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Indonesian propolis: chemical composition, biological activity and botanical origin

Boryana Trusheva[a]*, Milena Popova[a], Eko Budi Koendhori[b], Iva Tsvetkova[c], Christo Naydenski[c] and Vassya Bankova[a]

[a]*Institute of Organic Chemistry with the Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria;* [b]*Department of Microbiology, School of Medicine, Airlangga University, Surabaya, Indonesia;* [c]*Institute of Microbiology, Bulgarian Academy of Sciences, Sofia, Bulgaria*

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From a biologically active extract of Indonesian propolis from East Java, 11 compounds were isolated and identified: four alk(en)ylresorcinols (obtained as an inseparable mixture) (**1**–**4**) were isolated for the first time from propolis, along with four prenylflavanones (**6**–**9**) and three cycloartane-type triterpenes (**5**, **10** and **11**). The structures of the components were elucidated based on their spectral properties. All prenylflavanones demonstrated significant radical scavenging activity against diphenylpicrylhydrazyl radicals, and compound **6** showed significant antibacterial activity against *Staphylococcus aureus*. For the first time *Macaranga tanarius* L. and *Mangifera indica* L. are shown as plant sources of Indonesian propolis.

Keywords: propolis; alk(en)ylresorcinols; prenylflavanones; cycloartane triterpenes; *Macaranga tanarius*; *Mangifera indica*

1. Introduction

Propolis (bee glue) is a sticky, dark-coloured material that honeybees collect from living plants. They mix it with wax and use it in the construction and adaptation of their nests, mainly to fill out cracks in the beehive and for defence against pathogenic microorganisms. It has been reported that bee glue possesses valuable biological activities such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory and anticancer activities, mainly due to its aromatic acids, phenolic compounds and flavonoids (Awale et al., 2008; Gekker, Hu, Spivak, Lokensgard, & Peterson, 2005; Kujumgiev et al., 1999; Lustosa, Galindo, Nunes, Randau, & Rolim Neto, 2008; Scazzocchio, D'Auria, Alessandrini, & Pantanella, 2006). Owing to its valuable qualities, propolis is extensively used in complementary and alternative medicine, in food and beverages to improve health, and to prevent diseases such as inflammation, heart disease, diabetes and cancer (Bankova, de Castro, & Marcucci, 2000).

*Corresponding author. Email: bobi_tru@orgchm.bas.bg

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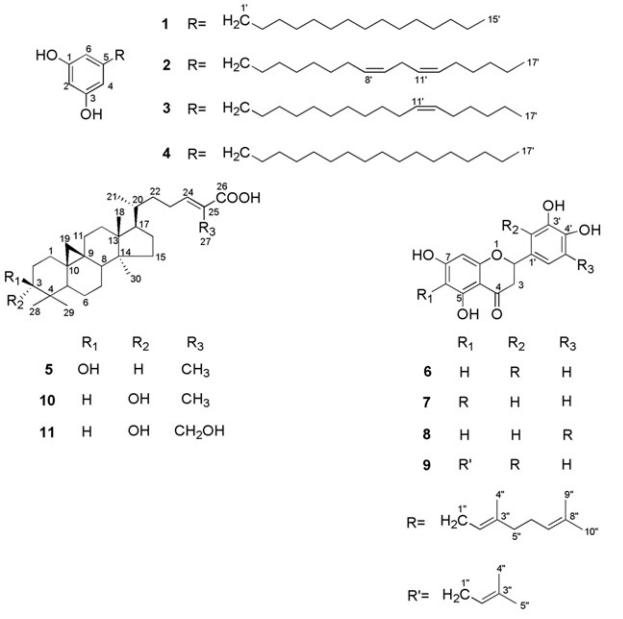
A single propolis sample can contain more than 300 components, as the plant source and respectively the chemistry of the bee glue depend on the specificity of the local flora at the site of collection, which differs at different geographic locations (Bankova, Trusheva, & Popova, 2008). This causes problems in the medicinal use and standardisation of propolis, and generates the necessity of more thorough investigations on the chemical composition, plant origin and biological activity of propolis from different regions of the world, especially from places yet to be investigated (Bankova, 2005). For this reason, tropical bee glue, in particular from regions of Southeast Asia, has become the subject of increasing interest, concerning its relatively unknown and highly varied chemistry and biological activity.

Indonesian propolis has received little attention to date and no data have been published on its detailed chemical composition and botanical origin (Syamsudin, Wiryowidagdo, Simanjuntak, & Heffen, 2009). In this article, we report our results on the constituents and plant sources of Indonesian propolis from Java. The antibacterial and radical scavenging activities of extracts and isolated compounds were determined.

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2. Results and discussion

The chloroform extract of the investigated propolis sample was subjected to column chromatography on silica gel, and several fractions were produced. After further purification by repeated column chromatography and PTLC one inseparable mixture, one complex mixture and four individual compounds were obtained (Figure 1). All of them are among the main sample components.



	R_1	R_2	R_3
5	OH	H	CH_3
10	H	OH	CH_3
11	H	OH	CH_2OH

	R_1	R_2	R_3
6	H	R	H
7	R	H	H
8	H	H	R
9	R'	R	H

Figure 1. Components identified in Indonesian propolis.

The inseparable mixture was analysed by 1D and 2D NMR and GC–MS. It contained the following alk(en)ylresorcinols: 5-pentadecylresorcinol (**1**), 5-(8′Z, 11′Z-heptadecadienyl)-resorcinol (**2**), 5-(11′Z-heptadecenyl)-resorcinol (**3**) and 5-heptadecylresorcinol (**4**). 5-(8′Z,11′Z-Heptadecadienyl)-resorcinol (**2**) was the most abundant. Other than the substances mentioned above, the mixture contained some minor C_{15}-, C_{17}- and C_{19}- substituted alk(en)ylresorcinols with different degrees of unsaturation. Because they were present in a very small amounts they remained unidentified. Isolated alk(en)ylresorcinols have recently been found in mango (*Mangifera indica* L.) peel and proved to have antioxidant and very strong cyclooxygenase (COX-1 and COX-2) inhibitory activity (Knoedler, Berardini, Kammerer, Carle, & Schieber, 2007; Knoedler et al., 2008). Compounds **1**–**4** were found for the first time in propolis.

The complex mixture was comprised of prenylflavanones, the flavonoid compounds with geranyl side chains, which were identified by means of GC–MS after silylation and comparison with authentic samples to be propolin C (**7**), propolin F (**8**) and propolin G (**9**). The most abundant among them was propolin C (**7**). One of the isolated pure compounds was also a prenylflavanone: propolin D (**6**). Compounds **6**–**9** were found for the first time in Indonesian propolis, but these constituents are very characteristic of *Macaranga* (pacific)-type propolis (Taiwanese and Okinawan), originating from the resinous surface material on the fruits of the tropical tree *Macaranga tanarius* L. (Chen et al., 2004; Kumazawa et al., 2004, 2008). Prenylated flavanones are known to possess antibacterial, antioxidant and antitumour activities (Shirataki et al., 2001).

Three individual cycloartane-type triterpenes were isolated and identified, based on comparison of their spectral properties with literature data: mangiferolic acid (**5**), isomangiferolic acid (**10**) and 27-hydroxyisomangiferolic acid (**11**). To date, the cycloartane triterpenes have been isolated from Brazilian and Myanmar propolis (Li et al., 2009; Silva et al., 2005).

Compounds **5**, **10** and **11** and alk(en)ylresorcinols **1**–**4** are characteristic components of *M. indica* and prenylflavanones of the fruits of *M. tanarius* (Anjaneyulu et al., 1989; Knoedler et al., 2007, 2008; Kumazawa et al., 2008). Moreover, both *M. indica* and *M. tanarius* are widely distributed in East Java, where the propolis used in this study was collected. Thus, the plant sources of Indonesian propolis from East Java are *M. indica* and *M. tanarius*, which distinguish it from the already known pacific-type propolis (Macaranga type), containing only prenylflavonoids (Popova, C. Chen, P. Chen, Huang, & Bankova, 2009).

The ethanol and chloroform extracts and isolated compounds were tested for their antimicrobial and radical scavenging activities. The results are presented in Table 1. No activity was found against *C. albicans* or *E. coli* (data not shown), similar to most propolis samples of different origin and constituents (Kujumgiev et al., 1999). The chloroform extract possessed good antibacterial activity against *S. aureus*, commensurable to that of the standard, due to the presence of alk(en)ylresorcinols **1**–**4** and mainly to propolin D (**6**), which showed the highest antibacterial activity.

The ethanol and chloroform extracts demonstrated low RSA against DPPH radicals (5.0% and 9.2%, respectively; Table 1), compared to the well-known antioxidant caffeic acid (75.5%), used as a positive control, but the isolated prenylflavanones **6**–**9** showed significant activity (more than 50% inhibition). Thus, the main biologically active components of Indonesian propolis are prenylflavanones.

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Table 1. Antibacterial and RSA of extracts and isolated compounds.

	Antibacterial activity against *S. aureus*	DPPH RSA
Sample	Inhibitory zone (mm)[a]	RSA (%)[a]
Ethanol extract	14 ± 0	5.0 ± 0.4
Chloroform extract	18 ± 1	9.2 ± 0.1
(1) + (2) + (3) + (4)	17 ± 1	2.7 ± 0.1
5	Not tested	2.4 ± 0.1
6	21 ± 1	52.5 ± 1.5
7 + 8 + 9	Not tested	56.7 ± 1.1
11	0	2.4 ± 0.2
Caffeic acid[b]	Not tested	75.5 ± 1.1
Bulgarian propolis extract[b]	20 ± 1	Not tested

Notes: [a]Tests were done in triplicate, values are mean ± standard deviation (SD). [b]Bulgarian propolis extract and caffeic acid are designated only as standards for antibacterial and DPPH RSA, respectively.

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Once again, the results demonstrate the fact that propolis, independently of its plant source and chemical composition, always possesses antimicrobial and antioxidant activity. The presence of biologically active constituents, like the alk(en)ylresorcinols and prenylflavonoids in the propolis from East Java, Indonesia is a confirmation of its potential for application in complementary and alternative medicine.

3. Experimental

3.1. *General experimental procedure*

NMR spectra were recorded on a Bruker AV 600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) in $CDCl_3$. The GC–MS analysis was performed after silylation of the samples with a Hewlett Packard gas chromatograph 5890 Series II Plus linked to a Hewlett Packard 5972 mass spectrometer system, equipped with a 23 m long, 0.25 mm i.d. and 0.5 μm film thickness HP-5 capillary column. The temperature was programmed from 100°C to 310°C at a rate of $5^{\circ}C\,min^{-1}$. Helium was used as a carrier gas, at flow rate $0.7\,mL\,min^{-1}$, split ratio 1 : 80, injector temperature 280°C and ionisation voltage 70 eV. For silylation, about 5 mg of the sample was mixed with 75 μL bis(trimethylsilyl)trifluoroacetamide and 50 μL of dry pyridine, heated at 80°C for 20 min and analysed by GC–MS. Column chromatography was performed on silica gel 60 (Merck, 63–200 μm), normal phase. Analytical TLC was performed on silica gel 60 F_{254} plates (Merck). Preparative TLC (PTLC) was performed on silica gel 60 F_{254} glass plates (Merck, 20 × 20 cm and 0.25 mm). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with vanillin–sulphuric acid in methanol (5 : 95 w/v vanillin : methanol solution, freshly mixed with a 5 : 95 v/v sulphuric acid : methanol solution), followed by heating at 100°C.

3.2. *Propolis sample*

Propolis was produced by *Apis mellifera* subsp. *mellifera* and was collected near Batu City, East Java Province, Indonesia, by scraping from the bee container.

3.3. *Extraction of propolis*

Propolis (1 kg) was cut into small pieces and extracted with 96% ethanol (1 : 10, w/v) at room temperature for 7 days; after filtration the ethanol extract was concentrated under reduced pressure to give a crude extract (152 g, yield 15.2%). The crude ethanol extract (152 g) was dissolved in ethanol–water and extracted successively with chloroform (three times). The chloroform extract was evaporated to give 68.5 g dry residue after evaporation.

3.4. *Isolation of compounds*

The chloroform extract was subjected to column chromatography on silica gel (350 g) with an *n*-hexane/ethyl acetate gradient (1 : 0.05/1 : 1) to produce 27 fractions (I–XXVII).

From fraction VI (95.8 mg), after additional purification by PTLC (silica gel, *n*-hexane/ethyl acetate 1 : 0.16, two-fold development), an inseparable mixture of alk(en)ylresorcinols (32.7 mg) was obtained. This mixture (after silylation) was analysed by GC–MS under the above-mentioned conditions. The following compounds were identified in the mixture: 5-pentadecylresorcinol (**1**) (21%), 5-(8′Z, 11′Z-heptadecadienyl)-resorcinol (**2**) (30%), 5-(11′Z-heptadecenyl)-resorcinol (**3**) (12%) and 5-heptadecylresorcinol (**4**) (12%) (Figure 1).

Fraction XIV (778 mg) was rechromatographed on a silica gel column eluted with *n*-hexane/acetone, gradient (1 : 0.2/1 : 0.8). Fraction XIV.7 (19.5 mg) after additional purification with PTLC (*n*-hexane/acetone 1 : 0.4) yielded mangiferolic acid (**5**) (3β-hydroxycycloart-24-en-26-oic acid; 7.1 mg). From the 11th fraction of this column XIV.11 (87 mg), propolin D (**6**) (23 mg) and a mixture of prenylflavanones were isolated after further separation by PTLC (silica gel, *n*-hexane/acetone 7 : 3). The mixture was analysed by GC–MS after silylation and propolin C (**7**) (42%), propolin F (**8**) (40%) and propolin G (**9**) (17%) were identified.

Fraction XIV.16 (5.2 mg) after further purification by PTLC (silica gel, *n*-hexane/acetone 7 : 3) yielded isomangiferolic acid (**10**) (3α-hydroxycycloart-24-en-26-oic acid; 1.5 mg).

Fraction XXIII (200 mg) was rechromatographed by PTLC, mobile phase chloroform/methanol/water 60 : 15 : 4, to afford 27-hydroxyisomangiferolic acid (**11**) (50 mg).

3.5. *Identification of compounds*

3.5.1. *Alk(en)ylresorcinols (**1–4**)*

Pink resin. Alk(en)ylresorcinols (**1**–**4**) as an inseparable mixture were identified based on their GC–MS analysis and comparison of their ^{1}H- and ^{13}C-NMR spectra with literature data (Gunstone, 1993; Knoedler et al., 2008).

- 5-Pentadecylresorcinol diTMS (**1**), retention time (RT) 35.3 min, MS (EI, 70 eV), *m*/*z* (relative intensity %): 464 M^+ (33), 449 $[M–CH_3]^+$ (9), 268 $[M–C_{14}H_{28}]^+$ (100), 73 (13).

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- 5-(8′Z,11′Z-Heptadecadienyl)-resorcinol diTMS (**2**), RT 37.6 min, MS (EI, 70 eV), *m/z* (relative intensity %): 488 M^+ (24), 473 $[M–CH_3]^+$ (6), 268 $[M–C_{16}H_{28}]^+$ (100), 73 (14).
- 5-(11′Z-Heptadecenyl)-resorcinol diTMS (**3**), RT 37.9 min, MS (EI, 70 eV), *m/z* (relative intensity %): 490 M^+ (25), 475 $[M–CH_3]^+$ (6), 268 $[M–C_{16}H_{30}]^+$ (100), 73 (13).
- 5-Heptadecylresorcinol diTMS (**4**), RT 38.0 min, MS (EI, 70 eV), *m/z* (relative intensity %): 492 M^+ (35), 477 $[M–CH_3]^+$ (8), 268 $[M–C_{16}H_{32}]^+$ (100), 73 (11).

3.5.2. *Mangiferolic acid (**5**), isomangiferolic acid (**10**) and 27-hydroxyisomangiferolic acid (**11**)*

White crystals. These compounds were identified based on their ^{1}H- and ^{13}C-NMR spectra, which are identical with literature data (Anjaneyulu, Ravi, Prasad, & Connolly, 1989; Silva, Cito, Chaves, & Dantas, 2005).

3.5.3. *Propolin D (**6**)*. Yellow resinous substance. Its EI–MS, ^{1}H- and ^{13}C-NMR spectra are identical with literature data (Chen, Weng, Wu, & Lin, 2004).

3.5.4. *Mixture of propolin C (**7**), propolin F (**8**) and propolin G (**9**)*

Yellow resin. The components of this mixture were identified by TLC and GC–MS comparison with an authentic sample.

3.6. *Antimicrobial tests*

For the investigation of the antibacterial and antifungal activity, the agar cup method was used with test strains *Staphylococcus aureus* 209 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia), *Escherichia coli* WF + (obtained from the Collection of ZIMET, Central Institute of Microbiology and Experimental Therapy, Jena, Germany) and *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, Institute for State Control of Drugs, Sofia; Spooner & Sykes, 1972). An inhibitory zone with a diameter <10 mm corresponds to lack of activity (10 mm is the diameter of the cup). The test solution (0.1 mL), containing 0.4 mg of each substance in ethanol, was applied to every cup (concentration of the test solution: 4 mg mL^{-1}). Control experiments with solvents showed that the solvent does not exhibit any activity.

3.7. *DPPH free radical scavenging activity*

The DPPH free radical scavenging activity (RSA) was measured according to a procedure previously described in the literature (Nenadis & Tsimidou, 2002). The decrease of the absorption at 516 nm of the DPPH solution after addition of the tested solution was measured. An aliquot (2000 μL) of 0.1 mmol ethanolic DPPH solution was mixed with 30 μL of a 2 mmol ethanolic solution of the tested substance.

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The RSA was expressed as percentage decrease with respect to a control value. Caffeic acid was used as a positive control.

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