Ethnopharmacological evaluation of Radal (leaves of *Lomatia hirsuta*) through metabolite profiling, and isolation of 2-methoxyjuglone.

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Introduction

Lomatia hirsuta (Lam.) Diels ex Macbr. (Proteaceae) is a wild tree growing in Chile from Coquimbo to Chiloe (IV-X Regions). It is distributed from the sea to sub mountain zones through 700-1200 m of altitude. Other *Lomatia* species in Chile are *L. ferruginea* (Cav.) R.Br. and L. *dentata* (R. et P.) R. Br. *L. hirsuta* is also present in Argentina, Ecuador and Peru. Products prepared from the leaves of *L. hirsuta* are used in traditional medicine in Chile under the common name of Radal. A tea of Radal is used for treatment of cough, bronchial troubles, and asthma. In a preliminary screening a methanol extract of the leaves in contrast to an extract of the stem revealed antifungal activity in particular against *Candida albicans* (Lauritsen & Jorgensen, 2001). Since a previous phytochemical study of the leaves did not explain the antifungal activity the present study was undertaken. *C. albicans* and *Aspergillus fumigatus* were chosen as test organisms together with *Penicillium expansum*, since these organisms increasingly cause severe infections in patients with reduced immune response, e.g. HIV. (http://www.cdc.gov/ncidod/dbmd/mdb/diseases.htm).
The earlier study of *L. hirsuta* confirmed the presence of the coumarins umbelliferone and scopoletin and the flavonoids quercetin, rhamnetin, iso-rhamnetin, and quercetrine (Erazo et al., 1997). An infusion of the leaves of *L. hirsuta* was found to possess a mild anti-inflammatory effect (Erazo et al., 1997).
Naphthoquinones, such as lomatiol, juglone and naphthazasin, are major metabolites in some *Lomatia* species (Moir and Thompson, 1973), including native plants such as *L. ferruginea*, and *L. dentata*, but have not previously been found in *L. hirsuta* leaves.

Results and discussion

A bioactivity guided fractionation lead to isolation of 8.5 mg of 2-methoxyjuglone (**1**) from a methanol extract of *Lomatia hirsuta*. Among the tested microorganism **1** was found only to be active against *Candida albicans* with a MIC of 8 µg/ml.
Species belonging to the Proteaceae are known to contain phenolic compounds. A GC-MS analysis confirmed the presence of cinnamic acid (**2**) (approx. 0.4 % w/w of the tea residue), ethyl cinnamate (**3**) (approx. 0.04 % w/w of the MeOH extract), 4-hydroxybenzoic acid (**4**) (approx. 0.05 % w/w of the MeOH extract), vanillic acid (**5**) (approx. 0.05 % w/w of the tea residue), methyl vanillate (**6**) (approx. 0.04 % w/w of the MeOH extract), isovanillic acid (**7**) (approx. 0.02 % w/w of the MeOH extract) and 4-hydroxyacetophenone (piceol) (**8**) (approx. 0.02 % w/w of the MeOH extract). Cinnamic acid was the major constituent in the polar extracts. The compounds **1**-**8** have not previously been identified in *L. hirsuta*. The presence of scopoletin and umbelliferone as major constituents (Erazo et al. 1997) was confirmed by the GC-MS analyses.
Cinnamic acid (**2**) is a chemical marker for Proteaceae, and derivatives of juglone are also commonly found in genus *Lomatia*, but have not been reported in *L. hirsuta*.
The initial screening revealed antifungal activity of the methanol extract against *C. albicans* and against *P. expansum*, whereas no antibacterial activity was seen. The presence of **1** - **8** and the compounds reported in Erazo et al. (1997) can explain the antifungal activity, since **2** and **3** are reported to have broad antifungal activities (Narasimhan et al., 2004). Umbelliferone (Rodríguez-Gamboa et al., 2000), scopoletin (Carpinella et al., 2005), **4**, and **5** (Moura et al., 2004) have all been shown to possess antifungal and/or antibacterial properties. The presence of this range of compounds explains the antimicrobial effects seen in the preliminary study (Lauritsen & Jorgensen, 2001).
Erazo et al. (1997) showed that their extract had mild anti-inflammatory effect. Piceol (**8**) is known to possess anti-inflammatory properties in mice (Alvarez et al., 2000).
The tea of the leaves from *L. hirsuta* is locally used for the treatment of cough, bronchial troubles and asthma. In the residue of the tea we identified the compounds **2** and **5**, whereas none of the minor constituents have been identified. Compounds **1** and **3** have been found to be toxic, but the absence of these compounds in the tea makes it likely that the tea is harmless. This idea was supported by the absence of toxicity of tea residue towards *Artemia salina* in a concentration range from 0.5 to 5 mg/ml. It still remains to be established whether the tea could be toxic to human cells.

Conclusion

The identification of the compounds **1** - **8** along with those previously identified (Erazo et al., 1997) combined with the results of the pharmacological tests encourage further studies on the beneficial effects of tea of the leaves of *Lomatia hirsuta*.

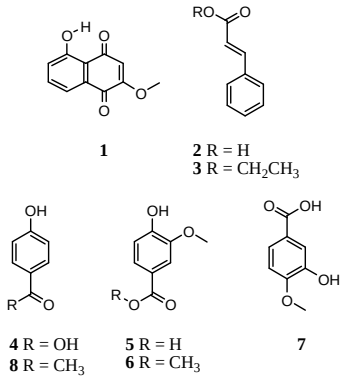

Fig 1. Identified compounds from *Lomatia hirsuta*

Fig 2. *Lomatia hirsuta (Lam.) Diels ex J.F. Macbr.*

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Plant material

The leaves of *Lomatia hirsuta* (Lam.) Diels ex J.F. Macbr. (Proteaceae) were collected next to the river Gol-Gol east of Osorno, Chile. The plants were identified in the field by Alfonso Guzman and verified by Professor Jaime Zapata from Universidad de Los Lagos, Osorno, Chile. Voucher specimens have been placed at Universidad de Los Lagos and at the Danish University of Pharmaceutical Sciences.

Extraction and isolation

For the initial screening, 1 g of the dried leaf powder was ultrasonicated with 15 ml of methanol for 30 minutes. The extract was concentrated after filtration to give an average yield of 20% w/w. The active constituents were isolated from 320 g of dried leaves, which were extracted with heptane and methanol to give after vacuum evaporation of the solvent 1.5 % w/w and 9.3 % w/w of a residue, respectively. The methanol extract was partitioned between EtOAc and H_2O, and the two phases were concentrated to yield 7.3 g and 21.5 g, respectively.
The residue of the EtOAc phase (7.3 g) was subjected to VLC (300 g silica gel, 0.063 - 0.2 mm, Merck, 10 x 10 cm column) using CH_2Cl_2, CH_2Cl_2-EtOAc 19:1 to 1:1, neat EtOAc, and neat MeOH as eluent. Repeated LC, of the CH_2Cl_2 fraction (performed on 80 g silica gel, 0.063 - 0.2 mm, Merck) yielded 8.4 mg of 2-methoxyjuglone eluting with CH_2Cl_2. The ^1H and ^{13}C NMR spectra (Varian 300 MHz at 25 °C, using TMS as standard) were similar to those reported (Moore & Scheuer, 1966). On TLC (Silica gel 60 F_{254}, Merck, eluent CH_2Cl_2) 2-methoxyjuglone elutes to Rf 0.4 and turns purple by colouring with KOH.
The tea of the leaves was prepared by mixing 200 ml of boiling water with 20 g of dried leaves and leaving the mixture for 30 minutes. The mixture was filtered and the filtrate concentrated by lyophilisation to give a yield of 9.4 w/w %.

Identification by GC-MS

All the obtained fractions, extracts, and the residue of the tea were analysed by GC-MS. The samples were dissolved in a appropriate solvent until a concentration of approximately 1 mg/ml was obtained. Initial temperature of the GC (Agilent 6890N) was hold at 50 °C for 2 minutes and then increased with a rate of 20 °C per minute until a final temperature of 300 °C that was hold for 5 minutes. Total run time was 20 minutes. The column was a capillary column (Agilent 19091S-433), length 30 m, diameter 250 µm, film thickness 0.25 µm. The flow was split by 1:100 before introductions into the MS detector (Agilent 5973, electron ionization (EI)). The EM voltage was 952.9 V. The obtained spectra were by Enhancen ChemStation, MSD ChemStation D.01.02.16 provided by Agilent compared with the spectra in the NIST/EPA/NIH Mass Spectral Library, Version 2.0 a ed. The library search was performed with the NIST Mass Spectral Search program. All samples were analysed three times, and cinnamic and vanillic acid were run as standards.

Antimicrobial screening

A direct bioautographic method was used to determine the activity against *Penicillium expansum* (IMI 285521) and *Aspergillus fumigatus* (IBT 25732). A thin-layer chromatographic agar overlay technique was used to determine the activity against *Candida albicans* (IMI 349010), *Bacillus subtilis* (ATCC 6633), *Pseudomonas aeruginosa* (ATCC 9027), *Staphylococcus aureus* (ATCC 6538) and *Escherichia coli* (ATCC 11229) (Simonsen et al., 2004). Minimum inhibitory amount (MIA) of the compounds was determined using amphotericin B as a positive control for *C. albicans* (MIA = 1.2 µg) and nystatin for *P. expansum* (MIA = 0.5 µg). A microplate method was used to determine minimum inhibitory concentration values (MIC) of pure compounds against *C. albicans*. The compounds were dissolved in DMSO and diluted with Sabouraud broth (SAB) to a final DMSO concentration of 2 %. To each well of a 96-well microtiter plate was added 50 µl of a test solution and 50 µl of a culture of *C. albicans* in SAB adjusted to mach a 0.5 MacFarland standard solution. The plates were incubated for 48 h at 30°C and the growth of the fungi assessed after addition of MTT and determination of absorbance at 560 nm with a Labsystems Multiscan EX microplate reader (Simonsen et al., 2004).

Artemia salina toxicity assay

The *Artemia salina* (brine shrimp) toxicity assay was performed as previously described (Solis et al., 1993). Six concentrations were tested in 96-well microplates in six fold, each well containing 10-20 nauplii.