Free Radical Scavengers from *Cymbopogon citratus* (DC.) Stapf Plants Cultivated in Bioreactors by the Temporary Immersion (TIS) Principle

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The biomass production of *Cymbopogon citratus* shoots cultivated in bioreactors according to the temporary immersion (TIS) principle was assessed under different growth conditions. The effect of gassing with CO_2-enriched air, reduced immersion frequency, vessel size and culture time on total phenolic and flavonoid content and free radical scavenging effect of the methanolic extracts was measured. From the TIS-culture of *C. citratus*, seven compounds were isolated and identified as caffeic acid (**1**), chlorogenic acid (**2**), neochlorogenic acid (**3**), *p*-hydroxybenzoic acid (**4**), *p*-hydroxybenzoic acid 3-*O*-β-D-glucoside (**5**), glutamic acid (**6**) and luteolin 6-*C*-fucopyranoside (**7**). The occurrence of compounds **1**–**7** and their variability in *C. citratus* grown under different TIS conditions was determined by HPLC. The free radical scavenging effect of the methanolic extract and compounds was measured by the discoloration of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). The main metabolites in 6- and 8-week-old cultures, both in 5 and 10 l vessels, were chlorogenic acid (**2**) (100–113 mg%) and neochlorogenic acid (**3**) (80–119 mg%), while in the cultures with CO_2-enriched air and reduced immersion frequency the main compound detected in the extracts was glutamic acid (**6**) (400 and 670 mg% for the green and white biomass and 619 and 630 mg% for the green and white biomass, respectively). The most active compounds, as free radical scavengers, in the DPPH discoloration assay were caffeic acid (**1**), chlorogenic acid (**2**), neochlorogenic acid (**3**) and the flavonoid luteolin 6-*C*-fucopyranoside (**7**).

Key words: Cymbopogon citratus, TIS Culture, Free Radical Scavengers

Introduction

The aromatic perennial grass *Cymbopogon citratus* (DC.) Stapf (Poaceae) is widespread in tropical and subtropical countries. In the Latin American countries it is widely used as infusion and decoction of the aerial parts (leaves) for medicinal purposes and as a source of the essential oil. In Argentina, the plant is recommended for treating digestive complaints (Hilgert, 2001) while in Cuba it is reputed as an antitussive, to relieve cough, as hypotensive and for the treatment of some gastric disorders. In Costa Rica, the leaf infusion is used as a carminative, expectorant and depurative, however, the most common use in Latin America is for treating digestive problems (Gupta, 1995). Recently, the free radical scavenging and antioxidant effect of *C. citratus* has been reported and the main active constituents were identified by assay-guided isolation (Cheel *et al*., 2005a).

Preliminary assays of methanolic extracts from different cultures of *C. citratus* obtained by the temporary immersion (TIS) principle showed a free radical scavenging effect measured by the discoloration of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). This report describes the occurrence of various active constituents of *C. citratus* and their production and variability under the TIS culture technology as well as the free radical scavenging effect of the whole extracts and single constituents.

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Materials and Methods

Chemicals

Plant growth regulators, nutrient media and salts used for tissue culture of *C. citratus* were obtained from Duchefa Biochemie BV (Haarlem, The Netherlands). The solid medium was Phytagel from Sigma Chemical Co. (USA). All solvents used were of analytical grade. Chloroform from Fisher (USA), ethanol and methanol from J. T. Baker (USA), acetonitrile from Caledon Lab. Ltd. (Canada) and formic acid from Merck (Germany) were used. The standards of caffeic acid and monosaccharides were from Sigma Chemical Co. (USA). TLC analysis was carried out on aluminum-coated silica gel (Sigma-Aldrich) and cellulose F_{254} plates from Merck (Germany). Folin-Ciocalteu phenol reagent, aluminum chloride hexahydrate and sodium carbonate were from Merck (Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was from Sigma Chemical Co. (USA).

Plant material

All samples of *C. citratus* included in this study are derived from *in vitro* shoot cultures. For the establishment of the *in vitro* cultures of *C. citratus*, plants cultivated in the medicinal botanical garden of the Central University of Las Villas, Santa Clara, Cuba were used. These plants are originated in Bayamo City, Cuba. A voucher herbarium specimen has been kept at the Herbarium of the Agricultural Faculty at the University of Granma, Cuba.

General in vitro culture conditions

For the establishment of the *in vitro* cultures, axillary buds as explants from greenhouse plants were surface-sterilized and first cultured in MS medium (Murashige and Skoog, 1962). For explant multiplication, the medium was supplemented with 0.3 mg/l BAP (6-benzylaminopurine) and 30 g/l sucrose. The solid medium was prepared adding 2.5 g/l Phytagel (Sigma Co.). The pH value was adjusted to 5.7 before sterilization by autoclaving. The culture temperature was 26 °C, under a light regime of 16 h at 50 μmol/(s m^2) provided by daylight fluorescent lamps (Philips TLT 40W/54 R. S.).

Standard conditions for C. citratus biomass production in temporary immersion systems (TIS)

Biomass production was performed using a bioreactor system working according to the TIS principle (Etienne and Berthouly, 2002). This culture system allows optimization of gas and nutrients exchange. The device consisted of several 5 l glass bottles as cultivation vessels equipped with culture rafts. Under standard conditions the system was run with 2.5 l medium that was changed every 2 weeks and plants were immersed every 4 h for 5 min. The inoculum amount was 20 g/vessel and the culture temperature and the light intensity were as given above.

For experiments on variation of the cultivation conditions *in vitro*, culture vessels of different size, a two stage culture model as well as longer culture periods were tested. The parameters were varied as given in Table I.

Another focus was on the influence of gassing with CO_2-enriched air on growth and metabolite content of shoot cultures of *C. citratus* cultivated in bioreactors according to the TIS principle. The gassing air was enriched with CO_2 by passing a washing bottle with a solution of 60.8 g/l K_2CO_3 and 156.2 g/l $KHCO_3$. A control bioreactor was operated in the same time under the same conditions but gassed with normal air.

Additionally, the influence of reduced immersion frequency on hyperhydricity, growth and metabolite content of shoot cultures of *C. citratus* cultivated in bioreactors was investigated. The plants were immersed and gassed 2 times a day for 5 min and additionally 2 times a day for 5 min. A control bioreactor was operated in the same time under standard conditions as described above. The harvested biomass was classified visually. If differences could be observed it was divided into a green and a white to light green part. All investigated samples including the varied parameters are summarized in Table I.

After harvesting, plant samples were dried in an air circulation oven at 40 °C. The air-dried, powdered plant material (1 g) was extracted under reflux using methanol (3×25 ml). The antioxidant profile as well as the flavonoid and phenolic content of the extracts was determined in the selected assays.

Determination of total phenolic and flavonoid content

The total phenolic content of the extracts was determined using the method described by Singleton *et al.* (1999). Briefly, the appropriate extract dilutions were oxidized with the Folin-Ciocalteu reagent and the mixture was neutralized with so-

Table I. Growth conditions for *C. citratus* shoots cultivated in bioreactors according to the temporary immersion (TIS) principle.

Dry weight [g]	Remarks (including variations from standard culture conditions)
409.35	Pool of samples produced in TIS under different conditions
86	Culture in 5 l vessels for 6 weeks without exchange of the culture medium, 2.5 l culture medium
48	Culture in 5 l vessels for 8 weeks without exchange of the culture medium, 2.5 l culture medium
118	Culture in 10 l vessels for 6 weeks (4 weeks cultivation on the standard medium, last 2 weeks cultivation on fresh prepared standard medium), 2.5 l culture medium
92	Culture in 10 l vessels for 6 weeks (4 weeks cultivation on the standard medium, last 2 weeks cultivation on a medium containing only micro- and macroelements according to Murashige and Skoog), 2.5 l culture medium
95	Standard culture conditions (see Materials and Methods)
	Influence of gassing with CO_2-enriched air
74	Gassing with normal air, white part of the biomass (white and green samples are from the same vessel)
53	Gassing with normal air, green part of the biomass (green and white samples are from the same vessel)
80.1	Gassing with CO_2-enriched air, white part of the biomass (white and green samples are from the same vessel)
68.2	Gassing with CO_2-enriched air, green part of the biomass (green and white samples are from the same vessel)
	Influence of a reduced immersion frequency
46	6 Immersions and gassings a day, green biomass, no white part
48	2 Immersions and 4 gassings a day, green part of the biomass (samples 1231 and 1232 are from the same vessel)
4	2 Immersions and 4 gassings a day, white part of the biomass (samples 1231 and 1232 are from the same vessel)

dium carbonate. The absorbance of the resulting blue color was measured at 700 nm after 30 min using a Heλios α V-3.06 UV-VIS spectrophotometer. Quantification was done on the basis of a standard curve of gallic acid. Results were expressed as gallic acid equivalent (%). Data are reported as mean ± standard deviation (s.d.) for at least three replicates.

The total flavonoid content in the samples was determined by the methodology of Chang *et al.* (2002). Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as quercetin equivalent (%). Data are reported as mean ± s.d. for at least three replicates.

Extraction and isolation of the active compounds

The air-dried plant material (140 g) from a pool of samples obtained by the TIS system was successively extracted with petroleum ether (PE, 3 × 1.5 l) and methanol (MeOH, 3 × 1.5 l) to afford 1.2 g of PE- and 54.93 g of MeOH-soluble extracts (w/w yield: 0.85 and 39.24%, respectively). Some 47 g of the MeOH extract were resuspended in water (1 l) and successively partitioned with ethyl ether (Et_2O, 3 × 0.5 l) and ethyl acetate (EtOAc, 3 × 0.5 l) yielding 2.63 g of Et_2O- (1.87%), 1.59 g of EtOAc- (0.75%) and 45.20 g of water-soluble compounds (Aq) (32.28%), respectively.

Some 700 mg of the EtOAc extract were resuspended in MeOH, applied to a Sephadex LH-20 column (40 × 2 cm) and permeated with MeOH to obtain 30 fractions of 18 ml each. After comparison by TLC (silica gel, EtOAc/AcOH/H_2O, 10:2:3, v/v/v, detection by UV light) and after application of diphenylboric acid ethanolamine complex reagent fractions with similar patterns were pooled into 5 groups: I (1–3); II (4–8); III (9–10); IV (11–16); V (17–30).

The fraction groups I–III not containing compounds of interest were discarded. Fraction IV (115 mg) afforded after preparative HPLC [column: Lichrospher RP-18, 250 mm × 25 mm, 7 μm; isocratic solvent system: MeOH/H_2O (0.1% formic acid), 8:2, pH 2.5; flow rate: 2.5 ml/min; detection: UV, 280 nm] 15 mg of compound **5** (R_t = 55 min). Fraction V (98 mg) afforded after preparative HPLC [column: Lichrospher RP-18, 250 mm × 25 mm, 7 μm; solvent system: MeOH/H_2O (0.1% formic acid), 8:2, pH 2.5; flow rate:

5 ml/min; detection: UV, 280 nm] 6 mg *p*-hydroxybenzoic acid (compound **4**, R_t = 22 min).

The water-soluble extract (6.29 g) was resuspended in MeOH and permeated on a Sephadex LH 20 column (33 × 5.5 cm i. d.; equilibrated with MeOH). Some 30 fractions of 200 ml each were obtained. After TLC comparison, fractions were pooled together as follows: I (1–5); II (6–8); III (9–11); IV (12–14); V (15–17); VI (18–19); VII (20–30). The fraction pools I–IV were not further investigated.

Fraction V (250 mg) yielded after permeation on a Sephadex LH 20 column (40 × 2 cm i. d.; equilibrated with MeOH) some 50 fractions of 10 ml each. After TLC comparison, fractions 23–25 afforded 25 mg compound **2** and fractions 35–40 yielded 22 mg compound **3**. Successively gel permeation of fraction VI on a Sephadex LH 20 column (40 × 2 cm) with MeOH afforded 4 mg caffeic acid (**1**).

Fraction VII (2 g) was submitted to high-speed preparative countercurrent chromatography (HSCCC) using a P. C. Inc. (USA) instrument equipped with a multilayer coil of 1.68 mm i. d. polytetrafluoroethylene (PTFE) tubing of approx. 80 ml and 240 ml with a total capacity of 320 ml. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 10.5 cm, and the β value was 0.76 ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The speed varied between $0 \rightarrow 1200$ rpm. The flow rate was controlled with a DC analytic gearmotor (Bodine Electric Company). The sample was injected with a P. C. Inc. injection module with a 10 ml sample injection loop. The solvent system used was $CHCl_3$/MeOH/EtOH/H_2O/AcOH (5:3:3:4:0.010, v/v/v/v). It was thoroughly equilibrated overnight in a separatory funnel at room temperature and the two phases were separated and degassed by sonication shortly before use. The solvent system provided an ideal range of the partition coefficient (K) for the applied sample and a desirable settling time (15 s). 57% of the stationary phase were retained in the coil. First, the coiled column was entirely filled with the stationary phase (upper phase). Then, the apparatus was rotated forward at 800 rpm, while the mobile phase (lower phase) was pumped into the column in a tail to head (T → H) direction at a flow rate of 1.7 ml/min. After the mobile phase front emerged and the hydrodynamic equilibrium was established in the column, about 10 ml of filtered sample solution (1 g of fraction VII, dissolved in 5 ml of upper phase and 5 ml of lower phase) were injected through the injection module. The effluent of the column was continuously monitored by TLC (silica gel, mobile phase EtOAc/AcOH/H_2O 10:2:3, v/v/v). The spots were observed under ultraviolet light (254 nm). Some 180 fractions of 8 ml each were collected and pooled together according to the TLC pattern. From fractions 98–107, 16 mg of compound **7** were obtained while fractions 124–130 afforded 40 mg glutamic acid (**6**).

HPLC analysis

The determination of flavonoids in the samples was performed according to Sánchez-Rabaneda *et al.* (2003) with some modifications. HPLC analysis was performed using a HPLC-DAD Merck-Hitachi (LaChrom, USA) equipment consisting of a L-7100 pump, a L-7455 UV diode array detector and D-7000 chromato-integrator. A C18-RP column (Phenomenex, 5 μm, 250 mm × 4.60 mm i. d.) was used. The compounds were monitored at 256 nm and the absorbance was measured between 200 and 400 nm. Gradient elution was carried out with water/0.1% formic acid (solvent A) and 20% solvent A in 80% acetonitrile (solvent B) at a constant flow rate of 1 ml/min. A linear gradient was used (Sánchez-Rabaneda *et al.*, 2003). Under our experimental conditions, R_t of the isolated compounds was as follows: compound **1**, 41.20 min; compound **2**, 35.50 min; compound **3**, 36.00 min; compound **4**, 26.64 min; compound **5**, 43.01 min; compound **6**, 38.37 min; compound **7**, 44.53 min. Calibration curves were performed to estimate the main active compounds content in the samples. The correlation between concentration/peak area was assessed by the ordinary least square regression model. The correlation coefficient was r^2 = 0.9998. The amount of the active principles was expressed as g per 100 g of dry material.

DPPH discoloration assay

The free radical scavenging effect of the extracts and compounds was assessed by the discoloration of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical as previously reported (Schmeda-Hirschmann *et al.*, 2003). The scavenging of free radicals by extracts and compounds was evaluated spectrophotometrically at 517 nm against the absorbance of the DPPH radical.

The percentage of discoloration was calculated as follows:

$$\text{percentage of discoloration} = \frac{1 - (\text{absorbance of compound/extract})}{\text{absorbance of blank}} \times 100.$$

The degree of discoloration indicates the free radical scavenging efficiency of the substances. Quercetin was used as a free radical scavenger reference. The free radical scavenging effect of crude extracts and compounds is reported as IC_{50} values.

Statistical analysis

All determinations were conducted in triplicate and all results were calculated as mean ± s.d. To determine whether there was any difference between activity or phenolic content of samples, variance analysis using the Anova test was applied. Values of $p < 0.05$ were considered as significantly different. The differences among means were determined using Tukey's multiple comparison test. To assess the relationship between the activities and the phenolic(s) content, Pearson's correlation coefficients were calculated with 95% confidence. The statistical package STATGRAPHICS PLUS for Windows version 2000 was used to analyze the data.

Structural identification of the compounds

Nuclear magnetic resonance (NMR) spectra were obtained using a Bruker spectrometer, operating at 400 MHz for ^1H and 100 MHz for ^{13}C NMR. Dimethyl sulfoxide (DMSO-d_6) and methanol (CD_3OD) were used as solvents. Chemical shifts are presented in ppm and coupling constants (*J*) in Hz. The UV spectra were obtained using a Heλios α V-3.06 UV-VIS spectrophotometer. MeOH was used as solvent. HPLC-DAD was used for identification and quantification of the main compounds in the crude extracts, fractions and for determining purity criteria of the isolated compounds before NMR measurements and assays.

Results and Discussion

TIS culture

After the establishment of shoot cultures (static cultures), plant material was maintained in the liquid temporary culture system. Shoots grown in microcontainers were used as inoculum for the cultivation in bioreactors. The first step was the optimization of the cultivation conditions for a high biomass productivity. Samples produced under different culture conditions were taken for an analytical investigation of secondary metabolites production. As demonstrated before, the biomass productivity in the TIS system was three times higher than in microcontainers (Wilken *et al.*, 2005).

When the effect of culture time and vessel volume was compared, higher extraction yield was found for the six weeks cultures in the 5 l vessels (26.88%), the yield decreased in the 8 weeks culture (16.90%) with similar activity as free radical scavengers measured by the DPPH bleaching assay and with similar chemical composition. The extraction yield for the 6 weeks and 8 weeks cultures in 10 l vessels was the same but the free radical scavenging activity was lower in the older cultures as well as the total phenolic content. The percent extraction yields for the six samples investigated (including the standard culture and the culture mixture) ranged between 16.90 and 26.88%. Large variations in total phenolic and flavonoid contents were observed, in the range 0.35–1.46 and 0.13–0.75%, respectively. The main metabolites in the 6 and 8 weeks cultures both in 5 and 10 l vessels were chlorogenic acid (**2**) (100–113 mg%) and neochlorogenic acid (**3**) (80–119 mg%) while in the cultures with CO_2-enriched air and reduced immersion frequency the main compound detected in the extracts was glutamic acid (**6**) (400 and 670 mg% for the green and white biomass and 619 and 630 mg% for the green and white biomass, respectively) (see Tables II and III).

Influence of gassing with CO_2-enriched air

Applying gassing with normal air, a growth from 20 g to 1225 g fresh weight (factor 61.3) compared to a growth from 20 g to 904 g fresh weight (factor 45.2) applying gassing with CO_2-enriched air was obtained within 52 days. In this case the gassing with CO_2-enriched air revealed no increase in biomass production but a slight decrease was observed. However gassing with CO_2-enriched air seems to have nearly no influence on the amount of harvested green biomass (444 g compared to 490 g fresh weight by gassing with normal air). For the white biomass a higher decrease was observed applying gassing with CO_2-enriched air (460 g compared to 735 g fresh weight by gassing with normal air).

Sample remarks	% MeOH extract	% Total phenolics	% Total flavonoids	DPPH IC_{50} [μg/ml]
Vessel volume and culture time				
5 l, 6 weeks	26.88	0.97 ± 0.02	0.17 ± 0.01	67 ± 3
5 l, 8 weeks	16.90	0.61 ± 0.02	0.13 ± 0.02	71 ± 2
10 l, 6 weeks	23.10	0.72 ± 0.02	0.17 ± 0.02	98 ± 2
10 l, 6 weeks	23.30	0.35 ± 0.02	0.16 ± 0.01	137 ± 4
Standard culture	24.56	0.97 ± 0.03	0.29 ± 0.01	81 ± 3
Culture mixture, 2001	19.80	1.46 ± 0.01	0.75 ± 0.02	58 ± 2
CO_2-enriched				
Standard culture, white biomass	21.64	0.52 ± 0.01	0.20 ± 0.02	50 ± 3
Standard culture, green biomass	30.12	0.82 ± 0.02	0.34 ± 0.01	94 ± 4
CO_2-enriched, white biomass	27.44	0.37 ± 0.01	0.13 ± 0.02	78 ± 3
CO_2-enriched, green biomass	15.76	0.40 ± 0.01	0.19 ± 0.01	90 ± 2
Reduced immersion frequency				
Standard culture, green biomass	27.96	0.54 ± 0.02	0.27 ± 0.01	87 ± 3
Reduced immersion, green biomass	23.60	0.85 ± 0.02	0.51 ± 0.02	70 ± 2
Reduced immersion, white biomass	28.64	1.08 ± 0.02	0.42 ± 0.03	68 ± 2

Table II. Percentual extraction yield (methanolic extract), total phenolic and flavonoid content (g/100 g dry starting material) and free radical scavenging activity of *C. citratus* shoots cultivated in bioreactors according to the temporary immersion (TIS) system. The data are presented as mean values ± s.d. of three determinations.

Table III. Content of compounds **1**–**7** in *C. citratus in vitro* cultures. The data (in mg%) are presented as mean values ± s.d.

Sample	Compound						
	1	**2**	**3**	**4**	**5**	**6**	**7**
5 l, 6 weeks	10.2 ± 0.6	113 ± 3	94 ± 2	19 ± 1	15.2 ± 0.3	ND	10.2 ± 0.6
5 l, 8 weeks	10.3 ± 0.6	100 ± 2	119 ± 2	40 ± 2	22 ± 1	ND	26 ± 2
10 l, 6 weeks	8.4 ± 0.2	112 ± 4	104 ± 6	30 ± 2	18 ± 0.4	ND	16 ± 1
10 l, 6 weeks	16.5 ± 0.3	100 ± 2	80 ± 2	29 ± 1	22 ± 0.4	ND	18.3 ± 0.5
Standard culture	7.2 ± 0.2	104 ± 3	68 ± 4	22 ± 2	13 ± 1	626 ± 10	13.6 ± 0.8
Culture mixture, 2001	15.3 ± 1.2	136 ± 2	55 ± 2	32 ± 1	9 ± 0.6	670 ± 50	25 ± 2
Standard culture, white biomass	9.1 ± 0.3	101 ± 2	65 ± 4	34 ± 2	11 ± 2	310 ± 20	10.3 ± 0.2
Standard culture, green biomass	12.4 ± 0.4	126 ± 1	66 ± 2	99 ± 3	15 ± 2	680 ± 20	15 ± 2
CO_2-enriched culture, white biomass	8.2 ± 0.2	109 ± 2	104 ± 2	77 ± 2	20 ± 0.4	670 ± 15	16.4 ± 0.6
CO_2-enriched culture, green biomass	5.5 ± 0.1	66 ± 3	47 ± 2	60 ± 2	7 ± 0.5	400 ± 10	7.1 ± 0.2
Standard culture, green biomass	8.0 ± 0.1	62 ± 2	84 ± 3	46 ± 2	8 ± 0.2	730 ± 10	16 ± 2
Reduced immersion, green biomass	9.3 ± 0.1	54 ± 1	106 ± 2	32 ± 2	16 ± 1	619 ± 12	19.4 ± 0.5
Reduced immersion, white biomass	10.2 ± 0.3	194 ± 2	54 ± 5	66 ± 2	11 ± 1	630 ± 5	20.2 ± 0.4

ND, not detected.

On a dry weight basis, large variation in extract yields for the green (15.76–30.12%) and the white (21.64–27.44%) biomass of the standard and CO_2-enriched culture was observed. Highest MeOH-soluble content was found for the green biomass of the standard culture (30.12%) as well as for the white biomass of the CO_2-enriched sample (27.44%).

Highest total phenolic and flavonoid content was found in the green biomass of the standard culture sample (0.82 and 0.34%, respectively). Highest free radical scavenging effect was found in the extractives of the white biomass rather than in the green plant part (see Table II). The main compound in all samples was metabolite **6**, with values ranging between 400 to 680 mg% for the green and 310 to 670 mg% for the white/colorless samples, respectively (Table III).

Reduced immersion frequency

Reducing the immersion frequency a growth from 17.9 g to 473 g fresh weight (factor 26.42) compared to a growth from 18.1 g to 273 g fresh weight under standard cultivation conditions was observed within 55 days. In both cases, nearly no white biomass was found by visual classification. Besides this increase in biomass production, the

reduction of the immersion frequency revealed a significant increase of the quality of the biomass *i.e.* while under standard conditions the plantlets showed hyperhydricity; this effect was completely avoided by reduction of the immersion frequency. Hyperhydricity is an often observed problem of *in vitro* plant propagation, considered a physiological disorder, related to malfunctioning stomata control, reduction in pigmentation and/or alteration during cell wall formation in epidermal cells (Kevers *et al.*, 2004) which reasons are not exactly known up today. The affected shoots turn vitreous, fragile and atrophied and plantlets are unable to adapt under non-sterile greenhouse conditions. *C. citratus* plantlets additionally showed leafs with turned petioles and a reduced leaf area. On a dry weight basis, higher total phenolic and flavonoid content was found in the reduced immersion samples.

Reduction of immersion frequency led to extraction yields of 23.60 and 28.64% for the green and white biomass, respectively, with similar effects as for free radical scavengers. Highest total phenolic content was observed for the white biomass (1.08%) but the higher flavonoid content was found in one of the green biomass samples (0.51%). The DPPH activity was similar for the green and white biomass of the TIR-reduced immersion samples. The main compound in the cultures was glutamic acid (**6**), with contents between 619 and 630 mg%. Highest content of **2** for all the samples was found in the white biomass of the reduced immersion samples (Tables II and III).

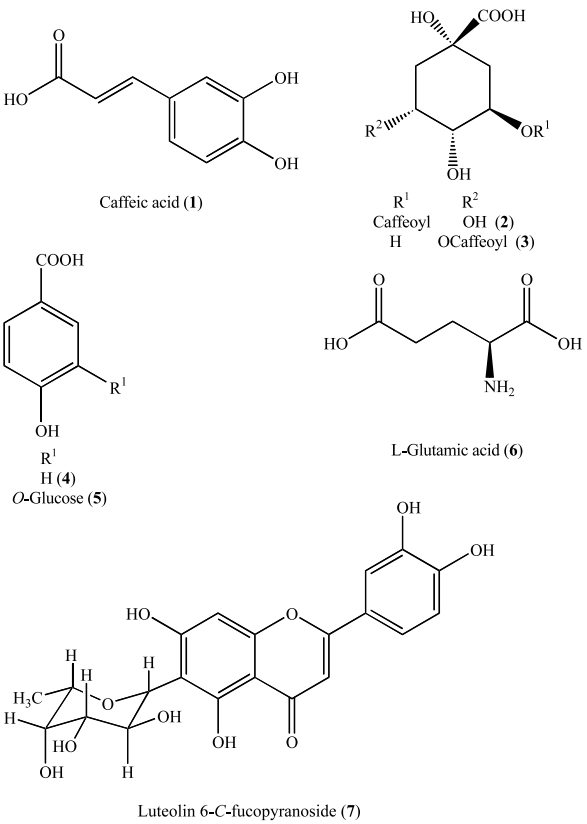

Fig. 1. Structures of compounds caffeic acid (**1**), chlorogenic acid (**2**), neochlorogenic acid (**3**), *p*-hydroxybenzoic acid (**4**), *p*-hydroxybenzoic acid 3-*O*-D-glucoside (**5**), glutamic acid (**6**) and luteolin 6-*C*-fucopyranoside (**7**) isolated from TIS-cultured *Cymbopogon citratus.*

Phenolic and flavonoid content, free radical scavenging effect and isolated compounds

From the TIS-cultured *C. citratus* plants, seven compounds were isolated and identified as caffeic acid (**1**) (Lim *et al.*, 2003), the chlorogenic acid isomers **2** and **3** (Wang *et al.*, 1999; Sefkow *et al.*, 2001), *p*-hydroxybenzoic acid (**4**), *p*-hydroxybenzoic acid 3-*O*-β-D-glucoside (**5**) (Klick and Herrmann, 1988), glutamic acid (**6**) and the flavonoid luteolin 6-*C*-fucopyranoside (**7**) (Mareck *et al.*, 1991; Snook *et al.*, 1995). The structure of the isolated compounds is presented in the Fig. 1. The spectroscopic data of the isolated compounds are in agreement with the literature.

The percentage variation of the total phenolic and flavonoid content of the different *C. citratus* samples as well as the free radical scavenging effect determined by the discoloration of the DPPH radical is summarized in Table II. Total phenolic content of the *in vitro* samples ranged from 0.35 to 1.08%, total flavonoid content from 0.13 to 0.51% (Table II).

The IC_{50} values of the crude methanolic extracts in the DPPH assay ranged between 50 and 137 μg/ml (Table II). The free radical scavenging effect of the compounds **1**–**7** (IC_{50} values in μg/ml) is as follows: caffeic acid (**1**), 2; chlorogenic acid (**2**), 16; neochlorogenic acid (**3**), 17; *p*-hydroxybenzoic acid (**4**), 122; *p*-hydroxybenzoic acid 3-*O*-β-D-glucoside (**5**), 196; glutamic acid (**6**), 200; luteolin 6-*C*-fucopyranoside (**7**), 20.

The content of compounds **1**–**7** in the *in vitro* cultures of *C. citratus* is presented in Table III. The main free radical scavenger found in the samples was chlorogenic acid (**3**). From the methanolic ex-

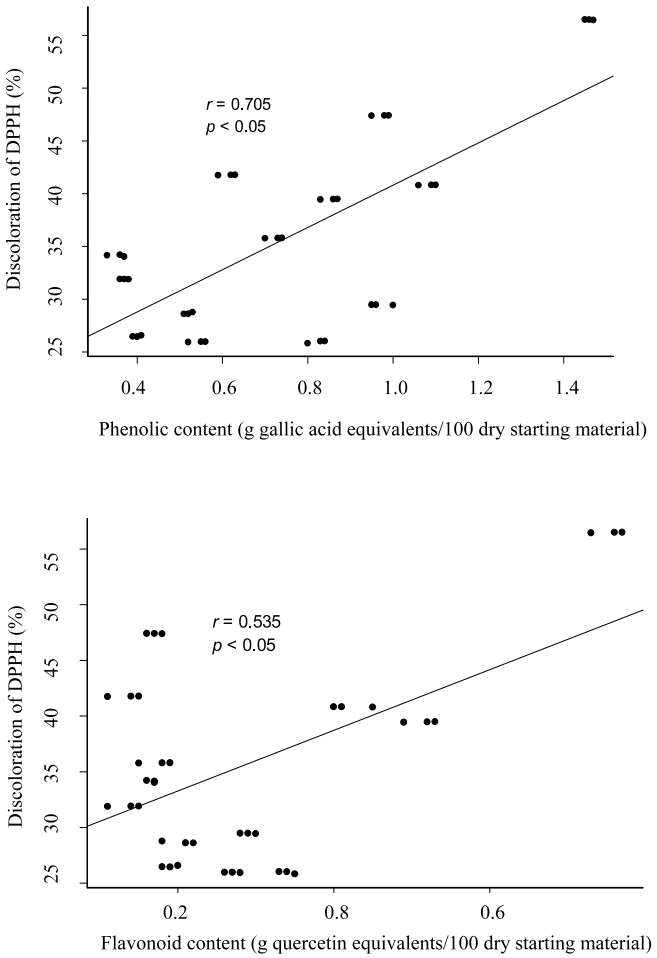

Fig. 2. Correlation between DPPH activity of *Cymbopogon citratus* samples and percentage phenolic and flavonoid content.

tract of *C. citratus* cultivated in Chile, the *C*-glycosylflavonoids: isoorientin, isoscoparin, swertiajaponin, isoorientin 2″-*O*-rhamnoside, orientin; as well as chlorogenic acid and caffeic acid were recently reported (Cheel *et al*., 2005a). Flavones, flavonoids *C*- and *O*-glycosides were reported by De Matouschek and Stahl-Biskup (1991) from a Peruvian sample of *C. citratus*; the plant growing in Chile contained only *C*-glycosylflavonoids in detectable amounts. No information was found on secondary metabolite production in *in vitro* cultures of *C. citratus*. The most active compounds from the samples investigated were caffeic acid (**1**), chlorogenic acid (**2**), and the flavonoid luteolin 6-*C*-fucopyranoside (**7**).

Flavonoid *C*-glycosides related to the luteolin *C*-glycoside obtained from *C. citratus* cultures have been isolated as constituents of fern and Poaceae species. The 6,8-di-*C*-rhamnoside of luteolin has been reported as constituent of the fern *Asplenium viviparum* (Imperato, 1992), while the same author (Imperato, 1994) isolated luteolin 8-*C*-rhamnoside-7-*O*-rhamnoside from the Pteridaceae *Pteris cretica*. The luteolin *C*-glycoside maysin was reported as an antifeedant compound toxic to the corn earworm *Heliothis zea* by Elliger *et al*. (1980). An HPLC methodology for the detection of this compound in corn silk was published by Snook *et al*. (1989). The C-4″-hydroxy derivatives of maysin and 3′-methoxymaysin from corn silks (*Zea mays*) were reported by Snook *et al*. (1995).

According to Liu *et al*. (2004), a glutamic acid derivative presented antioxidant and free radical scavenging activity with an IC_{50} value of 48 μM in the DPPH assay. Caffeic acid has been reported as a strong antioxidant (Lim *et al*., 2003) and chlo-

rogenic acid and its isomers are long known for their free radical/antioxidant effect (Niggeweg *et al.*, 2004). Olthof *et al.* (2001) have shown that chlorogenic and caffeic acid are effectively absorbed in the intestinal tract and can be detected in blood.

In our study with crude extracts a high and positive correlation between phenolic content and DPPH activity ($r = 0.705$, $p < 0.05$) was observed (Fig. 2). That association presented a determination coefficient of $R^2 = 0.497$ which suggested that 49.7% of the free radical scavenging activity of the studied extracts resulted from the contribution of the total phenolic content. Also, it can be concluded that antioxidant activity of the studied extracts is not limited to phenolics. A study performed with *Ocimum* accessions (Javanmardi *et al.*, 2003) showed that the antioxidant activity may also come from the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids, and vitamins, among others, which in our study contributed to 50.3% of the antioxidant capacity. In fact in our study, the aminoacid L-glutamic acid, which was isolated from *C. citratus* cultures, showed a weak antioxidant activity. Other studies carried out with a wild strawberry (Cheel *et al.*, 2005b) and *Smallanthus sonchifolius* root (Yan *et al.*, 1999) showed the relevant antioxidant activity of non-phenolic metabolites as L-tryptophan.

In relation to flavonoids, a weak and positive correlation was observed between total flavonoid content and DPPH activity ($r = 0.535$, $p < 0.05$) (Fig. 2) showing a determination coefficient of $R^2 = 0.286$. This result suggested that 28.6% of the free radical scavenging activity of the studied extracts resulted from the contribution of flavonoids. In a recent study carried out with the aerial parts of *C. citratus* from Chile (Cheel *et al.*, 2005a), some 76% of the free radical scavenging activity of the extracts resulted from the contribution of flavonoids. In the same study the five active flavonoids isolated would be the main contributors to the free radical scavenging activity of the plant which could be the result of synergies as explained by García-Alonso *et al.* (2004). In contrast, in our study the low contribution of the flavonoid content (28.6%) to the free radical scavenging activity in *Cymbopogon* cultures could be explained by the contribution of a single main flavonoid.

The micropropagation of *Cymbopogon* species has been reported by several authors. In a number of aromatic species belonging to the genus *Cymbopogon* (*i.e. C. flexuosus*, *C. nardus*, *C. citratus*, *C. winterianus*, *C. martini* and *C. jwarancusa*), callus cultures are easily established from a series of explants, including seeds, seedlings, culms, roots, inflorescences and rhizome explants (Jagadish Chandra and Sreenath, 1982). The present TIS culture conditions for *C. citratus* mainly favoured constant growth of the plantlets with rapid biomass increase. Despite rapid cell proliferation, TIS standard conditions favoured synthesis of some secondary metabolites as samples of *in vitro* plantlets presented a high percentage of total phenolic and flavonoid contents. Green-colored plantlets always showed a higher content of phenolics and flavonoids compared with the colorless sample. In the CO_2-enriched culture, however, the difference in total phenolics was not significant but total flavonoid content was higher in the green biomass. In the TIS-reduced immersion samples, however, total phenolic content was higher in the white biomass extract (Table II).

As described in the literature for *Thymus vulgaris*, a higher CO_2 level in the atmosphere during a culture period may result in a rise of the concentration of secondary metabolites (Tisserat and Vaughn, 2001). The carbon/nutrient balance (CNB)-model summarizes possible effects of a higher CO_2 level on the plant metabolism (Bryant *et al.*, 1983). According to this model the concentration of carbon-containing metabolites correlates positively and the concentration of nitrogen-containing compounds negatively with a higher C/N ratio. A higher atmospheric CO_2 concentration leads subsequently to a change in the chemical composition of plants. The dimension of this change depends mainly on the availability of nitrogen and carbon (Stitt and Krapp, 1999).

In conclusion, the present work shows that *in vitro* cultures of *C. citratus* produce variable amounts of the antioxidants/free radical scavengers caffeic acid (**1**), chlorogenic acid (**2**), *p*-hydroxybenzoic acid (**4**), glutamic acid (**6**) and the flavonoid luteolin 6-*C*-fucopyranoside (**7**).

Conclusion

As far as we know, this is the first report on the occurrence of bioactive compounds in *in vitro* cultures of *C. citratus*. In addition to previously described compounds for this species, a 6-*C*-luteolin glycoside was also isolated and identified as luteo-

lin 6-*C*-fucopyranoside. Additional studies are required to identify the minor constituents of the different TIS *in vitro* cultures of *Cymbopogon citratus*.

Under our experimental conditions, it was possible to obtain biomass in high yields with variable contents of well-known antioxidants and free radical scavengers which can be useful nutraceuticals. Further studies are needed to optimize the TIS culture conditions as well as to identify antioxidants other than phenolics present in the plant extracts.

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