 605935) were deposited at Herbarium of Fundación Miguel Lillo, Tucumán, Argentina. *Zuccagnia punctata* Cav. (Fabaceae) was authenticated by Lic. Nora Muruaga, Botany Department, Fundación Miguel Lillo.

2.3. Preparation of Zuccagnia punctata ethanolic extract (tincture)

Ground air-dried plant material was macerated in ethanol 80% (1 g/5 mL) for 7 days with stirring (40 cycles/min) at room temperature (Farmacopea Nacional Argentina, 1978). The extract was filtered through Whatman No. 4 filter paper. About 500 g of *Zuccagnia punctata* powder yielded 58 g of crude extract.

2.4. Fractionation of ethanol extract of Zuccagnia punctata

The EtOH was evaporated and the aqueous fraction was extracted with Et_2O. The organic fraction was subjected to column (180 mm × 30 mm) chromatography using silica gel (0.063–0.200 mm) and eluted stepwise with C_6H_6 and then with mixtures of C_6H_6–$CHCl_3$, $CHCl_3$, $CHCl_3$–CH_3OH and CH_3OH. Fractions of 100 mL were collected, evaporated to dryness in a rotary evaporator at 50 °C and the residue was dissolved in EtOH. The column fractions were analyzed by TLC. HPLC was used to identify 2′,4′-dihydroxychalcone by comparison with commercially available compounds. UV–vis absorption spectra (200–600 nm) were obtained using a Beckman DU 650 spectrophotometer (Mabry et al., 1970) while NMR spectra were recorded in a Bruker AC 200 (200 MHz) spectrometer in DMSO at room temperature (Zampini et al., 2005). Extracts dissolved in DMSO were used in all experiments.

Total phenolic content in the natural products was estimated by the Folin–Ciocalteau method (Singleton et al., 1999). Flavonoids were estimated using the method of Woisky and Salatino (1998). Quercetin was used as a standard.

2.5. General toxicity assay using Artemia salina (Brine shrimp)

Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial sea water. After 24 h the phototropic nauplii were collected. A microwell toxicity assay was carried out according to Solis et al. (1993). *Zuccagnia punctata* tincture or 2′,4′- dihydroxychalcone was evaporated and then dissolved in 3 μL DMSO prior to adding sea water. Serial dilutions were made in the wells of microplates in triplicate in 100 μL sea water. A suspension of nauplii containing 10–15 organisms (100 μL) was added to each well and the covered plate incubated at 22–29 °C for 24 h. Plates were then examined under a microscope and the number of dead (non-motile) nauplii in each well counted. One hundred microlitres of methanol was added to each well and after 15 min the total number of shrimp in each well was counted. LC_{50} values were then calculated by Probit analysis (Finney, 1971).

LC_{50} values were confirmed using 3-(4,5-dimethyl-2-yl)-2,5-dimethyltetrazolium bromide (MTT). After adding a solution of MTT in each well for 4 h, the amount of formazan formed was measured at 590 nm in a spectrophotometer Beckman DU 650 (Shimizu et al., 2004).

2.6. Cell cultures

HepG2 cells (ATCC HB 8065) were obtained from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Brescia, Italy.

The cells were grown as monolayer cultures in Eagle's minimum essential medium (MEM), with the addition of 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U/mL penicillin and 0.1 mg/mL streptomycin. Cell stocks were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. All experiments were performed on HepG2 cells at passages between 101 and 108. Cell stocks were routinely frozen and stored in liquid N_2.

2.7. Cell treatment for genotoxicity/antigenotoxicity studies

Genotoxicity/antigenotoxicity studies were carried out in HepG2 cells cultured in six-well tissue culture plates (Orange Scientific, Belgium) inoculated with 5 mL of culture medium containing 5×10^5 cells per well. The overall culture length was 72 h throughout the experiments.

In the co-treatment study (protocol A), cells maintained during 68 h in a culture medium were treated for the next 4 h as follows: (a) *Zuccagnia punctata* ethanolic extract (2.5, 5 and 10 μg/mL of phenolic compounds) or 2′,4′-dihydroxychalcone (0.01, 0.1 and 1 μg/mL) to check the absence of genotoxicity of this compound; (b) direct acting mutagen 4-NQO (3 μM); (c) simultaneous exposure to 4-NQO and *Zuccagnia punctata* ethanolic extract or 2′,4′-dihydroxychalcone.

In the pre-treatment study (protocol B) the cells were cultivated in a growth medium for 24 h and (a) exposed for 24 h to increasing concentrations of *Zuccagnia punctata* ethanolic extract (2.5, 5 and 10 μg/mL of phenolic compounds) or 2′,4′-dihydroxychalcone (0.01, 0.1 and 1 μg/mL) and then cultured during the remaining 24 h in fresh medium; (b) treated 48 h after seeding with the indirect acting mutagen B[*a*]P (20 μM) during the last 24 h culture; (c) treated for 24 h with increasing concentrations of *Zuccagnia punctata* ethanolic extract or 2′,4′-dihydroxychalcone (as above reported) and, during the last 24 h culture, treated with the indirect acting genotoxic compound B[*a*]P.

Negative (DMSO) controls were included in both protocols. After treatment, cells were washed with PBS, harvested and processed for the comet assay.

2.8. Cell viability

Cell viability was determined according to protocols described for genotoxicity/antigenotoxicity studies. The HepG2 cells were stained with fluorescein diacetate, a fluorescent dye that stains living cells and propidium iodide, a fluorescent dye that stains only dead cells (Jones and Senft, 1985). Then, the cells were examined with an Olympus BX41 fluorescence microscope (excitation filter 515–560 nm, emission filter 590 nm). Cell viability was calculated as a percentage of the respective control values.

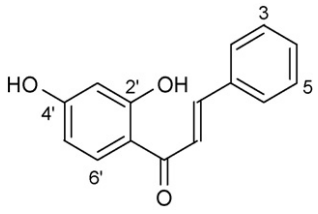

Fig. 1. Structure of main flavonoid present in *Zuccagnia punctata* Cav.: 2′,4′-dihydroxychalcone (DHC)

2.9. Alkaline single-cell gel-electrophoresis (SCGE) assay

2.9.1. Experimental procedure

The assay was performed according to the procedure of Moretti et al. (2002). Microscope slides were covered with a thin layer of 1% normal melting-point (NMP) agarose in Ca^{+2}- and Mg^{+2}- free PBS (Dulbecco's phosphate buffered saline) and allowed to dry overnight.

At the end of the treatments, cells were washed twice with 5 mL ice-cold PBS, pH 7.4, and trypsinized with 0.3 mL of a 0.025% solution. After 3 min, the process was stopped by adding complete culture medium. Cells were collected by centrifugation ($70 \times g$, 8 min, 4 °C) and the pellet was gently resuspended in 300 μL of 0.7% low melting-point (LMP) agarose at 37 °C. Then, 65 μL of cell suspension in agarose (1.5×10^5 cells) was rapidly layered onto a pre-coated slide. Coverslips were added and the agarose layers were allowed to solidify at 4 °C for 5 min.

The coverslips were gently removed and 75 μL aliquots of 0.7% LMP agarose were layered, covered again and maintained at 4 °C for 10 min. Coverslips were then removed and the slides immersed in cold, freshly prepared lysing solution (10 mM Tris–HCl pH 10, 1% sodium *N*-lauryl sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 10% DMSO, 1% Triton X-100) for at least 60 min at 4 °C. After lysis, the slides were immersed in ice-cold electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA; pH > 13) for 40 min to allow DNA unwinding. Electrophoresis runs were performed in an ice bath for 20 min by applying an electric field of 25 V (1 V/cm) and adjusting the current to 300 mA. Then, the slides were rinsed with neutralization buffer (0.4 M Tris–HCl, pH 7.5). For preservation, the slides were dehydrated in 70% ethanol and were allowed to dry at air temperature until staining. All steps were conducted under yellow light to prevent additional DNA damage.

2.10. Cell examination

Each slide was stained by rehydrating it with 60 μL of a 20 μg/mL propidium iodide solution and was covered with a coverslip. Fifty cells from each slide of three replicate slides were evaluated with an Olympus BX41 fluorescence microscope (excitation filter 515–560 nm, emission filter 590 nm) equipped with a CDD camera. A computerized image analysis system ("Comet Assay III", Perspective Instruments Ltd., Suffolk, UK) was employed to evaluate the DNA damage in individual cells. The mean tail intensity (percentage of DNA migrated in the comet tail) was used as a measure of DNA damage.

2.11. Statistical analysis

Each treatment consisted of three replications and was repeated twice. The average data obtained were analyzed statistically by Student *t*-test, comparing the treated cells with their controls at significance level of $p \leq 0.05$. The inhibition percentages of 4-NQO or B[*a*]P mediated genotoxicity were calculated as follows: % inhibition = [(tail intensity$_{mutagen}$ − tail intensity$_{sample}$)/tail intensity$_{mutagen}$] × 100.

3. Results

Zuccagnia punctata hydroalcoholic extract has showed high content of phenolic compounds (42 mg/g of dry plant material) and 2′,4′- dihydroxychalcone (15.8 mg/g dry material). *Zuccagnia* extract and 2′,4′- dihydroxychalcone (Fig. 1) were assayed to determine the genotoxic and antigenotoxic effects.

A general toxicity assay (lethality test of *Artemia salina*) was performed prior to genotoxicity/antigenotoxicity assays in order to select non-toxic concentrations of the *Zuccagnia punctata* extract and 2′,4′-dihydroxychalcone. The LC_{50} values of the *Zuccagnia punctata* extract was around 5 μg of phenolic compounds/mL. Otherwise, 2′,4′- dihydroxychalcone showed LC_{50} values of 3.5 μg/mL. Cellular viability studies using HepG2 cells were also performed prior to genotoxicity/antigenotoxicity studies. Over the concentration range tested, neither the extract (up to 10.0 μg of total phenolic compounds/mL medium) nor the isolated compound from aerial parts of *Zuccagnia punctata* (up to 1 μg/mL) was found to affect cell viability. Cell viability was always ≥90%.

The potential of both natural products to induce DNA damage was assessed using the comet assay. HepG2 cells were exposed to increasing concentrations of *Zuccagnia punctata* extract or 2′,4′-dihydroxychalcone, both in protocol A (4 h exposure) and protocol B (24 h exposure). Neither the extract nor the chalcone caused the formation of DNA breaks compared with non-supplemented cells (Tables 1 and 2), with tail intensity values very close to those of control cultures. Following co-treatment (4 h) of HepG2 cells with *Zuccagnia* hydroalcoholic extract and 4-NQO, a distinct, statistically significant reduction in the extent of DNA damage was observed (Table 1). In this protocol, the antigenotoxic activity of the tested extract increased with increasing concentrations in a dose–response manner and % inhibition values were 7.8%, 18.4% and 31.3% for the tested concentrations of 2.5, 5 and 10 μg/mL of phenolic compounds, respectively.

Cell pre-treatment (24 h) with the ethanolic extract decreased B[*a*]P-induced DNA damage, although at a less, not statistically significant level (Table 1).

In subsequent experimental series, HepG2 cells were exposed to 2′,4′-dihydroxychalcone alone (0.01, 0.1 and 1 μg/mL) and combined with the model mutagens (4-NQO or B[*a*]P), as in the aforementioned approach, in order to investigate whether the chalcone was responsible for the antigenotoxic effects of the crude extracts. An approximately U-shaped concentration–response curve was obtained in the co-treatment (4 h) protocol with a statistically significant reduction in the extent of DNA damage observed for the three tested concentrations (Table 2), with the highest effect observed at 0.1 μg/mL. In this case, % inhibition values were 18.1%, 23.5% and 13.1% for the tested concentrations of 0.01, 0.1 and 1 μg/mL, respectively.

Pre-treatment (24 h) of HepG2 cells with 2′,4′-dihydroxychalcone was highly effective in decreasing B[*a*]P-induced DNA damage at a statistically significant level, with an almost clear dose–response relationship (Table 2). Inhibition values were 28.2%, 41.2% and 43.9% for the tested concentrations of 0.01, 0.1 and 1 μg/mL, respectively.

4. Discussion

In conclusion, the absence of genotoxic response by *Zuccagnia* extract against HepG2 cells is a positive step forward in determining the safe use of these plants in traditional medicine. In the ethnopharmacological context, the lack of toxicity and genotoxicity are important for the whole population. The occurrence rate of cancer as well as diseases caused by genotoxic agents is increasing in the world. Antigenotoxic determination of plant extract is important in the discovery of new effective natural treatments.

Although an antigenotoxic effect found in a plant extract does not necessarily mean that it is an anticarcinogen, it hints at the possibility of acting as one.

Zuccagnia punctata hydroalcoholic extracts protected HepG2 cells against DNA damage induced by the direct-

Table 1
Genotoxic/antigenotoxic activity of *Zuccagnia punctata* hydroalcoholic extract in cultures of HepG2 cells

Effectors	*Zuccagnia punctata* extract (μg/mL)			
	0.0	2.5	5.0	10
Tail intensity				
DMSO	5.2	4.4	3.1	4.0
4-NQO (3 μM)	39.6	36.5	32.3	27.2
B[*a*]P (20 μM)	21.7	19.0	20.2	19.6
% Genotoxicity inhibition				
4-NQO (3 μM)	0.0	7.8	18.4[a]	31.3[a]
B[*a*]P (20 μM)	0.0	12.2	6.9	9.5

[a] Statistical significance ($p \leq 0.05$).

Table 2
Genotoxic/antigenotoxic activity of 2′,4′-dihydroxychalcone in cultures of HepG2 cells

Effectors	2′,4′-Dihydroxychalcone (μg/mL)			
	0.0	0.01	0.1	1.0
Tail intensity				
DMSO	5.2	3.8	6.2	5.9
4-NQO (3 μM)	39.6	32.4	30.3	34.4
B[*a*]P (20 μM)	21.7	15.6	12.7	12.1
% Genotoxicity inhibition				
4-NQO (3 μM)	0.0	18.1[a]	23.5[a]	13.1[a]
B[*a*]P (20 μM)	0.0	28.2[a]	41.2[a]	43.9[a]

[a] Statistical significance ($p \leq 0.05$).

acting (4-NQO) genotoxic compound. The antigenotoxic effect against 4-NQO could be explained for the presence of 2′,4′-dihydroxychalcone in this plant species (5 μg of extract in equivalent of phenolic compound contain 1 μg of chalcone). In our experimental conditions 1 μg of chalcone and 5 μg of phenolic compounds contained in *Zuccagnia* tincture produce similar percentage of 4-NQO genotoxicity inhibition. Otherwise, the inhibitory effect of *Zuccagnia* extract on genotoxicity by B[*a*]P is lower than the effect produced by chalcone (inhibition values of 10% and 43% with 5 and 1 μg, respectively). Differences observed for the tincture and the isolated chalcone in the inhibition patterns could be explained by the presence of weak or non-effective constituents in the extract.

Compounds with a chalcone-based structure have shown antiprotozoal, antifungal, antibacterial, antiinflammatory, interleukin-1, xanthine oxidase and tyrosinase inhibitory, phytoestrogenic, antipyretic, analgesic, cytoprotective, antioxidant and anticancer pharmacological properties (Anto et al., 1995; Edenharder and Tang, 1997; Ponce et al., 2000; Avila et al., 2001; López et al., 2001; De la Rocha et al., 2003; Nakamura et al., 2003; Uchiumi et al., 2003; Wu et al., 2003; Nerya et al., 2004; Zampini et al., 2004; Zampini et al., 2005; Nowakowska, 2007). Carbonyl compounds usually exert their action by direct interaction with some enzyme, usually through hydrogen bonds. In order to analyze the antigenotoxic activity of chalcone, it is reasonable to assume that such interaction is determined by the reactivity of the carbonyl group and hydroxyl groups at position C2′ and C4′.

This genotoxicological study suggests that the antigenotoxic properties of *Zuccagnia punctata* are of great pharmacological importance and might be beneficial for cancer prevention. However, further *in vitro* and *in vivo* experiments are required to better characterize the medicinal properties of this plant.

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