Gastroprotective Mechanisms of Centipedic Acid, a Natural Diterpene from *Egletes viscosa* LESS.

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This study was aimed to clarify the mechanisms of gastroprotection by centipedic acid (CPA), a natural diterpene from *Egletes viscosa* LESS. (Asteraceae) using ethanol-induced gastric mucosal damage in mice and gastric secretion in 4-h pylorus-ligated rats as model systems. In mice, intragastrically administered CPA (25, 50, 100 mg/kg) greatly reduced the mucosal lesions induced by 96% ethanol (0.2 ml, p.o.) by 18, 53, and 79%, respectively, whereas *N*-acetylcysteine (NAC, 300 mg/kg, i.p.), the reference compound produced a 50% inhibition. In 4-h pylorus-ligated rats, CPA (50 mg/kg) applied intraduodenally decreased both gastric secretory volume and total acidity. Similar to NAC, the plant diterpene effectively prevented the ethanol associated decrease in non-proteic sulfhydryls (NP-SH) and the elevated thiobarbituric acid-reactive substances (TBARS) in gastric tissue, suggesting that these compounds exert an antioxidant effect. Pretreatment of mice with indomethacin, the cyclooxygenase inhibitor but not with capsazepine, the transient receptor potential vanilloid-1 (TRPV1)-receptor antagonist greatly suppressed the gastroprotective effect of CPA. Furthermore, CPA gastroprotection was significantly attenuated in mice pretreated with *i*-NAME or glibenclamide the respective inhibitors of nitric oxide synthase and K\textsubscript{ATP} channel activation. These data suggest that CPA affords gastroprotection by different and complementary mechanisms, which include a sparing effect on NP-SH reserve, and roles for endogenous prostaglandins, nitric oxide, and TRPV1-receptor and K\textsubscript{ATP} channel activation.

Key words  *Egletes viscosa*; Asteraceae; centipedic acid; diterpene; gastroprotection; mechanism

Peptic ulcer disease and gastric dispepsia-associated with chronic use of therapeutics such as nonsteroidal anti-inflammatory drugs (NSAIDs) and anticancer agents are the two major causes that adversely affect the life quality. Presently used antisecretory agents like proton pump inhibitors may represent a key option in peptic ulcer therapy but their prolonged use seems to be associated with high incidence of hip fractures. Non-steroidal anti-inflammatory drugs (NSAIDs)-induced gastropathy remains a major clinical problem, which has not been solved through the introduction of selective inhibitors of cyclooxygenase-2 (COX-2) due to cardiac side effects. Similar to NSAIDs, many cancer chemotherapeutics such as cisplatin, and bisphosphonates like alendronate can induce gastric dyspepsia. In recent years, there is an active search to discover novel and alternative agents useful to combat gastric dyspepsia, and peptic ulcer disease.

Diterpenoids form a large class of secondary metabolites isolated from plants that possess a wide spectrum of pharmacological profile that include anti-inflammatory, antimicrobial, antispasmodic, cytotoxic, antitumor and gastroprotective properties. Of particular interest of gastroprotective diterpenes are the clerodanes (*trans*-dehydrotrocon from *Croton cajucara*, and aparistham from *Aparisthium cordatum*), the labdanes (*solidagenone* from *Solidago chilensis* and *15-acetoxylabd-8(17)-en-19-ol* as well as *15,19-diacetoxylabd-8(17)-en* from *Araucaria araucana*), abietane (*feruginol* from *Pseudopitys andina*, and Jatrophone from *Jatropha isabellii*), which may play a major role in drug discovery as well, in providing lead structures for the development of synthetic molecules.

In several parts of Brazil, tea or decoction prepared from the flower buds of *Egletes viscosa* (Asteraceae) is a popular remedy for the treatment of digestive and intestinal problems. Phytochemical studies on flower buds revealed the presence of diterpene compounds, centipedic acid (CPA) and 12-acetoxy-hawtriwaic acid lactone (tanabolin), and a tetra-methoxy flavone, ternatin. Pharmacological investigations identified the anti-inflammatory, hepatoprotective, antidiarrhoeal and gastroprotective properties of ternatin. In a previous study, we established the gastroprotective effects of both CPA and tanabolin against indomethacin and ethanol-induced gastric lesions, validating the traditional use of *E. viscosa*.

However, the gastroprotective mechanisms were not elucidated. Experimental and clinical evidence suggests that oxidative stress and increased acid secretion play a pivotal role in the etiopathology of gastric ulcer disease and antioxidants and antacid agents can afford gastric cytoprotection. The present study was mainly aimed to probe into the gastroprotective mechanisms of CPA, using the mouse model of gastric damage induced by 96% ethanol to study the role of oxidative stress and capsaicin-sensitive sensory afferents and the 4-h pylorus-ligated rat to verify whether or not it modulates gastric acid secretion.

MATERIALS AND METHODS

Plant Material and Isolation of CPA The flower buds of *Egletes viscosa* LESS. (Asteraceae) were collected from the experimental plantation pertaining to the Department of Agronomy, Federal University of Ceará, after its authentification by Prof. Edson de Paula Nunes, and a voucher specimen

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(#16327) has been deposited at the Herbário Prisco Bezerra of the same University. CPA was extracted and isolated from the dried plant material as per procedures described earlier. From this, the following drugs and chemicals were used: absolute ethanol (EtOH; BDH, U.K.); N^N-nitro-l-arginine methyl ester (l-NNAME), N-acetylcyesteine (NAC), capsaicin, capsazepine, clonidine, yohimbine, diazoxide, glibenclamide, glutathione, thiobarbituric acid (Sigma Chemical Company, St. Louis, MO, U.S.A.); indomethacin (Indocid®, Merck Sharp & Dohme, Brazil), misoprostal (Cytotec®, Continental Pharma, Italy), and cimetidine (Tagamet® SmithKline, Rio de Janeiro, Brazil). All other chemicals used were of analytical reagent grade (Merck). CPA was first dissolved in DMSO and subsequently diluted in distilled water and the resulting solutions of CPA did not exceed concentration of DMSO beyond 2%. Other drugs were dissolved either in physiological saline or distilled water. Drug concentrations were adjusted for treatment to give a volume of 10 ml/kg. In pilot studies 2% DMSO (vehicle) demonstrated no per se effect or influence on the ulcerogenicity of ethanol.

Animals Male Swiss albino mice (20—25 g) and male Wistar rats (150—160 g) obtained from the Central Animal House of Federal University of Ceará were used. They were housed in environmentally controlled conditions (22±2 °C, 12-h light–dark cycle), with free access to standard pellet diet (Purina do Brasil LTDA, São Paulo, Brazil) and water ad libitum. Animals were kept in cages with raised floors to prevent coprophagy. They were fasted over a period of 15 h and were habituated to the test environment for 2 h before the experimentation. The experimental protocols were approved by the Animal Care and Use Committee of the Federal University of Ceará in accordance with the ethical guidelines of National Institute of Health, Bethesda, U.S.A.

Effect of CPA on Ethanol-Induced Gastric Damage Acute gastric lesions were induced in mice (n=8/group) by intragastric administration of absolute ethanol (96%) in a volume of 0.2 ml using orogastric metal tube. CPA dissolved in DMSO (2% in distilled water as vehicle) was administered at oral doses of 25, 50, and 100 mg/kg, 60 min before ethanol application. Vehicle (2% DMSO), NAC (300 mg/kg, i.p.), and cimetidine (100 mg/kg, p.o.) or vehicle were administered intraduodenally, immediately after pylorus ligation. Four hours after pylorus ligation, the rats were killed, stomachs were removed, gastric contents were collected and centrifuged at 3500 rpm for 15 min. The supernatant volume (ml) was measured and total acidity was determined by titration with 0.1 N NaOH using 2% phenolphthalein indicator, and the results were expressed as μEq/h.

Data Analysis The data are presented as mean±S.E.M. of 6—8 animals per group and the statistical significance between groups was analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keul’s test. The differences between groups were regarded as significant at p<0.05.

RESULTS

Effect of CPA and NAC on Ethanol-Induced Gastric Damage The effect of CPA on gastric lesions induced by absolute ethanol is shown in Fig. 2. Ethanol induced an in-
tense gastric mucosal damage in the form of hemorrhagic streaks in control group of mice that received vehicle alone but not CPA. The lesion area (mm²) expressed as percentage in relation to total area of corpus in the control group was 63.78±4.37 mm². Compared to controls that received the vehicle, CPA-treated groups of mice displayed marked and dose-dependent protection against ethanol-damage. At the test doses of 25, 50, and 100 mg/kg, the reductions in gastric damage were in the order of 18, 53, and 79%, respectively. A higher dose (>100 mg/kg) of CPA did not cause further improvement in its gastroprotection against ethanol injury (data not shown). Among the reference standards included in the study, while NAC showed a 50% inhibition on ethanol-induced gastric lesions, cimetidine failed to produce any significant effect. However, the inhibitory effect of CPA at 100 mg/kg appeared much greater (79%) than to NAC, a known antioxidant tested at the dose of 300 mg/kg.

Effect of CPA on Ethanol-Induced Levels of Gastric NP-SH in Mice and on Gastric Secretion in Pylorus-Ligated Rats Since 50 mg/kg CPA offered a ca. 50% protection, this dose was selected to study its influence on ethanol-induced depletion of gastric NP-SH in mice and on gastric secretion in 4-h pylorus-ligated rats. Ethanol significantly depleted gastric NP-SH in control mice that received the vehicle (213.9±15.9 μg/g), when compared to basal value seen in saline-treated normal control (428.0±42.1 μg/g). (Fig. 3A). In contrast, ethanol-induced depletion of NP-SH was significantly less in animal groups treated with CPA (366.3±31.1 μg/g), or NAC (304.1±21.6 μg/g), a sulphydryl donor. The effect of CPA and NAC on ethanol-induced changes in TBARS content of gastric tissues is shown in Fig. 3B. While ethanol significantly enhanced the TBARS level (90.3±10.3 μM) as compared to values seen in normal controls (54.5±6.2 μM), pretreatment of animals with CPA (37.6±3.2 μM) or NAC (31.7±4.7 μM) resulted in marked suppression of ethanol-induced increases in TBARS, indicating that these agents suppress lipid peroxidation. Figure 4 depicts the effects of CPA (50 mg/kg, p.o.) and cimetidine (100 mg/kg, p.o.) on gastric secretory volume and total acidity in 4-h pylorus-ligated rats. In vehicle-treated controls, the gastric secretion volume and total acidity were 4.7±0.4 ml and 128.9±9.4 μEq/h), respectively. CPA (50 mg/kg) treatment caused marked diminutions in 4-h pylorus-ligation associated increases of gastric secretory volume (2.6±0.3 ml) and total acidity (55.5±15.6 μEq/h), whereas cimetidine decreased only the total acidity (27.3±3.7 μEq/h), but not the secretory volume (3.6±0.6 μEq/h).
DISCUSSION

The present investigation confirms our earlier observation on the gastroprotective effect of CPA, a linear diterpenoid isolated from the flower buds of \textit{Egletes viscosa}. At oral doses of 25—100 mg/kg, CPA dose-dependently prevented the ethanol-induced acute hemorrhagic mucosal lesions of the glandular region of the stomach. It has been firmly established that oxidative stress and impaired prostaglandin synthesis contribute to gastric mucosal damage in experimental models of gastric lesions induced by ethanol.\textsuperscript{24} In this study, CPA significantly replenished the ethanol-induced gastric wall NP-SH depletion and greatly diminished the lipid peroxidation as evidenced by decreased TBARS, suggesting its capacity to prevent oxidative stress. It was reported that in humans, a reduction in gastric glutathione can occur following ethanol consumption and glutathione pretreatment could subside the gastric damage.\textsuperscript{25} In this study, similar to NAC, a donor of sulfhydrls, CPA significantly replenished the ethanol-associated decrease in NP-SH (reduced glutathione). Therefore, it is conceivable that it is endowed with antioxidant property.

Some plant-derived substances have been shown to attenuate ethanol-and stress-induced gastric lesions via activation of prostaglandin, nitric oxide and sensory nerve pathways and thus improving the microcirculation.\textsuperscript{26,27} The results obtained in this study clearly show that CPA simulates the gastroprotection seen in mice treated with the TRPV1-receptor agonist capsaicin, prostaglandin analogue misoprostol, NOS-agonist \textit{l}-arginine, or K\textsuperscript{+}\textsubscript{ATP} channel opener diazoxide. On the basis of the results obtained, showing that CPA exerts a gastroprotective effect against ethanol-induced lesions, we studied the mechanisms involved in such activity. Capsaicin is the active component of red hot peppers, which modifies specifically the capsaicin-sensitive sensory afferent nerves. It has been shown that capsaicin-sensitive afferent fibers play a crucial role in acute gastroprotection. Activation of sensory neurons by compounds like capsaicin causes the release of neuropeptides such as calcitonin gene-related peptide (CGRP), increased production of nitric oxide through activation of constitutive nitric oxide synthase, with a consequent increase in mucosal blood flow that renders the stomach less susceptible to damage.\textsuperscript{28} However, in the present work, capsaizepine, a TRPV1-receptor antagonist produced no significant blockade of CPA gastroprotection, suggesting that additional mechanisms may be involved in its gastroprotective action.

Prostaglandins are of particular importance for the maintenance of gastric mucosal integrity when neuronal defense mechanisms are impaired.\textsuperscript{29} Among the various factors involved in the maintenance of gastric mucosal integrity and protection against ethanol-injury, we focused the attention on the contribution of endogenous nitric oxide (NO) or prostaglandins (PG) in the gastroprotective activity of CPA.
The ability of CPA to protect against indomethacin-induced gastric ulcers may be due to enhanced synthesis of prostaglandins, or reduced acid output observed in our pylorus-ligated rat model. It significantly reduced basal gastric secretory volume as well as titratable acidity, simulating the action of prostaglandin. Gastric acidity although does not have a prominent role in ethanol-induced mucosal injury, the present finding that CPA reduces gastric acidity has relevance for its protection against indomethacin. Since the present finding that CPA reduces gastric acidity has relevance for its protection against indomethacin, it is suggested that under these conditions endogenous prostaglandins and nitric oxide act as activators of K\textsubscript{ATP} channels, and or reduced acid output observed in our pylorus-ligated rat model. It significantly reduced basal gastric secretory volume as well as titratable acidity, simulating the action of prostaglandin. Gastric acidity although does not have a prominent role in ethanol-induced mucosal injury, the present finding that CPA reduces gastric acidity has relevance for its protection against indomethacin.

In this study, treatment with CPA afforded much greater gastroprotection than to NAC, a known antioxidant against ethanol damage, possibly due to some additional actions. The gastroprotective effect of CPA must be a result of an increased bioavailability of mucosal sulfhydryls, endogenous prostaglandins, nitric oxide release and potassium channel activation as it was largely mitigated in animals pretreated with L-NAME, glibenclamide, and indomethacin, the respective inhibitors of NOS, K\textsubscript{ATP} channels and prostaglandin synthesis. In contrast, the gastroprotective effect of NAC was largely mitigated by L-NAME, weakly suppressed by glibenclamide and almost unchanged by indomethacin treatments (data not shown). It implies that CPA has a much broader spectrum of activity in affording gastroprotection than to NAC.

In conclusion, the results of this study indicate a cytoprotective role for the diterpene, CPA from Egletes viscosa affording gastroprotection against gastric mucosal damage induced by ethanol. Its gastroprotective mechanism is a multifactorial that possibly involves an antioxidant effect through a sparing action on gastric NP-SH (reduced glutathione), stimulation of endogenous prostaglandins and nitric oxide release, activation of capsaicin-sensitive gastric afferents, and K\textsubscript{ATP} channels, accompanied by an increase in gastric microcirculation.

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