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Spasmolytic effects of *Baccharis conferta* and some of its constituents

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Abstract

The Nahua of the Mexican state of Veracruz use *Baccharis conferta* in the treatment of a variety of gastrointestinal illnesses, especially diarrhoea associated with gastrointestinal cramps. The aerial parts of *B. conferta* were investigated phytochemically and pharmacologically using the guinea pig ileum assay as a model (histamine, KCl and electric stimulation). The crude ethanolic extract showed a dose-dependent antispasmodic effect that was particularly strong in flavonoid-rich fractions (e.g. IC50 value for fraction E.3.1 from the ethyl acetate fraction, in histamine-induced contraction, 10 μg mL^{-1}). Several flavonoids (apigenin-4′,7-dimethylether, naringenin-4′,7-dimethylether, pectolinarigenin and cirsimaritin) were isolated, while others were identified in complex fractions by GC-MS. The flavonoids play an important role in the antispasmodic activity of this indigenous drug. Additionally, oleanolic acid and its methyl ester as well as erythrodiol were isolated. Oleanolic acid methyl ester shows weak antibacterial activity against *M. luteus* and *E. coli* (20 μg/spot in a TLC assay). The phytochemical as well as the pharmacological data provide some in-vitro evidence for the use of *B. conferta* in the treatment of gastrointestinal cramps.

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Introduction

The Nahua of the Sierra de Zongolica (Veracruz, Mexico) use *Baccharis conferta* Kunth (Asteraceae), commonly called *escoba* or *escobilla china*, for treating gastrointestinal problems, notably diarrhoea and dysentery as well as colics and gastrointestinal cramps (Weimann & Heinrich 1997; Heinrich et al 1998). Similar uses are also known from many other regions in Mexico. Additionally, a variety of other uses (e.g. urinary problems and fever with cramps in children) have been recorded in the ethnobotanical literature (Argueta & Zolla 1994). This species has not been investigated pharmacologically, but in the electrically stimulated ileum of the guinea pig *B. salicifolia*, *B. serraefolia*, *B. trinvervis* and *B. vaccinoides* were shown to possess spasmolytic activity (Tortoriello et al 1995). Only caryophyllen and some triterpenes (including oleanolic acid and erythrodiol) have so far been isolated from the aerial parts of this species, while the roots yielded matricariaester, angelicaester and two coumaranes (Bohlmann & Zdero 1976). Guerrero & Romo de Vivar (1973) described the diterpene bacchofertine. The extract did not show inhibitory activity on NF-κB activation or cytotoxic effects (Bork et al 1996). Baccharis is a large genus with about 200 species. While many other species have been studied in detail, the lack of studies on the ethnobotanically important *B. conferta* combined with the very strong spasmolytic activity reported for other

species merited the study of the antispasmodic activity of the aerial parts and the isolation of some of its main constituents.

Materials and Methods

Plant material

Aerial parts of *B. conferta* Kunth (Asteraceae, Tribus Astereae) were collected in the Sierra de Zongolica, Veracruz, Mexico during March 1996 and identified in collaboration with O. Tellez (MEXU). Voucher specimens (CWEI 65 and CWEI 330) were deposited at the Méxican National Herbarium (MEXU) of the UNAM, México, D.F., Mexico, the herbarium of the Instituto de Ecología (XAL), Xalapa, Veracruz, Mexico, the ethnobotanical herbarium of the Instituto Mexicano del Seguro Social (IMSS-M), Mexico, D.F., Mexico, at the Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, University of London, UK and in the village of Zongolica itself.

Phytochemistry: general procedures

All solvents with the exception of the ones used in HPLC were of laboratory grade and were purchased from Merck (Darmstadt, Germany) or Roth (Karlsruhe, Germany). For the flavonoids methanol (MeOH)/water (H_2O) (8:2) on reversed phase (Merck) was generally used as a TLC system. The following adsorbents were used: Sephadex LH 20 (Pharmacia, Uppsala, Sweden), MCI-gel (Mitsubishi Chem. Ind., White Plains, NY) and silica gel (0.063–0.2 mm, Merck, Darmstadt).

GC-MS

All experiments were conducted on a Finnigan GC-MS 4000 using an Optima-1 column with Helium (8 psi) (detector and injector 160°C, split ratio 24:1, ionization power applied 70 eV, flame ionization detector). The respective flavonoid fraction was dissolved in dimethylsulfoxide and separated using a temperature gradient from 150°C (3 min) to 300°C at a rate of 10°C/min. The compounds were identified by comparison with authentic compounds and/or with the help of an in-house MS library. In some instances ^{1}H NMR spectra were also obtained. The MS data of pectolaringenin and apigenin-4′,7-dimethylether were identical to those of the isolated substances.

Extraction and isolation

The air-dried aerial parts (417 g) were pulverized and extracted under reflux once with ethanol (EtOH) (96%) and twice with EtOH (70%). The filtrates were combined, the organic solvent evaporated under reduced pressure with a rotary evaporator and the resulting residue freeze-dried, yielding 132.3 g of crude extract. Of this extract 101.3 g was further separated using a liquid–liquid procedure with n-hexane, ethyl acetate (EtOAc) and H_2O, resulting in 1.7, 50.9 and 47.4 g of fractions H, E and W, respectively.

Fraction H was further separated using column chromatography on silica gel (toluene/EtOAc 7:3) and MCI-gel (MeOH 60–100% and 90–100%) and yielded the following compounds: oleanolic acid (**1**), pectolinarigenin (**5**) and apigenin-4′,7-dimethylether (**4**). Fraction E was separated over a Sephadex LH 20 column eluted with MeOH, fraction E.1 afterwards over silica gel (dichloromethane/MeOH/H_2O) and an RP18-MPLC-column and E.2 over RP18-MPLC and -HPLC-column. E.1 yielded the methyl ester of oleanolic acid (**2**) and erythrodiol (**3**), and E.2 and E.3 yielded cirsimaritin (**6**) and naringenin-4′,7-dimethylether (**7**) as well as their subfractions, which were studied by GC-MS.

Identification of the isolated compounds

The spectroscopic details of the compounds are summarized in detail in Weimann (2000). In general ^{1}H NMR, UV and EI-MS experiments were conducted, and if required ^{13}C NMR spectra were also recorded. In the case of the flavonoids, experiments observing UV bathochromic shifts after the addition of $AlCl_3$ were used to confirm the substitution pattern.

Pharmacological methods

For all experiments freshly killed guinea pigs were used. A 1-cm long segment of the ileum was prepared and expanded between two metal prongs in a small-volume organ bath as described previously (Claeson et al 1991). In the experiments the following controls were included: pure solvent (EtOH), papaverine (Apoteksbolaget, Sweden) and, in the case of the KCl-induced contractions only, apigenin (Roth, Karlsruhe, Germany). The data are expressed as percentage inhibition and for some fractions IC50 (electrical field stimulation (EFS) and histamine)HIST)) and RC50 values (concentration that results in a 50% relaxation; KCl) were determined (Claeson et al 1991). All experiments were carried out according to current Swedish legislation.

Electrical stimulation
Contractions were induced using a Grass S4 stimulator. Two electrodes were placed close to the ileum preparation. Contractions were induced with pulses of 0.1 Hz and a voltage of 50 V. The sample (maximal volume 10–20 μL) was applied once a regular deflection was reached. After washing with buffer the ileum was reused (Weimann 2000). Cumulative measurements were obtained for determining the dose–effect curves (van Rossum 1963).

Histamine-induced contraction
In this series of experiments the contraction was induced by applying histamine dihydrochloride (2×10^{-6} M, Merck). First a cumulative dose–effect curve of histamine without test sample was obtained. The submaximal dose of activation that resulted in an 80% activation (of the maximal value obtained) was then used in the experiment. The contraction that was achieved with this concentration was set as 100%. Samples were added 5 min prior to histamine. The inhibitory effect was calculated from the difference between the effect of histamine without and with test sample (Pongprayoon et al 1992).

KCl-induced contraction
KCl (60 mM) was used in order to achieve a continuous contraction of the ileum. The samples were added once the contractions were stable. The relaxation (including the RC50 values) was determined from the difference between the total tension of the piece of ileum after the addition of KCl and the reduced tension after addition of the sample (Claeson et al 1991).

Antibacterial activity
The antibacterial activity was determined as described previously using *Micrococcus luteus* DSM 348, *Escherichia coli* DSM 498 and *Staphylococcus aureus* ATCC 25933/25923/SG511 (Bork et al 1996; Hamburger & Cordell 1987; Weimann 2000).

Results and Discussion

Spasmolytic activity of the extract and of the main fractions

The crude extract of *B. conferta* (aerial parts) showed a dose-dependent inhibition of all three types of induced contraction of the guinea pig ileum (Figure 1). The IC50 values were 234 and 123 μg mL^{-1} for the EFS- and HIST-induced stimulation, respectively, and the RC50 value was 100 μg mL^{-1} for the KCl-induced continuous contraction. These data expand findings by Tortoriello et al (1995), who found a similar effect in the EFS-induced ileum with methanolic extracts of *B. salicifolia*, *B. serraefolia*, *B. trinervis* and *B. vaccinoides* (leaves).

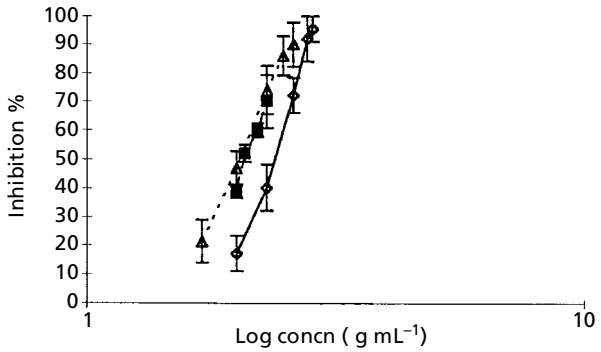

Figure 1 Dose–effect curve of the ethanolic crude extract (3 experiments). —■—, His; —◇—, electrical stimulation; --△--, KCl.

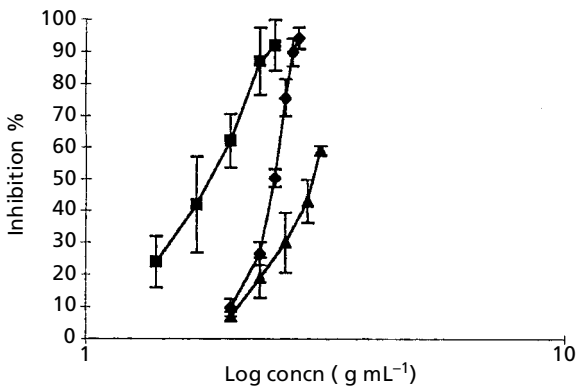

Figure 2 Dose–effect curve of the main fractions H, E, and W in the EFS-model (3 experiments). —■—, H (hexan fraction); —◆—, E (ethylacetate fraction); —▲—, W.

Liquid–liquid separation led to three main fractions: n-hexane (H), EtOAc (E) and H_2O (W) (Figure 2). While the H_2O fraction was completely inactive in the KCl and HIST experiments, and practically inactive in the EFS experiments, the n-hexane-fraction, and to a lesser degree the EtOAc fraction, showed inhibitory activity in the EFS experiments. Fraction E was also investigated in the KCl and HIST models and was shown to have relatively low IC50 and RC50 values (Table 1).

Two subfractions (E.2.1 and E.3.1), which were obtained from fraction E, were also evaluated in the assay and were shown to be nearly as active as the controls papaverine and apigenin (Table 1). These

Table 1 IC50 and RC50 values of the crude extract and of selected fractions in the guinea pig ileum assay (controls apigenin and papaverine).

	EFS ($\mu g/mL^{-1}$)	HIST ($\mu g/mL^{-1}$)	KCl ($\mu g/mL^{-1}$)
Crude extract	234	123	100
H (n-hexane)	63	n.d.	n.d.
E (EtOAc)	257	58	151
W (H_2O)	977	Inactive	Inactive
E.2.1	n.d.	18	15
E.3.1	23	10	11
Apigenin	n.d	n.d	8
Papaverine	4	6	2

EFS, electrical field stimulation; HIST, histamine; n.d., not determined.

fractions are particularly rich in flavonoids (see below). Fraction E.2.1 was shown to contain cirsimaritin (**6**) and naringenin-4′,7-methylether (**7**).

Phytochemical investigation

Compounds isolated

The following triterpenes were isolated from the aerial parts of *B. conferta*:

- oleanolic acid (**1**)
- oleanolic acid methyl ester (**2**)
- erythrodiol (12-oleanen-3,28-diol) (**3**)

EI-MS, ^{1}H NMR and ^{13}C NMR data for **1** and **2** are in agreement with published data (Carvalho & Seita 1993 (**1**), Ikuta & Itokawa 1986 (**2**)). The erythrodiol (12-oleanen-3,28-diol) (**3**) data (MW 442, EI-MS and ^{1}H NMR) are in correspondence with published data (Ahmad et al 1992).

The following flavonoids were also isolated:

- apigenin-4′,7-methylether (**4**)
- pectolinarigenin (5,7-dihydroxy-4′,6-dimethoxyflavone) (**5**)
- cirsimaritin (4′,5-dihydroxy-6,7-dimethoxyflavone) (**6**) (from E.2.1)
- naringenin-4′,7-methylether (**7**) (from E.2.1)

The ^{1}H NMR data of **4** are identical with published data (Herrera et al 1996). The EI-MS and ^{1}H NMR of **5** are identical with those of Seth et al (1982). The EI-MS and ^{1}H NMR indicated that **6** is identical with cirsimaritin, which was previously reported by Bosabalidis et al (1998). EI-MS and ^{1}H NMR were used to identify **7** (Mabry et al 1970).

All compounds, with the exception of **1** and **3**, are reported here for the first time for this species, but flavonols such as kaempferol and its derivatives as well as flavonones such as naringenin have been reported from other species of this genus. **1** is present in relatively large amounts in the extract ($> 0.6\%$ of the crude extract; Weimann 2000); each individual flavonoid is present in relatively low quantities ($< 0.1\%$) and the biological activity thus must be one of a complex phytotherapeutic mixture.

Other compounds identified by GC-MS only

Several biologically active fractions could not be analysed further with phytochemical methods such as HPLC because they have the same chemical structures, which differ only in single substitutes. Consequently, some of the fractions were investigated with GC-MS and the following additional compounds were identified (Weimann 2000):

- acacetin (apigenin-4′-methylether) (**8**) (E.3.1)
- salvigenin (5-hydroxy-4′,6,7-trimethoxyflavone) (**9**)
- kaempferol (**10**)
- kaempferid (kaempferol-4′-methylether) (**11**) (E.3.1)
- 6-methoxykaempferol (**12**)
- 6-methoxykaempferol-4′-methylether (**13**) (E.3.1)
- eupatilin (6-methoxykaempferol-7-methylether) (**14**)
- 6-methoxykaempferol-4′,7-dimethylether (**15**)
- naringenin (**16**)
- naringenin-4′-methylether (**17**)
- dihydrokaempferid (dihydrokaempferol-4′-methylether) (**18**)

Identification was possible by comparing the GC retention times as well as the mass spectra with the data of authentic samples. Additionally, apigenin-4′,7-dimethylether (**4**), pectolinarigenin (**5**), cirsimaritin (**6**) and naringenin-4′,7-dimethylether (**7**) were identified, and their mass spectra shown to be identical to those of **4**, **5**, **6** and **7**, which were isolated previously (see above).

The phytochemical studies in combination with the pharmacological assays allowed the identification of a

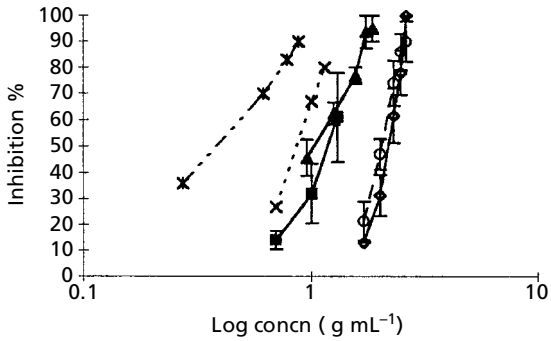

Figure 3 Dose–effect curve selected fractions in the KCl model. —◇—, EtOAc fraction; —■—, E2; —▲—, E3; —○—, crude extract; —-★-—, papaverine; —X—, apigenin.

complex mixture of flavonoids as active constituents. It is particularly noteworthy that fractions E.2 and E.3, which were tested in the guinea pig model (KCl experiments), showed similar dose–effect curves (Figure 3). Not a single compound but a relatively large number contribute to the in-vitro effect, preventing the evaluation of the pure compounds.

Antibacterial activity

Oleanolic acid methyl ester had very weak antibacterial activity against *M. luteus* and *E. coli* (6 mm inhibition zone at 20 μg/spot in the TLC assay; Weimann 2000), but this effects seem to be of little pharmacological relevance. It was inactive against *S. aureus* (Bork et al 1996). All other compounds were either inactive or could not be tested because they were isolated in insufficient amounts. In these cases the parent fraction generally showed no relevant antibacterial activity against *S. aureus*.

Conclusion

There exists a multitude of purely chemical and some purely pharmacological studies on species of this genus. In this investigation it was possible to isolate or identify compounds that contribute to pharmacological activity in the guinea pig ileum. This study thus provides in-vitro evidence for the species' pharmacological activity and is the first pharmacological and phytochemical study on this species.

The isolated guinea pig ileum model is a good tool for detecting drug effects. It is useful for screening plant extracts and monitoring the isolation of pharmacologically active compounds (Samuelsson 1991). It may help the evaluation of species with an indigenous use in gastrointestinal illnesses, a type of illness that is particularly common in developing countries (Heinrich 1998; Weimann & Heinrich 1998). The data provide good in-vitro evidence for the antispasmodic activity of the aerial parts of *B. conferta*, which may be used to further develop the Nahua ethnopharmacopoeia (Weimann et al 1998; Heinrich & Gibbons 2001).

Flavonoid-rich fractions had the strongest effect in the guinea pig ileum model. The effect is not due to a single compound and *Baccharis* thus provides an interesting example of a medicinal plant where a relatively large number of compounds contribute to this effect. While synergy was implicated in other cases (Williamson 1999), in this case an additive effect seems to be more likely. The study thus provides empirical evidence for the use of a complex extract as a drug, an issue that has received renewed interest in recent years. This group of compounds is well known for a variety of in-vitro effects and some experiments with quercetin indicate that they may act as spasmolytics via an antagonist effect on Ca^{+} channels (Capasso et al 1991). Detailed mechanistic and in-vivo studies will have to be conducted once we have a better understanding of the biotransformation and resorption of this important class of compounds in the gastrointestinal tract (Oliveira & Watson 2000).

As shown in this project on the medicinal plants of the Nahua of the Sierra de Zongolica, ethnopharmacology may give important information on the role of plants in a society (Weimann & Heinrich 1997; see also Ankli et al 1999) and on the potential of such resources for the local population. Further investigations including in-vivo animal and/or clinical studies are highly desirable, especially in order to evaluate potential adverse effects and the efficacy of this phytomedicine. The study of indigenous uses with the goal of improving the health care in marginalized areas has become an ever more important aspect of natural product biology.

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