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Communication

Inhibition of H^+/K^+ ATPase in the gastroprotective effect of *Baccharis illinita* DC

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Abstract

Baccharis illinita DC (Compositae) is used in folk medicine to treat gastric disturbances. Preliminary studies with other extracts of *B. Illinita* showed gastric protection against ethanol-, indometacin- and stress-induced ulcers and the inhibition of gastric secretion. Based on these data, the aim of this study was to verify the pathways involved in the inhibition of gastric secretion. The chloroform extract (CE) of flowers from *B. illinita* (3, 10, 30 and 100 mg kg^{-1} i.p.) tested on rats with pylorus ligature reduced the volume and the total acidity of gastric content by approximately 50% (ED50 = 69 mg kg^{-1}). Treatment with CE (100 mg kg^{-1} i.p.) reduced the gastric total acidity stimulated by histamine, bethanechol and pentagastrin to 42%, 27% and 57% of that in the stimulated control group, respectively. The CE (10, 30 and 100 μM) inhibited H^+/K^+ ATPase activity in-vitro, with an IC50 of 37 μM. The isolated flavonoid luteolin (1, 3, 10 and 30 μM) also inhibited H^+/K^+ ATPase activity by 50%, at a dose of 30 μM. Our results suggest that the reduction in gastric secretion occurs through inhibition of H^+/K^+ ATPase, which is the final step in acid secretion and therefore one of the most important steps.

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Introduction

For more than a century, peptic ulceration has been a major cause of morbidity and mortality. The pathophysiology of peptic ulcer centres on an imbalance between aggressive and protective factors in the stomach, such as acid-pepsin secretion, integrity of the mucosal barrier, mucus secretion, blood flow, cellular regeneration, prostaglandins and growth factors (Higham et al 2002). Borreli and Izzo (2000) revealed the extensive variety of chemical compounds with anti-ulcer activity isolated from medicinal plants. Plant extracts are some of the most attractive sources of new drugs and have shown promising results for the treatment of gastric ulcer (Alkofahi & Atta 1999).

The genus *Baccharis* belongs to the Asteraceae family and several species (e.g. *B. trimera*) are commercialized in Brazil. *B. illinita* DC is an annual sub-shrub with yellowish flowers that grows in natural grasslands. This plant is popularly known in the south of Brazil as 'chá ventura' or 'erva milagrosa' and has been used in folk medicine as an anti-inflammatory, for the healing of skin and mucosal wounds, and for its gastroprotective actions (Baggio et al 2003; Verdi et al 2004). Phytochemical investigation of *B. illinita* has demonstrated the occurrence of numerous flavonoids and diterpenes (Verdi et al 2004). In addition, preliminary studies by Baggio et al (2003) have shown that the aqueous and hydroalcoholic crude extracts obtained from several parts of *B. illinita* protected the gastric mucosa against ethanol-, indometacin- and stress-induced mucosal lesions and inhibited gastric acid secretion in rats. In the present study we investigated whether inhibition of H^+/K^+ ATPase by the chloroform extract (CE) of the flowers from *B. illinita* DC, as well as its isolated compounds, could be involved in the gastroprotective mechanisms previously mentioned.

Material and Methods

Plant material

B. illinita DC was collected in Serra do Falcão, Minas Gerais, Brazil, and identified by the botanist José Badini (Universidade Federal de Ouro Preto, Minas Gerais, Brazil). A voucher specimen has been deposited at the Herbarium of the Universidade Federal de Ouro Preto (voucher OUPR no. 3450).

Phytochemical characterization

The phytochemical characterization of the CE of *B. illinita* was described previously by our group (Verdi et al 2004) as follows. The air-dried flowers of *B. illinita* (500 g) were first extracted with chloroform and then with ethanol, at room temperature for 15 days. The crude CE (6.0 g, yield 1.2%) was suspended in EtOH:H_2O (10:90 v/v) and extracted with ethyl acetate to yield 1.3 g (22% from CE) of the EtOAc fraction. Chromatography fractionation on silica gel eluting with hexane–ethyl acetate–ethanol mixtures led to the isolation of the major flavonoids nobiletin 1 (4 mg, 0.31% yield), tangeretin 2 (3 mg, 0.23% yield), luteolin 3 (49 mg, 4% yield), kaempferol 4 (3 mg, 0.23% yield) and naringenin 5 (33 mg, 2.5% yield), as well as three diterpenes: 15-β-hydroxy-ent-kaur-16-en-19-oic acid 6 (6 mg, 0.5% yield), 15-β-senecioyloxy-ent-kaur-16-en-19-oic acid 7 (106 mg, 8% yield) and 17-hydroxy-ent-kaur-15-en-19-oic acid 8 (11 mg, 0.8%) (Figure 1). The flavonoids were identified by mass spectrometry and NMR spectral analysis and comparison with literature data (Agrawal 1989). The diterpenes were identified by interpretation of the spectral data (1H NMR, two-dimensional 1H–1H correlation spectroscopy, 13C-NMR, distortionless enhancment by polarization transfer (DEPT), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bound coherence (HMBC)) as well as by comparison of the data with literature values (Lobitz et al 1998; Amaro-Luis 1993). HMQC and HMBC are two-dimensional inverse H–C correlation techniques that allow determination of C–H connectivity: HMQC is selective for direct C–H coupling; HMBC will give longer range couplings (2–4 bond coupling).

Preparation of extract

For in-vivo experiments the CE was dissolved in 1% Tween 80 and saline. The vehicle used for this test was 1% Tween 80 and saline. For in-vitro experiments, the CE and flavonoids (luteolin, naringenin and kaempferol) were dissolved in DMSO (final DMSO concentration 5%). The vehicle used for this test was DMSO.

Animals

Female Wistar rats (180–200 g) were obtained from the animal facilities of the Universidade Federal do Paraná. A male rabbit (2 kg) was obtained from Cooperativa dos Produtores de Coelho do Estado do Paraná (Coelho Brasil; São José dos Pinhais, PR, Brazil). Animals were kept in a constant 12 h light–dark cycle

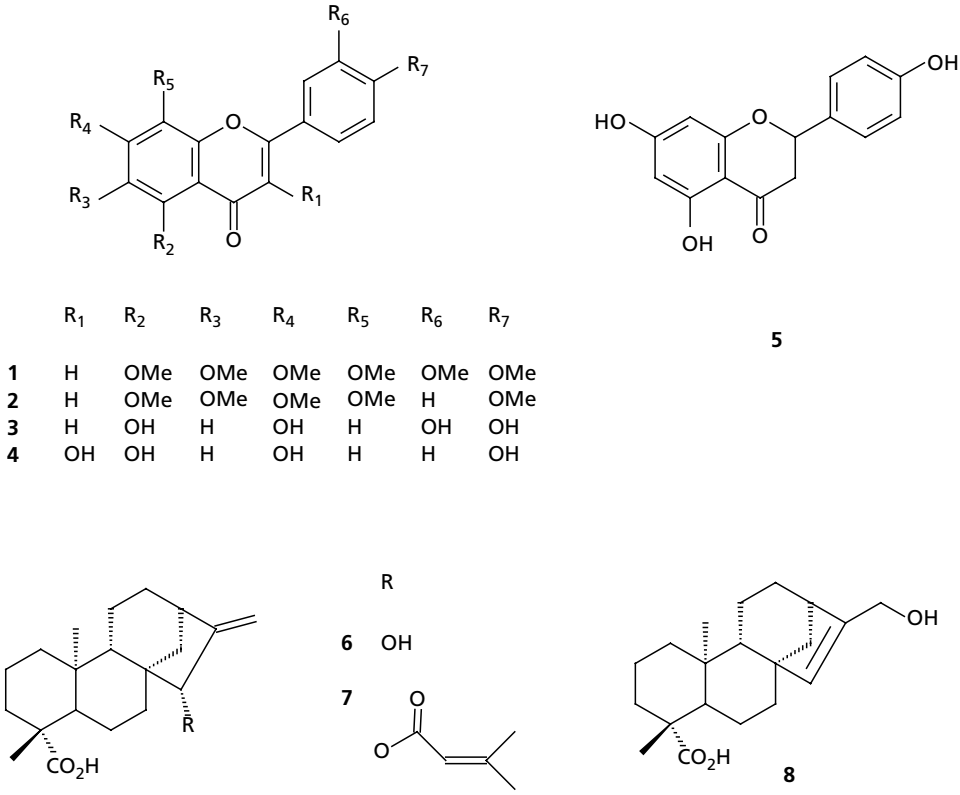

Figure 1 Chemical structures of the major compounds from the chloroform extract of *Baccharis illinita.*

with controlled temperature (22±2°C). Standard pellet food (Nuvital, Curitiba, PR, Brazil) and water were available ad libitum. The Institutional Ethics Committee of the Universidade Federal do Paraná approved all procedures (approval number 137).

Measurement of gastric secretion

A pylorus ligature was performed in fasted rats (n=6 per group) under anaesthesia according to the method of Shay et al (1945). Immediately after pylorus ligature, CE (3, 10, 30 and 100 mg kg^{-1} i.p.) or vehicle (0.1 mL 100 g^{-1}, i.p.) was administered. Omeprazole (40 mg kg^{-1} p.o.), used as a positive control, was administered 1 h before the pylorus ligature. Animals were killed after 4 h and the gastric secretion collected with a pipette. The final volume was determined directly after washing the mucosal side of the stomach with 3 mL distilled water. The total acidity of the gastric juice was titrated in a digital burette with 0.1 N NaOH using phenolphthalein (2%) as an indicator. The effects of CE were also tested on gastric secretion induced by bethanechol (2.5 mg kg^{-1} s.c.), histamine (20 mg kg^{-1} s.c.) or pentagastrin (400 μg kg^{-1} s.c.). All secretagogue agents were injected 1 h after surgery for pylorus ligature.

Preparation of rabbit gastric H^+/K^+ ATPase

The H^+/K^+ ATPase was prepared by ultracentrifugation and gradient separation as described previously (Kubo et al 1995). Gastric glandular tissues were homogenized in five volumes of homogenizing buffer (250 mM sucrose, 2 mM $MgCl_2$, 1 mM EGTA and 2 mM HEPES, pH 7.4, with protease inhibitor) and centrifuged (20 000 *g* for 20 min). The supernatant was centrifuged at 100 000 *g* for 1 h and the resulting pellet was homogenized in homogenizing buffer, placed on top of a 30% sucrose cushion and centrifuged at 100 000 *g* for 2 h. The gastric vesicular fraction containing H^+/K^+ ATPase was collected and stored at −70°C. All procedures were performed at 4°C.

Assay of H^+/K^+ ATPase

H^+/K^+ ATPase activity was assayed according to the method of Kubo et al (1995). The assay contained 5 μg protein, with or without CE (3–100 μg/mL^{-1}), luteolin, naringenin or kaempferol (1–30 μM). The reaction was initiated by addition of 1 mM ATP. Samples were incubated for 20 min at 37°C and the reaction was stopped by addition of ice-cold trichloroacetic acid (50%). The amount of inorganic phosphate (Pi) formed was quantified according to the method of Fiske and Subbarow (1925). Activity was calculated using the extinction coefficient of (ε=11 000 M^{-1} cm^{-1}). Experiments were performed in triplicate. Omeprazole (500 μM) was used as a positive control.

Protein determination

Protein concentration of gastric ATPase was determined in a 96-well plate using a commercial BCA protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin as the standard.

Statistical analysis

Results are presented as the mean ± s.e.m. and were compared using one-way analysis of variance followed by Bonferroni's test. *P* values of less than 0.05 were considered significant. The ED50 and EC50 values were calculated by fitting the data to the equation: Vi/Vo = 1/(1 + [I]/IC50) using the KhaleidaGraph 3.0 for Windows program (Synergy Software, PA, USA), where Vi = total activity, Vo = remaining activity and [I] = inhibitory concentration.

Results

Gastric acid secretion

Rats secreted 8.8±0.6 mL gastric juice, with a total acidity of 0.077±0.004 mEq [H^+] mL^{-1} at 4 h after application of the pylorus ligature (control group). The administration of CE from the flowers of *B. illinita* (30 and 100 mg kg^{-1}, i.p.) produced a dose-dependent inhibition of acid secretion, which was accompanied by reductions in both the volume of gastric secretion (by 43% and 54%, respectively) and its acidity (by 35% and 57%, respectively). The ED50 calculated for the latter was 69 mg kg^{-1}. The positive omeprazole reduced the volume of gastric juice produced by 58% and the gastric acidity by 90% (Figure 2).

Gastric acid secretion stimulated by secretagogues

Gastric total acidity was increased by histamine by 40% compared with the control group (0.114±0.004 vs 0.071±

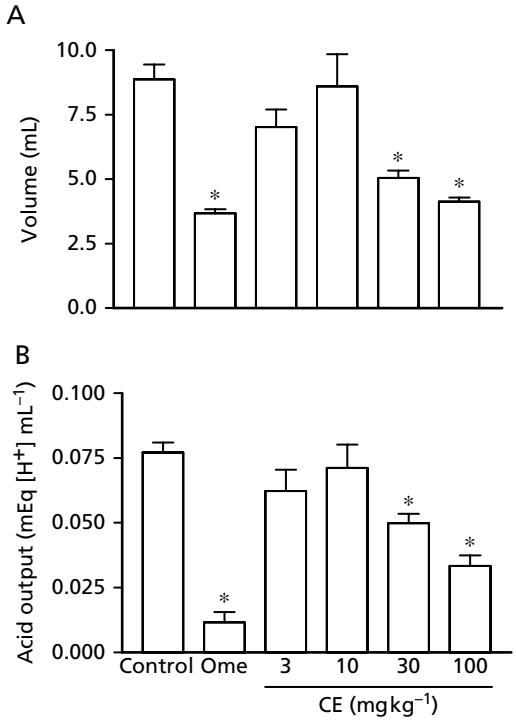

Figure 2 Gastric secretion inhibition by the chloroform extract of flowers from *B. illinita* (CE) after 4 h of pylorus ligation. The animals received vehicle (saline + Tween, 0.1 mL 100 g^{-1} i.p.; controls), CE (3–100 mg kg^{-1} i.p.) or omeprazole (Ome; 40 mg kg^{-1} p.o.). The volume (A) and acidity (B) of the gastric contents were determined after 4 h. All values are mean ± s.e.m. Statistical comparison was by analysis of variance followed by Bonferroni's test. *P < 0.05 vs control. The EC50 for acidity of gastric contents was 69 mg kg^{-1}.

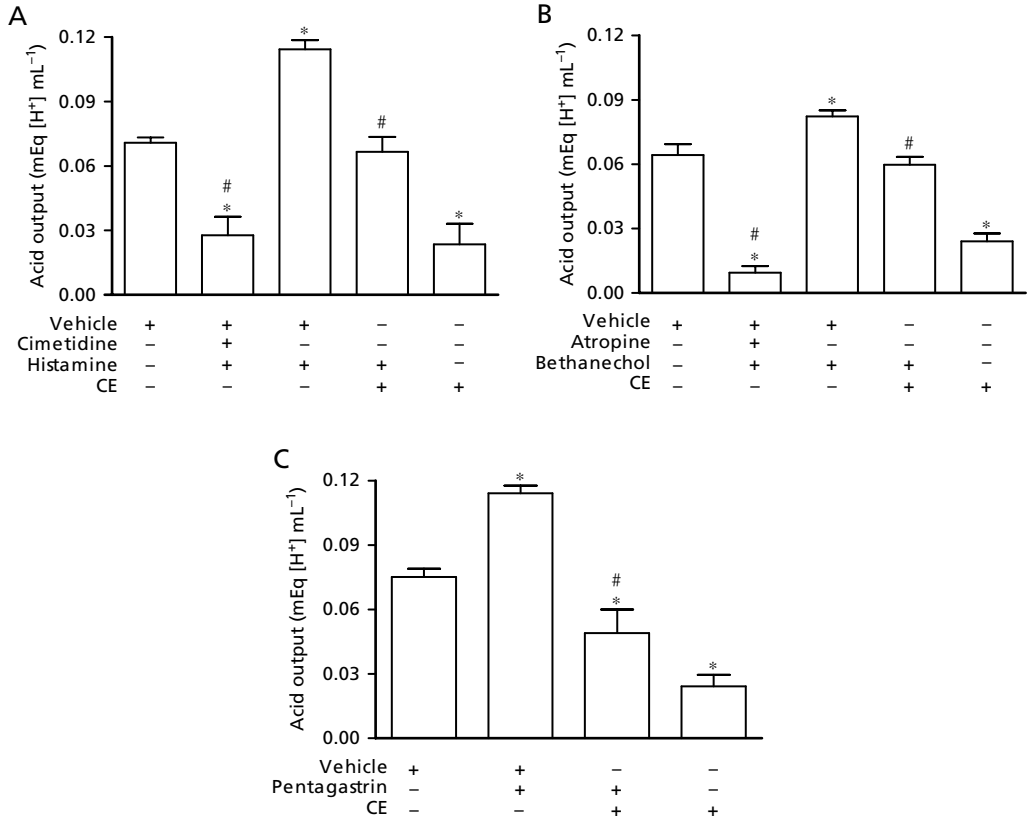

Figure 3 Inhibition of histamine- (A), bethanechol- (B) and pentagastrin-stimulated (C) gastric total acidity by the chloroform extract of flowers from *B. illinita* (CE) after 4 h of pylorus ligation. The animals received vehicle (saline + Tween, 0.1 mL 100 g^{-1} i.p.; controls), CE (100 mg kg^{-1} i.p.), cimetidine (60 mg kg^{-1} i.p.) or atropine (1 mg kg^{-1} s.c.) before stimulation with histamine (20 mg kg^{-1} s.c.), bethanechol (2.5 mg kg^{-1} s.c.) or pentagastrin (400 μg kg^{-1} s.c.). The acidity of the gastric contents was determined after 4 h. Values are mean ± s.e.m. Statistical comparisons were by analysis of variance followed by Bonferroni's test. *$P < 0.05$ vs vehicle control; $^{\#}P < 0.05$ vs stimulated group.

0.002 μEq [H^+] mL^{-1}). I.p. administration of CE (100 mg kg^{-1}) or cimetidine (60 mg kg^{-1}) blocked the increase in gastric total acidity by 42% and 75%, respectively (Figure 3A). Bethanechol increased the total acidity by 22% compared with the control group (0.082 ± 0.003 vs 0.064 ± 0.005 μEq [H^+] mL^{-1}). Treatment with CE (100 mg kg^{-1} i.p.) or atropine (1 mg kg^{-1} s.c.) reduced the increase in gastric total acidity by 27% and 89%, respectively (Figure 3B). Pentagastrin increased the total acidity by 35% compared with the control group (vehicle) (0.114 ± 0.003 vs 0.075 ± 0.004 μEq [H^+] mL^{-1}). Treatment with CE (100 mg kg^{-1}, i.p.) blocked the increase in gastric total acidity by 57% (Figure 3C).

H^+/K^+ ATPase activity

ATP hydrolysis by H^+/K^+ ATPase isolated from the gastric mucosa of rabbits treated with vehicle was 1.677 ± 0.094 μM Pi mg^{-1} min^{-1}. The CE (10, 30 and 100 μg/ml) reduced the H^+/K^+ ATPase activity by 31%, 44% and 66%, respectively, with an IC50 value of 37 μg mL^{-1} (Figure 4).

In the study of the effects of luteolin, naringenin and kaempferol from *B. illinita* on H^+/K^+ ATPase activity, ATP hydrolysis by H^+/K^+ ATPase isolated from the gastric mucosa of rabbits treated with vehicle was 2.191 ± 0.057 μM Pi mg^{-1} min^{-1}. Luteolin at concentrations of 1 and 3 μM enhanced the H^+/K^+ ATPase activity by 39% and 76%, respectively. However, at a

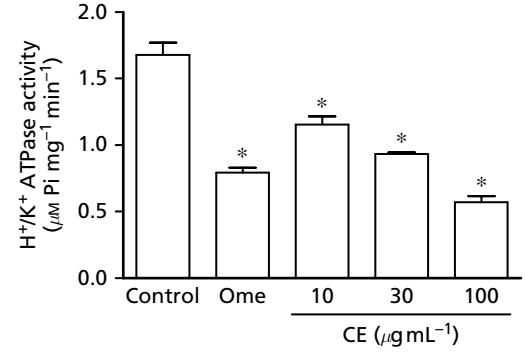

Figure 4 Inhibition of H^+/K^+ ATPase by the chloroform extract of flowers from *B. illinita* (CE). The CE (3–100 μg mL^{-1}) or vehicle (saline + DMSO) was incubated at 37°C for 20 min in triplicate. Omeprazole (Ome; 500 μM) was used as a positive control. Values are mean ± s.e.m. Statistical comparisons were by analysis of variance followed by Bonferroni's test. *$P < 0.05$ vs control group. The IC50 was 37 μg mL^{-1}.

Table 1 Inhibition of H^+/K^+ ATPase by luteolin, naringenin and kaempferol isolated from the chloroform extract of flowers from *Baccharis illinita*. The flavonoids (1–30 μM) or vehicle (saline + DMSO) were incubated at 37°C for 20 min in triplicate. Omeprazole was used as a positive control for the test

	Control	Omeprazole	1 μM	3 μM	10 μM	30 μM
Luteolin	2.19 ± 0.06	0.79 ± 0.03*	3.04 ± 0.14*	3.85 ± 0.21*	2.63 ± 0.17	1.07 ± 0.10*
Naringenin	2.40 ± 0.51	1.16 ± 0.05*	5.46 ± 0.03*	4.99 ± 0.20*	3.61 ± 0.48*	5.87 ± 0.16*
Kaempferol	1.66 ± 0.09	1.03 ± 0.04*	1.14 ± 0.06*	1.20 ± 0.08*	1.24 ± 0.07*	1.36 ± 0.11

Data are mean ± s.e.m. *$P < 0.05$ vs control (analysis of variance followed by Bonferroni's test).

concentration of 30 μM, luteolin reduced the H^+/K^+ ATPase activity by 51%. Kaempferol reduced the H^+/K^+ ATPase activity by about 30%, but we did not observe a concentration–response curve. Naringenin enhanced the H^+/K^+ ATPase activity at all concentrations tested (Table 1).

Discussion

Preliminary studies with the aqueous extracts of roots and flowers and hydroalcoholic extract of the leaves and stems of *B. illinita* showed protective activity against ethanol-induced ulcers (Baggio et al 2003). In this report, we demonstrated the main pathway involved in the gastroprotective activity of the CE of flowers from *B. illinita* in an experimental model of gastric hypersecretion using a pylorus ligature and H^+/K^+ ATPase activity.

The CE reduced gastric acid secretion of rats with their pylorus ligated. In addition, the extract blocked the induction of gastric acid secretion in response to the three major gastric acid secretagogues (histamine, bethanechol and pentagastrin), indicating a common pathway. We therefore concluded that inhibition of gastric acid secretion was by inhibition of the H^+/K^+ ATPase, the only common pathway to these three interactions. This conclusion was confirmed by the in-vitro inhibition of ATP hydrolysis by H^+/K^+ ATPase isolated from rabbit.

Gastric acid is produced by the H^+/K^+ ATPase enzyme in parietal cells, which are located deep in the gastric mucosa. This enzyme transports H^+ into the parietal cell canaliculus in exchange for K^+. Stimulation of the parietal cells occurs via muscarinic (M_3), gastrin (CCK_B) and/or histamine (H_2) receptors on the basolateral membrane. Such stimulation causes the movement of H^+/K^+ ATPase to the apical membrane of the cell, where it can exchange H^+ for K^+. Chloride ions enter the secretory canaliculi from the cytoplasm by a passive transport mechanism, resulting in the secretion of HCl (Kromer 1995; Sachs et al 1995).

Gastric acid is pathogenic in many gastrointestinal disorders, such as gastroesophageal reflux disease and peptic ulcer. Understanding the physiology of gastric secretion has led to numerous ways to inhibit acid production. Proton pump inhibitors (PPIs) are the antisecretory drugs of choice for serious acid-related conditions (Robinson 2005). Numerous trials have demonstrated the superior efficacy of the PPIs over other acid-inhibiting drugs and so-called cytoprotective agents (Mössner & Caca 2005).

Many compounds present in this plant may be responsible for the inhibitory actions observed in this study. Flavonoids, a group of phenolic compounds present in a wide variety of plant sources such as fruits and herbs, affect the activity of enzyme systems, including cyclic nucleotide phosphodiesterase, protein kinase C, ion-transport ATPase, hyaluronidase and others (Ferrell et al 1979; Shoshan & MacLennan 1981; Harbone 1988; Ferriola et al 1989; Kuppusamy et al 1990).

Murakami et al (1992) showed that naturally occurring phenolic compounds, including the most common flavonoid, quercetin, are inhibitors of H^+/K^+ ATPase, and the phenolic hydroxyl groups play a role in the inhibition of the enzyme. This finding is corroborated by our results, and suggests that *B. illinita* has wide potential for the treatment of gastric disturbances.

Conclusions

Collectively, the results show that the gastroprotective effects of the CE of *B. illinita* DC flowers involve the inhibition of gastric H^+/K^+ ATPase. Further studies will be carried out to elucidate the mechanisms underlying this activity and to identify the active principles associated with this effect.

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