Chair and Department of Pharmaceutical Botany[1], Collegium Medicum, Jagiellonian University, Toxicological Laboratory[2], Department of Laboratory Diagnostics, L. Rydygier's Hospital, Kraków, Poland

Coumarin compounds in *Ammi majus* L. callus cultures

H. Ekiert[1] and E. Gomółka[2]

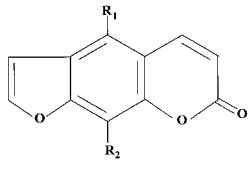
Callus cultures of *Ammi majus* L. (bishop's weed), *Apiaceae*, were maintained on variants of Linsmaier-Skoog's (L-S) medium differing in the content of the phytohormones, α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) (0.1–10.0 mg/l). The increments of callus tissue fresh weight showed considerable differences (1.4 bis 4.4-fold) during 4-week subcultures on the media tested. HPLC analysis revealed the presence of six compounds in the extracts of callus tissues, which are known metabolites in plants growing under natural conditions: the linear furanocoumarins psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin and their precursor umbelliferone. The total contents of coumarin compounds under examination showed marked differences dependent on the phytohormone concentrations in the medium. They ranged from 40.95 to 871.05 mg/100 g of dry weight. Imperatorin was the metabolite dominating among the furanocoumarins in calli from almost all tested variants of L-S medium (maximum content of 169.27 mg/100 g). Callus tissue cultured on one of the variants (0.1 mg/l NAA, 0.1 mg/l BAP) yielded in high content of xanthotoxin (145.33 mg/100 g). On the majority of media, umbelliferone was also accumulated at high quantities (maximum content of 536.29 mg/100g). The medium containing 0.1 mg/l NAA and 0.1 mg/l BAP was the best for the accumulation of analysed coumarins. This medium favoured the formation of embryogenic callus. Xanthotoxin also dominated quantitatively among the furanocoumarins in the tested vegetative organs and fruits of the plant grown under natural conditions (leaves: 26.10 mg, roots: 5.55 mg, fruits: 3010.41 mg/100 g). Maximum contents of this metabolite in *in vitro* culture were many times higher than those found in vegetative plant organs but manifold lower than in fruits. On the other hand, maximum contents of imperatorin obtained in *in vitro* culture were many times higher in comparison with those detected in vegetative organs and fruits (leaves: 14.10 mg, roots: 3.30 mg, fruits: 94.70 mg/100 g of dry weight.).
The *A. majus* L. callus culture, established in the course of the present experiments, can be considered a valuable model for studies of the biosythesis of coumarin compounds, and a potential source of the psoralen 8-alkoxy derivatives imperatorin and xanthotoxin and their precursor umbelliferone.

1. Introduction

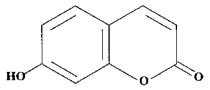
Ammi majus L., bishop's weed (*Apiaceae*) is a species growing mainly in the Mediterranean Basin. In Poland and other countries of Central and Eastern Europe, this species does not grow under natural conditions, though there have been attempts to grow it experimentally. In temperate climates, the fruits do not ripen in cooler and less sunny years. The plants are also frequently attacked by pathogens. *A. majus* L. fruits are considered the richest natural source of linear furanocoumarins, namely psoralen derivatives. The fruits of various origin were shown to contain about 20 compounds belonging to the aforementioned group [1–3]. Only a few reports demonstrated in the species under examination the occurrence of other secondary metabolites, mainly, coumarins, angular furanocoumarins, flavonoids [1, 2].
Among the linear furanocoumarins present in *A. majus* L. fruits, xanthotoxin, bergapten, isopimpinellin and imperatorin are the most important, in terms of their therapeutic potential. These furanocoumarins, psoralen derivatives, exhibit photosensitising and antiproliferative actions [4]. *A. majus* L. fruits are the basis for manufacturing most of the preparations used in dermatology (e.g. Meladinine® (Promedica), Oxsoralen®, Geralen® (Gerot), Psoraderm®-5 (Pharmacal)). Furanocoumarins can also act as calcium channel blockers [5]. For the aforementioned reasons, they were chosen as research object in this study.
A. majus L. was the research object for biotechnological studies aimed at both, the development of micropropagation methods [6–8] and the assessment of coumarin compounds production either endogenously [9] or stimulated by biotic and abiotic elicitors [10, 11]. Studies on determination of biosynthetic pathways of linear furanocoumarins were performed, too [12–14]. Recently, an attempt of its genetic transformation has been undertaken using *Agrobacterium rhizogenes* to enhance furanocoumarin production [15]. Studies on furanocoumarin accumulation in *in vitro* cultures of this species were performed in our laboratory as early as in the eighties [9, 16]. The present study was conducted on callus cultures characterised by a higher furanocoumarin production capacity derived from another parent material. This paper analyses the effect of the phytohormone concentration on the content of these metabolites in *in vitro* cultures. The results of HPLC analysis of extracts from tissues cultured on different variants of the medium as well as of extracts from vegetative parts and fruits of plants grown under natural conditions, used for comparison, are presented below.

2. Investigations, results and discussion

A. majus L. callus tissue was derived from hypocotyl parts of sterile seedlings. Linsmaier-Skoog's medium (L-S) [17] containing 2 mg/l NAA and 2 mg/l BAP proved valuable for both callus initiation and supporting tissue growth. These results are in agreement with our previous experiments on callus cultures of *Anethum graveolens* L. (unpublished) and *Pastinaca sativa* L. [18], and *Ruta graveolens* L. shoot cultures [19]. They are also similar to our findings on previously established *A. majus* L. cultures [9, 16]. On the other hand, for the initiation of cultures of this species, Purohit et al. [8] and Królicka et al. [10] proposed Murashige-Skoog's [20] medium that is richer in vitamins than L-S medium. Purohit at al. [8] proposed also other phytohormone composition, α-indoleacetic acid (IAA) and kinetin.
In the present study, we observed a 1.4 bis 4.4-fold increase in callus fresh mass during a culture period of 4 weeks on the tested variants of L-S medium. These data



	R_1	R_2
Psoralen	H	H
Xanthotoxin	H	OCH_3
Bergapten	OCH_3	H
Isopimpinellin	OCH_3	OCH_3
Imperatorin	H	$OCH_2 - CH = C(CH_3)_2$



Umbelliferone

are comparable with earlier published results [9, 16]. Mass increments were significantly higher than those observed in *Pastinaca sativa* L. callus cultures cultivated on the same variants of L-S medium for even longer subculture periods (1.5 bis 3.0 -fold increase over 6 weeks) [18].

In callus tissue extracts the presence of six compounds was demonstrated: psoralen, bergapten, xanthotoxin, isopimpinellin, imperatorin as linear furanocoumarins and umbelliferone, the biogenetic precursor of these metabolites, using HPLC (based on comparison with authentic standard compounds). The abovementioned compounds are well-known metabolites of the parent plant [1, 2, 21–23]. Fruit extracts, examined as a reference material, contained all six tested compounds. HPLC analysis showed qualitative and quantitative differences between the metabolite contents in the extracts of callus tissues cultured *in vitro* in comparison to plant extracts (Table). Significant differences were also observed in the contents of the compounds tested in callus tissues in dependence of the phytohormone composition of the medium (Fig.).

Only 3 metabolites: imperatorin, xanthotoxin and umbelliferone were detected in all analysed extracts of callus tissues, irrespective of the variant of L-S medium used. Isopimpinellin and bergapten were present in the tissues cultured on the majority of medium variants while psoralen, the parent compound in the group of linear furanocoumarins, occurred at very low quantities only in the calli grown on four variants of the L-S medium.

Imperatorin was the compound dominating among the furanocoumarins in tissues cultured on almost all tested variants of L-S medium. Its contents fluctuated in a wide concentration range, varying from 3.10 mg to 169.27 mg/100 g of dry weight (contents of all compounds are expressed in this way further on in this paper). Tissues cultured on one of the media tested (0.1 mg/l NAA, 0.1 mg/l BAP) accumulated also high amounts of xanthotoxin (145.33 mg/100 g). The contents of this metabolite varied considerably, ranging from 0.61 mg/100 g to 145.33 mg/100 g. The contents of the two remaining furanocoumarins, bergapten and isopimpinellin were low and showed lesser differences (1.17–14.36 mg/100 g and 0.39 to 6.37 mg/100 g, respectively). Psoralen, precursor of furanocoumarins, was detected only on four medium variants and its amounts did not exceed 1 mg/100 g. Umbelliferone was accumulated at high quantities on the majority of the examined media (13.28–536.29 mg/100 g).

Table: Contents of the analysed metabolites in fruits and vegetative parts of *Ammi majus* L. and their maximum amounts obtained in *in vitro* cultures on various media

Metabolites	Contents (mg/100 g of dry weight)			
	Callus tissues*	Fruits	Leaves	Roots
Psoralen	0.61	232.97	9.36	2.07
Bergapten	14.36	858.47	– **	4.92
Xanthotoxin	145.33	3010.41	26.10	5.55
Isopimpinellin	6.37	205.24	–	–
Imperatorin	169.27	94.70	14.10	3.30
Umbelliferone	536.29	592.69	7.77	5.93

* callus tissues from different variants of L-S medium
** content lower than 0.001 mg/100 g

In fruits, analysed for comparison, xanthotoxin was the dominating metabolite. Its content was exceptionally high, amounting to 3010.41 mg/100 g. The contents of bergapten and umbelliferone were also high (858.47 and 592.69 mg/100 g, respectively). The contents of psoralen and isopimpinellin were much lower (232.97 and 205.24 mg/100 g, respectively). Imperatorin was also accumulated to lower amounts (94.70 mg/100 g). The qualitative and quantitative furanocoumarin composition depends on the origin of fruits (crop location) and on the degree of their ripeness, among other factors [21–23]. *A. majus* L. fruits are the organ of the plant which is the richest source of the tested compounds. In vegetative plant organs, furanocoumarins are accumulated in significantly lower amounts [21, 22]. Our analyses of plant extracts confirmed earlier reports of other authors. The contents of coumarin compounds in leaf and root extracts were much lower than those found in fruits. Xanthotoxin was the dominating metabolite among the furanocoumarins (leaves: 26.10 mg/100g, roots: 5.55 mg/100 g). These amounts were also much lower than the maximum content obtained in *in vitro* cultures. A similar dependence was observed in the case of imperatorin. Its contents in vegetative plant organs and fruits were lower than in material from *in vitro* culture (leaves: 14.10 mg/100 g, roots: 3.30 mg/100 g, fruits: 94.70 mg/100 g).

During our previous studies, we obtained manifold lower maximum contents of the individual metabolites in *in vitro* cultures of *A. majus* L. (maximum 6.67–35.83 mg/100 g of dry weight). Furthermore, bergapten, the 5-methoxy derivative of psoralen was the metabolite, which dominated quantitatively [9, 16]. The contents of xanthotoxin and imperatorin were very low, reaching maximally 6.67 and 22.11 mg/100 g of dry weight, respectively. It appears that in the present study, we obtained a culture in which the biosynthetic pathway leading to 8-alkoxy derivatives dominated.

The *A. majus* L. callus culture, examined in the present study, was characterised by a high biosynthetic yield of certain metabolites in spite of the low degree of tissue differentiation, and without stimulation by elicitors. Previously we obtained high furanocoumarin contents in undifferentiated *Pastinaca sativa* L. callus cultures (isopimpinellin: 90.2–238.9 mg/100 g, xanthotoxin: 14.1–82.6 mg/100 g, and psoralen: 18.6–108.8 mg/100 g) [18]. It is also worth mentioning that our *A. majus* L. culture preserved its high biosynthetic potential in spite it its age (9 years).

We observed maximum contents of xanthotoxin, imperatorin and umbelliferone in cultures on the medium containing the lowest tested concentrations of phytohormones, 0.1 mg/l of both NAA and BAP. This medium favoured the formation of embryogenic callus. These results correspond with the data obtained earlier on the other *A. majus* L. culture [9, 16]. In those studies, the highest contents of the dominating metabolite bergapten were observed in cul-

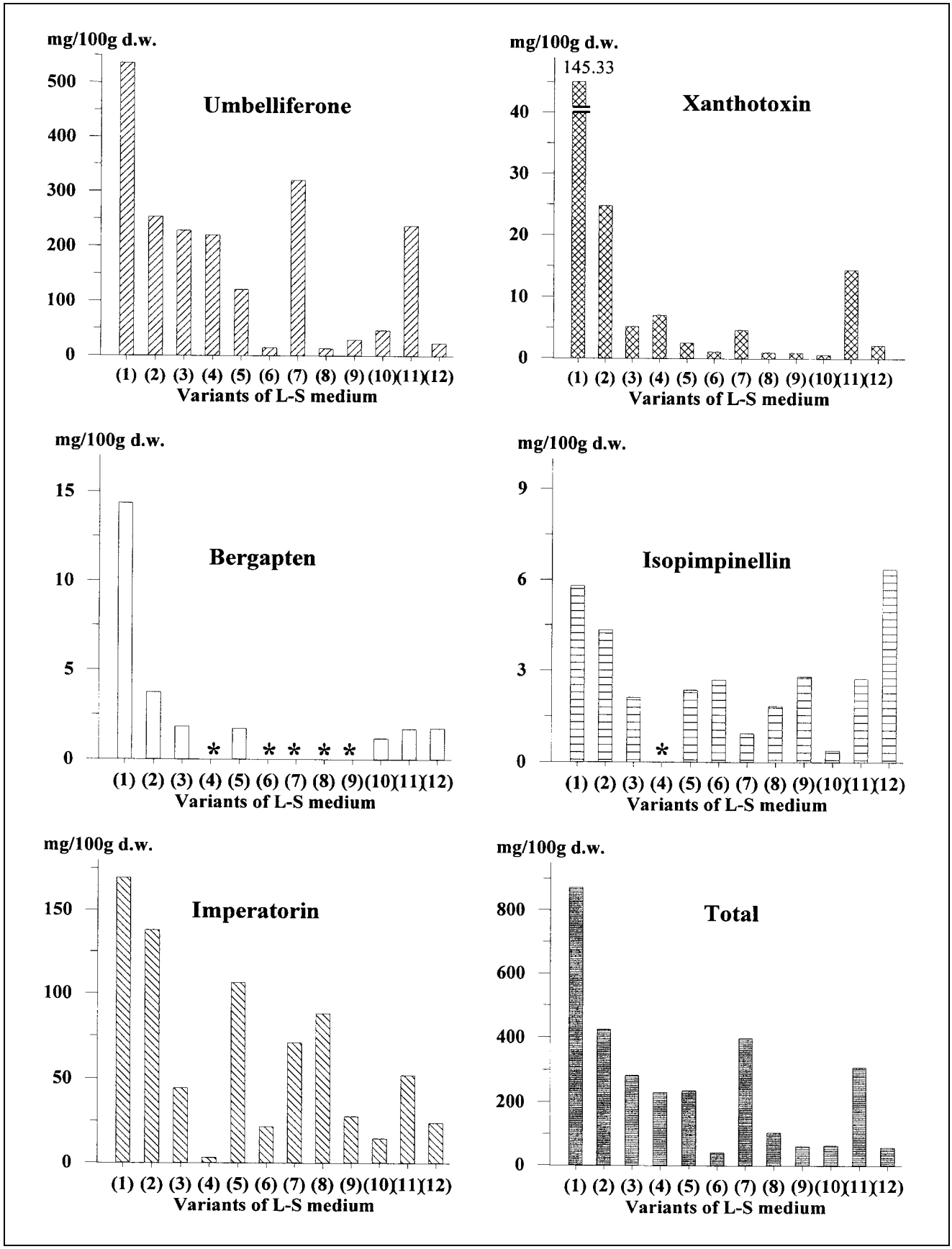


Fig.: Contents of the tested coumarin compounds (mg/100 g of dry weight) in *Ammi majus* L. callus tissues cultured on media containing various concentrations of phytohormones, NAA/BAP (mg/l): (1) 0.1/0.1; (2) 1.0/1.0; (3) 2.0/ 2.0; (4) 3.0/1.0; (5) 5.0/5.0; (6) 10.0/10.0; (7) 1.0/5.0; (8) 5.0/1.0; (9) 5.0/10.0; (10) 10.0/5.0; (11) 1.0/10.0; (12) 10.0/1.0. Due to low psoralen contents (0.13–0.61 mg/100 g) detected only in calli from four of the tested media, this metabolite was not included in the Fig.
* content lower than 0.001 mg/100 g

tures on the medium with an identical phytohormone composition as now. Purohit et al. [8] observed maximum contents of xanthotoxin (10.6 mg/g of dry weight) in regenerating callus of *A.majus* L. cultured on Murashige-Skoog's medium.

On the basis of the studies on *Pastinaca sativa* L. callus cultures, we proposed L-S medium supplemented with 3 mg/l NAA and 1 mg/l BAP as the best for the accumulation of the furanocoumarins [18]. However, it did not prove beneficial for the *A. majus* L. culture since the obtained total content of furanocoumarins was very low (only 10.12 mg/100 g) while the precursor quantity was elevated (219.96 mg/100 g).

3. Discussion

The present study demonstrated a marked effect of the phytohormone concentration on the contents of the analysed compounds. The influence of phytohormones on the accumulation of various groups of secondary metabolites is a well-known dependence [24]. We also showed such an effect in a *Pastinaca sativa* L. cultures [18]. However, Tirillini et. al. [25] were unable to document this dependence in their experiments with a *Heracleum sphondylium* ssp. sphondylium callus culture, possibly due to a too narrow concentration range of the phytohormones (0.1 to 1.0 mg/l) 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. Furthermore, in comparison with our culture, these authors obtained considerably lower contents of four furanocoumarins: bergapten, imperatorin, isopimpinellin and xanthotoxin, amounting to 0.7–5.1 μg/g of dry weight.

The maximum total content of the tested coumarins accumulated in our *A. majus* L. callus culture, amounting to 871.05 mg/100 g can be considered interesting from a practical point view.

The *A. majus* L. callus culture, studied in the present experiments, can be proposed as convenient material for studies of the biosynthesis of coumarin compounds, and as a potential source of imperatorin and xanthotoxin and their precursor umbelliferone. This possibility is made even more promising by the fact that the biosynthetic efficacy of the culture can be increased by an appropriate choice of phytohormones in the medium.

4. Experimental

4.1. Establishment of in vitro culture

The callus culture of *A. majus* L. was established in 1990. Callus was derived from hypocotyls of sterile seedlings (fruits were obtained from Botanischer Garten der Technischen Hochschule, Aachen). Callus initiation was observed on Linsmaier-Skoog's medium (L-S) [17], containing α-naphthaleneacetic acid (NAA) at 2 mg/l and 6-benzylaminopurine (BAP) at 2 mg/l under constant artificial light (900 lx) at a temperature of 25 ± 2 °C.

4.2. Experimental cultures

Callus cultures were maintained on agar variants of L-S medium containing varying amounts of the phytohormones NAA and BAP, in a concentration range from 0.1 to 10 mg/l: 0.1/0.1; 1.0/1.0; 2.0/2.0; 3.0/1.0; 5.0/5.0; 10.0/10.0; 1.0/5.0; 5.0/1.0; 5.0/10.0; 10.0/5.0; 1.0/10.0; 10.0/1.0 (12 variants). Cultures were cultivated under light conditions and temperature specified in p. 4.1. Callus cultures were subcultured every 4 weeks.

4.3. Extraction

Dried and ground material (approximately 1 g), callus tissues collected after 28 days of culture (subcultures from 153 to 162) as well as leaves, roots and fruits of the plants growing in open air (material was collected in September 1998 in the Botanical Garden of Jagiellonian University, Kraków, Poland), were extracted with two portions (50 ml) of boiling 96% ethanol in a Soxhlet's apparatus for 10 h. Extracts were combined and evaporated to dryness. The residue was quantitatively dissolved in 10 ml of 96% ethanol and analysed by HPLC.

4.4. HPLC

Contents of psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin and umbelliferone were determined according to a procedure developed in our laboratory. Analytical conditions were reported in detail earlier [18, 26]. Briefly, they were as follows.

HPLC apparatus: Ati Unicam, Cambridge; Pump: Crystal 200 (Ati Unicam, Cambridge); Column: Supelcosil LC-8 (4.6 mm/25 cm); Solvent system: methanol/water (1 : 1.2); in the case of imperatorin: methanol/water (2 : 1); Flow rate: 1 ml/min.; Detector UV: $\lambda = 310$ nm; Standards: manufactured by Carl Roth.

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References

1 Hegnauer, R.: Chemotaxonomie der Pflanzen. Bd. 6, Birkhäuser Verlag, Basel und Stuttgart 1973
2 Hoppe, A.: Drogenkunde. Walter de Gruyter, Berlin, New York 1975
3 Ivie, W. G.: J. Agric. Food Chem. **26**, 1394 (1978)
4 Pathak, M. A.; Parrish, J. A.; Fitzpatrick, T. B.: Farmaco, Ed. Sci. **36**, 479 (1981)
5 Härmälä, P.; Vuorela, H.; Nyiredy, Sz.; Törnquist, K.; Kaltia, S.; Sticher, O.; Hiltunen, R.: Phytochem. Anal. **3**, 42 (1992)
6 Sehgal, C. B.: Current Science **41**, 263 (1972)
7 Grewal, S.; Sachdeva, U.; Atal, C. K.: Indian J. Exp. Biol. **14**, 716 (1976)
8 Purohit, M.; Pande, D.; Datta, A.; Srivastava, P. S.: Planta Med. **61**, 481 (1995)
9 Ekiert, H.: in: Bajaj, Y. P. S. (Eds.): Biotechnology in Agriculture and Forestry, Vol. 21. Medicinal and Aromatic Plants IV. p.1, Springer Verlag, Berlin, Heidelberg 1993
10 Królicka, A.; Krauze-Baranowska, M.; Cisowski, W.; Łojkowska, E.; in: Abstracts of IX International Congress on Plant Tissue and Cell Culture, p. 92, Jerusalem, Israel, June 1998
11 Królicka, A.; Krauze-Baranowska, M.; Staniszewska, I.; Cisowski, W.; Szafranek, J.; Łojkowska, E.: in Abstracts of 7 th Conference on the Application of Chromatographic Methods in Phytochemical and Biomedical Analysis, p. 22, Lublin, Poland, June 1998
12 Hamerski, D.; Matern, U.: Eur. J. Biochem. **171**, 369 (1988)
13 Hamerski, D.; Matern, U.: FEBS Lett. **239**, 263 (1988)
14 Hamerski, D.; Beier, R. C.; Kneusel, R. E.; Matern, U.; Himmelspach, K.: Phytochemistry **29**, 1137 (1990)
15 Królicka, A.; Bielawski, K.; Łojkowska, E.; in: Abstracts of the Third International Symposium in the Series "Recent Advances in Plant Biotechnology", p.124, Stará Lesná, High Tatras, Slovak Republic, September 1999
16 Ekiert, H.: Doctor's Thesis, Medical Academy, Kraków, Poland, 1988
17 Linsmaier, E. M.; Skoog, F.: Physiol. Plant. **18**, 100 (1965)
18 Ekiert, H.; Gomółka, E.: Pharmazie, in press
19 Ekiert, H.; Kisiel, W.: Acta Soc. Bot. Polon. **66**, 329 (1997)
20 Murashige, T.; Skoog, F.: Physiol. Plant. **15**, 473 (1962)
21 Blažek, Z.; Stary, F.: Arch. Pharm. **35**, 117 (1965)
22 Blažek, Z.; Stary, F.: Sci. Pharm. **34**, 97 (1966)
23 Gheorghiu, M.; Pavel, M.; Tarpo, E.; Contz, O.: Grintescu, A.; Georgescu, V.: Farmacia (Bucharest) **13**, 541 (1965)
24 Mantell, S. H.; Smith, H.; in: Mantell, S. H.; Smith, H. (Eds.): Plant Biotechnology, p. 75, Cambridge University Press, Cambridge 1983
25 Tirillini, B.; Ricci, A.: Phytother. Res. **12**, S25 (1998)
26 Ekiert, H.; Gomółka, E.: Acta Soc. Bot. Polon. **68**, 197 (1999)

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Dr Halina Ekiert
Chair and Department of Pharmaceutical Botany,
Collegium Medicum, Jagiellonian University,
9 Medyczna Street
30-688 Kraków
Poland
mfekiert@cyf-kr.edu.pl