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Characterization of the pungent principles and the essential oil of *Zanthoxylum schinifolium* pericarp

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The pungent principles and the essential oil from the pericarp of *Zanthoxylum schinifolium* (Rutaceae) have been investigated and compared to those of *Z. bungeanum*, the primary source of the traditional Chinese drug Huajiao (Pericarpium Zanthoxyli). HPLC-MS and HPLC-NMR analyses revealed an alkylamide profile highly similar to that of *Z. bungeanum*, with hydroxy-α-sanshool and hydroxy-β-sanshool being in both plants the major constituents of the alkylamide fraction. GC-FID and GC-MS analyses of the essential oil showed that limonene was, like in *Z. bungeanum*, the main component (21%), followed by 4-terpineol, γ-terpinene, α-terpineol acetate, β-pinene, α-terpineol and β-linalool.

1. Introduction

Zanthoxylum schinifolium Sieb. & Zucc. (Syn. *Fagara schinifolia* Engler) is a spiny shrub distributed in China, Korea, Japan and Taiwan. The genus *Zanthoxylum* (Rutaceae) commonly called "prickly ash" comprises about 200 species of shrubs and trees predominantly distributed in the tropics. Several species have been used for centuries in culinary and medicinal practice. The dried pericarp of the ripe fruits produces a tingling sensation and is used throughout Asia as a pungent condiment known as Szechuan pepper. Depending on the geographical location, different species such as *Z. bungeanum*, *Z. piperitum* or *Z. schinifolium* are employed. In addition to being used as a spice, several *Zanthoxylum* species have found applications in traditional medicine, in particular for the treatment of disorders of the digestive organs, as local anaesthetic agent, anthelmintic and insecticide (Perry 1980). The constituents responsible for the tingling, anaesthetic and insecticidal properties are unsaturated fatty acid amides of sanshool- and bungeanool-types (Bryant and Mezine 1999; Xiong et al. 1997). The tingling sensation is apparently unique to this class of compounds, but poorly understood, as no specific receptor has been identified so far (G. Appendino, personal communication). In addition, the pericarp contains essential oil adding to the characteristic flavour of the fruit.

In China, the traditional drug Huajiao consists of the dried pericarp of the ripe fruits from *Z. bungeanum* or *Z. schinifolium* (Stöger and Friedl 2003). While there have been several studies on the alkylamides of various *Zanthoxylum* species, including *Z. bungeanum* (Mizutani et al. 1988; Xiong et al. 1997) and *Z. piperitum* (Hatano et al. 2004), information about the pungent principles of *Z. schinifolium* and the constituents of the fruit remains scarce and hardly accessible. TLC and HPLC fingerprint analyses have been presented as part of a monography on the drug Huajiao (Müller-Jakic et al. 2001). The coumarins bergapten and umbelliferone, and the quinoline alkaloids, skimmianine and schinifoline have been isolated from the fruit peel (Liu et al. 1991). In addition, a few analyses have been performed on the essential oil (Liu and Wei 1991; Lee 1998; Paik et al. 2005). In fact, investigations have rather focused so far on other plant organs: quinoline alkaloids and coumarins have been reported from the roots (Brader et al. 1993; Chang et al. 1997; Chen et al. 1995; Hong et al. 1992; Tsa et al. 1998; Tsa et al. 2000) and stems (Jo et al. 2002; Pan et al. 1993). A new phenolic acid amide, cis-fagaramide, together with coumarins, alkaloids and various terpenes have been isolated from the leaves (Cheng et al. 2002).

The scarcity of available data prompted us to reinvestigate the constituents of *Z. schinifolium* pericarp. Here, we report on the pungent principles and the essential oil. A comparison of the alkylamides and the oil composition of *Z. schinifolium* and *Z. bungeanum* by HPLC-UV-MS and GC analyses is presented.

2. Investigations and results

2.1. Pungent principles

In order to localize the pungent activity, the dried pericarps of *Z. schinifolium* and *Z. bungeanum* were submitted to different extraction procedures. Whole extracts were prepared by supercritical fluid extraction with CO_2, and CO_2 containing 5 and 10% of EtOH as a modifier. At the same time, the volatile constituents were selectively obtained by steam distillation. Organoleptic assessment of the SFE extracts revealed that the pungent principles were found mainly in the pure CO_2 extract, while no pungent effect at all was detected in the essential oil. TLC analysis of the CO_2 extracts of *Z. schinifolium* and *Z. bungeanum*

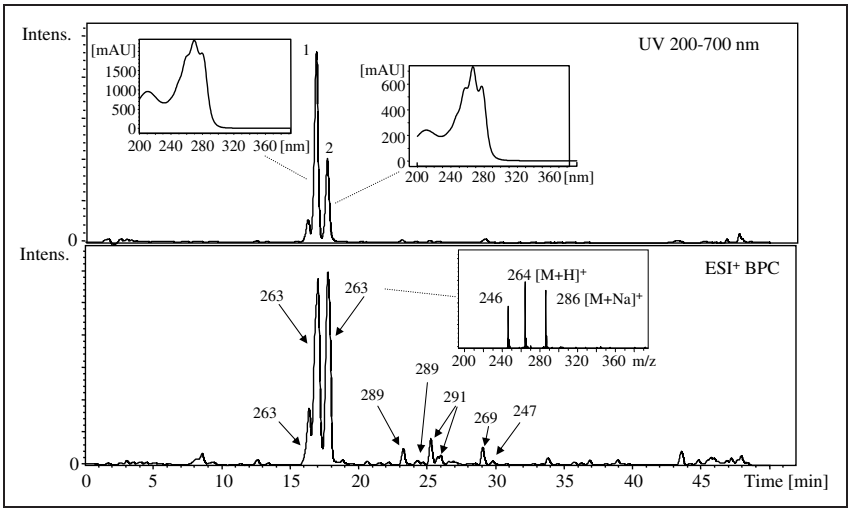
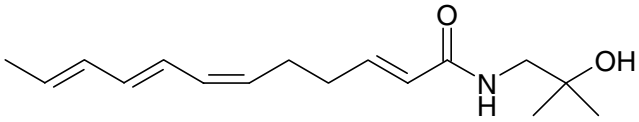


Fig. 1:
HPLC-UV/MS analysis of the CO_2 extract of *Z. schinifolium* pericarp

gave a highly similar pattern with, in both extracts, a prominent UV absorbing spot (Rf 0.42, system 1) which turned dark brown upon treatment with vanillin/sulphuric acid. HPLC-UV-MS analyses showed a group of three main peaks (Rt 16.3, 16.9, 17.7 min) with identical molecular mass (263 amu) (Fig. 1). In MS^2 experiments performed on the $[M + H]^+$ quasimolecular ions, the three compounds gave similar fragmentation patterns, with main fragments observed at m/z 246 and 147. The UV spectra exhibited three absorption maxima around 270 nm which were characteristic of an aliphatic conjugated double bond system and in good agreement with data reported for sanshool derivatives (Mizutani et al. 1988). Final proof was obtained by an HPLC-NMR analysis of the CO_2 extract from *Z. schinifolium* (Fig. 2) which at the same time enabled to identify both major peaks. Thus, the compounds eluting at 16.9 and 17.7 min are hydroxy-α-sanshool (**1**) and hydroxyl-β-sanshool (**2**), respectively. On the other hand, due to the low concentration and the incomplete chromatographic resolution, no structure could be conclusively assigned to the smaller peak at 16.3 min. The HPLC-UV-MS analysis of *Z. schinifolium* showed further minor components, the structures of which can be tentatively assigned to bungeanool and sanshool derivatives previously reported in *Z. bungeanum*. Thus, the UV and MS data of the peaks at Rt 23.2 and 24.2 min ($[M + H]^+$ 290) agreed well with hydroxy-γ-sanshool and hydroxy-γ-isosanshool, and those of peaks at Rt 25.2 and 25.9 min ($[M + H]^+$ 292) with bungeanool and isobungeanool. Finally, the peak at 29.8 min ($[M + H]^+$ 248) probably corresponds to α- or β-sanshool.



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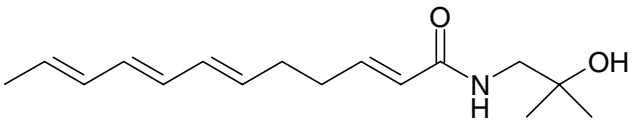

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2.2. Volatile constituents

The volatile constituents of *Z. schinifolium* and *Z. bungeanum* were compared by GC-MS and GC-FID analyses of the essential oil obtained by steam distillation. The com-

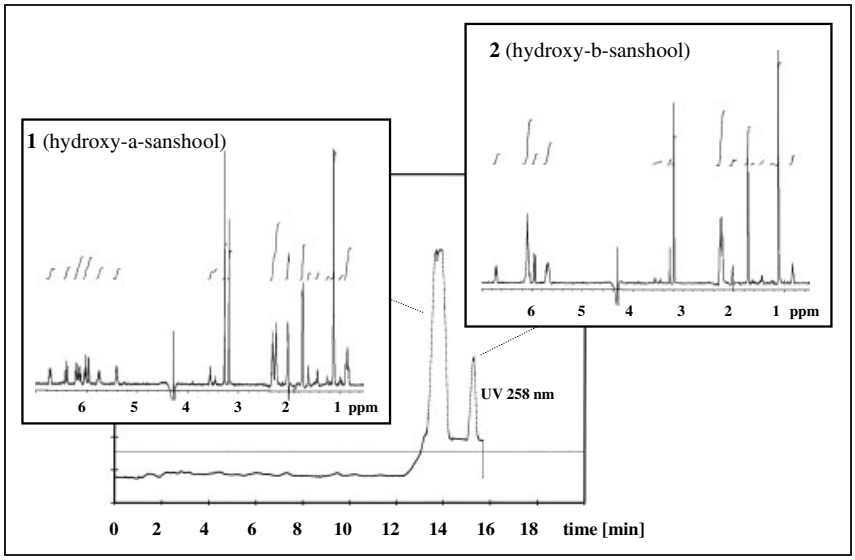

Fig. 2:
HPLC-NMR analysis of the CO_2 extract of *Z. schinifolium* pericarp. The chromatogram recorded for the analysis of **2** is shown. MeCN−D_2O (0.7% TFA) 36 : 64 to 50 : 50 in 25 min

Table: Composition of the essential oils of *Z. schinifolium* and *Z. bungeanum* from GC-FID analyses

Compound	RT (min)	*Z. schinifolium* Relative conc. (%)	*Z. bungeanum* Relative conc. (%)
α-Pinene*	4.36	1.04	0.74
Sabinene	4.89	1.94	1.87
β-Pinene*	5.08	5.07	4.31
α-Phellandrene	5.33	0.55	0.55
α-Terpinene*	5.52	2.74	4.37
p-Cymene	5.64	2.33	0.66
Limonene*#	5.77	21.63	18.48
β-*Z*-Ocimene	5.93	1.10	1.19
γ-Terpinene	6.17	6.01	8.19
4-Carene	6.63	1.49	1.81
β-Linalool*	6.74	4.32	5.18
4-Terpineol*	8.08	14.50	15.98
α-Terpineol*	8.24	4.55	4.34
Piperitone	9.17	1.97	2.55
Anethol*	9.60	1.98	0.59
α-Terpineol acetate*	10.55	5.33	5.13
Geraniol acetate	10.89	0.63	0.51
β-Elemene	11.25	1.13	0.74
β-Caryophyllene*	11.71	1.92	1.57
Cadinene	12.99	2.15	2.29

* Identification confirmed by comparison with a commercial standard.
# Limonene peak contains small amounts of incompletely resolved cineol and β-*E*-ocimene

position of the essential oil of both plants as determined by GC-FID is reported in the Table. The oils of the two species had a similar composition. Limonene was the main constituent, and large amounts of β-linalool, β-pinene, α-terpinene, γ-terpinene, 4-terpineol, α-terpineol and α-terpineol acetate were found. We investigated the effect of the extraction procedure on the composition of the volatile constituents and, therefore, analysed the CO_2 extract of *Z. schinifolium*. The GC-MS analysis showed that this extract contained, compared to the hydrodistillate, only traces of the highly volatile monoterpenes α- and β-pinenes, sabinene, α-phellandrene, α- and γ-terpinene and 4-carene, as well as less 4-terpineol, while the contents of β-*Z*-terpineol and 4-terpineol acetate were on the other hand much higher (Fig. 3).

3. Discussion

The alkylamide profile of *Z. schinifolium* agrees well with the chromatographic fingerprints previously reported in the Huajiao monography (Müller-Jakic et al. 2001). In the latter work, however, compound identification was only based on UV spectral data and remained therefore questionable. At the same time, in an earlier survey on Japanese *Zanthoxylum* species, no unsaturated aliphatic acid amides had been detected in the pericarp of *Z. schinifolium* (Yasuda et al. 1982). However, the latter finding appears rather doubtful when considering the well established pungent properties of the plant. The alkylamide patterns of *Z. schinifolium* and Z. *bungeanum* are almost identical. Hydroxy-α-sanshool and hydroxy-β-sanshool are by far the major constituents in both species. Since a *Z*-configuration in the alkyl chain is required for pungency (Galopin et al. 2004), hydroxy-α-sanshool (**1**) is the primary pungent principle found in the pericarp of both plants. On the basis of the TLC and HPLC-analyses, as well as from the organoleptic assessment, the alkylamide concentration appears however to be lower in *Z. schinifolium*.

The composition of the essential oil of *Z. bungeanum,* in particular the large amounts of limonene, 4-terpineol and β-pinene, is in accord with the results of previous ana-

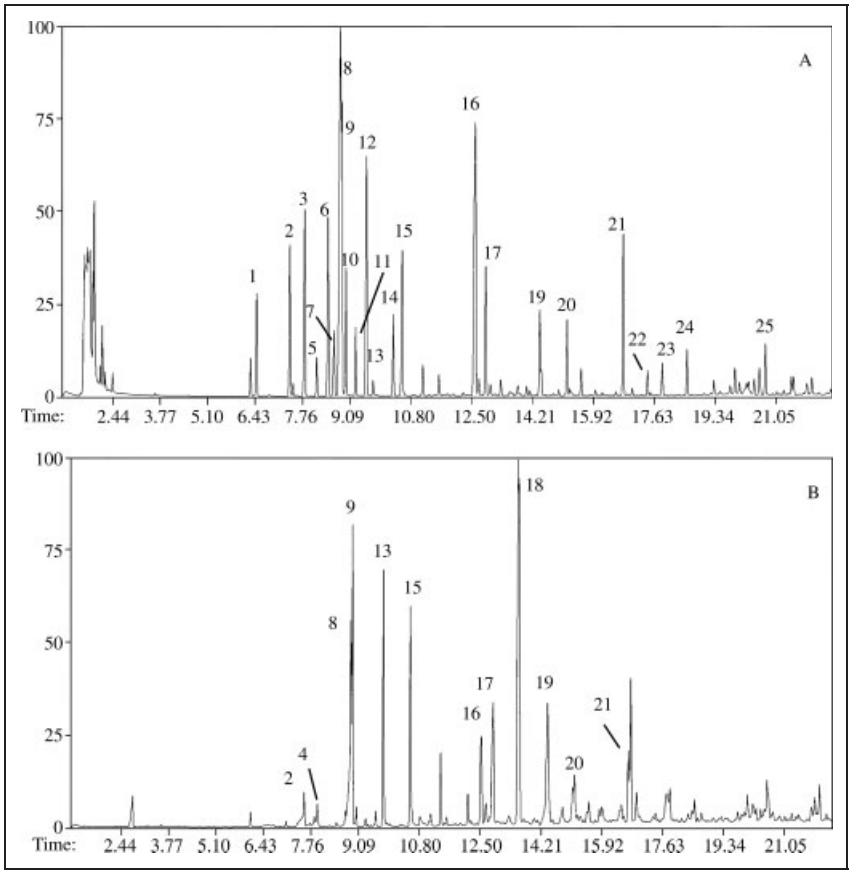

Fig. 3:
Capillary GC-MS chromatograms of the volatile components from the fruits of *Z. schinifolium* obtained by steam distillation (A) and supercritical CO_2 extraction (B). Compounds: 1 α-pinene*, 2 sabinene*, 3 β-pinene*, 4 β-myrcene*, 5 α-phellandrene, 6 α-terpinene*, 7 *p*-cymene*, 8 limonene*, 9 cineol*, 10 β-*E*-ocimene, 11 β-*Z*-ocimene, 12 γ-terpinene*, 13 β-*Z*-terpineol, 14 4-carene, 15 β-linalool*, 16 4-terpineol*, 17 α-terpineol*, 18 4-terpineol acetate*, 19 piperitone, 20 anethol*, 21 α-terpineol acetate*, 22 geraniol acetate, 23 β-elemene, 24 β-caryophyllene*, 25 α-cadinene. * Identification confirmed by comparison with a commercial standard

lyses (Li et al. 2001; Tirillini et al. 1991). The essential oils of *Z. schinifolium* and *Z. bungeanum* have a similar composition and differ primarily in the respective amounts of their constituents. Previous reports indicating the presence of predominant amounts of geranyl acetate and citronellal (Lee 1998; Paik et al. 2005) in the essential oil of *Z. schinifolium* could not be confirmed. Compared to the hydrodistillate, the supercritical CO_2 extract of *Z. schinifolium* contained less 4-terpineol but large quantities of 4-terpineol acetate. This observation suggests that the latter undergoes facile hydrolysis during steam distillation.

The highly similar alkylamide pattern and essential oil composition of Z. *schinifolium* and *Z. bungeanum* support the indistinctive use of both species as source of Huajiao in the Chinese traditional medicine. Further studies are, however, required to compare the alkylamide contents of both sources and evaluate their respective potency.

4. Experimental

4.1. General

TLC was carried out on silica gel 60 F_{254} precoated Al sheets (Merck, Darmstadt, Germany) with $CHCl_3$–MeOH 9 : 1 (System 1). Samples were applied using a Linomat IV (Camag, Muttenz, Switzerland). Detection was performed with vanillin-sulfuric acid reagent. HPLC-UV/MS analyses were carried out on an Agilent 1100 Series system consisting of binary pump, column oven and PDA detector, connected to a Gilson 215 injector (Gilson, Middleton, USA) and coupled to an Esquire 3000plus ion trap mass spectrometer (Bruker daltonic, Bremen, Germany). HPLC-NMR analyses were performed on a 600 MHz Varian INOVA spectrometer (Varian AG, Zug, Switzerland) coupled to a Varian HPLC system consisting of a HPLC pump 9012 and a diode array detector 9065.

4.2. Plant material

The dried pericarps of *Zanthoxylum bungeanum* Maxim. and *Z. schinifolium* Sieb. et Zucc. were purchased in October 2004 on a public market in Beijing. The identity of the plant material was confirmed by Prof. Yicun Huang, Chinese Academy of Science, Institute of Microbiology, Beijing, China. Voucher specimens have been deposited at the Institute of Pharmaceutical Biology, University of Basel, Switzerland.

4.3. Supercritical fluid extraction

Before extraction, the plant material was cryo-milled in an ultracentrifugal mill (Retsch ZM100, Haan Germany) under continuous cooling with liquid nitrogen. Extractions were carried out using an Isco (Lincoln, NE, USA) SFX 200/220 SFE system consisting of two syringe pumps, a two channel thermostated extraction module, a controller and a variable thermostated restrictor. An Isco polymer cartridge was filled with 2.5–3.0 g of a 1 : 1 mixture of powdered plant material and Kieselgur (Riedel-de Haën, Seelze, Germany). The cartridge was successively extracted for 2 h with pure CO_2 and CO_2–EtOH mixtures containing 5% and 10% of modifier, respectively. The pressure was set at 300 bars. The extraction temperature was 50 °C and the restrictor temperature was set at 100 °C (*Z. bungeanum*) or 70 °C (*Z. schinifolium*). Extracts were collected at the restrictor outlet in glass tubes containing 15 ml EtOH.

4.4. Steam distillation

Steam distillation was performed using a distillation device according to the European Pharmacopoeia. 10 g of grinded plant material were boiled in 100 ml of H_2O for 2 h at a distillation rate of 2–3 ml/min. The distillate was collected in 0.6 ml *n*-hexane, previously saturated with water for 30 min. For organoleptic assessments a separate sample was prepared using the same procedure without *n*-hexane.

4.5. Organoleptic assessment

3–5 µl of extract were spotted onto a 1 cm^2 piece of filter paper. After drying, the paper was placed onto the tongue of the test person and the sensation was scored. Each extract was assayed by a panel of three persons. Between two assays, a regeneration time of 30 min was allowed.

4.6. HPLC-UV-MS analyses

Separations were achieved on a Nucleodur® 100-5 C18 cartridge (4 × 125 mm, 5 µm, Macherey Nagel) with a gradient of acetonitrile in H_2O (35 : 65 to 75 : 25 in 40 min), flow rate 0.5 ml/min. The ESI-MS spectra were recorded in the positive ion mode. The capillary voltage was 4500 V, the capillary end voltage 112.8 V and the skimmer voltage 40.0 V. The nebulizer gas pressure was set to 30 psi, the dry gas flow to 10 l/min and the dry temperature to 350 °C.

4.7. HPLC-NMR

Analyses were performed on a Nucleosil® 120-5 C-18 column (4.6 × 150 mm, 5 µm, Macherey Nagel) with a gradient of acetonitrile in D_2O containing 0.07% TFA. The gradient was 38 : 62 to 58 : 42 in 25 min for compound **1** and 36 : 64 to 50 : 50 in 25 min for compound **2**. The flow rate was 1.0 ml/min and the injection volume 90 µl. Detection was at 258 nm. ^{1}H NMR-Spectra were recorded in the stop flow mode.

4.8. GC analyses of volatile oil constituents

Qualitative analyses were carried out on a GC-17 gas chromatograph coupled to a QI 5000 quadrupol mass spectrometer (Shimadzu, Kyoto, Japan). The separations were performed on an Optima® 5 MS capillary column (5% diphenyl-95% dimethylpolysiloxan; 30 m × 0.25 mm i.d., 0.25 µm) (Macherey-Nagel) with a gradient from 50 °C to 210 °C at a rate of increase of 6 °C/min. The temperature was held at 50 °C for 2 min and at 210 °C min for 1.3 min. The injector temperature was 160 °C and the interface temperature 230 °C. The gas flow was set at 1.7 ml/min with a splitting ration of 1 : 4. The detector voltage was 1.4 kV and the m/z range 100–300. 0.1–5.0 µl were injected. Compounds were identified by means of the NIST database (version 2.02a, Juli 2002). Identity of the main constituents was further confirmed by comparison with commercial standards.

Quantitative determinations were performed on a gas chromatograph 3600 with flame ionisation detector (Varian, Palo Alto, CA, USA). An identical column as above was used with a gradient of 75 °C to 210 °C at a rate of increase of 10 °C/min. The temperature was held at 75 °C for 2 min and at 210 °C for 4.5 min. The injector temperature was 220 °C and the interface temperature 230 °C. The gas flow was set at 15–30 ml/min. The essential oil was diluted to 1 : 100 with *n*-hexane and 5 µl were injected.

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ERRATUM

In the paper

Pharmazeutisches Institut der Universität[1], Bonn, Germany, Institute of Chemistry and Chemical Technology[2], MAS, Ulaanbaatar, Mongolia, Department of Biology and Pharmaceutical Botany[3], Medical University of Warsaw, Poland

Phytochemical and analytical studies of extracts from *Rhodiola rosea* and *Rhodiola quadrifida*

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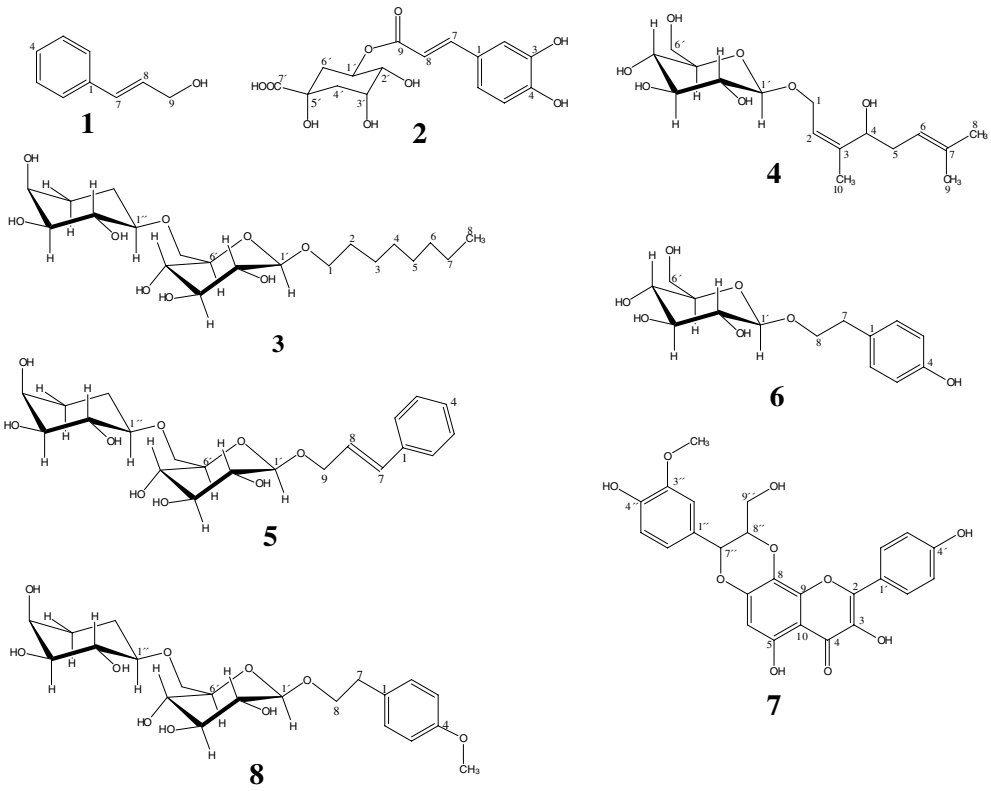
the sugar moieties were drawn incorrectly. The correct figures are shown here:



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