

1 **Establishment of *in vitro* tissue cultures from *Echinacea angustifolia* D.C. adult plants**  
2 **for the production of phytochemical compounds**

3 \* M. Lucchesini<sup>1</sup>, A. Bertoli<sup>2</sup>, A. Mensuali-Sodi<sup>3</sup>, L.Pistelli<sup>2</sup>

4

5 <sup>1</sup> Dipartimento di Biologia delle Piante Agrarie, Università di Pisa, Viale delle Piagge 23,  
6 Pisa, Italy

7 <sup>2</sup> Dipartimento di Chimica Bioorganica e Biofarmacia, Università di Pisa , via Bonanno 33,  
8 Pisa, Italy

9 <sup>3</sup> Scuola Superiore di Studi e Perfezionamento Sant'Anna, piazza Martiri della Libertà 33,  
10 Pisa, Italy

11 \* Corresponding author. Fax: +39 050 2216524 E-mail address: mlucchesini@agr.unipi.it

12

13

14

15

16

17

18

19

20

21

22

23

24 **Abstract**

25 The establishment of *in vitro* cultures of *Echinacea angustifolia* was obtained directly from  
26 section of flower stalks of adult plants. The vegetative propagation was obtained directly from  
27 section of flower stalks placed on a mineral MS basal medium with B5 vitamins  
28 supplemented with 0.5 mgL<sup>-1</sup> 6-benzylaminopurine (BA) while callus regenerative masses  
29 were established from leaf sections cultured on the same basal medium supplemented with 3  
30 mg L<sup>-1</sup> BA and 0.5 mg L<sup>-1</sup> indole-3-butyric acid (IBA). The secondary metabolite contents of  
31 shoots proliferating on different culture media and callus masses were compared with *in vitro*  
32 or *in vivo* seedlings. The quali-quantitative LC-DAD-ESI-MS analyses both on the *n*-hexanic  
33 and methanolic extracts demonstrated that significant production of caffeic acid derivatives,  
34 echinacoside and alkamides from different *in vitro* *E. angustifolia* tissues was possible.  
35 Choosing the appropriate plant material from different *in vitro* cultures, the plant metabolite  
36 pathway might be addressed towards the alkamides or the caffeic acid derivatives  
37 productions.

38

39 **Keywords:** alkamides; flavonoids; caffeic acid derivatives; *Echinacea angustifolia*; flower  
40 stalk; *in vitro* shoots; LC-DAD-ESI-MS.

41

42

43

44

45 **Introduction**

46

47 The genus *Echinacea* belongs to the *Asteraceae* family and has nine species (McGregor,  
48 1968). At present, only three species are used in phytotherapy: *E. angustifolia* D.C. (De  
49 Candolle) var. *angustifolia* (syn. *Rudbeckia angustifolia* L.), *E. pallida* (Nutt.) Nutt. and *E.*  
50 *purpurea* (L.) Moench.. *Echinacea* spp. are native of North America (McGregor, 1968) and  
51 belonged to the rich Pharmacopoeia of the native Americans, who had used them for  
52 hundreds of years for infections, inflammations and insect bites (Lloyd, 1921).

53 The chemistry of *Echinacea* species is well-known and caffeic acid derivatives, flavonoids,  
54 polyacetylenes, alkamides, pyrrolizidine alkaloids, polysaccharides and glycoproteins were  
55 isolated and characterized. (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and  
56 Reminger, 1989; Bauer *et al.*, 1989; Bauer *et al.*, 1988a).

57 In the last few years, the increased demand of natural remedies in Europe has caused an  
58 enhancing industrial request in the production of standardized plant material and extracts.

59 *Echinacea* is an Extraeuropean genus and the certified plant material for propagation of *E.*  
60 *angustifolia* is not available yet (Li, 1998). Moreover, plants of the genus *Echinacea* are  
61 characterized by their difficult germination caused mainly by seed dormancy (Baskin *et al.*,  
62 1992; Macchia *et al.*, 2001; Feghahati and Reese, 1994; Sari *et al.*, 2001).

63 Progress in medicinal plant clonal propagation has been requested, especially for species such  
64 as *Echinacea* with an agricultural production not sufficient for the growing pharmaceutical  
65 industry demand. For this purpose, it is important to develop a reproducible protocol to  
66 cloning *E. angustifolia*.

67 Till now only few reports showed attempts concerning the *in vitro* procedures for the  
68 establishment of *E. angustifolia* clones from adult plants (Harbage, 2001; Lakshmanan *et al.*,  
69 2001).

70 However there are no reports of regeneration using flower stalk sections as an explant source  
71 for this species.

72 Difficulties to promote *E. angustifolia* tissue cultures from selected adult plants were mainly  
73 caused by the rosette habit of this species. The short internodes and the vegetative apices  
74 located near the ground, caused initial contaminations difficult to eradicate. In this work a  
75 regeneration protocols from flower stalk explants was established. The use of this type of  
76 explants allowed to reduce contamination problems and could be linked to previous analysis  
77 of plant during their vegetative growth. The selection of important medicinal species is an  
78 essential step to improve the agronomic and pharmaceutical features in particular when a high  
79 phenotypic variability is present as in plant belonging to *Echinacea* family Regarding the  
80 biosynthesis of the typical secondary metabolites, the presence of echinacoside, caffeic acid  
81 derivatives, and polysaccharides were shown only in cell suspension cultures from seedling  
82 tissues of *E. angustifolia* (Smith *et al.*, 2002), but most of the works about the production of  
83 caffeic acid derivatives, alkamides and anthocyanins were carried on mainly on extracts of  
84 other species of the *Echinacea* family cultivated *in vitro* (Schollhorn *et al.*, 1993; Sicha *et al.*,  
85 1991 Luczkiewicz and Cisowski, 2001 Luczkiewicz *et al.* 2002). Moreover, no data on the  
86 influence of the origin of the *in vitro* plantlets and their multiplication over time on secondary  
87 metabolites are available in the literature.

88 In this work *E. angustifolia in vitro* cultures were established from adult plants.  
89 Extraction and LC-DAD-ESI-MS protocols were performed in order to evaluate the  
90 main secondary metabolites production in shoots collected in different phases of the *in*  
91 *vitro* culture. Secondary metabolites content was compared with that of greenhouse  
92 flowering plants and with *in vitro* germinated seedlings.

Deleted: ¶  
¶

## 93 Materials and methods

94

### 95 Plant material

96 Open field *E. angustifolia* mother plants were furnished by the Department of Agronomy of  
97 the University of Pisa. Plants were transferred to a greenhouse and conditioned (phase 0 of  
98 micropropagation process according with Debergh and Maene (1981) by spraying them twice  
99 with 0.10 g/l Benomyl fungicide (Du Pont Agricultural Products, Wilmington, Delaware, UK)  
100 every 15 days the shoots were cut after an additional period of five days without treatments  
101 (Mensuali-Sodi et al. 1997). Leaf explants and flower stalks were employed as starting  
102 material for tissue culture.

103

### 104 *Echinacea angustifolia* tissue cultures from adult plants

105 Leaves and flower stalks removed from mother plants were subjected to a first washing in tap  
106 water for 16 hours then, after reducing explant size, they were sterilized in a 15% of sodium  
107 hypochlorite (8% Cl active) aqueous solution stirred for 15 min followed by three final rinses  
108 in sterile distilled water. Under laminar flow cabinet the flower stalks were cut in slices 1-2  
109 mm thick and the leaf explant portions were excised containing the central vein (0.25 cm<sup>2</sup>).  
110 Each type of explants was placed in a climatic chamber 25 ± 1°C either in the darkness or at  
111 16 hours of photoperiod with irradiance of 50 µmol sec<sup>-1</sup>m<sup>-2</sup>. The culture medium employed  
112 was the basal medium, named CH, consisting of MS macro and micro elements, B5

113 Vitamines (Gamborg, 1968), 300 mg L<sup>-1</sup> reduced Gluthatione (GSH), 500 mg L<sup>-1</sup> 2-(N-  
114 Morpholino) ethanesulfonic acid (MES), 30 g L<sup>-1</sup> sucrose, 7 g L<sup>-1</sup> agar, pH 5.8. Two  
115 arrangements of growth regulators were used: 0.01 mg L<sup>-1</sup> 1-naphtaleneacetic acid (NAA)  
116 plus 1 mg L<sup>-1</sup> BA, and 0.5 mg L<sup>-1</sup> BA. Both media were added with 0.3% of *Plant*  
117 *Preservative Mixture*, Plant Cell Technology Inc., U.S.A. (PPM).

118 *E. angustifolia* regenerated shoots were sequentially subcultured in vessels containing CH  
119 medium with 0.25 mg L<sup>-1</sup> (initial proliferating shoots: IP shoots) and 0.5 mg L<sup>-1</sup> BA (axillary  
120 proliferating shoots: AP shoots) interleaved by the CH medium with half mineral strength,  
121 vitamins and hormones free, 15 g L<sup>-1</sup> sucrose and 5 g L<sup>-1</sup> active charcoal.

122 Leaves from *in vitro* shoots derived from flower stalk regeneration were excised and explants  
123 (0.5 cm<sup>2</sup>) were cut from the middle area of the lamina. Two different culture media were  
124 used named CHe and CHe\* containing basal medium CH added with 3 mg L<sup>-1</sup> BA and 0.5  
125 IBA or 6 mg L<sup>-1</sup> BA 1 mg L<sup>-1</sup> IBA respectively.

126 Regenerated shoot were subcultured on the same basal medium added with 0.5 mg L<sup>-1</sup> BA in  
127 Magenta vessels (LR).

128 All media tested in these experiments were sterilized by autoclaving at 121°C at 1 atm. for 20  
129 min.

130 *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80  
131 μmol sec<sup>-1</sup> m<sup>-2</sup> and photoperiod of 16 hours.

132

### 133 ***Echinacea angustifolia* greenhouse plants**

134 *Echinacea angustifolia* D.C. achenes were obtained from Gargini Sementi S.n.c. (Lucca,  
135 Italy). Achenes were sowed in Petri dishes and incubated at 25 ± 1°C with a 16 h photoperiod  
136 (cool white fluorescent light 70 μmol m<sup>-2</sup> s<sup>-1</sup>). To overcome seed dormancy the inoculated  
137 achenes were previously subjected to stratification at 4°C in the dark for 11 days in the  
138 presence of 1 mM ethephon (2-chloroethylphosphonic acid) (Macchia *et al.* 2001). After

139 germination seedlings were transplanted in multi-pots containing pit-perlite soil (50:50 v:v)  
140 under greenhouse conditions. Leaf samples were collected at the beginning of the flowering  
141 period.

142

#### 143 ***Echinacea angustifolia* tissue cultures from seedlings.**

144 Seeds from the same source above cited, pre-treated to avoid seed dormancy with the same  
145 procedure above described, were sterilized by immersion in 70% ethanol for 30 s followed  
146 by soaking in 15% sodium hypochlorite solution (8% active chlorine) containing two drops  
147 of Tween 20<sup>®</sup> for 18 min and followed by three rinses in sterile water. Achenes were sowed  
148 on half strength inorganic basic nutrient MS (Murashige and Skoog, 1965), sucrose (15 g L<sup>-1</sup>)  
149 <sup>1</sup>), agar (7 g L<sup>-1</sup>), without vitamins or growth regulators. The medium was sterilized by  
150 autoclaving at 121°C at 1 atm. for 20 min. After the cold stratification period sowed seeds  
151 were transferred to a growth chamber and maintained at 25 ±1°C with a 16 h photoperiod  
152 (cool white fluorescent light 70 μmol m<sup>-2</sup> s<sup>-1</sup>).

153 To induce shoot proliferation, *in vitro* germinated seeds were deprived of the root system and  
154 placed on the basal medium CH and, to promote shoot proliferation, 0.5 mg L<sup>-1</sup> BA was used.  
155 *In vitro* cultures were maintained in a growth chamber at 22 ± 1°C with an irradiance of 80  
156 μmol sec<sup>-1</sup> m<sup>-2</sup> and photoperiod of 16 hours.

157

#### 158 **Plant Experiments and Statistical analysis**

159 Explants used for shoot induction from adult plants were positioned in Petri ø 6 cm dishes (5  
160 explant/dish, 5 dishes/treatment). During the proliferation and growing phase explants were  
161 subcultured into 175 ml glass culture vessels (5 explants/vessel; 10 vessels/treatment) and in  
162 G7 Magenta vessels (6 explants/vessel; 5 vessels/treatment). Shoot number per explant and  
163 length during the proliferation were expressed as mean ± standard error.

164 All the experiments were repeated twice and all data were recorded after three weeks (one  
165 subcultures).

166 *In vitro* and *in vivo* germination data were recorded on the day 2, 4, and 6 after the pre-  
167 treatments which were used to avoid seed dormancy. Germination ability was expressed as  
168 germination percentages on the total seeds; mean germination time (MGT) was calculated as  
169 reported to Ellis e Roberts (1980):

$$170 \frac{\sum(t_i \cdot n_i)}{\sum n_i}$$

171 where  $t_i$  represents the day number from the root emission and  $n_i$  the seed number germinated  
172 in each time intervals (n=50; 5 seeds/Petri dishes). As regards shoot induction from aseptic  
173 seedlings, each explant was placed into a disposable 30 ml vial (n=25). Germination  
174 percentages, MGT, shoot number and length during the proliferation from *in vitro* seedling  
175 were recorded and reported as mean values  $\pm$  Standard Error (SE).

176

## 177 **Phytochemical investigation**

### 178 **Chemicals**

179 LC grade Water, Acetonitrile, Methanol and Formic acid (Backer) were used for LC-DAD-  
180 MS Liquid Chromatography Diode Array Detector Electrospray Ionization Mass analysis.  
181 Commercial compounds were used as some reference materials: caftaric acid (**1**) (10 mg,  
182 ChromaDex, lot: 01-03028-301), chlorogenic acid (**2**) (10 mg, Extrasynthese, lot: 327-97-9),  
183 echinacoside (**3**) (10 mg, ChromaDex, lot: 01-05020-101), cichoric acid (**4**) (10 mg,  
184 ChromaDex, lot: 00-03640-300) and, caffeic acid (**5**) (10 mg, Sigma Aldrich, lot: 60018). The  
185 flavonoids [quercetin (**7**), luteolin (**8**), apigenin (**9**), kaempferol (**10**), *p*-cumaric acid (**11**),  
186 betulinic acid (**12**), apigenin 7-*O*- $\beta$ -glucoside (**13**), isorhamnetin 3-rutinoside (**14**)] used as  
187 standard compounds were part of a home-made database of natural compounds, isolated and  
188 identified by NMR and MS experiments in our laboratory (HPLC purity grade 97-98%).

189 Alkamide (6) [dodeca-2E,4E,8Z,10E-tetraenoic acid isobuthylamide] was isolated and  
190 characterized during a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var.  
191 *bravado*) cultivated in Sanremo (Italy) (Table 1).

192

### 193 **Sample preparation and LC-DAD-ESI-MS analysis**

194 The aerial parts (1.3 g) of the *E.angustifolia* plant material were freeze-dried and extracted by  
195 ultrasonic apparatus with *n*-hexane and methanol in turn (100 ml x 2h, 3 times).

196 Each fraction was analysed by LC-DAD-ESI-MS. LC system consisted of a Surveyor  
197 Thermofinnigan liquid chromatograph pump equipped with an analytical Lichrosorb RP-18  
198 column (250 x 4.6 mm i.d., 5mm, Merck), a Thermofinnigan Photodiode Array Detector and  
199 an ion trap LCQ Advantage mass spectrometer. The analyses were carried out by a linear  
200 gradient using water with 0.1% HCOOH (solvent A), CH<sub>3</sub>CN (solvent B) from 10:90 v/v (B-  
201 A) to 70:30 (15 min) (flow 0.7 ml/min, run time 40 min). The spectral data from the DAD  
202 detector were collected during the whole run in the range 210-700 nm and the peaks were  
203 detected at 254 (alkamides, flavonoids) and 330 nm (caffeolquinic derivatives , flavonoids)  
204 for all analysed samples.

205 LC-ESI-MS analyses [negative ion mode for caffeoilquinic derivatives (1-5) and flavonoids  
206 (7-14), positive mode for alkamide (6)], SRM Selected Reaction Monitoring, TIC Total Ion  
207 Current , m/z 100 to 800 amu) were performed in the same chromatographic conditions using  
208 the specific ESI values for caffeic acid and alkamides (sheath gas flow-rate 62 arbitrary units,  
209 auxiliary gas flow 9 arbitrary units, capillary voltage -16 V and capillary temperature 280°C).  
210 The qualitative results of LC-DAD-ESI-MS were showed in Table 1.The amounts of the  
211 compounds (1-6) were estimated by using a multilevel external standard procedure:

212 Caftaric acid (1)                       $Y = 1.0347 + 2.5974 \text{ E-}05 X$                        $r = 0.9977$

213 Chlorogenic acid (2)                       $Y = 1.9601 + 1.6762 \text{ E-}05 X$                        $r = 0.9985$

214 Echinacoside (3)                       $Y = 0.1024 + 6.5431 \text{ E-}05X$                        $r = 0.9966$

215	Cichoric acid (4)	$Y = 2.1424 + 1.2889 \text{ E-}05 \text{ X}$	$r = 0.9988$
216	Caffeic acid (5)	$Y = 1.7444 + 8.4818 \text{ E-}06 \text{ X}$	$r = 0.9994$
217	Alkamide (6)	$Y = 1.668 + 1.2277 \text{ E-}05 \text{ X}$	$r = 0.9978$

218

219 | **Results**

Deleted: ¶

220

221 ***Echinacea angustifolia* tissue cultures from adult plants**

222 The contamination of *E. angustifolia* explants was a serious problem that required a lot of  
223 time consuming tentative to establish a protocol for the mother plant management to pull  
224 down the micro flora of these plants. Several treatments with fungicides on the *in vivo* mother  
225 plants and the presence of the biocide PPM in the culture medium gave 40% of non-  
226 contaminated explants, which was sufficient to start the culture. Callus formation was induced  
227 from leaf explants cultured on both tested media in the in light and darkness, but the callus  
228 developed on the BA medium was necrotic as that on the NAA with BA medium in the light.  
229 The flower stalk slices give rise to cell proliferation when cultured on  $0.5 \text{ mg L}^{-1}$  BA in the  
230 light (Table 2 and Fig. 1). These green calli showed purple spots which generally developed  
231 shoot primordial so they could be considered as markers of the regeneration process (Table  
232 2). The shoots derived from these spots were subcultured on the same medium (Table 3)  
233 maintaining, at the end of the second subculture, their morphogenetic capacity (Table 3 )  
234 with a sustained growth of regenerating callus. The leaf explants cultured on medium with  
235 NAA combined with BA in dark condition, developed a conspicuous white friable callus  
236 during the successive subculture but didn't show shoot regeneration at all (Table 3). During  
237 this induction phase, hyperhydricity occurred in the new developed shoots. To reduce this  
238 phenomenon, the shoots were subcultured in the same medium with half BA amount ( $0.25 \text{ mg}$   
239  $\text{L}^{-1}$ ) (initial proliferating shoots: IP shoots) (Table 4), but the successive subcultures showed a  
240 progressive culture deterioration which encountered a high hyperhydricity again (Figure 2A).

241 The successive culture on medium with active charcoal t lacking growth regulators  
242 determined the callus reduction at the shoot basal end, a drastic decrease of the shoot number  
243 and an increase in shoot length and quality (Table 4). When hyperhydricity was reduced,  
244 *Echinacea* shoots were cultured again on the initial medium with 0.5 mg l<sup>-1</sup> BA. During this  
245 multiplication phase the explants produced a low number of new axillary shoots (axillary  
246 proliferating shoots: AP shoots) (Table 4 and Figure 2B). These shoots, not only showed  
247 normal leaf features, no hyperhydric symptoms and less callus amount at the basal end but  
248 also were able to regenerate, *de novo* shoots from the central vein of the intact leaves (Figure  
249 3).

250 An analogous high morphogenetic potential was observed when leaf sections excised from  
251 AP shoots were cultured.

252 From the results summarized in Table 5 we can notice that CHe medium including BA 3  
253 mgL<sup>-1</sup> and IBA 0.5 mgL<sup>-1</sup> produced a high percentage of callus with purple spots which  
254 could be considered “differentiation spots” as above described. Growth regulators in a double  
255 concentration (CHe\*) didn’t improve the callus formation and shoot differentiation.  
256 Regenerated shoots from the callus cultures on CHe medium were subcultured (Figure 4) on  
257 the same basal medium CH with 0.5 mgL<sup>-1</sup> BA (leaf regenerated shoots: LR shoots). The  
258 results described in Table 5 demonstrated that shoot regeneration from *in vitro* growth leaves  
259 could provide a good regeneration rate (1 leaf portion: 16 new shoots) useful to increase the  
260 *E. angustifolia* shoot biomass.

261

### 262 ***Echinacea angustifolia* from seedlings**

263 To compare the phytochemical contents of *in vitro* cultures with those of tissues from *E.*  
264 *angustifolia* propagated by seeds, *in vitro* and *in vivo* seedling cultivations were established.  
265 For this purpose, germination was tested to provide the starting material for the greenhouse  
266 and the *in vitro* cultivation. Pre-treatment with ethephon together with stratification of *E.*

267 *angustifolia* achenes was useful as demonstrated by the high percentage of germination (  
268  $69.7 \pm 4.03$ ) and a low mean germination time (MGT,  $2.47 \pm 0.11$  ).  
269 New shoots (Proliferating Seedling shoots: PS shoots) was obtained culturing *E. angustifolia*  
270 seedling explants on the CH basal culture medium with 0,5 mg/l BA ( $2,1 \pm 0.3$  number of  
271 shoots per explants with an average length of  $1.72 \pm 0.24$  cm ).

Deleted: ¶

## 272 **Phytochemical analysis**

273 *Echinacea* plant material was investigated for the production of secondary metabolites as  
274 listed below:

- 275 • Shoots from flower stalk of *E. angustifolia* plants and collected in the different  
276 culture phases (IP shoots, AP shoots and LR shoots);
- 277 • Shoots from *in vitro* germinated seedlings named Proliferated Seedlings (PS shoots);
- 278 • Leaves from green-house plants collected at the beginning of the flowering period  
279 named (GH plants).

280 The *n*-hexane and methanolic extracts were obtained by ultrasonic apparatus from fresh plant  
281 material after freeze-drying. LC-DAD-ESI-MS analyses were performed in order to evaluate  
282 the production of alkamides, flavonoids and caffeolquinic derivatives.

283 The phytochemical screening was carried out by the comparison of the retention time, UV  
284 and MS spectra for each peak in the extract samples with those of the reference compounds  
285 (**1-14**) (Table 1). A summary of the quantitative results ( $\mu\text{g/g}$  dry plant material) for the selected  
286 constituents (**1-6**, Fig. 5) in the analysed samples is given in Table 7.

287 Alkamide (**6**) was detected as the main constituent in the *n*-hexane extracts of *in vitro* IP  
288 shoots and LR shoots. Phenolic acids (**1, 2, 4, 5**) and echinacoside (**3**) were produced in much  
289 lower amounts in comparison with the alkamide (**6**) in the same samples. An opposite result  
290 was observed in the AP shoots and *in vivo* GH plants which produced caffeic acid derivatives  
291 especially. The AP samples reduced the production of alkamide (**6**) by half in comparison  
292 with the IP samples, but they were characterized by a much larger amount of chlorogenic acid

293 (2), echinacoside (3), and cichoric acid (4). The *in vitro* proliferating seedlings (PS shoots)  
294 contained only cichoric acid (4).

295

## 296 **Discussion**

297

298 The aim of this work was to perform a standardized protocol for the massive multiplication of  
299 *E. angustifolia* plants able to synthesize their typical secondary metabolites.

300 In this work it was settled up the active shoot organogenesis of flower stalks from adult  
301 plants: the possibility to use this type of explant from a particular medicinal plant, previously  
302 selected for their yield during the vegetative phase, could be very convenient for the growers.

303 This method could be applied without causing damage or completely destroying individual  
304 plants as it occurred when the apical buds were excised from the rosettes. Plant propagation  
305 from flower stalks was employed for *in vitro* regeneration of several species (Bajaj et al,  
306 1983; Tan Nhut et al., 2001; Martin, 2005) but it is an unusual techniques for tissue culture of  
307 *Compositae* plants. Direct and indirect regeneration appeared at the same time on flower stalk  
308 explants exposed to light on media supplemented with 0.5 BA. This type of explant produced  
309 a a satisfied shoot proliferation comparable with those observed on petioles and leaves of *E.*  
310 *purpurea* (Choffe et al.,2000; Koroch et al. 2002). During these experiments this phenomenon  
311 occurred together with a high shoot hyperhydricity and with the development of abundant  
312 callus at the shoot basal end as also observed by Lakshmanan et al. (2001) on *in vitro* seedling  
313 leaves of different *Echinacea* species.

314 Hyperhydricity was considered as a physiological response to simultaneous stress factors  
315 determined by the *in vitro* culture conditions as high cytokinin treatments combined with the  
316 high relative humidity in the closed flask atmosphere (Kevers et al., 2003). Therefore, in order  
317 to obtain *E. angustifolia* plantlets more suitable to develop a continuous multiple shoot

318 production, the BA content was halved. The procedure gave a temporary improvement of the  
319 culture but the successive subcultures on the same medium showed again a progressive  
320 deterioration . A mid-step culture phase using active charcoal (Debergh and Maene, 1981)  
321 was necessary to restore shoot quality. These plantlets, cultured again on 0.5 mg L<sup>-1</sup> BA CH  
322 basal medium restore completely the shoot quality but maintained a poor multiplication  
323 capacity (1.7 shoots per explant). This multi-step protocol, improved the quality of the shoots,  
324 their elongation and it reduced the callus production at the basal end, giving suitable explants  
325 for the successive proliferation phases. The choice to increase the *in vitro* biomass and the  
326 previous observations concerning the direct organogenesis on shoot foliage, lead to perform  
327 leaf cultures on media with auxins and cytokinins . Auxins alone or in combination with  
328 cytokinins has been frequently associated with the induction and expression of regeneration in  
329 plant tissue culture (Steward et al., 1964). Zobayed and Saxena (2003), observed somatic  
330 embryogenesis from leaves of *E. purpurea* *in vitro* seedling when IBA (0.5 mgL<sup>-1</sup>) was used  
331 together with BA (2.5 mg L<sup>-1</sup>). In this work the combination of analogous IBA with BA  
332 concentrations in the CHe medium was useful to produce a conspicuous biomass of shoot  
333 regenerating callus confirming that the use of IBA might be effective in regeneration of  
334 recalcitrant species to the *in vitro* culture (Zobayed and Saxena, 2003). Summarizing, the *in*  
335 *vitro* propagation process of *E. angustifolia* from flower stalk consisted of three distinct  
336 phases: an adventitious regeneration phase from stalk sections, an axillary proliferation phase  
337 of the previous regenerated shoots and an adventitious regeneration phase from leaf pieces of  
338 the axillary shoots. The shoots sampled from these different differentiation process were  
339 subjected to the phytochemical analysis. Concerning to the analysis of the active constituents,  
340 LC-DAD-ESI-MS screening was carried out on the *in vitro* and *in vivo* plant material to  
341 evaluate the production of the typical antioxidant constituents (1-14) of *Echinacea* species  
342 (Table 1). The results showed that none of the well-known in *Echinacea* adult plants  
343 Flavonoids (7-14) (Bauer and Foster, 1991; Bauer and Wagner, 1991; Bauer and Reminger,

344 1989; Bauer *et al.*, 1989; Bauer *et al.*, 1988a) were present in the different plant material  
345 analysed in this work.

346 A significant difference between shoots from seedlings growing *in vitro* (PS shoots) and *in*  
347 *vivo* (GH) was showed. A lower secondary metabolite production was observed in PS (in  
348 vitro proliferating seedlings) shoots in comparison with the GH plants and it could be related  
349 to the *in vitro* growth conditions. In particular, between all the antioxidant constituents (**1-14**),  
350 the *in vitro* seedling accumulated only cichoric acid.

351 The *in vitro* shoots deriving from adult plants in the axillary proliferation phase (AP shoots)  
352 were characterized by a high amount of caffeic acid derivatives. They showed the production  
353 of caftaric, chlorogenic, cichoric acids, and echinacoside but no caffeic acid. AP shoots are  
354 plantlets well developed and showed phenotypic features similar to the greenhouse plants , for  
355 this reason, both cultures produced a significant amount of caffeolquinic derivatives. The  
356 yields of these secondary metabolites were similar or higher than those reported in leaves of  
357 *E. angustifolia* adult plants showed in Table 7. In particular, echinacoside (**3**), the main active  
358 constituent found only in wild or cultivated *E. angustifolia* roots (Bauer and Wagner, 1991;  
359 Bauer, 1998), was accumulated in a similar amount in the leaves of the AP shoots. In addition  
360 it could be remarked that cichoric acid was the main bioactive constituent in AP shoots and it  
361 showed much higher yields (3.05 %) in comparison with those reported in Table 7 from  
362 different organs of *E. angustifolia* adult plants (Bauer and Wagner, 1991; Bauer, 1998).

363 On the contrary, shoot at the end of the initial proliferating phase (IP shoots) accumulated a  
364 lower amount of phenolic metabolites in comparison with AP shoots and with the greenhouse  
365 plants but were able to produce a large quantity of alkamide (**6**). In addition, it was also  
366 evident that leaf regenerating shoots (LR) lost the ability to synthesize caffeic acid derivatives  
367 since they produced almost exclusively alkamide (**6**). It is well-known that alkamides are  
368 present in the roots, leaves, and stalks of wild or cultivated *E. angustifolia* plants (Bauer, *et al.*  
369 1989). In this work the initial shoots from flower stalk and the regenerating shoots from leaf

370 explants (IP and LR) supplied an amount (0.023 and 0.036 % respectively) of alkamides (6)  
371 comparable to the typical content of wild *E. angustifolia* leaves and stalks showed in Table 7.  
372 This behaviour might be due to the IP and LR shoots physiological status in which a primary  
373 metabolism, directed towards the regeneration process, was dominant rather than a secondary  
374 one. Moreover, the higher propagation rates observed in IP and LR, might cause an  
375 hyperhydric status as observed by other Authors (Kevers et al. 2003; Hazarika, 2006) .  
376 Hyperhydricity, considered as a stress response, might lead IP and LR shoots towards several  
377 biochemical changes associated with a different pattern of metabolite accumulation opposite  
378 to AP shoots and GH plants.

379 The AP shoots drastically reduced the production of alkamide (6) in comparison with the IP  
380 and LR shoots. Furthermore, the leaves of *E. angustifolia* GH plants produced at least ten  
381 folds lower amounts of alkamide than the IP and LR shoots.

382 In conclusion, from our knowledge, this is the first report on significant production caffeic  
383 acid derivatives and alkamides from *in vitro* regenerated shoots of *E. angustifolia*. It was  
384 pointed out how the micropropagation of *E. angustifolia* plantlets from adult plants and the  
385 careful development of the proper multiplication procedures could allow us to get plant  
386 biomass able to produce active compounds at a rate comparable to that of the original plants.  
387 The different *in vitro* conditions, affect the plant metabolite pathway operating as a switch  
388 eliciting for the alkamide or the caffeic acid derivatives production. Therefore, the shoot  
389 regeneration protocols developed in the current study permit to choice the best culture phase  
390 to produce either caffeic acid derivatives or alkamides.

391

## 392 **Acknowledgments**

393  
394 The research was financed by The Sixth Framework Programme of European Commission-  
395 Food Quality and Safety Project- titled “Ready to eat food for breakfast and sport activity

396 with high content of nutraceuticals reducing a disease risk and promoting public health” (Nutra-  
397 Snacks) and by the research Project of the Italian Space Agency (A.S.I.) “Medicine and  
398 Biotechnology - Biotechnological Applications” Feasibility Study Contract (June 2006):  
399 “From Molecules To Man: Biotechnological Applications of Space Research” (MoMa).

Deleted: -----Page Break-----

400

Formatted

## 401 **References**

- 402 Bajaj, Y. P. S., Sidhu, M. M. S., Gill, A. P. S., 1983. Some factors affecting the *in vitro*  
403 propagation of gladiolus. *Scientia Horticulturae* 18, 269-275.
- 404 Baskin, C.C., Baskin, J.M., Hoffman, G.R., 1992. Seed dormancy in the prairie forb  
405 *Echinacea angustifolia* var. *Angustifolia* (*Asteraceae*): After ripening pattern during  
406 cold stratification. *Int J Plant Sci.* 153, 239-243.
- 407 Bauer, R., 1998. *Echinacea*: Biological effects and active principles. In Lawson, L.D., Bauer  
408 R., (eds.), *Phytomedicines of Europe: Chemistry and Biological activity*, ACS  
409 Symposium Series 691. American Chemical Society, Washington, DC, pp. 140-157.
- 410 Bauer, R., Foster, S., 1991. Analysis of Alkamides and Caffeic Acid Derivatives from  
411 *Echinacea simulata* and *E. paradoxa* roots. *Planta Med.* 57, 447-449.
- 412 Bauer, R., Khan, I.A., Wagner, H., 1988a. TLC and HPLC analysis of *Echinacea pallida* and  
413 *E. Angustifolia* roots. *Planta Med.* 54, 426-430.
- 414 Bauer, R., Remiger, P., Wagner, H., 1989. Alkamides from the roots of *Echinacea*  
415 *angustifolia*”, *Phytochemistry* 28, 505-508.
- 416 Bauer, R., Reminger, P. 1989. TLC and HPLC analysis of Alkamides in *Echinacea* Drugs.  
417 *Planta Med.* 55, 367-371.
- 418 Bauer, R., Wagner, H., 1991b. *Echinacea* species as potential immunostimulatory drugs. In:  
419 Farnsworth, N. R. and Wagner, H. (eds.) *Economic and medicinal plant research* 5. New  
420 York, NY: Academic Press, pp. 253-321.

- 421 Choffe, K.L., Victor, J.M.R., Murch, S.J., Saxena, P.K., 2000. In vitro regeneration of  
422 *Echinacea purpurea* L.: direct somatic embryogenesis and indirect shoot organogenesis  
423 in petiole culture. Vitro Cell Dev Biol Plant. 36, 30-36.
- 424 Debergh, P.C., Maene, L.J., 1981. A scheme for commercial propagation of ornamental plants  
425 by tissue culture. Scientia Hort. 14, 335-345.
- 426 Ellis, R.H., Roberts, E.H., 1980. The influence of temperature and moisture on the seed  
427 viability period in barley (*Horedum disticum* L.). Ann Bot. 45: 31-37.
- 428 Feghahati, S.M.J., Reese, R.N., 1994. Ethylene-, Light-, and Prechill-enhanced Germination  
429 of *Echinacea angustifolia* Seeds. J Amer Soc Hort Sci. 119, 853-858.
- 430 Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrient requirements of suspension cultures  
431 of soybean root cells. Exp Cell Res. 50, 151-158;
- 432 Harbage, J.F., 2001. Micropropagation of *Echinacea angustifolia*, *E. pallida* and *E. purpurea*  
433 from Stem and Seed Explants. HortScience 36, 360-364.
- 434 Hazarika, B.N., 2006. Morpho-physiological disorders in *in vitro* culture of plant. Sci. Hortic.  
435 108, 105–120.
- 436 Kevers, C., Franck, T., Strasser, R.J., Dommès, J., Gaspar, T., 2003. Hyperhydricity of  
437 micropropagated shoot: a typically stress-induced change of physiological state. Plant  
438 Cell Tissue and Organ Cult. 77, 181-191.
- 439 Koroch, A., Juliani, H.R., Kapteyn, J., Simon, J.E., 2002. *In vitro* regeneration of *Echinacea*  
440 *purpurea* from leaf explants. Plant Cell Tissue Organ Cult. 69, 79-83.
- 441 Lakshmanan, P., Danesh, Taji, M., 2001. Production of four commercially cultivated  
442 *Echinacea* species by different methods of *in vitro* regeneration. Journal of Horticultural  
443 Science and Biotechnology 77, 158-163.
- 444 Li, T.S.C., 1998. *Echinacea*: Cultivation and medicinal value. HortTechnology. 8, 122-129.

445 Lloyd, J.U., 1921. A Treatise on *Echinacea*. In: Drug Treatise No. 30. Lloyd Brothers,  
446 Pharmacists, Inc., Cincinnati Ohio, USA.

447 Luczkiewicz, M., Cisowski, W., 2001. Optimization of the second phase of a two phase  
448 growth system for anthocyanin accumulation in callus cultures of *Rudbeckia hirta*. Plant  
449 Cell Tissue Organ Cult. 65, 57-68.

450 Luczkiewicz, M., Zarate, R., Migas, W.D., Migas, P., Verpoorte, R. 2002. Production of  
451 *pulchelin E* in hairy roots, callus and suspension cultures of *Rudbeckia hirta* L. Plant  
452 Sci. 163, 91–100.

453 Macchia, M., Angelini, L.G., Ceccarini, L., 2001. Methods to overcome seed dormancy in  
454 *Echinacea angustifolia* D.C. Scientia Horticulturae 89, 317-324.

455 Martin, K. P., Geevarghese, J., Joseph, D., Madassery, J., 2005. *In vitro* propagation of  
456 *Dendrobium* hybrids using flower stalk node explants. Indian J. Exp. Biol. 43, 280-5.

457 McGregor, R.L., 1968. The Taxonomy of the Genus *Echinacea* (*Compositae*). Univ. Kansas  
458 Sci Bull 48, 113-142;

459 Mensuali Sodi, A., Lucchesini, M., Mitterpergher, L., 1997. Elm tissue culture:  
460 micropropagation of clones resistant to Dutch elm disease. Acta Hort. 457, 235-241.

461 Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with  
462 tobacco tissue cultures. Physiol. Plant. 15: 473-493.

463 Sari, A. O., Morales, M.R., Simon, J. E., 2001. Ethephon can overcome seed dormancy and  
464 improve seed germination in Purple coneflower species *Echinacea angustifolia* and  
465 *Echinacea pallida*. HortTechnology 11, 202-205.

466 Schollhorn, C., Schecklies, E., Wagner, H. 1993. Immunochemical investigations of  
467 polysaccharides from *E. purpurea* cell suspension cultures. Planta Med. 59, A662-A663.

468 Sicha, J., Becker, H., Dusek, J., Hubia, J., Siatka, T., Hrones, I., 1991. Callus cultures of the  
469 genus *Echinacea*. Effect of phenylalanine on the growth of cultures and the production  
470 of cinnamic acids. *Pharmazie* 46, 363-364.

471 Smith, M. A. L., Kobayashi, H., Gawienowski, M., Briskin, D. P., 2002. An *in vitro* approach  
472 to investigate medicinal chemical synthesis by three herbal plants. *Plant Cell Tissue*  
473 *Organ Cult.* 70, 105-111.

474 Steward, F. C., Mapes, M. O., Kent, A., Holsten, R. D., 1964. Growth and development of  
475 cultured plant cells. *Science* 143, 20–27.

476 Tan Nhut, D., Van Le, B., Tanaka, M., Tran Thanh Van, K., 2001. Shoot induction and plant  
477 regeneration from receptacle tissues of *Lilium longiflorum*. *Sci. Hort.* 87, 131-138.

478 Zobayed, S. M., Saxena, P. K., 2003. *In vitro* regeneration of *Echinacea purpurea* L. :  
479 enhancement of somatic embryogenesis by indolbutyric acid and dark pre-incubation. *In*  
480 *vitro* Cell Dev. Biol. 39 , 605-612.

Deleted: ¶  
-----Page Break-----

Deleted: Table 1 ¶  
Home-made database of natural compounds, alkamide (6), flavonoids and caffeic acid derivatives (1-14), isolated and identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).¶  
N°

... [1]

488 **Figure captions**

489

490 **Fig. 1** The *E. angustifolia* flower stalk slices give rise to cell proliferation and shoot  
491 regeneration when cultured on CH basal medium with 0.5 mg L<sup>-1</sup> BA in the light.

Formatted

492  
493 **Fig 2** Distinct type of *E. angustifolia* shoots during the multi-step propagation phases.

Formatted

494 **A** : Initial proliferating shoots (IP shoots); **B**: Axillary proliferating shoots (AP shoots);

Formatted

495  
496 **Fig. 3** *E. angustifolia* shoot regeneration from the leaf central vein. Shoots were cultured on  
497 CH basal medium with 0.5 mg L<sup>-1</sup> BA.

498

499 **Fig. 4** Leaf regenerating shoots (LR shoots) on the CH basal medium with BA (3 mg L<sup>-1</sup>) and  
500 IBA (0.5 mg L<sup>-1</sup>) named CHe.

Formatted

Deleted: .

Formatted

501

502 **Fig. 5** Chemical structures of the selected constituents (1-6) of *E. angustifolia* leaf analysed  
503 samples.

Formatted

Table 1

Home-made database of natural compounds, alkamide (**6**), flavonoids and caffeic acid derivatives (**1-14**), isolated and identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV ( $\lambda$ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)
1	caftaric acid	6.80	210, 240, 294, 329sh	312	[M-H] <sup>-</sup> 311.1	179.1 [M-H-132] <sup>-</sup>	23	135.2 [M-H-132-CO <sub>2</sub> ] <sup>-</sup>	32		
2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	[M-H] <sup>-</sup> 353.1	191.1 [M-H-162] <sup>-</sup>	25				
3	echinacoside	15.1	220, 247sh, 292sh, 330	786	[M-H] <sup>-</sup> 785.3	623.0 [M-H-glc] <sup>-</sup>	30	477.1 [M-H-glc-rha] <sup>-</sup>	32		
4	cichoric acid	18.8	210, 244, 295, 330sh	474	[M-H] <sup>-</sup> 473.1	310.8 [M-H-162] <sup>-</sup>	23	179.0 [M-H-162-132] <sup>-</sup>	23	135.1 (44) [M-H-162-132-CO <sub>2</sub> ] <sup>-</sup>	32
5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	[M-H] <sup>-</sup> 179.1	135.2 [M-H-CO <sub>2</sub> ] <sup>-</sup>	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	M+H] <sup>+</sup> 248.3	149.1 [M+H-99] <sup>-</sup>	33	121.1 [M+H-99-C <sub>2</sub> H <sub>4</sub> ] <sup>-</sup>	30	105.9 (15) [M+H-99-C <sub>2</sub> H <sub>4</sub> -CH <sub>3</sub> ] <sup>-</sup>	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	[M-H] <sup>-</sup> 301.2	179.1 [M-H-122] <sup>-</sup>	40	151.0 [M-H-122-CO] <sup>-</sup>	38		
8	luteolin	30.2	253, 267, 242sh, 291sh, 349	286	[M-H] <sup>-</sup> 285.2	241.2 [M-H-CO <sub>2</sub> ] <sup>-</sup>	48	199.7 [M-H-CO <sub>2</sub> -41] <sup>-</sup>	43		
9	apigenin	33.3	267, 269sh, 336	270	[M-H] <sup>-</sup> 269.4						
10	kaempferol	14.9	253sh, 266, 294sh, 322sh, 367	286	[M-H] <sup>-</sup> 285.3						
11	p-coumaric acid	52.0	223, 286	164	[M-H] <sup>-</sup> 163.2	119.1 [M-H-CO <sub>2</sub> ] <sup>-</sup>	31				

12	betulinic acid	52.0	220, 307	456	[M-H+HC OOH] <sup>-</sup>				
13	apigenin 7 O β glucoside	32.7	286, 333	432	[M-H] <sup>-</sup>	269.3	35		
14	isorhamnetin 3 O rutinoside	18.6	253, 267sh, 306sh, 326sh, 370	624	[M-H] <sup>-</sup>	431.1			
									623.1

**Table 2**

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

PGR	explant	regeneration tendency		callus induction		callus amount*		callus colour	
		dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	-	+++	-	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	-	-	-	-
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

\*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ =

medium developed covering half of the explant, +++ = largely developed covering all the explant

**Table 3**

Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions.

Data are presented as means  $\pm$  SE.

<b>Explants and growth conditions</b>	<b>Subcultures</b>	<b>N°shoots/exp</b>	<b>Length (cm)</b>	<b>Callus amount*</b>	<b>Colour, texture</b>
Flower stalk- Light (0.5 mg L <sup>-1</sup> BA)	I	2.67 $\pm$ 0,33	0.67 $\pm$ 0,20	+++	green, compact
	II	3.00 $\pm$ 0.58	0.73 $\pm$ 0.15	+++	green, compact
Leaf - Dark (0.01 mg L <sup>-1</sup> NAA+ 1 mg L <sup>-1</sup> BA)	I	1.67 $\pm$ 0.33	0.50 $\pm$ 0.01	++	white, friable
	II	0.00	/	++	white, friable

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

**Table 4**

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means  $\pm$  standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>IP shoots:</u> BA (0.25 mgL <sup>-1</sup> )	2,36 ± 0,40	1,31 ± 0,14	+ /+++	friable light green
Active charcoal (5 g/L)	1,10 ± 0,06	1,63 ± 0,25	+	compact green
<u>AP shoots:</u> 0.5 mg L <sup>-1</sup> BA	1,77 ± 0,79	1,66 ± 0,39	+	compact green

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Page Break

**Table 5**

Callus formation and shoot regeneration of *in vitro* *E. angustifolia* leaf portions on CH basal medium with different amounts of growth regulators (CHe and CHe\*) and proliferation of shoots derived from Che medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L<sup>-1</sup> BA.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>CHe</u> (3 mg L <sup>-1</sup> BA + 0.5 mg L <sup>-1</sup> IBA)	3.98 ± 0.69	0.54 ± 0.10	+++	Friable white + purple spots
<u>CHe*</u> (6 mg L <sup>-1</sup> BA + 1 mg L <sup>-1</sup> IBA)	2.72 ± 1.57	0.42 ± 0.08	+++	Friable white
<u>LR shoots</u> (0.5 mg L <sup>-1</sup> BA)	3.60 ± 0.54	2.65 ± 0.36	+	Friable at the shoot base

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

Page Break

**Table 6**

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS: Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

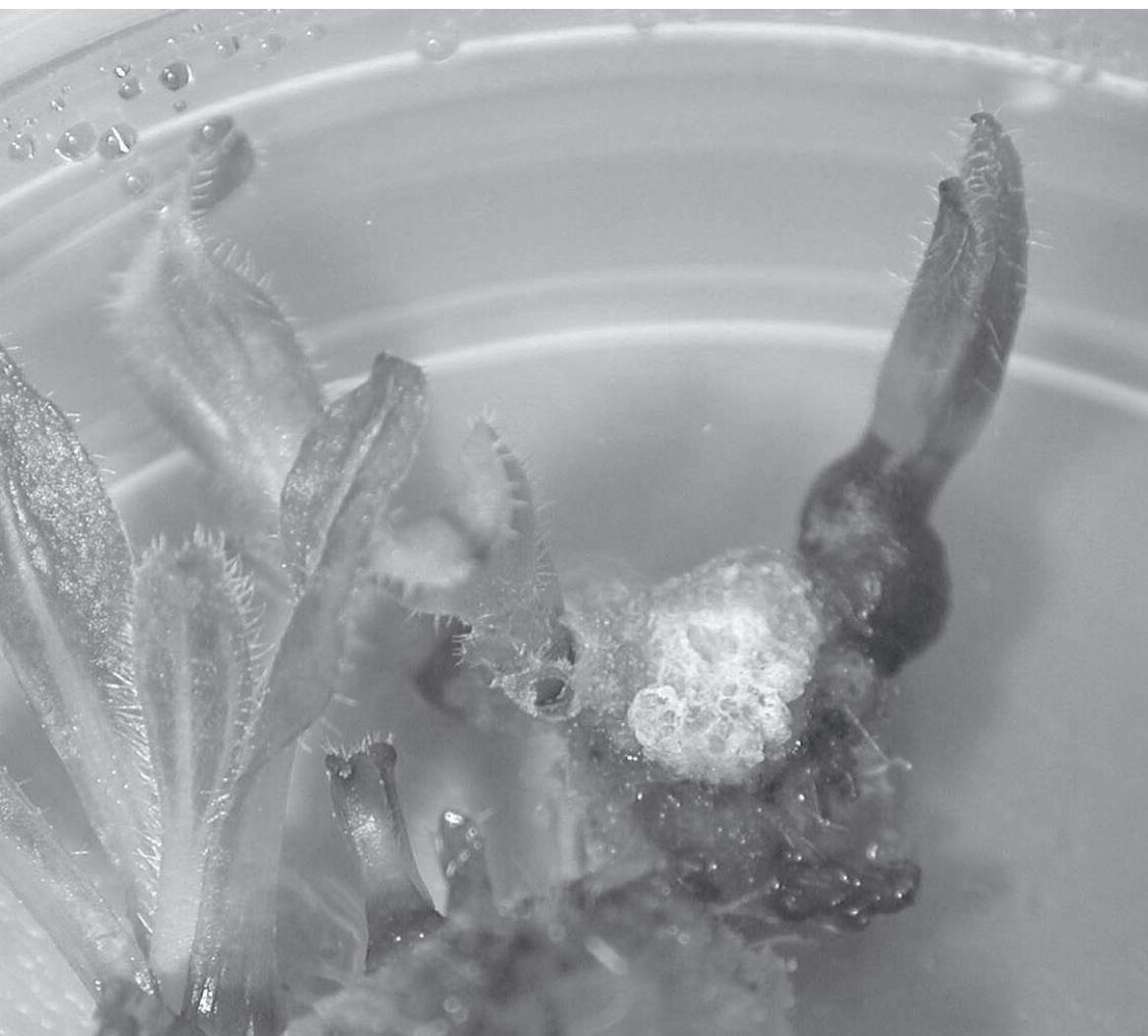
COMPOUNDS ( $\mu\text{g/g}$ dried plant)	<i>In vitro</i> proliferation from flower stalk			seedlings	
	IP shoots	AP shoots	LR shoots	PS shoots	GH plants
caftaric acid (1)	16.54 $\pm$ 0.17	2551.5 $\pm$ 33.6	–	–	4283.7 $\pm$ 24.3
chlorogenic acid (2)	54.61 $\pm$ 2.11	11230.5 $\pm$ 145.5	–	–	1176.4 $\pm$ 6.5
echinacoside (3)	47.34 $\pm$ 0.98	5813.3 $\pm$ 53.7	–	–	5991.1 $\pm$ 46.1
cichoric acid (4)	46.13 $\pm$ 1.23	30530.8 $\pm$ 456.0	–	110.63 $\pm$ 1.32	1534.3 $\pm$ 10.6
caffeic acid (5)	4.11 $\pm$ 0.09	–	16.78 $\pm$ 0.16	–	116.4 $\pm$ 2.45
alkamide (6)	235.63 $\pm$ 9.86	107.35 $\pm$ 3.39	367.95 $\pm$ 10.75	–	26.83 $\pm$ 0.94

**Table 7**

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (4; 29).

<b>Plant material</b>	<b>Echinacoside</b>	<b>Cichoric acid</b>	<b>Alkamides</b>	<b>Flavonoides</b>	<b>Glicoproteines /polisaccarides (µg/mg)</b>	<b>Essential oil (%/fresh plant material)</b>
<b>leaves</b>		0.1	0.001-0.03	0.38		< 0.1
<b>flowers</b>	0.1-1.0	0.15	0.001-0.03			< 0.1
<b>stalks</b>		0.05	0.001-0.03			< 0.1
<b>roots</b>	0.3-1.3	traces	0.009-0.151		220.01	< 0.1

Figure



Figure



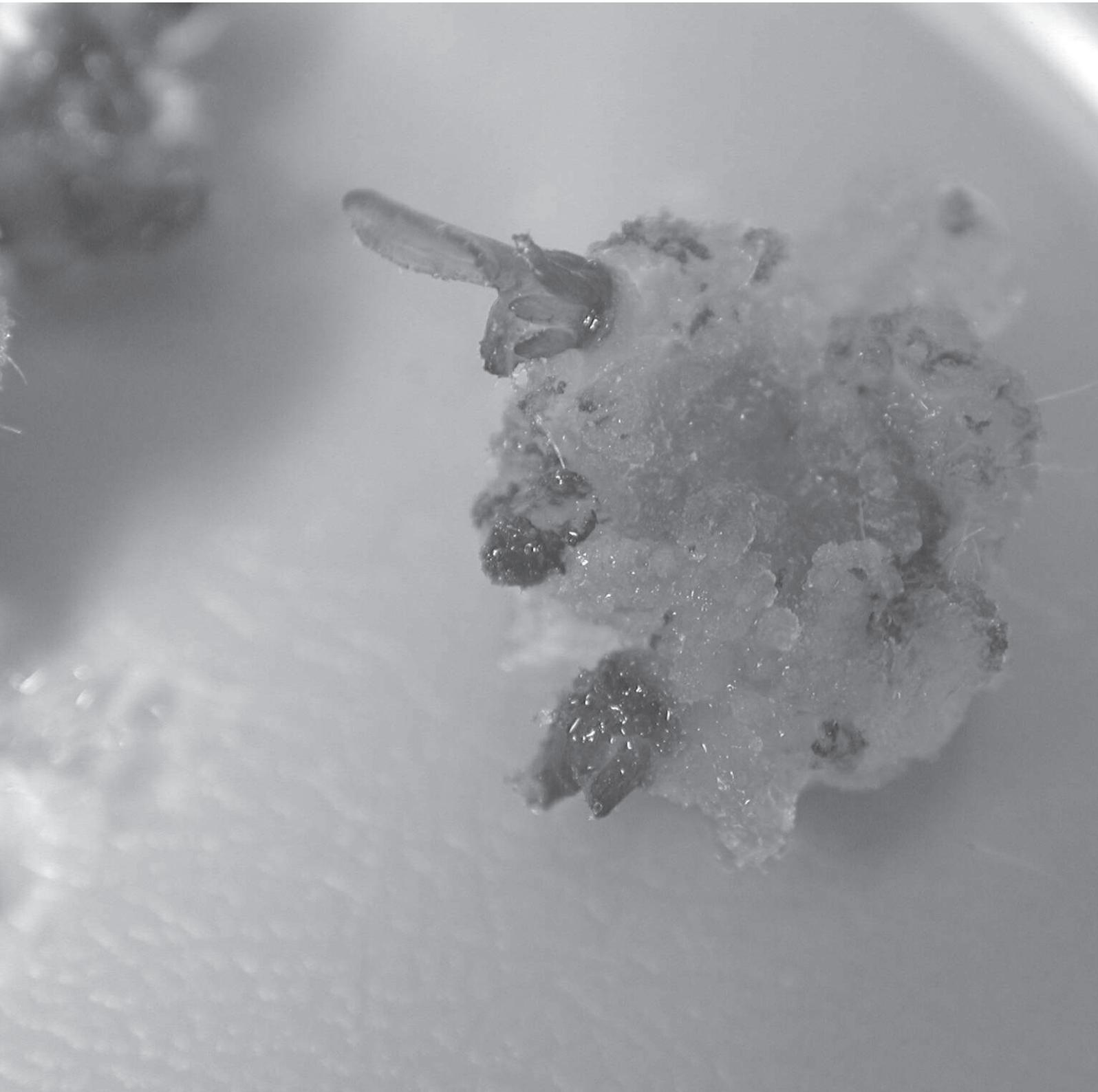
Figure



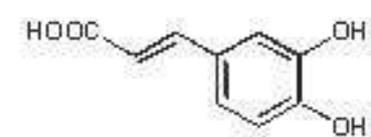
Figure



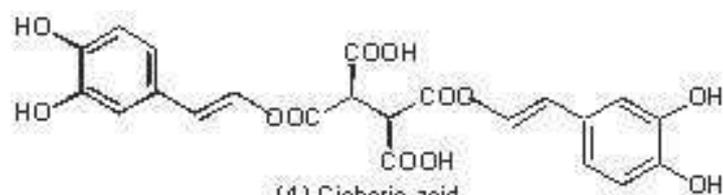
Figure



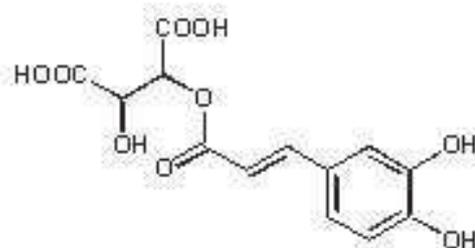
Figure



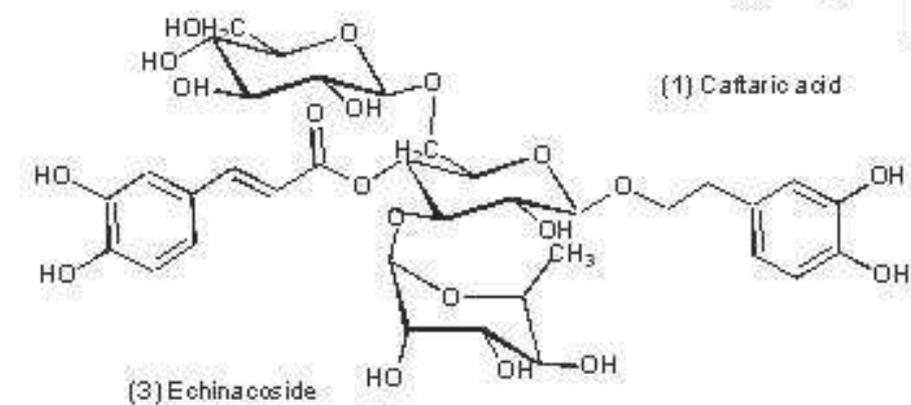
(5) Caffeic acid



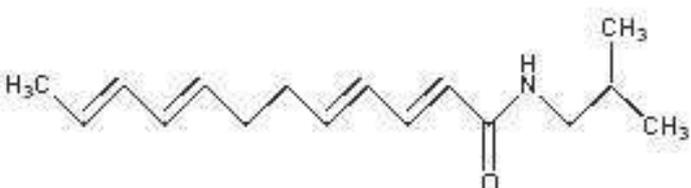
(4) Cichoric acid



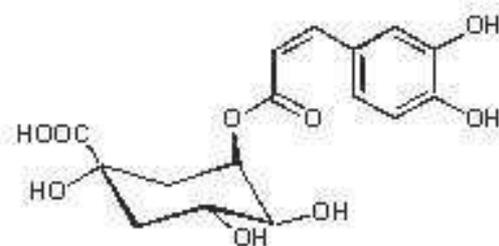
(1) Caffeoyl tartaric acid



(3) Echinacoside



(6) Dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide



(2) Chlorogenic acid

**Table 1**

Home-made database of natural compounds, alkamide (**6**), flavonoids and caffeic acid derivatives (**1-14**), isolated and identified by NMR and MS experiments by a phytochemical investigation of *E. purpurea* plants (*E. purpurea* var. *bravado*) cultivated in Sanremo (Italy).

N°	COMPOUND	Rt (min)	UV ( $\lambda$ nm)	MW	Base peak m/z	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)
1	caftaric acid	6.80	210, 240, 294, 329sh	312	[M-H] <sup>-</sup> 311.1	179.1 [M-H-132] <sup>-</sup>	23	135.2 [M-H-132-CO <sub>2</sub> ] <sup>-</sup>	32		
2	chlorogenic acid	10.2	234sh, 244, 297sh, 328	354	[M-H] <sup>-</sup> 353.1	191.1 [M-H-162] <sup>-</sup>	25				
3	echinacoside	15.1	220, 247sh, 292sh, 330	786	[M-H] <sup>-</sup> 785.3	623.0 [M-H-glc] <sup>-</sup>	30	477.1 [M-H-glc-rha] <sup>-</sup>	32		
4	cichoric acid	18.8	210, 244, 295, 330sh	474	[M-H] <sup>-</sup> 473.1	310.8 [M-H-162] <sup>-</sup>	23	179.0 [M-H-162-132] <sup>-</sup>	23	135.1 (44) [M-H-162-132-CO <sub>2</sub> ] <sup>-</sup>	32
5	caffeic acid	13.4	220, 247sh, 292sh, 330	180	[M-H] <sup>-</sup> 179.1	135.2 [M-H-CO <sub>2</sub> ] <sup>-</sup>	33				
6	dodeca-2E,4E,8Z,10E-tetraenoic acid isobutylamide	45.4	235, 260	247	M+H] <sup>+</sup> 248.3	149.1 [M+H-99] <sup>-</sup>	33	121.1 [M+H-99-C <sub>2</sub> H <sub>4</sub> ] <sup>-</sup>	30	105.9 (15) [M+H-99-C <sub>2</sub> H <sub>4</sub> -CH <sub>3</sub> ] <sup>-</sup>	33
7	quercetin	29.6	255, 267, 301sh, 298sh, 370	302	[M-H] <sup>-</sup> 301.2	179.1 [M-H-122] <sup>-</sup>	40	151.0 [M-H-122-CO] <sup>-</sup>	38		
8	luteolin	30.2	253, 267, 242sh, 291sh, 349	286	[M-H] <sup>-</sup> 285.2	241.2 [M-H-CO <sub>2</sub> ] <sup>-</sup>	48	199.7 [M-H-CO <sub>2</sub> -41] <sup>-</sup>	43		
9	apigenin	33.3	267, 269sh, 336	270	[M-H] <sup>-</sup> 269.4						
10	kaempferol	14.9	253sh, 266, 294sh, 322sh, 367	286	[M-H] <sup>-</sup> 285.3						
11	p-coumaric acid	52.0	223, 286	164	[M-H] <sup>-</sup> 163.2	119.1 [M-H-CO <sub>2</sub> ] <sup>-</sup>	31				
12	betulinic acid	52.0	220, 307	456	[M-H+HC-OOH] <sup>-</sup> 501.2						
13	apigenin 7 O $\beta$ glucoside	32.7	286, 333	432	[M-H] <sup>-</sup> 431.1	269.3 [M-H-glc] <sup>-</sup>	35				
14	isorhamnetin 3 O rutinoside	18.6	253, 267sh, 306sh, 326sh, 370	624	[M-H] <sup>-</sup> 623.1						

**Table 2**

Influence of the plant growth regulators (PGR) and the growth conditions on the regeneration tendency and callus initiation from leaves and flower stalks of *E. angustifolia* adult plants.

PGR	explant	regeneration tendency		callus induction		callus amount*		callus colour	
		dark	light	dark	light	dark	light	dark	light
BA	flower stalk	none	direct from purple spots	none	yes	-	+++	-	green with purple spots, compact
NAA+BA	flower stalk	none	none	none	none	-	-	-	-
BA	leaf	none	none	yes	yes	++	++	necrotic	necrotic
NAA +BA	leaf	direct etiolated	none	yes	yes	++	++	white, friable	necrotic

\*Scale to quantify callus amount; += scarcely developed at the explant margins, ++ = medium

developed covering half of the explant, +++ = largely developed covering all the explant

**Table 3**

Shoot regeneration (number and length of new formed shoots) and callus formation (colour and texture) from flower stalk and leaf explants in different growth conditions. Data are presented as means  $\pm$  SE.

<b>Explants and growth conditions</b>	<b>Subcultures</b>	<b>N°shoots/exp</b>	<b>Length (cm)</b>	<b>Callus amount *</b>	<b>Colour, texture</b>
Flower stalk- Light (0.5 mg L <sup>-1</sup> BA)	I	2.67 $\pm$ 0,33	0.67 $\pm$ 0,20	+++	green, compact
	II	3.00 $\pm$ 0.58	0.73 $\pm$ 0.15	+++	green, compact
Leaf - Dark (0.01 mg L <sup>-1</sup> NAA+ 1 mg L <sup>-1</sup> BA)	I	1.67 $\pm$ 0.33	0.50 $\pm$ 0.01	++	white, friable
	II	0.00	/	++	white, friable

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

**Table 4**

Shoot proliferation (shoot number and length of new formed shoots), and callus development at the basal end of *E. angustifolia* shoots in different phases of the propagation process. Data are presented as means  $\pm$  standard error. AP: Growth Phase shoots; IP: Proliferation Phase shoots.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>IP shoots:</u> BA (0.25 mgL <sup>-1</sup> )	2,36 $\pm$ 0,40	1,31 $\pm$ 0,14	+ / ++	friable light green
Active charcoal (5 g/L)	1,10 $\pm$ 0,06	1,63 $\pm$ 0,25	+	compact green
<u>AP shoots:</u> 0.5 mg L <sup>-1</sup> BA	1,77 $\pm$ 0,79	1,66 $\pm$ 0,39	+	compact green

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

**Table 5**

Callus formation and shoot regeneration of *in vitro* *E. angustifolia* leaf portions on CH basal medium with different amounts of growth regulators (CHe and CHe\*) and proliferation of shoots derived from Che medium (LR shoots). Shoot proliferation was performed on CH basal medium with 0.5 mg L<sup>-1</sup> BA.

	N° shoot/exp	Length (cm)	Callus Amount*	Callus quality
<u>CHe</u> (3 mg L <sup>-1</sup> BA + 0.5 mg L <sup>-1</sup> IBA)	3.98 ± 0.69	0.54 ± 0.10	+++	Friable white + purple spots
<u>CHe*</u> (6 mg L <sup>-1</sup> BA + 1 mg L <sup>-1</sup> IBA)	2.72 ± 1.57	0.42 ± 0.08	+++	Friable white
<u>LR shoots</u> (0.5 mg L <sup>-1</sup> BA)	3.60 ± 0.54	2.65± 0.36	+	Friable at the shoot base

\*Scale to quantify callus amount; += scarcely, ++ = medium, +++ = largely

**Table 6**

LC-DAD-ESI-MS analysis of the aerial part of *E. angustifolia* selected plant material.

IP: initial proliferating shoots; AP: axillary proliferating shoots; LR: Leaf Regenerated shoots; PS:

Proliferating seedlings; GH plants: plants cultivated in greenhouse. Standard error (n = 3)

COMPOUNDS ( $\mu\text{g/g}$ dried plant)	<i>In vitro</i> proliferation from flower stalk			seedlings	
	IP shoots	AP shoots	LR shoots	PS shoots	GH plants
caftaric acid (1)	16.54 $\pm$ 0.17	2551.5 $\pm$ 33.6	–	–	4283.7 $\pm$ 24.3
chlorogenic acid (2)	54.61 $\pm$ 2.11	11230.5 $\pm$ 145.5	–	–	1176.4 $\pm$ 6.5
echinacoside (3)	47.34 $\pm$ 0.98	5813.3 $\pm$ 53.7	–	–	5991.1 $\pm$ 46.1
cichoric acid (4)	46.13 $\pm$ 1.23	30530.8 $\pm$ 456.0	–	110.63 $\pm$ 1.32	1534.3 $\pm$ 10.6
caffeic acid (5)	4.11 $\pm$ 0.09	–	16.78 $\pm$ 0.16	–	116.4 $\pm$ 2.45
alkamide (6)	235.63 $\pm$ 9.86	107.35 $\pm$ 3.39	367.95 $\pm$ 10.75	–	26.83 $\pm$ 0.94

**Table 7**

Data from literature on the main active substance yields (%) in different organs of *Echinacea angustifolia* plants (4; 29).

<b>Plant material</b>	<b>Echinacoside</b>	<b>Cichoric acid</b>	<b>Alkamides</b>	<b>Flavonoides</b>	<b>Glicoproteines /polisaccarides (µg/mg)</b>	<b>Essential oil (%/fresh plant material)</b>
leaves		0.1	0.001-0.03	0.38		< 0.1
flowers	0.1-1.0	0.15	0.001-0.03			< 0.1
stalks		0.05	0.001-0.03			< 0.1
roots	0.3-1.3	traces	0.009-0.151		220.01	< 0.1