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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC STUDY OF *CASIMIROA EDULIS*

I. DETERMINATION OF IMIDAZOLE DERIVATIVES AND RUTIN IN AQUEOUS AND ORGANIC EXTRACTS*

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SUMMARY

The imidazolic derivatives present in the seeds and leaves of *Casimiroa edulis* (Rutaceae), a antihypertensive popular remedy, were successfully separated and estimated by high-performance liquid chromatography. Dimethylhistamine, methylhistamine and casimiroedine were separated by paired ion chromatography while histamine was derivatized and determined in the reversed-phase mode using fluorimetric detection. In addition, the flavonoid glycoside rutin, an important constituent of the leaves, was determined by reversed-phase high-performance liquid chromatography.

INTRODUCTION

Casimiroa edulis Llave et Lex (Rutaceae) is a tree distributed in the temperate zones of Mexico and Central America (popularly called "zapote blanco") which has been known since prehispanic times for its interesting sedative-like effect and its use as an sleep inducer[1–3]. In the folk medicine, a concoction of leaves and less frequently of seeds is administered for this purpose. Furthermore, the seeds are used in the treatment of dermatological conditions[4].

The hypotension following intravenous administration of the infusion of seeds was noticed by García Carmona[5] and later by De Lille[6], the latter being the first to report the effect of the crude drug on blood pressure. In early phytochemical studies a hypnotic substance was assumed to be responsible for the pharmacological effects but a search for it was unsuccesful.

Major and Dursch[7] and Ling *et al.*[8] in 1958 isolated N^{α},N^{α}-dimethylhistamine (DMH) from the seeds and confirmed the marked hypotensive activity of this substance, whose physiological action was already known from the work of Huebner *et al.*[9,10].

* Taken in part from the M.Sc. Thesis of M.L.R.

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A number of reports describing a wide variety of chemical structures isolated from the bark and seeds are available[11–13], but the phytochemical constituents of the leaves have not been studied. Of the known compounds, casimiroedine (CM) is the most abundant metabolite present in the seed, although it apparently does not significantly affect the blood pressure[11,14].

Our interest was in developing a chromatographic method to determine DMH and the closely related imidazolic derivatives N^{α}-methylhistamine (MHI), histamine (HI) itself and CM in both aqueous and organic extracts of seeds and leaves.

No high-performance liquid chromatographic (HPLC) method is available for DMH, although it has been determined chromatographically by methods used for other alkyl imidazoles, such as paper chromatography[15], adsorption and partition chromatography[16], bioassay[17] and radio-immunoassay[18].

A paired-ion HPLC method was succesfully applied to the simultaneous determination of DMH, MHI and CM, and HI was determined by derivatization and reversed-phase HPLC using fluorimetric detection[19], became the former method was less sensitive and presented problems of overlapping peaks. It should be noted that in previous work, the search for imidazolic compounds had not revealed the presence of HI in the seeds.

Additionally, the flavonoid glycoside rutin, a major component of the leaves, which had not been reported before in *C. edulis*, was determined by a reversed-phase HPLC method.

EXPERIMENTAL

Equipment

A Varian Model 5000 liquid chromatograph with a Rheodyne injector Model 7125 (20-μl sample loop) attached to a Varian UV-50 variable wavelength UV detector operated at 220 nm and controlled by a Vista 401 CDS printer was used. In fluorimetric determinations a detector Fluorichrom was used with excitation–emission filters at 340–380 and above 400 nm, respectively.

Columns

For paired-ion and reversed-phase chromatography, two separate Micropack MCH-10 30 cm × 0.4 cm I.D. columns were used. Both columns had guard-columns of length 3 cm from Waters filled with Vydac reversed-phase packing.

Reagents

Methanol and water were HPLC grade from Merck and were filtered prior to use through 0.45-μm pore-size membranes from Millipore. Analytical grade reagents were used from the following sources: hydrochloric acid, perchloric acid, sodium sulphate, citric acid, sodium hydroxide and boric acid from Baker; *o*-phthalaldehyde and mercaptoethanol from Aldrich; sodium lauryl sulphate from Sigma. Reference substances were: histamine dihydrochloride and free base from Sigma; DMH and MHI from SmithKline & French. CM was isolated and characterized from the phytochemical work-up of seeds[20].

Derivatization reagent

Boric acid (0.5 g) was dissolved in 20 ml of water and the pH was adjusted to 10.4 ± 0.1 with 10% sodium hydroxide. *o*-Phthalaldehyde (50 mg) was dissolved in 200 μl of methanol and added to this solution. The resulting solution was kept at 4°C until use.

*Preparation of extracts**

Dry finely ground seeds (63 g) were exhaustively defatted by maceration with hexane at room temperature and further extracted in a similar way with methanol until practically no residue was observed after vacuum removal of the solvent. The amount of extract obtained was 11.42 g, and aliquots thereof were taken for sample preparation. The infusion of seeds was prepared by boiling 6 g of dry ground seeds in 50 ml of HPLC water for 10 min. The solution was filtered through Whatman No. 2 filters and freeze-dried. The residue (0.532 g) was used for sample preparation. The organic extract of leaves was prepared by macerating 10 g of dry finely ground leaves in methanol in a similar way to the seeds; 1.1833 g of residue was obtained after elimination of the solvent. Rutin was determined from this extract. A second organic aqueous extract was prepared for DMH and MHI determination. Thus, 2 g of dry ground leaves were macerated exhaustively in 0.1 *N* hydrochloric acid–methanol (25:27), the organic solvent removed under vacuum and the remaining solution was freeze-dried. The residue (0.225 g) was used completely for sample preparation. The infusion of leaves was prepared by boiling 2 g of dry ground leaves in 50 ml of HPLC water for 10 min. After the usual work-up, the sample was made up with 0.112 g of residue.

Preparation of samples

Aliquots of methanolic or aqueous extract of seeds and aqueous or aqueous-methanolic extract of leaves were dissolved in 0.1 *N* perchloric acid, clarified in a Millipore system with 0.22-μm pore-size filters and made up to 10 ml in volumetric flasks. Reference compounds were used in the same way to prepare the corresponding standard solutions. A mixture of imidazolic metabolites, including histidine (HIS), was used for monitoring resolution in the paired-ion mode (Fig. 1).

Chromatographic methods

HI was determined in a reversed-phase mode previously described, which involves derivatization with *o*-phthalaldehyde[19]. The mobile phase was $2.5 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate buffer–methanol (1:1). For DMH, MHI and CM the mobile phase was methanol–water (55:45) containing 10^{-3} *M* sodium lauryl sulphate as counter-ion, $2 \cdot 10^{-2}$ *M* sodium sulphate and 0.2% v/v perchloric acid. In all experiments a flow-rate of 1 ml/min and column temperature of 50°C were used unless otherwise specified. Calibration curves were obtermined for all reference compounds (Fig. 2).

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* The vegetable material was available from a local market and further botanically classified at the IMSSM Herbarium.

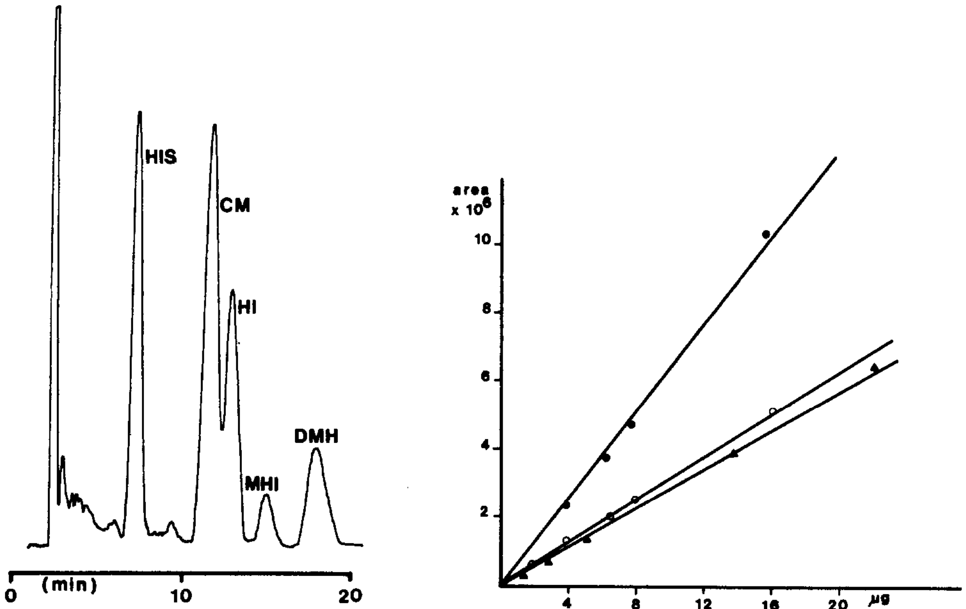


Fig. 1. Paired-ion chromatogram of an artificial mixture of imidazolic metabolites from *C. edulis.*

Fig. 2. Calibration curves for CM (●), DMH (O) and MHI (▲).

Derivatization

An aliquot of an extract of either seeds or leaves was dissolved in water and the pH adjusted to 7. The *o*-phthalaldehyde reagent was added and the solution extracted with ethyl acetate. For the standard solution 0.496 mg of histamine in water and 3 ml of reagent were stirred vigorously for 5 min. After three extractions with ethyl acetate the organic layers were collected and made up to 10 ml in a volumetric flask.

Rutin determination

Rutin was determined in the organic extract of leaves in the reversed-phase mode using methanol–$5 \cdot 10^{-2}$ citrate buffer (50:50) as mobile phase with UV readings at 360 nm. Both sample and reference compound were dissolved in methanol.

RESULTS

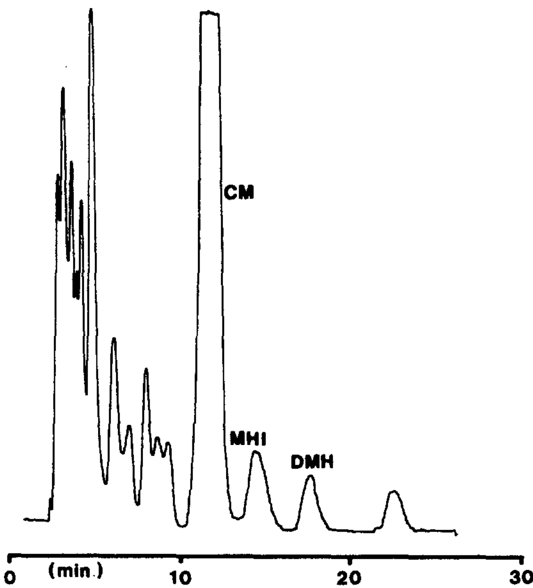
The secondary metabolites found in the various extracts are summarized in Table I. Paired-ion chromatography of the standard solution of imidazolic compounds resulted in a good resolution of these compounds (Fig. 1). Histidine was added because of its structural relationship to the known metabolites, although it could not be clearly assigned in the chromatogram of the extracts. All the calibration graphs were linear within the concentrations range used (Fig. 2; the curves for HI and rutin are omitted for clarity). The chromatographic profile of the methanolic extract of seeds (Fig. 3) was almost identical with that of its aqueous extract. HI was detected in both methanolic and aqueous extracts of leaves and seeds, but better

TABLE I

METABOLITE CONTENTS IN SEEDS AND LEAVES OF *C. edulis*

Metabolite	*Seeds**,**		*Leaves**,**			*MDQ**** *(ng)*	*k'*§
	Methanol ext. (% wt.)	*Infusion (mg/ml)*	*Methanol ext. (% wt.)*	*Mt. aq. ext. (% wt.)*	*Infusion (mg/ml)*		
CM	0.92 ± 0.041	0.21 ± 0.016	–	–	–	70	3.01
HI	0.0035 ± 0.0004	0.0011 ± 0.0001	–	–	–	0.1[19]	2.99
MHI	0.16 ± 0.011	0.050 ± 0.004	–	0.15 ± 0.023	–	240	4.24
DMH	0.12 ± 0.01	0.047 ± 0.003	–	0.115 ± 0.010	0.029 ± 0.002	328	5.25
Rutin	–	–	1.24 ± 0.074	–	–	60	0.48

* Confidence limits are given for a confidence level of 95% with $n = 3$ and $\bar{\mu} = \bar{x} \pm ts$.
** Percentages refer to total content by weight in the dry vegetable material.
*** The minimum detectable quantity (MDQ) was established as S/N = 4.
§ Calculated as $\frac{t_R - t_0}{t_0}$.



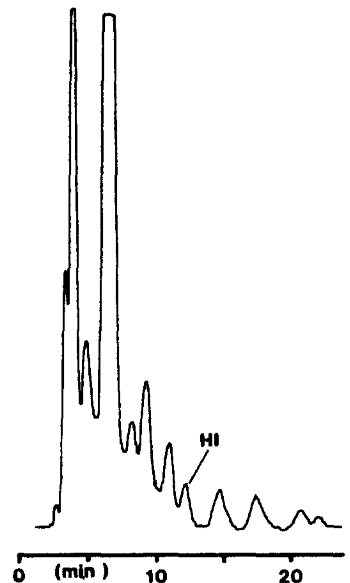


Fig. 3. Paired-ion chromatogram of the methanolic extract of seeds.

Fig. 4. Reversed-phase chromatogram of the derivatized extract of seeds. Temperature, 40°C.

resolution and quantitative results were obtained from either extract of seeds (Fig. 4). In HI determination an average 106% recovery was obtained on addition of a standard to the corresponding extrtact.

DISCUSSION

Owing to the relatively large amounts of imidazolic metabolites involved, paired-ion chromatography is a suitable HPLC method for the determination of DMH, as well as the related derivatives MHI and CM. It is advantageous in the sense that no derivatization at a free amino group was possible as a means to increase the sensitivity to spectroscopic detection. However, there is less histamine in the extract so a derivatization reaction and fluorimetric detection became necessary. The contents of CM and DMH were found to be similar to those previously reported for these metabolites isolated from the seeds. On the other hand, HI and MHI had not been reported before in this plant.

Under the conditions used in the paired-ion experiments the stabilization time was long; this could be attributed to inadequacy of the lauryl radical for achieving equilibrium in the chemically bonded matrix. Therefore, a smaller counter-ion seems necessary to overcome this inconvenience.

The analysis of the phytochemical constituents of leaves by HPLC is of importance because this is the part of the plant most frequently used for the treatment of insomnia and ailments related to hypertension. All imidazolic metabolites known from the seeds, and in addition the flavonoid glycoside rutin, were found in the leaves.

Further studies with the leaves seem necessary before a more complete picture of the structure–activity relationships is available, and for this purpose the analytical application of HPLC in its various modes should provide a powerful and versatile tool.

ACKNOWLEDGEMENTS

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