Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2014; 52(7): 835–840
 DOI: 10.3109/13880209.2013.871638

informa
healthcare

ORIGINAL ARTICLE

Analytical methodology optimization to estimate the content of non-flavonoid phenolic compounds in Argentine propolis extracts

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Abstract

Context: Traditionally, the content of total phenolics (flavonoid phenolics (FP) and non-flavonoid phenolics (NFP)) and flavonoids (flavone/flavonol and flavonone/dihydroflavonol) in propolis has been determined by different methodologies. Until now, the percentage of total phenolic (TP) compounds that corresponds to FP and NFP, expressed in the same units by a spectrophotometric method, has not been determined.

Objective: The current study proposes a quick and simple methodology that separates FP and NFP in propolis samples and determines TP, FP, and NFP by the same method.

Materials and methods: Propolis samples from five Argentine provinces (Tucumán, Santiago del Estero, Salta, Misiones, and Jujuy) were used. Extraction of TP from the propolis samples was carried out by maceration with 80% ethanol and quantified by Folin–Ciocalteu reagent (FC-R). Then, FP was precipitated with formaldehyde in acid medium. After centrifugation, NFP were determined in the supernatant using FC-R. FP content was calculated as the difference between the content of TP and NFP. The method was also validated using commercial flavonoids and chalcones.

Results: FP recovery in all experiments was between 85.95% and 98.29%. Propolis from Tucumán had significantly higher amounts of total phenols than propolis from other provinces. SE5 showed higher content of FP (81.52%) followed by SA1 (74.75%). The propolis from TUC4, SA4, SE3, and MI showed the lowest FP content and highest content of NFP.

Conclusions: This method provides a simple, reliable, and specific spectrophotometric assay to estimate the content of NFP, FP, and TP in propolis samples.

Keywords

Bee glue, flavonoid phenolic precipitation, total phenolic compounds content

History

Received 4 August 2013
Revised 11 October 2013
Accepted 13 November 2013
Published online 6 February 2014

Introduction

Argentine propolis has been used extensively in folk medicine since it possesses antifungal, antibacterial, anti-inflammatory, and antioxidant properties among others (Isla et al., 2001, 2005, 2009, 2012a,b; Ordóñez et al., 2011; Solórzano et al., 2012; Vera et al., 2011). Bee glue chemical composition depends on the local flora and geographic and climatic characteristics of the collection site (Bankova et al., 2006; Choi et al., 2006; Isla et al., 2009, 2012a,b; Solórzano et al., 2012). In Argentina, 80% ethanol is the solvent of choice for the extraction of biologically active components of propolis (mainly phenolics, including different types of flavonoids), using the traditional method of maceration (Norma IRAM-INTA 15935-2, 2008).

Rapid routine chemical characterization of propolis requires the development of a suitable methodology. Characterizing and standardizing Argentine propolis preparations require the determination of total phenolic compounds by the colorimetric method using Folin–Ciocalteu reagent (FC-R) while flavone and flavonol contents are established by colorimetric methods using aluminum chloride (Norma IRAM-INTA 15935-2, 2008). The 2,4-dinitrophenylhydrazine (DNP) method is used to quantify flavanones and dihydroflavonols (Popova et al., 2003, 2004). The sum of the content of both flavonoid types does not represent the real content of total flavonoids because they are determined by different methodologies and expressed in equivalents of different reference compounds (quecetin and naringenin for flavones/flavonol and flavanone/dihydroflavonols, respectively).

Up to the present, the percentage of phenolic compounds that correspond to flavonoid phenolics (FP) and non-flavonoid phenolics (NFP) expressed in the same units by a spectrophotometric method have not been found. This paper reports the optimization of an analytical methodology to measure NFP and FP compound contents in propolis samples in the same units using FC-R and to establish the relation between FP and NFP in each sample.

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Materials and methods

Samples

Propolis samples from apiaries of five Argentine provinces: Tucumán (five samples, TUC1–TUC5), Santiago del Estero (seven samples, SE1–SE7), Salta (five samples, SA1–SA5), Misiones (one sample, MIS), and Jujuy (one sample, JUJ) were used (Table 1).

Reference compounds were from Sigma and Indofine Chemical Co. (St. Louis, MO)

Propolis extract preparation

Propolis (2 g) was mixed with 25 mL of 80% ethanol and shaken at 70 °C for 30 min. After extraction, the mixture was centrifuged at 3000*g* for 10 min, and the supernatants were used for further analysis and named propolis ethanol extract (PEE).

Flavonoid determination

Total flavone and flavonol content. An aliquot (0.1 mL) of different dilutions of each extract, 0.4 mL methanol, and 0.4 mL 5% $AlCl_3$ in methanol were mixed (Woisky & Salatino, 1998). The mixture was left for 30 min at room temperature and the absorbance at 425 nm was measured. The results were expressed as mg equivalent of quercetin per mL of extract (mg QE/mL) and mg QE/g crude propolis. Three measurements were performed for each extract.

Total flavanone and dihydroflavonol content. Flavanone and dihydroflavonol contents were determined using DNP in acid media according to Popova et al. (2004) with modifications. An aliquot of each extract sample was diluted with 80% ethanol to a volume of 0.25 mL and 0.5 mL of DNP solution (1 g DNP in 2 mL 96% sulfuric acid, diluted to 100 mL with methanol) was added and heated at 50 °C for 50 min. After cooling to room temperature, 0.3 mL of the previous mixture was diluted to 1 mL with 10% KOH. The resulting solution was centrifuged at 1500*g* for 10 min and 0.25 mL of the supernatants was diluted to 1.5 mL with methanol. Absorbance was measured at 492 nm. The results were expressed as mg equivalent of naringenin per mL of extract (mg NE/mL) and mg NE/g crude propolis. Three measurements were performed for each extract.

Analyses of phenolic compounds

Total soluble phenolic (flavonoids and non-flavonoids) determination. Total phenolic compound concentration in selected propolis samples was determined spectrophotometrically according to the Folin–Ciocalteu (F-C) colorimetric method (Singleton et al., 1999). An aliquot of each extract solution was mixed with 0.2 mL of the Folin–Ciocalteu reagent, and 0.8 mL of 15.9% sodium carbonate. The final volume was made to reach 3 mL with distilled water. The sample was left for 20 min at room temperature and the absorbance at 760 nm was measured. Calibration was performed using gallic acid as a reference compound. The results were expressed as mg of gallic acid equivalent per mL (mg GAE/mL) and mg GAE/g crude propolis. Three measurements were performed for each extract.

Table 1. Phenolic compounds and flavonoids contents in ethanol extracts of propolis from different regions of Argentina.

Propolis samples	Geographical origin	Total phenolic compounds (mg GAE/*g* propolis)	Flavones and flavonols (mg QE/g propolis)	Flavanones and dihydroflavonols (mg NE/*g* propolis)
TUC 1	Tucumán, Amaicha del Valle (Departmento Tafi del Valle, Sample 101-INTA)	235.75 ± 2.35	274.87 ± 2.10	128.25 ± 1.40
TUC 2	Tucumán, Tafí del Valle (Departmento Tafi del Valle, Sample 102-INTA)	210.37 ± 2.20	182.75 ± 1.60	95.75 ± 0.70
TUC 3	Tucumán, El Siambón (Departmento Trancas, Sample 103-INTA)	85.00 ± 0.80	32.25 ± 0.25	30.00 ± 0.30
TUC 4	Tucumán, Cachi Yaco (Departmento Leales, Sample 206-INTA)	17.25 ± 1.60	2.37 ± 0.02	51.37 ± 0.40
TUC 5	Tucumán, Finca Medina (Departmento Famaillá, Sample 192-INTA)	132.25 ± 1.20	53.70 ± 0.60	45.75 ± 0.40
SA1	Salta, El Galpón (Departmento Metán, Sample 163-INTA)	49.50 ± 0.50	16.87 ± 0.20	13.87 ± 0.15
SA2	Salta, El Barquillo (Sample 350-INTA)	177.62 ± 1.10	160.87 ± 1.60	85.62 ± 0.8
SA3	Salta, Joaquín V. González (Departmento de Anta, Sample 193-INTA)	142.87 ± 1.00	32.50 ± 0.30	56.75 ± 0.5
SA4	Salta (Departmento de Anta, Sample 349-INTA)	144.75 ± 1.00	25.62 ± 0.30	86.25 ± 0.8
SA5	Salta, Cachi (Departmento Cachi, Sample 104-INTA)	144.37 ± 1.00	42.62 ± 0.30	47.50 ± 0.4
JUJ	Jujuy, Yuto (Departmento Ledesma, Sample 100-INTA)	178.50 ± 1.60	3.87 ± 0.20	104.62 ± 1.0
SE1	Santiago del Estero (Departamento Capital, Sample 200-UNSE)	34.82 ± 2.60	66.01 ± 1.51	3.66 ± 0.36
SE2	Santiago del Estero (Departmento Figueroa, Sample 201-UNSE)	37.87 ± 1.74	49.71 ± 1.62	4.32 ± 0.43
SE3	Santiago del Estero (Departmento Banda, Sample 202-UNSE)	31.82 ± 2.74	56.85 ± 2.51	7.19 ± 0.40
SE4	Santiago del Estero (Departmento Copo, Sample 203-UNSE)	40.91 ± 3.42	34.60 ± 0.87	5.70 ± 0.30
SE5	Santiago del Estero (Departmento Banda, Sample 204-UNSE)	27.32 ± 1.94	21.28 ± 0.12	4.77 ± 0.47
SE6	Santiago del Estero (Departmento Los Romanos, Sample 205-UNSE)	27.76 ± 0.41	29.80 ± 0.62	7.13 ± 0.60
SE7	Santiago del Estero (Departmento Figueroa, Sample 206-UNSE)	42.36 ± 1.09	46.61 ± 0.76	5.59 ± 0.40
MIS	Misiones, Apóstoles (Departamento de Apóstoles, Sample 299-INTA)	73.50 ± 0.60	1.37 ± 0.20	29.00 ± 1.00

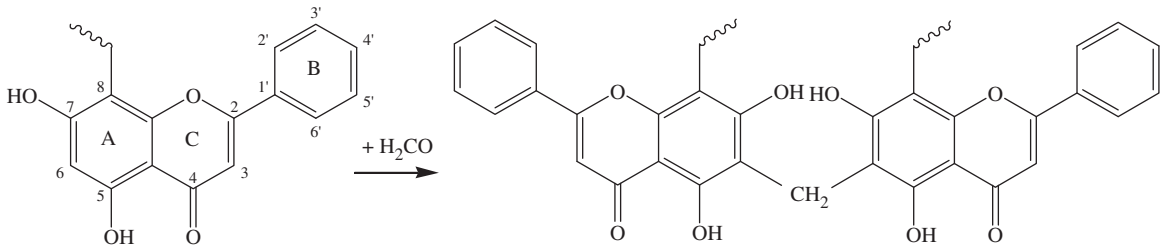

Figure 1. Chemical reaction between flavonoid and formaldehyde.

NFP determination. Formaldehyde reacts with the 6- or 8-position on the flavonoids forming a methylol derivative that will attach to another 6- or 8-position on another flavonoid, and so on (Figure 1). Flavonoid polimerization time was referred to the working time of flavonoids toward formaldehyde from a colloidal solution to become a solid or semi-solid gel. These condensed molecules could be removed by centrifugation at 9000*g*. The residual NFP content of propolis samples could be analyzed by Folin–Ciocalteu method in the supernatant using the methodology described by Kramling and Singleton (1969), for wines with the following modifications.

Methodology

An aliquot (0.125 mL) of different dilutions of each propolis extract was mixed with 0.375 mL of ethanol and acidified with 0.5 mL of HCl (1:3, v:v). Then, different volumes of formaldehyde 8 g/L (0.125 mL, 0.25 mL, or 0.50 mL) were added and mixed. The mixture was maintained at room temperature and at normal atmospheric pressure for different times: 0, 30, 60, 120, 240 min, 24 and 48 h. The mixtures were centrifuged at 9000*g* for 5 min. Each pellet and supernatant were reserved and named as FP fraction (FP-fraction) and NFP fraction (NFP-fraction), respectively.

The pellet was solubilized by two methods:
Method 1: the pellet was solubilized with 0.5 mL of ethanol, sonicated for 5 min and neutralized with KOH 0.1 N.
Method 2: the pellet was washed three-fold with 0.5 mL of distilled water and centrifuged. Then, the washed pellet was solubilized with 0.5 mL of ethanol.

Each NFP-fraction and FP-fraction were used to determine the phenolic content by F–C according to Singleton et al. (1999).

The amount of FP was also calculated as the difference between total free phenols (TP) and NFP in propolis.

$$[\mathrm{FP}] = [\mathrm{TP}] - [\mathrm{NFP}]$$

The presented data are the average of three measurements.

Method validation

The positive controls were realized using solutions (1 mg/mL) of luteolin (flavone), hesperetin (flavanone), quercetin (flavonol), and 2′-4′-dihydroxychalcone (chalcone).

The methodology described previously was used (0.5 of sample, 0.25 mL of formaldehyde, 0.5 mL of HCl, and 24 h of incubation) to obtain flavonoid precipitation. Then, the phenolic content was determined in the supernatant by F–C.

Thin-layer chromatography (TLC)

PEE, NFP-fraction, and FP-fraction (10 µg GAE) were spotted on TLC plates (Kieselgel 60 F254 0.2 mm, Merck, Darmstadt, Germany). They were developed in ascending direction using chloroform:ethyl acetate (80:20). The separated components were also visualized under ultraviolet light (254 and 365 nm, UV Lamp Model UVGL-58 Mineralight Lamp, Upland, CA) followed by spraying with NP reagent (Wagner et al., 1984).

Statistical analysis

Sampling and analyses were performed in triplicate, and the data are presented as mean ± standard deviation. The correlation between two variants by Pearson test was realized using Infostat software package (Di Rienzo et al., 2012). with the level of significance set at $p < 0.05$.

Results and discussion

According to Singleton and Rossi (1965), various phenolic compounds have different responses in this assay. This method's molar response is roughly proportional to the number of phenolic hydroxyl groups in a given substrate, but the reducing capacity is enhanced when two phenolic hydroxyl groups are oriented *ortho* or *para* (Frankel et al., 1995). The advantages of this spectrophotometric method are its simplicity, good repeatability, and acceptable accuracy. In previous reports (Isla et al., 2001, 2005, 2012a,b; Nieva et al., 2005; Ordoñez et al., 2011; Vera et al., 2011; Solórzano et al., 2012), a wide range of phenolic compound concentrations in propolis samples from different phytogeographical provinces of Argentine (Isla et al., 2012b) have been described.

Total phenolic and flavonoid contents in Argentine propolis samples

Eighteen propolis samples were analyzed. The content of total phenolics by FC-R showed values between 17.25 and 235.75 mg GAE/g crude propolis. The content of flavones and flavonols (using $AlCl_3$) in Argentine propolis showed values between 1.37 and 274.87 mg QE/g crude propolis. Flavanones and dihydroflavonols (using DNP) showed values between 13.87 and 128.25 mg NE/g crude propolis (Table 1). The propolis from Amaicha del Valle (Tucumán) showed the highest phenolic, flavonoid, flavone, and flavonol content; but with this methodology, it was not possible to determine the relation between FP/NFP because different methodologies were used for each chemical group.

Table 2. Effect of contact time of formaldehyde with phenolic compounds in acid medium on the content of non-flavonoid phenolic and flavonoid phenolic compounds of ethanolic extracts of propolis from Tucumán (T1), Argentina.

	Phenolic compound content			
Contact time (h)	NFP (FC-R) mg GAE/mL propolis extract	NFP (FC-R) mg GAE/g propolis	FP (FP = TP-NFP) mg GAE/mL propolis extract	NFP (FC-R) mg GAE/g propolis
0.0	17.79 ± 0.50^{a}	222.37 ± 5.00^{a}	0.00 ± 0.00^{c}	
0.5	10.50 ± 0.50^{b}	131.25 ± 2.20^{b}	7.29 ± 0.10^{b}	91.12 ± 1.00^{b}
1.0	10.00 ± 0.40^{b}	$125.00 \pm 1.20^{b,c}$	$7.79 \pm 0.30^{a,b}$	$97.37 \pm 1.00^{a,b}$
2.0	9.50 ± 0.30^{b}	118.75 ± 1.10^{c}	8.29 ± 0.40^{a}	103.62 ± 5.00^{a}
24	9.50 ± 1.00^{b}	118.75 ± 1.10^{c}	8.30 ± 0.50^{a}	103.75 ± 5.00^{a}
48	9.50 ± 1.00^{b}	118.75 ± 1.10^{c}	8.30 ± 0.50^{a}	103.75 ± 3.00^{a}

FP, flavonoid phenolic fraction; NFP, non-flavonoid phenolic fraction; TP, total free phenols. TP = 235.75 ± 2.35 mg GAE/g propolis. Formaldehyde = 250 µL. Values (mean ± SEM, $n = 3$) in the same column followed by a different letter are significantly different (Tukey's, $p \leq 0.05$).

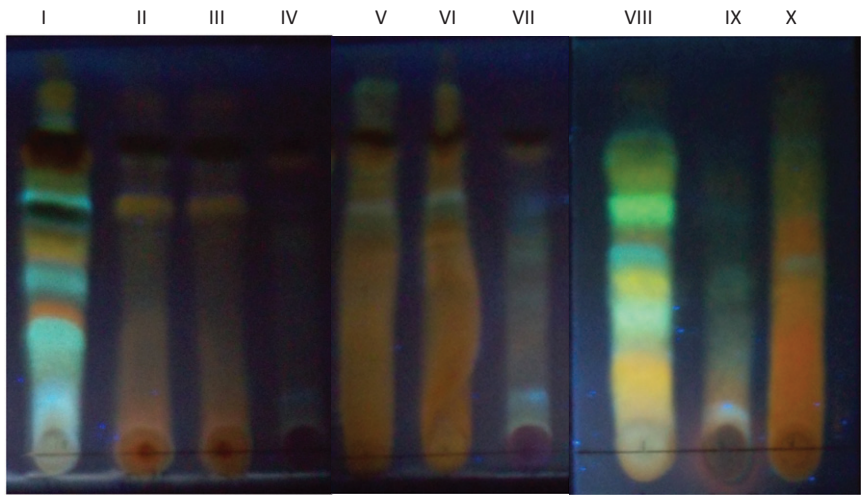

Figure 2. TLC of PEE, supernatant, and residue after Treatment 1: PEE + 250 µL formaldehyde, centrifugation or Treatment 2: PEE + 500 µL formaldehyde, centrifugation. Line I: PEE from TUC I, Line II: residue of Treatment 1 neutralized with KOH; Line III: residue of Treatment 2 neutralized with KOH; Line IV: supernatant of Treatment 1; Line V: residue of Treatment 1 after washing with water and resuspending with EtOH; Line VI: residue of Treatment 2 after washing with water and resuspending with EtOH; Line VII: supernatant of Treatment 1; Line VIII PEE from SA2; Line IX: supernatant of Treatment 1; Line X: residue of Treatment 1 after washing with water and resuspending with EtOH.

Optimization of the FP extraction to determine NFP with FC method

The influence of two parameters (i.e., reaction time and concentration of the chemical reagents) on formaldehyde-induced FP polymerization in acid medium was examined using propolis from Tucumán (T1), Argentina (Table 2).

Three concentrations of formaldehyde were experimented (125, 250, and 500 µL of formaldehyde) during different contact times (0 until 48 h). The results obtained showed an increase of polymerization with 250 µL of formaldehyde. A longer contact time between formaldehyde (250 µL) and phenolics of propolis samples showed better precipitation of FP. No significant yield increase was observed using 500 µL of formaldehyde and longer reaction times (48 h). As expected, the analysis of the recovered supernatant at the end of the processes showed a decrease in the total phenolic content compared to the raw material by the removal of FP. It is noteworthy to mention that in all cases, the flavonoids extracted exhibited a very low solubility in water. Consequently, it was possible to use water to eliminate the acid medium. The FP-fraction was soluble in 500 µL of ethanol. A rapid TLC fingerprinting of the solubilized residue (FP) by methods 1 and 2 confirmed the presence of flavonoids by UV fluorescence after spraying with NP (Figure 2).

Table 3. Validation of method of determination of non-flavonoid phenolic content using commercial flavonoids and commercial phenolic acids.

Standard (1 mg/mL)	Recovery of flavonoid (%)
Luteolin (flavone)	87.45
Hesperetin (flavonone)	98.29
Quercetin (flavonol)	91.21
2′,4′-Dihydroxychalcone (chalcone)	85.95

The method of precipitation of FP (Figure 3)and determination of NFP were validated by using commercial flavonoids (flavone, flavonol, flavanone, and dihydroflavanone) and chalcones, all propolis components, and preparing them in the same way as all propolis samples. The recovery of flavonoids in all experiments was between 85.95 and 98.29% and is presented (Table 3).

NFP content of North Argentine propolis

Propolis from Tafi del Valle, Tucumán, had significantly higher amounts of total phenols than propolis from other provinces. SE5 showed higher content of FP (81.52%) followed by SA1 (74.75%), TUC 1(72.71%), SE4 (69.33%),

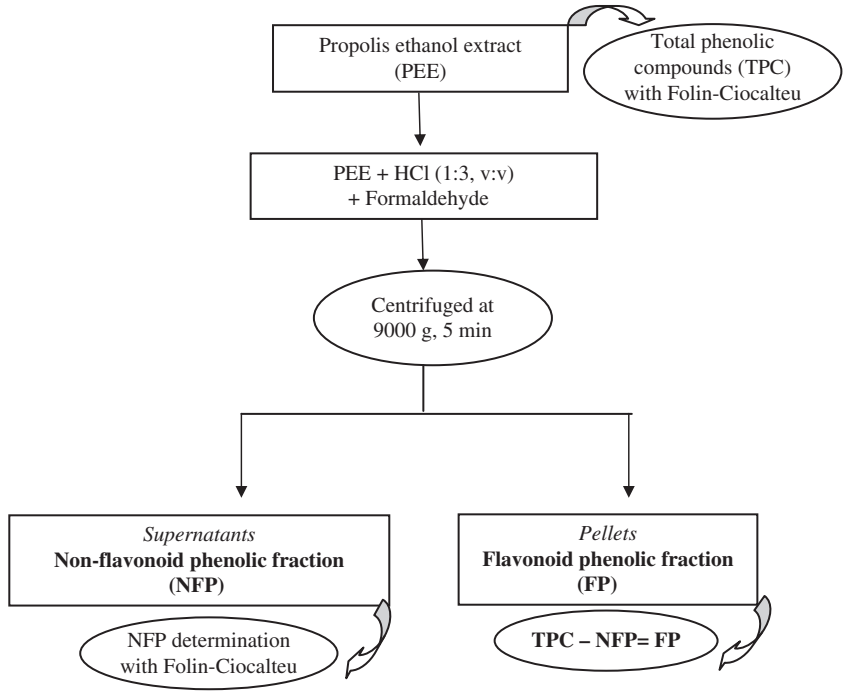

Figure 3. Analytical methodology for separating the polyphenols of propolis samples in two fractions, one enriched in flavonoid phenolic compounds and other fraction enriched in non-flavonoid phenolic compounds.

Table 4. Content of non-flavonoid phenolic and flavonoid phenolic compounds of ethanol extracts of propolis from different regions of Argentina.

	Phenolic compounds (mg EAG/mL)			
Propolis samples	TP	NFP	FP = TP-NFP	% FP
TUC 1	17.79 ± 0.50	10.3 ± 1.00	7.49 ± 1.00	72.71
TUC 2	16.83 ± 0.50	6.66 ± 0.50	10.17 ± 0.50	60.43
TUC 3	6.80 ± 0.20	3.39 ± 0.50	3.41 ± 0.50	50.41
TUC 4	1.38 ± 0.20	1.00 ± 0.50	0.38 ± 0.50	27.54
TUC 5	6.97 ± 0.30	3.87 ± 0.20	3.10 ± 0.20	44.47
SA1	3.96 ± 0.30	1.00 ± 0.10	2.96 ± 0.10	74.75
SA2	14.21 ± 0.50	6.90 ± 0.10	7.31 ± 0.10	51.44
SA3	11.43 ± 0.50	4.33 ± 0.20	7.10 ± 0.10	62.11
SA4	11.58 ± 0.50	7.70 ± 0.20	3.88 ± 0.10	33.50
SA5	11.55 ± 0.50	6.51 ± 0.10	5.04 ± 0.10	43.64
JUJ	14.28 ± 0.50	4.63 ± 0.10	9.65 ± 0.10	67.58
SE1	13.93 ± 0.30	6.99 ± 0.10	6.94 ± 0.10	49.82
SE2	15.15 ± 0.50	5.35 ± 0.10	9.80 ± 0.10	64.69
SE3	12.73 ± 0.30	8.30 ± 0.20	4.43 ± 0.20	34.80
SE4	16.37 ± 0.50	5.02 ± 0.10	11.35 ± 0.10	69.33
SE5	10.93 ± 0.50	2.03 ± 0.10	8.91 ± 0.10	81.52
SE6	11.10 ± 0.50	3.45 ± 0.10	7.65 ± 0.10	68.92
SE7	16.95 ± 0.50	5.50 ± 0.10	11.45 ± 0.10	67.55
CH1	2.45 ± 0.30	1.98 ± 0.10	0.47 ± 0.10	19.18
CH2	1.46 ± 0.40	1.23 ± 0.10	0.23 ± 0.10	15.75
CH3	1.68 ± 0.50	1.05 ± 0.20	0.63 ± 0.20	37.50
MIS	5.88 ± 0.50	4.9 ± 0.10	0.98 ± 0.10	16.66

TP, total phenolic compounds; NFP, non-flavonoid phenolic compounds; FP, flavonoid phenolic compounds. Values (mean ± SEM, $n = 3$).

SE6 (68.92%), JU (67.58%); SE7 (67.55%), SA3 (62.11%), TUC 2 (60.43%), Table 4. The propolis from TUC4, SA4, SE3, and MI showed the lowest FP content and highest content of NFP.

Conclusions

This method provides a simple and specific spectrophotometric assay for NFP determination in propolis samples. The possible use of formaldehyde to precipitate the FP compounds has been proposed for propolis in the present paper for the first time.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. This research was supported by grants from Consejo de Investigación de la Universidad Nacional de Tucumán, Argentina (CIUNT), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), and Agencia Nacional de Promoción Científica y Técnica (ANPCyT).

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