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Chemical composition, botanical evaluation and screening of radical scavenging activity of collected pollen by the stingless bees
*Melipona rufiventris* (Uruçu-amarela)

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**ABSTRACT**

Stingless bees in Brazil are indigenous and found all over the country. Bee pollen is used for its nutritional value in the human diet. It is made up of natural flower pollen mixed with nectar and bee secretions. In order to evaluate the chemical composition, free radical scavenging activity, and botanical origin, sample of pollen loads from stingless bee, *Melipona rufiventris* (Uruçu amarela) was studied. The EtOAc extract of pollen of *Melipona rufiventris* yielded the following compounds: p-hydroxycinnamic acid, dihydroquercetin, isorhamnetin, isorhamnetin-3-O-(6″-O-E-p-coumaroyl)-β-D-glucopyranoside, luteolin, and quercetin. This is the first report of the isolation of isorhamnetin-3-O-(6″-O-E-p-coumaroyl)-β-D-glucopyranoside from pollen. The free radical-scavenging activities of different solvent extracts of pollen were determined using DPPH assay. This activity decreases in the order: EtOAc > EtOH > Hexane extract. It appears that the EtOAc extract of the pollen is a good scavenger of active oxygen species. The botanical evaluation of pollen loads showed the composition by two pollen types, with the dominant type (97.3%) being *Scoparia dulcis* (L.) (Scrophulariaceae) and the minor one *Senna obtusifolia* (L.) Irwin & Barneby (Fabaceae). This suggests a specific foraging behavior in *Melipona rufiventris* bees, even in an environment with such a rich botanical diversity as the Northeastern Brazil.

**Key words:** antioxidant activity, bee pollen, flavonoids, *Melipona rufiventris*, Uruçu amarela.

**INTRODUCTION**

The Meliponin is a bee group of more than 300 species encountered around the world. They are characterized as having social and bearing an atrophied and non-functional sting, which justifies their popular name of stingless bees (Roubik 1989). Stingless bees are found in tropical and sub-tropical regions of South and Central Brazil, they are indigenous and present all over the country, although species differ from region to region (Nogueira-Neto 1997, Lima-Verde and Freitas 2002).

Besides their importance as major pollinators of most wild plants and some cultivated species, honey and bee pollen of meliponins are also source of food and medicines (geopropolis) and income to rural populations. There is a strong culture of bee keeping and using their products in Brazil and other parts of the world where these bees are native (Freitas 1999).
One of their products, bee pollen, is used for its nutritional value in the human diet. It is made up of natural flower pollen mixed with nectar and bee secretions, and is rich in sugars, proteins, lipids, vitamins and flavonoids (3–5% dry weight) (Tomas-Lorente et al. 1992). Although bee pollen found in the market is harvested from honey bee (Apis mellifera) colonies, many other social bee species also collect and use pollen, such as the stingless bees (Nogueira-Neto 1997).

During ancient times, people throughout the world commonly used pollen, for the goodness and medicinal properties. Some of the reasons why ancient people used bee-pollen are the same for its use today. To date, no scientific evidence has been cited to disprove the claimed properties of bee-pollen (Campos et al. 1997). In recent years, the physiological functionality of natural foods has received much attention, due to increasing interest in human health. Among natural products, honey bee-derived apicultural products such as pollen and propolis have been applied for centuries in traditional medicine as well as in food diets and supplementary nutrition (Kroyer and Hegedus 2002). Much work has been conducted on the chemistry and properties of propolis and pollen. Hundreds of chemical compounds have been identified from them. In general, compared to many standard human foods, pollens are rich in protein, low in fat and possess a wealth of minerals and vitamins (Campos et al. 1996).

Reactive oxygen species (ROS) are produced in all mammalian cells as the result of normal cellular metabolism and due to the activation of oxidant-producing enzymes in response to exogenous stimuli. The balance between ROS production and antioxidant defenses determines the degree of oxidative stress. Generation of ROS has been associated with cell signaling, stress responses, cell proliferation, aging, and cancer development. The ability of ROS to induce cellular damage and to cause cell death opens the possibility to utilize this property in the treatment of cancer through a free radical mediated mechanism (Villamor et al. 2004). It has been reported that both pollen and propolis extracts and their respective isolated compounds have free radical scavenging activity (Campos et al. 2003, Kumazawa et al. 2004).

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The major aim of the present study is to determine the antiradical activity of the crude residue and its fractions derived from pollen loads collected and processed by stingless bee Melipona rufiventris. In a previous work the chemical composition of the closely related solitary bee species Melipona subnitida Ducke was studied (Silva et al. 2006). Both species, among others, are endangered by extinction, mostly because their native environments are being destructed, and is well documented the sharp foraging range of these species (Gathmann and Tscharntke 2002).

MATERIALS AND METHODS

SAMPLE

Pollen loads of Melipona rufiventris (67.6 g) were extracted with EtOH in an ultrasound bath and the extract filtered and concentrated under reduced pressure. The crude residue was dissolved in MeOH:H2O (7:3) and fractioned with n-hexane and then with EtOAc. These solvents were evaporated to dryness. The EtOAc fraction (6.1 g) was subsequently submitted to column chromatography using Sephadex® LH-20 and MeOH as elution system. The collected flavonoid fractions were analyzed by commercial TLC aluminum sheets (Merck silica gel 60 F254), with the spot visualization done with spraying diphenylboryloxyethylamine (NP) solution in MeOH and observing under UV light (366 nm). The EtOAc extract of the pollen of Melipona rufiventris yielded the phenolics, 1 (1.1 mg), 2 (10.0 mg), 3 (28.0 mg), 4 (15.0 mg), 5 (5.0 mg), and 6 (10.0 mg).

FREE RADICAL-SCAVENGER ACTIVITY

The free radical-scavenger activity was determined by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, as described previously (Silva et al. 2006). The antiradical activity of the EtOH extract, hexane and EtOAc fractions...
mixing of 2 mL of DPPH solution (100µM in ethanol) with an appropriate amount of extract or compound, followed by homogenization. After 30 min, quantity of the remaining DPPH radicals was recorded from absorption at λ 517 nm. Three analytical replicates (n = 3) were carried out on each extract for antiradical activity determinations. Measurements were averaged, and the results are given as mean ± standard deviation (S.D.). Antiradical efficiency was established using regression analysis at a 95% significance level (P < 0.05). Results are presented in EC_{50} values, which represent the concentration of sample required to scavenge 50% of the DPPH radicals available (Table I). The reference standard were ascorbic acid (EC_{50} = 2.48 ± 0.03µg/mL) and butylated hydroxyl toluene BHT (4.47 ± 0.08µg/mL).

**RESULTS AND DISCUSSION**

**IDENTIFICATION OF THE COMPOUNDS**

The NMR spectra were used to identify the structures of the phenolics isolated from pollen loads of *Melipona rufiventris* as *p*-hydroxycinnamic acid (1), dihydroquercetin (2), isorhamnetin (3), isorhamnetin-3-*O*-(6-*E*-p-coumaroyl)-β-D-glucopyranoside (4), luteolin (5), and quercetin (6), Figure 1. Their spectral data, particularly 13C NMR, were in close agreement with literature values, for dihydroquercetin, isorhamnetin, luteolin and quercetin (Agrawal 1989, Markham and Chari 1982), and for isorhamnetin-3-*O*-(6-*E*-p-coumaroyl)-β-D-glucopyranoside (4) (Romussi et al. 1988). Table I showed the 1H and 13C-NMR data for compound 4.

Flavonoids are widely recognized as chemotaxonomic markers in plants in general (Emereciano et al. 2001, Harborne and Turner 1984). Recently, this evidence was obtained for pollen flavonoids (Campos et al. 1997) and has been recognized previously as potentially useful taxonomic markers (Meurer 1988, Tomas-Lorente 1992). In addition, pollen flavonoids are arguably quality factors in terms of their phytotherapeutic value as antioxidants and radical scavengers (Pietta 2000, Cook and Samman 1996), and their involvement as essential components in pollen germination and pollen tube grown (Ylstra et al. 1992).

With the exception of the acylated flavonoid, isolated (45% < X > 3%) and isolated (< 3%) (Maurizio and Louveaux 1965).

**BOTANICAL ORIGIN OF THE POLLEN LOADS**

Samples of pollen loads (2.0 g) were hydrated in water in order to free pollen grains from the loads mass. After being completely homogenized, they were washed, centrifuged and mounted in glycerin jelly according to the method of Maurizio and Louveaux (1965). Five slides with glycerin jelly were mounted from each sample. Pollen grains were counted (500 per slide, at least) to establish the identity and frequency of the pollen types. Pollen types were identified by comparison with slides from the palynotheca of the Bee Laboratory (Universidade Federal do Ceará, Brazil). Pollen types were classified according to their frequency in the sample into dominant (> 45%), accessory (45% < X < 3%) and isolated (< 3%) (Maurizio and Louveaux 1965).

Fig. 1 – Compounds isolated from the pollen loads of stingless bee *Melipona rufiventris*.
<table>
<thead>
<tr>
<th>No.</th>
<th>C</th>
<th>H (J Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>155.98</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>132.91</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>177.17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>162.50</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>99.18</td>
<td>6.12 (s)</td>
</tr>
<tr>
<td>6</td>
<td>165.62</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>93.83</td>
<td>6.34 (s)</td>
</tr>
<tr>
<td>8</td>
<td>103.44</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>120.93</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>113.26</td>
<td>7.86 (d, 2.0)</td>
</tr>
<tr>
<td>1'</td>
<td>120.93</td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>149.64</td>
<td></td>
</tr>
<tr>
<td>3'</td>
<td>146.92</td>
<td></td>
</tr>
<tr>
<td>4'</td>
<td>55.71</td>
<td>3.83 (s)</td>
</tr>
<tr>
<td>5'</td>
<td>115.26</td>
<td>6.87 (d, 7.6)</td>
</tr>
<tr>
<td>6'</td>
<td>122.27</td>
<td>7.50 (d, 8.2)</td>
</tr>
<tr>
<td>OCH₃</td>
<td>74.28</td>
<td>3.26 (m)</td>
</tr>
<tr>
<td>1''</td>
<td>76.25</td>
<td>3.28 (m)</td>
</tr>
<tr>
<td>2''</td>
<td>70.07</td>
<td>3.18 (m)</td>
</tr>
<tr>
<td>3''</td>
<td>74.28</td>
<td>3.41 (m)</td>
</tr>
<tr>
<td>4''</td>
<td>62.99</td>
<td>4.27 (m)</td>
</tr>
<tr>
<td>5''</td>
<td>124.85</td>
<td></td>
</tr>
<tr>
<td>6''</td>
<td>130.22</td>
<td>7.35 (d, 8.0)</td>
</tr>
<tr>
<td>1'''</td>
<td>115.84</td>
<td>6.77 (d, 8.4)</td>
</tr>
<tr>
<td>2'''</td>
<td>161.13</td>
<td></td>
</tr>
<tr>
<td>3'''</td>
<td>130.22</td>
<td>7.35 (d, 8.0)</td>
</tr>
<tr>
<td>4'''</td>
<td>144.78</td>
<td>7.30 (d, 16.2)</td>
</tr>
<tr>
<td>5'''</td>
<td>113.46</td>
<td>6.03 (d, 16.0)</td>
</tr>
<tr>
<td>6'''</td>
<td>166.27</td>
<td></td>
</tr>
</tbody>
</table>

This is however the first report of isorhamnetin-3-O- (6''-O-E-p-coumaroyl) -β-D-glucopyranoside (4) in bee pollen.

Antiradical activity of EtOH extract, and hexane and EtOAc fractions of bee pollen according to the DPPH radical-scavenging method.

The free radical scavenging activities of different solvent extracts of pollen loads of *Melipona rufiventris* were determined using the DPPH assay. The results, expressed as EC₅₀ values, were calculated by regression analysis as present in Table I (the correlation coefficients of the regression curves are also present in the Table). The EtOAc fraction showed potent free radical-scavenging activity on the DPPH radical compared to EtOH extract. The hexane extract was inactive (EC₅₀ > 500μg/mL).

The polyphenolics present in the EtOAc extract could be responsible for the activity. Quercetin, luteolin (Nessa et al. 2004), isorhamnetin and dihydroquercetin (Edenharder and Grunhage 2003) are already tested for the antiradical activity.

The complete assignment for compound (4) is showed in Table II and the corresponding chemical structure is shown in Figure 1.

**TABLE II**

<table>
<thead>
<tr>
<th>Solvents extracts of bee pollen</th>
<th>EC₅₀ (μg/mL)±SDa</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtOH</td>
<td>104.1 ± 1.2</td>
<td>0.99</td>
</tr>
<tr>
<td>Hexane</td>
<td>&gt; 500</td>
<td></td>
</tr>
<tr>
<td>EtOAc fraction</td>
<td>15.3 ± 0.4</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Mean ± SD (n = 3); a The concentration sufficient to obtain 50% of a maximum scavenging capacity (procedure described in materials and methods). EC₅₀ values were calculated from regression curves, r represents the correlation coefficient.

**BOTANICAL ORIGIN**

Bee pollen studied here, collected directly from hives, consisted of a mixture of pollen types of different species. This profile of flavonoid distribution has been shown also in other stingless bee-pollens as *Melipona subnitida* (Silva et al. 2006), and *Scaptotrigona bipunctata* (Lins et al. 2003).
Mixture of different pollen types in one pollen bee is not common in social bees. *Apis mellifera*, a polylectic species, is known for its high fidelity to individual sources of pollen (Roubik 1989). Stingless bees are less generalist than *A. mellifera* in their foraging behavior (Nogueira-Neto 1997). Not surprisingly, only two pollen types were present in the pollen loads studied, suggesting that *Senna obtusifolia* contributed with little pollen and *Scoparia dulcis* was the major source of pollen to *Melipona rufiventris* in the observed period, even though the colonies were placed in an environment with a rich and varied flora, as in Brazilian Semi-Arid Northeast. This fact corroborates the foraging specificity to *Melipona rufiventris*.

CONCLUSION

In pollen loads of *Melipona rufiventris* the EtOAc mainly comprised of *p*-hydroxycinnamic acid, dihydroquercetin,isorhamnetin,isorhammetinisorhammetin-3-O-(6"-O-E-p-coumaroyl)-β-D-glucopyranoside, luteolin, and quercetin. To the best of our knowledge, this is the first report of the isolation of isorhamnetinisorhamnetin-3-O-(6"-O-E-p-coumaroyl)-β-D-glucopyranoside from pollen. The botanical evaluation of bee pollen showed that it was composed by two pollen types, with the dominant type (97.3%) being *Scoparia dulcis* and the isolated one, *Senna obtusifolia*. This result suggests a specific foraging behavior in *Melipona rufiventris* bees, even in an environment as rich in diversity as the Northeastern Brazilian flora. The free radical scavenging effectiveness of the extracts showed that EtOAc extract was the most active. This is the first study of pollen loads from stingless bee *Melipona rufiventris*, a native species of Northeastern Brazil. The results of these trials will be helpful for the commercial production of the stingless bee-pollen for pharmaceutical or nutritional use. Other bioactivity determinations are now being carried out in order to give us more information about the potentiality of these pollens. It suggests that the extracts of the pollen are good scavengers of active oxygen species. This property could be useful in the preparation of the manuscript.

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RESUMO

As abelhas sem ferrão são espécies indígenas encontradas todo o Brasil. Seu pólen é utilizado devido ao seu valor nutricional na dieta humana. É produzido a partir de pólen misturado com néctar e líquidos secretados pelas abelhas, sendo avaliar a composição química, a atividade sequestradora de radicais livres e a origem botânica foi estudado no coletado pela abelha sem ferrão *Melipona rufiventris* (amarela). Do extrato acetato de etila foram isolados os compostos: ácido *p*-hidroxicinnâmico, dihidroquercetina, isorhamnetina, 3-O- (6"-O-E-p-coumaro)-β-D-glicopiranossidoisoflavona, luteolina e quercetina. Esta é a primeira vez que a *3-O- (6"-O-E-p-coumaro)-β-D-glicopiranossidoisoflavona* da lada de pólen apical. A atividade sequestradora de radicais livres de vários extratos com solventes diferentes foi avaliada pelo teste com DPPH (difenilpicril-hidrazida). A avaliação botânica palínica mostrou que o pólen era composto de dois tipos, um majoritário (97.3%) proveniente de *Scoparia dulcis* (L.) (Scrophulariaceae) e outro minoritário proveniente de *Senna obtusifolia* (Fabaceae). Estes resultados sugerem o cultivo de forragem bastante específico exibido pela *Melipona rufiventris*, mesmo em um ambiente tão rico em diversidade vegetal como o Nordeste do Brasil.


