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Quinoline alkaloids in honey: Further analytical (HPLC-DAD-ESI-MS, multidimensional diffusion-ordered NMR spectroscopy), theoretical and chemometric studies

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ABSTRACT

The wound-healing properties of honey are well established and it has been suggested that, among its pharmaco-active constituents, kynurenic acid (KA) exerts antinociceptive action on injured tissue by antagonizing NMDA at peripheral GABA receptors. The aim of this study was to investigate the quantitative profile of KA and of two recently identified, structurally related derivatives, 3-pyrrolidinyl-kynurenic acid (3-PKA) and its γ-lactamic derivative (γ-LACT-3-PKA), by examining their mass spectrometric behavior, in honeys from different botanical sources. We used a combination of HPLC-DAD-ESI-MS and NMR techniques (one-dimensional ^{1}H NMR and diffusion-ordered spectroscopy NMR).

Chestnut honey constantly contained KA (2114.9–23 g/kg), 3-PKA (482.8–80 mg/kg) and γ-LACT-3-PKA (845.8–32 mg/kg), confirming their reliability as markers of origin. A new metabolite, 4-quinolone (4-QUIN), was identified for the first time in one chestnut honey sample (743.4 mg/kg). Small amounts of KA were found in honeydew, sunflower, multifloral, almond and eucalyptus honeys, in the range of 23.1–143 mg/kg, suggesting contamination with chestnut honey. Total phenol content (TPC) was in the range from 194.9 to 1636.3 mg_{GAE}/kg and total antiradical activity (TAA) from 61 to 940 $mg/_{GAE}$/kg), depending on the botanical origin.

Principal component analysis (PCA) was then done on these data. The three different clusters depicted: (i) antinociceptive activity from KA and/or its derivatives, typical of chestnut honey; (ii) antioxidant/radical scavenging activity by antioxidants responsible for the antiinflammatory action (dark honeys); (iii) peroxide-dependent antibacterial activity due to H_2O_2 production by glucose oxidase in honey.

The PCA findings provide useful indications for the dermatologist for the treatment of topical diseases, and the profiling of KA and its derivatives may shed light on new aspects of the kynurenine pathway involved in tryptophan metabolism.

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1. Introduction

Kynurenic acid (KA) is a tryptophan metabolite formed through the kynurenine pathway and is considered ubiquitous in living systems. Since its discovery in dog urine in 1853 [1], it has been found in the central nervous system of mammals [2] and insects [3], in plants [4], and in biological fluids and organs [5,6]. It has recently been detected in edible vegetables, meat, and honeybee products, arousing even more interest in its biochemistry and biological functions [7,8].

KA (Fig. 1) is the only known endogenous antagonist of all subtypes of ionotropic glutamate receptors [9]. It is preferentially active not only at the glycine allosteric site of the N-methyl-D-aspartate (NMDA) receptor, but also acts as a non-competitive antagonist of α7 nicotinic acetylcholine receptors (AR) and mediates α-amino-3-hydroxy-5 methyl-4-isoxazole propionic acid (AMPA) receptor responses [10].

We have already identified KA in honey from different botanical origins through a combination of spectrometric techniques (multidimensional NMR and high-resolution mass spectrometry—HRMS),

Abbreviations: KA, kynurenic acid; 3-PKA, 3-(2′-pyrrolidinyl)-kynurenic acid γ-LACT-3-PKA; TPC, total phenol content; ARA, antiradical activity; IDO, indoleamine-2,3-dioxygenase; GAE, gallic acid equivalent; DOSY, dispersion ordered spectroscopy; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; SIM, single ion monitoring.

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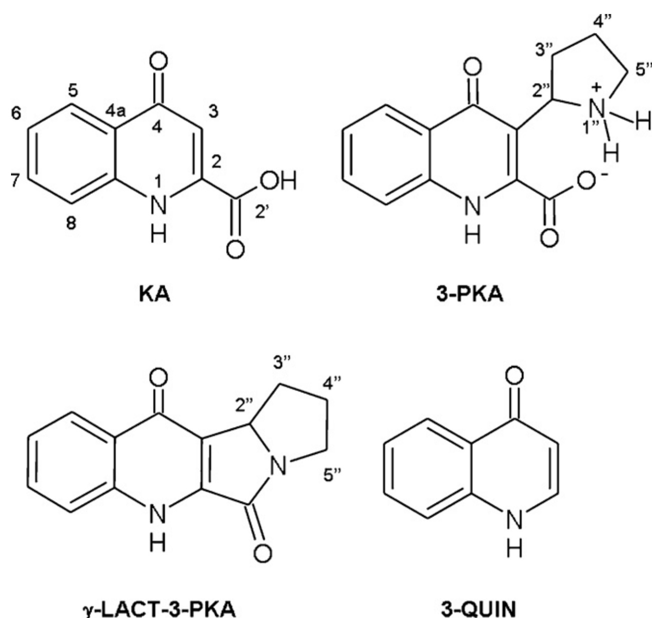

Fig. 1. Chemical structure of the quinoline alkaloids identified by HPLC-DAD, HPLC–MS and dispersion ordered spectroscopy (DOSY) NMR in chestnut honey: kynurenic acid (KA), 3-pyrrolidinyl kynurenic acid (3-PKA), the γ-lactam derivative of 3-PKA (γ-LACT-3-PKA) and 4-quinolon (3-QUIN).

with the highest content in chestnut honey. More recently we found two new KA derivatives in the same honey, which we studied by a multidimensional NMR approach [11]. This led us to propose for the first of these, 3-PKA, a pyrrolidinyl moiety at the C-3 of KA, and for the second the structure of the γ-lactam derivative of 3-PKA, γ-LACT-PKA, arising from intramolecular condensation between the C2 carboxyl moiety of KA and the aminic nitrogen of the pyrrolidinyl side chain (Fig. 1). However, no MS study of these derivatives has been reported.

Interest in the use of honey as a skin dressing for the treatment of wounds and burns in clinical practice [12] is based on: (i) its peroxide-dependent antibacterial activity; (ii) the presence of an array of specific antiinflammatory constituents; (iii) the possibility that KA might have antinociceptive action on injured tissue by antagonizing NMDA at peripheral GABA receptors [8,13,14].

The aims of this work were therefore: (i) to investigate the quantitative profile of KA, 3-PKA and γ-LACT-3-PKA (after theoretical assessment of their ability to bind at the glycine allosteric site of the NMDA receptor), in honeys from different botanical sources, and of the newly discovered derivative of KA, 4-quinolone (4-QUIN); (ii) to evaluate the total phenol content (TPC) and total antiradical activity (TAA) of different honeys. Honey components among which phenolic acids, flavonoids, Maillard products, carotenoids are involved in attenuation of the inflammatory process since they participate in COX-1 and COX-2 inhibition, in scavenging ROS and free radicals, and in the action of inflammatory mediators (cytokines) [15]; (iii) to apply a standard chemometric approach (principal component analysis, PCA) to predict the potential antinociceptive and antiinflammatory activity.

The results provide information that might help explain the therapeutic properties of honeys from floral or arboreal sources, and assist the dermatologist in deciding the most appropriate type of honey for clinical use. In addition they shed light on an interesting connection between KA and its derivatives 3-PKA, γ-LACT-3-PKA and 4-QUIN which could open up new metabolic perspectives on KA turnover in honeybees, mediated by the kynurenine pathway.

2. Material and methods

2.1. Chemicals

All chemicals, reagents and KA were of analytical grade, purchased from Sigma-Fluka-Aldrich Chemical Co. (Milan, Italy). HPLC-grade, analytical-grade organic solvents and deuterated dimethylsulphoxide (DMSO-d^6) were also purchased from Sigma-Fluka-Aldrich (Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. Honey samples

Commercial honey samples were purchased from local stores or from beekeepers in the region around Milan, authenticated and processed with the procedures previously described [16].

2.3. Sample preparation and calibration curves

Samples for HPLC-DAD-ESI-MS and diffusion-ordered spectroscopy (DOSY) NMR were prepared as previously reported [8,17]. Ten grams of honey were diluted to 100 mL with distilled water, stirred until completely dissolved, filtered through cotton to remove solid particles, and the pH was adjusted to 2.5 with 6N HCl. The filtrate was passed through a RP-SPE cartridge filled with solid phase (3 g of silica-bonded C-18 resin, Discovery® DSC-18, Supelco, Bellefonte, PA, USA), and after washing first with acidic water (HCl, 50 mL, pH 3) then with neutral water (50 mL), the substances retained in the column were recovered by elution with methanol (20 mL). The methanol fraction was dried under a gentle stream of nitrogen, and the residue was taken up in 600 μL of DMSO-d^6 for NMR analysis or with a 7:3 H_2O/acetonitrile mixture for HPLC–MS.

Samples for HPLC analysis were prepared by diluting 1 g of honey with 1 mL of acidic water (CF_3COOH, pH 3); this mixture was thoroughly mixed and methanol was added to 10 mL. The suspension was centrifuged (6000 rpm × 6 min), the supernatant filtered on 0.45-μm nylon filters (Millex HV, PVDF membrane, 13 mm, Millipore, Vimodrone, Milan, Italy) and 10 μL were injected into the HPLC-DAD apparatus. HPLC-DAD analysis was done according to the method proposed by Hervé et al., with minor modifications [18].

3-PKA was quantified using a calibration curve ($y = 0.0085x + 0.7493$, $R^2 = 0.9998$, constructed by triplicate injections of 3-PKA, purified by semipreparative HPLC [11], from 0.1 μg to 10 μg. The limit of detection (LOD, calculated as $3\sigma/S$, where σ indicates the standard deviation of the response and S the sensitivity obtained from the slope of the analytical calibration curve) was 0.01 μg injected, and the limit of quantification (LOQ) 0.03 μg. The recovery range, determined on an acacia honey sample (used as blank since it contains no detectable quinoline alkaloids) spiked with known amounts of 3-PKA (10, 50, 100, 200, 500, 1000 mg/kg) and processed as above, was 97.56–103.65%.

4-QUIN and γ-LACT-4-PKA were determined by comparison of the signal integrals deduced from the ^{1}H NMR spectra, following the procedure reported by Bradamante et al., with minor modifications [19]. In view of the chromatographic overlap of KA and γ-LACT-3-PKA, this latter was quantified applying the following equation:

$$C_{\gamma\text{-LACT-3-PKA}} = \frac{C_{\text{3-PKA}}}{\text{MW}_{\text{3-PKA}}} \times \frac{[\text{I}_{\text{H-5 3-PKA+H-5}\gamma\text{-LACT-3-PKA}} - \text{I}_{\text{H-6 3-PKA}}]}{\text{I}_{\text{H-6 3-PKA}}} \times \text{MW}_{\gamma\text{-LACT-3-PKA}}$$

where $C_{\text{3-PKA}}$ is the concentration of 3-PKA determined by HPLC-DAD expressed as mg/kg, $\text{I}_{\text{H-5 3-PKA H-6 3-PKA + H-5 }\gamma\text{-LACT-3-PKA}}$ is the cumulative integral value of the H-5 NMR signals (centered at 8.15 ppm) from 3-PKA and γ-LACT-3-PKA, and $\text{I}_{\text{H-6 3-PKA}}$

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