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Alkamides are one of the most important constituents of lipophilic extracts of Echinacea angustifolia roots. These compounds play an important role in the versatile pharmacological actions of this plant. The present study aimed to compare the concentrations of isomeric dodeca-2,4,8,10-E/Z-tetraenoic acid isobutylamides (DTAI) in brain and periepididymal fat tissues and blood plasma of rats. Thirty, 60, 240 and 720 min after the oral administration of E. angustifolia root extract, tissue and plasma concentrations were determined by reversed-phase HPLC with ESI-MS/MS detection. The calculated terminal t½ of the mixture of DTAI was 8.28 h, which indicates a relatively slow elimination. In the 0.5–4 h period the brain/plasma and fat/plasma concentration ratios were continuously above 3 and 18, respectively, followed by equilibrium at 12 h. Our results indicate substantial accumulation of alkamides in lipid-rich tissues, which presumably contributes to a maintained pharmacological action.

**Keywords:** HPLC-MS/MS, Pharmacokinetics, Echinacea angustifolia, Alkamide, Dodeca-2,4,8,10-E/Z-tetraenoic acid isobutylamides.

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Echinacea species are the most widely used immunomodulatory plants in the treatment of common cold. Pharmacologically active constituents of Echinacea species are alkamides, alkylketones, caffeic acid derivatives and polysaccharides [1, 2]. Isomeric dodeca-2,4,8,10-E/Z-tetraenoic acid isobutylamides (DTAI) (Figure 1) are the main alkamides in the roots of E. purpurea, E. angustifolia and E. pallida and in the herbs of E. purpurea and E. angustifolia [3].

![Figure 1: Chemical structures of dodeca-2,4,8,10-E/Z-tetraenoic acid isobutylamides.](image)

Alkamides may play an important role in the medicinal benefits of Echinacea extracts. Alkamides of E. purpurea stimulated, dose-dependently, the phagocytic activity of alveolar macrophages in rats [4]. Two Echinacea-derived alkamides suppressed the ability of activated Jurkat T cells to produce IL-2 in a dose-dependent manner [5]. The mixture profile purified from E. angustifolia decreased significantly the lipopolysaccharide stimulated NF-kB level, TNF-α and NO production in macrophages in vitro [6]. Polyunsaturated alkamides isolated from Achillea species and E. angustifolia roots were shown to possess inhibitory activity in vitro cyclooxygenase and 5-lipoxygenase assays [7]. These data suggest that alkamides may have not only immunostimulatory but also anti-inflammatory activity. DTAI have been found to inhibit both cyclooxygenase and 5-lipoxygenase at micromolar concentrations [8]. Extract of E. angustifolia decreased anxiety in the elevated plus-maze, social interaction and social avoidance tests in rodents [9]. The extract of the plant can significantly regulate excitatory, but not inhibitory, synaptic transmission in the hippocampus, and this action might be involved in its anxiolytic effects observed in behaviour tests [10]. Certain alkamides act as cannabinomimetics at both the cannabinoid CB1 and CB2 receptors [11-13]. Alkamides of E. angustifolia extract may contribute to decreased mild anxiety symptoms since CB1 receptors are implicated in anxiety [14]. Data concerning the absorption, metabolism, bioavailability, and bioactivity of natural products and their metabolites are primarily important to link results from in vitro assays and clinical studies. Woelkart et al. evaluated the pharmacokinetics of DTAI in rat plasma, as well as in liver and brain tissues [15]. Goey et al. have described a validated HPLC-MS/MS procedure for quantitative analysis of DTAI in human plasma [16]. Although lipid soluble substances may be deposited in fat tissues which may fundamentally influence the pharmacokinetic behaviour of such compounds, no data are available concerning the concentrations of DTAI in depot fat.

In this research, we studied the concentration changes of DTAI in brain and periepididymal adipose tissues of rats using an HPLC-MS/MS method, and compared them with those in plasma of the animals. Terminal t½ of DTAI was also calculated.

Oral treatment of rats with 50 mg/kg E. angustifolia root extract was performed in order to obtain data concerning the pharmacokinetics of the active constituents both in plasma and lipid-rich brain and fat tissues. DTAI concentration was maximal in lipid-rich brain and fat tissues which may fundamentally influence the pharmacokinetic behaviour of such compounds, no data are available concerning the concentrations of DTAI in depot fat.
18–24 times higher than in the plasma in the period 0.5–4 h and reached equilibrium by 12 h (Table 1). High concentrations were found in the brain too but the tissue/plasma ratios were lower (2–6-fold) and also decreased substantially by 12 h.

**Figure 2:** DTAI concentrations in the plasma (●), brain (○) and fat tissue (●) samples of rats treated with 50 mg/kg *Echinacea* extract (N: 6).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Brain/plasma ratio</th>
<th>Fat/plasma ratio</th>
</tr>
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<tbody>
<tr>
<td>0.5</td>
<td>7.10</td>
<td>30.23</td>
</tr>
<tr>
<td>1</td>
<td>3.05</td>
<td>20.61</td>
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<tr>
<td>4</td>
<td>4.60</td>
<td>35.22</td>
</tr>
<tr>
<td>12</td>
<td>0.51</td>
<td>0.96</td>
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</table>

DTAI concentrations in plasma, brain and fat tissue samples obtained from rats were determined by means of HPLC-MS/MS 0.5–12 h after oral treatment with *Echinacea* extract (Figure 3). Since the main purpose of our study was the comparison of concentrations in relevant tissues (brain and fat) with those in plasma the timing of the sample collection is not optimal for a complete pharmacokinetic analysis. However, the calculated terminal t½ of the analyte was 8.27 h, which indicates a substantially complete pharmacokinetic analysis. However, the calculated plasma timing of the sample collection is not optimal for a concentration in relevant tissues (brain and fat) with those in plasma.

**Figure 3:** MRM chromatogram of periepididymal adipose tissue 4 hours after *Echinacea* extract administration (solid line) and the 0.8 ng/20 µL concentration calibration standard (dotted line).

Highly lipid-soluble substances tend to accumulate in fat tissue and this may basically influence the disposition of the drug. However, none of the previously published studies were extended to the determination of DTAI in adipose tissue. This is the first report presenting direct comparison of DTAI concentrations in plasma, brain and fat. Our data indicate that DTAI reaches considerably high concentration, presumably maximal in the body in adipose tissue. DTAI deposited in the fat may contribute to a sustained brain concentration. If such redistribution is operative in humans it may implicate that the relative fat weight may exert a profound action on the disposition of the agents and hence on the duration of their action too.

**Experimental**

**Chemicals and reagents:** DTAI was isolated from *E. angustifolia* by Hohmann et al. [12] and its identity and purity (95%) was investigated by 1H NMR (500 MHz, CDCl3). Benzalnilde (purity 99.9%) and methylcellulose were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), acetonitrile (LiChrosolv HPLC grade) from Merck KGaA (Darmstadt, Germany), and formic acid and methanol (analytical grade) from Renal Zrt. (Budapest, Hungary). Millipore Direct-Q UV3 (Millipore, Bedford, MA, USA) clarifier was used to produce purified water for HPLC-MS measurements. The *E. angustifolia* root extract (extraction: ethanol 85%, v/v; ratio of herbal drug - native extract: 6.5–8.1; excipient: maltodextrin 30%; marker: echinacoside 4%) was purchased from Euromed SA (Millet del Valles, Spain; Batch No. 419061).

**Calibration standards and quality control samples:** Standard stock solution was prepared by dissolving solid DTAI in water-acetonitrile (6:4) in a concentration of 1 µg/20 µL. Working standard solutions were prepared by serial dilutions using water-acetonitrile (6:4) from the stock solution to final concentrations that covered the concentration range of the assay. The benzalnilde internal standard (IS) stock solution, 4.12 ng/20 µL, was prepared using water-acetonitrile (6:4). Its concentration was 1 ng/20 µL in the calibration standards and in the samples. Quality control samples (QC) were prepared using the procedure of sample preparation at the concentrations of 0.023 ng/20 µL (LC) and 0.920 ng/20 µL (HC). The IS concentration was 1 ng/20 µL.

**Animals and treatment:** Animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII. section 243/1998). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008). Male Wistar rats of 200–240 g body weight (Toxi-Coop, Budapest, Hungary) were housed in temperature (20–23°C), humidity (40–60%) and light (12 h of light, 12 h of dark) regulated rooms, with water and standard rodent food (Bioplan, Isaszeg, Hungary) intake available ad libitum. Animals were fasted for 16 h before oral treatment with 50 mg/kg *E. angustifolia* extract suspended in 0.25% methylcellulose in a dosing volume of 5 mL/kg. Six animals were randomized into each group.

**Sample collection and preparation:** After 30, 60, 240 and 720 min rats were anesthetized with isoflurane inhalation and blood samples were collected by cardiac puncture. Blood coagulation was prevented and plasma was prepared by centrifugation (4000 RPM, 10 min, 4°C). Plasma samples of 200 µL were extracted 2 times with 1200 µL methanol and 15 µL IS solution was added. The sample was homogenised by vortexing for 90 sec. After both extractions the samples were centrifuged (6200 RPM, 15 min, 4°C) and the collected supernatants were evaporated under nitrogen at room temperature. Whole brains were removed and periepididymal adipose tissues from both sides were collected and wet weights were recorded. Tissues were homogenized in a double volume of methanol, vortexed for 90 sec and centrifuged (6200 RPM, 15 min, 4°C). Fifteen µL IS solution was added to 500 µL supernatant and evaporated as mentioned before. Samples were dissolved in 200 µL water-acetonitrile (6:4) in an ultrasonic bath and then filtered using...
a 0.45 μm pore size syringe filter into chromatographic sample bottles. Pharmacokinetic parameters were calculated by means of PKSolver in noncompartmental analyses [18].

**HPLC-MS/MS conditions:** LC analyses were performed using a Shimadzu HPLC system (Shimadzu, Tokyo, Japan), equipped with 2 LC-20AD pumps, CBM-20A controller, SPD-20A UV-Vis detector, SIL-20A autosampler, DGU-20A3 degasser and CTO-20AC column thermostat, coupled to an API 2000 triple quadrupole mass spectrometer, equipped with an electrospray (ESI) interface (AB SCIEX, Framingham, MA, USA). Chromatographic separation was achieved with a Phenomenex Kinetex XB-C18 column (2.1 × 50 mm; 2.6 μm) and a C18 guard column (both supplied by Gen-Lab Kft., Budapest, Hungary). Eluent A was 0.1% formic acid in acetonitrile and eluent B was 0.1% formic acid in water at a flow rate of 500 μL/min. The elution gradient started with 40% of eluent A, keeping isocratic conditions for 0.5 min. Then, eluent A increased to 90% in 1.0 min and was held for 1.0 min. Finally, initial conditions were reached again in 1.2 min, with a reequilibration time of 1.7 min in order to restore the column. The sample injection volume was set at 20 μL. The ion source temperature was 325°C. Measurements were carried out in positive ionization mode and the quantification was accomplished by using multiple reaction monitoring (MRM) with transitions of m/z 248→152 for DTAI and m/z 198→105 for benzaniilde. Data acquisition and evaluation were performed using Analyst 1.5.1 software.

**Method validation:** The applied HPLC-MS method, described above, was used and validated by Woelkart et al. [15] to quantitate DTAI in rat plasma and other tissues, except periepididymal fat. In this assay, validation was carried out only for periepididymal fat samples. The R² value for the standard curve was 0.9992 and the linear range was determined between 0.021–1.050 ng/20 μL. Limit of detection and limit of quantification were 0.0043 and 0.0214 ng/20 μL, respectively. Relative standard deviations of the replicate measurements were 19.8% for LC and 0.87% for HC samples. Extraction recovery was 75% for LC and 58% for HC samples.

**Acknowledgments** - The publication is supported by the European Union and co-funded by the European Social Fund (TÁMOP-4.2.2.A-11/1/KONV-2012-0035).

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Three New Isoflavonoids from *Erythrina caffra*
Zelalem Yibrailign Desta and Runner R. T. Majinda

Bijayasaline: A New C-Glucosyl-α-hydroxydihydrochalcone from the Heartwood of Bijayasal (*Pterocarpus marsupium*)
Khem Raj Joshi, Hari Prasad Devkota and Shoji Yahara

Nelumal A, the Active Principle of *Ligularia nelumbifolia*, is a Novel Aromatase Inhibitor
Francesco Epifano, Salvatore Genovese, Serena Fiorito, Chantal Magné Nde and Colin Clyne

Chemical Constituents of *Dendrobium venustum* and their Antimalarial and Anti-herpetic Properties
Prapapan Sukphan, Boonchoo Sriratalak, Wanwimon Mekboonsonglarp, Vinolmas Lipinpun and Kittisak Likhitwityawuid

HPLC-PDA Simultaneous Determination and Protective Effect of *Anemarrhena asphodeloides* Against Acute Renal Failure
Chang-Seb Seo, Hye Kyung Ha, Young-Jung Kim and Ju-Young Jung

New Rocaglamide Derivatives from Vietnamese *Aglaia* species
Ngoc Tu Duong, RuAngelie Edrada-Ebel, Rainer Ebel, Wenhan Lin, Anh Tuan Duong, Xuan Quy Dang, Ngoc Hieu Nguyen and Peter Proksch

Oxirapentyns A, B and E from the Marine-Derived Strain of *Isaria felina KKM 4639* as Stimulators of Initial Stages of Development of Agricultural Plants
Mikhail M. Anisimov, Elena L. Chaikina, Olga F. Smetanina and Shamil Sh. Afiyatullov

Concerning the Structure of Islandoquinone Isolated from the Lichen *Cetraria islandica*

Methodology for Porphyrin Isolation by High-Performance Countercurrent Chromatography
Amaro C. Ramos, Fernanda S. Neves, Maria Raquel G. Vega, Edmilson J. Maria and Rodrigo R. Oliveira

Possible Role of Fat Tissue in the Pharmacokinetics of Dodeca-2,4,4,8,10E,Z-tetraenoic Acid Isobutylamides after Oral Administration of *Echinacea angustifolia* Extract in Rats
Nikoletta Jedlinszki, Dóra Rédei, József Haller, Tamás F. Freund, Judit Hohmann and István Zupkó

Ascidian Tunicate Extracts Attenuate Rheumatoid Arthritis in a Collagen-induced Murine Model

Dialyzable Leukocyte Extracts Activate TLR-2 on Monocytes

The Volatile Constituents of *Parquetina nigrescens* from Southwestern Nigeria
Moses S. Owolabi, Oladipupo A. Lawal, Rebecca M. Hauser and William N. Setzer

Seasonal Variations in the Composition of the Essential Oils of *Lavandula angustifolia* (Lamiaceae)
Branislava Lakušić, Dmitar Lakušić, Mihailo Ristić, Mirjana Marčetić and Violeta Slavkovska

Chemical Composition and Antimicrobial Activity of the Essential Oil from *Allium hookeri* Consumed in Xishuangbanna, Southwest China
Ren Li, Yuan-Fei Wang, Qian Sun and Hua-Bin Hu

Chemical Composition, and Cytotoxic, Antioxidant and Antibacterial Activities of the Essential Oil from *Ginseng* Leaves
Rui Jiang, Liwei Sun, Yanbing Wang, Jianzeng Liu, Xiaoan Liu, Hao Feng and Daqing Zhao

Essential Oil from Leaves of *Liquidambar formosana* Ameliorates Inflammatory Response in Lipopolysaccharide-activated Mouse Macrophages
Kuo-Feng Hua, Tzu-Jung Yang, Huan-Wen Chiu and Chen-Lung Ho

Accumulation of Silicon in Cacti Native to the United States: Characterization of Silica Bodies and Cyclic Oligosiloxanes in *Stenocereus thurberi*, *Opuntia littoralis*, *Opuntia ficus-indica*, and *Opuntia stricta*
Cynthia R. Wright, Emanuel A. Waddell and William N. Setzer

Review/Account

A Phytochemical, Pharmacological and Clinical Profile of *Paederia foetida* and *P. scandens*
Liang Wang, Yiping Jiang, Ting Han, Chengjian Zheng and Luping Qin
## Contents

### Original Paper

<table>
<thead>
<tr>
<th>Title</th>
<th>Authors</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A New Source of (R)-Limonene and Rotundifolone from Leaves of <em>Lippia pedunculosa</em> (Verbenaceae) and their Trypanocidal Properties</td>
<td>Leociley Rocha Alencar Menezes, Nilmarra Nunes Santos, Cássio Santana Meira, Jâmyle Andrade Ferreira dos Santos, Elisalva Teixeira Guimaraës, Milena Botelho Pereira Soares, Angelita Nepel, Andersson Barison and Emmanoel Vilaça Costa</td>
<td>737</td>
</tr>
<tr>
<td>Improved Synthesis of (±)-Trichodiene – A Volatile Marker for Trichothecene Mycotoxins</td>
<td>Julian Gebauer, Martina Wernburg and Matthias Koch</td>
<td>741</td>
</tr>
<tr>
<td>Synthesis and Insecticidal Activities of Novel Nitrogenous Derivatives of Celangulin-V</td>
<td>Jiwen Zhang, Lihui Cui, Longbo Li, Zhan Hu, Qunliang Zhang, Zhaonong Hu and Wenjun Wu</td>
<td>745</td>
</tr>
<tr>
<td>An Easy Way to Pyrimidine Based Nucleoterpenes</td>
<td>Serena Fiorito, Salvatore Genovese and Francesco Epifano</td>
<td>149</td>
</tr>
<tr>
<td>Diasteroselective Addition of Diazomethane to Zaluzanin A</td>
<td>Adriana Ortiz-León, J. Martín Torres-Valencia, J. Jesús Manríquez-Torres, José G. Alvarado-Rodríguez, Uvaldo Hernández-Balderas, Carlos M. Cerda-García-Rojas and Pedro Joseph-Nathan</td>
<td>753</td>
</tr>
<tr>
<td>Tauroarenarones A and B, New Taurine-containing Meroterpenoids from the Marine Sponge <em>Dysidea</em> sp.</td>
<td>Natalia K. Utkina and Vladimir A. Denisenko</td>
<td>757</td>
</tr>
<tr>
<td>Stereo and Regioselective Microbial Reduction of the Clerodane Diterpene</td>
<td>Jair Muñozoli, Maria C. F. Oliveira, José R. Puiva, António H. Sousa, Mary A. S. Lima, José N. Silva Júnior, Francisco G. Barbosa, E. M. Kihlsiri Wijeratne and A. A. Leslie Gunatilaka</td>
<td>759</td>
</tr>
<tr>
<td>Scalarane Sesterterpenes from the Paracel Islands Marine Sponge <em>Hyríos</em> sp.</td>
<td>Fan Yang, Jian-Hong Gan, Xiaoyan Liu and Hou-Wen Lin</td>
<td>763</td>
</tr>
<tr>
<td>Novel Cucurbitane Triterpenoids and Anti-cholinesterase Activities of Constituents from <em>Momordica charantia</em> L.</td>
<td>Wichuta Khuainth, Thanmarat Aree, Surachai Pornpakakul and Pattara Sawasdee</td>
<td>765</td>
</tr>
<tr>
<td>Chromatographic Fingerprint Combined with Content of Asperosaponin VI and Antioxidant Activity for Quality Evaluation of Wine-fried Dipasic Radix</td>
<td>Li Song, Shaojun Wang, Xiaoju Duan, Xinhua Liu, Zhaofang Li, Lei Nie and Guangyi Chu</td>
<td>773</td>
</tr>
<tr>
<td>Morphological, Chemical and Molecular Characterization of <em>Centella asiatica</em> Germplasms for Commercial Cultivation in the Indo-Gangetic Plains</td>
<td>Archana Prasad, Sunita S. Dhawan, Ajay K. Mathur, Om Prakash, Madan M. Gupta, Ram K. Verma, Raj K. Lai and Archana Mathur</td>
<td>779</td>
</tr>
<tr>
<td>Ring A Conformation of Aconine and Pseudaconine in CDCI, Hong-Ying Deng, Qiao-Hong Chen and Feng-Peng Wang</td>
<td>785</td>
<td></td>
</tr>
<tr>
<td>Revised NMR Data for 9-O-Demethylgalanthine: an Alkaloid from <em>Zephyranthes robusta</em> (Amaryllidaceae) and its Biological Activity</td>
<td>Marcela Šafratová, Zdeněk Novák, Andrea Kulhánková, Jiří Kuneš, Martina Hrabinová, Daniel Jun, Kateřina Macáková, Lubomír Opletal and Lucie Cahliková</td>
<td>787</td>
</tr>
<tr>
<td>Structure Revision of N-Mercapto-4-formylcarbostyril Produced by <em>Pseudomonas fluorescens</em> G308 to 2-(2-Hydroxyphenyl)thiazole-4-carbaldehyde [aeruginaldehyde]</td>
<td>Lumeng Ye, Pierre Cornelis, Karel Guillemyn, Steven Ballet, Carsten Christophersen, and Ole Hammerich</td>
<td>789</td>
</tr>
<tr>
<td>Accumulation and Function of Trigonelline in Non-leguminous Plants</td>
<td>Hiroshi Ashihara and Shin Watanabe</td>
<td>795</td>
</tr>
<tr>
<td>Metabolites of the Endophytic Fungus <em>Penicillium</em> sp. FJ-1 of <em>Acanthus ilicifolius</em></td>
<td>Jian-Fang Liu, Wei-Jie Chen, Ben-Ru Xin and Jie Lu</td>
<td>799</td>
</tr>
<tr>
<td>Overexpression of Cinnamate 4-Hydroxylase and 4-Coumaroyl CoA Ligase Promoted Flavone Accumulation in <em>Scutellaria baicalensis</em> Hairy Roots</td>
<td>Young Seon Kim, Yeon Bok Kim, Yeli Kim, Mi Young Lee and Sang Un Park</td>
<td>803</td>
</tr>
<tr>
<td>New Isoflavone Glycosides from the Stems of <em>Dalbergia vietnamensis</em></td>
<td>Pham Thanh Loan, Hoang Le Tuan Anh, Nguyen Thi Cuc, Duong Thi Hai Yen, Dan Thi Thuy Hang, Tran Minh Ha, Nguyen Xuan Nghiem, Nguyen Van Du, Tran Huy Thai, Chau Van Minh and Phan Van Kiem</td>
<td>809</td>
</tr>
<tr>
<td>A Characterization of Content, Composition and Scavenging Capacity of Phenolic Compounds in Parsnip Roots of Various Weight</td>
<td>Nada Č. Nikolić, Miodrag M. Lazić, Ivana T. Karabegović, Gordana S. Stojanović and Zoran B. Todorović</td>
<td>811</td>
</tr>
<tr>
<td>Variability of Procyanidin type A- and -B Trimers Content in Aerial Parts of Some <em>Vaccinium</em> Species and Cultivars</td>
<td>Peeter Toomik, Tõnu Püssa and Ain Raal</td>
<td>815</td>
</tr>
</tbody>
</table>

*Continued inside backcover*