Antioxidant and anti-inflammatory activities of phenolic compounds isolated from Melipona beecheii honey

Jorge Carlos Ruiz-Ruiz a, Angel Jesus Matus-Basto a, Pablo Acereto-Escoffie b and Maira Rubí Segura-Campos b

aDivisión de Estudios de Posgrado e Investigación, Instituto Tecnológico de Mérida, Mérida, Yucatán, México; bFacultad de Ingeniería Química, Universidad Autónoma de Yucatán, Mérida, Yucatán, México

ABSTRACT
Methanolic extract was obtained from Melipona beecheii honey and quantitative analysis of components like total amount of phenolic compounds, flavonoids and flavonols were estimated. Phenolic acids and flavonoids were fractionated by reverse phase HPLC and assessed for antioxidant and anti-inflammatory activities by in vitro methods. Total phenol content was estimated as 63.22 mg of Gallic acid equivalents/100 g of honey. Total flavonoids and flavonols were found to be 3.61 and 3.16 mg of Catechin or Naringenin equivalents/100 g of honey, respectively. The flavonoids fraction exhibited the highest antioxidant activity, with IC50 values of 0.33, 0.92 and 0.36 mg/mL for radical scavenging activity, chelating effects and reducing power, respectively. Inhibition of albumin denaturation, membrane stabilization and proteinase inhibition assays indicated that flavonoids extract showed anti-inflammatory activity. Phenolic compounds like phenolic acids and flavonoids present in the M. beecheii honey could be partially responsible for their antioxidant and anti-inflammatory activities.

Introduction
Therapeutic use of honey for the treatment of chronic wounds, diabetic ulcers, eye and gastric ailments has been documented. This beneficial role of honey was attributed to its antibacterial activity. However, since some of these diseases have been recognized as being a consequence of free radical damage, it seems that part of the therapeutic role of honey is due to its antioxidant activity. The role of free radicals and reactive oxygen species has been emphasized in a number of diseases, including cancer, cardiovascular disease, cataracts, macular degeneration, impaired wound healing, gastrointestinal inflammatory diseases and other inflammatory processes (Aljadi & Kamaruddin, 2004).

Inflammation is a complex process, which is frequently associated with pain and involves occurrences such as: the increase of vascular permeability, increase of protein denaturation and membrane alteration. When tissue cells become injured they release...
kinins, prostraglandins and histamine. These substances act as chemical messengers that attract some of the body’s natural defense cells, a mechanism known as chemotaxis. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process (Leelaprakash & Mohan-Dass, 2011).

Among the compounds with biological activity that are present in honey, the compounds that display antioxidant capacity, such as phenolic acids and flavonoids, have received special attention from research groups, due to their role in the prevention of diseases associated with oxidative stress (Álvarez-Suarez et al., 2012; Arunachalam, Parimalazhagan, & Saravanan, 2011). Phenolic compounds are the major functional components of honey and may significantly contribute to its biological activity (Hung et al., 2004; McLoone, Warnock, & Fyfe, 2016). One of the potential benefits of phenolic compounds is to stabilize cell membranes by reducing lipid peroxidation and scavenging free radicals (Chaudhuri, Banerjee, Basu, Sengupta, & Sengupta, 2007). Data from in vitro experiments have recently revealed that the antioxidant properties of phenolic compounds could lie in their localization in lipoprotein domains and cell membranes, which generally serve as targets for lipid peroxidation, suggesting a protective interaction with lipid bilayers.

The results of diverse studies (Kuçuk et al., 2007; Lee, Eun Jeong Choi, Park, & Kim, 2014; Saxena, Gautam, & Sharma, 2010) demonstrated that by combining different antioxidant tests and determining the levels of total phenols it is possible to characterize the antioxidant potential of honey and understanding of its biological properties and possible therapeutic applications. In this sense, Duarte et al. (2012) correlated the content of phenols and flavonoids with the antioxidant activity of honey from Africanized and stingless bees in Alagoas (Brazil). On the other hand, Borsato et al. (2014) evaluated the topical anti-inflammatory activity of a metahnolic extract from honey of Melipona marginata.

In this research, an effort is made to corroborate the specific mechanics involved in the antioxidant and anti-inflammatory activities of phenolic compounds of honey from stingless bees. These compounds were extracted from Melipona beecheii honey and fractionated in phenolic acids and flavonoids using RP-HPLC, their biological activities were evaluated using different in vitro specific assays.

**Materials and methods**

**Melipona beecheii honey sample**

Samples of M. beecheii honey were collected directly from beehives in April 2016 at Mani, which is in the state of Yucatan in Mexico. The collection was performed with 20 mL sterile disposable syringes, and was transferred to storage polyethylene bottles (500 mL), which were stored at 8°C until analysis.

**Honey extraction for phenolic analysis**

Methanolic extract of honey was used for phenolic and bioactivity analyses. 7.5 g honey was placed in a falcon tube (50 mL) and 25 mL 99% methanol was added. The mixture was continuously stirred with a shaker at room temperature for 24 h. Particles were
removed with filter paper. The final volume (25 mL) of the solution was adjusted with methanol. The methanolic extract was divided into two parts, the first being used for quantitative analysis of total amount of phenolic compounds, flavonoids and flavonols and the second for the fractionation of the methanolic extract by reverse phase HPLC.

Quantitative phenolic analysis

The phenolic content was determined using the Folin–Ciocalteu method, based on the reduction of phosphomolybdate and phosphotungstate complex by phenolics compounds (Georgé, Brat, Alter, & Amiot, 2005). Absorbance was determined at 760 nm. The data were calculated by a comparison between a standard curve of Gallic acid and the absorbance of each sample. The total amount of phenolic compounds was determined in milligrams of Gallic acid equivalents/100 g of honey. The flavonoid content was determined using the aluminum chloride method (Lee, Kim, Lee, & Lee, 2003). Absorbance was measured at 510 nm. The data were calculated by a comparison between a standard curve (0–100 μg/mL of quercetin) and the absorbance of each sample. The total amount of flavonoid compounds was determined in milligrams of quercetin equivalents/100 g of honey. Total flavonols were estimated as mg of naringenin equivalents/100 g of honey by the method of Popova, Silici, Kaftanoglu, and Bankova (2005). The absorbance was determined at 510 nm.

HPLC analysis of honey phenolic compounds profile

Honey polyphenol fractionation was carried out using a liquid chromatograph (Agilent 1100) with binary analytical system. With a diode array detector (DAD), the column used was a C18 Hi-Pore RP-318, 250 mm × 10 mm BIO-RAD, operated at 35°C. The mobile phase consisted of solvent A (water and acetic acid, 99:1) and solvent B (water, acetonitrile and acetic acid, 59:40:1). The gradient program was as follows: from 25% to 100% B in 30 min, and then isocratic by 10 min. The injection volume was 100 μL, and the flow rate was 1 mL/min. Chromatograms for the phenolic acids were recorded at 270 nm and for flavonoids at 360 nm (Lianda, Sant’Ana, Echevarria, & Castro, 2012).

Assessment of in vitro antioxidant activity of methanolic extract and fractions

Radical scavenging assay

The antiradical activity of methanolic extract, fractions or ascorbic acid was evaluated using a dilution series to obtain five concentrations (0.0–3.0 mg/mL). This process involved mixing the 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (60 mM in ethanol) with an appropriate amount of methanolic extract or fractions, followed by homogenization. After 30 min, the remaining DPPH radicals were quantified by measuring the absorption at 517 nm. The percentage of inhibition was given by the formula: percent inhibition (%) = \([A_0 - A_1]/A_0 \times 100\), where \(A_0\) was the absorbance of the control (reactive + distilled water) solution and \(A_1\) was the absorbance in the presence of methanolic extract, fractions or standards. IC\(_{50}\) (concentration providing 50% of radical inhibition)
values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding scavenging effect. Ascorbic acid was used as standard (Silva et al., 2006).

**Chelating effect**

The chelating effect of the honey methanolic extract and its fractions was evaluated using a dilution series to obtain five concentrations (0.0–3.0 mg/mL). Briefly, 1.0 mL of sodium acetate buffer (100 mM, pH 4.9), 100 µL of Cu (II) standard solution (1.0 mg/mL) and 100 µL of methanolic extract, fractions or standard were homogenized in a test tube. The mixture was allowed to react for 5 min at room temperature and 25 µL of a pyrocatechol violet solution (4.0 mmol/L) was then added. Absorbance was determined at 632 nm. Chelating effect was calculated as follows: Chelating effect (%) = (1 − sample absorbance/blank absorbance) × 100. IC\(_{50}\) (concentration providing 50% of chelating effect) values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding chelating effect. Ascorbic acid was used as standard (Saiga, Tanabe, & Nishimura, 2003).

**Reducing power**

The reducing power was evaluated using a dilution series to obtain five concentrations (0.0–3.0 mg/mL). First, 200 µL of methanolic extract, fractions or standard, 500 µL of phosphate buffer (0.2 M, pH 6.6) and 500 µL of potassium ferricyanide (1%) were homogenized in a test tube. The test tube was then incubated at 50°C for 20 min. Subsequently, 500 µL of trichloroacetic acid 10% (w/v) were added; then the test tube was centrifuged at 3000 \(\times\) g for 10 min. An aliquot of 500 µL of the supernatant was dissolved in an equal amount of distilled water, immediately 500 µL of ferric chloride (0.1%) were added. Absorbance was determined at 700 nm. IC\(_{50}\) (concentration providing 50% of reducing power) values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding reducing power. Ascorbic acid was used as standard (Yen & Chen, 1995).

**Assessment of in vitro anti-inflammatory activity of methanolic extract and fractions**

**Inhibition of albumin thermal denaturation**

The inhibition of albumin thermal denaturation was evaluated using a dilution series of methanolic extract, fractions or standard to obtain five concentrations (0.0–3.0 mg/mL). The reaction mixture consisted of methanolic extract, fractions or standard at different concentrations and 1% aqueous solution of ovoalbumin. The pH of the reaction was adjusted to 7.0 using small amount of 1.0 mol/L HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 20 min. After cooling the samples, the turbidity was measured spectrophotometrically at 660 nm. The experiment was performed in triplicate. Percent inhibition of protein denaturation was calculated as follows: Percentage inhibition of albumin thermal denaturation = (absorbance blank − absorbance
sample) \times 100/\text{absorbance blank}. The \text{IC}_{50} (concentration providing 50\% of inhibition of albumin thermal denaturation) values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding inhibition of albumin thermal denaturation. Ascorbic acid was used as control. Acetylsalicylic acid was used as standard (Mizushima & Kobayashi, 1968).

**Membrane stabilization test**

Fresh whole human blood (10 mL) was collected and transferred to the heparinized centrifuged tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed three times with equal volume of isosaline solution (the same concentration of salts as blood plasma). The volume of the blood was measured and reconstituted as a 10\% v/v suspension with isosaline solution (Sadique, Al-Rqobahs, Bughaith, & Gindi, 1989). The inhibition of membrane stabilization was evaluated using dilution series of methanolic extract, fractions or standard to obtain five concentrations (0.0–3.0 mg/mL). The reaction mixture (700 µL) consisted of 350 µL of methanolic extract, fractions or standard and 350 µL of 10\% red blood cells suspension; instead of sample only an isosaline solution was added to the blank test tube. All the centrifuge tubes containing the reaction mixture were incubated in a water bath at 56°C for 30 min. After incubation, the tubes were cooled and the reaction mixture was centrifuged at 2500 rpm for 5 min and the absorbance of the supernatant was determined at 560 nm. The experiment was performed in triplicate. Percent membrane stabilization activity was calculated by the next formula: Membrane stability = (absorbance blank – absorbance sample) \times 100/\text{absorbance blank}. The \text{IC}_{50} (concentration providing 50\% of membrane stabilization) values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding membrane stabilization effect. Acetylsalicylic acid was used as standard (Shinde et al., 1999).

**Proteinase inhibitory action**

The inhibition of proteinase inhibitory action was evaluated using dilution series of methanolic extract, fractions or standard to obtain five concentrations (0.0–3.0 mg/mL). The reaction mixture (350 µL) contained 0.06 mg of alcalase, 20 mmol/L of Tris HCl buffer (pH 7.4) and 350 µL of methanolic extract, fractions or standard at different concentrations. The mixture was incubated at 37°C for 5 min and then 350 µL of 0.8\% (w/v) casein was added. The mixture was incubated for an additional 20 min. 700 µL of 10\% trichloroacetic acid was added to terminate the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant (protein hydrolyzed) was determined at 210 nm against the buffer as a blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated according to the formula: Percentage proteinase inhibitory action = (absorbance blank – absorbance sample) \times 100/\text{absorbance blank}. The \text{IC}_{50} (concentration providing 50\% of proteinase inhibitory action) values were calculated using the dose inhibition curve in linear range by plotting the sample concentration versus the corresponding proteinase inhibitory action. The concentration used ranged from 0 to 3 mg/mL. Acetylsalicylic acid was used as standard (Oyedepo & Femurewa, 1995).
**Statistical analysis**

All results were analyzed using central tendency and dispersion measures. One-way ANOVAs and LSD multiple range were run to evaluate biological activities of methanolic extract and fractions. All analyses were processed with the Statgraphics Plus version 5.1 software.

**Results and discussion**

*Phenolic extraction and quantitative phenolic analysis*

Phenolic compounds are known to possess the ability to reduce oxidative damage acting as antioxidants. They can trap the free radicals directly or scavenge them through a series of coupled reactions with antioxidant enzymes (Sakat, Juvekar, & Gambhire, 2010). Some studies associated the antioxidant capacity of phenolic compounds with an important role in stabilizing lipid peroxidation (Álvarez-Suarez et al., 2012). Methanolic extract of *M. beecheii* honey exhibited a total phenolic content of 63.22 mg of GAE/100 g of honey (Table 1). Can et al. (2015) investigated the total phenolic content of Turkish honeys, the values of which varied widely, between 16.02 and 120.04 mg GAE/100 g. According to this authors monofloral honeys have higher phenolic content than multifloral and honeydew honeys. Most of honeys range between 30 and 60 mg GAE/100 g. In this study, the total phenolic content of *M. beecheii* honey is intermediate to those reported by Can et al. (2015).

Flavonoids and flavonols are an important sub-branch of the polyphenol family, synthesized by plants. In this study, total flavonoids and total flavonols were found to be 3.61 and 3.16 mg of Catechin and Naringenin equivalents/100 g of honey, respectively. Flavonoids and flavonols represented 5.71% and 4.99% of the total phenolic content. Total phenolic content is extensively used as an antioxidant test; however, results of presents study suggested that could be used as marker of honey’s antioxidant capacity.

*Fractionation of methanolic extract by RP-HPLC*

Based on the chromatogram obtained for the methanolic extract, it was possible to delimitate the chromatogram regions where the peaks related to the phenolic acids and flavonoids appear. The peaks associated with retention times of between 0 and 18 min correspond to compounds of greater polarity, since the eluent used during the beginning of HPLC fractionation has a polar nature (Table 2). As the time of the chromatographic

| Table 1. Phytochemical and antioxidative components of methanolic extract of *M. beecheii* honey. |
|---------------------------------------------------------------|
| Parameters | Compounds extracted from *M. beecheii* honey |
| Extractive yield | 10.01% |
| Total phenol content | 63.22a |
| Total flavonoids content | 3.61b |
| Total flavonols content | 3.16c |

*mg of Gallic acid equivalent/100 g of honey.
*mg of Catechin equivalent/100 g of honey.
*mg of Naringenin equivalent/100 g of honey. Values represent in the results are mean of three replicates.
run increases the solvent polarity decreases and therefore, the peaks that appear correspond to less polar molecules, with retention times of between 20 and 35 min.

The HPLC gradient began with 25% water–acetonitrile–acetic acid, 59:40:1 and this percentage increased over the 30 min run time, reaching 100%. Considering this solvent gradient, the polar compounds were released from the column after shorter retention times (Table 2). According to Ferreres et al. (1994), with this method, peaks that appear between retention times of 20 and 45 min are probably the peaks related to flavonoids. Figure 1 illustrates the chromatogram regions and corresponding polarities for the methanolic extract of the *M. beecheii* honey sample used in this study.

These components could be derived from phenolic acids at 290 nm or flavonoids at 340 nm. In this sense peaks 1–11 were grouped and designed as phenolic fraction (PF), peaks 12–16 were grouped and designed as flavonoid fraction (FF). The antioxidant and anti-inflammatory activities of the methanolic extract, phenolic fraction and flavonoid fraction were determined using in vitro assays.

### Table 2. Retention time, height and area of phenolic compounds of *M. beecheii* honey obtained by reverse phase high performance liquid chromatography.

| Peak | \(t_R\) (min) | Peak height (mUA) | Peak area (%) |
|------|--------------|------------------|--------------|
| Polar phenolic compounds (phenolic acids, 270 nm) | | | |
| 1 | 2.156 | 25.0894 | 10.7337 |
| 2 | 2.771 | 12.4679 | 6.6411 |
| 3 | 2.942 | 22.2936 | 13.9667 |
| 4 | 3.392 | 3.0951 | 1.7149 |
| 5 | 3.773 | 3.9841 | 4.1921 |
| 6 | 4.221 | 4.1052 | 3.6952 |
| 7 | 5.017 | 27.8478 | 33.9559 |
| 8 | 8.364 | 1.9477 | 2.9224 |
| 9 | 9.536 | 2.9842 | 5.202 |
| 10 | 10.82 | 0.7780 | 1.2162 |
| 11 | 18.469 | 0.5290 | 1.3187 |
| Non-polar phenolic compounds (flavonoids, 360 nm) | | | |
| 12 | 21.762 | 0.9620 | 23.9273 |
| 13 | 24.475 | 0.1610 | 3.7919 |
| 14 | 26.222 | 0.1370 | 2.7485 |
| 15 | 29.589 | 0.5940 | 17.6874 |
| 16 | 35.170 | 0.0455 | 0.7322 |

Note: \(t_R\), retention time.

**Assessment of in vitro antioxidant activity of methanolic extract and fractions**

**Radical scavenging assay**

Many methods have been proposed to evaluate the antioxidant activity, or capacity, of phenolic compounds and phenolic-rich extracts. These methods have been classified according to the mechanism of radical deactivation, according to the physiological relevance of the free radical, or according to the competitive or direct approach of the reaction (Prior, Wu, & Schaich, 2005). Radical scavenging assay is widely used for screening antioxidant activity, since it can accommodate many samples in a short period and detect bioactive extracts or compounds at low concentration (Piao et al., 2004). The decrease in absorbance of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical caused by antioxidant was due to the scavenging of the radical by hydrogen donation. Positive control (ascorbic
acid), methanol extract and both fractions showed scavenging activity in a concentration-dependent manner (Table 3).

The methanolic extract exhibited the lowest radical scavenging activity with an IC$_{50}$ value that required a concentration of 3.7783 mg/mL. Both fractions exhibited radical scavenging activity with FF with the highest value (Table 3). Flavonoid antioxidants function as scavengers of free radicals by rapid donation of a hydrogen atom to radicals. In general, the radical-scavenging activity of flavonoids depends on the molecular structure

**Figure 1.** Chromatograms of methanolic extract of *M. beecheii* honey obtained by reverse phase high performance liquid chromatography.

| Sample | IC$_{50}$ values (mg/mL) | Radical scavenging assay | Chelating effect | Reducing power |
|--------|---------------------------|--------------------------|-----------------|----------------|
| ME     | 3.7783$^d$                | 2.9814$^d$               | 2.6277$^e$      |
| PF     | 0.5243$^c$                | 0.6240$^b$               | 0.7067$^d$      |
| FF     | 0.3381$^{1b}$             | 0.9216$^c$               | 0.3697$^b$      |
| AA     | 0.0502$^a$                | 0.0541$^a$               | 0.0584$^a$      |

Notes: Each value represents the mean of three determinations. Experimental group were compared with control. Different superscripts indicate statistical significance. ME = methanolic extract; PF, phenolic acid fraction 1; FF, flavonoid fraction, AA, ascorbic acid.
and the substitution pattern of hydroxyl groups, that is, on the availability of phenolic hydrogens and on the possibility of stabilization of the resulting phenoxy radicals via hydrogen bonding or by expanded electron delocalization (Amic, Davidovic-Amic, Beslo, & Trinajstic, 2003).

**Chelating effect**

Test sample chelating activity is significant in deciding the antioxidant status of an extract or compound because it reduces the concentration of metal ions catalyzing lipid peroxidation (Mohan, Balamurugan, Salini, & Rekha, 2012). Chelating effect exhibited by methanolic extract of *M. beecheii* honey and its fractions showed a concentration-dependent manner, comparable with the control (Table 3). The methanolic extract exhibited the lowest chelating effect with an IC$_{50}$ value that required a concentration of 2.98 mg/mL. Phenolic acid fraction and flavonoid fraction exhibited higher activity than methanolic extract, with IC$_{50}$ values that required 4.7 and 3.2 times less concentration, respectively. Catechol and gallol and the many functionalized derivatives, including most polyphenol compounds, are effective chelators. Metal ions that exhibited octahedral geometry, such as Cu$^{2+}$ and Cu$^{3+}$, can coordinate up to three catecholate or gallate groups. Because of this, it might be expected that polyphenols with catechol or gallol groups would always bind iron in a 3:1 fashion (Figure 2).

However, since polyphenol compounds are so structurally varied and the complexes formed are pH dependent, they often exhibit variable coordination modes. Despite pK$_a$ values in the range of 7–9 for the most acidic phenolic hydrogen, polyphenols are easily deprotonated at or below physiological pH in the presence of iron and form very stable complexes (Mohan et al., 2012). This would explain the behavior observed in the present study, with the fraction of phenolic acids having the highest chelating capacity.

**Reducing power**

Reduced power was measured in methanolic extracts and its fractions by the transformation of Fe (III) to Fe (II). In this assay, an increase in absorbance indicates an increase in reduction of Fe (III) to Fe (II). This assay is widely used to evaluate the antioxidant activity of polyphenols. It is associated with the presence of reductons, which shows antioxidant actions by donating a hydrogen atom and so breaking the free radical chain (Saha, 2012).

![Figure 2. Octahedral coordination geometry of general iron–polyphenol complexes. Gallols, R = OH; catechols, R = H. Coordination requires deprotonation of the polyphenol ligands.](image-url)
Rahman, Shahriar, Azad, & Das, 2013). The reduced power of an extract or a compound is related with its electron transfer ability and therefore may serve as a significant indicator of its antioxidant activity. In this study, the methanolic extract and its fractions displayed a positive concentration-dependent antioxidant potential. Ascorbic acid had much higher activity than methanolic extract and its fractions (Table 3). The methanolic extract exhibited the lowest reducing power with an IC$_{50}$ value that required a concentration of 2.62 mg/mL. Both fractions exhibited reducing power with FF with the highest value.

**Assessment of in vitro anti-inflammatory activity of methanolic extract and fractions**

**Inhibition of albumin denaturation**

Protein denaturation is a process in which proteins lose their quaternary, tertiary and secondary structure by application of external stress or compounds. Most biological proteins lose their biological function when denatured (Angel, Vimala, & Nambisan, 2013). Denaturation of proteins in tissues, may be caused by auto-antigens in certain arthritic diseases. So it may be said that tissue protein denaturation is a marker for inflammatory and arthritic diseases (Angel et al., 2013). In this study, as part of the investigation on the mechanism of the anti-inflammation activity, ability of methanolic extract of *M. beecheii* honey and its fractions to inhibit protein denaturation was determined. Flavonoid fraction was effective in inhibiting heat-induced albumin denaturation. Inhibition of 50% was observed at 0.11 mg/mL. Acetylsalicylic acid (a standard anti-inflammation drug) showed an inhibition of 50% at a concentration of 0.05 mg/mL. This indicates that its anti-inflammatory activity is greater compared to the extract and its fractions (Table 4).

IC$_{50}$ values indicate that the control (acetylsalicylic acid) is approximately 46 times more potent than the methanolic extract and 2.3 times more potent than flavonoid fraction, respectively. With respect to the methanolic extract flavonoid fraction is showing to be 95.89% more potent. Hence, it is possible that if flavonoid fraction is purified, the activity will increase significantly.

**Membrane stabilization test**

Membrane stabilization has been used as a method to study the in vitro anti-inflammatory activity because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may well stabilize lysosomal membranes.

### Table 4. Effect of phenolic extract and fractions of *M. beecheii* honey on albumin denaturation, membrane stabilization and proteinase inhibition.

| Sample | IC$_{50}$ values (mg/mL) | Albumin denaturation | Membrane stabilization | Proteinase inhibition |
|--------|--------------------------|----------------------|------------------------|----------------------|
| ME     | 2.9019$^d$               | 1.6915$^d$           | 3.4428$^d$             |
| PF     | 0.5810$^c$               | 0.7197$^c$           | 2.5630$^d$             |
| FF     | 0.1192$^{b}$             | 0.4288$^b$           | 1.7955$^b$             |
| AcA    | 0.0505$^a$               | 0.0783$^a$           | 0.0923$^a$             |

Notes: Each value represents the mean of three determinations. Experimental group were compared with control. Different superscripts indicate statistical significance. ME, methanolic extract; PF, phenolic acid fraction 1; FF, flavonoid fraction; AcA, acetylsalicylic acid.
Stabilization of lysosomal is important in limiting the inflammatory response by preventing the release of lysosomal constituents of activated neutrophil, such as bacterial enzymes and proteases, which causes further tissue inflammation and damage upon extra cellular release. The lysosomal enzymes released during inflammation produce various disorders. The extra cellular activity of these enzymes are said to be related to acute or chronic inflammation (Leelaprakash & Mohan-Dass, 2011). The results showed that flavonoid fraction at a concentration of 0.42 mg/mL protect the erythrocyte membrane against lysis induced by heat (Table 4). Acetylsalicylic acid 0.07 mg/mL offered a 50% of protection against the damaging effect of heat. The mode of action of the methanolic extract, its fractions or a standard anti-inflammatory drug could be connected with binding to the erythrocyte membranes with subsequent alteration of the surface charges of the cells. This might have prevented physical interaction with aggregating agents or promote dispersal by mutual repulsion of like charges which are involved in the hemolysis of red blood cells. It has been reported that certain flavonoids exerted profound stabilizing effect on lysosomal membrane both in vivo and in vitro (Oyedapo, Akinpelu, & Orefuwa, 2004).

**Proteinase inhibitory action**

Methanolic extracts and their fractions exhibited proteinase inhibition activity as shown in Table 4. Flavonoid fraction showed an inhibition of 50% at 1.79 mg/mL. Acetylsalicylic acid showed an inhibition of 50% at 0.09 mg/mL. Flavonoids are able to inhibit enzymes with different catalytic activity; this inhibition is related to the biological effect of flavonoids on pathophysiology of different diseases. According to Leelaprakash and Mohan-Dass (2011), neutrophils are known to be a rich source of serine proteinase and are localized at lysosomes. It was previously reported that leukocyte proteinase plays an important role in the development of tissue damage during inflammatory reactions and significant level of protection was provided by proteinase inhibitors (Leelaprakash & Mohan-Dass, 2011), like flavonoids. Prostaglandins and nitric oxide biosynthesis are involved in inflammation, and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these mediators. It has been demonstrated that flavonoids are able to inhibit both enzymes, as well as other mediators of the inflammatory process such as reactive C protein or adhesion molecules. Modulation of the cascade of molecular events leading to the overexpression of those mediators include inhibition of transcription factors such as nuclear factor kappa B and AP-1, through the inhibition of protein kinases involved in signal transduction. Increased antioxidant defenses through activation of the NF-E2-related factor 2 (Nrf2) also contribute to the anti-inflammatory capacity of flavonoids.

**Conclusions**

Honey contains different phytochemical compounds and thus exhibits multiple biological activities. From this study, it can be concluded that phenolic acids and flavonoids present in honey from M. beecheii honey are related to its antioxidant and anti-inflammatory properties. The results of this research corroborate the therapeutic use made of honeys produced by stingless bees, for the treatment of various diseases such as cancer,
neurological disorder, aging and inflammation. Results from in vitro assays suggested that phenolic acids and flavonoids chemical properties play a major role in promoting these bioactivities. Antioxidant and free radical scavenging potential is clearly revealed in DPPH assay, chelating effect of prooxidant cations and reducing power assay. Anti-inflammatory potential was depicted clearly in protein denaturation inhibition, membrane stabilization and protease inhibition assay. Further studies can be performed for the isolation and identification of individual phenolic compounds of the fractions. In vivo studies can also be applied to understand the mechanisms of antioxidant and anti-inflammation properties exerted by honey from *M. beecheii*.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Notes on contributors**

*Jorge Carlos Ruiz-Ruiz*, research-professor at Instituto Tecnológico de Mérida in Mexico.

*Angel Jesus Matus-Basto*, masters student at Instituto Tecnológico de Mérida in Mexico.

*Pablo Acereto-Escoffié*, professor at Universidad Autónoma de Yucatán in Mexico.

*Maira Rubi Segura-Campos*, research-professor at Universidad Autónoma de Yucatán in Mexico.

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