Fitoterapia 81 (2010) 621–626

Contents lists available at ScienceDirect

Fitoterapia

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

Nepetalactone content in shoot cultures of three endemic *Nepeta* species and the evaluation of their antimicrobial activity

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ARTICLE INFO

Article history:
Received 10 September 2009
Accepted in revised form 1 March 2010
Available online 20 March 2010

Keywords:
Nepeta rtanjensis
Nepeta sibirica
Nepeta nervosa
Nepetalactone
Antimicrobial activity

ABSTRACT

Rapid micropropagation of *Nepeta rtanjensis*, *N. sibirica* and *N. nervosa* was performed. Qualitative and quantitative nepetalactone content in methanol extracts of *in vitro* grown plants was analysed by reverse-phase HPLC coupled with UV and MS detection. Only *trans,cis*-nepetalactone was detected in shoots of *N. rtanjensis*, while *cis,trans*-nepetalactone stereoisomer was present in *N. sibirica*. No nepetalactone was observed in shoots of *N. nervosa*. The antimicrobial activity of methanol extracts, against eight bacterial and eight fungal species, was evaluated. All the tested extracts showed significant antibacterial and strong antifungal activity. However, *N. rtanjensis* extract exhibited the best antimicrobial potential.

1. Introduction

The genus *Nepeta* (fam. *Lamiaceae*) is comprised of approximately 250 annual and perennial species which are native to temperate Europe, Asia, North Africa, and the mountainous regions of tropical Africa [1]. Some of these species are well known for their medicinal properties and are widely used in folk medicine. The pharmacological properties and various biological activities are usually ascribed to nepetalactone compounds primarily found in the essential oils of the *Nepeta* species.

Nepetalactone is an iridoid monoterpenoid which exists in the form of eight stereoisomers, four diasteroisomers and their corresponding enantiomers. With a few exceptions, the 7 S diasteroisomers are found in natural sources [2]. Even though these nepetalactone isomers differ only in the orientation of a single chemical bond, they still show differential biological activities. Dawson et al. [3] and Hardie et al. [4] reported that the configuration at C-7 is crucial for the biological activity on aphids. Bates and Sigel [5] found that cats are attracted to *cis,trans*- (4aα,7α,7aα-nepetalactone), and especially to the *trans,cis*-nepetalactone (4aα,7α,7aβ-nepetalactone) stereoisomer. The *trans,cis* stereoisomer is highly toxic to some insects [6], and acts as a stronger repellent against cockroaches than the *cis,trans* stereoisomer [7,8], while both nepetalactones exhibited mosquito repellent activity [9,10]. Of particular interest to human health, the *cis, trans*-nepetalactone was found to be more active than the *trans,cis* stereoisomer against *Helicobacter pylori* [11].

The objective of this study was to compare the antibacterial and antifungal activities of three *Nepeta* species differing in their qualitative and quantitative nepetalactone content. Selected *Nepeta* species included *N. rtanjensis* Diklić & Milojević, *N. sibirica* L. and *N. nervosa* Royle & Bentham. *N. rtanjensis* Diklić & Milojević, an endemic and critically endangered perennial in Serbia, was previously reported to contain much higher amounts of *trans,cis*-nepetalactone than *cis,trans*-nepetalactone [12,13]. *N. sibirica* L. is endemic to central Asia, Mongolia and southern Siberia [14], and possesses high amounts of *cis,trans*-nepetalactone [14,15]. *N. nervosa* Royle & Bentham, a plant species endemic to

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0367-326X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2010.03.007

Kashmir [16], contains nepetalactones only in trace amounts [17]. Some literature regarding the biological activity of their essential oils including antimicrobial potential has been published [13,18]. However, to our knowledge, this is the first characterization of the antibacterial and antifungal activity of their leaf extracts. Sufficient plant material was obtained by efficient *in vitro* propagation of these endemic species.

2. Material and methods

2.1. Plant material

Seeds of *N. sibirica* and *N. nervosa* were provided from Grugapark Essen (International Seed Exchange, Essen, Germany). The seeds of *N. rtanjensis* were collected in August 2004 from greenhouse grown plants.

2.2. Sterilization and plant culture conditions

Seeds were surface sterilized in 20% solution of commercial bleach for 10 min and rinsed 5 times in sterile distilled water. The germination of seeds was induced by 24 h treatment with 1 mM solution of GA_3 containing 500 mg l^{-1} nystatin. Seeds were rinsed in sterile distilled water 3 more times and subsequently transferred on a half-strength MS medium [19], supplemented with 100 mg l^{-1} myo-inositol, 20 g l^{-1} sucrose and 7 g l^{-1} agar. Media were brought to pH = 5.8 (pH = 7.0 for *N. rtanjensis*) before sterilizing by autoclaving at 114 °C for 25 min. Cultures were grown in 350 ml glass jars closed with transparent polycarbonate caps, with 60 ml culture medium in each. Growth conditions were as previously described by Mišić et al. [20].

All the experiments were performed using a single clone of each species (*N. rtanjensis*, *N. nervosa* and *N. sibirica*). Four weeks after the onset of the experiments the following parameters were recorded: the mean length of shoot, the mean number of nodes and axillary buds per shoot, rooting percentage, fresh and dry weights of shoots. In order to obtain sufficient plant material, all the experiments were repeated three times, using 40 explants each.

2.3. Preparation of plant extracts for the HPLC–UV and HPLC–MS analyses

The plant material was air-dried and stored in paper bags at room temperature until use. Each sample (250 mg, dried and powdered) was spiked with 10 µl of geraniol (Haarman Reimer, Germany), which was used as an internal standard, and extracted with 10 ml of methanol. All samples were filtered through 0.45 µm nylon filters (Spartan-3NY, S & S Biopath, USA) and stored at 4 °C until use.

2.4. HPLC conditions for the analyses of nepetalactone

The analyses of *cis,trans*- and *trans,cis*-nepetalactone content in extracts of *N. rtanjensis*, *N. nervosa*, and *N. sibirica* shoots were performed by using a modification of the procedure described by Ganzera et al. [21], as previously reported by Mišić et al. [22]. All the analyses were performed on a Hewlett Packard HPLC system, model 1100 with DAD. The column used for the nepetalactone analysis was Hypersil BSD-C18 (5 µm), 125 × 2 mm I.D. An additional peak confirmation was made by a peak spectra evaluation via HP Chemstation chromatographic software (Palo Alto, USA) also used for the data acquisition and method/run control.

Standard solutions were prepared by dissolving *N. rtanjensis* essential oil, containing 79.89% *trans,cis*-nepetalactone and 6.30% *cis,trans*-nepetalactone, in methanol (10 µl/10 ml methanol). Further calibration levels were prepared by diluting the stock with methanol (correlation coefficient $R^2 = 0.99877$).

2.5. MS conditions for the analyses of nepetalactone

In order to confirm the identified nepetalactone stereoisomers, samples were analysed by HPLC–MS, using modified procedure described by Wang et al. [23]. Reverse phase HPLC analysis was carried out on Waters Breeze HPLC system (Waters, Milford, MA, USA) with EMD 1000 mass detector in positive ESI mode. Signals were recorded in single ion mode for nepetalactone at m/z 167 and the MS scan for mass span from 100 to 400 amu. ESI source parameters were 3.5 kV for capillary and 35 V for cone voltage, respectively. Source temperature was 130 °C and desolvation temperature was 400 °C. Separations were performed on a Waters Xterra MS C-18 column 2.1 × 50 mm with 3.5 µm particle size. Mobile phases were 0.1% formic acid (mobile phase A) and 30% acetonitrile (mobile phase B) with the following gradient profile: the first 20 min from 30 to 70% B, followed by 15 min reverse to 30% B with additional 5 min of equilibration time. Acetonitrile (J. T. Baker, USA), methanol (Carbo Reagenti, Milano) and p.a. grade formic acid were used. The data acquisition and evaluation were carried out using Waters Empower 2 Software (Waters, Milford, MA, USA).

2.6. Preparation of plant extracts for the antimicrobial activity evaluation

Aerial parts of *Nepeta rtanjensis*, *N. sibirica* and *N. nervosa* were extracted with methanol (1:1 = w:v), evaporated and the residues were solubilized in dimetylsulphoxide (DMSO) (1:1 = w:v).

2.7. Microorganisms, culture conditions and tests for antimicrobial activity

The following Gram-negative bacteria were tested: *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Listeria monocytogenes* (NCTC 7973), *Enterobacter cloacae* (human isolate) and the following Gram-positive bacteria: *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Staphylococcus aureus* (ATCC 6538). Antifungal bioassays were performed against following fungal species: *Aspergillus flavus* (ATCC 9643), *Aspergillus fumigatus* (human isolate), *Aspergillus niger* (ATCC 6275), *Fusarium sporotrichoides* (ITM 496), *Fulvia fulvum* (TK 5318), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Trichoderma viride* (IAM 5061). The organisms were obtained from the Mycological Laboratory,