ACCUMULATION OF P-COUMARIC ACID AND OTHER BIOACTIVE PHENOLIC ACIDS IN IN VITRO CULTURE OF RUTA GRAVEOLENS SSP. DIVARICATA (TENORE) GAMS.

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Abstract

Shoot-differentiating callus culture of Ruta graveolens ssp. divaricata (Tenore) Gams was maintained on four variants of Linsmaier-Skoog (L-S) medium containing different concentrations of plant growth regulators, 1-naphthaleneacetic acid and 6-benzylaminopurine, ranging from 0.1 do 3.0 mg/l. Methanolic extracts of biomass cultured in vitro were used to determine the contents of free phenolic acids with an HPLC method. Out of eight compounds under analysis, six were shown to be present in the samples: caffeic acid, chlorogenic acid, p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid and syringic acid. Total contents of the compounds under study ranged from 124.9 to 138.7 mg/100g d.w., depending on the L-S medium variant. p-Coumaric acid was the main compound in the extracts from biomass cultured on all tested medium variants and its amounts were interesting from practical perspective (97.3-112.9 mg/100g d.w.). This compound was isolated, purified and its identity was confirmed by spectral methods. The contents of other compounds did not exceed 11.1 mg/100g d.w.

Extracts of the above-ground parts of plants growing in vivo (stems, leaves, herb), analyzed for comparison, contained three phenolic acids: protocatechuic acid (52.0-88.2 mg/100g d.w.), vanillic acid (2.6 – 17.8 mg/100g d.w.) and trace amounts of syringic acid.

This is the first report on the effect of growth regulators on the accumulation of free phenolic acids in biomass of R.graveolens cultured in vitro. This is also the first report documenting the isolation of p-coumaric acid from biomass of this subspecies cultured in vitro and also from any plant in vitro culture. In addition, for the first time in the above-ground parts of the plants growing in vivo phenolic acids were analyzed.

Keywords: Rutaceae, Common rue subspecies, Shoot-differentiating callus culture, p-Coumaric acid

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Introduction

Phenolic acids are a group of plant metabolites very important from therapeutically point of view. Compounds belonging to this group possess valuable biological properties, e.g. anti-inflammatory, immunostimulating, cholagogic, hepatoprotective, hypolipemic, hypocholesterolemic, spasmolitic and antyaggregatory action [1-4]. In addition, more recent studies proved antioxidant, antiradical and anticancer activities of some compounds from this group, e.g. protocatechuic acid, caffeic acid and chlorogenic acid [5-8]. Several publications emphasized valuable therapeutic and also cosmetic properties of *p*-coumaric acid, e.g. its antioxidant and antigenotoxic effect, and its potentially useful as hypopigmenting agent [9-11].

There are different opinions about taxonomic classification of *Ruta graveolens ssp. divaricata* (Tenore) Gams. According to Engler [12] this plant is a variety of *Ruta graveolens* L. Other authors, Hegi [13] and Hoppe [14] describe it as *Ruta graveolens* L. subspecies. The later view has been represented by research team from Würzburg University which started

with the professionaly investigation of chemical composition of this plant and its *in vitro* cultures [15, 16]. This view is also accepted by our research team.

Ruta graveolens (Fig.1) common rue or herb-of-grace has a limited distribution range. It is native only to Italy, Adriatic Sea shores (the Nanos Mountains) and the Balkan Peninsula [14, 15, 17].

Fig.1. Ruta graveolens [18]

Chemical composition of this plant is not known in detail. Its above-ground parts contain volatile oil with terpene hydrocarbons being the main components (Kubeczka K.H. op. cit. Abou-Mandour [15]). Our research team investigated the composition of coumarin compounds in this subspecies and confirmed for the first time the presence of the dimmer coumarine-daphnoretin methyl ether, some linear furanocoumarins and dihydro-furanocoumarin-rutamarin.

Studies investigating *R.graveolens ssp. divaricata in vitro* cultures conducted so far are scarce. They examined growth of cells and tissues under different culture conditions and their ability to differentiation and regeneration [15, 16]. Our research team investigated capability of accumulation of coumarin compounds in different types of cultures and the dynamics of accumulation of these metabolites during growth cycles [19, 20]. In addition, we demonstrated a marked potential of cells to transform exogenous phenolic compounds (hydroquinone and *p*-hydroxybenzoic acid) into arbutin and other glucosylation products.

The above-ground parts of *Ruta graveolens* L. (common rue), apart from volatile oil, many coumarin compounds, alkaloids and flavonoids, contain in addition, phenolic acids [21]. In our studies, we confirmed the presence of the latter group of metabolites both in shoots cultured *in vitro* and plants growing *in vivo* [22].

Considering taxonomic relationship between *R.graveolens ssp. divaricata* and *R. graveolens* and chemotaxonomic guidelines, we included also the phenolic acids into our analysis on the investigating subspecies. Our preliminary experiments demonstrated capability of biosynthesis of phenolic acids by *R.graveolens ssp. divaricata* cells cultured *in vitro* [21].

Concentrations of growth regulators in culture media belong to the most important factors influencing the accumulation of secondary metabolites in plant *in vitro* cultures

[23]. Therefore, in the present study we examined the effect of concentrations of the selected plant growth regulators, 1-naphthaleneacetic acid (NAA, auxin) and 6-benzylaminopurine (BAP, cytokinin) in the Linsmaier-Skoog medium [24], in the concentration range from 0.1 to 3.0 mg/l on the accumulation of free phenolic acids. We aimed to optimize culture conditions beneficial for the production of these compounds and to compare biosynthetic capabilities of cells growing *in vitro* with plants cultivated *in vivo*. The studies were also designed to propose *in vitro* cultures as a potential biotechnological source of some bioactive phenolic acids. This is the first report on the effect of growth regulators on phenolic acids accumulation in *R.graveolens ssp. divaricata* shoot-differentiating callus culture. These are also the first data on analysis of phenolic acids in the above-ground parts of the plants growing in vivo.

Materials and Methods

Origin of in *vitro* culture

The shoot–differentiating callus culture of *Ruta graveolens ssp. divaricata* (Tenore) Gams was established at the Institute for Biosciences, Würzburg University (Germany). Starting material for establishment of the culture originated from the plants harvested in natural sites in the Nanos Mountains and grown subsequently at the Botanical Garden of above mentioned institute. *In vitro* culture was derived from fragments of young leaves and stems.

Experimental *in vitro* cultures

Experimental stationary liquid shoot-differentiating callus culture was maintained on four Linsmaier and Skoog – LS medium [24] variants differing in concentration of plant growth regulators, NAA and BAP [mg/l]: 0.1 and 0.1; 1.0 and 1.0; 2.0 and 2.0; 3.0 and 1.0. The cultures (three series) were grown under constant artificial light (4 W/m^2, LF-40 W lamp, daylight, Piła) at 25 ± 2 C during four weeks growth cycles.

Plant material

Plant material harvested in Botanical Garden of Institute for Biosciences, Würzburg University (Germany) in 2009 analyzed for comparison, comprised: above-ground vegetative parts (stems, leaves and herb) of *Ruta graveolens ssp. divaricata.*

RP-HPLC analysis

Biomass from *in vitro* cultures collected after four week growth cycles (three series) and plant material were extracted twice with boiling methanol for 6 h. In the methanolic extracts, chromatographic quantification of eight phenolic acids was performed according to the RP-HPLC method developed by Ellnain-Wojtaszek and Zgórka [25] with our modifications. Separation was performed using Purospher 100 RP-18e analytical column (4 × 250mm) with the solvent system composed of methanol and 0.5% acetic acid (1:3 v/v). The flow rate was 1 ml/min. Detection was carried out using UV detector at λ=254 nm. Quantification was made by comparison with standards: caffeic, chlorogenic, protocatechuic, syringic acids (Sigma), *p*-coumaric, ferulic, *p*-hydroxybenzoic, vanillic acids (Fluka).

Isolation and identification of *p* – coumaric acid

The isolation and purification of the main metabolite was performed with chromatographic method using prep. TLC plates (Merck, Art. 1.11844) and HP- TLC plates (Merck, Art. 1.05631), respectively. Spectral analysis was performed on ^{1}H-NMR spectrum (300 MHz, DMSO, NMR spectrometer MSL-300, Bruker) and EI-MS spectrum (70 eV, LKB-2091 mass spectrometer, Sweden).

Results

Biomass increments

Within four-week growth cycles on the tested Linsmaier-Skoog (L-S) medium [24] variants, dry biomass grew 5.2-6.3 times. The biomass increments on the three testing medium variants, these contain growth regulators at concentrations from 1.0 mg/l to 3.0 mg/l were high and almost identical from 6.2- to 6.3-fold. The lowest biomass increments were obtained on the medium containing 0.1 mg/l NAA and 0.1 mg/l BAP.

Accumulation of free phenolic acids in extracts of biomass from *in vitro* culture

Five of eight assayed phenolic acids: caffeic acid, chlorogenic acid, *p*-coumaric acid, protocatechuic acid and syringic acid were determined in extracts from biomass of *R.graveolens ssp. divaricata* shoot-differentiating callus culture cultivated on all L-S medium variants. *p*-Hydroxybenzoic acid was present only in extracts from biomass growing on the L-S medium supplemented with 3mg/l NAA and 1mg/l BAP. None of extracts contained vanillic acid and ferulic acid.

The contents of individual compounds were diverse and ranged from 1.2 to 112.9 mg/100g d.w. (Fig. 2).

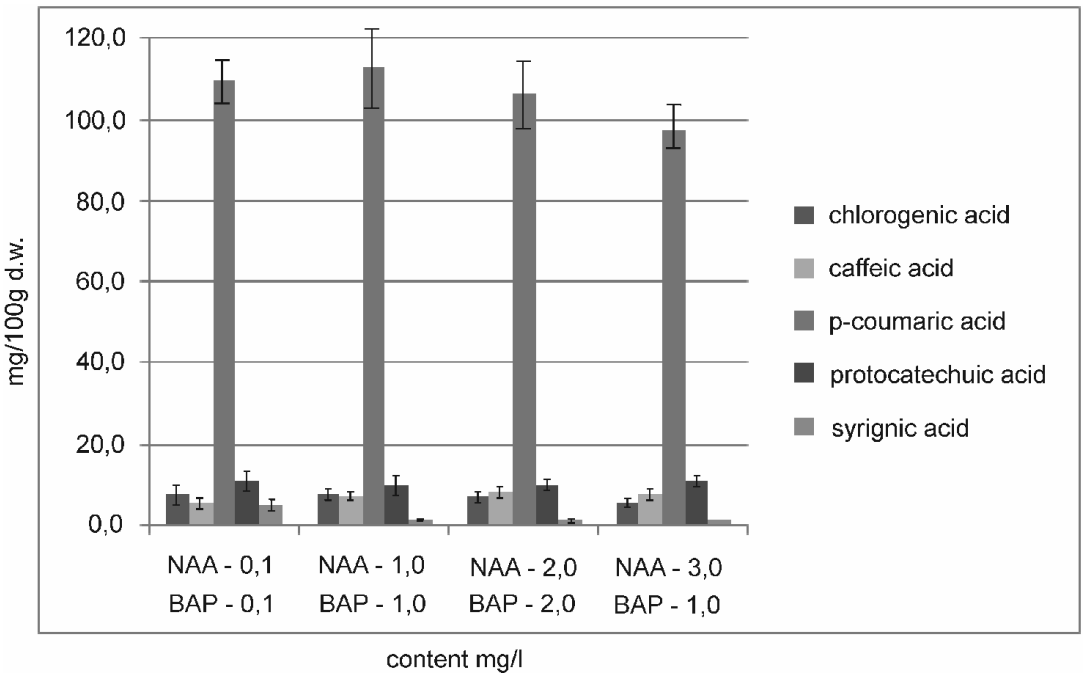

Fig. 2 Contents (mg/100 g d. w.) of the studied phenolic acids in extracts from biomass of *R. g. ssp. divaricata* cultured *in vitro* on Linsmaier and Skoog medium variants with different concentration of plant growth regulators (mg/l of medium). Data are presented as the mean of three series ± SEM. *p*-Hydroxybenzoic acid was not included into the figure, because it was detected only in extracts from LS medium containing 3mg/l NAA, and 1 mg/I BAP (1.8 mg/100 g d.w.)

The contents changed from 1.16– to 4.0-fold in dependence of the concentrations of growth regulators in culture medium. The maximum contents of syringic acid amounting to 4.8mg/100g d.w. were obtained on the L-S medium containing 0.1mg/l NAA and 0.1mg/l BAP. The maximum contents of caffeic acid and chlorogenic acid were almost identical and equaled 8.0 and 7.9mg/100g d.w., and were observed on the L-S medium variants enriched in different compositions of growth regulators, namely 2mg/l NAA and 2mg/l BAP, and 1mg/l NAA and 1mg/l BAP, respectively. The highest contents of protocatechuic acid reaching 11 mg/100 g d.w. were accumulated on two L-S medium variants (containing 0.1mg/l NAA and 0.1mg/l BAP, and 3mg/l NAA and 1mg/l BAP).

Among all analyzed phenolic acid, *p*-coumaric acid was the quantitatively dominating compound. Its content in extracts from biomass cultured on all L-S medium variants was high, ranging from 97.3 to 112.9mg/100g d.w. The maximum content of this compound was assayed in extracts from biomass containing 1mg/l NAA and 1mg/l BAP.

The total content of phenolic acids in biomass extracts depended on L-S medium variant and amounted from 124.9 to 138.7 mg/100g d.w. The highest contents were seen on three tested L-S medium variants supplemented with auxin and cytokinin at concentrations from 0.1 to 2.0 mg/l.

Accumulation of phenolic acids in extracts from the above-ground parts of plants growing *in vivo*.

Extracts from the above-ground plants analyzed for comparison (stems, leaves, herb) contained less phenolic acids and their composition differed in comparison with extracts of biomass from *in vitro* cultures (Table 1).

Table 1. Contents (mg/100 g d.w.) of phenolic acids in different vegetative parts of the plants harvested in Botanical Garden of Würzburg University and their maximal amounts obtained in biomass cultured *in vitro*

Metabolites	**Herb**	**Leaves**	**Stems**	**Biomass from *in vitro* culture** [a]
Caffeic acid	_ [b]	_	_	8.0
Chlorogenic acid	_	_	_	7.9
p-Coumaric acid	_	_	_	112.9
Ferulic acid	_	_	_	_
p-Hydroxybenzoic acid	_	_	_	1.8
Protocatechuic acid	88.2	85.1	52.0	11.1
Syringic acid	traces [c]	traces	traces	4.8
Vanillic acid	13.5	17.8	2.6	_
Total content	101.8	102.8	54.6	186.5 [d]

[a] - biomass cultured on various LS medium variants
[b] – not detected
[c] - contents lower than 0.001mg/100 g d.w.
[d] - sum of maximal contents of the estimated metabolites

Out of eight analyzed compounds, the analyzed extracts from organs of plants contained three metabolites: protocatechuic acid (52.0 – 88.2 mg/100g d.w.), vanillic acid (2.6 – 17.8mg/100g d.w.) and trace amounts of syringic acid.

Extracts from organ of plants growing *in vivo* did not contain even trace quantities of *p*-coumaric acid, which was the main compound in *in vitro* cultures.

Isolation and identification of *p*-coumaric acid

The main metabolite was isolated from biomass cultured *in vitro* and purified using chromatographic methods (preparative TLC, HP-TLC). The identity of this compound was also confirmed by spectral analysis (^{1}H-NMR, EI-MS spectra) and comparison with literature data [26].

Discussion

The present study demonstrated the effect of concentrations of growth regulators in the Linsmaier and Skoog (L-S) medium [24] on phenolic acid accumulation in shoot-differentiating callus culture of *Ruta graveolens ssp. divaricata*.

The total contents of phenolic acids in biomass extracts were diverse and ranged from 124.9 to 138.7 mg/100g d.w. depending on L-S medium variant. The contents of individual compounds also differed from 1.16- to 4.0-fold.

The influence of concentrations of growth regulators in culture media on the accumulation of different groups of secondary metabolites is a known phenomenon [23]. We documented such effect in our studies on the accumulation of coumarin compounds in *Ammi majus* and *Pastinaca sativa* cultures [27, 28] and recently in research on lignan accumulation in *Schisandra chinensis* culture [29]. We also evidenced the dependence of phenolic acid accumulation on the concentrations of growth regulators in *Ruta graveolens* [22], and recently in *Schisandra chinesis* [30] in vitro cultures.

In the present studies, the maximum total contents of free phenolic acids and the main metabolite, *p*-coumaric acid were confirmed on the L-S medium supplemented with 1mg/l NAA and 1 mg/l BAP. The greatest 6.3-fold dry biomass increments were also obtained on this L-S medium variant. Hence, this medium variant can be proposed as a "universal medium", i.e. both biomass growth-promoting and productive medium. The same experiments with *R. graveolens* shoot culture identified other variants of L-S medium as the best productive media, namely those containing 2mg/l NAA and 2mg/l BAP and 3mg/l NAA and 1mg/l BAP [23].

The obtained results revealed differences in composition and contents of phenolic acid between biomass from *in vitro* cultures and the above-ground organs of plants growing *in vivo*. Most importantly, *p*-coumaric acid was the main compound in biomass from *in vitro* cultures, whereas protocatechuic acid prevailed in native plant. Changes in biosynthetic pathways have been often observed in *in vitro* cultures and our results also confirmed this phenomenon. Our earlier studies on *R.graveolens ssp. divaricata* shoot-diffrentiating callus culture demonstrated significant biosynthetic differences in comparison with plants growing *in vivo*. Namely, xanthotoxin and isopimpinellin dominated in the *in vitro* cultured biomass while xanthotoxin and imperatorin prevailed in above-ground parts of plant. Plant material did not contain even traces of isopimpinellin, one of the main metabolites accumulated *in vitro* [19].

The metabolic changes noted in shoot-differentiating callus culture of *R.graveolens ssp. divaricata* are undoubtedly associated with a relatively low degree of differentiation of biomass from this *in vitro* culture.

On the other hand, our earlier studies indicated much smaller differences in phenolic acid biosynthetic pathways between *R. graveolens* shoots cultured *in vitro* and plants harvested *in vivo*. The same compound, i.e. protocatechuic acid dominated both in *in vitro*-cultured biomass and in plant material (*Herba Rutae*) [22].

In the present study, *R.graveolens ssp. divaricata* biomass was shown to contain large amounts of p-coumaric acid (97.3 – 112.9 mg/100g d.w.). These amounts are interesting from practical perspective. It should be noted that this compound has important therapeutic values and cosmetic potential [9-11].

The most of articles in plant biotechnology dealing with the accumulation of phenolic acids have concentrated on rosmaric acid [21]. Reports focused on *p*-coumaric acid are rare. The presence of this compound was confirmed e.g. in *Penstemon barbatus* and *Lavandula angustifolia* cultures [31]. We confirmed the presence of this metabolite in *Ruta graveolens* shoot cultures [22] and recently in *Schisandra chinensis in vitro* cultures [30].

Since the contents of *p*-coumaric acid in *R.graveolens ssp. diva*ricata biomass were high, we were able to isolate and purify this compound and to confirm its identity using spectral methods [26]. Our studies are the first to report the isolation of *p*-coumaric acid from plant *in vitro* cultures. The results of this research indicated that in vitro culture of *R.graveolens ssp. divaricata* could be a potential rich biotechnological source of *p*-coumaric acid and other bioactive phenolic acids.

In addition, for the first time in the above-ground parts of the plant growing *in vivo* phenolic acids were analyzed.

Resumo

Ŝoso-diferencigantaj kalusaj kulturoj de Ruta graveolens ssp. divaricata (Tenore) Gams estis kreskigantaj sur kvar variantoj de Linsmaier-Skoog (L-S) mediumo, kiuj enhavis diversajn koncentradojn de regulatoroj de planta kreskado, t.e. 1-naftalenaceta acido kaj 6-benzylaminopurino, inter 0,1 kaj 3,0 mg/l. Metanolaj ekstraktoj de la biomaso kulturitaj in vitro estis uzitaj por eltrovi la kvanton de liberaj fenolaj acidoj per HPLC-metodo. El ok analizitaj substancoj, ses estis trovitaj en la specimenoj: kafea acido, klorogena acido, p-kumara acido, p-hidroksibenzoa acido, protokatehhua acido kaj siringa acido. La kompleta enhavo de la esploritaj substancoj estis inter 124,9 kaj 138,7 mg/100g seka pezo, depende de la varianto de la L-S-mediumo. P-kumara acido estis la ĉefa substanco en la ekstraktoj de biomaso kulturita sur ĉiuj variantoj de la mediumo kaj ĝia kvantoj estis interesaj el la praktika vidpunkto (97,3 - 112,9 mg/100g seka pezo). Tiu ĉi substanco estis apartigita, purigita kaj ĝia identeco estis konfirmita per spektraj metodoj. La enhavo de la aliaj substanco ne transpasis 11,1 mg/100g seka pezo.

Ekstraktoj de la suprateraj partoj de plantoj kiuj kreskis in vivo (trunkoj, folioj, herbo), kompare analizitaj, enhavis tri fenolajn acidojn: protokatehhuan acidon (52,0-88,2 mg/100g seka pezo), vanilan acidon (2,6–17,8 mg/100g seka pezo) kaj minimumajn kvantojn de siringa acido.

Tiu ĉi estas la unua raporto pri la efiko de kreskadregulatoroj al la akumulado de liberaj fenolaj acidoj en biomaso de R. graveolens kulturita in vitro. Tiu ĉi plue estas la unua raporto kiu dokumentas la apartigadon de p-kumara acido el biomaso de tiu subspeco kulturita in vitro kaj samtempe de iu planta in vitro kulturo. Plie unuafoje fenolaj acidoj estis analizitaj en la suprateraj partoj de plantoj kiuj kreskis in vivo.

References:

1 De Graff, W. G.; Myers, L. S. J.; Mitchell, J. B.; Hahn, S. M.; Int J Oncol. 2003, 23, 159-163.

2 Ekiert, H.; Czygan, F. C.; Biotechnology - Secondary metabolites. Plants and microbes (Ramawat, K. G. and Merillon, J. M. ed) 445-482, Science Publ. Inc. USA, Jersey, Plymouth, UK 2007.

3 Nakamura, Y.; Torikai, K.; Ohigashi, H.; Free Radical Bio Med. 2001, 30, 967-978.
4 Sroka, Z.; Cisowski, W.; Food Chem Toxicol. 2003, 41, 753-758.
5 Chlopčikova, Š.; Psotova, J.; Miteková, P.; Soušek, J.; Lichnovský V.; Šimánek, V.; Phytother Res. 2004,18, 408-413.
6 Jiang, Y.; Kusama, K.; Satoh, K.; Takayama, E.; Watanabe, S.; Sakagami, H.; Phytomedicine. 2000, 7, 483-491.
7 Kono, Y.; Shibata, K.; Kodama, Y.; Sawa, Y.; Biochem J. 1995, 312, 947-953.
8 Olthof, M. R.; Hollman, P. C.; Katan M. B.; J Nut. 2001, 131, 66-71.
9 Ferguson, R. M.; Shuo-tun, Z.; Philip, J. H.; Mol Nut Food Res. 2005, 49, 585-5903.
10 Abdel-Wahab M.H.; El-Mahdy M.A.; Abd-Ellah M.F.; Helal G.K.; Khalifa F.Hamada F.M.; Pharmacol Res. 2003,48, 461-465.
11 An, S. M;, Koh, J. S.; Boo, Y. C.; Phytother Res. 2010, 24,1175-1180.
12 Engler A.; *Syllabus der Pflanzenfamilien* Bd.2. Berlin-Nikolassee, Gebrüder Borrntraeger Verlag 1964.
13 Hegi, G.; Illustrierte Flora von Mittel-Europa. München: Carl Hanser Verlag 1965.
14 Hoppe, H. A.; Drogenkunde. Berlin, New York: Walter de Gruyter 1975.
15 Abou-Mandour, A. A.; Planta Med. 1982, 46, 105-109.
16 Hartung, W.; Abou-Mandour, A. A.; Angewandte Botanik. 1996, 70, 221-223.
17 Tutin, T. G.; Heywood, V. H.; Burges, N. K.; Moore D.M.; Valentine D.H.; Walters J.H. Webb D.A. ; Flora Europaea vol. 2., Cambridge, Cambrige University Press 1968.
18 Kresanek J. Atlas Liecivych Rastlin a Lesnych Plodov. Osveta 1977.
19 Ekiert, H.; Abou-Mandour, A. A.; Czygan, F.; Pharmazie.2005, 60, 66-68.
20 Ekiert, H.; Czygan, F.; Pharmazie. 2005, 60, 623-626.
21 Ekiert, H.; Czygan, F.; Biotechnology - Secondary metabolites. Plants and microbes (Ramawat, K. G. and Merillon, J. M. ed). 445-482, Science Publ. Inc. USA, Jersey, Plymouth, UK 2007.
22 Ekiert, H.; Szewczyk, A.; Kuś, A.; Pharmazie. 2009, 64, 694-696.
23 Ramawat, K. G.; Mathur, M.; Biotechnology - Secondary metabolites. Plants and microbes (Ramawat, K. G. and Merillon, J. M. ed) 59-102, Science Publ. Inc. USA, Jersey, Plymouth, UK 2007.
24 Linsmaier, E. M.; Skoog, F.; Physiol Plantarum. 1965, 18, 100-127.
25 Ellnain- Wojtaszek, M.; Zgórka, G.; J Liquid Chromatography Relatated Technics. 1999, 2, 1457-1471.
26 Yoshizawa, H.; Shan, J.; Tanigawa, S.; Kitamura, Y.; Colloid Polym Sci. 2004, 282, 583-588.
27 Ekiert, H.; Gomółka, E.; Pharmazie, 2000, 55, 684-687.
28 Ekiert, H.; Gomółka, E.; Pharmazie, 2000, 55, 618-620.
29 Szopa, A.; Ekiert, H.; Pharmazie. 2011, 66, 633-634.
30 Szopa, A.; Ekiert, H.; Appl Biochem Biotech. 2012, 166: 1941-1048. 30. Wysokińska, H.; Świątek, L.; Acta Pol Pharm. 1986, 43, 623-629.
31 Bantrope, D. V.; Bilyard H. J.; Watson, D. G.; Phytochem. 1985, 24, 2677-2680.

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