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FULL PAPER

Assessment of the Mutagenicity of Propolis Compounds from the Brazilian Cerrado Biome in Somatic Cells of *Drosophila melanogaster*

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Abstract:

Few information on the biological properties of the chemical constituents of propolis produced by *Apis mellifera* from the Cerrado of Midwest Brazil has hitherto been reported. In the present work, the mutagenic properties of five compounds isolated from a sample of brown propolis from the aforesaid biome, namely, caffeic (**1**), p-coumaric (**2**), dihydro-p-coumaric (**3**), and acetylisocupressic (**4**) acids, and aromadendrin (**5**) have been assessed by performing the somatic mutation and recombination test (SMART) on wing cells of *Drosophila melanogaster*, using standard (ST) and high bioactivation (HB) crosses. This is the first report of assessment of the mutagenic potential of **2**-**4**, and using the SMART assay for **5**. No mutagenicity was induced by **1**-**3** and **5** in the descendants from the ST and HB crosses at all evaluated concentrations. However, **1** and **2**, unlike **3**, were shown to be toxic to descendants of both ST and HB crosses. A structure-activity relationship established among **1**-**3** revealed that a C-7/C-8 unsaturation is responsible for the toxic effect of **2** compared to **3**, while an additional *ortho*-dihydroxy substitution at C-3 and C-4 confers to **1** the highest toxicity to *D. melanogaster* flies. Diterpene **4** proved mutagenic only after P450 activation, suggesting that it may act as a promutagen compound.

Keywords: Brazilian brown propolis; genotoxicity; mutagenic activity; promutagenicity; SMART

1. Introduction

Propolis is an essential product to the protection and survival of bees [1]. Due to its several biological properties, propolis samples have been employed in pharmaceutical and food industries, as well as in traditional medicine as nutraceuticals and functional foods, to improve health and for treatment or prevention of a vast range of diseases [2, 3]. Since the chemical composition of propolis samples, as well as the concentrations of their constituents are dependent on local flora, the evaluation of the biological activities of their components has been the subject of continuous investigation, including assessment of cytotoxic, anti-inflammatory, antimicrobial, antitumor, mutagenic, and antioxidant activities, among others [2-8]. Brazilian propolis produced by *Apis mellifera* was classified into 13 groups according to their phytogeographical origins and physicochemical characteristics, comprising brown, yellow, green and red types of propolis, the occurrence of the first two groups being also reported in Mato Grosso do Sul state (Midwest Brazil) [9-12]. Although the chemical composition and biological properties of representatives of these groups have been consistently investigated, few information on the biological properties of the chemical constituents of propolis from the Cerrado of Midwest Brazil has hitherto been reported [12-14]. In a previous study, we

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investigated the mutagenicities of the hexane and ethanol extracts of a sample of brown propolis collected from the Cerrado biome in Mato Grosso do Sul state, using the somatic mutation and recombination test (SMART), an in vivo short-term assay to access genetic lesions in *Drosophila melanogaster* [12]. An expressive total phenol concentration was found for the ethanol extract, and both the ethanol and hexane extracts proved non-mutagenic to the descendants from the ST and HB crosses at the doses tested. Highly conserved DNA sequences between mammals and *D. melanogaster* ensure this organism as an effective model to understand human biology and disease processes [15]. Herein, we performed the wing-spot assay (SMART) using standard (ST) and high bioactivation (HB) crosses to investigate the *in vivo* mutagenic and toxic potentials of the cinnamic acid derivatives caffeic (**1**), *p*-coumaric (**2**), and dihydro-*p*-coumaric (**3**) acids, the labdane-type diterpene acetylisocupressic acid (**4**) and the flavanonol aromadendrin (**5**), isolated from the hexane (**4**) and the ethanol (**1**-**3** and **5**) extracts of a sample of brown propolis obtained from the same aforementioned source.

2. Results and Discussion

Five compounds were obtained from the chemical study of a sample of brown propolis produced by *Apis mellifera* in the Cerrado landscape of Central-West Brazil: the phenylpropanoids caffeic (**1**), *p*-coumaric (**2**) and dihydro-*p*-coumaric (**3**) acids, and the flavanonol aromadendrin (**5**) from the ethanol extract, in addition to the diterpene acetylisocupressic acid (**4**) from the hexane extract (Figure 1). The structures of these known compounds were established on the basis of their ^{1}H and ^{13}C NMR data, which were in accordance with those published [16-20].

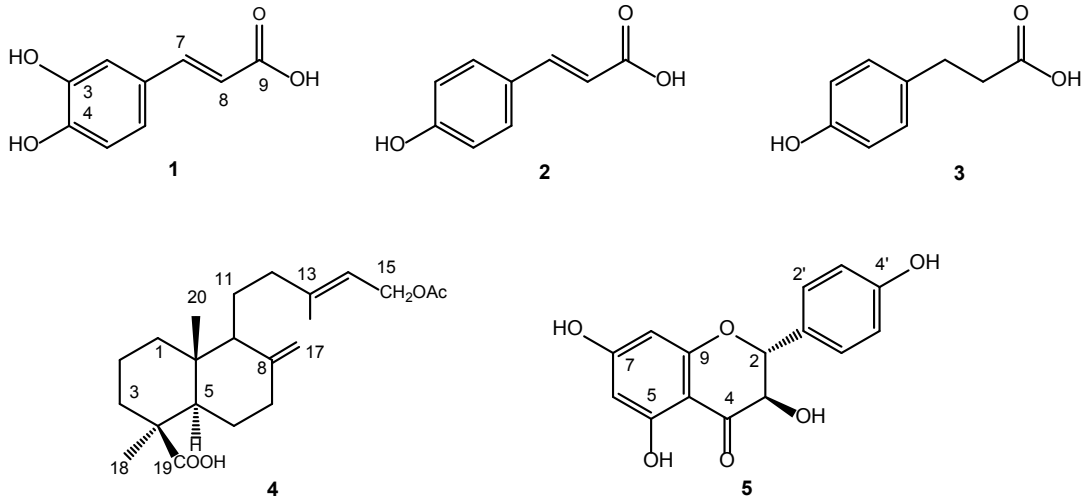

Figure 1. Chemical structures of caffeic (**1**), *p*-coumaric (**2**), and dihydro-*p*-coumaric (**3**) acids, acetylisocupressic acid (**4**) and aromadendrin (**5**).

In a previous study of a sample of yellow propolis collected in the Pantanal ecosystem of Mato Grosso do Sul, its ethanol extract showed cytotoxic activity against ovarian (OVCAR-8) tumor cell line, but was inactive against Gram-negative and Gram-positive bacteria. In addition, ^{1}HNMR, and GC-EIMS analyses revealed the absence of phenolic compounds and the presence of 15 pentacyclic triterpenes, together with mixtures of other aliphatic compounds [11].

The occurrence of **1**, **2** and **3** was formerly detected in a number of propolis samples worldwide [2, 21]. Compound **4** was identified as acetylisocupressic acid, a labdane-type diterpene previously reported in Greek and Brazilian propolis [20, 22]. In Brazil, diterpenes found in propolis are typical of some Araucaria species and thus indicate a possible plant source of Brazilian propolis samples which synthesize a resin rich in labdane-type diterpenes [22]. In addition, the *Baccharis* genus (Asteraceae), the main botanical source of Brazilian green propolis, probably contributes to the occurrence of

diterpenes in propolis from the Central-Western and Southeastern regions [23]. Flavonoid compounds, albeit mostly described in European and Chinese propolis, have also been detected in some samples from Brazil [24]. However, flavonoids bearing a flavanonol-type skeleton, like that of aromadendrin (**5**), are of less common occurrence [25, 26].

The ethanol extract of the propolis sample evaluated in the present investigation did not show any statistically significant mutagenicity against ST or HB larvae at the concentrations tested (1.0, 2.0 and 4.0 mg/mL), like the ethanol extract of the formerly investigated sample [12]. This was revealed by the frequencies of clone formation per cell division, which ranged from 0.20 x 10^{-5} to 0.61 x 10^{-5} for the ST cross and from 0.82 x 10^{-5} to 1.53 x 10^{-5} for the HB cross, when compared with their concurrent negative controls (1.84 x 10^{-5} for ST and 1.23 x 10^{-5} for HB crosses) (Table S1, Supplementary Material). Likewise, no mutagenicity was induced by the cinnamic acid derivatives **1**, **2**, and **3** and the flavanonol **5** in the descendants from the ST and HB crosses at all assessed concentrations, since no statistically significant differences in the induction of mutant spots were observed between the larvae treated with those compounds and their corresponding negative controls (Tables S2 and S3, Supplementary Material). However, a toxic effect of **1** and **2** was observed in the flies after exposure of both ST and HB larvae to these cinnamic acid derivatives at 20 and 40 mmol.L^{-1}, and 40 mmol. L^{-1}, respectively (Table S2, Supplementary Material), and mortality of flies was observed in the following order: dihydro-*p*-coumaric (**3**, non-toxic) < *p*-coumaric acid (**2**) < caffeic acid (**1**). Diterpene **4**, on the other hand, proved mutagenic at the highest concentration tested (2.76 mmol.L^{-1}) only after P450 bioactivation (Table S3, Supplementary Material). The aforementioned results are summarized in Figure 2.

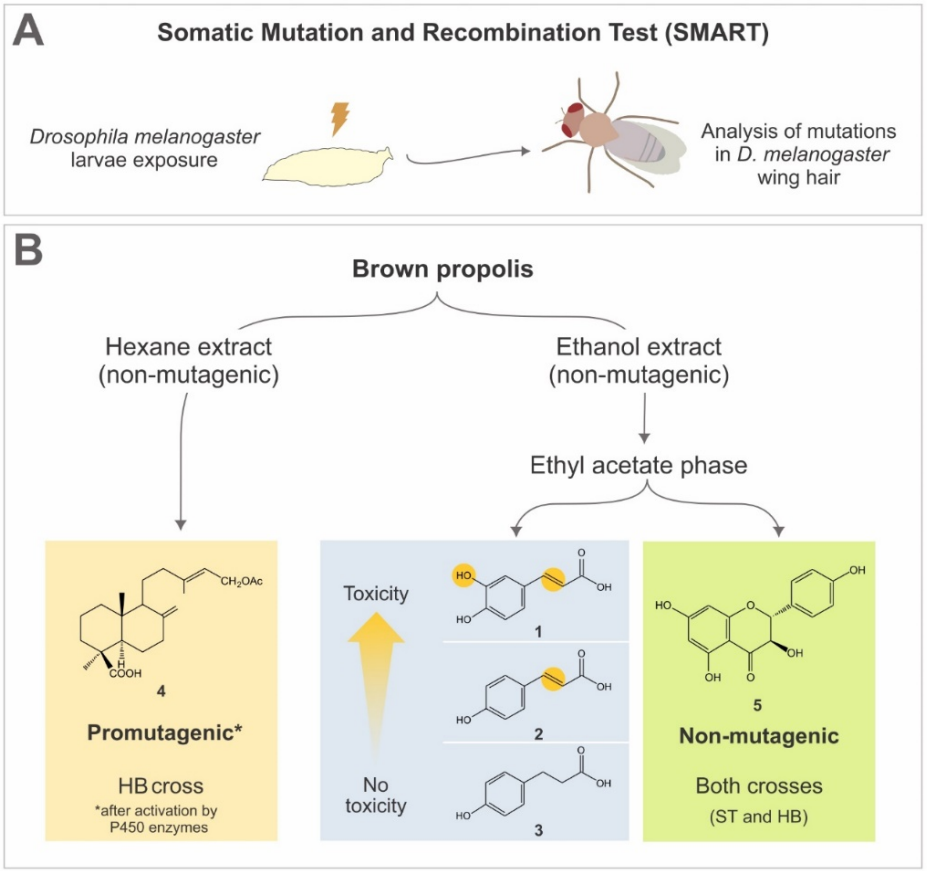

Figure 2. Mutagenic potential of compounds **1**-**5** to *D. melanogaster* MH descendants (ST and HB crosses).

In our previous report, a great radical-scavenging ability was shown by the ethanol extract and ethyl acetate fraction from a sample of brown propolis obtained from the same source as

that of the present investigation [12]. Therefore, the *p*-coumaric acid derivatives (**1**, **2** and **3**), as well as flavonoid **5** isolated in the present study might contribute to this effect. Regarding the previous results of the mutagenic potential of the ethanol and hexane extracts, and of the ethyl acetate fraction, neither of them proved mutagenic to *D. melanogaster* larvae originated from both ST and HB crosses [12]. When associated with the mutagen doxorubicin, the ethyl acetate fraction showed inhibitory effect of doxorubicin-induced genetic damage only at the lowest concentration tested (0.69 mg/mL; ST cross), with lack of dose-response [12]. This effect appears to be mediated by phenolic compounds, like **1**-**3**, that in high concentration can act as pro-oxidant (toxic), while at low concentration show antioxidant effect (nontoxic) [27, 28]. Literature has already reported this ambiguous behavior (toxic or protective), known as "Janus" phenomenon, exhibited for caffeic acid (**1**), as well as for some propolis extracts, depending on their concentration and cell types [12, 29].

According to the literature, the cytotoxic activity of **2** was associated with the presence of a *p*-hydroxyl group [30]. In addition, our data revealed that the presence of a double bond in the side chain (Δ^7) is responsible for the toxic effect of **2** when compared to **3**. Furthermore, this same double bond in association with an *o*-dihydroxy substitution pattern at C-3 and C-4 confer to **1** the highest toxicity. A relevant structure-activity relationship can thus be established among **1**-**3**, indicating that the toxicity to *D. melanogaster* flies increases as a C7/C-8 unsaturation followed by an additional hydroxylation at C-3 are introduced in the structure of these *p*-coumaric acid derivatives.

Biological activities have been reported for the labdane-type diterpene acetylisocupressic acid (**4**), such as antimicrobial properties and abortifacient effect in beef cattle, but its mechanism of action was not entirely elucidated [20, 31]. In the present study, **4** showed a mutagenic activity at the highest concentration tested after bioactivation by P450 enzymes (HB cross), indicating that this diterpene acts as a promutagen. Promutagen compounds produce DNA damage because of being metabolically activated by enzymatic systems [32]. Indeed, metabolism is required for **4** to be abortifacient in cattle [31].

Among the wide range of biological activities depicted by flavonoid compounds, their ability to reduce free radical formation and to scavenge free radicals is mostly known [33]. Literature reports for aromadendrin (**5**) its protective effect on human embryonic kidney (HEK-293) cells from oxidative stress by inhibiting the production of reactive oxygen species [34]. In addition, this flavonoid did not show any toxicity to *Salmonella typhimurium* TA100 strain, and modulated a weak antimutagenicity against AFB1 [35]. It was postulated that the structural features essential for the antimutagenicity of flavonoids on AFB1 using the Ames test were the presence of free hydroxyl groups at positions C-5 and C-7 [35]. Likewise, our data showed that **5** also proved neither mutagenic nor toxic to the offspring of both ST and HB crosses (Table S3, Supplementary Material).

3. Material and Methods

3.1 Propolis sample

Brown propolis produced by *Apis mellifera* L. was purchased in resin form (500 g) from Apiário Vovô Pedro® located in Campo Grande county, Mato Grosso do Sul State – Brazil, in January 2016.

3.2 General experimental procedures

Chromatography procedures were performed using silica gel (70-230 and 230-400 mesh particle size, Merck®), silica gel 60 RP-18 (40-63 µm, Merck®) and Sephadex LH-20 (100 µm, Sigma®). The TLC chromatographic plates were revealed with cerium sulfate spray solution (2% solution in H_2SO_4 2N).^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or CD_3OD (Cambridge Isotope Laboratories, Inc.) on a Bruker DPX-300 spectrometer, operating at 300.13 MHz (^{1}H)/75.47 MHz (^{13}C).

3.3 Extraction and isolation of compounds 1-5

The hexane and ethanol extracts of propolis and the ethyl acetate fraction resulting from partitioning of the latter were previously obtained as described by Fernandes et al. [12].

Part of the hexane extract (75.0 g) was chromatographed on a silica gel column (70-230

mesh) using step gradient elution with hexane, hexane-ethyl acetate, ethyl acetate and ethyl acetate-methanol to give 65 fractions of 125 mL each). Acetylisocupressic acid (**4**, 18.9 mg) was isolated from fraction 40 (500mg), after repeated column chromatography procedures on Sephadex LH-20 [hexane-CH_2Cl_2 (2:8)].

An aliquot (1.0 g) of the ethyl acetate fraction was submitted to RP-18 silica gel column chromatography using methanol-H_2O (2:8, 4:6, 8:2) and methanol as eluents to yield 10 fractions of 200 mL each. Fraction 3 (0.72 g), eluted with methanol-H_2O (4:6), was further separated by column chromatography on Sephadex LH-20 (methanol) to give five fractions. *p*-Coumaric acid (**2**, 8.2 mg) and dihydro-*p*-coumaric acid (**3**, 10.6 mg) were obtained from fractions 4 and 5, respectively, while fraction 3 gave caffeic acid (**1**, 8.5 mg). Aromadendrin (**5**, 6.7 mg) was isolated after purification of fraction 2 by column chromatography on silica gel (230-400 mesh) using $CHCl_3$-methanol mixtures with increasing methanol content (5-30%).

*Caffeic acid (**1**):* White amorphous powder. ^{1}H NMR (300 MHz, CD_3OD): δ 6.20 (*d*, *J* = 15.0 Hz, H-8), 6.75 (*d*, *J* = 9.0 Hz, H-5), 6.91 (*dd*, *J* = 9.0, 3.0 Hz, H-6), 7.01 (*d*, *J* = 3.0 Hz, H-2), 7.51 (*d*, *J* = 15.0, 8.4 Hz, H-7). ^{13}C NMR (75 MHz, CD_3OD): δ115.1 (C-8), 115.7 (C-2), 116.5 (C-5), 122.8 (C-6), 127.8 (C-1), 146.7 (C-3), 147.0 (C-7), 149.4 (C-4), 171.1 (C-9).

*p-Coumaric acid (**2**):* White amorphous powder. ^{1}H NMR (300 MHz, CD_3OD): δ 6.28 (*d*, *J* = 15.0 Hz, H-8), 6.81 (*d*, *J* = 9.0 Hz, H-3,5), 7.44 (*d*, *J* = 9.0 Hz, H-2,6), 7.59 (*d*, *J* = 15.0 Hz, H-7). ^{13}C NMR (75 MHz, CD_3OD): δ 115.7 (C-8), 116.1 (C-4), 116.5 (C-3,5), 127.2 (C-1), 131.1 (C-2,6), 146.6 (C-7), 171.2 (C-9).

*Dihydro-p-coumaric acid (**3**):* White amorphous powder. ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$): δ 2.52 (*t*, *J* = 6.0 Hz, H-8), 2.80 (*t*, *J* = 6.0 Hz, H-7), 6.69 (*d*, *J* = 8.0 Hz, H-3,5), 7.04 (*d*, *J* = 8.0 Hz, H-2,6).^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$): δ 31.3 (C-8), 37.4 (C-7), 116.0 (C-3,5), 130.2 (C-2,6), 133.0 (C-1), 156.5 (C-4).

*Acetylisocupressic acid (**4**):* White amorphous powder. ^{1}H NMR (300 MHz, $CDCl_3$): δ0.57 (*s*, H-20), 0.98-1.80 (*m*, H-1, H-12), 1.21 (*s*, H-18), 1.28 (*brs*, H-5), 1,42-1.55 (*m*, H-11), 1,47-1.77 (*m*, H-2), 1.49 (*m*, H-9), 1.80 (*m*, H-6), 1.77-2.38 (*m*, H-7), 2.13 (*m*, H-3), 4.49 (*brs*, H-17a), 4.55 (d, J = 9.0 Hz, H-15), 4.82 (*brs*, H-17b),5.28 (*brt*, *J* = 6.0 Hz, H-14).^{13}C NMR (75 MHz, $CDCl_3$): δ12.8 (C-20), 16.5 (C-16), 19.9 (C-2), 21.8 (C-11), 26.0 (C-6), 29.0 (C-18), 37.9 (C-3), 38.4 (C-12), 38.7 (C-7), 39.1 (C-1), 40.4 (C-10), 44.2 (C-4), 55.4 (C-9), 56.3 (C-5), 61.4 (C-15), 106.5 (C-17), 118.1 (C-14), 142.9 (C-13), 147.9 (C-8), 183.5 (C-19), 21.1/ 171.2 (-$OCOCH_3$).

*Aromadendrin (**5**):* White amorphous powder. ^{1}H NMR (300 MHz, CD_3OD): δ4.54 (*d*, *J* = 12.0 Hz, H-3), 4.98 (*d*, *J* = 12.0 Hz, H-2), 5.88 (*s*,H-6,8), 6.84 (*d*, *J* = 9.0 Hz, H-3',5'), 7.35 (*d*, *J* = 9.0 Hz, H-2',6').^{13}C NMR (75 MHz, CD_3OD): δ73.5 (C-3), 84.9 (C-2), 96.4 (C-6,8), 101.8 (C-10), 116.2 (C-3',5'), 129.3 (C-1'), 130.4 (C-2',6'), 159.0.0 (C-4'), 164.6 (C-9), 165.1 (C-5), 168.9 (C-7), 198.4 (C-4).

3.4 Mutagenic assessment by SMART assay

3.4.1 Strains, crosses, and analysis

Three mutant *Drosophila melanogaster* strains were used*:* (i) *multiple wing hairs* (*mwh*), (ii) *flare-3*(*flr^3*) and (iii) *ORR; flare^3,* which has chromosomes 1 and 2 characterized by a high level of enzymes of the cytochrome P450 (CYP450) complex, conferring high sensitivity to promutagens and procarcinogens [36].

Two crossings were performed, (1) *standard* (ST), in which virgin *flr^3*females were selected and crossed with *mwh* males, and (2) *high bioactivation* (HB), from virgin *ORR/flr3* females and *mwh* males according to Graf et al. [37] and Graf & van Schaik [38] protocols, respectively. For chronic exposure, third-instar larvae from ST and HB crosses were carefully collected and transferred to glass vials containing 1.5 g of alternative culture medium (instant mashed potato flakes, Yoki Alimentos, Brazil) rehydrated with caffeic (**1**), *p*-coumaric (**2**) and dihydro-*p*-coumaric (**3**) acids (5, 10, 20 and 40 mmol.L^{-1}), acetylisocupressic acid (**4**) (0.69, 1.38 and 2.76 mmol.L^{-1}), and aromadendrin (**5**) (0.35, 0.70 and 1.4 mmol.L^{-1}) solutions. For all assays, a negative control group (Milli-Q® water with 1% Tween-40 and 3% ethanol) was included.

The following offspring were obtained: marker-heterozygous (MH) (*mwh*+/+*flr^3*) flies, with wild wing phenotype, and balancer-heterozygous (BH) (*mwh*+/+*TM3*, *Bd^S*) flies. After feeding exposure

and completing the metamorphosis cycle, the emerging adults were collected and fixed in 70% ethanol. Both sexes from MH individuals were separated and their respective wings were arranged in pairs on slides, fixed in Faure's solution, and their ventral and dorsal surfaces were observed under an optical microscope (400× magnification)in order to analyzing spots-per-fly mutations: small and large single spots, as well as twin spots.

3.4.2 Statistical analysis

Data were analyzed according to Frei & Würgler [39]. This statistical method (χ^2-test) allows to decide whether mutations scored in SMART assay indicate a positive (+), negative (-), or inconclusive (i) results to mutagenicity. For assessment of mutagenic effects, the frequencies of each type of mutant spot per wing in a treated series were compared with its concurrent negative control series, using Kastenbaum & Bowman's binomial conditional test [40] with significance levels set at α = β= 0.5.

4. Conclusions

This is the first report of assessment of the mutagenic potential of *p*-coumaric (**2**), dihydro-*p*-coumaric (**3**), and acetylisocupressic (**4**) acids, and using the SMART assay for aromadendrin (**5**). The foregoing results revealed that **4** may act as a promutagen compound. In addition, these findings allowed the establishment of the structure-activity relationship among the cinnamic acid derivatives **1**, **2** and **3**, indicating that the presence of a double bond at C-7 plus an *ortho*-dihydroxylation pattern at C-3/C-4 is responsible for an increase of their toxic effects to *D. melanogaster* flies.

Since the concentration of the chemical constituents of propolis samples may vary according to the geographic region and plant sources, the toxicological effects identified in the present study show that special attention should be given to the use of propolis samples with a high content of *p*-coumaric acid derivatives as an alternative medicine or as a food supplement. Further research on the toxic effects of these constituents is therefore needed, so that their mechanisms of action can be fully established.

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