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**An ethnopharmacological survey conducted in the Bolivian
Amazon, and identification of *N*-alkylamides and lignans
from *Lepidium meyenii* and *Heliopsis helianthoides* var.
scabra with effects on the central nervous system**

Summary of Ph.D. Thesis

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INTRODUCTION

Porvenir is a Bolivian indigenous community in the Bajo Paraguá Communal Territory of Origin, home to the Chiquitano mestizos and the Guarasug'we indigenous nation. This region provided a good research area, because no ethnomedicinal fieldwork had previously been conducted in Porvenir.

Numerous South-American plant species are traditionally used for central nervous system (CNS) disturbances, which are at the main focus of neuroscientific research, and therefore we aimed a phytochemical and pharmacological investigation of two traditionally used plants (*Lepidium meyenii* and *Heliopsis helianthoides* var. *scabra*) with possible effects on the brain. The hypocotyls of *L. meyenii* (Maca, Brassicaceae) are widely consumed as a common vegetable and have a multiplicity of other uses in the Peruvian and Bolivian highlands, among them fertility enhancement being the most popular. Maca has been found to contain certain metabolites characteristic of the species, such as the *N*-alkylamide (NAA) macamides. Some species of the *Heliopsis* genus (Asteraceae) are also used in North-American traditional medicines, and have been reported to contain NAAs and lignans. Among these species, *H. helianthoides* var. *scabra* has not been studied in detail. With the discovery of the functional interaction of plant NAAs with the endocannabinoid system (ECS), these compounds have become important as lead compounds of drug development. The promising anti-metastatic potential of several lignans underlines the significance of these compounds as potential tools in cancer treatment. Our studies on *L. meyenii* and *H. helianthoides* var. *scabra* focused on the phytochemical analysis of these species to isolate and identify NAAs and lignans and to carry out detailed pharmacological analyses.

The traditional knowledge of medicinal plants is also utilized in the food industry. Certain South-American plants, such as Maca are marketed in Europe, but their

utilization partially differs from the traditional way. This difference raises the suspicion that the quality of certain products cannot meet the requirements, and an analytical study of Maca containing preparations was therefore also proposed.

AIMS OF THE STUDY

The aims of our study were

- to describe the medicinal plants applied in the traditional medicine of Porvenir, Bolivia, and the phytochemical and pharmacological evaluation of the plant species used for CNS disturbances, by comparison of the folk-medicinal use with the available scientific literature data;
- the isolation and structure elucidation of *N*-alkylamides from *L. meyenii* and *H. helianthoides* var. *scabra* and, in the frame of cooperation, to test these compounds on different targets within the ECS;
- the isolation and structure elucidation of lignans from *H. helianthoides* var. *scabra* and, in the frame of cooperation, to evaluate their potential antimetastatic activity in the brain;
- to develop an analytical protocol for the qualitative and quantitative analysis of *L. meyenii*-containing food supplements and to screen selected products for the presence of synthetic adulterants (phosphodiesterase inhibitors).

MATERIALS AND METHODS

During the 5-months field work in Porvenir ethnobotanical data were recorded. Techniques of cultural anthropology were applied and the traditionally used medicinal plants were collected, and botanically identified. The knowledge on medicinal plants was analyzed by means of *A. H. Gentry's* method. The traditional use of herbs was evaluated whether it correlates with the relating scientific data.

The roots of *H. helianthoides* var. *scabra* (Dunal) Fernald 'Asahi' were obtained from a nursery (Hegede Flower Nursery Ltd., Kecskemét, Hungary) in

September 2009. The yellow dry hypocotyl powder of *L. meyenii* Walp originated from Peru and was purchased from Raw Organic Maca Powder, EverTrust Ltd, UK (batch number M-010177-11-220312).

For the isolation of NAAs and lignans, several chromatographic methods [vacuum liquid chromatography (VLC), medium pressure liquid chromatography (MPLC), preparative thin-layer chromatography (PLC), rotational planar chromatography (RPC), high pressure liquid chromatography (HPLC) and centrifugal partition chromatography (CPC)] were applied. Structure elucidation was carried out by means of 1D and 2D NMR and HRSIMS methods.

For the quantification of macamide content of dietary supplements, the macamide *N*-benzyl-(9Z,12Z)-octadecadienamide that we isolated was used as chemical marker. The products were analysed by a HPLC-DAD method developed by us. For the presence of phosphodiesterase inhibitors, multicomponent preparations were analyzed by TLC and HPLC-DAD according to a protocol developed in our laboratory.

The isolated NAAs were examined for their possible activities on different targets within the endocannabinoid system (ECS) by *Jürg Gertsch et al* (Institute of Biochemistry and Molecular Medicine, NCCR TransCure, University of Bern, Bern, Switzerland).

All of the isolated lignans of *H. helianthoides* were examined for their possible activities on melanomas brain metastases formation by *János Haskó et al*. (Institute of Biophysics, Biological Research Centre, Hungarian Academy of Sciences, Szeged, Hungary).

MAIN RESULTS

Ethnopharmacological fieldwork

A total of 145 medicinal plant species were registered in Porvenir, among which 37 species are used for diseases of the CNS, pain and fever. The ethnobotanical data that do exist in the literature correspond closely with the utilization of the plants in Porvenir, but the great majority of the species used have not been widely investigated from phytochemical or pharmacological points of view, and they are therefore worthy for further investigations.

Isolation from *Lepidium meyenii*

Dried *L. meyenii* hypocotyl powder (1.2 kg) was extracted with *n*-hexane, which was the most adequate among the different solvents tested for the extraction of NAAs. Further purification was carried out with centrifugal partition chromatography (CPC) combined with HPLC. CPC was used by us for the first time to isolate NAAs and, since it has no solid stationary phase, it proved to be very useful in the isolation of highly unstable compounds such as alkamides. A two-phase solvent system consisting of *n*-hexane–EtOAc–MeOH–H₂O 9:1:9:1 was used in the ascending mode. Nine main fractions were obtained, two of which were purified again with CPC (MeCN–*n*-hexane, 1:1, descending mode) and the alkylamide-containing fractions were then subjected to RP-HPLC systems (MeCN–H₂O, 9:1 and MeCN–H₂O, 95:5). Three compounds (**1-3**) were yielded in 5.0–92.0 mg.

Isolation from *Heliopsis helianthoides* var. *scabra*

The fresh roots (9 kg) were extracted with MeOH, which was suitable for the extraction of both lipophilic and polar compounds. And then solvent–solvent extraction with CHCl₃ was applied in order to separate the apolar constituents. The purification was continued with more selective methods (VLC, RP-VLC, MPLC, RPC, PLC and HPLC). VLC and MPLC afforded crude fractionation of the main components. RPC, PLC and HPLC were the most effective and most selective

methods. The most used solvent system was *n*-hexane–EtOAc followed by *n*-hexane–Me₂CO, benzene–CH₂Cl₂–Et₂O and MeCN–H₂O. Ten compounds (**4**–**13**) were isolated from the plant yielding 2.0–15.0 mg.

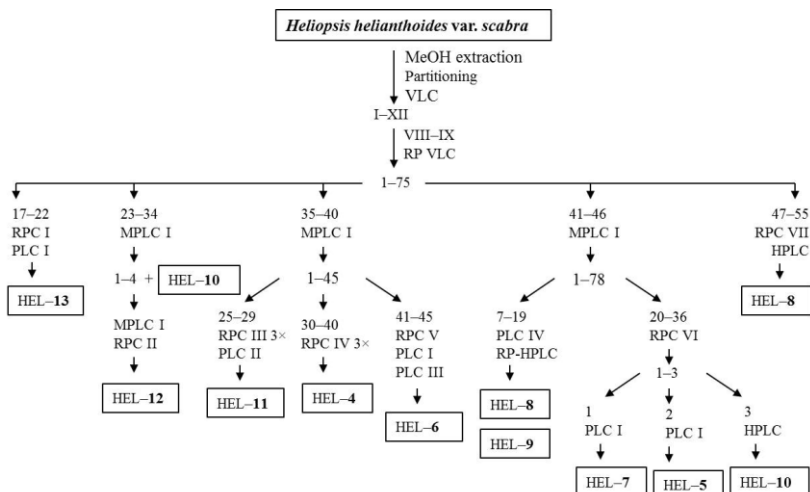


Figure 2. Isolation of compounds from *H. helianthoides* var. *scabra*

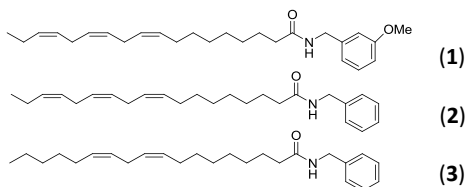
Structure elucidation of the isolated compounds

The structure determination of compounds **1**–**3** isolated from *L. meyenii* led to the identification of previously described polyunsaturated aromatic diene and triene amide macamides, which contain 18 carbon atoms in their aliphatic chain: *N*-(3-methoxybenzyl)-(9*Z*,12*Z*,15*Z*)-octadecatrienamamide (**1**), *N*-benzyl-(9*Z*,12*Z*,15*Z*)-octadecatrienamamide (**2**) and *N*-benzyl-(9*Z*,12*Z*)-octadecadienamamide (**3**).

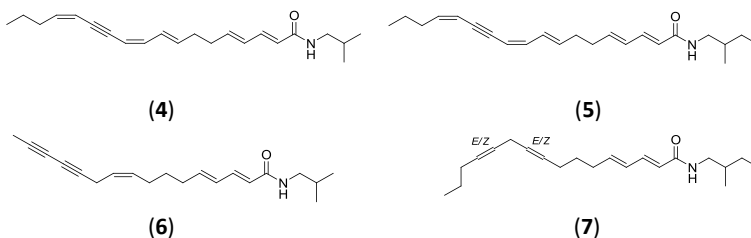
From *Heliopsis helianthoides* var. *scabra*, three new (**5**, **6**, **7**) and one known (**4**) aliphatic isobutyl- and methylbutylamides with 16 and 18 carbon atoms and three to six double and triple bonds on their fatty acid chain were identified.

Octadeca-2*E*,4*E*,8*E*,10*Z*,14*Z*-pentaen-12-ynoic acid isobutylamide (**4**) was isolated previously from *H. helianthoides*, but its ¹³C NMR data are reported by us for the first time. In the cases of the three new natural products octadeca-2*E*,4*E*,8*E*,10*Z*,14*Z*-pentaen-12-ynoic acid 2'-methylbutylamide (**5**), hexadeca-2*E*,4*E*,9*Z*-triene-12,14-diynoic acid isobutylamide (**6**), and hexadeca-2*E*,4*E*,9,12-tetraenoic acid 2'-methylbutylamide (**7**), two of them contain acetylene bonds as well.

N*-Alkylamides isolated from *Lepidium meyenii



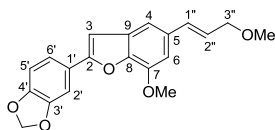
N*-alkylamides isolated from *Heliopsis helianthoides* var. *scabra



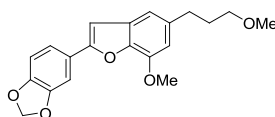
Moreover, two new arylbenzofuran neolignans, 1''-dehydroegonol 3''-methyl ether (**8**) and egonol 3''-methyl ether (**9**) were determined from *H. helianthoides* var. *scabra*. Four known lignan derivatives (**10-13**) were identified on the basis of the good agreement of measured and previously reported data. Helioxanthin (**10**), an arylnaphthalene derivative, was identified earlier from the root of this species and from thirteen other plants. The dibenzylbutane (7*E*)-7,8-dehydroheliobuphtalmin

(11) and heliobupththalmiin (12) were isolated for the first time from *H. helianthoides* var. *scabra*, but earlier from *H. bupththalmoides*. Three more dibenzylbutane derivatives and the dibenzylbutyrolactone 7-acetoxyhinokinin (13) were identified for the first time from *H. helianthoides* var. *scabra*, but earlier from *Ruta pinnata* L.

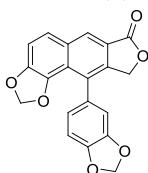
Lignans isolated from *Heliopsis helianthoides* var. *scabra*



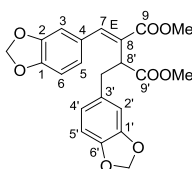
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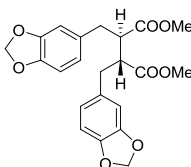
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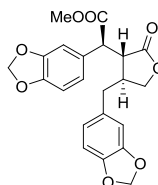
(10)



(11)



(12)



(13)

Analysis of dietary supplements

A total of 14 Maca containing mono- (5) and multicomponent (9) preparations were randomly selected and purchased. The presence of Maca was confirmed in 8 products. The Maca powders contained 28.0-225.8 μg *N*-benzyl-(9*Z*,12*Z*)-octadecadienamide (3) /g. In 6 preparations, the concentration of the marker

macamide was below the detection limit and 1 preparation was adulterated with a synthetic phosphodiesterase inhibitor (thiosildenafil).

Activities of *N*-alkylamides on the endocannabinoid system

From the studied compounds (**1**, **3-7**) macamide **3** showed a low inhibition of FAAH and caused a significant inhibition of AEA uptake, which was even more potent than the inhibition measured with the reference inhibitors. Compound **3** also showed a significant binding affinity toward CB receptors, with an unexpected tenfold selectivity towards CB₁. Among the NAAs from *H. helianthoides* var. *scabra* only **7** showed a potent binding interaction with the CB₁ receptor. See Table 1.

Table 1. Summary of the effects of the isolated NAAs on ECS targets.^a

Cpd.	AEA cell uptake IC ₅₀ (μM)	Effi- cacy (%)	FAAH IC ₅₀ (μM) (95% CI)	Effi- cacy (%)	CB ₁ K _i (μM) (95% CI)	CB ₂ K _i (μM) (95% CI)
1	>100	44	19.1 (13.8–28.18)	85	8.7 (4.66–13.75)	>50
2	84.4 (72.44–>100)	53	11.5 (6.81–18.78)	85	8.9 (3.79–10.19)	44.0 (24.16–>50)
3	0.7 (0.47–0.97)	73	4.1 (2.95–5.62)	83	0.5 (0.33–0.67)	4.1 (3.11–5.66)
4	2.5 (1.28–4.70)	75	17.8 (13.18–25.70)	84	8.6 (2.39–10.92)	9.2 (5.41–9.84)
6	4.3 (2.69–6.97)	75	12.3	79	>20 (6.28–>20)	22.6 (12.68–25.30)
7	2.2 (0.87–5.31)	51	20.0 (12.59–33.11)	80	0.31 (0.18–0.59)	1.2 (0.90–1.71)
OMDM-2	4.12 (2.01 – 12.19)	72	23.29 (10.72 – 48.98)	82	n.d.	n.d.
UCM707	1.46 (1.18 – 1.80)	67	7.24 (6.03 – 13.18)	79	n.d.	n.d.

^aOMDM-2 and UCM-707 are positive controls for AEA uptake. n.d., not determined.

These results provide additional evidence of the structural and functional similarity between NAAs and endocannabinoids, potentially interlinking the use of NAA-containing medicinal plants and botanical dietary supplements with the ECS as a potentially major site of action.

Pharmacological effects of lignans on melanomas brain metastasis formation

Helioxanthin (**10**) and (7E)-7,8-dehydroheliobuphthalmin (**11**) exhibited various effects on melanoma and brain endothelial cells, with the potential to interfere with different steps of metastasis formation. These findings indicate that these compounds impede the migration of melanoma cells, and inhibit the adhesion of melanoma cells to the brain endothelial cells. Both compounds also enhanced the barrier function and decreased the migratory properties of cerebral endothelial cells. These effects might be instrumental in preventing the transendothelial migration of melanoma cells and the vascularization of tumors.

SUMMARY

- The traditional medicine of Porvenir was evaluated in detail. Altogether 107 known complaints or symptoms were described, 11 of them relating for CNS illnesses or symptoms, for which 37 plant species are used. The majority of the species are worthy for further phytochemical and pharmacological investigations.
- Phytochemical analysis of *L. meyenii* led to the isolation and structure elucidation of previously described macamides, *N*-(3-methoxybenzyl)-(9Z,12Z,15Z)-octadecatrienamide (**1**), *N*-benzyl-(9Z,12Z,15Z)-octadecatrienamide (**2**) and *N*-benzyl-(9Z,12Z)-octadecadienamide (**3**).
- For the isolation of macamides a new CPC – HPLC method was developed by us.
- Phytochemical investigation of *H. helianthoides* var *scabra* led to the isolation and structure elucidation of four *N*-alkylamides, octadeca-

2*E*,4*E*,8*E*,10*Z*,14*Z*-pentaen-12-ynoic acid isobutylamide (**4**), octadeca-2*E*,4*E*,8*E*,10*Z*,14*Z*-pentaen-12-ynoic acid 2'-methylbutylamide (**5**), hexadeca-2*E*,4*E*,9*Z*-triene-12,14-diyenoic acid isobutylamide (**6**) and hexadeca-2*E*,4*E*,9,12-tetraenoic acid 2'-methylbutylamide (**7**). Compounds **5-7** are new natural products, while ¹³C NMR data of **4** was published for the first time by us.

- Further phytochemical investigation of *H. helianthoides* var. *scabra* resulted in the isolation of six lignans: two new arylbenzofuran neolignans, 1"-dehydroegonol 3"-methyl ether (**8**) and egonol 3"-methyl ether (**9**), and four known lignan derivatives, helioxanthin (**10**), (7*E*)-7,8-dehydroheliobupthalmin (**11**), heliobupthalmin (**12**) and 7-acetoxihinokinin (**13**).
- As a result of a pharmacological assay on different targets of the ECS with the isolated *N*-alkylamides, the *N*-methylbutylamide **7** and the *N*-benzylamide **3** were identified as perspective compounds for further pharmacological analysis and as potential lead compounds.
- As a result of a pharmacological assay with the isolated lignans, helioxanthin (**10**) and (7*E*)-7,8-dehydroheliobupthalmin (**11**) exhibited various effects on melanoma and brain endothelial cells, with the potential to interfere with different steps of metastasis formation.
- A simple and reliable analytical protocol for the qualitative and quantitative analysis of the Maca content of dietary supplements was developed.
- According to our method two-third of the analyzed multicomponent products were of inferior quality, without any presence of Maca. Moreover, one of them was adulterated with the synthetic phosphodiesterase inhibitor, thiosildenafil.

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. Hajdu Z, Hohmann J. An ethnopharmacological survey of the traditional medicine in the community Porvenir, Bajo Paraguá Indian Reservation, Bolivia. *Journal of Ethnopharmacology* 2012, **139**: 838-857.

If: 2.47

II. Hajdu Z, Nicolussi S, Rau M, Lorántfy L, Forgo P, Hohmann J, Csupor D, Gertsch J. Identification of endocannabinoid system-modulating *N*-alkylamides from *Heliopsis helianthoides* var. *scabra* and *Lepidium meyenii*. *Journal of Natural Products* 2014, **77**(7): 1663-1669.

If*: 3.29

III. Hajdu Z, Haskó J, Krizbai I, Wilhelm I, Jedlinszki N, Fazakas, C, Molnár J, Forgo P, Hohmann J, Csupor D. Evaluation of lignans from *Heliopsis helianthoides* var. *scabra* for their potential antimetastatic effects in the brain. *Journal of Natural Products*, 2014, *under review*

If*: 3.29

IV. Hajdu Z, Lorántfy L, Jedlinszki N, Boros K, Hohmann J, Csupor D. Quality control of Maca-containing (*Lepidium meyenii* Walp) dietary supplements. *Acta Alimentaria*, 2015, **44**(3)

If*: 0.43

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Hajdu Z, Csupor D, Szendrei K. Maca – egy hazánkban is népszerű dél-amerikai gyógynövény. 2013 *Gyógyszerészet* 57: 156-169.

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Hajdu Z, Lorántfy L, Hohmann J, Csupor D. Alkilamidok izolálása *Lepidium meyenii*ből és *Heliopsis helianthoides*ből. Fialat Gyógynövénykutatatók Fóruma, Budakalász, 2014

Hajdu Z. Maca (*Lepidium meyenii* Walp) és maca tartalmú termékek fitokémiai vizsgálata. Clauder Ottó Emlékverseny, Budapest, 2013

* based on calculation of 2013

Hajdu Z. An ethnopharmacological evaluation of the plants used for gastro-intestinal complaints by inhabitants of Porvenir, Bajo Paraguá Indian Reservation, Bolivia. XX Symposium of Brazilian Medicinal Plants & X International Congress of Ethnopharmacology, Sao Paulo, Brazil, 2008

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Hajdu Z, Lorántfy L, Jedlinszki N, Hohmann J, Csupor D. Quality Control of Maca-Containing (*Lepidium meyenii*, Walp) Dietary Supplements. Balaton Symposium on High-Performance Separation Methods, Siófok, Hungary, 2013

Lorántfy L, **Hajdu Z,** Hohmann J, Németh L, Csupor D. Alkilamidok izolálása a *Lepidium meyenii* kivonatából. Vegyészkonferencia 2013) Hajdúszoboszló, Hungary, 2013

Hajdu Z, Csupor D, Forgó F, Hohmann J. Phytochemical investigation of *Heliopsis helianthoides*, a North American traditional medicinal plant. 13th International Congress of the Society for Ethnopharmacology, Graz, Austria, 2012

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