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Spasmolytic and antidiarrhoeal properties of the Yucatec Mayan medicinal plant *Casimiroa tetrameria*

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Abstract

The Maya of the Yucatán peninsula commonly use the leaves of *Casimiroa tetrameria* for treating gastrointestinal disorders, notably diarrhoea and dysentery, as well as gastrointestinal cramps. The phytochemical investigation resulted in the isolation of 13 compounds: eight polymethoxylated flavonoids (two as minor components with a main constituent), four flavonoid glycosides and one furanocoumarin. In this study we used two well-established models in order to assess the gastrointestinal effects of *C. tetrameria* extracts and isolated compounds: the USSING-chamber, a pharmacological model for diarrhoea, and the isolated guinea pig ileum, a model for modulatory effects on ileum contraction. Extracts and the class of polymethoxylated flavonoids showed strong inhibitory effects in both models, which provides ex-vivo evidence for the use of this botanical drug in the treatment of several gastrointestinal problems, most notably diarrhoea. The crude extract, polymethoxylated flavonoid-rich fractions and the polymethoxylated flavonoids tested showed prominent antisecretory activity. Polymethoxylated flavonoid-rich fractions also inhibited the histamine-induced contractions in the guinea pig model. The effects are not due to a single compound, but to a large number of structurally related compounds that all contribute to the effect.

Introduction

The Yucatec Maya (México) use *Casimiroa tetrameria* Millsp. (Rutaceae), commonly called *Yuy*, for treating gastrointestinal problems, notably diarrhoea and dysentery, as well as gastrointestinal cramps. The use of this plant, as well as numerous other species, has been documented in a detailed ethnobotanical study (Ankli et al 1999). Traditional forms of treating illness among the Yucatec Maya of México, who still use diverse locally available plant resources, are of considerable importance. Their medical system and knowledge is without doubt a vital part of their culture. Ankli et al (1999) systematically recorded and investigated the medico-botanical knowledge of Mayan communities south of Valladolid.

C. tetrameria is one of about six currently recognized species in the genus, but a systematic re-evaluation of the genus would be highly desirable (Vibrans, pers. commun.). However, *C. tetrameria* is relatively well described because of its characteristic five-lobed leaves in combination with a fruit that contains only one 13–14-mm long seed (Martínez 1951). The closely related *C. edulis* yields an economically important fruit, the white sapote/Mexican apple.

Diarrhoea is still one of the major health threats to populations in tropical and subtropical poor countries. The WHO has estimated that 3–5 billion cases occur each year (1 billion in children < 5 years) and that approximately 5 million deaths are due to diarrhoea annually (2.5 million in children < 5 years). An effective, but simple and cheap, treatment exists: oral rehydration therapy using sugar/salt solutions, but in many communities healers and patients still rely on locally available phytomedicines. A study by Martinez et al (1998), for example, looked at what form of treatment is administered by the primary care-takers of young children, i.e. their mothers, and demonstrated that herbal treatments are still important in the home treatment of diarrhoea. Consequently, an evaluation of commonly used home remedies such as

C. tetrameria is an opportunity to develop culturally acceptable forms of treatment and to provide scientific evidence for such indigenous uses.

In this study we used two well-established ex-vivo models in order to assess the gastrointestinal effects of *C. tetrameria* extracts and isolated compounds: the USSING-chamber, a pharmacological model for diarrhoea, and the isolated guinea pig ileum, a model for modulatory effects on ileum contraction.

Materials and Methods

General experimental 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).

Plant material

C. tetrameria was collected from the villages and surroundings of Chikindzonot, Ekpeds and Xcocmil, Yucatán, México (1994–1996). Authenticated voucher specimens were deposited at the Herbarium of the National Herbarium of México (MEXU), the Centro de Investigaciones Científicas de Yucatán (CICY) in Mérida, the Instituto Nacional Indigenista in Valladolid, Yucatán, University of Zurich Herbarium and the Centre for Pharmacognosy and Phytotherapy, The School of Pharmacy, London. The material was identified by A.A. in collaboration with researchers at CICY.

Extraction and isolation

The air-dried, pulverized leaves (530 g) were 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 122 g of crude extract. Of this extract 110 g was further separated using a liquid–liquid procedure with petroleum ether (PE), ethyl acetate (EtOAc) and H_2O, resulting in 20, 10 and 80 g of fractions P, E and W, respectively. Fraction E was further fractioned using column chromatography on Sephadex LH 20 (methanol (MeOH) 100%), RP_{18} (various systems of acetonitrile/MeOH/H_2O and MeOH/H_2O), Si_{60} (EtOAc/toluol) and yielded the compounds described in the section on the constituents of *Casimiroa tetrameria* (for details see Heneka et al 2005).

In order to prepare an extract similar to the one used by the Maya, 10–15 g (exactly weighed) was powdered and 500 mL of distilled H_2O was added. The mixture was slowly heated to 80–90°C and the temperature was kept at this level for 30 min. The resulting filtrate was freeze-dried and later re-dissolved for use in the experiments.

Pharmacological methods

All experiments were ex vivo and appropriate facilities were used. The experiments were conducted after sacrificing the animals in accordance with all legal requirements and specifically the animal handling guidelines of the respective institutions (University of Freiburg and Pentapharm).

Antisecretory effects in the USSING chamber model

The USSING chamber is a model for the investigation of ion transport mechanisms across the epithelia in the gastrointestinal system and the experiments were conducted as described previously (Greger et al 1991; Hoer et al 1995) using the mucosa of the distal colon, which had been stripped of the underlying musculature. Importantly, only PGE_2 was used as a stimulant. Briefly, the exposed area (0.64 cm^2) of mucosa between the two chambers filled with electrolyte Ringer solution supplemented with indometacin (to prevent endogenous prostaglandin synthesis) and maintained at 37 °C and circulated by gas lift (95% O_2 and 5% CO_2) served as a means for establishing the transepithelial potential. Two pairs of Ag/AgCl electrodes were used for current injection and voltage measurement, respectively. After initial equilibration amiloride (0.1 mM) was added to the mucosal bath to block Na^+ re-absorption. Afterwards, PGE_2 was added (1 μM, serosal) as a secretagogue. A few minutes later the extract, fraction or compound (normally predissolved in DMSO) was added. Figure 1 shows a typical experiment; the time points when the various reagents were added are indicated by arrows. Previous experiments showed that DMSO at the maximum concentration used (0.3%) had no significant effect.

In order to calculate the tissue resistance (transepithelial resistance, R_{te} ($\Omega\,cm^2$)), rectangular current pulses (0.1 Hz) with an amplitude of 100 μA and a duration of 10 ms were applied (I_{test}) and the voltage deflection (V_{te}) caused by the tissue input resistance was measured. All

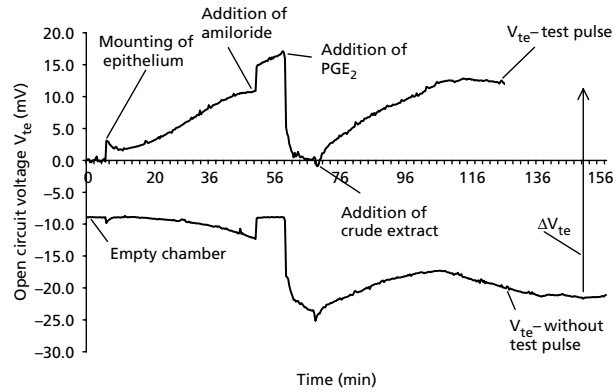

Figure 1 A typical experiment indicating the change in transepithelial potential (V_{te} (mV)) and the antisecretory effect of the crude extract (500 μg mL^{-1}, serosal). Fifty minutes after the start of the recording the sodium resorption is blocked by application of amiloride (0.1 mM). The transepithelial chloride secretion is initiated at minute 60 by adding PGE2 (1 μM), the addition of the crude extract (serosal application, 500 mg) at minute 67 results in a change in the transepithelial potential.

data were calculated from V_{te}, $\Delta V_{te}{}^0$ and ΔV_{te}. The resistance (R_{te}) was obtained by:

$$R_{te} = (\Delta V_{te} - \Delta V_{te}{}^0) \times A/I_{test}\ (\Omega\,cm^2)$$

where $\Delta V_{te}{}^0$ is the voltage deflection of the USSING chamber before the tissue is mounted, A is the surface area of tissue and I_{test} is the pulse applied (100 μA, 10 ns, 0.1 Hz). From the open-circuit voltage (V_{te}) and R_{te}, the short-circuit current (I_{sc}) was calculated by Ohm's law:

$$I_{sc} = V_{te}/R_{te}\ (A/cm^2)$$

The short circuit is equivalent to the electrogenic transepithelial ion transport and is thus a measure for the transtissue electrolyte transport.

Isolated guinea pig ileum

For all experiments, freshly sacrificed albino guinea pigs of both sexes were used. Segments of the ileum (2–4 cm long) were prepared and expanded between two metal prongs in a small-volume organ bath as commonly practised (Williamson et al 1997). Histamine (HIS, 1 μM) was used to induce contractions. The degree of contraction was measured using an isotonic measuring device and recorded using a Perkin-Elmer recorder. In the experiments pure solvent (EtOH) and DMSO were included as controls. The system is used routinely by Pentapharm and has been validated internally. The data are expressed as percentage inhibition and for some fractions and compounds IC50 values were determined (Williamson et al 1997; Heneka 2000; Weimann et al 2002).

Statistical analysis

All values are given as mean values (± s.d.). Because of the need to reduce the number of ex-vivo experiments and the overall costs, and because of time limitations, the number of experiments varied but was generally ≥4. Statistical analysis of the ex-vivo pharmacological experiments described in the sections on the antisecretory effects in the USSING chamber model and isolated guinea pig ileum was conducted using the WILCOXON sign rank test. A significance level of $P < 0.05$ was considered to be statistically significant. Because of the nature of the fractions/compounds available (mixtures), it was decided that a statistical comparison between individual fractions was not meaningful.

Activity against *Helicobacter pylori* (ATCC 43504)

These experiments were conducted as described previously (Ankli et al 2002) using the disc diffusion technique on Wilkins–Chalgren agar plates or, for determining the MIC values, in a modified minimal Nedenskov medium. The density was determined photometrically. The MIC concentration was defined as the extract concentration that did not produce any visible growth (< 0.03 as compared to 1.2 for the control).

Results and Discussion

Constituents of *Casimiroa tetrameria*

The phytochemical investigation resulted in the isolation of 13 compounds, eight polymethoxylated flavonoids (5,6,2′,3′,5′,6′-hexamethoxyflavone and 5,6,2′,3′,6′-pentamethoxyflavone (two new natural products) as well as the known 5,6,2′,3′,4′,6′-hexamethoxyflavone, 5,6,3′,4′,5′-pentamethoxyflavone (cerrosillin B), 5,6,2′,3′,4′-pentamethoxyflavone (Af-2), 5,6,2′,6′-tetramethoxyflavone (zapotin), 5,6,3′,4′-tetramethoxyflavone (Af-1), 5,6,3′,5′-tetramethoxyflavone (cerrosillin)), four flavonoid glycosides (quercetin-3-O-glucoside, quercetin-3-O-rutinoside, kaempferol-3-O-glucoside and kaempferol-3-O-rutinoside) and one furanocoumarin, the new natural compound 5-methoxy-8-(5″-hydroxy)-prenyloxy-psoralene (Heneka 2000; Heneka et al 2005).

Since fresh leaves were not available, a phytochemical investigation of a tea from air-dried material prepared in a way similar to the method used by the Maya (boiling in water) was conducted. This indicated that the flavonoid-containing fraction represents only 1.1% of the total aqueous extract and that cerrosillin B (5,6,3′,4′,5′-pentamethoxyflavone) is the main flavone in this extract (58% of the flavonoid fraction based on an HPLC quantification). Two other constituents were also identified: AF-1 (5,6,2′,3′,4′-pentamethoxyflavone, 17%) and AF-2 (5,6,3′,4′-tetramethoxyflavone, 6%).

Antisecretory activity

The initial screening (500 $\mu g\,mL^{-1}$) of six species used by the Maya (*Bidens squarosa* Less, Asteraceae, *C. tetrameria, Hylocereus undatus* (L.) Britton and Rose, Cactaceae) and Nahua (*Bacharis conferta* Kunth, Asteraceae; *Lysiloma divaricata* (Jacq.) MacBr, Mimosaceae, *Tagetes erecta* L. Asteraceae) in the USSING chamber system identified *C. tetrameria* as the most active extract. If applied mucosally it inhibited Cl^- secretion by 23%, if applied serosally by >50%. All other species were inactive mucosally (with the exception of the phytochemically relatively well known *T. erecta*, which showed a reversion of the Cl^- secretion of 44% (mucosal application) and 42% (serosal application). *L. divaricata* was only active if applied serosally (39%).

We next investigated whether or not the antisecretory effect of *C. tetrameria* crude extract is dose dependent. The extract showed a statistically significant dose-dependent antisecretory effect if tested at concentrations of 500 (52%), 400 (40%), 300 (31%), 200 (30%), 100 (25%) and 50 (20%) $\mu g\,mL^{-1}$ ($n \geq 4$ for each dose). A typical experiment is shown in Figure 1 (500 $\mu g\,mL^{-1}$). Both the EtOAc and the PE fraction were evaluated as part of the initial steps of a bioassay-guided fractionation procedure. The EtOAc fraction is somewhat more active at all

concentrations and was more active than the crude extract (data not shown). The H_2O fraction was only marginally active at the highest concentrations (10% at 400 and 300 $\mu g\,mL^{-1}$).

This prompted us to initiate a phytochemical investigation of the EtOAc fraction. A bioassay-guided fractionation in the strict sense was not possible since the test systems require relatively large amounts of sample, but whenever feasible a fraction was tested. The most active fraction was one that contained four of the eight polymethoxylated flavonoids isolated. However, again it was difficult to isolate the active constituents in sufficient amounts. We therefore decided to test the fractions that contained defined proportions of selected constituents and one pure compound at 100, 50 and 25 $\mu g\,mL^{-1}$ (see Table 1). Overall it is noteworthy that the only pure constituent (5,6,3′,4′,5′-pentamethoxyflavone) is less active than the mixtures and that all fractions tested showed prominent antisecretory activity, which does, however, not differ significantly between the fractions tested.

The tea prepared from the air-dried leaves (500 $\mu g\,mL^{-1}$) showed an antisecretory effect of 34% (± 4) within 30 min.

Antispasmodic activity

These experiments were conducted after completing the phytochemical study of the species and we were interested in testing the crude extract, the main fractions and selected fractions with defined percentages of polymethoxylated flavonoids in this system. HIS-induced contraction of the guinea pig ileum was inhibited in a dose-dependent way and simultaneously the extract induced spontaneous contractions (Figure 2). Crude extract (300 $\mu g\,mL^{-1}$) resulted in a nearly complete inhibition of HIS-induced contractions and 100 $\mu g\,mL^{-1}$ in approximately 50% reduction. Subsequently, the main fractions obtained from the crude extract (P, E and W) were tested at 100 $\mu g\,mL^{-1}$. Interestingly, only the H_2O fraction strongly induced spontaneous contractions, while the EtOAc and PE fractions drastically reduced the HIS-induced contractions (Table 2). It was not possible to study the induction of spontaneous contractions induced by the H_2O fraction further, since it had not been studied phytochemically. Similar to the experiments in the USSING chamber, we next tested fractions with defined proportions of specific polymethoxylated flavonoids (Table 3). All flavonoid fractions tested as well as the pure compound 5,6,3′,4′,5′-pentamethoxyflavone have a comparable effect in this system. No statistically significant differences were found for the fractions tested, indicating that all these compounds contribute more or less equally to the inhibition of the HIS-induced contractions.

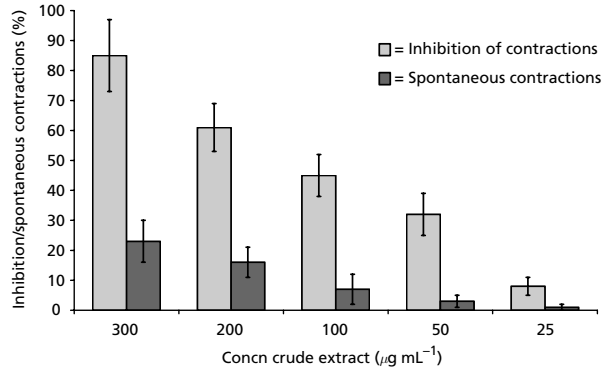

Figure 2 Dose-dependent inhibition of the HIS (1 μM) induced contractions (left) and induction of spontaneous contractions (right) resulting from applying various concentrations of the crude extract (n = 6). The contraction after adding 1 μM histamine is set at 100%.

Table 1 Comparison of the antisecretory effect of polymethoxylated flavonoids in the USSING chamber

Concentration of fraction tested ($\mu g\,mL^{-1}$)	100	50	25
(5,6,3′,4′-tetra-/5,6,3′,4′,5′-penta-methoxyflavone → 4:1)	73 ± 2	54 ± 10	39 ± 14
(5,6,3′,4′-tetra-/5,6,3′,4′,5′-penta-methoxyflavone → 3:7)	70 ± 5	65 ± 14	40 ± 9
5,6,3′,4′,5′-/5,6,2′,3′,4′-penta-methoxyflavone → 19:1)	71 ± 19	48 ± 5	29 ± 6
(5,6,3′,4′,5′-/5,6,2′,3′,4′-penta-methoxyflavone → 1:9)	83 ± 5	43 ± 5	29 ± 3
(5,6,3′,5′-tetra-/5,6,2′,3′,4′-penta-methoxyflavone → 3:17)	58 ± 10	30 ± 13	26 ± 5
(5,6,2′,6′-tetra-/5,6,2′,3′,6′-penta-/5,6,2′,3′,5′,6′-hexa-/5,6,2′,3′,4′,6′-hexa-methoxyflavone → 3:17:35:46)	96 ± 14	71 ± 22	47 ± 7
5,6,3′,4′,5′-pentamethoxyflavone	40 ± 18	26 ± 2	16 ± 3

The data show the reversion of the PGE_2 (1 μM, serosal) induced negative short-circuit current I_{sc} (%) for the values of PGE_2 only; the data were obtained 30 min after adding the samples (n = 4 for each fraction and concentration).

In-vitro effects against *Helicobacter pylori*

The crude extract (600 μg) showed an inhibitory zone of 4 mm and an MIC value of 3 $\mu g\,mL^{-1}$ (ATCC 43504), and showed a weak inhibition of 25% in a clinical

Table 2 Inhibition of HIS (1 μM) induced contractions and spontaneous contractions comparing the crude extract, and the H_2O, EtOAc and PE phases at 100 $\mu g\,mL^{-1}$

Fraction tested	Inhibition of contraction (%)	Spontaneous contraction (%)	Relaxation (%)
Crude extract (n = 6)	45 ± 7	7 ± 5	–
H_2O fraction (n = 4)	10 ± 5	40 ± 8	–
EtOAc fraction (n = 4)	79 ± 9	–	5 ± 2
PE fraction (n = 4)	74 ± 5	–	2 ± 1

Table 3 Comparison of the inhibitory effect of polymethoxylated flavonoids using guinea pig ileum

Concentration of fraction tested ($\mu g\,mL^{-1}$)	25	12.5	6.25
(5,6,3′,4′-tetra-/5,6,3′,4′,5′-pentamethoxyflavone → 4:1)	86 ± 5	64 ± 4	n.t.
(5,6,3′,4′-tetra-/5,6,3′,4′,5′-pentamethoxyflavone → 3:7)	93 ± 4	70 ± 17	n.t.
5,6,3′,4′,5′-/5,6,2′,3′,4′-pentamethoxyflavone → 19:1)	92 ± 3	64 ± 6	n.t.
(5,6,3′,4′,5′-/5,6,2′,3′,4′-pentamethoxyflavone → 1:9)	85 ± 9	64 ± 5	n.t.
(5,6,3′,5′-tetra-/5,6,2′,3′,4′-pentamethoxyflavone → 3:17)	87 ± 7	75 ± 4	n.t.
(5,6,2′,6′-tetra-/5,6,2′,3′,6′-penta-/5,6,2′,3′,5′,6′-hexa-/5,6,2′,3′,4′,6′-hexamethoxyflavone → 3:17:35:46)	91 ± 4	88 ± 8	34 ± 9
5,6,3′,4′,5′-pentamethoxyflavone	83 ± 4	79 ± 13	34 ± 4

The data show the inhibition of the contractions induced with histamine, 1 μM (= 100%) (n = 4 for each fraction and concentration). n.t. = not tested.

isolate. The EtOAc and the PE extracts showed a complete inhibition of the growth of the clinical isolate at 10 $\mu g\,mL^{-1}$, while the H_2O fraction and a polymethoxylated subfraction of the EtOAc fraction were inactive.

Conclusion

In a previous publication we showed that the crude extract of *C. tetrameria* inhibits the growth of several pathogenic bacteria and also has antiprotozoal effects (Ankli et al 2002). In this publication we have demonstrated that extracts and the class of polymethoxylated flavonoids show effects that provide ex-vivo evidence for the use of this botanical drug in the treatment of several gastrointestinal problems, most notably diarrhoea. Polymethoxylated flavonoid-rich fractions had the strongest effect in both models. Importantly, there is no significant difference between the level of activity of the polymethoxylated flavonoid-rich fraction and the compounds isolated from it, indicating that the effect is not due to a single compound. Thus *C. tetrameria* provides an interesting example of a medicinal plant where a relatively large number of compounds contribute to this effect. Synergistic effects may well explain the data observed in our study (Williamson 2001), but this requires further investigation. The study thus provides empirical evidence for the use of a complex extract as a drug, an issue that has received renewed interest only in recent years. Although flavonoids have been studied in detail for their pharmacological effects and have been active in a variety of biological test systems (e.g. Harborne 1994), very little pharmacological information on 5,6-methoxylated flavonoids is available.

Consequently, this study contributes to developing potential phytomedicines for local use (cf. Tortoriello et al 1995) and for understanding the role of medicinal plants in Mexican indigenous cultures (Heinrich 2003, Leonti et al 2003). Medicinal plants used for infectious diseases and most notably diarrhoea remain one of the least studied botanical resources and this example highlights the potential of such studies.

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