



Original Article

 Constituents from the bark resin of *Schinus molle*

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ABSTRACT

A total of five terpenes was isolated from the bark resin of *Schinus molle* L., Anacardiaceae, and their structures were determined by spectroscopic techniques. Among these compounds the sesquiterpene hydrocarbon terebinthene showed significant growth inhibitory activity against human colon carcinoma HCT-116 cells. Furthermore, terebinthene and pinicolic acid (**5**) also showed antibacterial activity against *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633.

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Introduction

The family Anacardiaceae is of pantropical occurrence and includes a few representatives in temperate regions. The family comprises about 70 genera and 600 species. Many of them are used traditionally as healing, stomachic and antidiarrheal agents, due to the presence of tannins and oil resins (Duarte et al., 2006). In this family, *Schinus molle* L. (also known as Californian pepper and pink pepper) was introduced from South America to most tropical and subtropical areas of the world, as well as the Mediterranean (Rhouma et al., 2009). In Peruvian traditional medicine *S. molle* is used as antibacterial, topical antiseptic, digestive and purgative diuretic (Duke, 2002), for toothache, wound healing, rheumatism, and menstrual disorders, as well as stimulant and antidepressant (Machado et al., 2007), for respiratory and urinary infections (Perez and Anesini, 1994), and as analgesic and central depressant (Barrachina et al., 1997). Extracts of *S. molle* showed promising anti-tumoral effects and cytotoxic activity (IC₅₀ 50 ± 7 µg/ml) against a human hepatocellular carcinoma Cell Line, Hep G was also reported (Ruffa et al., 2002). Recent studies revealed that *S. molle* essential

oil was cytotoxic in several cell lines, and it was more effective on breast carcinoma and leukemic cell lines (Díaz et al., 2008).

Extensive investigations of the fruit and leaf essential oils of *S. molle* identified a broad variety of constituents (Abdel-Sattar et al., 2010; Bendaoud et al., 2010), showing that the ingredients are similar, but with differences in their percentage depending on the region in which they are grown. Main components are monoterpenes hydrocarbons and oxygenated monoterpene hydrocarbons. No reports were found concerning isolation and characterization of secondary metabolites from the bark resin. Therefore, we decided to concentrate our effort in *S. molle* resin.

Material and methods

NMR and MS infrastructure and methods

NMR spectra (¹H, ¹³C, APT, NOESY1D, *H,H*-COSY, edited HSQC, and HMBC) were recorded on a Varian Mercury 400 plus (400 MHz for ¹H, 100 MHz for ¹³C) and a Varian Mercury 300 plus (300 MHz for ¹H, 75 MHz for ¹³C) spectrometer, respectively, at 26 °C and with CDCl₃ as a solvent. The chemical shifts were reported relative to the residual solvent peak, used as an internal reference (¹H 7.26 ppm, ¹³C 77.16 ppm). Chemical shifts are given in δ values, coupling constants *J* in Hz.

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Plant material

The resin bark of “molle” was collected in March 2009 from Trujillo-Peru and the species was identified as *Schinus molle* L., Anacardiaceae, by Eric F. Rodríguez Rodríguez at Herbarium Truxillense (HUT), National University of Trujillo, Peru. A voucher specimen under No. 58335 (HUT) documenting the collection was deposited at Herbarium Truxillense (HUT) in Peru.

Extraction and isolation

Spot tests were used for the qualitative determination of secondary metabolites present in the resin of *S. molle* (Dominguez, 1973; Harborne, 1984). We identified small amounts of steroids and triterpenoids (dark green) by the Liebermann-Burchard's test, and flavonoids (red) by the Shinoda's test. No alkaloids were detected using Dragendorff's test.

The resin of *S. molle* (10.14 g; still containing pieces of wood and insoluble materials) was extracted with CH_2Cl_2 and hexane (250 ml each). Both extracts were combined yielding 2.63 g after removal of the solvents. This residue was fractionated by column chromatography on silica gel eluting with hexane, called fraction 1 (0.92 g) and by using CH_2Cl_2 system to give fraction 2 (1.50 g). Fraction 1 was subjected to column chromatography with hexane/ CH_2Cl_2 (100:0 \rightarrow 99.5:0.5 \rightarrow 99:1) system to give germacrene D (**1**) (264 mg; R_f : 0.81) and terebinthene (**2**) (18.4 mg; R_f : 0.91).

Fraction 2 was subjected to column chromatography with the eluent system $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ (1:0 \rightarrow 2:0.1 \rightarrow 3:0.1 \rightarrow 4:0.2 \rightarrow 5:0.3) to furnish isomasticadienonic acid (**4**) (120 mg; R_f : 0.65) and to $\text{CH}_2\text{Cl}_2/\text{EtOAc}$ (5:0.3 \rightarrow 3:1 \rightarrow 2:1.5 \rightarrow 1:2) resulting in the isolation of isomasticadienoic acid (**3**) (336 mg; R_f : 0.6) and pinicolic acid (**5**) (18.6 mg; R_f : 0.2).

Bioactivity assays

Cytotoxicity screen

The human HCT-116 colon carcinoma cell line (ACC-581) was obtained from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung für Mikroorganismen und Zellkulturen, DSMZ) and was cultured under conditions recommended by the depositor. Cells were seeded at 6×10^3 cells per well of 96-well plates in 180 μl complete medium (90% McCoy's 5A + 10% FBS) and treated with sesquiterpene hydrocarbon terebinthene (**2**) in serial dilution after 2 h of equilibration. Terebinthene (**2**) was tested in duplicate as well as the internal solvent control. After 5 d incubation, 20 μl of 5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in PBS (phosphate-buffered saline; pH 7.4) was added per well and it was further incubated for 2 h at 37 °C. The medium was then discarded and cells were washed with 100 μl PBS before adding 100 μl 2-propanol/10 N HCl (250:1) in order to dissolve formazan granules. The absorbance at 570 nm was measured using a microplate reader (Tecan Infinite M200Pro), and cell viability was expressed as percentage relative to the respective solvent control. IC₅₀ values were determined by sigmoidal curve fitting and values represent the average \pm SD of two independent measurements.

Bacteria strains assay

The strains used were *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* (isolated at the Hospital Regional Docente de Trujillo, Peru). The bacterial cultures were grown in Mueller-Hinton agar media incubated at 37 °C for 24 h following literature (CLSI, 2012).

Agar-well diffusion methods: The agar-well with diameter of 5.5 mm were inoculated to a concentration equivalent to

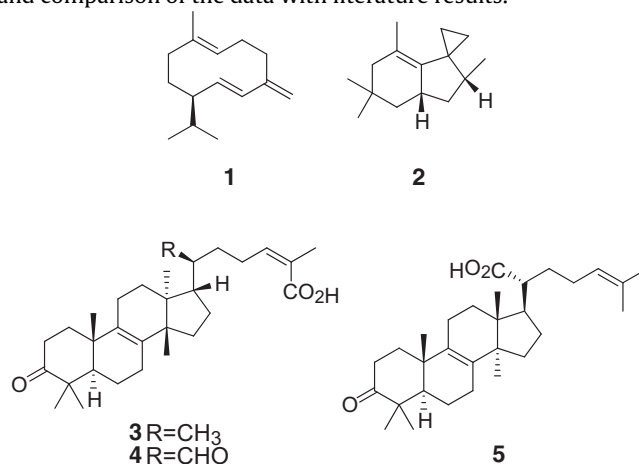
0.5 McFarland. Compounds **2** and **5** were inoculated at 40 μl in DMSO, at concentrations of 5 and 10 mg/ml. After bacterial diffusion for 10 min, those petri plates were incubated at 37 °C for 22 h.

Broth macrodilution MIC (minimal inhibition concentration)

Dry extract CH_2Cl_2 (**3–5**) was diluted in DMSO, tween 80 and water in ratio 2:1:3 according to the following concentrations: 0.062, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg/ml. Each MIC test was repeated twice and all tubes were incubated for 22 h at 37 °C. After incubation MIC results were determined as minimum concentration which showed no visible bacterial growth. Then 50 μl of resazurin sodium salt (Sarker et al., 2007) were added to each tube to a final concentration of 0.02%, and the samples were incubated at 37 °C for 1 h. MIC was confirmed as minimum concentration when no color change of the resazurin sodium salt (from blue to pink) happened.

Results and discussion

The structure of all five already known compounds germacrene-D (**1**), terebinthene [(6*R*,8*R*)-9-spiro(cyclopropa)-2,4,4,8-tetramethylbicyclo[4.3.0]non-1-ene(**2**)], isomasticadienoic acid (**3**), isomasticadienonic acid (**4**), and pinicolic acid [3-oxo-5 α -lanosta-8,24-diene-21-oic acid (**5**)] was determined after careful purification of the compounds using spectroscopic methods and comparison of the data with literature results.



Germacrene D is a biogenetic precursor of many other sesquiterpene structures (Bülow and König, 2000), terebinthene was isolated from *S. terebinthifolius* (Richter et al., 2010), the triterpenoid acids **3** and **4** were found in the berries or the stem exudate of *S. molle* (Pozzo-Balbi et al., 1978; Abdel-Sattar et al., 2007) and **5** was identified as anti-inflammatory triterpenoid from *Ganoderma* genus (Ko et al., 2008). Surprisingly, there are no reports in literature for sesquiterpene hydrocarbon **2** as an ingredient of *S. molle*, but **2** was isolated for the first time from *S. terebinthifolius* (Richter et al., 2010). Compound **5** was not found to be active against any type of cancer, but induced platelet aggregation (Mosa et al., 2011).

Terebinthene (**2**): (6*R*,8*R*)-9-spiro(cyclopropa)-2,4,4,8-tetramethylbicyclo[4.3.0]non-1-ene: ¹H NMR (400.1 MHz, CDCl_3): δ = 0.49 (1H, m, H-10a), 0.53 (1H, m, H-11a), 0.60 (1H, m, H-10b), 0.69 (3H, s, CH_3 -15), 0.71 (1H, s, H-11b), 1.00 (1H, m, H-7a), 1.06 (3H, s, CH_3 -13), 1.08 (1H, H-5a), 1.10 (3H, s, CH_3 -14), 1.28 (3H, m, CH_3 -12), 1.63 (1H, m, J = 18.5 Hz, H-5b), 1.83 (1H, m, H-7b), 2.00 (1H, m, H-8), 2.12 (1H, m, H-3a), 2.12 (1H, m, H-3b), 2.65 (1H, br, H-6); ¹³C NMR (100 MHz, CDCl_3): δ = 6.33 (C-10), 7.36 (C-11), 13.00 (C-12), 17.29 (C-15), 24.46 (C-9), 29.66 (C-13), 30.70 (C-14), 34.24 (C-8), 37.15 (C-4), 38.27 (C-7), 41.33 (C-6), 45.80 (C-3), 48.57 (C-5), 126.04 (C-2), 139.25 (C-1).

Table 1
Diameters of inhibition zones at concentrations of 5 and 10 mg/ml.

Compounds	Concentration (mg/ml)	Diameter (mm)			
		<i>S. aureus</i> ATCC 25923	<i>B. subtilis</i> ATCC 6633	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i>
2	10	9.3	9.3	0	0
	5	8.0	8.6	0	0
5	10	7.1	9.0	0	0
	5	6.8	8.8	0	0

Table 2
MIC from the bark resin of *Schinus molle*.

Resin	MIC	
	<i>S. aureus</i> ATCC 25923	<i>B. subtilis</i> ATCC 6633
CH ₂ Cl ₂ extract	8.0 mg/ml	0.125 mg/ml

Cytotoxicity on HCT-116 cells

The *in vitro* cytotoxicity of **2** was evaluated in a tetrazolium salt-based assay (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) against human HCT-116 colon carcinoma cells. Compound **2** displayed a moderate inhibition of HCT-116 cells with IC₅₀ 14.23 ± 1.3 µg/ml.

Antibacterial activity

Additionally, we determined the antibacterial activity of compounds **2** and **5** by screening against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* (Table 1). The other constituents (**1**, **3** and **4**) are well known for their antibacterial activity and pharmacological properties (Duarte et al., 2006; Rhouma et al., 2009; Chantraine et al., 1998). The MIC of CH₂Cl₂ extract was reported in Table 2.

Conclusions

Here we report the isolation and characterization of five constituents from the bark resin of *S. molle*: germacrene *D* (**1**), terebinthene (**2**), isomasticadienoic acid (**3**), isomasticadienonic acid (**4**), and pinicolic acid (**5**). The resin of *S. molle* contained 10% of germacrene *D* (**1**), which is very high compared with other natural sources (Kapoor et al., 2009). Terebinthene (**2**) was identified as a constituent of *S. molle* for the first time and it showed significant cytotoxic activity against a human colon carcinoma cell line.

Authors' contributions

GRMG contributed running the laboratory work, and drafted the paper; LH did the NMR investigations; RWB contributed in collecting plant samples and revised the paper; MLGY and API carried out biological activity tests and wrote one part of the manuscript.

All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2016.07.004.

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