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Extracts and compounds active on TRP ion channels from *Waldheimia glabra*, a ritual medicinal plant from Himalaya

Annamaria Giorgi[a], Angela Bassoli[b], Gigliola Borgonovo[b], Sara Panseri[c], Alessandra Manzo[a], Daniela Pentimalli[a,*], Aniello Schiano Moriello[d], Luciano De Petrocellis[d]

[a] *Centre of Applied Studies for the Sustainable Management and Protection of Mountain Areas (Ge.S.Di.Mont.), University of Milan, Via Morino 8, 25048 Edolo, Brescia, Italy*
[b] *Department of Food, Environmental and Nutritional Sciences, University of Milan, Via Celoria 2, 20133 Milan, Italy*
[c] *Department of Health, Animal Science and Food Safety (VESPA), University of Milan, Via Celoria 10, 20133 Milan, Italy*
[d] *Endocannabinoid Research Group, Institute of Biomolecular Chemistry, CNR, Pozzuoli, Napoli, Italy*

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ABSTRACT

Background: : *Waldheimia glabra* (Decne.) Regel is a wild plant from the Himalayan Mountains, commonly known as Smooth Ground Daisy. This plant is traditionally used by local populations in religious rituals (incense) or in traditional herbal medicine to treat skin diseases, headache, joint pain and fever. In literature few data are available on the investigation of this aromatic plant.
Purpose: The present work aims at deepening knowledge about the chemical composition of *W. glabra* extracts and incense, as well as its activity on TRP ion channels.
Methods: Extracts and incense of *W. glabra* were analyzed by using HS-SPME GC/MS, GC/MS and NMR analysis. Tests on the activity of *W. glabra* extracts and isolated compounds (+)-ludartin **1** and B-ring-homo-tonghaosu **2** on TRP channels were also performed.
Results: Some extracts and pure compounds from *W. glabra* showed an interesting activity in terms of efficacy and potency on rat TRPA1, an ion channel involved in several sensory mechanisms, including pungency, environmental irritation and pain perception. Activity is discussed and compared with that of other known TRPA1 natural agonists with different chemical structures. All compounds showed only a negligible inhibition activity on rat TRPM8 ion channel.
Conclusions: Our findings demonstrate that *W. glabra* is involved in the receptor activation mechanism and therefore represents a new natural product potentially useful in pharmaceutical and agrifood research.

Introduction

The Himalaya region is rich in Asteraceae species, many of which are confined in Alpine zones. Asteraceae is one of the biggest families of flowering plants and Anthemideae is the large tribe of this family (Bremer, 1994).

Waldheimia glabra (Decne.) Regel or *Allardia glabra* (Decne) belongs to this tribe and is a typical aromatic species able to grow in the Alpine zones of the Himalaya up to 4000–5400 m above sea level (a.s.l.) in particular in the north of Pakistan, Kashmir, Tibet, Nepal and Bhutan (Abid and Qaiser, 2009). Some species are mainly used for their analgesic, anti-inflammatory, antiseptic, gastrointestinal or immunostimulatory properties and even as ingredients for perfumes and cosmetics (Bhellum, 2013).

Locals normally use the plant to deal with skin diseases like itching skin or they apply it to wounds as an antiseptic paste (Ghimire et al., 2009). This therapeutic property was confirmed by our study conducted in 2012 on secondary metabolites of dried and powdered samples of *W. glabra*. In particular, we detected the presence of seychellene, a volatile compound characteristic of Patchouli essential oil (Giorgi et al., 2012) for the first time. Patchouli oil is known to be used as an antifungal agent and for skin infection (Kalra et al., 2006). Therefore, seychellene content can explain on the one hand the property of *W. glabra,* and on the other hand why locals normally use the plant for skin diseases. In

Abbreviations: TRP, Transient receptor potential; AITC, allyl isothiocyanate; EC_{50}, half maximal effective concentration; IC_{50}, half maximal inhibitory concentration

* Corresponding author.
E-mail addresses: anna.giorgi@unimi.it (A. Giorgi), angela.bassoli@unimi.it (A. Bassoli), gigliola.borgonovo@unimi.it (G. Borgonovo), sara.panseri@unimi.it (S. Panseri), alessandra.manzo@unimi.it (A. Manzo), daniela.pentimalli@unimi.it (D. Pentimalli), aniello.schianomoriello@icb.cnr.it (A. Schiano Moriello), luciano.depetrocellis@icb.cnr.it (L. De Petrocellis).

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addition, our recent study demonstrated an antibacterial activity of *W. glabra* essential oil against *E. coli* and *S. aureus* and cytotoxicity on human breast adenocarcinoma cells MCF-7 and MDA-MB-231 (Manzo et al., 2015).

Even during religious rituals the incense produced from the burning leaf plant is spread out in the air for its aromatic properties.

"Ghaan poe" or "high-mountain incense" are the two synonyms that represent the plant *W. glabra* (Rana Man and Samant, 2011; Gairola et al., 2014; Angmo et al., 2012; Ghimire and Aumeeruddy-Thomas, 2009).

There is a lack of information about the properties and effects of this aromatic plant in literature (Giorgi et al., 2012) and the present work aims at deepening the knowledge about the chemical composition and the pain relieving activity of *W. glabra* extracts and incense in order to support the traditional uses of the plant.

W. glabra extracts and incense were examined by GC/MS and HS-SPME GC/MS analysis and tests on the activity of *W. glabra* extracts on TRPA1 and TRPM8 channels were also performed. TRPA1 is a polymodal sensor involved in the somatosensory perception of several stimuli among which cold, pungent compounds, airborne irritants and cannabinoids as well as in audition, proprioception, neurogenic inflammatory responses and pain perception (Zygmunt and Högestätt, 2014; Nilius et al., 2012; Preti et al., 2015). TRPM8 is activated by cold and agents like menthol (an active ingredient of peppermint) and several other chilling compounds such as icilin, which induce a cool sensation. TRPM8 antagonists have therapeutic potential in neuropathy, inflammatory models (Knowlton et al., 2011; Calvo et al., 2012) inhibiting bladder reflexes (Lashinger et al., 2008) and can be beneficial in the treatment of aberrant cold sensitivity (Patel et al., 2014).

Material and methods

Plant material and extraction procedures

The aerial parts of *W. glabra* (Decne.) Regel were collected at 5050 m a.s.l. in the Khumbu Valley, Sagarmatha National Park, near the Pyramid Laboratory Observatory (27.95° N, 86.80° E) at the end of October 2009. A specimen was matched for confirmation of identity with the reference voucher number NP29 and it was preserved in the Herbarium of the Department of Agricultural and Environmental Sciences, University of Milan (Milan, Italy).

An aliquot of 10.00 g of dry aerial parts of *W. glabra* was extracted at room temperature for 5 h with magnetic stirring with five solvents (100 ml) of increasing polarity: hexane, Et_2O, CH_2Cl_2, MeOH, and H_2O. Each fraction was evaporated to dryness, except for the aqueous fraction, which was freeze-dried. The following extracts (recovery, g) were obtained: E1 (hexane) (0.25); E2 (Et_2O) (0.4); E3 (CH_2Cl_2) (0.2); E4 (MeOH) (0.8); E5 (H_2O) (0.5).

HS-SPME GC/MS analysis of W. glabra incense

2 g of dry aerial parts of *W. glabra* were enclosed in a customised aerated glass cage manufactured by COLAVER s.r.l. (Vimodrone, MI, Italy) and then burned. A manual SPME holder was inserted into the glass gage in order to extract the headspace. Volatile compounds were collected using a 50/30 μm divinylbenzene/Carboxen™/polydimethylsiloxane (DVB/CAR/PDMS) StableFlex™ fiber (Supelco, Bellefonte, PA, USA). The fiber was exposed to the incense for 4 h.

Analysis was performed using a Trace GC Ultra (Thermo-Fisher Scientific; Waltham, MA, USA) Gas Chromatograph coupled with a quadrupole Mass Spectrometer Trace DSQ (Thermo-Fisher Scientific; Waltham, MA, USA) and equipped with an Rtx-Wax column (30 m; 0.25 mm i.d.; 0.25 μm film thickness, Restek, PA, USA). The oven temperature program was: from 35 °C, hold 8 min, to 60 °C at 4/min, then from 60 °C to 160 °C at 6 °C/min and finally from 160 °C to 200 °C at 20 °C/min. Carry over and peaks originating from the fiber were regularly assessed by running blank samples. After each analysis, fibers were immediately thermally desorbed in the GC injector for 5 min at 250 °C in order to prevent contamination. Injections were performed in split mode ratio (20:1). The carrier gas was helium at a constant flow of 1 ml/min. The transfer line to the mass spectrometer was maintained at 230 °C and the ion source temperature was set at 250 °C. Mass spectra were obtained by using a mass selective detector with electronic impact of 70 eV and multiplier voltage of 1456 V. Data were collected at a rate of 1 scan/s over the *m/z* range of 30–350. Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analyzed under the same conditions, when available. The identification of MS fragmentation patterns was performed either by comparison with those of pure compounds or by using the National Institute of Standards and Technology (NIST) MS spectral database. Quantification of volatile compounds from *W. glabra* incense was carried out by peak area normalization (expressed in %). Analyses were performed in triplicate.

GC/MS analysis of W. glabra extracts

Extracts of *W. glabra* were injected into GC/MS at a concentration of 2 $\mu g/ml^{-1}$ in dichloromethane. Triple quadrupole mass spectrometry (QqQ) with electronic impact (EI) mode was used for the detection of volatile compounds in plant extracts.

A GC Trace 1310 chromatograph coupled to a TSQ8000 triple quadrupole mass detector (Thermo Fisher Scientific, Palo Alto, CA, USA) with a fused-silica capillary column Rt-5MS Crossbond-5% diphenyl 95% dimethylpolysiloxane (35 m × 0.25 mm i.d., 0.25 μm film thickness, Restek, Bellefonte, PA, USA) were used. The oven temperature program was as follows: initial temperature of 80 °C, hold for 3 min, and increased up to 170 °C at 10 °C/min; then increased from 170 °C to 190 °C at 3 °C/min, and raised to 240 °C at 2 °C/min, before being ramped to 280 °C at 3 °C/min and finally from 280 °C to 310 °C at 10 °C/min and hold at this temperature for 5 min. The carrier gas (helium, purity higher than 99.999%) was in constant flow mode at 1.0 ml/min^{-1}. A volume of 1 μl was injected using a programmed temperature vaporiser injector (PTV) in splitless mode with a 1-min splitless period and the following inlet temperature programme: 80 °C (0.05 min), 14.5 °C/s to 200 °C (1 min) and 4.5 °C/s to 320 °C (12 min of cleaning phase). A baffle liner (2 mm × 2.75 mm × 120 mm, Siltek-deactivated; Thermo Fisher Scientific) was used. The transfer line was maintained at 270 °C and the ion source at 250 °C. Mass spectra were obtained by using a mass selective detector with electronic impact of 70 eV, a multiplier voltage of 1456 V. Data were collected at a rate of 1 scan/s over the *m/z* range of 30–350. Compounds were identified by comparing the retention times of the chromatographic peaks with those of authentic compounds analyzed under the same conditions, when available. The identification of MS fragmentation patterns was performed either by comparison with those of pure compounds or by using the NIST MS spectral database. Quantification of volatile compounds from *W. glabra* extracts was carried out by peak area normalization (expressed in %). Analyses were performed in triplicate.

Structural determination of (+)-ludartin ***1****, [(+)-3α,4α-epoxyguaia-1(10),11(13)-dieno-12,6α-lactone]*

1D and 2D spectra were recorded on Bruker AMX-300 and Bruker Avance-600 instruments, using TMS as an internal standard; J values are given in Hertz. IR spectra were recorded on Perkin Elmer 1310 spectrometer. UV spectra were obtained from Perkin-Elmer Lamba 40 VU–vis spectrometer. Specific rotation was measured at room temperature by using Perkin-Elmer 141 polarimeter and the rotation was observed at 549 nm (D line of Na).

TLC was carried out on silica gel 60 F254 cards, 0.25 mm. Flash silica gel was used for column chromatography. Analytical HPLC was performed by Varian SD200 liquid chromatograph system with