In vitro Propagation and Chemical and Biological Studies of the Essential Oil of *Salvia przewalskii* Maxim.

Ewa Skała[a,*], Danuta Kalemba[b], Anna Wajs[b], Marek Różalski[c], Urszula Krajewska[c], Barbara Różalska[d], Marzena Więckowska-Szakiel[d], and Halina Wysokińska[a]

[a] Department of Biology and Pharmaceutical Botany, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland. Fax: 0484267791 60. E-mail: botanika@pharm.am.lodz.pl
[b] Institute of General Food Chemistry, Technical University of Łódź, Stefanowskiego 4/10, 90-924 Łódź, Poland
[c] Department of Pharmaceutical Biochemistry, Medical University of Łódź, Muszyńskiego 1, 90-151 Łódź, Poland
[d] Department of Immunology and Infectious Biology, Institute of Microbiology and Immunology, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

* Author for correspondence and reprint requests

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The procedure of *Salvia przewalskii* shoot multiplication and the ability of regenerated plants to produce essential oil is reported. The essential oil was obtained by hydrodistillation from leaves and flowering stems of field-grown plants, and their chemical composition was examined by GC, GC-MS and ^{1}H NMR. The differences in yield as well as qualitative and quantitative composition between the oils isolated from *in vitro* and *in vivo* plants were observed. *S. przewalskii* essential oil was tested for its antimicrobial and cytotoxic properties. It was found that cytotoxicity against human leukemia HL-60 cells and antimicrobial activity (especially, against *Staphylococcus aureus* and *S. epidermidis* strains) of oils isolated from *in vitro* plants were higher than those for oils from *in vivo S. przewalskii* plants.

Key words: Salvia przewalskii, Essential Oil, Antimicrobial and Cytotoxic Activity

Introduction

The genus *Salvia* includes about 900 species and is considered as one of the largest genera of the Lamiaceae family. Since ancient times *Salvia* species have been used in folk medicine due to their antibacterial, antioxidant, antitumour and antidiabetic properties (Dweck, 2000).

One of the medicinally important *Salvia* species is *S. przewalskii*. It is a herbaceous plant, endemic to north-western China (Li *et al*., 1991). The dried roots of *S. przewalskii*, locally called "Hong Qin Jiao", are used in traditional and folk Chinese medicine as a substitute for *Salvia miltiorrhiza*, for the treatment of coronary heart diseases (Wang *et al*., 1988). The most important biologically active constituents of *S. przewalskii* roots are tanshinones, abietane diterpenoids, such as tanshinone I, tanshinone IIA, dihydrotanshinone, cryptotanshinone (Li *et al*., 1991; Weng-Sheng *et al*., 2003). Apart from tanshinones, also other bioactive compounds, such as triterpenoids (przewanoic acid A and B, oleanolic and ursolic acids) (Wang *et al*., 1988; Weng-Sheng *et al*., 2003) and phenolic derivatives (lithospermic acid B esters) (Zhijun *et al*., 1999), have been isolated from the roots of *S. przewalskii*. Recently, we have reported tanshinone accumulation in roots of *S. przewalskii* micropropagated plants (Skała and Wysokińska, 2005). In the present study, we have developed the system for *S. przewalskii* multiple shoot formation using shoot tips and leaves as explants. The shoots were rooted and plantlets grown in soil as described in our previous work (Skała and Wysokińska, 2005).

The yield and chemical composition of the essential oil from *S. przewalskii* micropropagated plants are described in this paper. For comparison, essential oil from *S. przewalskii* plants propagated from seeds was also isolated and analyzed. All the plants grown in the field under the same conditions were collected at the same developmental stage. Additionally, essential oils isolated from the *in vitro*- and *in vivo*-derived plants were tested for their cytotoxic and antimicrobial activity.

In the literature, there are two reports on the production of essential oil in the aerial parts of *S. przewalskii*. Liu *et al.* (2006) studied the essential oil from flowers and leaves of *S. przewalskii* originating from Tibet and noted that oils obtained by hydrodistillation contained 69 and 43 components, respectively. The volatile oil yield from leaves was 0.2% v/w; it was higher than that from the flowers (0.08%) (Liu *et al.*, 2006). In another study, only 24 and 27 constituents in essential oils from flowers and leaves of *S. przewalskii* growing in a Botanical Garden in the Siberia region were identified (Bajkova *et al.*, 2002).

Essential oils have potential application in medical procedures and in the cosmetic, food and pharmaceutical industries (Chalchat *et al.*, 1998). The antimicrobial, anti-inflammatory and antioxidant activities of essential oils of many plant species have been shown (Kalemba and Kunicka, 2003). Also, essential oils of various *Salvia* species are known to have pharmacological properties (Veličković *et al.*, 2003; Komatou *et al.*, 2005). For example, essential oil of *S. lavandulaefolia* and some of its chemical constituents have anticholinesterase effects and these properties are relevant to the treatment of Alzheimer's disease (Perry *et al.*, 2001). However, biological activity of essential oil of *S. przewalskii* has not been studied yet.

Experimental

Plant material

Leaves and flowering stems of *S. przewalskii* plants obtained by micropropagation and plants propagated from seeds (received from the Botanical Garden of Poznań, Poland) were used for the essential oil analysis. The former will be referred to as *in vitro* plants and the latter as *in vivo* plants. *In vitro* and *in vivo* plants were grown in the Medicinal Plant Garden of the Medical University of Łódź (Poland) and were collected at the flowering stage in July 2006. The species identity was confirmed in our department based on data of Li and Hedge (1994). A voucher specimen was deposited in the Department of Biology and Pharmaceutical Botany, Medical University of Łódź (Poland).

Establishment of shoot culture

Shoot tips and leaves of 4-week-old shoots aseptically grown on MS (Murashige and Skoog, 1962) agar (0.7%) medium supplemented with indole-3-acetic acid (IAA) (2.8 μM) and 6-benzylaminopurine (BA) (4.4 μM) were used as explants. The shoots were obtained from shoot tips of 4-week-old seedlings of *S. przewalskii* as described previously (Skała and Wysokińska, 2005).

To evaluate the effect of cytokinins on shoot multiplication, shoot tips (0.5 cm in length) with two leaves were cultured on MS agar (0.7%) medium supplemented with IAA (0.6 μM) and various concentrations of BA (2.2–8.9 μM), thidiazuron (TDZ) (2.3–9.1 μM) or kinetin (KIN) (2.3–9.2 μM). Subcultures were carried out every 28 d. Each treatment consisted of 11–14 explants and was repeated three times, by three successive passages using *in vitro*-derived shoot tip explants, representing a total of 33–42 observations per treatment. The efficiency of each combination of cytokinin was determined by recording the number of explants forming axillary shoots divided by the total number of explants (in %), the multiplication rate (number of shoots obtained per explant), shoot length and frequency of hyperhydricity (%) (Table I).

The analysis of variance and Duncan's multiple range test were used for comparison among treatment means.

For the experiments on induction of adventitious shoots, the leaves (about 2 cm in length) from the aseptically grown shoots were cut into laminae (about 1×0.5 cm) and petioles (1 cm in length). The explants were cultured on MS agar medium supplemented with BA (0.9, 1.8 or 4.4 μM) and one of the auxins (IAA; IBA, indole-3-butyric acid; or NAA, α-naphthaleneacetic acid) at the concentrations of 1.1 μM and 2.7 μM. After 4 weeks the response of leaves was expressed in terms of percentage of explants forming adventitious shoot buds and number of shoots per explant. The experiments were replicated three times using 7–13 explants in each treatment.

Culture conditions

The pH values of all media were adjusted to 5.6–5.8. The media were stream-sterilized in an autoclave under 1.5 kg/cm^3 and 121 °C for 17 min. The cultures were grown at (26 ± 2) °C with a 16/8 h (light/dark) photoperiod in a growth room fitted with a cool white-fluorescent lamp providing a PPFD level of 40 μmol m^{-2} s^{-1}.

Rooting, hardening and field cultivation

For rooting, individual axillary shoots developed from shoot tip explants were used. Rooting of *S. przewalskii* shoots and hardening as well as field

cultivation of micropropagated plants were described in our previous work (Skała and Wysokińska, 2005).

Isolation of essential oil

The essential oil was obtained by hydrodistillation of air-dried plant material (110–160 g) using a Clevenger-type apparatus for 4 h.

GC and GC-MS analyses

GC analysis of the essential oil samples was performed using a Carlo-Erba Vega 6000 apparatus equipped with an FID detector and a capillary column CP Sil 5 CB (30 m × 0.32 mm i. d., film thickness 0.25 μm, Quadrex Corporation, New Haven). The oven temperature was programmed as follows: 60–300 °C at 4 °C/min and the final temperature was held for 10 min; injector temperature 320 °C; detector temperature 310 °C; carrier gas N_2 at a flow rate of 1.5 ml/min. Peaks were measured by electronic integration. The percentage composition of the oil samples was computed from the peak areas using the normalization method. An alkane mixture was run under the same conditions for relative retention indices (RI) determination.

GC-MS analysis was carried out with a Fisons MD 800 mass spectrometer (ion source 200 °C, EI 70 eV) connected to a GC 8000 gas chromatograph; helium served as the carrier gas with a flow rate of 0.8 ml/min. A CP Sil 5 CB column was used at the same parameters as described for GC.

NMR spectra

^{1}H NMR spectra were recorded with a Bruker DPX 250 instrument; solvent $CDCl_3$; TMS served as internal standard.

Identification of components

The identification of compounds was based on the comparison of their RIs and MS spectra with those of authentic samples, computer libraries NIST and MassFinder 3 or literature data (Adams, 1995; Joulain and König, 1998). The identification of isolated sesquiterpenes was confirmed by comparison of their ^{1}H NMR spectra with literature data.

Isolation of components

The sample of essential oil of plants regenerated *in vitro* (360 mg) was subjected to vacuum distillation. The fraction of monoterpenes was separated. The residue (170 mg) consisting of sequiterpenes was subjected to flash chromatography (FC) on Silica gel 60 (0.040–0.063 mm, Merck) with pentane and pentane with increasing amounts of diethyl ether: 5%, 10%, 20% and finally 50%. The separation was monitored by TLC and GC.

Antimicrobial susceptibility testing

The susceptibility of microorganisms to essential oils was determined by a standard CLSI (Clinical and Laboratory Standards Institute) microdilution method. Sterile stock solutions of each oil at the concentration of 160 mg/ml were prepared in dimethyl sulphoxide (DMSO). The agent concentration range used in the antimicrobial tests was 2.5–15 mg/ml (the final concentration) prepared for bacteria in Mueller-Hinton broth (Difco) and for the yeast in RPMI-1640 medium supplemented with L-glutamine and $NaHCO_3$ (Biomed, Poland). To specify the minimal inhibitory concentration (MIC), turbidometric (OD_{600}) studies were carried out using the multifunction counter Victor2 (Wallac, Finland).

Cell and cytotoxicity assay

Cell cultures

Human leukemia promyelocytic HL-60 and lymphoblastic NALM-6 cell lines were used. Leukemia cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated foetal bovine serum (Invitrogen, Paisley, UK) and antibiotics (100 μg/ml streptomycin and 100 U/ml penicillin). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Cytotoxicity assay by MTT

Cytotoxic activity of essential oils was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, USA] assay (Hansen *et al.*, 1989). Exponentially growing leukemia cells were seeded at 8×10^3/well on 96-well plates (Nunc, Roskilde, Denmark). Stock solutions of the analyzed compounds were freshly prepared in DMSO and diluted with complete culture medium to obtain the concentration range from 1 to 1000 μg/ml. Cells were exposed to the test compounds for 46 h, then the MTT reagent was added and incubation was continued for 2 h. After incubation, MTT-formazan crystals were dissolved in 20% SDS and 50% DMF at pH

4.7 and the absorbance was read at 562 and 630 nm on an ELISA-plate reader (ELX 800, Bio-Tek, USA). As a control, cultured cells were grown in the absence of drugs. The IC_{50} values (the concentration of the tested oil required to reduce the cells survival fraction to 50% of the control) were calculated from concentration-response curves and used as a measure of cellular sensitivity to a given treatment. Data points represent means of at least 6 repeats ± SD.

Results and Discussion

Shoot multiplication

The efficiency of shoot induction from shoot tip explants was tested on MS agar medium supplemented with different concentrations of BA, KIN or TDZ (range from 2.2 to 9.2 μM) in combination with IAA (0.6 μM) (Table I). The percentage of responding explants was high (80–100%) in the presence of BA or TDZ. The highest multiplication rate [(3.9 ± 0.03) shoots/explant within 4 week] was obtained on the medium containing 4.5 μM TDZ, followed by 2.3 μM TDZ (3.3 ± 0.13) and 2.2 μM BA (3.2 ± 0.23) without significant differences between them. A significant decrease in the number of shoots per explant was observed, when higher concentrations of BA (> 4.4 μM) or TDZ (6.8 or 9.1 μM) were used (Table I). The higher concentrations resulted also in shorter shoots and an increased percentage of cultures with hyperhydric shoots (Table I). The third cytokinin tested, kinetin, had a positive effect on the length of *S. przewalskii* shoots when compared to BA or TDZ, but the shoot multiplication efficiency decreased in the presence of this cytokinin. Moreover, the frequency of hyperhydricity was found to be high. For example, 41.5% of cultures with hyperhydric shoots were observed when kinetin at the concentration of 9.2 μM was used (Table I).

Considering the shoot number, their length and quality (the lowest percentage of shoots with hyperhydricity symptoms), BA at a concentration of 2.2 μM in combination with IAA (0.6 μM) proved to be the most effective for axillary shoot proliferation when the shoot tips were used as the explants. Therefore, this medium was selected and used for successive subcultures. The effective response of BA in promoting shoot formation had also been earlier reported for several other species of *Salvia* (Mederos *et al.*, 1997; Santos-Gomes *et al.*, 2002; Skała and Wysokińska, 2004). The cytokinin was used in combination with either IAA, *i.e.* in the case of *Salvia nemorosa* (Skała and Wysokińska, 2004), or other auxins such as 2,4-dichlorophenoxyacetic acid (*Salvia officinalis*) (Santos-Gomes *et al.*, 2002) and NAA (*S. canariensis*) (Mederos *et al.*, 1997). Liu *et al.* (2000) regenerated *S. sclarea* shoots *via* organogenesis on MS medium containing BA (2.2 μM) and IAA (2.8 μM).

The organogenic ability of *S. przewalskii* leaves removed from 4-week-old aseptic shoots was also

Table I. Effect of cytokinins on multiple shoot formation through shoot tip explants of *Salvia przewalskii* cultured for 4 weeks on MS medium supplemented with 0.6 μM IAA.

Type and concentration of cytokinin [μM]	Number of explants	Explants producing shoots (%)	Mean number of shoots/explant ± SE	Mean length of shoots ± SE [cm]	Frequency of hyperhydricity (%)
BA 2.2	40	97.5	3.17 ± 0.23 ^{acd}	1.42 ± 0.08	3.94
BA 4.4	42	90.48	2.97 ± 0.12 ^{abc}	1.31 ± 0.07	12.39
BA 6.7	35	94.28	2.24 ± 0.18 ^{bf}	1.13 ± 0.05	4.05
BA 8.9	35	97.14	2.44 ± 0.15 ^{abf}	1.04 ± 0.04	10.84
TDZ 2.3	34	94.12	3.25 ± 0.13 ^{cd}	1.17 ± 0.06	11.54
TDZ 4.5	35	100	3.86 ± 0.03 ^{d}	1.32 ± 0.08	16.3
TDZ 6.8	34	79.41	2.26 ± 0.22 ^{bf}	0.87 ± 0.03	34.43
TDZ 9.1	33	90.91	1.47 ± 0.16 ^{e}	0.9 ± 0.04	40.9
KIN 2.3	35	68.57	2.33 ± 0.12 ^{bf}	1.11 ± 0.03	8.93
KIN 4.6	35	85.71	2.57 ± 0.13 ^{bcf}	1.2 ± 0.06	16.88
KIN 6.9	35	91.43	2.13 ± 0.02 ^{f}	1.51 ± 0.08	20.59
KIN 9.2	34	70.59	2.21 ± 0.23 ^{bf}	1.46 ± 0.08	41.51

The data were recorded as means ± standard error. Means followed by the same letters are not significantly different ($p \leq 0.05$) using Duncan multiple range test.

investigated. The explants were divided into laminae and petioles and inoculated individually into MS agar medium containing different auxins (IAA, NAA or IBA) at a concentration of 1.1 μM or 2.7 μM in combination with 0.9, 1.8 or 4.4 μM BA. The adventitious shoot buds ($\leq$ 0.5 cm) were directly induced from explants tested but at low frequency. As estimated after a period of 4 weeks only 10% of the petioles developed about 4 shoot buds per explant when MS medium supplemented with 1.1 μM NAA and 4.4 μM BA was used. The shoot bud formation was restricted to the distal cut end of the petioles. Shoot organogenesis also occurred on medium containing other auxins (IAA or IBA) but at lower frequency (6–9%) and less number of shoot buds per petiole explant (1.0–2.3). A further decrease in frequency of bud regeneration was observed, when leaf laminae were used as explants. Under the best conditions tested (MS medium with 2.7 μM NAA and 0.9 μM BA) only 4% of the explants developed an average of 2.0 shoot buds per explant. The buds were formed mostly on the whole surface of the lamina close to the midrib. Among the *Salvia* species, shoot induction directly from leaf explants has been already reported in *S. nemorosa* (Skała and Wysokińska, 2004) and *S. canariensis* (Mederos-Molina, 2004).

Our results clearly indicate that attempts to micropropagate *S. przewalskii via* organogenesis have limited success due to poor regeneration frequency and formation of unelongated small buds, mostly less than 0.5 cm in length. In contrast, micropropagation of *S. przewalskii* through shoot tip culture is an efficient method, both in terms of producing multiple shoots and their quality. The multiple shoots were rooted within 4 weeks on full- or half-strength MS media alone or in the presence of 0.5 μM IBA (Skała and Wysokińska, 2004). Rooted plantlets were transplanted to soil with 90% success. Using this plant production system up to 900 plantlets could be generated from a single explant (shoot tip) within 4 months. After acclimatization the plants grew well in the field and flowered in the second year of vegetation. They were similar in morphology to *S. przewalskii* plants cultivated by the conventional method (from seeds). Preliminary studies using flow cytometric analysis of the nuclear DNA content did not show any differences in ploidy levels between *in vitro*- and *in vivo*-developed plants (data not shown).

Essential oil composition

The ability of *S. przewalskii* micropropagated plants at the flowering stage to produce volatile oil was investigated. The oil was obtained by hydrodistillation of the leaves (LV sample) and flowering stems (FSV sample) in yields of 0.29% v/w and 0.04% v/w, respectively (Table II). The chemical composition of essential oils of the leaves and flowering stems was analyzed by GC and GC-MS. In leaf oil and flowering stem oil, 80 and 81 components were identified, which were 99.2% and 97.8% of the total oil, respectively. The components together with their percentages are listed in order of their elution from a CP Sil 5 CB column in Table II. 13 monoterpene hydrocarbons (45–47%), 20 oxygenated monoterpenes (15–21%), 23 sesquiterpene hydrocarbons (23–25%), 21–22 oxygenated sesquiterpenes (9–13%) and 3 other components (0.1–0.3%) were identified in the investigated oils. Among the monoterpenes limonene and β-phellandrene (23.6%) were identified as the main constituents of the leaf oil. Unfortunately, the compounds could not be separated on the column used in the study. The other major monoterpenes (*i. e.* these that were detected in relative amounts greater than 5%) in the oil were α-pinene (6.4%), camphene (5.8%) as well as the monoterpene alcohol borneol (7.8%) and its acetate (8.2%), whereas the sesquiterpene fraction was dominated by β-caryophyllene (16.5%). In the sample of oil isolated from flowering stems, higher relative amounts of β-phellandrene and limonene (25.5%) and α-pinene (15.1%) were found but the percentages of borneol (4.1%), bornyl acetate (5.6%) and β-caryophyllene (13%) were lower than those in the leaf oil. For comparative purposes, the essential oil from seed-derived plants of *S. przewalskii* was also analyzed. The plants grew under the same conditions and were collected at the same time as micropropagted plants. The hydrodistillation of leaves (L) and flowering stems (FS) from seed-grown plants gave oils in 0.59% (v/w) and 0.26% (v/w) yields, respectively (Table II). In the oils we identified 77 components, about 40 of which are reported for the first time in *S. przewalskii* species. The chemical profiles of oils of leaves and flowering stems were similar. β-Phellandrene and limonene (up to 36%), α-pinene (17–21%) and β-caryophyllene (6–7%) were the main components. A significant difference was observed only in α-pinene contents. As it was already found in the oils of regenerated plants, the amount

Table II. Constituents of essential oils from *Salvia przewalskii* listed in order of elution from a CP Sil 5 CB column.

Peak No.	Constituent	LV[a] (%)	FSV[a] (%)	L[a] (%)	FS[a] (%)	RI[b]
1.	α-Thujene	0.7	0.1	0.2	0.2	923
2.	α-Pinene	6.4	15.1	16.9	21.4	933
3.	Camphene	5.8	1.5	0.7	1.1	948
4.	Sabinene	t	t	t	t	970
5.	β-Pinene	3.6	1.3	0.8	1.1	973
6.	Myrcene	1.1	0.8	0.8	0.9	981
7.	α-Phellandrene	1.5	0.5	0.4	0.4	998
8.	α-Terpinene	0.8	0.6	0.4	1.0	1112
9.	p-Cymene	t[c]	t	0.5	t	1115
10.	Limonene	23.6	25.5	36.1		1024
11.	β-Phellandrene					1024
12.	β-(*Z*)-Ocimene	–	–	0.1	t	1028
13.	β-(*E*)-Ocimene	0.7	0.3	0.2	0.2	1041
14.	γ-Terpinene	0.8	0.5	0.7	0.6	1055
15.	*trans*-Sabinene hydrate	t	t	t	t	1060
16.	*trans*-Linalooloxide (furanoid)	0.1	t	0.1	0.1	1062
17.	*cis*-Linalooloxide (furanoid)	0.1	0.1	0.1	0.1	1076
18.	Terpinolene	0.3	0.2	0.4	0.3	1081
19.	*cis*-Sabinene hydrate	t	t	t	t	1087
20.	Linalool	0.5	0.6	0.7	0.8	1087
21.	Oct-3-en-1-yl acetate	0.1	0.3	0.5	0.5	1095
22.	*trans*-*p*-Menth-2-en-1-ol	0.3	0.3	0.3	0.3	1106
23.	*trans*-*p*-Mentha-2,8-dien-1-ol	0.3	0.3	0.3	0.3	1120
24.	*cis*-*p*-Menth-2-en-1-ol	0.2	0.2	0.1	0.1	1124
25.	Camphor	0.2	0.1	0.1	0.1	1126
26.	*trans*-Pinocarveol	t	0.1	t	t	1128
27.	Borneol	7.8	4.1	0.2	0.7	1155
28.	Terpinen-4-ol	1.6	1.2	1.3	1.1	1167
29.	α-Terpineol	0.7	1.3	2.7	2.2	1178
30.	Myrtenol	0.2	0.3	0.4	0.4	1184
31.	*cis*-*p*-Mentha-1,8-dien-2-ol	0.1	t	t	0.1	1208
32.	Bornyl formate	t	0.1	0.1	0.1	1210
33.	Piperitone	t	t	0.1	t	1232
34.	Bornyl acetate	8.2	5.6	2.1	2.3	1273
35.	*p*-Menth-1-en-9-ol	0.2	0.1	0.1	t	1283
36.	Myrtenyl acetate	t	0.1	0.2	0.2	1306
37.	α-Cubebene	0.1	0.1	0.1	0.1	1354
38.	α-Ylangene	t	0.1	0.1	0.1	1375
39.	α-Copaene	0.4	0.5	0.5	0.5	1379
40.	β-Bourbonene	t	0.1	t	t	1383
41.	β-Cubebene	0.1	0.2	0.2	0.2	1385
42.	Italicene	0.1	0.1	0.2	0.2	1409
43.	α-Gurjunene	0.1	0.1	0.2	0.2	1413
44.	β-Caryophyllene[d]	16.5	13.0	7.1	6.4	1420
45.	Selina-3,6-diene	1.1	0.7	–	–	1425
46.	*trans*-α-Bergamotene	0.6	0.6	0.2	0.2	1433
47.	*trans*-Calarene	t	t	–	–	1434
48.	α-Humulene	1.0	1.3	0.5	0.6	1453
49.	(*Z*,*Z*)-α-Farnesene	–	–	0.4	0.4	1460
50.	Selina-4,7-diene	0.5	1.4	–	–	1468
51.	γ-Muurolene	0.2	t	t	t	1472
52.	*ar*-Curcumene[d]	0.4	0.5	2.9	2.8	1473
53.	γ-Curcumene	–	–			1473
54.	Clovene	t	t	–	–	1476
55.	δ-Selinene	1.2	1.4	–	–	1485
56.	(3*Z*,6*Z*)-α-Farnesene	0.2	0.1	–	–	1488
57.	α-Muurolene	–	–	1.2	1.0	1496
58.	(*E*,*E*)-α-Farnesene	–	–	0.2	0.1	1498
59.	β-Curcumene	–	–	1.8	1.3	1505
60.	γ-Cadinene[d]	0.5	1.1	–	–	1512

Table II (continued).

Peak No.	Constituent	LV[a] (%)	FSV[a] (%)	L[a] (%)	FS[a] (%)	RI[b]
61.	δ-Cadinene[d]	0.8	1.2	1.5	1.1	1516
62.	Eremophila-1(10),7(11)-diene	–	–	0.3	0.2	1525
63.	Selina-4(15), 7(11)-diene	0.4	0.5	–	–	1534
64.	Selina-3,7(11)-diene	0.4	0.4	0.3	0.2	1540
65.	(*E*)-Nerolidol[d]	0.9	0.7	–	–	1552
66.	Germacrene B	0.1	0.1	t	t	1556
67.	Spathulenol[d]	1.7	2.0	0.3	0.2	1566
68.	β-Caryophyllene epoxide[d]	1.3	1.2	1.0	1.5	1570
69.	Clovenol	t	t	0.1	0.1	1575
70.	Globulol	0.2	0.4	0.4	0.4	1590
71.	Ledol	0.1	0.2	0.1	0.1	1600
72.	Eremoligenol[d]	1.0	1.4	1.4	1.1	1621
73.	γ-Eudesmol[d]	0.3	1.0	1.8	1.4	1624
74.	T-Cadinol[d]	0.6	1.1	1.4	1.3	1630
75.	β-Eudesmol[d]	0.5	0.8	2.9	2.1	1639
76.	α-Cadinol[d]	1.1	1.0	4.7	3.3	1642
77.	α-Eudesmol[d]		2.3			1646
78.	Eudesm-4(15)-en-6-ol	0.1	t	0.1	0.1	1656
79.	β-Bisabolol	0.3	0.6	0.4	0.6	1658
80.	14-Hydroxy-9-epi-(*E*)-caryophyllene	0.1	0.1	0.1	0.1	1664
81.	Eudesma-4(15),7-dien-1β-ol	0.1	0.1	0.1	0.1	1672
82.	Muurola-4,10(14)-dien-1β-ol	t	t	t	t	1675
83.	Eudesm-3-en-6-ol	t	t	t	t	1679
84.	(*E*,*E*)-Farnesol[d]	0.2	0.2	0.1	0.1	1717
85.	Isovalencenol	t	t	0.1	0.1	1777
86.	(*E*,*E*)-Farnesyl acetate	0.2	0.3	0.1	0.2	1823
87.	2α-Acetoxyamorpha-4,7(11)-diene	t	t	t	t	1796
88.	Manool	t	t	t	t	2070
	Monoterpene hydrocarbons	45.3	46.5	58.2	61.9	
	Oxygenated monoterpenes	20.5	14.6	8.9	8.9	
	Sesquiterpene hydrocarbons	24.7	23.4	17.9	15.6	
	Oxygenated sesquiterpenes	8.7	13.4	15.1	12.8	
	Others	0.1	0.3	0.5	0.5	
Total identified		99.2	97.8	99.9	99.2	
Yield v/w dry weight (%)		0.29	0.04	0.59	0.26	

[a] L, leaves from *in vivo* plants; FS, flowering stems from *in vivo* plants; LV, leaves from *in vitro* plants; FSV, flowering stems from *in vitro* plants.
[b] RI, relative retention index.
[c] t, trace (percentage value less than 0.05%).
[d] Identification confirmed by ^{1}H NMR.

of the monoterpene hydrocarbon was higher in the flowering stem than in the leaf. Comparison of these results with those mentioned in the literature indicated that β-phellandrene (26%) and β-caryophyllene (11%) were also dominant in oil isolated from leaves of *S. przewalskii* plants growing wild in Tibet (Liu *et al.*, 2006). The oil was also characterized by a relative high quantity (9.7%) of the sesquiterpene germacrene D, which has not been detected in our samples. On the other hand, in the group of oxygenated sesquiterpenes apart from alcohols with a cadinane skeleton, mentioned earlier (Liu *et al.*, 2006), we identified some alcohols with the eudesmane skeleton (Table II), not previously found in *S. przewalskii*.

We revealed marked differences in the yields as well as the qualitative and quantitative composition of oils from *in vitro* and *in vivo S. przewalskii* plants, although all investigated oils were characterized by the presence of common major constituents. The micropropagated plants accumulated essential oils at reduced yield (15 to 50% of the levels of seed-derived plants). The differences in the qualitative composition of the investigated oils concerned mainly sesquiterpenes. Most hydrocarbons with the eudesmane skeleton [selina-3,6-diene, selina-4,7-diene, selina-4(15),7(11)-diene and δ-selinene] as well as clovene, γ-cadinene and (*E*)-nerolidol were identified only in *in vitro*-regenerated plants. On the other hand, (*Z*,*Z*)- and (*E*,*E*)-α-farnesene, β- and γ-curcumene, eremophila-1(10),7(11)-diene and α-muurolene were found only as constituents of *in vivo* plants. From the quantitative point of view, reduced amount of mo-

noterpene hydrocarbons with a corresponding increase in oxygenated monoterpene and sesquiterpene hydrocabon fractions in the oils isolated from *in vitro* plants was observed. Moreover, the amounts of borneol, bornyl acetate and β-caryophyllene in oils from micropropagated plants were at least twice higher than those found in oils from *in vivo* plants.

The results indicate that the plant regeneration process can affect to a certain degree the biosynthetic capacity and essential oil profile in mature plants of *S. przewalskii*. The variations might be attributed to culture conditions used before regenerated plants were transferred to the field. Similar observations were made in *S. przewalskii* roots in respect to the production of tanshinones (data not shown) and iridoids in micropropagated plants of *Harpagophytum procumbens* (Levielle and Wilson, 2002).

Biological activity of essential oils

The oil samples from leaves and flowering stems of *S. przewalskii in vitro* and *in vivo* plants (LV, FSV, L and FS) were evaluated for their cytotoxicity and antimicrobial activity. Among the bacteria tested *Staphylococcus aureus* and *S. epidermidis* were the most sensitive microorganisms to *S. przewalskii* essential oils (MIC values ranged from 2.5 to 10 mg/ml). The best results (MIC = 2.5 mg/ml) were observed for oil samples from *in vitro* regenerated plants (Table III). *Enterococcus faecalis* and *Escherichia coli* exhibited lower susceptibility against the oils tested (MIC values of 5–10 mg/ml), while *Pseudomonas aeruginosa* was the most resistant strain to the oils with MIC values >15 mg/ml (Table III). The results are in agreement with observations of other authors that Gram-negative bacteria are not sensitive or less sensitive to sage essential oils when compared with the sensitivity of Gram-positive bacteria (Maruzzella and Henry, 1958; Yousef and Tawil, 1980). The essential oils of *S. przewalskii* showed also antifungal activity against the yeast *Candida albicans* with MIC values of 5 mg/ml (for oils isolated from *in vitro* plants) and 10 mg/ml (for oils from *in vivo* plants).

The stronger antimicrobial activity shown by the oil from *in vitro S. przewalskii* plants can be associated, at least in part, with the relative high percentage of oxygenated monoterpenes. The antimicrobial activity of the oxygenated compounds is usually stronger than that of hydrocarbons (Kalemba and Kunicka, 2003). Borneol, which is the main constituent of the oxygenated monoterpene fraction from *in vitro S. przewalskii* plants was previously reported to possess a significant antibacterial activity (Dorman and Deans, 2000; Tabanca *et al.*, 2001). Activity against Gram-positive bacteria has also been found for bornyl acetate (Dorman and Deans, 2000), the second main component of the oxygenated monoterpene fraction. However, activity of the essential oils may be also due to the existence of other components present in lesser amounts and the combined or even synergistic biological effect between them (Kalemba and Kunicka, 2003).

The essential oils of *S. przewalskii* were also assayed for their cytotoxicity against two leukemia cell lines (Table IV). Based on the dose-response curves, IC_{50} values were calculated. As shown in Table IV all samples tested had almost no cytotoxic activity against the NALM-6 cell line (IC_{50}

Microorganism	MIC [mg/ml]			
	LV	FSV	L	FS
Staphylococcus aureus (ATCC 29213)	2.5	2.5	10.0	5.0
Staphylococcus epidermidis (ATCC 12228)	2.5	2.5	10.0	5.0
Enterococcus faecalis (ATCC 29212)	10.0	5.0	10.0	5.0
Escherichia coli (NCTC 8196)	10.0	10.0	10.0	5.0
Pseudomonas aeruginosa (NCTC 6749)	>15.0	>15.0	>15.0	>15.0
Candida albicans (ATCC 10231)	5.0	5.0	10.0	10.0

Table III. Antimicrobial activity of essential oils of *Salvia przewalskii*. MIC (minimal inhibitory concentration) values were determined by the microdilution assay.

L, essential oil of leaves from *in vivo* plants; FS, essential oil of flowering stems from *in vivo* plants; LV, essential oil of leaves from *in vitro* plants; FSV, essential oil of flowering stems from *in vitro* plants.

Table IV. Cytotoxicity of essential oils from *Salvia przewalskii* towards human leukemia HL-60 and NALM-6 cells.

Essential oil[a]	HL-60	NALM-6
	IC_{50} [μg/ml][b]	
LV	105.7 ± 19.8	347.7 ± 32.2
FSV	67.9 ± 15.4	385.6 ± 40.4
L	223.7 ± 24.9	345.9 ± 23.0
FS	160.7 ± 21.8	349.7 ± 31.1

[a] L, leaves from *in vivo* plants; FS, flowering stems from *in vivo* plants; LV, leaves from *in vitro* plants; FSV, flowering stems from *in vitro* plants.
[b] IC_{50}, the concentration of the tested oil required to reduce the cell survival fraction to 50% of the control.

values ranged from 346 μg/ml to 386 μg/ml). Promyelocytic leukemia HL-60 cells were more sensitive to the essential oils (IC_{50} values ranged from 70 μg/ml to 224 μg/ml). As it was reported with regard to the antimicrobial activity, the cytotoxic effect of essential oils obtained from *in vitro* plants was significantly higher in comparison to essential oils from *in vivo* plants. The highest cytotoxicity was observed for the flowering stem essential oil (FSV); the IC_{50} value for the FSV oil sample was 2.3 times lower than that for the FS sample (Table IV). It seems possible that the differences in the chemical composition of the essential oils tested are associated with higher cytotoxicity of the oils of *in vitro S. przewalskii* plants.

In conclusion, our study provides useful information for the micropropagation of *S. przewalskii* and on the biological activity of essential oil from the micropropagated plants.

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