

## Research Article

# Antifungal and Antibacterial Metabolites from a French Poplar Type Propolis

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During this study, the *in vitro* antifungal and antibacterial activities of different extracts (aqueous and organic) obtained from a French propolis batch were evaluated. Antifungal activity was evaluated by broth microdilution on three pathogenic strains: *Candida albicans*, *C. glabrata*, and *Aspergillus fumigatus*. Antibacterial activity was assayed using agar dilution method on 36 Gram-negative and Gram-positive strains including *Staphylococcus aureus*. Organic extracts showed a significant antifungal activity against *C. albicans* and *C. glabrata* (MIC<sub>80</sub> between 16 and 31 µg/mL) but only a weak activity towards *A. fumigatus* (MIC<sub>80</sub> = 250 µg/mL). DCM based extracts exhibited a selective Gram-positive antibacterial activity, especially against *S. aureus* (SA) and several of its methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) strains (MIC<sub>100</sub> 30–97 µg/mL). A new and active derivative of catechin was also identified whereas a synergistic antimicrobial effect was noticed during this study.

## 1. Introduction

Propolis is a resinous natural substance collected by honeybees from buds and exudates of various trees and plants, mixed with beeswax and salivary enzymes. Bees generally use it as a sealer, to smooth out the internal walls of the hive, as well as a protective barrier against intruders. Propolis has been used in folk medicine since ancient times due to its pharmacological potential associated with antioxidant [1–3], antifungal [4, 5], antibacterial [6–8], and anti-inflammatory [9] properties.

Propolis is generally composed of 50% of resin and balm (including polyphenolic compounds), 30% of wax and fatty acids, 10% of essential oils, 5% of pollen, and 5% of various organic and inorganic compounds. However, the composition of propolis deeply depends on the vegetation at the site of collection [10]. Indeed, propolis from temperate climatic zones, like in Europe, North America, or nontropical regions of Asia, mainly originates from the bud exudates

of *Populus* species (*Salicaceae*) and consequently is rich in flavonoids and phenolic acids and their esters [11]; however tropical propolis, originating from regions where neither poplars nor birches grow, is rich in prenylated derivatives of *p*-coumaric acids, benzophenones, or terpenoids [12, 13].

The antifungal, antibacterial properties and chemical composition of propolis from many countries all over the world have been widely studied [6, 8, 14–20] but few reports were already given for European propolis [21, 22]. In 1990, Grange and Davey [23] highlighted for the first time the bactericidal activity of a French propolis against Gram-positive strains whereas later on, in 2000, Hegazi et al. [22] could associate this antibacterial activity with the presence of benzyl caffeate, pinocembrin, and *p*-coumaric acid.

During a previous study, we have evaluated the antioxidant and anti-AGEs activities of different solvents extracts [water; 95% EtOH; 70% EtOH; MeOH; dichloromethane (DCM) and DCM/MeOH/H<sub>2</sub>O (31/19/4)] obtained from a French propolis batch and identified their active constituents

[24]. Here we have investigated the *in vitro* antifungal and antibacterial activities of these extracts. The antifungal activity was studied on three fungal strains (two yeasts, *Candida albicans* and *C. glabrata*, and one filamentous fungus, *Aspergillus fumigatus*). 36 strains of Gram-positive (including *Staphylococcus aureus*) and Gram-negative (including *Escherichia coli*) bacteria were used for the antibacterial assays. During this study, a new secondary metabolite was isolated, namely, 8-[(E)-phenylprop-2-en-1-one]-5-methoxy-(±)-catechin.

## 2. Materials and Methods

**2.1. Reagents and Standards.** Formic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, 3,4-dimethoxycinnamic acid, and prenyl caffeate were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, Saint-Quentin-Fallavier, France). Caffeic acid and chrysin were obtained from Acros Organics (Geel, Belgium). Galangin was purchased from Extrasynthese (Genay, France). Pinocembrin and pinobanksin-3-acetate were isolated from the DCM extract of propolis.

**2.2. Instrumentation.** Optical rotation was measured on a JASCO P-2000 polarimeter. IR spectra were recorded on a Bruker Vertex 70 spectrophotometer. NMR spectra (1D and 2D) were recorded on a Bruker Avance spectrometer at 500 MHz for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$ . MS analyses were performed on an ESI/APCI Ion Trap Esquire 3000+ from Bruker. UV absorbances were obtained from a Tecan Infinite M200 microplate spectrophotometer.

**2.3. Propolis Samples.** In order to analyze a typical French batch, that is, exhibiting an average chemical composition, a mixture of samples (10 g of each), collected in apiaries originating from different regions of France, was used for this study. These samples were provided by "Ballot-Flurin Apiculteurs," a company specialized in organic beekeeping. Indeed, even collected in the same geographical region, propolis profiles may differ between apiaries and even inside the same apiary from one hive to another one [25]. Keeping in mind any potential economic development, it then appeared more appropriate to study an industrial end-product, that is, a mixture, exhibiting an average chemical composition associated with an average antimicrobial activity, rather than a specific sample. Therefore, 24 batches of propolis collected over two years (2010 and 2011) from different places in France (cf. supporting information 1; see Supplementary Material available online at <http://dx.doi.org/10.1155/2015/319240>) were homogeneously mixed to undergo this study.

**2.4. Extractions.** The extraction processes have been already described [24]. Briefly, the propolis batch was homogeneously pulverized in the presence of liquid nitrogen and divided into 1 g samples. Four different extractions were then carried out on 1 g samples with water (E1), 95% EtOH (E2), 70% EtOH (E3), and MeOH (E4). Then, two extractions, preceded by a cyclohexane wax elimination, were independently performed on 1 g samples with DCM (E5) and

a mixture of DCM, MeOH, and  $\text{H}_2\text{O}$  (31/19/4) (E6). For E1, a decoction of 1 g of propolis powder was boiled in 20 mL  $\text{H}_2\text{O}$  at  $100^\circ\text{C}$  for 15 min. After cooling, the solidified wax and the residue were removed by filtration, and the filtrate was evaporated to dryness. For other solvents, 1 g of propolis powder (or residue obtained from a previous extraction) was macerated in  $3 \times 20$  mL of solvent. After stirring for  $3 \times 2$  h at room temperature, the mixture was filtered. The filtrates were gathered and evaporated under vacuum. Extraction yields (dried extract/100 g) were as follows: E1 7%; E2 68%; E3 65%; E4 68%; E5 50%; and E6 59%.

**2.5. Antifungal Activity.** Antifungal activity was assayed on human pathogenic fungi, including two common yeasts (*Candida albicans* ATCC 66396 and *C. glabrata* LMA 90-1085) and an opportunistic mould (*Aspergillus fumigatus* CBS 11326). The strains were obtained from the Parasitology and Mycology Laboratory at the University Hospital Center of Angers, France. Microorganisms were cultivated at  $37^\circ\text{C}$  on yeast extract-peptone-dextrose-agar (YPDA) containing 0.5 g/L chloramphenicol for two (*C. albicans* and *C. glabrata*) or three (*A. fumigatus*) days. Tests were performed according to a procedure described by Alomar et al. [27], following the guidelines of the approved reference method of the National Committee for Clinical Laboratory Standards (NCCLS) for yeasts [28] and filamentous fungi [29]. Briefly, the yeast suspensions were prepared in RPMI-1640 culture medium and adjusted spectrophotometrically at 630 nm to reach a final concentration of  $ca. 0.5 \times 10^3$  to  $2.5 \times 10^3$  cells/mL. The tests were performed using sterile 96 flat shaped well microtiter plates. Serial twofold sample dilutions were made in DMSO. Sample solutions were dispensed at a volume of  $5 \mu\text{L}$  in triplicate into the wells to obtain final concentrations from 250 to  $1.95 \mu\text{g/mL}$ . After 48 h at  $37^\circ\text{C}$  for *C. albicans* and *C. glabrata* and 72 h for *A. fumigatus*, the spectrophotometric MIC endpoint was calculated from the turbidimetric data as the lowest sample concentration causing a growth inhibition equal to or greater than 80% of the control ( $\text{MIC}_{80}$ ). Amphotericin B was used as a positive control.

**2.6. Antibacterial Activity.** Antibacterial activity was evaluated on 36 human pathogenic bacterial strains collected by the Laboratory of Bacteriology at the University Hospital Center of Angers, France: seven strains of *Acinetobacter baumannii* (RCH, SAN008, 12, AYE, CIP7034, 107292, and 5377), two of *Escherichia coli* (ATCC25922 and a clinical isolate), three of *Pseudomonas aeruginosa* (ATCC27853 and two clinical isolates), and 4 clinical isolates of *Enterobacter cloacae*, *E. aerogenes*, *Klebsiella oxytoca*, and *Salmonella enteritidis* (phage type 4) for Gram-negative bacteria; thirteen strains of *Staphylococcus aureus* (ATCC25923, six methicillin-susceptible clinical isolates, six methicillin-resistant clinical isolates), two clinical isolates of *S. epidermidis* (*methiS* and *methiR*), three clinical isolates of *Enterococcus faecalis* and one of *E. faecium*, and one clinical isolate of *Corynebacterium striatum* for Gram-positive bacteria. Tests were performed using the methodology described by Alomar et al. [30]. Briefly, a stock solution of each sample was prepared in

triplicate at 20 mg/mL in DMSO under sterile conditions. Serial dilutions were prepared (sample concentrations: 10, 20, 30, etc., to 100  $\mu\text{g/mL}$ ) and 0.1 mL of each dilution was added to 19.9 mL of Mueller-Hinton agar (Merck, Germany) and transferred to Petri plates. Bacterial strains ( $2 \times 10^4$  CFU/mL) were suspended in sterile NaCl aqueous solution (0.15 M) and inoculated on the different Petri plates using the multipoint inoculator (AQS, England). After 24 h of incubation at 37°C, the minimum inhibitory concentration (MIC<sub>100</sub>,  $\mu\text{g/mL}$ ) against bacterial strains was defined as the lowest concentration of each sample that inhibited visible growth. A blank was made inoculating the strains on Mueller-Hinton agar without any extract or compound. Oxacillin was used to distinguish the methicillin-resistant from the susceptible staphylococcal strains.

**2.7. HPLC-DAD and HPLC-MS Procedures.** Dry extracts were dissolved in MeOH (5 mg/mL for the aqueous extract and 10 mg/mL for the organic solvents ones) and centrifuged at 13000 rpm for 10 min prior to injection (10  $\mu\text{L}$ ) into the HPLC system. Analytical HPLC was run on a 2695 Waters separation module equipped with a diode array detector 2996 Waters. Separation was achieved on a LiChrospher column 100 RP-18 (125  $\times$  4 mm i.d., 5  $\mu\text{m}$ ) protected with a LiChrocart 4-4 guard cartridge (4  $\times$  4 mm i.d.) at a flow rate of 1 mL/min. The mobile phase consisted of 0.1% formic acid in water (solvent A) and MeOH (solvent B) and the separation was performed using the linear gradient: 25–100% B in 40 min. UV detection was achieved at two wavelengths: 254 and 280 nm.

The mass analyses were performed with an ESI interface coupled to an ion trap mass analyzer in both positive and negative modes, with the following conditions: collision gas, He; collision energy amplitude, 1.3 V; nebulizer and drying gas, N<sub>2</sub>, 7 L/min; pressure of nebulizer gas, 30 psi; dry temperature, 340°C; flow rate, 1.0 mL/min; solvent split ratio 1:9; scan range,  $m/z$  100–1000.

**2.8. Identification of Propolis Constituents.** **18** and **22** were directly identified in the DCM extract by HPLC/UV/MS and comparison with the literature data [31, 32], whereas **3**, **6**, **7**, **8**, **10**, and **32** were compared with authentic standards (Sigma-Aldrich and Acros organics, cf. Section 2.1). A flash chromatography was then carried out in order to identify the other phenolic constituents. 50.0 g of pulverized propolis was firstly extracted with cyclohexane (3  $\times$  200 mL, 2 h, room temperature) to eliminate waxes. After filtration, the residue was extracted with DCM (5  $\times$  200 mL, 2 h, room temperature) to give 25.0 g of dry DCM extract (50% yield). 21.0 g of this extract was fractionated using a CombiFlash Teledyne ISCO apparatus and a prepacked silica gel column (Interchim PF-50SI HC/300 g, 50  $\mu\text{m}$ ), at a flow rate of 100 mL/min and with the following gradient elution system: cyclohexane (C<sub>6</sub>H<sub>12</sub>) 100% (2.0 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 90:10 (1.7 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 90:10 to 80:20 (2.2 L), 80:20 to 70:30 (2.5 L), C<sub>6</sub>H<sub>12</sub>:EtOAc 70:30 to 60:40 (2.2 L), and C<sub>6</sub>H<sub>12</sub>:EtOAc 60:40 to 50:50 (3.0 L) then DCM:MeOH 96:4 (2.2 L). UV detection ( $\lambda$  254 and 280 nm) and TLC monitoring allowed collecting

21 fractions (*F1–21*). **48** [33] was identified in *F1*, **14** [34] and **17** [31] were identified in *F11*, **1** and **2** [19] were identified in *F13*, and finally **23** [31] was identified in *F15* by HPLC/UV/MS and comparison with the literature data. The remaining constituents were isolated and identified through 1D and 2D NMR analysis (cf. Section 2.2). 200 mg of *F1* was chromatographed on a silica gel column (Grace, 24 g) by flash chromatography at a flow rate of 25 mL/min with a mixture of C<sub>6</sub>H<sub>12</sub> and EtOAc (B) [gradient: 1% B (30 min), 2% B (5 min), 2–5% B (2 min), 5% B (2 min), 5–30% B (1 min), and 30% B (5 min)] to give **46** [33, 35] (5 mg), **47** [33, 36] (3 mg), and **49** [37] (5 mg). *F2* (1.5 g) gave **43** [38] whereas *F4* (696 mg) and *F5* (384 mg) allowed us to, respectively, identify **44** [37, 39] and **45** [40]. 500 mg of *F6* (1.6 g) was chromatographed on reverse-phase- (RP-) Flash chromatography (Interchim column PF-30C18 HC/6 g, 30  $\mu\text{m}$ ) at a flow rate of 15 mL/min with water and MeOH (B) [gradient: 25–30% B (20 min), 30–40% B (2 min), 40% B (8 min), 40–45% B (1 min), 45% B (12 min), 45–50% B (2 min), and 50% B (20 min)] to give **9** [31] (10 mg), **25** [38] (126 mg), and a mixture of **35** and **36** [34] (15 mg). Similarly 500 mg of *F7* was fractionated [gradient: 30–50% B (25 min), 50–60% B (25 min), and 60–65% B (20 min)] to give **11** [41] (2 mg), **33** [31, 42] (128 mg), **34** [38] (65 mg), and **42** [31] (41 mg) whilst 500 mg of *F8* [gradient: 30–45% B (20 min), 45% B (30 min), 45–48% B (5 min), 48–55% B (5 min), 55% B (8 min), 55–60% B (1 min), and 60% B (12 min)] gave **13** [38] (3 mg), **28** [31, 43] (224 mg), and **41** [34] (28 mg). 500 mg of *F9* [gradient: 30–60% B (50 min) and 60–65% B (5 min)] yielded mixture of **4** [34] and **5** [44, 45] (8 mg), **15** [43] (33 mg), **24** [32, 46] (16 mg), **32** [38] (150 mg), and another mixture of **37** [16] and **38** [32] (3 mg). **27** [34] and **29** [31] were identified from *F10* (1.3 g). 500 mg of *F11* [flow rate of 20 mL/min, gradient: 25–75% B (55 min)] gave **26** [47] (82 mg), **31** [31] (56 mg), and **39** [32] (70 mg). 300 mg of *F13* [flow rate of 15 mL/min, gradient: 30–45% B (20 min), 45% B (20 min), 45–50% B (10 min), 50–60% B (5 min), and 60% B (5 min)] gave **10** [31] (2 mg) and **16** [38] (3 mg). **30** [48] was directly identified in *F17* (543 mg). 500 mg of *F18* [gradient: 40% B (7 min), 40–50% B (2 min), and 50% B (30 min)] gave a mixture of **8** and **12** (cf. *F19*) together with the new compound **40** (12 mg). 500 mg of *F19* was chromatographed [gradient: 25% B (25 min), 25–35% B (1 min), and 35% B (20 min)] to give **8** (8 mg) and **12** (26 mg) [31]. Finally 500 mg of *F20* allowed us to isolate [gradient: 30–40% B (20 min), 40–43% B (20 min), and 43–50% B (10 min)] **19** [32] (3 mg) and a mixture of **20** [49] and **21** [50] (7 mg).

### 3. Results and Discussion

**3.1. Antifungal and Antibacterial Activities.** Table 1 shows the minimum inhibitory concentration of at least 80% of fungal growth (MIC<sub>80</sub>) obtained with E1–6 extracts for *Candida albicans*, *C. glabrata*, and *Aspergillus fumigatus*. E1 did not exhibit any interesting antifungal activity (MIC<sub>80</sub> > 250  $\mu\text{g/mL}$  for the three strains) whereas E2–6 showed significant antifungal activities (MIC<sub>80</sub> between 16 and 31  $\mu\text{g/mL}$ ) on both *C. albicans* and *C. glabrata*. These results are in agreement with those previously obtained for an Argentinian propolis



TABLE 1: Antifungal activity against *Candida albicans*, *C. glabrata*, and *Aspergillus fumigatus*.

| Extract | Solvent          | <i>C. albicans</i>        | <i>C. glabrata</i> | <i>A. fumigatus</i> |
|---------|------------------|---------------------------|--------------------|---------------------|
|         |                  | MIC <sub>80</sub> (μg/mL) |                    |                     |
| E1      | H <sub>2</sub> O | >250                      | >250               | >250                |
| E2      | 95% EtOH         | 31.25                     | 15.63              | 250                 |
| E3      | 70% EtOH         | 31.25                     | 31.25              | 250                 |
| E4      | MeOH             | 31.25                     | 31.25              | 250                 |
| E5      | DCM              | 31.25                     | 31.25              | 250                 |
| E6      | Mixed solvents   | 15.63                     | 31.25              | 250                 |
|         | Amphotericin B   | 0.125                     | 0.125              | 6                   |

on several *Candida* species (MIC<sub>100</sub> in a range of 31 to 125 μg/mL) [51] as well as with Greece and Cyprus ones (MIC<sub>100</sub> 20 μg/mL) [21]. E2–6 also exhibited a weak activity towards *A. fumigatus* (MIC<sub>80</sub> 250 μg/mL).

According to Ríos and Recio [52] a MIC<sub>100</sub> < 100 μg/mL should be considered as a promising value for a crude extract (versus 10 μg/mL for pure compounds). This is the reason why Table 2 gives the results of the antibacterial activity of E1–6 at the concentration of 100 μg/mL for 28 strains of Gram-negative and Gram-positive bacteria.

Results showed that Gram-negative bacteria were not susceptible to E1–6 at this concentration. In contrast, organic solvents extracts were active on several Gram-positive bacteria such as *Corynebacterium striatum* (sometimes involved in pleuropulmonary infections) (E2–5) and especially *Staphylococcus aureus*, including for the latter several methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) clinical isolates (E5–6). Sometimes called “golden staph,” *S. aureus* is the most pathogenic species of *Staphylococcus* genus. It might cause food poisoning, skin infections, abscesses, and diseases like pneumonia, meningitis, and sepsis. *S. aureus* is additionally one of the major causes of hospital-acquired infections, and the treatment of some multiresistant strains has become quite problematic. Among them, MRSA appears in France as one of the most commonly multiresistant strains encountered in hospitals.

MIC<sub>100</sub> of E1–6 were determined on the 6 susceptible Gram-positive strains as well as on 8 other MRSA and MSSA strains. Results are given in Table 3.

E1 did not show any interesting activity on the 14 studied strains (MIC<sub>100</sub> > 100 μg/mL). E2–6 showed interesting activities against *Corynebacterium striatum* with MIC<sub>100</sub> ranging from 63 to 90 μg/mL. E5 and E6 exhibited the best antibacterial activities against the *Staphylococcus* strains with MIC<sub>100</sub> up to 57 and 30 μg/mL, respectively. Among the alcoholic extracts, only E4 showed a moderate activity (MIC<sub>100</sub> 90 μg/mL) against *S. aureus* and one MRSA whereas E2 and E3 appeared as inactive. These overall activities therefore appeared to be better than those reported by Grange and Davey for the antibacterial activity of a French propolis on *S. aureus* and MRSA (MIC<sub>100</sub> 188–375 μg/mL) [23]. Our global antibacterial activity against MRSA and MSSA could be compared with those reported for propolis collected in Solomon Islands, exhibiting MIC<sub>100</sub> between

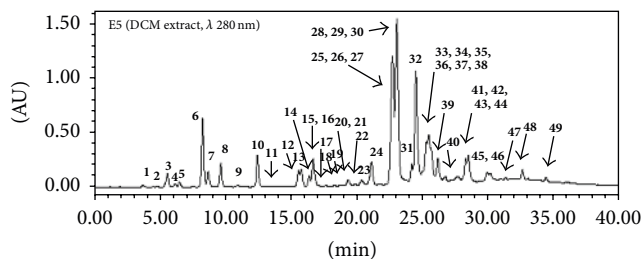


FIGURE 1: HPLC chromatograms of E5: 1 3,4-dihydroxybenzaldehyde, 2 4-hydroxybenzoic acid, 3 caffeic acid, 4 vanillin, 5 4-hydroxyacetophenone, 6 *p*-coumaric acid, 7 ferulic acid, 8 isoferulic acid, 9 benzoic acid, 10 3,4-dimethoxycinnamic acid, 11 3-phenylpropanoic acid, 12 pinobanksin-5-methyl ether, 13 cinnamic acid, 14 4-methoxycinnamic acid, 15 pinobanksin, 16 naringenin, 17 quercetin, 18 quercetin-3-methyl ether, 19 pinocembrin-5-methyl ether, 20 1,3-di-*p*-coumaroylglycerol, 21 1-*p*-coumaroyl-3-feruloylglycerol, 22 kaempferol, 23 apigenin, 24 cinnamylidene acetic acid, 25 pinocembrin, 26 benzyl caffeate, 27 isopent-3-enyl caffeate, 28 pinobanksin-3-acetate, 29 prenyl caffeate, 30 2-acetyl-1,3-dicoumaroylglycerol, 31 phenylethyl caffeate (CAPE), 32 chrysin, 33 benzyl *p*-coumarate, 34 galangin, 35 benzyl ferulate, 36 prenyl ferulate, 37 kaempferide, 38 rhamnocitrin, 39 cinnamyl caffeate, 40 8-[(*E*)-phenylprop-2-en-1-one]-5-methoxy-(±)-catechin (new), 41 cinnamyl isoferulate, 42 cinnamyl *p*-coumarate, 43 pinostrobin, 44 alpinone-3-acetate, 45 tectochrysin, 46 benzyl cinnamate, 47 cinnamyl benzoate, 48 cinnamyl cinnamate, and 49 cinnamyl cinnamylidene acetate.

64 and 128 μg/mL [6]. Similarly E4 was more active than a methanolic propolis extract from Jordan (585 μg/mL against *S. aureus* and 4700 μg/mL against MRSA) [20].

These results suggested that antifungal and antibacterial activities of propolis extracts could be related to their flavonoids contents [24]. Indeed, whereas E1–6 exhibited high total polyphenol contents (239–281 mg GAE/g), only those showing both high flavone/flavonol (FF) and flavanone/dihydroflavonol (FD) contents (i.e., E5–6) were active on the studied strains. In addition the higher the cumulative contents FF+FD were, the stronger the antibacterial activity was, as shown with E5 (254 mg/g) and E6 (236 mg/g) > E2–4 (220–228 mg/g). These results are in agreement with those reported by Velazquez et al. [15] for different Mexican propolis collected in Sonora State where EEP from the areas of Ures (410 mg/g), Caborca (332 mg/g), and Pueblo de Alamos (209 mg/g) showed MIC<sub>100</sub> against *S. aureus* of 100, 200, and >400 μg/mL, respectively.

**3.2. Chemical Composition.** Figure 1 shows the HPLC chromatogram of the DCM extract E5. 48 compounds were identified by comparison with the literature data (UV/MS) and pure standards or, when needed, through <sup>1</sup>H and <sup>13</sup>C (1D and 2D) NMR analysis after compound isolation.

Additionally a new flavan-3-ol was identified as the 8-[(*E*)-phenylprop-2-en-1-one]-5-methoxy-(±)-catechin **40** (Figure 2).

Compound **40** was obtained as a yellow amorphous solid (0.6 μg/g of DCM extract). The molecular formula was

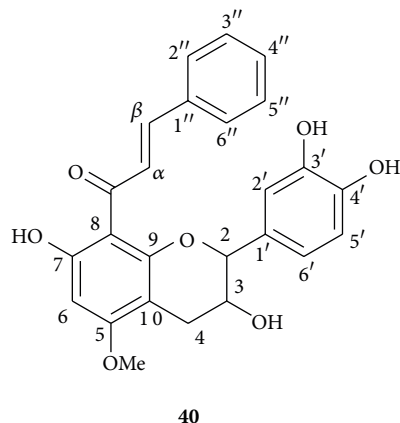
TABLE 2: Antibacterial activity of E1-6 against 28 Gram-negative and Gram-positive strains.

| Number                | Bacterial strains                                      | Extracts (100 µg/mL) |              |              |          |         |                    |
|-----------------------|--------------------------------------------------------|----------------------|--------------|--------------|----------|---------|--------------------|
|                       |                                                        | E1: H <sub>2</sub> O | E2: 95% EtOH | E3: 70% EtOH | E4: MeOH | E5: DCM | E6: mixed solvents |
| <i>Gram-negative:</i> |                                                        |                      |              |              |          |         |                    |
| 1                     | <i>Acinetobacter baumannii</i> (RCH)                   | -                    | -            | -            | -        | -       | -                  |
| 2                     | <i>Acinetobacter baumannii</i> (SAN008)                | -                    | -            | -            | -        | -       | -                  |
| 3                     | <i>Acinetobacter baumannii</i> (12)                    | -                    | -            | -            | -        | -       | -                  |
| 4                     | <i>Acinetobacter baumannii</i> (AYE)                   | -                    | -            | -            | -        | -       | -                  |
| 5                     | <i>Acinetobacter baumannii</i> (CIP7034)               | -                    | -            | -            | -        | -       | -                  |
| 6                     | <i>Acinetobacter baumannii</i> (CIP107292)             | -                    | -            | -            | -        | -       | -                  |
| 7                     | <i>Acinetobacter baumannii</i> (CIP5377)               | -                    | -            | -            | -        | -       | -                  |
| 8                     | <i>Enterobacter cloacae</i> (0705A1743)                | -                    | -            | -            | -        | -       | -                  |
| 9                     | <i>Enterobacter aerogenes</i> (0705A0867)              | -                    | -            | -            | -        | -       | -                  |
| 10                    | <i>Escherichia coli</i> (ATCC25922)                    | -                    | -            | -            | -        | -       | -                  |
| 11                    | <i>Escherichia coli</i> (0705A0434)                    | -                    | -            | -            | -        | -       | -                  |
| 12                    | <i>Klebsiella oxytoca</i> (0705C0187)                  | -                    | -            | -            | -        | -       | -                  |
| 13                    | <i>Pseudomonas aeruginosa</i> (ATCC27853)              | -                    | -            | -            | -        | -       | -                  |
| 14                    | <i>Pseudomonas aeruginosa</i> (0704C0134)              | -                    | -            | -            | -        | -       | -                  |
| 15                    | <i>Pseudomonas aeruginosa</i> (0703C0259)              | -                    | -            | -            | -        | -       | -                  |
| 16                    | <i>Salmonella enteritidis</i> (4)                      | -                    | -            | -            | -        | -       | -                  |
| <i>Gram-positive:</i> |                                                        |                      |              |              |          |         |                    |
| 17                    | <i>Corynebacterium striatum</i> (56)                   | -                    | +            | +            | +        | +       | +                  |
| 18                    | <i>Enterococcus faecalis</i> (11003508001)             | -                    | -            | -            | -        | -       | -                  |
| 19                    | <i>Enterococcus faecalis</i> (11003492701)             | -                    | -            | -            | -        | -       | -                  |
| 20                    | <i>Enterococcus faecalis</i> (11004774001)             | -                    | -            | -            | -        | -       | -                  |
| 21                    | <i>Enterococcus faecium</i> (11502441401)              | -                    | -            | -            | -        | -       | -                  |
| 22                    | <i>Staphylococcus aureus</i> (ATCC25923)               | -                    | -            | -            | +        | +       | +                  |
| 23                    | MRSA (0706C0025)                                       | -                    | -            | -            | +        | +       | +                  |
| 24                    | MRSA (0702E0196)                                       | -                    | -            | -            | -        | +       | +                  |
| 25                    | MSSA (0703H0036)                                       | -                    | -            | -            | -        | -       | -                  |
| 26                    | MSSA (0701A0095)                                       | -                    | -            | -            | -        | +       | +                  |
| 27                    | <i>S. epidermidis methiS<sup>a</sup></i> (12004523201) | -                    | -            | -            | -        | -       | -                  |
| 28                    | <i>S. epidermidis methiR<sup>b</sup></i> (12552599902) | -                    | -            | -            | -        | -       | -                  |

-: no antibacterial activity, +: antibacterial activity, <sup>a</sup>*methicillin-Susceptible*, <sup>b</sup>*methicillin-Resistant*.

TABLE 3: MICs of E1-6 against 14 Gram-positive strains including MRSA and MSSA.

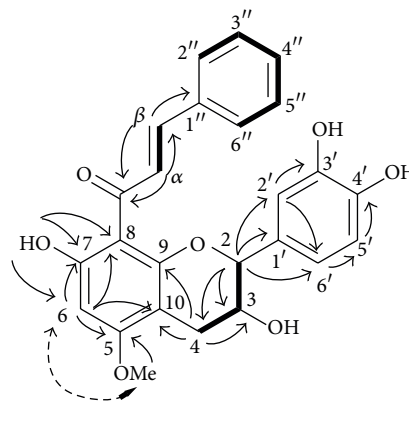
| Number | Bacterial strains                        | MIC <sub>100</sub> (µg/mL) |              |              |          |         |                    |           |
|--------|------------------------------------------|----------------------------|--------------|--------------|----------|---------|--------------------|-----------|
|        |                                          | E1: H <sub>2</sub> O       | E2: 95% EtOH | E3: 70% EtOH | E4: MeOH | E5: DCM | E6: mixed solvents | Oxacillin |
| 17     | <i>Corynebacterium striatum</i>          | >100                       | 83 ± 6       | 90 ± 0       | 77 ± 12  | 63 ± 15 | 87 ± 21            | —         |
| 22     | <i>Staphylococcus aureus</i> (ATCC25923) | >100                       | >100         | >100         | 90 ± 0   | 60 ± 10 | 67 ± 15            | ≤0.25     |
| 23     | MRSA (0706C0025)                         | >100                       | >100         | >100         | 90 ± 0   | 57 ± 12 | 30 ± 0             | ≥4        |
| 24     | MRSA (0702E0196)                         | >100                       | >100         | >100         | >100     | 80 ± 10 | 77 ± 23            | ≥4        |
| 25     | MSSA (0703H0036)                         | >100                       | >100         | >100         | >100     | >100    | >100               | ≤0.25     |
| 26     | MSSA (0701A0095)                         | >100                       | >100         | >100         | >100     | 87 ± 6  | 83 ± 29            | ≤0.25     |
| 29     | MRSA (11004533801)                       | >100                       | >100         | >100         | >100     | 80 ± 0  | 87 ± 21            | ≥4        |
| 30     | MRSA (11004691801)                       | >100                       | >100         | >100         | >100     | 77 ± 6  | 67 ± 23            | ≥4        |
| 31     | MRSA (11004787401)                       | >100                       | >100         | >100         | >100     | 97 ± 6  | >100               | ≥4        |
| 32     | MRSA (11006153901)                       | >100                       | >100         | >100         | >100     | 77 ± 6  | 73 ± 29            | ≥4        |
| 33     | MSSA (11004327701)                       | >100                       | >100         | >100         | >100     | 77 ± 6  | 73 ± 12            | 0.25      |
| 34     | MSSA (11004480701)                       | >100                       | >100         | >100         | >100     | 80 ± 0  | 97 ± 12            | 0.5       |
| 35     | MSSA (11004691801)                       | >100                       | >100         | >100         | >100     | 77 ± 6  | 90 ± 17            | 0.5       |
| 36     | MSSA (11004010401)                       | >100                       | >100         | >100         | >100     | 77 ± 6  | 90 ± 0             | ≤0.25     |



40

FIGURE 2: Chemical structure of the new compound **40**.

determined as  $C_{25}H_{22}O_7$  by HRESIMS (found for  $[M+H]^+$  435.1436; calculated 435.1438). The UV spectrum showed an absorption maximum at 350 nm. The IR spectrum indicated the presence of OH ( $3400\text{ cm}^{-1}$ ) as well as conjugated ketone carbonyl ( $1610\text{ cm}^{-1}$ ) groups. The  $^1\text{H}$  NMR spectrum exhibited signals due to a hydrogen-bonded OH at  $\delta_{\text{H}}$  14.49, two *trans*-olefinic protons ( $\delta_{\text{H}}$  8.06 and 7.63, 2 d,  $J = 15.7\text{ Hz}$ ), aromatic rings (9H,  $\delta_{\text{H}}$  6.15–7.30), and one methoxyle ( $\delta_{\text{H}}$  3.92). It also showed the characteristic signals of a flavan-3-ol moiety at  $\delta_{\text{H}}$  4.68 (1H, d,  $J = 8.9\text{ Hz}$ ,  $H_2$ ), 4.21 (1H, m,  $H_3$ ), 3.07 (1H, dd,  $J = 16.2, 5.7\text{ Hz}$ ,  $H_{4a}$ ), and 2.53 (1H, dd,  $J = 16.2, 9.5\text{ Hz}$ ,  $H_{4b}$ ). The  $^{13}\text{C}$  NMR and HMQC spectra confirmed the presence of 25 carbons with typical flavan-3-ol signals at  $\delta_{\text{C}}$  84.2 ( $C_2$ ), 66.8 ( $C_3$ ), and 30.6 ( $C_4$ ). In the  $^1\text{H}$  NMR spectrum, the signals at  $\delta_{\text{H}}$  7.11 (1H, d,  $J = 1.4\text{ Hz}$ ), 6.96 (1H, dd,  $J = 8.4, 1.4\text{ Hz}$ ), and 6.91 (1H, d,  $J = 8.4\text{ Hz}$ ) suggested the presence of a 1',3',4'-trisubstituted ring B whereas a singlet at  $\delta_{\text{H}}$  6.15 (1H) indicated a pentasubstituted ring A. Two multiplets at  $\delta_{\text{H}}$  7.17 (2H) and 7.29 (3H) revealed the presence of a phenyl residue. The HMBC spectrum showed a long-range correlation between the two *trans*-olefinic protons [ $\delta_{\text{H}}$  7.63 (1H, d,  $J = 15.7, H_{\alpha}$ ) and 8.06 (1H, d,  $J = 15.7, H_{\beta}$ )] and the ketone carbon at  $\delta_{\text{C}}$  193.2. This correlation revealed the presence of an  $\alpha,\beta$ -unsaturated ketone group. The *trans*-olefinic proton  $H_{\beta}$  at  $\delta_{\text{H}}$  8.06 was also correlated with the phenyl quaternary carbon at  $\delta_{\text{C}}$  136.2 ( $C_{1''}$ ). This correlation implied the presence of a (2*E*)-4-phenylprop-2-en-1-one moiety. A correlation between the methoxyle protons ( $\delta_{\text{H}}$  3.92) and the carbon at  $\delta_{\text{C}}$  165.1 ( $C_5$ ) proved that the  $\text{OCH}_3$  was attached to  $C_5$ . The NOESY spectrum showed that this methoxyle was spatially close to the proton at  $\delta_{\text{H}}$  6.15 ( $H_6$ ), whereas a long-range COSY indicated a correlation between  $H_6$  and one of the hydroxyl groups at  $\delta_{\text{H}}$  14.49 ( $\text{OH}_7$ ). Therefore a (2*E*)-4-phenylprop-2-en-1-one moiety was located at  $C_8$  ( $\delta_{\text{C}}$  105.9). Finally, it appeared that the aromatic ring B was substituted at  $C_{3'}$  and  $C_{4'}$  by two hydroxyl groups (NMR spectra cf. supporting information 2).  $^1\text{H}$  and  $^{13}\text{C}$  NMR data together with 2D NMR correlations for **40** are summarized in Table 4 and Figure 3.



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FIGURE 3: 2D NMR studies of compound **40**: COSY (bold lines), selected HMBC (solid arrows:  $^1\text{H} \rightarrow ^{13}\text{C}$ ), and NOESY (dashed arrows) correlations.TABLE 4:  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of the new compound **40** (in acetone- $d_6$ ).

| Position          | <b>40</b>                                |                             |
|-------------------|------------------------------------------|-----------------------------|
|                   | $\delta_{\text{H}}$ , mult. ( $J$ in Hz) | $\delta_{\text{C}}$ , mult. |
| 2                 | 4.68, d (8.9)                            | 84.2, CH                    |
| 3                 | 4.21, m                                  | 66.8, CH                    |
| 4                 | a 3.07, dd (16.2, 5.7)                   | 30.6, $\text{CH}_2$         |
|                   | b 2.53, dd (16.2, 9.5)                   |                             |
| 5                 |                                          | 165.1, qC                   |
| 6                 | 6.15, s                                  | 93.5, CH                    |
| 7                 |                                          | 168.1, qC                   |
| 8                 |                                          | 105.9, qC                   |
| 9                 |                                          | 157.6, qC                   |
| 10                |                                          | 102.4, qC                   |
| 1'                |                                          | 130.7, qC                   |
| 2'                | 7.11, d (1.4)                            | 116.2, CH                   |
| 3'                |                                          | 146.6, qC                   |
| 4'                |                                          | 146.2, qC                   |
| 5'                | 6.91, d (8.4)                            | 116.0, CH                   |
| 6'                | 6.96, dd (8.4, 1.4)                      | 121.3, CH                   |
| 1''               |                                          | 136.2, qC                   |
| 2''               | 7.28, m                                  | 129.2, CH                   |
| 3''               | 7.16, m                                  | 129.7, CH                   |
| 4''               | 7.28, m                                  | 130.8, CH                   |
| 5''               | 7.16, m                                  | 129.7, CH                   |
| 6''               | 7.28, m                                  | 129.2, CH                   |
| OH-7              | 14.49, s                                 |                             |
| $\alpha$          | 7.63, d (15.7)                           | 143.1, CH                   |
| $\beta$           | 8.06, d (15.7)                           | 128.4, CH                   |
| C=O               |                                          | 193.2, qC                   |
| $\text{OCH}_3$ -5 | 3.92, s                                  | 56.5, $\text{CH}_3$         |

**40** had no optical rotation and, thus, was isolated here as a racemate mixture of 8-[(*E*)-phenylprop-2-en-1-one]-2*R*,3*S*)-5-methoxycatechin (**40a**) and 8-[(*E*)-phenylprop-2-en-1-one]-2*S*,3*R*)-5-methoxycatechin (**40b**). Sha et al.

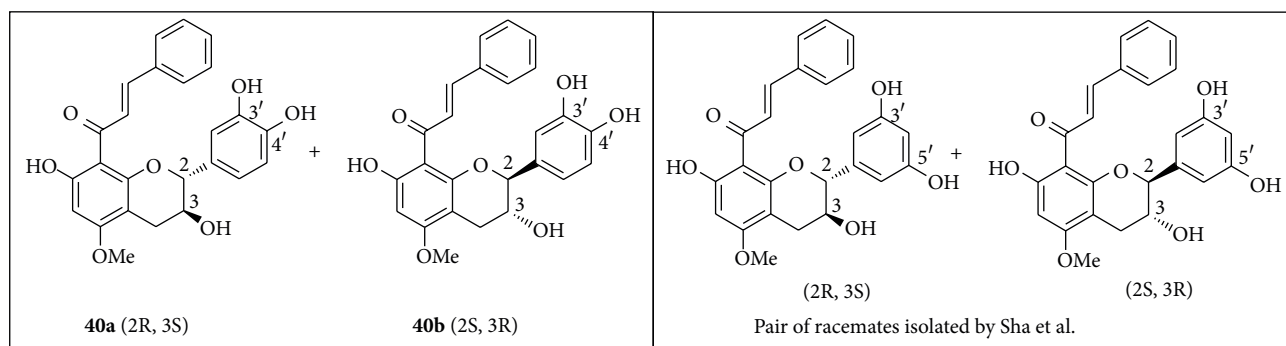


FIGURE 4: Structures of 8-[(E)-phenylprop-2-en-1-one]-(2R,3S)-5-methoxycatechin (**40a**) and 8-[(E)-phenylprop-2-en-1-one]-(2S,3R)-5-methoxycatechin (**40b**) and a similar pair of racemates isolated by Sha et al. 2009 [26].

TABLE 5: Antifungal and antibacterial activities of **40**.

| Antifungal activity |                           |        | Antibacterial activity       |                            |
|---------------------|---------------------------|--------|------------------------------|----------------------------|
| Fungal strains      | MIC <sub>80</sub> (μg/mL) | Number | Bacterial strains            | MIC <sub>100</sub> (μg/mL) |
| <i>C. albicans</i>  | >250                      | 22     | <i>S. aureus</i> (ATCC25923) | >100                       |
| <i>C. glabrata</i>  | >250                      | 23     | MRSA (0706C0025)             | ≤10                        |
| <i>A. fumigatus</i> | >250                      | 24     | MRSA (0702E0196)             | ≤10                        |
|                     |                           | 25     | MSSA (0703H0036)             | >100                       |
|                     |                           | 26     | MSSA (0701A0095)             | >100                       |

already isolated a similar compound, only differing from **40** by a 1',3',5'-trisubstituted aromatic ring B, from a Chinese propolis [26] (Figure 4).

**3.3. Antifungal and Antibacterial Activities of 40.** The new flavan-3-ol **40** did not show any antifungal activity on the three strains studied (Table 5). However, though active neither on *S. aureus* nor on MSSA, its MIC<sub>100</sub> on MRSA numbers 23 and 24 were lower or equal to 10 μg/mL (close to oxacillin: ≥4 μg/mL).

**3.4. Major Compounds Activities.** Antifungal and antibacterial activities were then individually evaluated for the five major compounds identified in E2–6, namely, pinobanksin-3-acetate (**28**), pinocembrin (**25**), chrysin (**32**), galangin (**34**), and prenyl caffeate (**29**) [24]. Their MIC<sub>80</sub> towards *C. albicans*, *C. glabrata*, and *A. fumigatus* as well as their MIC<sub>100</sub> towards *S. aureus*, MRSA, and MSSA are given in Table 6.

Pinobanksin-3-acetate (**28**), chrysin (**32**), and galangin (**34**) appeared as inactive. Pinocembrin (**25**) showed a moderate activity towards *Candida albicans*, *C. glabrata* (MIC<sub>80</sub> 62–125 μg/mL), and *S. aureus* (MIC<sub>100</sub> 100 μg/mL). Overall prenyl caffeate (**29**) exhibited the best activities (MIC<sub>100</sub> up to 16 μg/mL against *C. glabrata* and up to 63 μg/mL against *S. aureus* and MRSA). Even so it appeared that these compounds were not individually as active as it could be expected from E5–6 results (MIC<sub>100</sub> 30–97 μg/mL). As far as *S. aureus* and MRSA are concerned, this kind of synergistic effects was recently pointed out by Darwish et al. [20] who evaluated the antibacterial activities of pinobanksin-3-acetate, pinocembrin, and chrysin isolated from a Jordanian propolis. Therefore these results are also in agreement with Kujumgiev

et al. stating that, in spite of a great chemodiversity, no specific compounds can be associated with the antimicrobial activities of propolis extracts whereas, obviously, different flavonoid combinations are essential for these activities [7]. The antimicrobial of propolis extracts most probably involves a complex mechanism. It can be attributed to the synergistic effects of phenolic compounds such as cinnamic acid and ester derivatives, including caffeic acid and CAPE, as well as flavonoids including quercetin and naringenin [17, 53, 54]. Indeed, each of these compounds would be able to increase membrane permeability and inhibit bacterial mobility [54], thus contributing to the antimicrobial activity of propolis but also to its synergism with other antibiotics [53, 55, 56]. It is the reason why Stepanović et al. could notice the antibacterial and synergistic actions of propolis extracts with ampicillin, ceftriaxone, and doxycycline towards *Staphylococcus aureus* and with nystatin towards *Candida albicans*, stating that the bacterial resistance to antibiotics had no influence on the susceptibility to propolis extracts [57]. *In vitro* studies of synergism carried by Fernandes Jr. et al. also revealed synergistic effects of EEP with chloramphenicol, gentamicin, netilmicin, tetracycline, vancomycin, and clindamycin [58]. Therefore our findings are in total accordance with these results and, now that antibiotic resistance to bacteria has become a major public health concern [59], could bring valuable knowledge to develop new antimicrobial drugs for challenging *S. aureus* infections.

## 4. Conclusions

On the basis of these results, it may be concluded that organic solvents extracts of a French poplar type propolis are

TABLE 6: Antifungal and antibacterial activities of five major compounds.

| Antifungal strains |                              | MIC <sub>80</sub> (μg/mL) |              |               |                      |                |       |
|--------------------|------------------------------|---------------------------|--------------|---------------|----------------------|----------------|-------|
|                    | Pinobanksin-3-acetate (28)   | Pinocembrin (25)          | Chrysin (32) | Galangin (34) | Prenyl caffeate (29) | Amphotericin B |       |
|                    | <i>Candida albicans</i>      | 250                       | 62           | >250          | >250                 | 62             | 0.125 |
|                    | <i>Candida glabrata</i>      | 250                       | 125          | >250          | >250                 | 16             | 0.125 |
|                    | <i>Aspergillus fumigatus</i> | >250                      | 250          | >250          | >250                 | 125            | 6     |

| Bacterial strains |                                          | MIC <sub>100</sub> (μg/mL) |                  |              |               |                      |           |
|-------------------|------------------------------------------|----------------------------|------------------|--------------|---------------|----------------------|-----------|
| Number            | Bacterial strains                        | Pinobanksin-3-acetate (28) | Pinocembrin (25) | Chrysin (32) | Galangin (34) | Prenyl caffeate (29) | Oxacillin |
| 22                | <i>Staphylococcus aureus</i> (ATCC25923) | >100                       | 100 ± 0          | >100         | >100          | 63 ± 6               | ≤0.25     |
| 23                | MRSA (0706C0025)                         | >100                       | >100             | >100         | >100          | 70 ± 0               | ≥4        |
| 24                | MRSA (0702E0196)                         | >100                       | >100             | >100         | >100          | 70 ± 0               | ≥4        |
| 25                | MSSA (0703H0036)                         | >100                       | >100             | >100         | >100          | 93 ± 6               | ≤0.25     |
| 26                | MSSA (0701A0095)                         | >100                       | >100             | >100         | >100          | 93 ± 6               | ≤0.25     |

associated with a good antifungal activity towards *Candida albicans* and *C. glabrata*, correlated with high flavonoid contents. However only DCM based extracts (E5-6) showed a significant antibacterial activity against both methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* strains. Unfortunately these extracts are not compatible with a pharmaceutical use because of their toxicity, whereas EtOH based extracts were not as active as expected. Therefore it would be interesting to develop some alternative extraction of propolis using a nontoxic solvent such as subcritical water. In addition, it should be noticed that, as an intrinsic polytherapy, propolis may also circumvent the development of drug resistance by bacteria [60].

## Abbreviations

|          |                                                           |
|----------|-----------------------------------------------------------|
| APCI:    | Atmospheric pressure chemical ionization                  |
| ATCC:    | American Type Culture Collection                          |
| AYE:     | Patient code                                              |
| CAPE:    | Caffeic acid phenylethyl ester                            |
| CBS:     | Centraal Bureau voor Schimmelcultures                     |
| CIP:     | Collection de l'Institut Pasteur                          |
| COSY:    | Correlation spectroscopy                                  |
| DCM:     | Dichloromethane                                           |
| DMSO:    | Dimethyl sulfoxide                                        |
| EEP:     | Ethanol extract of propolis                               |
| ESI:     | Electrospray ionization                                   |
| FD:      | Flavanone/dihydroflavonol                                 |
| FF:      | Flavone/flavonol                                          |
| GAE:     | Gallic acid equivalent                                    |
| HMBC:    | Heteronuclear multiple bond correlation                   |
| HMQC:    | Heteronuclear multiple quantum correlation                |
| HPLC:    | High performance liquid chromatography                    |
| HRESIMS: | High resolution electrospray ionization mass spectrometry |
| IR:      | Infrared                                                  |

|        |                                                      |
|--------|------------------------------------------------------|
| LMA:   | Laboratoire de Mycologie d'Angers                    |
| MIC:   | Minimum inhibitory concentration                     |
| MRSA:  | Methicillin-resistant <i>Staphylococcus aureus</i>   |
| MS:    | Mass spectrometry                                    |
| MSSA:  | Methicillin-susceptible <i>Staphylococcus aureus</i> |
| NCCLS: | National Committee for Clinical Laboratory Standards |
| NMR:   | Nuclear magnetic resonance                           |
| NOESY: | Nuclear Overhauser effect spectroscopy               |
| RCH:   | Patient code                                         |
| RP:    | Reversed phase                                       |
| RPMI:  | Roswell Park Memorial Institute                      |
| UV:    | Ultraviolet                                          |
| YPDA:  | Yeast peptone dextrose agar.                         |

## Conflict of Interests

This study was financed by Ballot-Flurin Apiculteurs Cie. There are no other competing interests.

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