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Antiulcerogenic effect and cytotoxic activity of semi-synthetic crotonin obtained from *Croton cajucara* Benth.

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Abstract

Trans-dehydrocrotonin, the major diterpene isolated from the bark of *Croton cajucara*, has good antiulcerogenic activity which, however, is accompanied by toxic effects. On the basis of these results, a semi-synthetic crotonin, named 4SRC, was prepared to determine whether this substance has similar antiulcerogenic activity with lower or no toxicity. The natural crotonin was also isolated from the bark of *C. cajucara* but was not used due to the small amount obtained. The cytotoxic effect of semi-synthetic crotonin, expressed as cell viability, was assessed in (a) lung fibroblast cell line (V79) derived from Chinese hamsters, a system commonly used for cytotoxicity studies, and (b) rat hepatocytes isolated from male Wistar rats. After treatment, cell viability was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction (MTT reduction), total acid content and neutral red uptake assays. To evaluate V79 cell viability, different concentrations of semi-synthetic crotonin were incubated with the cells. To evaluate the antiulcerogenic effects of semi-synthetic crotonin (50, 100 and 200 mg/kg), we used the models of gastric ulcer induced by ethanol/HCl, stress, indomethacin/bethanechol, and ethanol in male Swiss mice and male Wistar rats. The substance had an $IC_{50} = 500$ μM in the neutral red uptake and MTT reduction tests and an $IC_{50} = 200$ μM in the nucleic acid content test. With regard to hepatocyte viability after treatment with semi-synthetic crotonin at different concentrations, semi-synthetic crotonin had an $IC_{50} = 10-500$ μM in the nucleic acid content and MTT reduction tests and an $IC_{50} = 120$ μM in the neutral red uptake test. In another experiment, V79 cells were incubated with the metabolites produced by hepatocytes treated with different concentrations of semi-synthetic crotonin. After a 4-h incubation, semi-synthetic crotonin had an $IC_{50} = 500$ μM in the MTT reduction and neutral red uptake tests and an $IC_{50} = 370$ μM in nucleic acid content test. The substance had significant antiulcerogenic activity in all models studied, suggesting the presence of a possible antisecretory effect combined with a cytoprotective effect. For this reason, the effect of semi-synthetic crotonin was also evaluated on biochemical parameters of gastric juice and gastric wall mucus, both obtained from pylorus-ligated mice. No significant differences were observed in these parameters between semi-synthetic crotonin-treated and control animals. The results obtained with semi-synthetic crotonin are promising, with a significant preventive effect against gastric ulcer induced by different agents. Our data also show that semi-synthetic crotonin was less toxic than dehydrocrotonin and that the cytotoxic effects decreases with the time that isolated hepatocytes were in culture.

Keywords: Crotonin; Semi-synthetic; V79 cell; Cytotoxicity; Hepatocyte toxicity; Gastric ulcer

1. Introduction

Croton cajucara Benth. occurs widely in the Amazon region of northern Brazil, where it is popularly known as ‘‘sacaca’’. This plant has a history of safe use in folk medicine in the form of a tea for ailments such as diarrhea,

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diabetes, gastrointestinal disorders and inflammation of the liver (Di Stasi et al., 1989). Previous studies with "sacaca" have shown that the major component of the bark is *t*-dehydrocrotonin (Itokawa et al., 1989, 1990; Ichihara et al., 1992), and among the minor constituents is clerodane *t*-crotonin dehydrocrotonin (Itokawa et al., 1989, 1990). The nor-clerodane diterpene *trans*-dehydrocrotonin is present in sacaca bark tea, suggesting an important role for these compounds in the traditional preparations (Souza-Brito et al., 1998). Souza-Brito et al. (1998) have shown that dehydrocrotonin has significant antiulcerogenic activity, probably related to its antisecretory and gastroprotective actions (Hiruma-Lima et al., 1999). However, it was also demonstrated that dehydrocrotonin has cytotoxic effects (Rodriguez and Haun, 1999). The natural crotonin, present in the bark, has a chemical structure similar to that of dehydrocrotonin. However, it was not used because only a small amount was isolated, which it was tested in the ethanol/HCl ulcer induced model that presented significative protection (Hiruma-Lima et al., 2002). For this reason, semi-synthetic crotonin, named here 4SRC, was prepared to determine whether it has the antiulcerogenic activity of dehydrocrotonin combined with diminished cytotoxicity.

2. Materials and methods

2.1. Animals

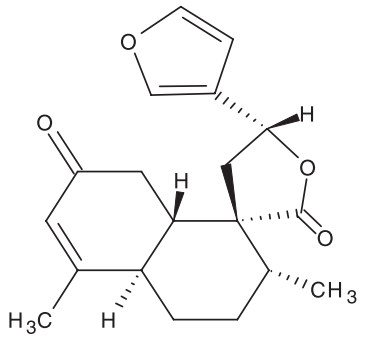
Male Wistar rats (150–200 g) and male Swiss mice (30–35 g), all obtained from the Central Animal House of the State University of Campinas (CEMIB/UNICAMP), were used in these experiments. Animals were fed normal rodent chow (NUVILAB CR-a®), with free access to tap water. They were fasted before the experiments because the drugs or test substances were always administered orally, except for the animals submitted to pylorus ligature ulcer. All experimental protocols were approved by the Ethics Committee for Animal Experimentation (CEEA) of the Institute of Biology (IB), State University of Campinas (UNICAMP).

2.2. Preparation of semi-synthetic crotonin

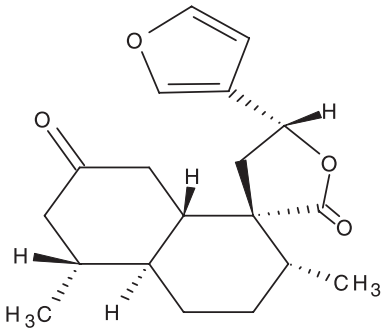
The bark of *C. cajucara* Benth. was collected from an experimental plantation in Benfica, near Belém, state of Pará, Brazil, and was identified by Dr. Nelson A. Rosa. A voucher herbarium specimen was deposited in the herbarium of the Museu Paraense Emílio Goeldi (accession number 247). Dehydrocrotonin was isolated from the bark as described by Souza-Brito et al. (1998). To obtain *trans*-crotonin, 12 g dehydrocrotonin was submitted to reduction at room temperature for 12 h using 4 atm H_2, $CHCl_3$ as solvent and paladium over carbon (10%) as the catalyst. The product of this reaction was purified through a silicagel 60 column (50 × 3 cm) eluted with a mixture of hexane/ethyl acetate (75/25), using an air pressure of 4 psi to maintain eluent flow at about 30 ml/min; 33 fractions of 50 ml were collected. Pure *trans*-crotonin was isolated from fractions 3 to 13. It eluted as a single spot under several thin layer chomatography conditions and showed very good agreement with the published physicochemical properties of the natural diterpene (Itokawa et al., 1989). The semi-synthetic crotonin was named 4SRC (Fig. 1).

2.3. V79 fibroblast cultures

The cytotoxic effect of semi-synthetic crotonin, expressed as cell viability, was assessed in a lung fibroblast cell line (V79) derived from Chinese hamsters. A cell line that is commonly used for cytotoxicity studies (Souza-Brito et al., 1998; Rodriguez and Haun, 1999). The V79 fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal calf serum, 100 IU penicillin/ml and 100 μg streptomycin/ml in a humidified incubator with a 5% CO_2 atmosphere at 37 °C. The cells were plated onto 96-well plates at a density of 3×10^4 per ml. The medium was removed 48 h after cell seeding and replaced with one containing semi-synthetic crotonin (80–400 μM), initially dissolved in methanol and then diluted in DMEM. The final concentration of methanol in the test and control media was 1%. The cells were exposed for 24 h to the test medium with or without semi-synthetic crotonin (control). Each drug



A. Dehydrocrotonin (DHC) - PM = 352



B. Crotonin (4SRC) - PM = 354

Fig. 1. Chemical structure of dehydrocrotonin (DHC) and crotonin (4SRC).

concentration was tested in eight replicates and in three experiments. At the end of incubation, three independent endpoints for cytotoxicity, MTT reduction, total acid content and neutral red uptake, were evaluated and each one is presented in the form of IC_{50}.

2.3.1. Nucleic acid content

The number of cells in control and treated wells was estimated from their total nucleic acid content according to Cingi et al. (1991). The cells were washed twice with cold phosphate-buffered saline (PBS) and a soluble nucleotide pool was extracted with cold ethanol. The cell monolayers were then lysed by incubation in 0.5 M NaOH for 1 h at 37 °C. The absorbance of the NaOH fraction at 260 nm was used as an index of cell number (Bianchi and Fortunat, 1990).

2.3.2. 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide reduction (MTT reduction)

The tetrazolium reduction assay was performed using the method of Denizot and Lang (1986). Briefly, fibroblasts were washed once with phosphate-buffered saline (PBS) before addition of 0.1 ml of serum-free medium containing MTT (1 mg/ml) to each well. Following incubation for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 1 ml of ethanol with stirring for 15 min in a microtitre plate shaker and absorbance was read at 570 nm.

2.3.3. Neutral red uptake

The neutral red uptake assay was performed as described by Borefreund and Puerner (1984). After 4 h of incubation with serum-free medium containing 50 μg of neutral red/ml, the cells were washed quickly with PBS and then 0.1 ml of an aqueous solution of 1% (v/v) acetic acid: 50% (v/v) ethanol was added to each well to extract the dye. After rapid shaking in a microtitre plate shaker, absorbance was read at 540 nm.

2.4. Primary culture of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (200–250 g) by the two-step collagenase perfusion method described by Guguen-Guillouzo and Guillouzo (1986). Cell viability measured by the Trypan blue exclusion test was greater than 90%. The cells were seeded onto 96-well plates at a density of 3×10^5 cells/ml in Ham F-12 medium supplemented with 10% fetal bovine serum, 0.2% bovine serum albumin, 0.1 IU bovine insulin/ml, 10^{-6} M dexamethasone, 50 IU penicillin/ml and 50 μg streptomycin/ml and incubated at 37 °C in a humidified 5% CO_2 atmosphere. After 4 h for cell attachment, the medium was changed to serum-free medium containing different concentrations of semi-synthetic crotonin followed by incubation for 20 h; each concentration was tested in eight replicates in three different experiments. In all cases wells containing serum-free medium without semi-synthetic crotonin were used as controls. At the end of the incubation period, three endpoints for cytotoxicity (MTT reduction, neutral red uptake and nucleic acid content) were evaluated. The cytotoxicity of semi-synthetic crotonin on "aged" hepatocyte cultures was also studied. In this case, hepatocytes were plated onto three 96-well plates and 4 h after seeding the medium was replaced with serum-free medium and 24 h later the cells were incubated with different concentrations of semi-synthetic crotonin for 24 h. After treatment, MTT reduction, nucleic acid content and neutral red uptake were determined as described above.

2.5. V79 fibroblasts cultured with conditioned medium from rat hepatocytes

The study was performed as described by Rodriguez and Haun (1999). Rat hepatocytes were isolated and cultured as described above. Four hours after plating, the cells were incubated with serum-free medium containing different concentrations of semi-synthetic crotonin. After 20 h, the medium was removed and used to treat semiconfluent cultures of V79 cells for 24 h as described above. After treatment, cell viability was determined by the MTT reduction, neutral red uptake and nucleic acid content assays.

2.6. Acute gastric lesions

The antiulcerogenic activity of different doses of semi-synthetic crotonin (50, 100 or 200 mg/kg) was assessed in four experimental models of acute gastric ulcer. In all experiments the doses of semi-synthetic crotonin were diluted in 12% Tween 80 and a positive control group was used. Semi-synthetic crotonin was orally administered because it is the most common method of drug administration. After the animals were killed, the stomachs were removed and opened along the greater curvature to determine the ulcerative index (UI) as described by Szelenyi and Thiemer (1978).

2.6.1. Ethanol-induced ulcers

This ulcer assay was performed in groups of seven rats each using the method of Morimoto et al. (1991). Rats were randomly divided into two groups and fasted for 24 h before the experiment. One milliliter of 99.5% ethanol was orally administered to rats treated with semi-synthetic crotonin (100 mg/kg) or with vehicle, 12% Tween 80 (10 ml/kg), 1 h before. One hour after the administration of ethanol, the rats were killed, the stomachs were removed, opened and the Lesion Index was determined as previously described.

2.6.2. Hypothermal-restraint stress ulcers

The antiulcerogenic activity of semi-synthetic crotonin was assessed in the hypothermic restraint stress-induced gastric ulcer model in mice according to the method of Levine (1971), with some modifications. Mice were fasted

for 36 h and then received an oral dose of semi-synthetic crotonin (50, 100 or 200 mg/kg), cimetidine (100 mg/kg) or vehicle, 12% Tween 80 (10 ml/kg). One hour after the treatments, gastric ulceration was induced by immobilizing the animals in a closed cylindrical cage at 4 °C. After 3 h, the mice were killed by cervical dislocation and the stomachs were removed and examined for ulcers as described previously.

2.6.3. Nonsteroidal anti-inflammatory drugs-induced gastric ulcers in cholinomimetic-treated mice

A total of 35 mice divided into five groups were fasted for 24 h with free access to water. Thirty minutes after the oral administration of semi-synthetic crotonin (50, 100 or 200 mg/kg), cimetidine (100 mg/kg) or vehicle, 12% Tween 80 (10 ml/kg), an indomethacin solution (30 mg/kg, dissolved in 5% sodium bicarbonate), was administered subcutaneously to each group as described by Rainsford (1978). Acetyl-β-methylcholine chloride (bethanechol) was administered intraperitoneally 15 min before indomethacin. The animals were killed 4 h later, the stomachs were removed and opened, and the gastric lesions were determined as described above.

2.6.4. Pylorus ligature

Male Swiss mice were fasted for 24 h. Thirty minutes after intraduodenal treatment with cimetidine (100 mg/kg), semi-synthetic crotonin (50, 100 or 200 mg/kg) or vehicle, 12% Tween 80 (10 ml/kg), the pylorus was ligated by the method of Shay (1945). The intraduodenal route was used to verify the systemic action of the substances. All animals were killed 4 h later, the stomachs were removed and the contents were drained into a graduated centrifuge tube via a small incision. The volume of gastric fluid was recorded, the pH was determined and the total acid output was calculated by titrating the pH to 7.0 with 0.05 N NaOH.

2.6.5. Gastric wall mucus determination

Alcian blue binding to the gastric wall mucus was determined by the method of Corne et al. (1974), with minor modifications. The test drugs, semi-synthetic crotonin (50 and 100 mg/kg) or vehicle, 12% Tween 80 (10 ml/kg), were given intraduodenally to pylorus-ligated mice. The mice were killed 4 h after surgery and the glandular segments from the stomachs were removed and weighed. Each segment was transferred immediately to 0.1% Alcian blue solution (0.16 M sucrose in 0.05 M sodium acetate, pH 5.8), and incubated for 24 h. The segments were centrifuged at 3000 rpm for 10 min and the absorbance of the supernatants was measured at 615 nm. The amount of Alcian blue extracted per gram of glandular tissue was then calculated.

2.7. Statistical analysis

The results of in vitro experiments are expressed as IC_{50} values (concentration that produced a 50% inhibitory effect on the evaluated parameter). These values were calculated by expressing the results as percentages of the controls and then determining the values graphically from the dose–response curves. The results of in vivo experiments are expressed as the means ± S.D. Statistical significance was determined by Student's *t*-test or by one-way analysis of variance (ANOVA) followed by Dunnett's test, with the level of significance set at $P < 0.05$, depending on the number of groups used.

3. Results

A broad range of substance concentrations were used in the viability assays, which measure the "killing capacity" or the "metabolic capacity" of a given chemical, as is usual when starting toxicity studies with an unknown compound (Cingi et al., 1991). The cytotoxic effects of semi-synthetic crotonin were measured by the MTT reduction, nucleic acid content and neutral red uptake assays in V79 cells and hepatocytes. The results indicated that there was a loss of viability of V79 fibroblasts after 24 h of exposure to semi-synthetic crotonin (Fig. 2). This loss of viability was dose-dependent, causing cell detachment and death. Also, as shown in Fig. 2, semi-synthetic crotonin toxicity was greater in the nucleic acid content assay ($IC_{50} = 200$ μM) than in the neutral red uptake assay ($IC_{50} = 500$ μM) assay and was apparently absent in V79 fibroblasts as evaluated by MTT reduction.

To assess the involvement of cytochrome *P*450 in the bioactivation of semi-synthetic crotonin, 24-h-old hepatocyte cultures were treated with different doses of semi-synthetic crotonin for 20 h, as described by Hammond and Fry (1996). The cytotoxicity observed in the MTT reduction

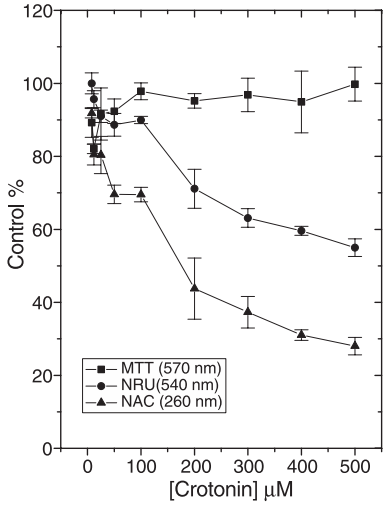


Fig. 2. Viability of V79 fibroblasts after treatment with semi-synthetic crotonin for 24 h. Endpoints evaluated: neutral red uptake (NRU), nucleic acid content (NAC) and MTT reduction (MTT). Each point represents the mean ± S.D. of three experiments in eight replicates.

(IC_{50} = 10–500 μM) and nucleic acid content (IC_{50} = 10–500 μM) tests in the 4-h rat hepatocyte culture was similar to that observed in 24-h-old V79 fibroblasts after exposure to semi-synthetic crotonin (Table 1). The IC_{50} was 10 μM in the MTT reduction assays 500 μM in the nucleic acid content assay, and 120 μM in the neutral red uptake assay. A significant loss of cytotoxicity was also observed as the hepatocytes aged.

To investigate whether or not the metabolites of semi-synthetic crotonin are potentially toxic to other cell types, a co-culture system of hepatocytes and V79 cells was used. The co-culture technique is a useful tool for assessing the toxicity of products resulting from hepatic bioactivation in target cells (Fry et al., 1995). The medium from cultured hepatocytes treated with semi-synthetic crotonin had the same toxic effect on V79 fibroblasts as semi-synthetic crotonin itself, as judged in the nucleic acid content and neutral red uptake tests. The results of this experiment are illustrated in Fig. 3. A slight stimulatory effect on the ability of V79 cells to reduce MTT was observed after incubation with the medium from hepatocytes cultured for 4 h.

To establish a general profile of the antiulcerogenic activity of semi-synthetic crotonin, the compound was administered orally at three doses (50, 100 or 200 mg/kg) in different gastric ulcer models in mice and rats. Table 2 presents the antiulcerogenic actions of semi-synthetic crotonin, cimetidine or lanzoprazole used as positive control on the gastric lesions induced by indomethacin–bethanechol, hypothermic restraint stress, and absolute ethanol.

Pretreatment with a single dose of semi-synthetic crotonin (100 mg/kg) before absolute ethanol administration significantly inhibited ulcer formation by 79%, confirming the cytoprotective properties previously observed (Table 2). When these results were compared with those obtained with dehydrocrotonin in this model (Souza-Brito et al., 1998), we observed a considerable decrease in the gastroprotective effect of dehydrocrotonin (55%) against ethanol lesions.

We also investigated the possible gastroprotective activity of semi-synthetic crotonin in the NSAID-induced gastric ulcer model (Table 2). In this model, semi-synthetic crotonin (50, 100 and 200 mg/kg) significantly inhibited ulcer formation by 16%, 47% and 41%, respectively. In contrast, dehydrocrotonin did not show antiulcerogenic activity when tested against NSAIDs such as indomethacin (Souza-Brito et al., 1998).

In order to verify this point, we measured the gastric wall mucus of rats submitted to the Shay method (Table 3). In contrast to our expectations, semi-synthetic crotonin (50 or 100 mg/kg) did not modify the free mucus produced by the gastric mucosa, as opposed to indomethacin, a classic cyclooxygenase inhibitor.

Table 1
Influence of hepatocyte culture age on the cytotoxicity of semi-synthetic crotonin

Hepatocyte age (h)	NAC (IC_{50}) (μM)	NRU (IC_{50}) (μM)	MTT (IC_{50}) (μM)
4	10–500	120	10–500
24	>500	>500	>500

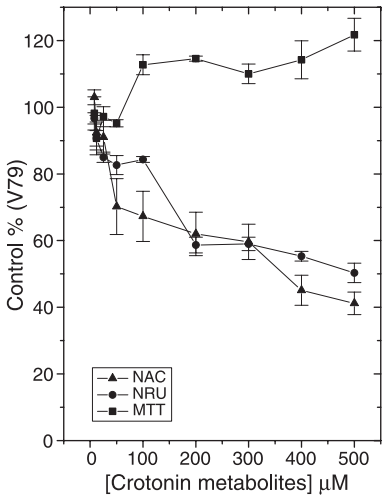


Fig. 3. Viability of V79 fibroblasts after treatment with conditioned medium from rat hepatocytes cultured for 4 h. Endpoints evaluated: neutral red uptake (NRU), nucleic acid content (NAC) and MTT reduction (MTT). Each point represents the mean ± S.D. of three experiments in eight replicates.

The next step was to evaluate semi-synthetic crotonin in terms of its antisecretory activity. This effect was determined in hypothermic restraint stress-induced ulcer in mice. Pretreatment with semi-synthetic crotonin at doses of 50, 100 or 200 mg/kg significantly inhibited the formation of gastric lesions induced by hypothermic restraint-stress by 60%, 81.5% and 74%. These data can be seen in Table 2. Cimetidine (100 mg/kg), the reference antiulcerogenic agent, significantly inhibited (66%) the ulceration induced in this model.

Finally, we investigated the effects of semi-synthetic crotonin on the biochemical parameters of gastric acid

Table 2
Effects of semi-synthetic crotonin (4SRC) on models of gastric lesions induced in mice and rats

Method	Treatment	*n*	Dose (mg/kg)	ILU	Inhibition (%)
Ethanol (rats)	Control	6	–	30.3 ± 13.3	–
	4SRC	5	100	6.2 ± 6.9[a]	79.6
Indomethacin	Control	9	–	8.44 ± 2.2	–
(mice)	Cimetidine	10	100	3.7 ± 1.8[b]	56.2
	4SRC	11	50	7.1 ± 2.6	16.0
		10	100	4.5 ± 2.1[c]	46.7
		9	200	5.0 ± 1.6[c]	40.8
Hypothermic	Control	8	–	8.75 ± 1.6	–
restraint	Cimetidine	6	100	3.0 ± 0.81[b]	65.7
stress	4SRC	8	50	3.5 ± 2.1[c]	60.0
(mice)		8	100	1.62 ± 0.99[b]	81.5
		7	200	2.28 ± 1.3[b]	74.0

The results are reported as means ± S.D.
[a] $P < 0.01$ compared to control (Student's test).
[b] $P \leq 0.001$ compared to control (Dunnett's test).
[c] $P < 0.01$ compared to control (Dunnett's test).

Table 3
Effects of semi-synthetic crotonin (4SRC) on the biochemical parameters of gastric juice obtained from pylorus-ligated mice

Method	Treatment	Dose (mg/kg)	pH	Total gastric acid (mEq/ml/3 h)	Gastric juice volume (ml)	Mucus (mg/g)
Pyloric ligation	Control	–	3.83 ± 0.34	9.42 ± 2.8	0.424 ± 0.348	–
	Cimetidine	100	4.68 ± 0.36[a]	4.47 ± 2.2[a]	0.440 ± 0.183	–
	4SRC	50	3.57 ± 0.38	8.14 ± 1.5	0.412 ± 0.168	–
		100	3.77 ± 0.34	12.09 ± 5.4	0.282 ± 0.162	–
		200	3.68 ± 0.36	9.08 ± 4.6	0.348 ± 0.108	–
Gastric wall mucus	Control	–	3.0 ± 0.8	–	0.690 ± 0.84	2.61 ± 0.5
	Cimetidine	100	4.5 ± 0.7[a]	–	0.559 ± 0.168	3.00 ± 0.6
	4SRC	50	3.2 ± 1.0	–	0.401 ± 0.150	2.13 ± 0.7
		100	2.4 ± 0.7	–	0.467 ± 0.221	1.98 ± 0.5

The results are reported as means ± S.D.
[a] $P < 0.05$ compared to control (Dunnett's test).

secretion (pH, total gastric acid, gastric juice volume) using the pylorus ligature method (Shay et al., 1945). As shown in Table 3, the volume, pH and total acid output of gastric secretions were not significantly affected by semi-synthetic crotonin (50, 100 and 200 mg/kg), whereas cimetidine (100 mg/kg) increased the pH and reduced the acid output of this secretion.

4. Discussion

The balance between the therapeutic versus the toxicological effects of a compound is an important parameter for the determination of its applicability as an antiulcer or any other pharmacological agent. The toxicological studies described here were conducted on cultured cells, which can be used to evaluate cytotoxicity (Ekwall and Ekwall, 1988) and target organ toxicity. In some cases, cultured cells may also provide information about the lethal dose in vivo (Shrivastava et al., 1991). We used V79 fibroblasts in our assays because this cell line is well characterized and commonly used in mutagenicity and toxicity studies (Cingi et al., 1991).

A broad range of concentrations were tested in the viability assays, which measure the "killing capacity" or the "metabolic capacity" of a given chemical, as is usual when starting toxicity studies of an unknown compound (Cingi et al., 1991). The cytotoxic effects of semi-synthetic crotonin were measured by the MTT reduction, nucleic acid content and neutral red uptake assays in V79 cells and hepatocytes. The results indicated that there was a loss of viability of V79 fibroblasts after 24 h of exposure to semi-synthetic crotonin. The cytotoxic effect of semi-synthetic crotonin was greater in the nucleic acid content assay than in the neutral red uptake assay and was apparently absent in V79 fibroblasts, as evaluated by MTT reduction.

Primary cultures of mammalian hepatocytes suffer a rapid and gradual loss of cytochrome *P*450 content without there being a severe initial decline in the levels of phase II enzymes following isolation. Hepatocyte cultures of different ages (24 and 72 h) are used to determine the involvement of cytochrome *P*450 in the toxic effects of chemicals. To assess the involvement of cytochrome *P*450 in the bioactivation of semi-synthetic crotonin, 24-h-old hepatocyte cultures were treated with different doses of semi-synthetic crotonin for 20 h as described by Hammond and Fry (1996). The cytotoxic effect of semi-synthetic crotonin in these assays suggests that semi-synthetic crotonin, like dehydrocrotonin (Rodriguez and Haun, 1999), is bioactivated by the cytochrome *P*450 microsomal system and generates less toxic metabolites as a function of time.

The results obtained with co-culture system with hepatocytes and V79 cells indicate that the toxicity of the metabolites was similar to that of semi-synthetic crotonin itself. The hepatic metabolism of xenobiotics is known to generate superoxide anion and free radicals, with the former being able to reduce tetrazolium compounds such as MTT (Andrews et al., 1997). Our findings support the presence of MTT-reducing metabolites in the medium from hepatocytes treated with semi-synthetic crotonin and confirm the results obtained by Rodriguez and Haun (1999) with dehydrocrotonin.

Pretreatment with semi-synthetic crotonin (100 mg/kg) was more effective in inhibiting ulcer formation than dehydrocrotonin in the ethanol-induced ulcer model, suggesting an increase in the gastroprotective effect. The intragastric application of absolute ethanol consistently produced severe lesions in all the control rats. However, diluted ethanol produced no more than minute lesions in the control rats (Sikiric et al., 1999). For this reason, absolute ethanol was used. It has been proposed that ethanol is responsible for the stimulation of gastric and oral secretion and gastrin and histamine release in a reflex way through sensitive terminals present in the gastric and oral mucosa, consequently stimulating gastric secretion. Based on this mechanism of ethanol injury, we suggest that semi-synthetic crotonin may act on the gastric mucosa as an antisecretory agent. Gastric acid is not a primary cause but plays a permissive role in the gastric mucosal damage induced by ethanol (Tarnawski et al., 1983). Previous studies have demonstrated that most antiulcer agents, such as proton pump inhibitors (omeprazole, lanzoprazole) and histamine H_2 receptor

anatagonists (cimetidine), have antisecretory activity (Larsson et al., 1983; Yamamoto et al., 1984; Lindberf et al., 1990; Kinoshita et al., 1997). Another action promoted by ethanol is its ability to damage the gastric mucosa by mechanical injury. Thus, semi-synthetic crotonin may also act as a cytoprotective agent because drugs like misoprostol are also active in this model (Morimoto et al., 1991).

The presence of gastric acid, the overproduction of leukotrienes, the inhibition of prostaglandin (PG) synthesis and the consequent disruption of the gastric mucosal barrier are the main factors involved in the pathogenesis of ulcer induced by indomethacin, a non-selective cyclooxygenase inhibitor (Dajani and Agrawal, 1995). In the indomethacin-induced ulcer model, semi-synthetic crotonin significantly inhibited ulcer formation at high doses. However, dehydrocrotonin did not show antiulcerogenic activity in this model (Souza-Brito et al., 1998). The effect of semi-synthetic crotonin on ulcers induced by indomethacin suggested a possible involvement of prostaglandins, because there is evidence of a relationship between the levels of certain prostaglandins and the resistance of the gastric mucosa to ulcerogenic agents (Wallace and Granger, 1996). According to Whittle and Vane (1987) and Motilva et al. (1996), among the probable antiulcer mechanisms, prostaglandins may make the mucosa more resistant to damage by (a) stimulating bicarbonate and mucus production, (b) maintaining an adequate blood flow, (c) inhibiting the removal of mast cell-derived inflammatory mediators, and (d) decreasing the production of free radicals. The action of semi-synthetic crotonin on prostaglandin synthesis is being studied.

The antisecretory activity of semi-synthetic crotonin could be observed in hypothermic restraint stress-induced ulcer. Stress-induced ulcers are probably caused by the release of histamine, by enhanced acid secretion and by a reduction in mucus production (Pal and Nagchaudhury, 1991). The involvement of histamine in hypothermal-restraint stress-induced ulceration was strengthened by the observation that cimetidine, an H_2 receptor antagonist, completely inhibited the ulceration induced by hypothermic restraint stress. Probably, the cytoprotective action of semi-synthetic crotonin is due to antagonism at histaminergic and/or cholinergic receptors. When the lesion is provoked by stress model, the best result obtained with dehydrocrotonin, at 100 mg/kg, was a 65% reduction (Souza-Brito et al., 1998). In this experiment, we also observed that semi-synthetic crotonin was more effective than dehydrocrotonin.

The effects of semi-synthetic crotonin on the biochemical parameters of gastric acid secretion (pH, total gastric acid, gastric juice volume) using the pylorus ligature method suggest that the antisecretory mechanism of intraduodenally administered semi-synthetic crotonin is opposite to that of dehydrocrotonin, which inhibits gastric acid secretion (Souza-Brito et al., 1998; Hiruma-Lima et al., 1999).

In conclusion, the results show that semi-synthetic crotonin has a better cytoprotective activity and a slightly lower cytotoxicity than dehydrocrotonin (Souza-Brito et al., 1998; Hiruma-Lima et al., 1999). However, it seems that the mechanisms involved in the antiulcerogenic action are different for semi-synthetic crotonin and dehydrocrotonin, probably due to the different spatial configuration of the compound under study. Further studies are necessary to investigate other possible mechanism(s) involved in this antiulcerogenic action of semi-synthetic crotonin and they are now in progress in our laboratory.

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