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Anti-secretory, anti-inflammatory and anti-*Helicobacter pylori* activities of several fractions isolated from *Piper carpunya* Ruiz & Pav.

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ABSTRACT

Ethnopharmacological relevance: The leaves of *Piper carpunya* Ruiz & Pav. (syn *Piper lenticellosum* C.D.C.) (Piperaceae), are widely used in folk medicine in tropical and subtropical countries of South America as an anti-inflammatory, anti-ulcer, anti-diarrheal and anti-parasitical remedy as well as an ailment for skin irritations.
Aims of the study: To study the anti-inflammatory, anti-secretory and anti-*Helicobacter pylori* activities of different fractions isolated from an ethanolic extract of the leaves of *Piper carpunya*, in order to provide evidence for the use of this plant as an anti-ulcer remedy. Moreover, to isolate the main compounds of the extract and relate their biological activity to the experimental results obtained with the fractions.
Materials and methods: Sixteen fractions were obtained from the ethanolic extract (F I–XVI) and 16 pure compounds were isolated and identified from these fractions. We studied the effects of the fractions (0.1–400 μg/mL) on the release of myeloperoxidase (MPO) enzyme from rat peritoneal leukocytes, on rabbit gastric microsomal H^+, K^+-ATPase activity and anti-*Helicobacter pylori* anti-microbial activity using the microdilution method (MM). The main compounds contained in the fractions were isolated and identified by ^{1}H- and ^{13}C NMR spectra analysis and comparison with the literature data.
Results: Eight fractions showed inhibition of MPO enzyme (F I–IV, X, XII, XIV and XV). The highest inhibition was observed with F XIV (50 μg/mL, 60.9%, $p < 0.001$). F X and XII were the most active ones, inhibiting the gastric H^+, K^+-ATPase activity with IC_{50} values equal to 22.3 μg/mL and 28.1 μg/mL, respectively. All fractions, except F XV, presented detectable anti-*Helicobacter pylori* activity, with a diameter of inhibition zones ranging from 11 mm up to 50 mm. The best anti-*Helicobacter pylori* activity was obtained with F III and V. Both fractions killed *Helicobacter pylori* with lowest concentration values, about 6.25 μg/mL. Sixteen pure compounds were isolated, five of them are flavonoids that possess strong anti-oxidant and free radical scavenging activity, e.g. vitexin, isovitexin, and rhamnopyranosylvitexin. Terpenoids like sitosterol, stigmasterol and phytol, which have shown gastroprotective activity, and dihydrochalcones, like asebogenin, with anti-bacterial activity, were also isolated. Furthermore, the rare neolignan **1**, that is a DNA polymerase β lyase inhibitor, and (6S, 9S)-roseoside, that shows strong anti-bacterial activity, were isolated, for the first time, from the genus Piper.
Conclusions: We suggest that the flavonoids isolated from F I and II (vitexin, isovitexin, rhamnopyranosylvitexin and isoembigenin) contribute to the anti-MPO activity, as well as to their anti-*Helicobacter pylori* activity. These flavonoids may also be responsible for the important inhibition of H^+, K^+-ATPase activity. Also the phytosterols and phytol obtained from F XIV and XV could be involved in these gastroprotective activities. These results encourage us to continue phytochemical studies on these fractions in order to obtain full scientific validation for this species.

1. Introduction

The leaves of *Piper carpunya* Ruiz & Pav. (syn *Piper lenticellosum* C.D.C.) (Piperaceae), known with the popular name of "guavid-uca" in Ecuador are widely used in folk medicine in tropical and subtropical countries of South America, as an anti-inflammatory,

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anti-ulcer, anti-diarrheal and anti-parasitical remedy as well as an ailment for skin irritations (Díaz and Dorado, 1986). The anti-inflammatory activity has been confirmed in rat models like the carrageenan-induced paw edema and the results obtained support its use in the traditional medicine of Ecuador (De las Heras et al., 1998). Recently, this plant has been shown to protect against gastric ulcers induced by non-steroidal anti-inflammatory drugs (NSAID) in rats (Trabadela et al., 2008). However, there are only few reports about the mechanisms involved in the gastroprotective and anti-inflammatory effects of this species.

Nowadays it is very well-known that *Helicobacter pylori* colonization of the gastric mucosa is one of the most common chronic bacterial infections in humans and the most important ethiopathogenic agent for gastric and duodenal ulcers. *Helicobacter pylori* produces progressive gastric damage, such as gastrointestinal ulcers and it is also associated to gastric cancer (Ecclissato et al., 2002). The infection is typically acquired in early childhood and once established commonly persists throughout life unless treated. The route of transmission is uncertain, though the gastro-oral, oral-oral and faecal-oral routes are considered highly possible. Numerous plant remedies have shown to be active against *Helicobacter pylori* infection, such as the oil extract of *Chamomilla recutita* (Shikov et al., 2008), the ethanolic extract of *Cuminum cyminum* L., Propolis (Nostro et al., 2005) as well as the methanolic extract of *Alchornea triplinervia* (Spreng.) Müll. Arg., which exhibited anti-secretory, anti-*Helicobacter pylori* and gastroprotective effects (Lima et al., 2008).

On the other hand, the proton-pump inhibitors, mainly omeprazole, constitute an important strategy in the prevention of NSAID-induced gastroenteropathy, in the treatment of gastroesophageal reflux disease (GERD), chronic gastritis and also, together with antibiotics, in the *Helicobacter pylori* eradication treatment. However, these therapies are not always successful because of antibiotic resistance and poor availability of these drugs in some countries. Thus, in countries with important biodiversity resources there is an increasing trend to use anti-secretory and anti-ulcer preparations from medicinal plants as alternative remedies for the efficient treatment of gastric hypersecretion and gastroduodenal ulcers.

These facts have stimulated our interest to investigate the anti-inflammatory, antacid and anti-*Helicobacter pylori* activities of *Piper carpunya*. In order to understand the mechanisms involved in these properties, we studied the effects of different fractions of the ethanolic extract of this plant on the release of myeloperoxidase (MPO) enzyme from rat peritoneal leukocytes, on the rabbit gastric H^+, K^+-ATPase activity and its anti-*Helicobacter pylori* anti-microbial activity using the microdilution method (MM).

Previous phytochemcal studies of *Piper carpunya* have reported the essential oil composition of the leaves and spikes (Vargas et al., 2004; Díaz et al., 1986; Calle and Ferreira, 1973), and the isolation of two new C6–C3 and C6–C1 compounds (Díaz et al., 1986).

2. Materials and methods

2.1. Plant material

The plant was collected at Tandapi, Cantón of Santo Domingo, in the province of Pichincha (Ecuador), in January 2005, and was identified by the botanist Dr. Cerón. A voucher specimen has been deposited at the Herbarium of the School of Pharmacy and Biochemistry, Faculty of Chemical Sciences, the Central University of Quito (Ecuador) and kept under reference number 188.

2.2. Preparation of the extract

Ground, air-dried leaves of *Piper carpunya* were soaked in 95% aq. EtOH for 2 h at room temperature. The extract was concentrated in vacuo to a thick syrup (ca 8.4 g). 2.7 g of this extract were subjected to column chromatography (CC) (RP-18, 40 g) and eluted with 80% aq. MeOH yielding (TLC monitoring) 16 main fractions (F I–XVI).

2.3. Isolation of pure compounds

F XVI was constituted mainly by chlorophylls and carotenoids. CC (silica gel, 4 g) of F VI (143 mg) and VII (63 mg), eluted with a hexane–EtOAc gradient (1–50% EtOAc), gave neolignan **1** (67 mg), followed by **2** (20 mg). CC (silica gel, 12 g) of F IX (0.24 g), eluted with a hexane–Et_2O gradient (5–50% Et_2O) followed by a hexane–EtOAc gradient (10–50% EtOAc) afforded acid **3** (141 mg) and ester **4** (18 mg). CC (silica gel, 4 g) of F IV (96 mg), eluted with a hexane–EtOAc gradient (20–33% EtOAc) afforded nervogenic acid **5** (25 mg), followed by asebogenin **6** (33 mg). CC (RP-18, 3 g) of F XI, eluted with MeOH–H_2O (6:1), gave 10 (5 mg). Sitosterol **7** (35 mg), stigmasterol **8** (12 mg) and phytol **9** (21 mg) were isolated by repeated CC (silica gel; different hexane–Et_2O gradient mixtures) from F XIV and XV. Structures **1**–**9** are reported in Fig. 1.

CC (RP-18, 60 g) of combined F I and II (0.86 g) eluted with a MeOH–H_2O gradient (80–100% MeOH) afforded **9** main subfractions. Subfraction **1** was constituted by sugars, while main constituents of subfractions **2**–**8** (0.23 g totally) were flavonoids (TLC). Repeated HPLC separations of the latter fractions on semipreparative (BDS HYPERSIL C18) and analytical columns (PUROSPHER STAR RP-18), using different MeOH–H_2O gradient mixtures, eventually afforded (6S, 9S)-roseoside **11** (12.5 mg), vitexin **12** (3.5 mg), isovitexin **13** (2.0 mg), 4′-O-methyl-2″-O-α-L-rhamnopyranosylvitexin **14** (25 mg), traces of 4′-O-methyl-2″-O-α-L-rhamnopyranosylisovitexin **15**, and 4′,7-di-O-methylvitexin (isoembigenin) **16** (5.7 mg). Structures **10**–**16** are reported in Fig. 1.

The compounds were identified by ^1H and ^{13}C NMR spectra analysis and confirmed by comparison with the literature data for **1** (Chaturvedula et al., 2004); **2** and **6** (Hermoso et al., 2003); **3**, **4**, **5**, and **10** (Oryala et al., 1993); **11** (Yamano and Ito, 2005); **13** (Masuoka et al., 2003); **14** and **15** (Kumamoto et al., 1985); **12** and **16** (Mahling et al., 1995).

2.4. Animals

For the in vitro assays a suspension of polymorph nuclear leukocytes (PMNs) and mononuclear cells were obtained from male Wistar rats (200–300 g body weight). Microsomal gastric H^+, K^+-ATPase was prepared from rabbit gastric mucosal homogenates. All experiments followed a protocol approved by the local animal ethics committee and the local government and were in accordance with the recommendations of the European Union and with the Guide for the Care and Use of Laboratory Animals of the USA NIH.

2.5. In vitro experimental protocols

2.5.1. Leukocyte isolation

A suspension of leukocytes containing approximately 85% polymorph nuclear leukocytes (PMNs) and 15% mononuclear cells were obtained from male rats by an i.p. injection of 10 mL of a solution of 6% oyster glycogen in saline followed 16–20 h later by 60 mL ice-cold modified Hank's balanced salt solution (HBSS) free of Ca^{2+} and Mg^{2+} (Moroney et al., 1988; De la Puerta et al., 1999) .The mixed peritoneal leukocytes were resuspended in complete HBSS at 2.5×10^6 cells/mL containing 1.26 mM Ca^{2+} and 0.9 mM Mg^{2+}. Cell viability based on Trypan blue exclusion was greater than 95%.