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The amoebicidal aqueous extract from *Castela texana* possesses antigenotoxic and antimutagenic properties

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Abstract

Due to long-term treatment toxicity and clinical resistance to drugs commonly used against *E. histolytica*, new drugs against amoebiasis are urgently needed. *Castela texana* (''chaparro amargo'') is a shrub taken traditionally in teas and capsules of dry plant to treat intestinal amoebic infections. An aqueous extract was prepared and its mutagenic, genotoxic and cytotoxicity properties were evaluated in prokaryotic and eukaryotic systems. This extract was neither mutagenic when evaluated with the Ames test in *Salmonella typhimurium* strains TA98, TA100 and TA102, nor genotoxic in unscheduled DNA synthesis in hepatocyte cultures, even at the highest concentrations tested. In fact, *C. texana* extract showed antimutagenic activity on *S. typhimurium* strains TA98 and TA100 in the Ames test. Furthermore, it was capable of protecting liver cell cultures against unscheduled DNA synthesis induced by 2-acetylaminofluorene at a concentration of 6.77 μg/ml. A free-radical scavenging test was used in order to explore the antioxidant capacity of *C. texana* extract with *S. typhimurium* strain TA102 pretreated with norfloxacin, a free radical producer. This extract showed a free radical withdrawal effect. The effective chemoprotective activity of this extract could be due to the antioxidant capacity of the *C. texana* extract components. In this paper it is shown that the antiamoebic natural product, *C. texana*, is also antimutagenic and protects against induction of preneoplastic lesions in rat liver. These results justify further studies to extend it use to human beings.

Keywords: Amoebicidal; Antigenotoxic; Antimutagenic; Antioxidant; *Castela texana*; Chemoprotection

1. Introduction

Amoebiasis is an intestinal and hepatic disease caused by *Entamoeba histolytica*, a parasitic protozoan of worldwide distribution. Epidemiological studies concerning the prevalence of this infection indicate that 90% of infected individuals remain as asymptomatic carriers, whereas the other 10% develop a clinically overt disease. *E. histolytica* is responsible for approximately 70,000 deaths per year (Walsh, 1986; WHO, 1998).

Drugs like metronidazole and emetine are the most widely used amoebicidal medicaments. Long-term treatment is often required and clinical cases of toxicity and resistance to the drugs have increased (Cervantes, 1972; Albach and Booden, 1978; Knight, 1980; Samarawickrema et al., 1997; Wassman et al., 1999). Therefore, there

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is an urgent need to develop new, effective, and safe drugs against amoebiasis. The screening of medicinal plants is a very effective way of finding new active substances.

Castela texana (T. & G.) Rose (syn. *C. tortuosa* Liebm, *C. nicholsonii* Hook) is a shrub commonly known in México as "chaparro amargo", traditionally used in the treatment of amoebic intestinal infection. *C. texana* belongs to the Simaroubaceae family, of which some members have been reported useful as antitumorals, particularly in leukaemia (Domınguez et al., 1979). Several quassinoids have been isolated of the methanolic extract obtained from the root or the aerial part of this plant (Geissman and Ellestad, 1962; Davidson et al., 1965; Domínguez et al., 1979; Chaudhuri and Kubo, 1992; Dou et al., 1996), and the amebicidal effect is mainly attributed to one of them named chaparrin (Calzado-Flores et al., 1986). Six compounds but not chaparrin showed potent non-selective cytotoxic activity in human cell lines (Dou et al., 1996). In contrast, a methanolic extract was administered intragastrically to rats, and they showed no signs of intoxication or changes in muscular and nervous systems (Calzado-Flores et al., 1998, 2002).

Several plant products have been characterized in vitro for their anticarcinogenic capacity due to the presence of different substances with antimutagenic activity (García-Gasca et al., 1998; Ramírez-Mares et al., 1999; Pérez-Carreón et al., 2002; González-Avila et al., 2003). These substances include vitamins, carotenoids, flavonoids and terpenoids that have been tested for antimutagenic effects in bacterial systems applying the Ames test (Maron and Ames, 1983). This screening test for mutagens uses bacteria to detect possible carcinogens, based on the fact that most compounds induce cancer just because they are mutagens. The Ames test determines the compound ability to mutate the genetically modified DNA of some *Salmonella typhimurium* strains (Ames et al., 1973b). It has been found in almost every instance that chemicals able to mutate bacteria were also carcinogenic to animals. Chemicals often are not mutagenic by themselves, but become mutagens as they are metabolized in liver. To mimic this liver activation process, the Ames test usually includes an extract of mammalian liver enzymes called S-9 mix (Ames et al., 1973a).

Another test has been adapted using eukaryotic cells, the unscheduled DNA synthesis (UDS) in primary culture of rat hepatocytes (Mendoza-Figueroa et al., 1979; Butterworth et al., 1987; Von der Hude et al., 1990). In a system in which replication DNA is blocked, the induced DNA repair is measured for assessing the ability of a chemical to reach and alter the DNA; this DNA damage is quantified by the amount of thymidine incorporated into nuclear DNA. UDS test has proven to be particularly valuable to know the genotoxicity and potential carcinogenicity of chemicals, and representative of the patterns of activation and detoxification occurring in hepatocytes (Hyun et al., 1987; Butterworth et al., 1987; Von der Hude et al., 1990).

Natural product extracts, many of them with antioxidant characteristics, are widely recommended in traditional medicine to cure parasitic or bacterial infections, in addition to their capacity to prevent cancer. This work describes for first time the use of the *C. texana* aqueous extract. This extract was studied because people often take this medicinal plant in teas. In contrast with commonly used antiamoebic drugs, *C. texana* extract showed antimutagenic properties. It is therefore highly justified to adopt this plant product for amoebiasis treatment, and to characterize its biological active components.

2. Materials and methods

2.1. Reagents

Norfloxacin (100% purity) was kindly donated by Applications Pharmaceutics, SA, Méx. City, México). 2-Acetyl-aminofluorene (AAF), collagenase type IV, hydroxyurea (HU), trypsin, perchloric acid (PCA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2-aminoanthracene (2AA), picrolonic acid (PA), *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine (ENNG), and mitomycin C were from Sigma-Aldrich Co. (St Louis, MO, USA). Fetal bovine serum (FBS) was from GIBCO (Grand Island, NY, USA). Tritiated deoxythymidine (^{3}H-dThy) was from Amersham Life Sciences (Buckinghamshire, UK). Cyclophosphamide (CP) was from SSA, México.

2.2. Castela texana extract preparation

The aqueous extract was obtained from *C. texana* dried roots, which were bought at Guadalajara, México, local market. The plant material was ground before its extraction. This material (250 g) was placed in 1 l of boiling distilled water for 1 h. The liquid was filtered and dried in a Buchi mini spry dryer B-280 (inlet temp. 180 °C, outlet temp. 100 °C, spry flow 100 ml/min) yielding 6 g of a yellowish powder.

2.3. Mutagenicity test

C. texana aqueous extract was assayed as mutagen in the Ames tube-incorporation test. *S. typhimurium* (His$^-$) strains TA98, TA100 and TA102, were grown in nutrient broth (NB) liquid medium for 16 h at 37 °C in agitation (90 rpm). A suspension of 100 μl of TA98 and TA100 strains was transferred to sterile screw-top tubes with 2 ml of 0.6% soft-agar and the *C. texana* extract

was added at different concentrations between 3.12 and 100 μg/plate. The assay was performed with or without 500 μl of mammalian liver-enzymes extract obtained from male Wistar rats, induced with 10% of Aroclor-1254 (S-9 mix) (Ames et al., 1973a). The total tube content was spread immediately onto plates with Vogel–Bonner medium and incubated for 48 h at 37 °C. All experiments were done twice and in triplicate. The number of revertants to histidine was determined in a Fisher colony counter. The reversion rate was compared to control plates without mutagen (spontaneous reversion), and to a positive control with a known mutagen (induced reversion): for TA98, PA (50 μg/plate) without S-9 mix, and 2AA (20 μg/plate) with S-9 mix; and for TA100, ENNG (10 μg/plate) without S-9 mix, and CP (500 μg/plate) with S-9 mix. *C. texana* extract was considered mutagenic if induced twice the spontaneous revertant number and a dose-response curve could be plotted (Maron and Ames, 1983).

To detect possible weak mutagenic activity of the *C. texana* extract, the preincubation method was used (Maron and Ames, 1983). Samples (100 μl) of *S. typhimurium* strains TA98 and TA100 grown in NB for 16 h at 37 °C in agitation (90 rpm) were exposed to the *C. texana* extract at concentrations of 3.12–100 μg/tube with or without S-9 mix. The mixture was then incubated at 37 °C for 60 min with agitation. Afterwards, 2 ml of 0.6% soft-agar were added and then spread over plates containing Vogel–Bonner medium. Plates were treated as described above. The positive controls used were the same as for the incorporation test, but the concentration of 2AA was of 10 μg/plate.

2.4. Antimutagenicity test

The antimutagenicity test was performed using the preincubation method mentioned above but with *S. typhimurium* strain TA102 (Maron and Ames, 1983), and using the free-radical inductor norfloxacin as a control of induced mutations (Arriaga-Alba et al., 1998; Arriaga-Alba et al., 2000). Norfloxacin was employed at 0.07 ng/plate and *C. texana* aqueous extract at 250, 500, and 1000 μg/plate. All tubes were added with S-9 mix, and the content spread over plates with Vogel–Bonner medium and incubated 48 h at 37 °C; revertants were counted as described above. Mitomycin C (2 ng/plate) was used as positive control.

2.5. Genotoxicity assay by unscheduled DNA synthesis (UDS) in primary hepatocyte culture

Hepatocytes from male Fisher rats (180–200 g weight) fasted during 18–20 h, were obtained by the collagenase perfusion method (Mendoza-Figueroa et al., 1979; Von der Hude et al., 1990). Three replicates of cells (8×10^5 each) were seeded onto 35 mm culture dishes in Eagle's medium modified by Dulbecco-Vogt, with 7% of FBS and 5 μg/ml insulin, and placed in a humidified incubator under CO_2 atmosphere at 37 °C. After 1 h of incubation, detached cells were removed and dishes refilled with medium containing 10 μM HU, 5 μCi ^{3}H-dThy, 1.25 μM AAF and *C. texana* extract (0.00271–108.4 μg/ml), and incubated 4 h more. Hepatocytes were then detached, incubated for 15 min at 37 °C with 0.25% trypsin, centrifuged 5 min at 2500 *g*, and the pellet was treated as described (Mitchell et al., 1983), adding 15 μl of PBS with 7% FBS and lysing cells by strong agitation. After that, 0.4 M PCA was added and incubated 30 min on ice to precipitate nucleic acids and proteins, centrifuged for 5 min at 4000 *g*, this was repeated twice. The pellet was suspended in 250 μl of 1 M PCA and incubated 30 min at 70 °C. Afterwards, tubes were centrifuged for 5 min at 6000 *g*, and DNA concentration was determined on supernatant by the diphenylamine assay (Leyva and Kelley, 1974). Data obtained were compared with a salmon DNA standard curve. DNA damage was determined by measuring the ^{3}H-dThy incorporated in 200 μl aliquots of each tube, in a liquid scintillation counter (Beckman).

2.6. Cytotoxicity of the C. texana extract

Cytotoxicity was measured by a colorimetric assay based on the reduction of MTT (Alley et al., 1988), with some modifications: after rat liver perfusion, hepatocytes were treated as above but dishes were refilled with medium containing *C. texana* extract at concentrations between 6.77 and 108 μg/ml, plus 1.25 μM AAF, and incubated 4 h more. Fresh medium was added and plates were incubated for 24 h prior to the addition of 0.4 mg/ml MTT. After 1 h of incubation, MTT was discarded and 1 ml of DMSO was added to each plate, transferred to tubes and absorbance read at 540 nm.

3. Results

3.1. Castela texana extract was not mutagenic and showed antimutagenic effect due to its antioxidant capacity

The Ames test was applied to determine if the *C. texana* aqueous extract caused damage to bacterial DNA. Results are shown in Fig. 1 for the incorporation method and Fig. 2 for the preincubation method that detects weak mutagens, both methods assayed with *S. typhimurium* TA98 and TA100 strains, with or without S-9 mix. Since the number of revertants obtained for all extract concentrations tested was similar to that of negative controls, it was inferred that *C. texana* aqueous extract did not damage the bacterial DNA.

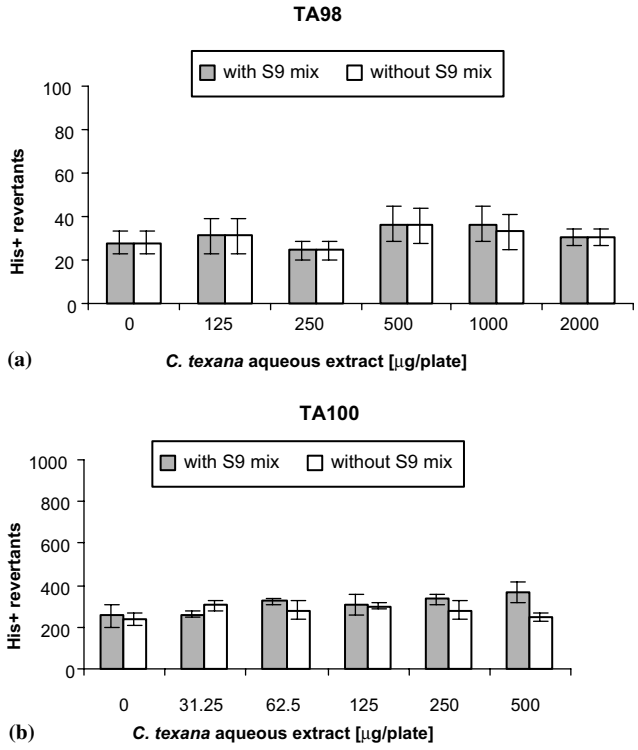

Fig. 1. Mutagenicity assay of *C. texana* aqueous extract by the Incorporation method in to *S. thyphimurium* TA98 (a) and TA100 (b) strains with and without S9 mix. Bacteria were incubated with the indicated concentration of extract. Number of spontaneous His^+ revertants was 25.3 ± 2.5 for TA98 and 278.5 ± 32 for TA100. Number of induced His^+ revertants was: *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine 2781 ± 432.5, cyclophosphamide 556.7 ± 121.7 for TA98; picrolonic acid 163.3 ± 32.8 and 2-aminoanthracene 3052 ± 118.3 for TA100. Each value represents the mean of two experiments in triplicate ±SD.

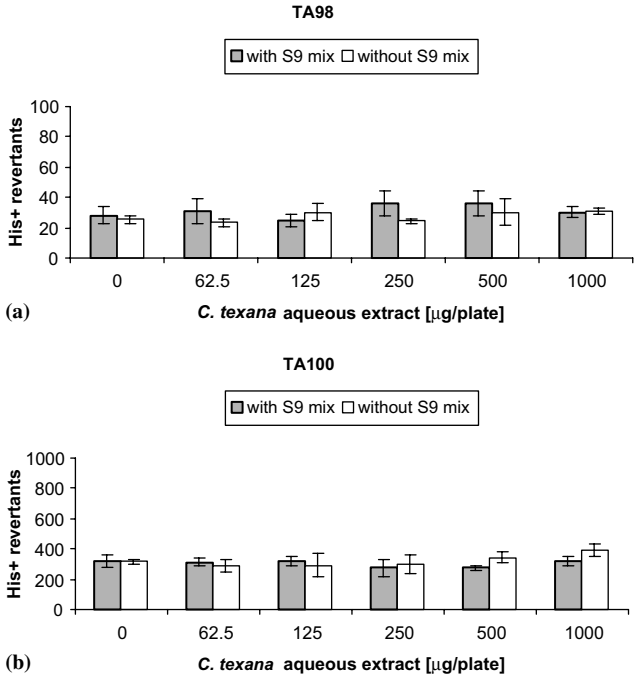

Fig. 2. Mutagenicity assay of *C. texana* aqueous extract by the Preincubation method. *S. typhimurium* strains TA98 (a) and TA100 (b) with or without S9 mix, were preincubated 1 h with *C. texana* extract with the indicated concentration. Number of spontaneous His^+ revertants was 26 ± 7 for TA98 and 196.7 ± 10.6 for TA100. Number of induced His^+ revertants was: picrolonic acid 128.6 ± 15.9 and 2-Aminoanthracene 2332.3 ± 220.1 for TA98; and for TA100 was *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine 2227.6 ± 133.9 and cyclophosphamide 592.6 ± 12.4. Each value represents the mean of two experiments in triplicate ±SD.

The antimutagenic capacity of the *C. texana* extract was tested for its antioxidant characteristics, which prevent mutations induced by the fluoroquinolone norfloxacin. We found that 250 μg/plate of *C. texana* extract diminished mutations by 31% (Fig. 3). This result, that is statistically significant, with $0.001 \geqslant P \leqslant 0.005$, indicates that the extract has a protective action, very likely, against the free radicals produced.

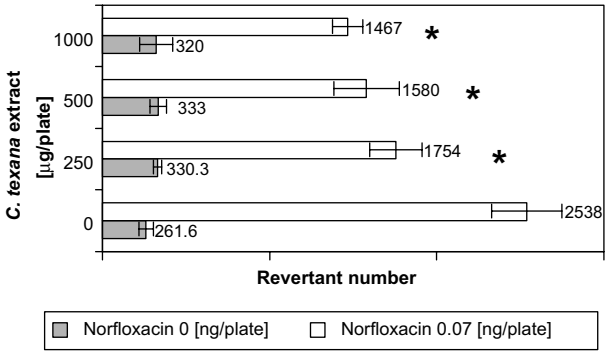

Fig. 3. Antimutagenic effect of *C. texana* aqueous extract in the presence of the free-radical inducer norfloxacin. Number of spontaneous His^+ revertants was 254 ± 24 and the number of induced His^+ revertants due to mitomycin C was 2422.2 ± 297.3. Data were analyzed statistically by student "*t*" test and significant values (*) $0.001 \geqslant P \leqslant 0.005$.

3.2. Antigenotoxic and chemoprotective effects of C. texana aqueous extract

The possible genotoxic damage caused by *C. texana* aqueous extract was evaluated by the UDS test in a primary culture of rat hepatocytes. Fig. 4a shows that the extract had no effect on hepatic cells, as the quantity of incorporated deoxythymidine was similar to that of untreated cells, at all concentrations tested.

Hepatocyte sensitivity to AAF was determined to evaluate the antigenotoxic potential of the *C. texana* aqueous extract. AAF concentration of 1.25 μM induced the highest ^{3}H-dThy incorporation (data not shown). Thus, different *C. texana* extract concentrations (0.00271–108.4 μg/ml) were tested in the presence of 1.25 μM AAF, and its antigenotoxic property was evaluated by ^{3}H-dThy incorporation. A complete protector effect of *C. texana* was found in concentrations of 6.77 μg/

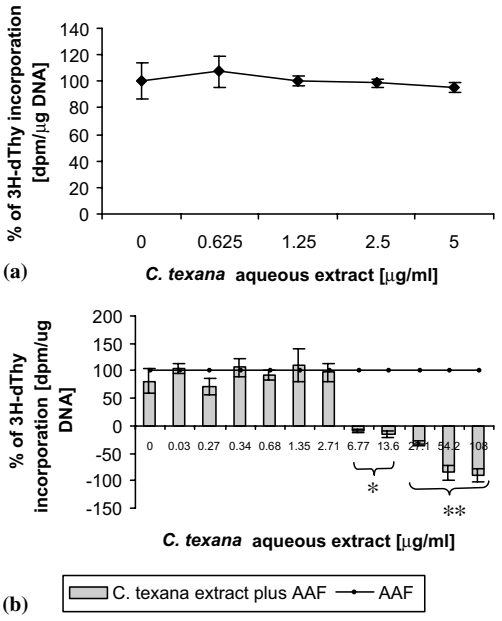

Fig. 4. Unscheduled DNA synthesis assay for *C. texana* aqueous extract in (a) non-treated cells, and (b) cells treated with 1.25 μM AAF. Three replicates of 8×10^5 liver cells were incubated with different concentrations of *C. texana* aqueous extract. One set of samples was treated only with water for basal incorporation of ^{3}H-dThy (100%) (a), or with 1.25 μM AAF (100%) (b). Results are expressed as percent of dpm of ^{3}H-dThy incorporated per μg of DNA with respect to the control. Each value represents the average of three independent experiments in triplicate. Data were anlyzed by student "*t*" test (*) $P \leqslant 0.05$ (**) $P \leqslant 0.001$.

ml and onwards in the next four concentrations tested up until 108.4 μg/ml. It is noteworthy that differences from AAF treated sample are statistically significant using the student "*t*" test analysis, $0.001 \geqslant P \leqslant 0.005$. These data are shown in Fig. 4b.

To verify that the lowering in d-Thy incorporation was due to the protective effect of the *C. texana* extract and not to a cellular death due to some toxic effect, hepatocyte viability was evaluated 24 h after treatment with the extract plus AAF, with the MTT test. As shown in Fig. 5, neither 1.25 μM AAF nor the *C. texana* extract plus AAF affected the hepatocyte viability even at the highest extract concentration. Viability was also tested at 4 and 8 h for 2.71 μg/ml of *Castela* extract and cellular alterations were not observed (data not shown).

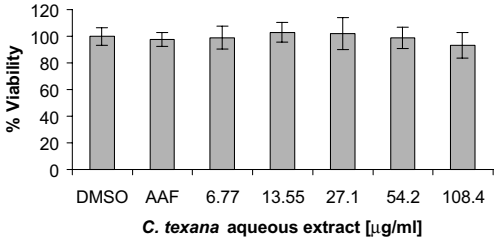

Fig. 5. Viability of the hepatocytes treated with 1.25 μM AAF plus *C. texana* aqueous extract at different concentration, measured after 24 h. Each value represents the means of three experiments in triplicate. The negative control was DMSO.

4. Discussion

Undoubtedly, natural products have a prominent future in the search for new and selective agents for treatment of different diseases. The present study is an evaluation of an aqueous extract of the antiamoebic *C. texana*, testing its alternative use as antioxidant. Although *C. texana* has long been used in traditional medicine in teas or capsules with dry plant to treat patients with amoebic dysentery, little is known about its mutagenic, toxic or antigenotoxic effects.

C. texana belongs to the Simaroubaceae family of which some members contain active principles, that have been reported as anticancerigenic products, such as a potent antileukaemia quassinoid from *Brucea antidysenterica* used in Ethiopia (Kupchan et al., 1975; Domínguez et al., 1979). Besides the antiplasmodial (Dou et al., 1996), antitrichomonic (Calzado-Flores and Segura-Luna, 1995; Calzado-Flores et al., 1998) and antiamoebic activities of *C. texana* (Calzado-Flores et al., 1986), contradictory results about a cytotoxic effect of the methanolic extract have been reported (Dou et al., 1996; Calzado-Flores et al., 1998, 2002). In this sense, it is important to use a harmless extract. The aqueous extract is the natural form generally taken by people with amoebiasis symptoms. This avoids the use of solvents which could make it less safe. The cytotoxic compounds found in the methanolic extract are probably not present in the aqueous extract.

The microbiological test described by Ames (Ames et al., 1973b; Maron and Ames, 1983) to identify chemical carcinogens and mutagens, showed that the *C. texana* aqueous extract was neither mutagenic with the incorporation nor with the preincubation methods that detect all kinds of mutagens. When this extract was assayed with the S9 mix for products that need to be activated by enzymes present in liver, results were similar, that is, *C. texana* did not induce mutations. Quite the opposite, it was antimutagenic, because it diminished the number of mutations induced by norfloxacin, a fluoroquinolone that produces free radicals which are rapidly detected by *S. typhimurium* strain TA102. Therefore, we propose the *C. texana* extract as an antioxidant product that could prevent the action of free radicals derived from other pharmacological mutagens or compounds produced by diet metabolic processes.

To confirm the beneficial effect of the *C. texana* extract, UDS was induced as an indicator of DNA antigenotoxicity in rat hepatocytes. The UDS results support the present argument, since this assay is mediated by the activation-dependent free-radical producer

carcinogen AAF. Very likely the preventive mechanism of the *C. texana* aqueous extract is due to the capture of free radicals. The extract by itself was not able to damage hepatocyte DNA. When extract was added together with the highest toxic concentration of AAF used, the potential damage to DNA was diminished. The *C. texana* aqueous extract showed efficient protective action against AAF genotoxicity, we are therefore proposing this extract as a reliable chemoprotector. This effect was found at concentrations where the biological activity against amoebas was also observed. Recent reports have shown that certain anti-mutagenic compounds inhibit pro-mutagenic and pro-carcinogenic activation and they are recommended as chemoprotectors, such as *Rhoeo discolor*, *Ardisia compressa*, *Calendula officinalis* and *Capsicum spp*. (García-Gasca et al., 1998; Ramírez-Mares et al., 1999; Pérez-Carreón et al., 2002; González-Avila et al., 2003) and other foods of plant or dairy origin (Abdelali et al., 1995; Wattenberg, 1992; Constable et al., 1996; Rogers et al., 1998).

Toxicity tests are of particular importance, because complex mixtures as such extracts contain a wide variety of constituents that could be cytotoxic by themselves or could have a potentially toxic effect. To determine if the aqueous extract was cytotoxic, cellular viability was measured in primary hepatocyte culture in the presence of different concentrations of the extract. Cell viability was not affected, not even at the highest concentrations tested.

The exact molecular mechanism involved in the antigenotoxic effect of *C. texana* extract is unknown. A detailed explanation of the extract's protective effect against induction of hepatic genotoxicity by AAF will be possible when the constituents of the extract be analysed.

In conclusion, we suggest that the aqueous extract of *C. texana* is antimutagenic, antioxidant and antigenotoxic. It could be safely tested for it use against amoebic infections, and also for chemoprotection against cancerigenic compounds.

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