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Antibacterial mono- and sesquiterpene esters of benzoic acids from Iranian propolis

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Abstract

Background: Propolis (bee glue) has been used as a remedy since ancient times. Propolis from unexplored regions attracts the attention of scientists in the search for new bioactive molecules.

Results: From Iranian propolis from the Isfahan province, five individual components were isolated: the prenylated coumarin suberosin **1**, and four terpene esters: tschimgin (bornyl *p*-hydroxybenzoate) **2**, tschimganin (bornyl vanillate) **3**, ferutinin (ferutinol *p*-hydroxybenzoate) **4,** and tefernin (ferutinol vanillate) **5**. All of them were found for the first time in propolis. Compounds **2** - **5** demonstrated activity against *Staphylococcus aureus*.

Conclusions: The results of the present study are consistent with the idea that propolis from unexplored regions is a promising source of biologically active compounds.

Background

Propolis (bee glue) has been used as a remedy since ancient times. At present, propolis is a popular remedy in the folk medicine of many nations and a row material for numerous over-the-counter preparations, health foods and beverages. Propolis has been proved to possess valuable biological activities: antimicrobial, antiviral, anti inflammatory, antioxidant, antitumor, etc. [1,2]. Bee glue is a plant derived product: bees collect it from resinous plant parts, and its chemical composition strongly varies in different eco-geographic zones. Despite the chemical variability however, it always demonstrates considerable biological activity, especially antimicrobial activity [3,4]. For this reason, propolis from unexplored regions attracts the attention of scientists in the search for new bioactive molecules [5]. In this article we report on the isolation of antibacterial terpene esters of phenolic acids, new propolis constituents, from bee glue originated from the central part of Iran.

Results and Discussion

The total ethanol extract of Iranian propolis and the light petroleum fraction of this extract demonstrated significant antibacterial activity against *Staphylococcus aureus*, similar to the activity of the poplar type propolis. For this reason, GC-MS chemical profiling of this propolis was performed.

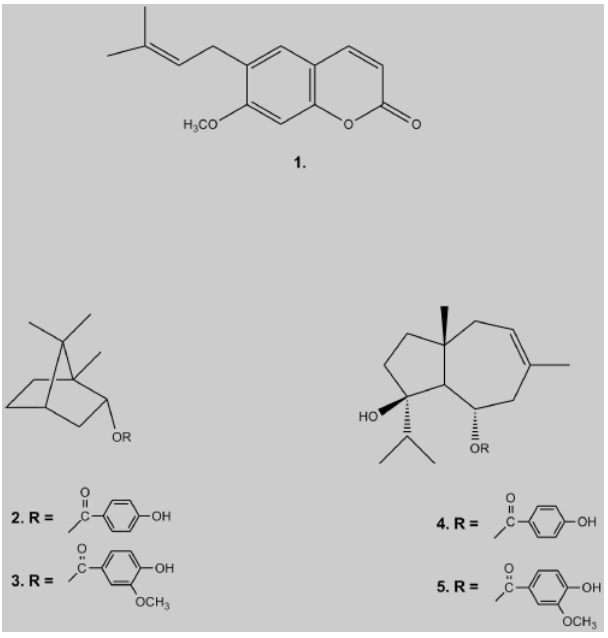
The GC-MS analysis of the ethanol extract after silylation (data not shown) revealed the presence of the poplar bud metabolites that are characteristic for poplar type propolis: pinocembrin, pinobanksin acetate, pentenyl caffeates, caffeic acid phenethyl ester (CAPE) [6]. However, the GC-MS profile demonstrated also the peaks of several terpene esters of substituted benzoic acids among the major components. Such compounds have never been found in propolis till now and for this reason, more detailed chemical studies of this propolis sample were performed.

The light petroleum fraction of the ethanol extract afforded, after repeated chromatographic procedures, five pure individual compounds. One of them was identified as the prenylated coumarine suberosin **1** (Scheme 1), by comparison of its spectral properties (MS, UV, NMR) with literature data [7]. It was found for the first time in propolis. This coumarin has been isolated earlier from numerous plant species belonging to different genera, e. g. *Citrus* [7], *Peucedanum* [8], *Heracleum* [9], etc. Thus, it cannot be used as taxonomic marker for the source plant of this propolis type.

The other four compounds were esters of mono- and sesquiterpene alcohols with *p*-hydroxybenzoic and vanillinic acids (Scheme 1), according to their mass spectra. Based on the detailed analysis of their mass and NMR

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Scheme 1 Constituents of Iranian propolis.

spectra (including 2D NMR) these compounds were identified as tschimgin (bornyl *p*-hydroxybenzoate) **2** [10], tschimganin (bornyl vanillate) **3** [11], ferutinin (ferutinol *p*-hydroxybenzoate) **4** [12] and tefernin (ferutinol vanillate) **5** [13], new propolis constituents. These compounds have been found in different *Ferula* species. The presence of sesqui- and diterpene esters of oxygenated benzoic acids is a characteristic feature of the metabolic profile of the plants of genus *Ferula* (Apiaceae) [14]. Several *Ferula* species grow in Iran, *F. gumosa* (an endemic to Iran) and *F. asafetida* being the most widespread [15,16]. They have flowering stems with a number of schizogenous ducts in the cortex containing the resinous gum [17], which is a potential propolis source. Thus, the source of compounds **2** - **4** in propolis has to be a plant species of the genus *Ferula* and the studied sample has two plant sources: poplar and ferula.

The total extract and the light petroleum fraction demonstrated low radical scavenging activity against DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (5.3 and 2.4%, respectively), compared to the well-known antioxidant caffeic acid (75.5%), used as a positive control. The individual compounds showed practically no antiradical activity (lower than 1%).

The antimicrobial activity of the isolated compounds was tested and compared to the activities of the total extracts (Table 1). No activity was found against *Candida albicans* and *Escherichia coli*, similar to most propolis samples of different origin and their constituents

Table 1 Antimicrobial activities against *S. aureus* (zones of inhibition) of propolis extract and the isolated components (at 400 μg in the cup).

Substance	Zone of inhibition (mm)
Ethanol extract	21 ± 1
Light petroleum fraction	21 ± 1
1 suberosin	0
2 tschimgin	24 ± 0
3 tschimganin	23 ± 1
4 ferutinin	24.3 ± 0.6
5 teferin	21.7 ± 0.6
Bulgarian propolis extract	20 ± 1
Streptomycin[a]	30 ± 1

Tests were done in triplicate, values are mean ± S.D.
[a] 100 μg in the cup.

[3]. The total ethanol extract, the light petroleum fraction and its constituents **2** - **5** were active against *S. aureus*. The most active compounds were **2** and **4**, the esters of *p*-hydroxybenzoic acid. Till now, antibacterial activity has been documented for **4** and**5** only [18], but for the bornyl esters **2** and **3** this is the first report on antibacterial activity. The studied propolis type of mixed origin (poplar & ferula) demonstrated slightly higher antibacterial activity compared to propolis from poplar origin (the Bulgarian sample) but the difference was not statistically significant.

Conclusion

The results of the present study are consistent with the idea that propolis from unexplored regions is a promising source of biologically active compounds.

Experimental

General

UV-VIS: Helios Gamma spectrophotometer, spectra were taken in methanol. IR: Bruker IFS 113v, spectra were taken in KBr pellets ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz), Bruker AV 600; spectra were taken in $CDCl_3$ (deuterated chloroform). MS: Hewlett Packard 5972 mass spectrometer, ionization voltage 70 eV. Column and flash chromatography were performed on Silica gel 60 (Merck, 63-200 μm), normal phase. Analytical thin-layer chromatography (TLC) was performed on Silica gel 60 F_{254} plates (Merck). Preparative thin-layer chromatography (PTLC) was performed on Silica gel 60 F_{254} glass plates (Merck, 20 × 20 cm, 0.25 mm). Detection of the spots was achieved under UV light (254 and 366 nm) and by spraying with vanillin- sulfuric acid in methanol (5:95 w/v vanillin:methanol solution, freshly mixed with a 5:95 v/v sulfuric acid:methanol solution), followed by heating at 100°C.

Propolis

Propolis sample was collected from several hives at an apiary near Isfahan, Isfahan province, Iran, in autumn 2008, by scraping.

Extraction and isolation

Propolis (31 g) was cooled in a refrigerator, ground and extracted twice with 70% ethanol (1:10, w:v) at room temperature for 24 h. A small part of this extract (10 ml) was evaporated to dryness and subjected to GC-MS analysis after silylation (5 mg of the residue was mixed with 50 μL of dry pyridine and 75 μL of BSTFA (bis-(trimethylsilyl)-trifluoroacteamide) and heated at 80°C for 20 min). The gas-chromatography - mass spectrometry (GC-MS) analysis was performed with a Hewlett Packard Gas Chromatograph 5890 Series II Plus linked to Hewlett Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm id and 0.5 μm film thickness HP5-MS capillary column; temperature program from 100 to 300°C at a rate of 5°C/min. carrier gas helium, flow rate 0.7 mL/min. Split ratio 1:20, injector temperature 280°C, interface temperature 300°C, ionization voltage 70 eV.

The ethanol extract was concentrated *in vacuo* and extracted successively with light petroleum (40 - 60°C) 3 times. The light petroleum extract was evaporated *in vacuo* to dryness to give 4.3 g dry residue.

The dry light petroleum extract was subjected to flash chromatography on silica gel, mobile phase light petroleum - EtOAc (ethyl acetate) (100% light petroleum to 100% EtOAc) and 10 fractions (A - J) were obtained. Fractions D and E (1.131 g, eluted with light petroleum - EtOAc 70:30- and 60:40) were re-chromatographed on a silica gel column eluted with light petroleum - EtOAc (90:10 to 100% EtOAc) and 27 fractions were obtained. Fraction 4 (55.7 mg, eluted with light petroleum - EtOAc 80:20) was subjected to PTLC (silica gel, mobile phase methylene chloride - EtOAc 99:1) to yield 16.5 mg **2** (tschimganin). Fraction 6 (66 mg, eluted with light petroleum - EtOAc 70:30) was subjected to PTLC (silica gel, mobile phase methylene chloride - EtOAc 99:1) and gave two pure compounds: 2.8 mg of **1** (suberosin) and 2.7 mg of **3** (tschimgin). Fraction 15 (11.3 mg, eluted with light petroleum - EtOAc 45:55) was further purified by PTLC (silica gel, mobile phase methylene chloride - EtOAc 20:1, two-fold development) to afford 5.8 mg ferutinin **4** and 2.1 mg teferin **5**.

Antimicrobial tests

For the investigation of the antibacterial activity, the agar cup method [19] was used with test strains *Staphylococcus aureus* 209, *Escherichia coli* WF+ and *Candida albicans* 562 (obtained from the Bulgarian Type Culture Collection, institute for State Control of Drugs, Sofia). An inhibitory zone with a diameter less than 10 mm corresponds to lack of activity (10 mm is the diameter of the cup). 0.1 ml of test solution containing 0.4 mg of each extract and substance in ethanol was applied to every cup (concentration of the test solution 4 mg/ml). Control experiments with solvent showed that it does not have any activity. Streptomycin and nystatin were used as positive controls.

DPPH free radical scavenging activity

DPPH free radical scavenging activity was measured according to the procedure described in [20]. In brief, the extracts and tested compounds were dissolved in ethanol (0.36 mg/ml), and to 2 ml DPPH solution(0.1 mM in ethanol) 30 μl of the analyzed solution was added. The resulting solution was thoroughly mixed and absorbance was measured at 516 nm after 20 min. The scavenging activity was determined by comparison of the absorbance with blank (100%), containing only DPPH and solvent. Caffeic acid was used as positive control.

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Authors' contributions

BT participated in the separation and identification of the compounds and performed DPPH tests. IT performed performed the extractions and some of the chromatographic separations. MN and HN performed the antimicrobial tests. AD performed sample collection. VB conceived of the study, participated in its design and coordination and contributed to drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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