Journal of Ethnopharmacology 155 (2014) 776–784

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research paper

Topical analgesic, anti-inflammatory and antioxidant properties of *Oxybaphus nyctagineus*: Phytochemical characterization of active fractions

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ARTICLE INFO

Article history:
Received 20 March 2014
Received in revised form
8 May 2014
Accepted 8 June 2014
Available online 16 June 2014

Chemical compounds studied in this article:
Patuletin-3-*O*-robinobioside (PubChem CID: 44575854)
Corchorifatty acid B (PubChem CID: 22956671)
9-hydroxy-10,12,15-octadecatrienoic acid (PubChem CID: 6439873)
9-hydroxy-10*E*,12*Z*-octadecadienoic acid (PubChem CID: 16061059)

Keywords:
Oxybaphus nyctagineus
Analgesic activity
Anti-inflammatory activity
6-methoxyflavonol diglycosides
Hydroxy-polyenoic fatty acids

ABSTRACT

Ethnopharmacological relevance: *Oxybaphus nyctagineus* (Michx.) Sweet has traditionally been used by several Native American tribes predominantly as a topical anti-inflammatory and analgesic agent.
Aim of the study: To evaluate the antioxidant, analgesic and anti-inflammatory activity of the extracts prepared from the aerial parts of *Oxybaphus nyctagineus* and to characterize the major chemical constituents of the bioactive extracts.
Materials and methods: Crude polar and apolar extracts (PCE and ACE) of the herb of *Oxybaphus nyctagineus* were prepared and tested in the models of the CFA-induced hyperalgesia in rat knee and carrageenan-induced paw edema in rat. To identify the active compounds, subfractions were prepared by column chromatography and subjected *in vitro* assays, such as antioxidant assays (DPPH, peroxynitrite ($ONOO^-$) scavenging), and the LPS-induced IL-1β release test in human monocytes. Preparative HPLC was employed for the isolation of active substances, while phytochemical analysis was performed by mean of LC–MS/MS and NMR.
Results: The topically administered PCE and ACE of *Oxybaphus nyctagineus* demonstrated a significant analgesic and anti-inflammatory effect in the inflammation animal models. The subfraction A4 of ACE and the subfraction P5 of PCE considerably inhibited the LPS-induced IL-1β release in human monocytes, while the strongest activity was localized in the subfraction P5 in the antioxidant assays. The HPLC–MS/MS and NMR analysis revealed that 6-methoxyflavonol diglycosides, namely patuletin-3-*O*-robinobioside (**1**), 6-methoxy-kaempferol-3-*O*-robinobioside (**2**), spinacetin-3-*O*-robinobioside (**3**), and hydroxy-polyenoic fatty acids, namely corchorifatty acid B (**4**), 9-hydroxy-10*E*,12*Z*,15*Z*-octadecatrienoic acid (9-HOT acid) (**5**), and 9-hydroxy-10*E*,12*Z*-octadecadienoic acid (9-HOD acid) (**6**) were present in PCE, and in ACE as major compounds.
Conclusion: The results of this study established a pharmacological evidence for the traditional use of *Oxybaphus nyctagineus* as an anti-inflammatory agent used topically, and provided data on its phytochemical composition for the first time.

1. Introduction

Oxybaphus nyctagineus (Michx.) Sweet (syn.: *Allionia nyctaginea*, *Mirabilis nyctaginea*) commonly known as heart-leaf four-o'clock or heart-leaf umbrella-wort belongs to the family Nyctaginaceae. The common names of this perennial plant refer to the physiology of blooming and the morphology of the flowers. The small, pink flowers of wild four-o'clock open in the late afternoon (around 4 p.m.) from May to September, which are covered by large papery bracts forming 'umbrellas' (Cruden, 1973; Cardina et al., 2009). The wild four o'clock is native to North America (Spellenberg, 2004), but by now, due to its ornamental value (Carrol, 2006), spreading strategy and high tolerance (Doll, 2002), it is widely distributed as an invasive species in several European countries, e.g.: in Austria (Melzer, 1971), in Romania (Culita, 2007), in Poland (Ceynowa-Gieldon, 1988), and in Hungary (Solymosi, 2008).

Native American tribes utilized different parts of *Oxybaphus nyctagineus* in diverse ways to treat wide scale of diseases. Among the Ponca it was called Maka^n-wasek ('strong medicine') and its

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http://dx.doi.org/10.1016/j.jep.2014.06.020
0378-8741/

chewed root was used as a remedy for wounds (Gilmore, 1919). The Pawnees knew this plant as Kahtstakat ('yellow medicine') and applied the dried root to cure sore mouth in babies, while women drank as decoction after childbirth to relieve abdominal swelling (Gilmore, 1919; Foster and Duke 1999). The Sioux drank the same decoction to reduce fever (Gilmore, 1919; Cardina et al., 2009). For the external treatment of fracture and swelling of the limbs, a mixture from the dried herb and soft grease was prepared by the Sioux (Densmore, 1918; Gilmore, 1919). The Ojibwe Indians also used this plant (called 'goko' coadji' bik', *i.e.*, pig root) to reduce sprains and swellings (Smith, 1932). The Navajo Indians applied its root and aerial part externally as 'fire medicine' for burns, scalds, swellings and sores in form of dusting powder, poultice and ointment (Wyman and Harris, 1941; Elmore, 1944). Interestingly, the Acoma and Laguna Indians smoked its leaves as tobacco (Swank, 1932), while the Sioux combined and boiled it with *Echinacea angustifolia* and taken as a vermifuge (Gilmore, 1919). Internally, the leaf or root tea was used for bladder ailments (Foster and Duke, 1999). Despite the above summarized extensive and diverse therapeutic uses of wild four-o'clock, to the best of our knowledge, no data regarding neither the phytochemical composition, nor the bioactivity of this species has been reported in the literature to date.

Considering that *Oxybaphus nyctagineus* was traditionally used to treat fractures and sprains, to reduce swellings, fever, inflammation and for wound healing, the basic purpose of our research was to investigate the antioxidant, analgesic and anti-inflammatory activity of the crude extracts of *Oxybaphus nyctagineus*. In parallel, we aimed to characterize the major chemical constituents of the bioactive fractions in order to verify its beneficial ethnopharmacological effects. In addition, the significant spreading of *Oxybaphus nyctagineus* as a weed in Hungary strengthened the relevance of our study.

2. Materials and methods

2.1. Chemicals and reagents

Analytical grade of dimethylsulfoxide, methanol, dioxane, and trifluoroacetic acid was obtained from Merck (Darmstadt, Germany). Thiopental was purchased from Sandoz (Kundl, Austria). Diclofenac gel (topical formulation of diclofenac) with active substance content of 10 mg/g was purchased from Ratiopharm (product no. L09783, Ulm, Germany). All other solvents, reagents and reference compounds were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA). Purified water (18 mΩ) was obtained from a Millipore (Bedford, MA, USA) Milli-Q water-purification system.

2.2. Plant material

Flowering shoots of *Oxybaphus nyctagineus* (Michx.) Sweet (*Nyctaginaceae*) were collected in the beginning of August 2011 from the Phylogenetic Plant Collection of the Botanical Garden belonging to the Centre for Ecological Research of Hungarian Academy of Sciences (HAS), Vácrátót, Hungary. The herb was authenticated by Erzsébet Fráter biologist, the curator of the Phylogenetic Plant Collection of the Botanical Garden in Vácrátót. Herbarium specimens (I.D.: Engel-July-2011) have been deposited at the Herbarium Collection of the Centre for Ecological Research, HAS, Vácrátót, Hungary. Plant material was dried to constant weight at 40 °C in a drying oven OP30 (Lab-Ex, Hungary).

2.3. Extraction and fractionation

For mimicking the lipophilic milieu of the traditionally prepared agent (*i.e.*, dried herb mixed with soft grease (Densmore, 1918)), the dried and ground aerial parts of *Oxybaphus nyctagineus* (55 g) were ultrasonically extracted twice with $CHCl_3$ (450+150 mL) for 30 min. The extract was filtered through a filter paper (apolar crude extract=ACE, yield: 5.55 g). The remaining and re-dried residue was re-extracted with 70% aqueous MeOH under the same conditions. The extract was filtered through a filter paper (polar crude extract=PCE, yield: 8.02 g). Both crude extracts were evaporated to dryness under reduced pressure at 60 °C in a rotary vacuum evaporator (Rotavapor R-200, BÜCHI, Switzerland). To retain chlorophyll, the apolar residue was subjected to polyamide column chromatography (MP Polyamid, cat. no. 0209602, MP Biochemicals, Germany). The apolar residue was dissolved in a minimum amount of $CHCl_3$, adsorbed on 1.5-fold polyamide powder and chromatographed over polyamide powder (3.5-fold) packed in a glass column. The column was eluted with 70–95% aqueous MeOH (150-fold by volume). The gained chlorophyll free extracts were pooled and re-evaporated to dryness. The residue was dissolved in $CHCl_3$ and mixed with 2.5-fold silica gel (Silica gel 60, 0.063–0.200 mm, cat. no. 1.07734.1000 Merck, Germany). This suspension was subjected to silica gel column chromatography (40-fold silica gel by volume) eluted fractionally with EtOH–$CHCl_3$ (A1=1:30, A2=2:45 and A3=1:15, v/v) and $CHCl_3$–MeOH–H_2O (A4=90:10:1, A5=90:30:3 and A6=45:30:3, v/v) to separate six fractions (25 mL eluent for each 10 g silica gel). The yield of the subfraction A4 was 116 mg.

The polar residue was directly fractionated with silica gel column chromatography. The residue was dissolved in 70% aqueous MeOH and adsorbed on 2.5-fold silica gel, which was homogenized with $CHCl_3$. This suspension was chromatographed on silica gel (20-fold silica gel by volume). $CHCl_3$–MeOH (P1=20:1, v/v), and $CHCl_3$–MeOH–H_2O (P2=90:10:1, P3=45:10:1, P4=30:10:1, P5=45:20:2, P6=90:50:5, P7=15:10:1 and P8=9:8:2, v/v) were used as eluents to get eight fractions. The yield of the subfraction P5 was 109 mg. To isolate the major components of P5, the extraction and chromatographic procedure described above was repeated starting with 165 g of dried and ground plant material, and subfractions of P5 were simply obtained by collecting it into four equivalent volumes (P5/4=**1** (95 mg); P5/2=**2** (95 mg); P5/1=**3** (133 mg)). All fractions and subfractions were separately evaporated to dryness under reduced pressure in a rotary vacuum evaporator at 60 °C.

For *in vivo* and *in vitro* experiments, the DMSO stock solutions (50 mg/mL) of the purified apolar extract (referred as ACE) and the pool of the polar fractions (referred as PCE) were prepared and applied.

2.4. in vitro antioxidant assays

2.4.1. DPPH radical scavenging activity

The free radical scavenging effect of the crude extracts and subfractions was assessed according to the method described by Blois (1958) and modified by Hu and Kitts (2005) for microplate format. The purple colored 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) is a stable free radical, which is reduced to the yellow 2,2-diphenyl-1-picrylhidrazine when allowed to react with an antioxidant. 250 μL of DMSO solution of samples in six concentrations, ranging from 0.5 to 300 μg/mL, and 50 μL of 0.3 mM DPPH ethanolic solution were mixed in a 96-well polypropylene plate (Agilent, Waldbronn, Germany). Pure DMSO and quercetin were used to provide the negative and positive controls, respectively. Absorbance of each well was measured at 517 nm after 30 min incubation at room temperature by an UV–visible plate reader (Multiskan Spektrum, Thermo Labsystem). The degree of scavenging activity was determined by the following formula: Inhibition (%)=$[(Abs_{control}-Abs_{sample})/Abs_{control}]\times 100$. EC_{50} values, the concentrations of the samples required to scavenge 50% of DPPH radical, were calculated by Prism 4 for Windows (Graph Pad