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Research Article

Comparative quantitative profiling of rare justicidin B in *in vitro* cultivated *Linum* species

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Abstract

This research investigates the production and optimization of justicidin B from *L. austriacum* and *L. alpinum in vitro* cultures, aiming for high yields of this pharmacologically significant arylnaphthalene lignan. Various cultivation techniques were employed, including shoot, root, and callus cultures, with different media formulations to induce justicidin B biosynthesis. *In vitro* shoots, callus, and root cultures of both *Linum* species were successfully established to enhance justicidin B production. Quantification of the lignan was performed using LC-HRESI-MS analysis, comparing the quantities to those of isolated and identified justicidin B from *L. leonii* hairy root extract. The results show that the *in vitro* root cultures of *L. alpinum* produced 1.3 times more justicidin B than those of *L. austriacum* (7.24 µg/mg DW and 5.31 µg/mg DW, respectively). Additionally, the shoot cultures of *L. alpinum* yielded 4.5 times more justicidin B than those of *L. austriacum* (4.34 µg/mg DW and 0.96 µg/mg DW, respectively).

Keywords

Linum alpinum, *Linum austriacum*, *in vitro* cultures, justicidin B, LC-MS analysis

Introduction

The genus *Linum* (Linaceae), encompassing over 200 species, is classified into various sections according to morphological characteristics (Ockendon and Walters 1968). Notably, these species are recognized for their lignan production (Konuklugil et al. 2007). Lignans have attracted substantial interest due to their diverse pharmacological activities, serving as key structures for developing new therapeutic drugs (Saleem et al. 2005). The presence of arylnaphthalene lignans has been documented in several *Linum* species, including *L. lewisii*, *L. altaicum* (Konuklugil et al. 2007), *L. leonii* (Vasilev et al. 2004), *L. narbonense* (Ionkova et al. 2013), and *L. glaucum* (Mohagheghzadeh et al. 2009).

Despite the potential of these secondary metabolites, their production in natural conditions is often limited (Chaudhari et al. 2023). This challenge is addressed through *in vitro* culture techniques combined with biotechnological methods, which greatly enhance the pharmaceutical industry's ability to produce bioactive phytochemicals with diverse medicinal properties (Hasnain et al. 2022). *In vitro* cultivation offers controlled and sustainable production of these valuable compounds, overcoming the limitations of natural conditions and ensuring consistent quality and yield. This approach also allows for the manipulation of different growth parameters to further boost the synthesis of target metabolites, making it an invaluable tool for pharmaceutical research and development (Espinosa-Leal et al. 2018). Established *in vitro*

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cultures of *L. austriacum*, including callus (CC), cell suspension (CSC), root (RC), and hairy root cultures (HRC), have been successfully used for the isolation, structural elucidation, and quantification of arylnaphthalene lignans (Mohagheghzadeh et al. 2002). Additionally, studies on justicidin B accumulation in various *in vitro* cultures such as CC, RC, and HRC using elicitors further underscore the potential of these techniques (Mascheretti et al. 2021).

Research indicates that justicidin B effectively inhibits the proliferation of malignant cells in various leukemic cell lines (Vasilev et al. 2006) and demonstrates significant growth-inhibitory properties on HeLa cells (Gertsch et al. 2003). Additionally, justicidin B is a potent inhibitor of bone resorption, highlighting its promise as a lead compound for new antirheumatic medications (Ilieva et al. 2014). Its efficacy against SARS-CoV-2 further underscores its potential as a potent antiviral agent (Tóth et al. 2023). In terms of anti-inflammatory effects, justicidin B's strong inhibition of bone resorption suggests its viability in developing new antirheumatic drugs (Apers et al. 2003). It also exhibits significant antifungal and antiprotozoal properties, effectively suppressing strains like *Aspergillus fumigatus* and *Candida albicans* and showing potent activity against Trypanosoma species (Gertsch et al. 2003). The antibacterial activity of justicidin B presents mixed results; some studies report its effectiveness against *Bacillus cereus* and *Escherichia coli* (Apers et al. 2003), while others indicate a lack of activity against bacteria such as *B. cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *E. coli* (Gertsch et al. 2003). The above-listed versatile biological activities of justicidin B underscore its potential as a candidate for developing new therapeutic agents through innovative drug development (Hemmati and Seradj 2016). In this study, using biotechnological methods, several *in vitro* cultures of *L. austriacum* and *L. alpinum* were established, and the production of the arylnaphthalene lignan justicidin B was assessed.

Materials and methods

Plant material

Shoot cultures (SC) of *L. austriacum* and *L. alpinum* were initiated from sterilized seeds using general procedures (Ionkova et al. 2010). The SC developed from seedlings was cultivated on MS medium (Murashige and Skoog 1962). RC propagation was successfully accomplished using modified MS medium supplemented with naphthylacetic acid (NAA) (2 mg/L) (MS-Li). CC of *L. austriacum* and *L. alpinum* were initiated by cultivating SC on modified MS medium supplemented with kinetin (2 mg/L), indole acetic acid (IAA) (5 mg/L), 2,4-dichlorophenoxyacetic acid (2,4-D) (5 mg/L), and casein (1 g/L) (G48) (Ionkova et al. 2010). The hairy root cultures of *L. leonii* were obtained by genetic transformation using the agropine-type strain *Agrobacterium rhizogenes* 15834 (Vasilev et al. 2006).

General experimental procedures and analytical methods

All solvents used were of at least analytical grade from Fischer Scientific (Loughborough, UK). Previously optimized LC-HRESI-MS analyses were used to determine the quantity of justicidin B, and a calibration curve was built for quantification purposes. A semi-preparative HPLC system (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) consisting of a Knauer Azura P 6.1L pump, a variable wavelength UV-Vis detector (Knauer UVD 2.1L), a hand injector, a fraction collector (FOXY R1), an AZURA column thermostat (CT 2.1), and YL Clarity software was used for the isolation of justicidin B. NMR spectra were recorded on a Bruker AVII+ 600 spectrometer (Bruker, Karlsruhe, Germany) in $CDCl_3$ (99.95%, Deutero GmbH) with TMS as the internal standard, operating at a proton NMR frequency of 600.13 MHz for ^{1}H and at 150.90 MHz for ^{13}C spectra. LC-MS analysis was performed at Thermo Scientific Q Exactive Plusquadrupole-Orbitrap mass spectrometer used in ultra-high-resolution mode (70 000, at *m/z* 200) coupled with a UPLC Dionex Ultimate 3 000 RSLC system equipped with a RP-18 Kinetex column (2.10 mm × 100 mm, 2.6 µm, Phenomenex (Corporation, Torrance, CA, USA). MS-grade solvents ACN and H_2O were used (Fischer). Gradient elution (0' 5% ACN, 3' 10% ACN; 16–17' 20% ACN; 19' 40% ACN, 20' 50% ACN, 22.50' 95% ACN, 24.50' 95% ACN) of filtered and degassed ACN/H_2O solution of FA 0.1% (v/v); column temperature 40 °C; a flow rate of about 300 µL/min was used during the analysis. The operating conditions of the HR-ESI source ionization device were: 3.5 kV voltage, 320 °C capillary temperature, 25 units of carrier gas flow, and 5 units of dry gas flow. The scan range was between 100 and 1 500 *m/z* at 70 000 resolution, and the MS/MS scan was set at 17 500 resolution, with AGC target 1e^5, maximum IT, a scan range of 100 to 1 500 *m/z*, an isolation window of 2.0 *m/z*, and (N) CE 20, 40, and 60. Nitrogen was used to atomize the samples. All the other parameters were set to obtain the most intense signal for $[M+H]^+$. All data were recorded and processed using Xcalibur software, version 2.0 (Thermo Fisher).

Isolation and structure elucidation of justicidin B

We utilized the justicidin B isolated in our laboratory from the HRC extract of *L. leonii* by semi-preparative HPLC. For the isolation, the EtOAc fraction of 70% MeOH extract was subjected to semi-preparative HPLC isolation, performed with HPLC-grade ACN in H_2O, each containing 0.1% FA. For the separation, an RP-18 Ascentis column (25 cm × 4.6 mm, 10 µm) at a flow rate of 5 mL/min was used. The mobile phase gradient is as follows: 0'–15' min 25% ACN, 15'–30' 60%, 30'–34' 100%, until 35' back to 25% ACN. HPLC analyses conducted at UV detection of 254 nm resulted in the isolation of justicidin B with Rt 21.17' (Suppl. material 1: fig. S1).

Chemistry

Justicidin B was isolated as a white powder, and during LC-HRESI-MS analysis, it was observed as a protonated molecular ion at *m/z* 365.1014 $[M+H]^+$, corresponding to the molecular formula $C_{21}H_{17}O_6^+$ (calc. for *m/z* 365.1020). NMR spectral data for justicidin B: ^1H NMR ($CDCl_3$) δ, ppm: 3.79 (s, 3H, s, $-CH_3$), 4.03 (s, 3H, s, $-CH_3$), 5.36 (s, 2H, $-CH_2-$), 6.03, 6.07 (d, 2H, *J* = 1.39, $-CH_2-$), 6.81 (d, 1H, Ar, *J* =7.86, –CH=), 6.84 (d, 1H, Ar, *J* = 1.68, –CH=), 6.95 (d, 1H, Ar, *J* =7.94, –CH=), 7.09 (s, 1H, Ar, –CH=), 7.16 (s, 1H, Ar, –CH=), 7.68 (s, 1H, Ar, –CH=) (Suppl. material 1: fig. S2). ^{13}C NMR ($CDCl_3$) δ, ppm: 56.07 (C^{12}, $-CH_3$), 56.31 (C^{11}, $-CH_3$), 68.30 (C^9, $-CH_2-$), 101.48 (C^{10}, $-CH_2-$), 106.07 ($C^{5'}{}_{arom}$), 106.23 ($C^{6}{}_{arom}$), 108.45 ($C^{3}{}_{arom}$), 110.79 ($C^{2'}{}_{arom}$), 118.49 ($C^{7}{}_{arom}$), 118.73 ($C^{8'}{}_{arom}$), 123.69 ($C^{6'}{}_{arom}$), 128.62 ($C^{1'}{}_{arom}$), 129.07 ($C^{2}{}_{arom}$), 139.73 ($C^{8}{}_{arom}$), 139.87 ($C^{7'}{}_{arom}$), 133.38 ($C^{1}{}_{arom}$), 147.75 ($C^{4'}{}_{arom}$), 147.79 ($C^{3'}{}_{arom}$), 150.29 ($C^{5}{}_{arom}$), 152.07 ($C^{4}{}_{arom}$), 170.20 ($C^{9'}$, =C=O) (Suppl. material 1: fig. S3).

Extraction of plant material and sebsequent quantification analysis of justicidin B

The powdered dried plant material from *in vitro* cultures of *L. alpinum* CC, SC, and RC and *L. austriacum* SC and RC was subjected to exhaustive extraction using 70% MeOH (3 × 30 mL). The obtained extracts were concentrated using a rotary vacuum evaporator and appropriately diluted with 50% MeOH/H_2O to about 100 µg/mL final concentration. An aliquot of each sample was subjected to LC-HRESI-MS analysis.

Results and discussion

In vitro cultivation

In vitro cultures of *L. austriacum* and *L. alpinum* were successfully established and maintained in our laboratory. For the initiation of SC in *L. austriacum* and *L. alpinum*, we used sterilized seeds. The SC that developed from seedlings was placed on MS culture medium. CC of *L. alpinum* was initiated by cultivating SC on modified MS medium supplemented with kinetin (2 mg/L), IAA (5 mg/L), 2,4-D (5 mg/L), and casein (1 g/L) (G48) (Ionkova et al. 2010). The RCs of *L. austriacum* and *L. alpinum* were cultivated on several different media. Both *Linum* species were cultivated on MS-Li medium and on modified MS-Li medium supplemented with NAA (1 ml/L) and IAA (0.4 ml/L) (MS-LiZ). *L. alpinum* RC was also cultured on MS medium enriched with IAA (5 mg/L), kinetin (2 mg/L), and casein (1 g/L).

Identification of justicidin B

Identification of justicidin B was based on its ^1H, ^{13}C NMR, and LC-HRESI-MS spectra compared to previously published data (Fig. 1) (Da Silva et al. 2007).

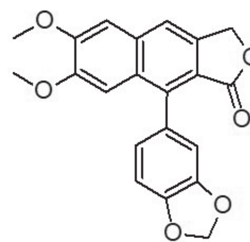

Figure 1. Chemical structure of justicidin B.

Calibration model

The quantity of justicidin B was determined by LC-HRESI-MS analysis after plotting a calibration curve using the isolated justicidin B from the HRC of *L. leonii*. The determination shows that the method is linear for all the standards (Fig. 2). The determination coefficient for justicidin B is $r^2 = 0.9974$. After the linearity was investigated, the regression analysis was performed. The determination coefficients and regression equations are presented in Table 1.

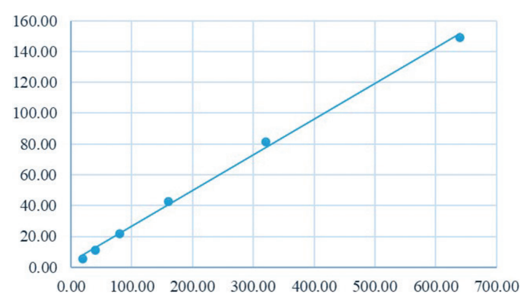

Figure 2. Calibration curve of justicidin B.

Table 1. Determination coefficient and regression equations of justicidin B.

	justicidin B
Determination coefficient	$r^2 = 0.9974$
Linear range (ng/mL)	20–600
Regression equations	Y = 3.37252e + 006 + 231587*X
Number of standards	6
Rt (min)	3.76

Quantification of justicidin B

The amount of justicidin B is calculated due to the calibration equation formula Y = 3.37252e+006+231587*X within each of the derived extracts from *in vitro* cultures of *L. alpinum and L. austriacum*. When comparing the two species, the *in vitro* RC of *L. alpinum* from G56 medium exhibits the highest overall concentration of justicidin B, reaching 7.24 µg/mg DW. In contrast, the CC from *L. alpinum* in G48 medium presents the lowest concentration of justicidin B, at merely 0.20 µg/mg DW. For *L. austriacum*, the highest concentration of justicidin B is found in the RC grown on MS-LiZ medium, which attains a value of 5.31 µg/mg DW. Although this concentration is lower than that observed in the highest-producing *L. alpinum* sample, it remains a sustainable alter-

native. These results underscore the exceptional efficacy of the G56 *in vitro* RC condition in *L. alpinum* for the biosynthesis of justicidin B, while also demonstrating that the MS-LiZ medium is particularly effective for justicidin B production in *L. austriacum*. This comparison highlights the critical importance of optimizing species-specific and culture-specific conditions to maximize the yield of valuable secondary metabolites such as justicidin B. The findings suggest targeted approaches in biotechnological applications to enhance metabolite production, leveraging the most effective culture conditions for each species.

Regarding the content of justicidin B in *L. alpinum*, results indicate that *in vitro* RC exhibits the highest yield, particularly those cultivated on G56 medium, with a concentration of 7.24 µg/mg DW. Another viable and sustainable source is *in vitro* SC grown on MS medium, which also demonstrates a significant justicidin B content of 4.34 µg/mg DW. In contrast, CC produces a very low amount of just 0.20 µg/mg DW (Fig. 3).

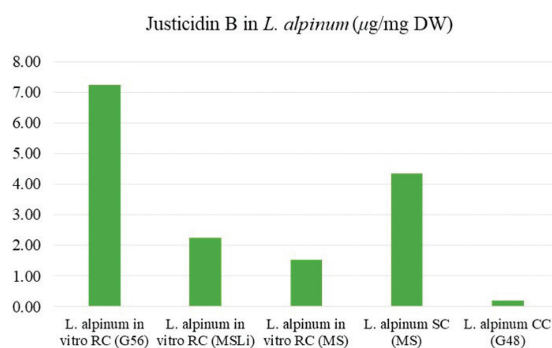

Figure 3. The amount of justicidin B in *in vitro* RC, SC, and CC from *L. alpinum* is represented as µg/mg DW on different mediums.

For *L. austriacum*, the *in vitro* RC cultivated on MS-LiZ medium exhibits the highest concentration of justicidin B, with a value of 5.31 µg/mg DW. This suggests that the MS-LiZ medium is particularly appropriate for justicidin B biosynthesis in *L. austriacum*. In comparison, the *in vitro* RC cultivated on MS-Li medium reached justicidin B production of 2.63 µg/mg DW, which indicates this model as a suitable alternative. Unfortunately, the concentration of justicidin B in *L. austriacum* SC grown on MS medium is the lowest among tested extracts of *L. austriacum* (0.96 µg/mg DW). This significant difference implies that SC is less efficient for justicidin B production compared to *in vitro* RC (Fig. 4).

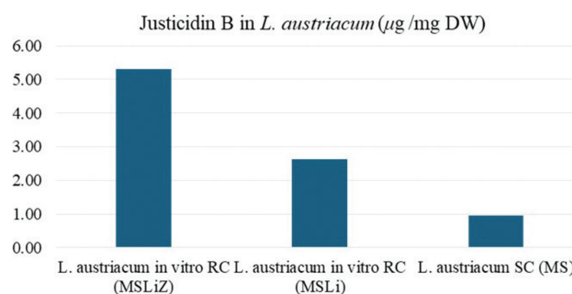

Figure 4. The amount of justicidin B in *in vitro* RC and SC from *L. austriacum* is represented as µg/mg DW on different mediums.

Conclusion

The focus of the study is optimizing the production of justicidin B, a bioactive rare arylnaphthalene lignan with diverse pharmacological properties, from *in vitro* cultures of two *Linum* species, *L. austriacum* and *L. alpinum*. The research successfully established various *in vitro* cultures, including SC, RC, and CC, using multiple culture media to induce justicidin B biosynthesis. The cultivation conditions significantly influenced the concentration of justicidin B, with *L. alpinum* RC on G56 medium and *L. austriacum* RC on MS-LiZ medium demonstrating the highest yield (7.24 µg/mg DW and 5.31 µg/mg DW, respectively).

Results showed varying concentrations of justicidin B across different *in vitro* cultures, underscoring the importance of specific cultivation methods in optimizing its production. These findings not only enhance our understanding of biotechnological strategies for secondary metabolite production but also highlight the potential of *in vitro* cultures of *Linum* species as sources of valuable bioactive compounds with potential as inhibitors of bone resorption and bone metastases.

Future research could focus on further optimizing cultivation techniques to scale up justicidin B production. These insights highlight the critical influence of cultivation methods and specific media conditions on optimizing the production of secondary metabolites like justicidin B. By concentrating on the most productive plant parts and species, further studies and practical applications can be better guided for efficient extraction and utilization of justicidin B.

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References

Apers S, Vlietinck A, Pieters L (2003) Lignans and neolignans as lead compounds. Phytochemistry Reviews 2: 201–217. https://doi.org/10.1023/B:PHYT.0000045497.90158.d2

Chaudhari GR, Prakash K, Patel SR, Chavda D, Shekhawat N, Ragi S, Ds SR (2023) Secondary metabolites production in plant tissue culture. The Pharma Innovation 12: 2198–2208. https://doi.org/10.22271/tpi.2023.v12.i9Sac.23144

Da Silva R, Maia Ruas M, Marcos Donate P (2007) Complete assignments of ^{1}H and ^{13}C NMR spectral data for arylnaphthalene lignan lactones. Magnetic Resonance in Chemistry 45: 902–904. https://doi.org/10.1002/mrc.2065

Espinosa-Leal CA, Puente-Garza CA, García-Lara S (2018) *In vitro* plant tissue culture: means for production of biological active compounds. Planta 248: 1–18. https://doi.org/10.1007/s00425-018-2910-1

Gertsch J, Tobler R, Brun R, Sticher O, Heilmann J (2003) Antifungal, antiprotozoal, cytotoxic and piscicidal properties of justicidin B and a new arylnaphthalide lignan from *Phyllanthus piscatorum*. Planta Medica 69: 420–424. https://doi.org/10.1055/s-2003-39706

Hasnain A, Naqvi SAH, Ayesha SI, Khalid F, Ellahi M, Iqbal S, Hassan MZ, Abbas A, Adamski R, Markowska D, Baazeem A, Mustafa G, Moustafa M, Hasan ME, Abdelhamid MMA (2022) Plants *in vitro* propagation with its applications in food, pharmaceuticals and cosmetic industries; current scenario and future approaches. Frontiers in Plant Science 13: 1009395. https://doi.org/10.3389/fpls.2022.1009395

Hemmati S, Seradj H (2016) Justicidin B: A Promising Bioactive Lignan. Molecules 21: 820. https://doi.org/10.3390/molecules21070820

Ilieva Y, Zhelezova I, Atanasova T, Zaharieva MM, Sasheva P, Ionkova I, Konstantinov S (2014) Cytotoxic effect of the biotechnologically-derived justicidin B on human lymphoma cells. Biotechnology Letters 36: 2177–2183. https://doi.org/10.1007/s10529-014-1612-4

Ionkova I, Antonova I, Momekov G, Fuss E (2010) Production of podophyllotoxin in *Linum linearifolium in vitro* cultures. Pharmacognosy Magazine 6: 180. https://doi.org/10.4103/0973-1296.66932

Ionkova I, Sasheva P, Ionkov T, Momekov G (2013) *Linum narbonense*: A new valuable tool for biotechnological production of a potent anticancer lignan Justicidine B. Pharmacognosy Magazine 9: 39. https://doi.org/10.4103/0973-1296.108138

Konuklugil B, Ionkova I, Vasilev N, Schmidt TJ, Windhövel J, Fuss E, Alfermann AW (2007) Lignans from *Linum* species of sections *Syllinum* and *Linum*. Natural Product Research 21: 1–6. https://doi.org/10.1080/14786410600798385

Mascheretti I, Alfieri M, Lauria M, Locatelli F, Consonni R, Cusano E, Dougué Kentsop RA, Laura M, Ottolina G, Faoro F, Mattana M (2021) New Insight into Justicidin B Pathway and Production in *Linum austriacum*. International Journal of Molecular Sciences 22: 2507. https://doi.org/10.3390/ijms22052507

McDill J, Repplinger M, Simpson BB, Kadereit JW (2009) The Phylogeny of *Linum* and Linaceae Subfamily Linoideae, with Implications for Their Systematics, Biogeography, and Evolution of Heterostyly. Systematic Botany 34: 386–405. https://doi.org/10.1600/036364409788606244

Mohagheghzadeh A, Schmidt TJ, Alfermann AW (2002) Arylnaphthalene Lignans from *in Vitro* Cultures of *Linum austriacum*. Journal of Natural Products 65: 69–71. https://doi.org/10.1021/np0102814

Mohagheghzadeh A, Dehshahri S, Hemmati S (2009) Accumulation of Lignans by *in vitro* Cultures of Three *Linum* Species. Zeitschrift für Naturforschung C 64: 73–76. https://doi.org/10.1515/znc-2009-1-213

Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15: 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Ockendon DJ, Walters SM [Eds] (1968) Flora Europaea. Vol. 2: Linaceae. paperback print. Cambridge University Press, Cambridge, 469 pp.

Saleem M, Kim HJ, Ali MS, Lee YS (2005) An update on bioactive plant lignans. Natural Product Reports 22: 696. https://doi.org/10.1039/b514045p

Tóth G, Horváti K, Kraszni M, Ausbüttel T, Pályi B, Kis Z, Mucsi Z, Kovács GM, Bősze S, Boldizsár I (2023) Arylnaphthalene Lignans with Anti-SARS-CoV-2 and Antiproliferative Activities from the Underground Organs of *Linum austriacum* and *Linum perenne*. Journal of Natural Products 86: 672–682. https://doi.org/10.1021/acs.jnatprod.2c00580

Vasilev N, Nedialkov P, Ionkova I, Ninov S (2004) HPTLC densitometric determination of justicidin B in *Linum in vitro* cultures. Die Pharmazie 59: 528–529.

Vasilev N, Elfahmi, Bos R, Kayser O, Momekov G, Konstantinov S, Ionkova I (2006) Production of Justicidin B, a Cytotoxic Arylnaphthalene Lignan from Genetically Transformed Root Cultures of *Linum leonii*. Journal of Natural Products 69: 1014–1017. https://doi.org/10.1021/np060022k

Supplementary material 1

Supplementary information

Authors: Rada Nedelcheva, Yancho Zarev, Iliana Ionkova

Data type: pdf

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