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Chemical and Biological Characterization of Cinnamic Acid Derivatives from Cell Cultures of Lavender (*Lavandula officinalis*) Induced by Stress and Jasmonic Acid

ASTRID NITZSCHE,[†] SERGEY V. TOKALOV,[§] HERWIG O. GUTZEIT,[§] AND JUTTA LUDWIG-MÜLLER*,[†]

Institut für Botanik and Institut für Zoologie, Technische Universität Dresden, D-01062 Dresden, Germany

Cell cultures of lavender (*Lavandula officinalis*) were analyzed for the metabolite profile under normal growth conditions and under stress as well as after jasmonic acid treatment. The main compound synthesized was rosmarinic acid, which was also secreted into the culture medium. Different solvent extraction methods at different pH values altered the profile slightly. Anoxic stress induced the synthesis of a cinnamic acid derivative, which was identified as caffeic acid by gas chromatography−mass spectrometry. Caffeic acid was also induced after treatment of the cell cultures with jasmonic acid. Although the antioxidative activity of both compounds, rosmarinic acid and caffeic acid, was confirmed in an assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH), it was demonstrated that both substances have a low cytotoxic potential in vitro using acute myeloid leukemia (HL-60) cells. The potential of the system for finding new bioactive compounds is discussed.

KEYWORDS: Caffeic acid; cinnamic acid derivatives; elicitation; HL-60 cells; jasmonic acid; *Lavandula officinalis*; rosmarinic acid; stress

INTRODUCTION

Within the past few decades there has been increasing interest in the discovery of either novel plant secondary metabolites with distinct biological activity or re-evaluation of already known substances with new biological activities. The characterization of such compounds has high priority within medicine and pharmacology in the search for new and more efficient compounds for the treatment of various diseases, in particular in cancer therapy. Screens using high-performance liquid chromatography (HPLC) coupled to efficient testing of bioactive compounds (activity profiling) has already led to the identification of promising natural compounds that could be chemically modified to increase their activity (*1*).

The isolation of bioactive compounds from whole plants is laborious and often an obstacle for pharmacological studies. Cell cultures provide an attractive alternative because of the defined cell type, the reproducible production of substances of interest, the facilitated isolation of the compounds, and the possibility of upscaling the process. Furthermore, certain metabolites may be more concentrated in cell cultures compared to plant material (*2*). A further advantage of cell cultures is the possibility to quantitatively and qualitatively alter the metabolite patterns by the addition of elicitors or by exposure of the cells to stress conditions.

Lavender is potentially a rich source of bioactive compounds. From different lavender species (*Lavandula officinalis*, *L. angustifolia*, *L. latifolia*, and *L. stoechas*) the most commonly analyzed compounds are volatile substances in the flowers. The most important compounds are linalool and linalyl acetate, which have interesting properties as local anesthetics (*3*). In addition, the sedative properties are also well documented (*4*) and are used in aroma therapy and for the treatment of cancer patients. Acute postoperative trauma can also be treated with lavender oil (*5*).

In addition to volatile plant substances, lavender flowers contain also other plant metabolites with putative biological activity such as phytosterols and phenolic substances including cinnamic acid derivatives and flavonoids (*6*). Polyphenols in general are believed to possess antioxidative properties. Flavonoids have been shown to be effective scavengers of reactive oxygen species (ROS), and it has been suggested that flavonoid anticancer activities depend heavily on their antioxidant and chelating properties (*7*, *8*). Flavonoids are also important for the plant as pigments, phytoalexins, and UV protectors (*9*) and have a variety of important biological activities that make them useful for the treatment of several diseases. They have been reported to exert multiple biological effects including anti-inflammatory, antiallergic, antiviral, and anticancer activities (*10*). Epidemiological studies suggest that a diet rich in fruit and vegetables is associated with a decreased incidence of cancer (*11*) and beneficial effects concerning coronary heart diseases (*12*) and neurodegenerative diseases (*13*).

* Corresponding author (telephone +49-351-463 33939; fax +49-351-463 37032; e-mail Jutta.Ludwig-Mueller@mailbox.tu-dresden.de).
[†] Institut für Botanik.
[§] Institut für Zoologie.

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Lavender also produces cinnamic acid derivatives such as rosmarinic acid in high amounts (*14*, *15*). The antimicrobial, antiviral, and antiphlogistic effects of rosmarinic acid have been documented (*16*). In addition, it is also a potent antioxidative substance (*17*). The anti-inflammatory properties are thought to be based on the inhibition of lipoxygenases and cyclooxygenases (*18*). In addition, inhibition of T cell proliferation can also account for the anti-inflammatory properties of the substance (*19*). HIV integrase is effectively inhibited by rosmarinic acid with an IC_{50} value below 10 μM (*20*). Beneficial effects of rosmarinic acid on suppression of collagen-induced arthritis in mice could also be demonstrated (*21*). Repeated administration of rosmarinic acid dramatically reduced the arthritic index and number of affected paws. The substance shows very low toxicity in mice after intravenous application (*18*).

Rosmarinic acid occurs throughout the Boraginaceae, but was also detected in species within other plant families such as Lamiaceae or Apiaceae (*22*). In addition to lavender, high concentrations of rosmarinic acid were detected in a variety of other herbal plant species such as *Rosmarinus officinalis* (*23*), *Ocimum basilicum* (*24*), *Salvia officinalis* (*25*), and *Perilla frutenscens* (*26*).

In our study we have used cell cultures of *L. officinalis* that were already selected for the production of high amounts of rosmarinic acid (*27*). Compared to plants cultivated under field conditions, the liquid cell culture medium contained a 6−10-fold higher amount of rosmarinic acid. The culture can be also grown in a bioreactor so that, in principle, the production of secondary plant metabolites can be scaled up considerably. We show in this paper that stress and elicitor treatment alters the metabolite profile of these cell cultures, which can therefore provide a promising source for "activity profiling" of cell extracts using human acute myeloid leukemia (HL-60) cell cultures.

MATERIALS AND METHODS

Plant Material. Cell cultures of *L. officinalis* were obtained from SIAB (Sächsisches Institut für Angewandte Biotechnologie, Leipzig, Germany). Cells were grown in liquid culture using Murashige and Skoog salts (*28*) and Gamborg's vitamins (*29*) obtained from Duchefa (Murashige and Skoog salts including Gamborg vitamins, 4.4 g/L). In addition, the medium contained 3% sucrose, 2 mg/L glycine, 0.25 mg/L 2,4-dichlorophenoxyacetic acid, 0.25 mg/L 1-naphthylacetic acid, and 0.25 mg/L kinetin. The pH was adjusted to 5.8. A 500 mL flask contained 125 mL of medium including cells. Cells were cultivated in flasks by rotating at 100 revolutions/min in the dark at 28 °C. The cells were routinely transferred to new flasks every 5 days (25 mL of cell suspension was transferred to 100 mL of fresh medium) or harvested at the same age for extraction if not indicated otherwise. Anoxic stress was created by leaving 3-day-old cell cultures for 3 days without shaking at 28 °C. Jasmonic acid (JA) was added at a final concentration of 10 μM to 5-day-old cell cultures. JA was dissolved in ethanol (final concentration in the medium $\leq$ 0.1%), and the cell cultures were exposed to the substance for 30, 60, and 270 min under standard conditions (constant shaking). Cells were harvested as described below.

Determination of Fresh Weight of Cell Culture. Cells were harvested by passing the culture volume through a Büchner funnel. The funnel was covered with a paper filter (d = 55 mm; no. 597). The cells were collected from the filter paper and stored at −80 °C until extraction. For fresh weight determination, 1 mL of cell suspension was removed from the culture using a cut pipet tip. The cells were shaken before each sample was taken. Fresh weight was recorded by first weighing the filter paper (wet) and then weighing the paper plus cells.

Extraction of Plant Cells and Culture Medium. *A. Extraction of Lavender Cells.* Cells were harvested as described above. The extraction of the tissue was carried out with 70% methanol (1 mL of methanol per 1 g of fresh weight). Extraction was performed with an Ultraturrax (13500 units/min; 10 min). The homogenate was then centrifuged for 5 min at 5000*g*. The supernatant was filtered, and the filtrate was evaporated to the aqueous phase. The pH of the aqueous phase was then adjusted to pH 3 (with acetic acid), pH 7 (original pH of H_2O extract), and pH 10 (with NH_3). The aqueous phase was then partitioned at least three times with the appropriate organic solvent (ethyl acetate, chloroform, or butanol). The organic phases were combined, evaporated to dryness, and resuspended in a small volume of 100% methanol for HPLC analysis. Prior to injection, the sample was centrifuged to remove particles.

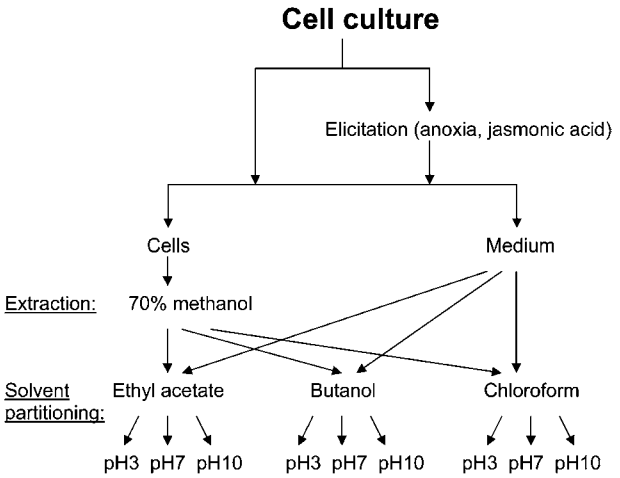


Figure 1. Extraction scheme for lavender cell cultures used in this study.

B. Extraction of the Culture Medium. The medium kept after the cells had been removed was adjusted to the desired pH value and extracted with different solvents as described for the cell homogenate. The extraction scheme (adapted from ref *30*) is shown in **Figure 1**.

Thin-Layer Chromatography (TLC) of Extracts from Different Lavender Tissues. All reagents used were as specified by Ph. Eur./DAB. The TLC plates were silica gel F254 (Merck). If not stated otherwise, the methanolic extract of the respective tissue was used for TLC analysis. Lavender flowers were extracted as follows: 0.75 g of dried flowers was heated (60 °C) in 5 mL of MEOH + 1 mL of H_2O for 10 min, removed, and heated a second time in 100% methanol. After filtration, the methanolic extracts were combined and evaporated. The samples were dissolved in 100% methanol and analyzed by TLC.

A. Alkaloids. Detection was with Dragendorff's reagent according to the manufacturer's instructions. TLC solvent was methanol/H_2O/chloroform/NH_3 (66:22:11:1). Alkaloids were visible as yellow, orange-red, or orange-brown spots.

B. Saponins. Saponins were detected with anisaldehyde reagent R (10 mL of anisaldehyde R was mixed with 90 mL of ethanol and, after the addition of 10 mL of H_2SO_4, mixed again). Approximately 10 g of plant material was extracted in 70% methanol at 100 °C, filtered, and used for TLC analysis. TLC solvent was ethyl acetate/H_2O/butanol (25:50:100). After evaporation of the solvent, the plate was sprayed with 10 mL of anisaldehyde reagent and then heated to 105−110 °C for 5−10 min. Saponins showed brown to gray-violet spots.

C. Anthraquinones. The substances were identified using KOH reagent (50 g of KOH/L). About 5 g of plant material was extracted with 5 mL of 70% ethanol and heated to 80 °C. After centrifugation for 10 min at 10000*g*, the supernatant was filtered and the filtrate used for TLC analysis. TLC solvent was H_2O/methanol/ethyl acetate (13:17:100). After evaporation of the solvent for a maximum of 5 min, the plate was sprayed with the reagent and then heated to 100−105 °C for 15 min. The spots were visualized under UV light at 365 nm as brownish-yellow spots.

D. Tannins. Tannins were visualized using Fast Blue Salt B solution (50 mg dissolved in 10 mL of H_2O). Approximately 5 g of plant material was incubated under vigorous shaking with 10 mL of H_2O for 10 min and then filtered. The filtrate was extracted twice with ethyl acetate, dried with 6 g of H_2O-free $NaSO_4$, and evaporated to dryness. The

residue was resuspended in 1 mL of ethyl acetate and used for TLC analysis. TLC solvent was 98% acetic acid/ether/ethyl acetate (20:20:40). After evaporation of the solvent after ~10−15 min at room temperature, the plate was sprayed with Fast Blue Salt B solution. The red-brown color could be intensified by additional treatment with NH_3.

E. Phenols. Phenols were identified by two different methods. *Method I.* Detection was achieved with dichlorchinonchlorimide reagent (10 g/L in methanol) and H_2O-free Na_2CO_3 solution (20 g/L). About 5 g of plant material was extracted for 10 min with 5 mL (1:1 methanol/H_2O) at 100 °C, then filtered, and the residue was washed with the same solvent (final volume = 5 mL). TLC solvent was H_2O-free formic acid/H_2O/ethyl acetate (6:6:88). Formic acid was evaporated by heating to 105−110 °C. The plate was sprayed with dichlorchinonchlorimide followed by spraying with Na_2CO_3 solution. Phenols showed a light blue to brownish-blue color.

Method II. The plate was sprayed with $FeCl_3$ solution [2% (w/v) in 96% ethanol]. Phenols had a blue to violet color.

F. Flavonoids. These compounds were detected using diphenylboryloxyethylamine solution (10 g/L in methanol) and Macrogol 400 solution (50 g/L methanol). For extraction, 10 g of plant material was heated to 60 °C for 5 min in 10 mL of methanol and filtered after cooling. The filtrate was used for TLC analysis. TLC solvent was (a) H_2O-free formic acid/ethyl methyl ketone/ethyl acetate (10:30:50) or (b) H_2O-free formic acid/H_2O/ethyl acetate (6:9:90). The plate was dried for 10 min at 100−105 °C and then sprayed with diphenylboryloxyethylamine followed by spraying with Macrogol solution. After 30 min, the spots were visualized under UV light at 365 nm. Flavonoids could be seen as yellow-orange, yellow-brown, or yellow-red spots. Anthraquinones showed red fluorescence and caffeic acid light blue fluorescence.

HPLC Separation of Metabolites. For HPLC separation the evaporated organic phases were weighed directly in the flask and dissolved in methanol (30 mg/mL). These methanolic extracts were then further diluted (1:6) prior to HPLC analysis. This resulted in comparable amounts of extract for further analysis. From the methanolic extract 100 μL was subjected to HPLC (Jasco BT 8100 pumps) coupled to an autosampler (Jasco AS-1550), equipped with a 4 mm × 125 mm Lichrosorb-100 C_{18} 5 μm, reverse phase column and a multiwavelength diode array detector (Jasco MC-919) set between 200 and 400 nm. As solvent 1% aqueous acetic acid (solvent A) and 100% methanol (solvent B) were used. The methanol concentration (solvent B) was changed as follows: 20% (0 min), 50% (20 min), 100% (40 min), 20% (5 min). Flow rate was 0.7 mL min^{-1}. Borwin chromatography software (JMBS Developments Software for Scientists) was used to process chromatograms. Identification of substances was achieved by comparing the retention times and respective peak spectra with authentic standards.

Gas Chromatography−Mass Spectrometry (GC-MS) Identification of Caffeic Acid. The peak corresponding to caffeic acid was collected, evaporated to dryness, and resuspended in ethyl acetate. To 50 μL of ethyl acetate containing caffeic acid was added 950 μL of ethereal diazomethane (*31*). The sample was incubated for 15 min at room temperature, the ether was evaporated, and the sample was again resuspended in ethyl acetate. The standard was treated likewise. Of each sample 2 μL was injected for further analysis. GC-MS analysis was carried out on a Varian Saturn 2100 ion-trap mass spectrometer using electron impact ionization at 70 eV, connected to a Varian CP-3900 gas chromatograph equipped with a CP-8400 autosampler (Varian, Walnut Creek, CA). Full-scan mass spectra were recorded. For the analysis 2.5 μL of the sample methylated for 15 min at room temperature with ethereal diazomethane dissolved in 20 μL of ethyl acetate was injected in the splitless mode (splitter opening 1:100 after 1 min) onto a Phenomenex ZB-5 column, 30 m × 0.25 mm × 0.25 μm, using He carrier gas at 1 mL min^{-1}. Injector temperature was 250 °C, and the temperature program was 60° for 1 min, followed by an increase of 20 °C min^{-1} to 310 °C, and then 5 min isothermically at 310 °C. Transfer line temperature was 280 °C. Scan rate was 0.6 s $scan^{-1}$, multiplier offset voltage 200 V, emission current 30 μA, and trap temperature 200 °C. Caffeic acid from the HPLC-purified sample was identified according to the retention time on GC compared with an authentic methylated standard and by recording the respective mass spectrum.

Table 1. Occurrence of Different Classes of Secondary Plant Compounds in Different Tissues of *L. officinalis* Determined by Thin-Layer Chromatography and Detection with Various Reagents[a]

	plant material				
substance class	cell culture	cell culture 3 days without shaking[b]	cell culture medium	cell culture medium 3 days without shaking[b]	flower pods
alkaloids	±	±	−−	±	−−
anthraquinones	++	++	−−	−−	±
flavonoids	−−	−−	−−	−−	++
saponins	−−	−−	−−	−−	±
tannins	++	±	−−	++	−−
phenols	++	++	++	++	++

[a] ++, present in larger amounts; ±, present; −−, not detectable. [b] Low oxygen stress.

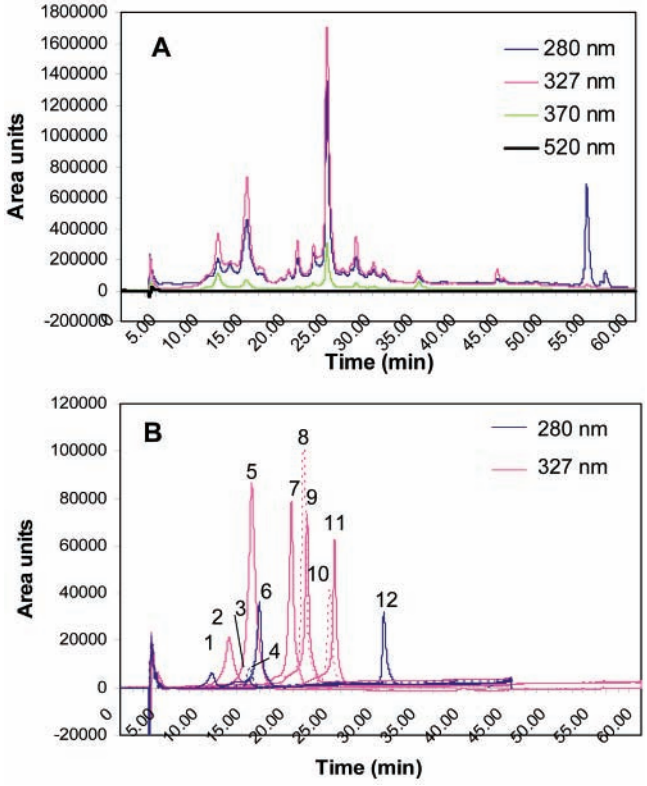


Figure 2. (**A**) HPLC traces of a typical ethyl acetate extract (pH 3) of lavender cells at different wavelengths. At 280 nm catechins, epicatechins, flavones, isoflavones, proanthocyanidins are detected, at 325 nm cinnamic acids and cinnamate esters are detected, at 370 nm flavonols are detected, and at 520 nm anthocyanidins are detected. No absorption was found in the samples at 520 nm. (**B**) Selected standards for cinnamic acid derivatives and catechins at different wavelengths. Peaks: 1, catechin (t_R 10.8 min); 2, chlorogenic acid (t_R 14.5 min); 3, vanillic acid (t_R 15.0 min); 4, epicatechin (t_R 15.1 min); 5, caffeic acid (t_R 15.5 min); 6, syringic acid (t_R 17.0 min); 7, *p*-coumaric acid (t_R 21.0 min); 8, sinapinic acid (t_R 21.5 min); 9, ferulic acid (t_R 21.7 min); 10, *o*-coumaric acid (t_R 25.0 min); 11, rosmarinic acid (t_R 25.2 min); 12, cinnamic acid (t_R 30.5 min).

In Vitro Cytotoxicity. *A. Cell Culture.* HL-60 cells (DSMZ, Germany) were maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Gibco, France). Cells were grown at 37 °C in a humidified 5% CO_2 atmosphere and maintained at a density of 2 × 10^5 to 1 × 10^6 cells/mL.

B. Exposure. HL-60 cells (0.5 × 10^6 cells mL^{-1}, 10 mL per flask) were cultured for 24 h in the presence of rosmarinic acid (RA), caffeic

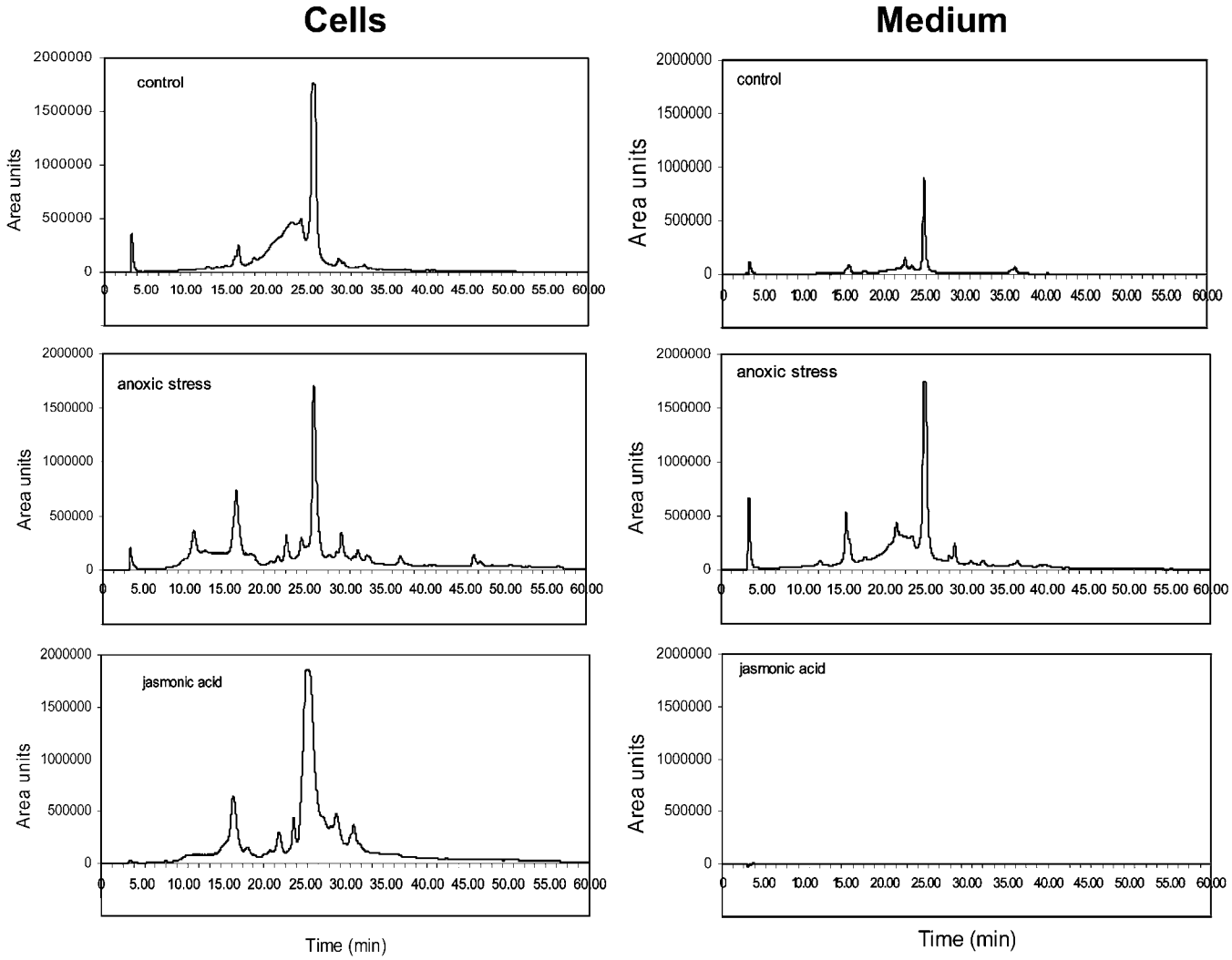


Figure 3. Extracts from lavender cells and culture medium obtained after solvent partitioning with ethyl acetate at pH 3. Control conditions (upper panels) were compared to cells and medium after treatment with anoxic stress (middle panels) and jasmonic acid (lower panels).

acid (CA), and total plant cell extract (LP) and purified 15 min (P15) and 25 min (P25) fractions of the HPLC eluates and the well-characterized bioactive flavonoid quercetin (Q) for comparison (1.0, 2.0, 5.0, 10.0, 20.0, 40.0, and 80.0 μM). Because the stock solutions of all compounds were prepared in DMSO, all cultures were made 0.1% in DMSO including the control sample.

C. Flow Cytometry. Flow cytometry was carried out as described previously (*32*). Briefly, the same volumes of the suspension cultures were harvested and the cells washed in phosphate-buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. Fixed cells were spun down, and the pellet was resuspended in PBS containing 50 μg/mL propidium iodide (PI, Sigma, Germany) and 0.2 mg/mL RNase (Sigma, Germany). After staining for at least 45 min at room temperature, the cells were analyzed by flow cytometry (CyFlow, Partec, Germany). The excitation wavelength was 473 nm, and red fluorescence (>590 nm) was recorded. For each sample the fluorescence of 100,000 cells was quantified. The percentage of cells in sub-G1 peak (apoptotic cells), G0+G1, S, and G2+M phases of cell cycle were calculated using CyFlow software (Partec).

Antioxidative Potential. Free radical scavenging activities of caffeic and rosmarinic acid were determined in a chemical reaction using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH, Fluka, Germany) as described by ref *33*. The flavonol quercetin was analyzed in parallel as positive control. The assays were performed in a 300 μL reaction mixture containing 200 μL of freshly made 0.1 mM DPPH solution in 96% ethanol, 90 μL of 50 mM Tris-HCl buffer (pH 7.4), and 10 μL of test compounds in DMSO at final concentrations of 0 (control), 1.0, 2.0, 5.0, 10, 20, and 40 μM. After 30 min of incubation at room temperature, the absorbance was measured in 96-well plates at 517 nm using a Spectra Rainbow photometer (Labinstruments GmbH, Austria).

Statistical Methods. Each extraction of cells and medium was done in duplicate for each condition, and similar results were obtained.

The experimental data for the antioxidative potential and apoptosis are expressed as the mean ± SD of several independent experiments (see caption of **Figure 6** for details). The significance of the recorded effects was assessed by analysis of variance (ANOVA).

RESULTS

Metabolite Profiles from Cell Extracts and Culture Medium. The presence of different classes of secondary metabolites in flower pods and cultured cells of lavender was demonstrated by TLC (**Table 1**). Whereas alkaloids were present in only minor amounts, the concentrations of phenolic compounds were high in most tissues. Tannins were induced under anoxic stress and were secreted into the medium. Anthraquinones were found in lavender cells, whereas flavonoids were more abundant in lavender flower pods. This is in agreement with the finding that detection of metabolites at different wavelengths showed that the majority of compounds absorbed with a maximum at 280 or 325 nm (**Figure 2**). Therefore, all chromatograms presented in this study were recorded at 325 nm, where cinnamic acid derivatives show a maximum.

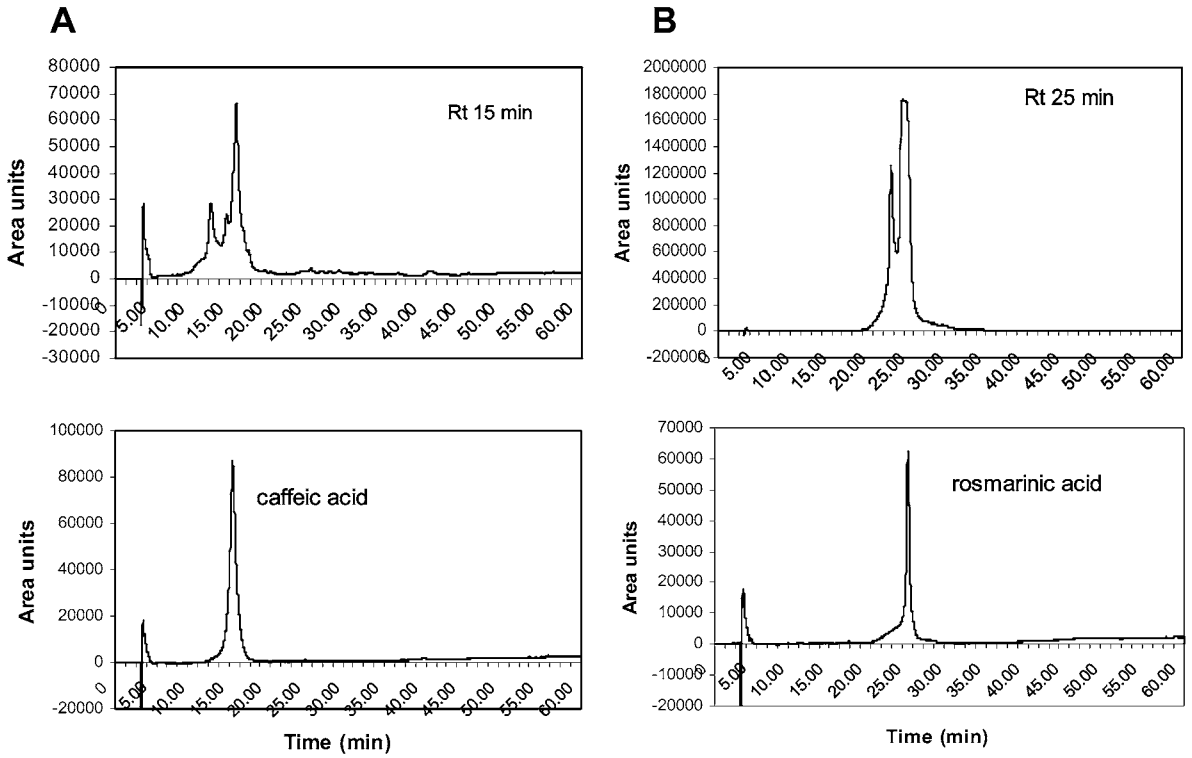


Figure 4. HPLC purification of caffeic acid (**A**) and rosmarinic acid (**B**) peaks used for (a) identification and (b) test of biological activity. The upper panels show the HPLC-purified extract and the lower panels the respective standard chromatogram. All chromatograms were recorded at 325 nm.

Solvent partitioning of lavender cells extracted with 70% methanol and medium for HPLC profiling was performed with different organic solvents at three different pH values (**Figure 1**). In all experiments the plant tissue used was in the exponential growth phase at the time point of harvest (data not shown). Synthesis of rosmarinic acid in *L. officinalis* and *L. vera* cell cultures was associated with the exponential growth phase (*27*, *34*). The main peak under normal growth conditions that could be extracted with different organic solvents was rosmarinic acid (**Figure 3**) as already shown in a previous study (*27*). This compound was also found to be secreted into the culture medium. Otherwise, there were differences in the metabolites found in cell extracts and culture medium under the same extraction conditions.

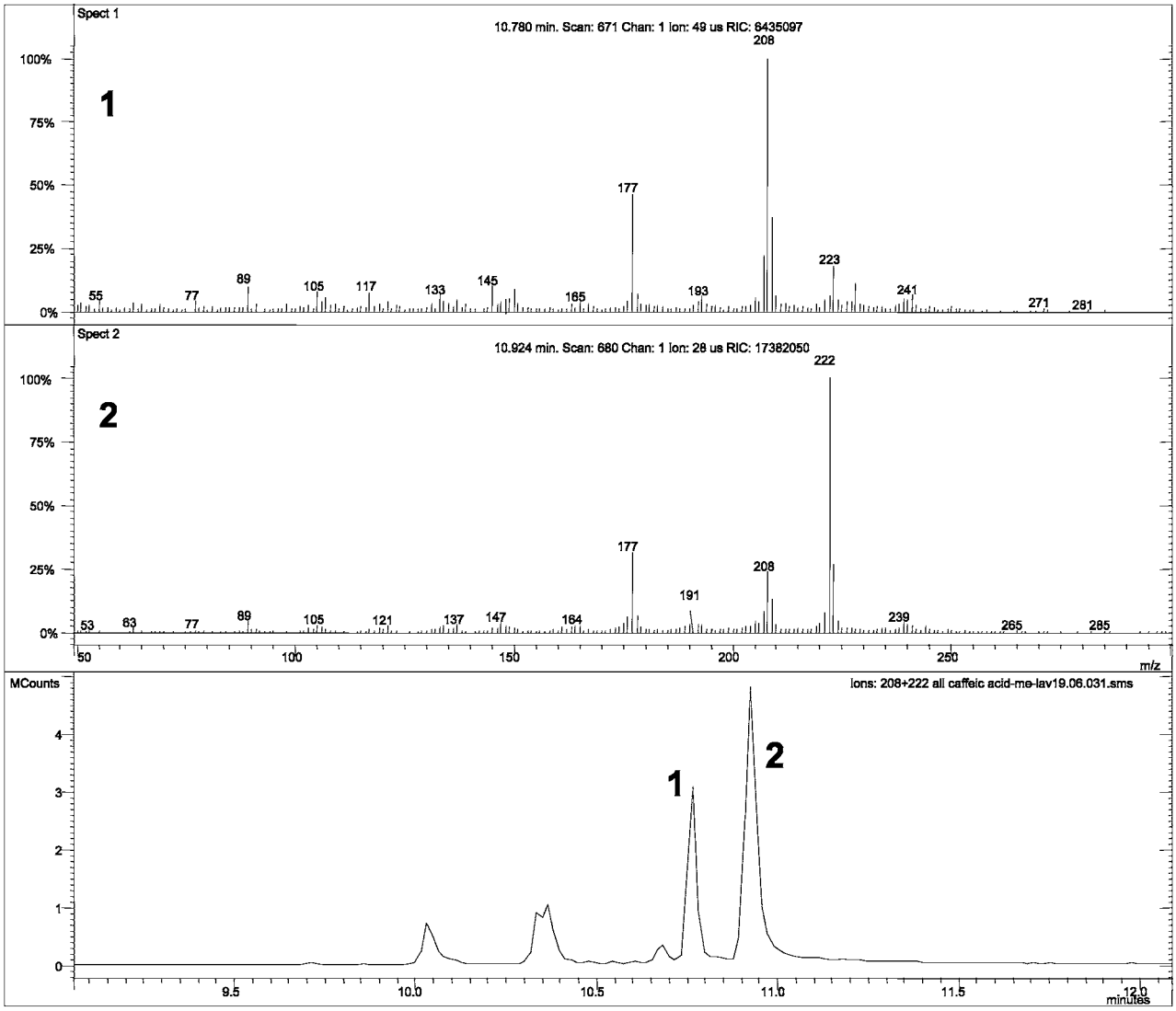
In general, the metabolite profile changed slightly according to the conditions during solvent extraction (solvent and pH value; data not shown). The best extraction conditions for solvent partitioning were found at pH 3 with ethyl acetate (**Figure 3**). The recovery of rosmarinic acid from untreated lavender cell extracts was below 1% with ethyl acetate at pH 7 and 10, respectively, compared to pH 3, whereas for caffeic acid the recovery was ~10% for the same conditions compared to pH 3. Chloroform showed the least differences to ethyl acetate as solvent, whereas with butanol the lowest number of metabolites was extractable. Other pH values may be used to selectively isolate different metabolites, because ethyl acetate as solvent at pH 7 extracted a peak from the medium of control cells chromatographing at t_R 21 min (data not shown). According to standard substances this peak could be *p*-coumaric acid, but probably also sinapinic acid or ferulic acid. However, during the course of this study this compound was not further characterized. At pH 10 with all solvents the least amount of secondary metabolites was extractable and the results between experiments were more variable (data not shown).

In addition to rosmarinic acid, a number of additional smaller peaks were seen in control extracts. According to standard substances the peak at t_R 15 min is tentatively identified as caffeic acid and the peak at t_R 30 min is likely to be cinnamic acid (**Figure 2B**). Another group of peaks with t_R values of 20−21 min was not well resolved and therefore could not be further analyzed with the techniques used in this study. Several phenolics chromatographed at this retention time, among them ferulic acid, sinapinic acid, and *p*-coumaric acid.

Identification of a Stress-Inducible Compound. After the cell cultures were subjected to stress caused by O_2 depletion, the peak at t_R 15 min increased significantly when the cells were extracted with ethyl acetate (**Figure 3**). The compound was also secreted into the culture medium. In addition to anoxic stress the compound at t_R 15 min was also inducible after treatment with jasmonic acid (JA), but in this case it was not found in the culture medium. The compound at t_R 30 min, which could probably be cinnamic acid, was also slightly induced by anoxic stress and JA. For further analysis the peak at t_R 15 min was collected, reanalyzed by HPLC, and shown to contain only minor impurities (**Figure 4**). The substance was then subjected to derivatization with ethereal diazomethane and subsequently analyzed by GC-MS. Authentic caffeic acid standard was treated likewise. The two hydroxyl groups of caffeic acid were converted into ether groups yielding caffeic acid dimethyl ether (CADE; **Figure 5B**), and the carboxyl group was methylated to yield the methyl ester of the former compound. The GC chromatogram showed two peaks with retention times of 10.78 and 10.92 min for both the standard (not shown) and HPLC-purified sample from stressed lavender cells (**Figure 5A**), which corresponded to the two caffeic acid derivatives with molecular ions of 208 (caffeic acid dimethyl ether) and MW 222 (methyl ester of CADE). The respective patterns of daughter ions (main daughter ion at *m*/*z* 177) for CADE corresponded to spectra found in the NIST library. Therefore, we conclude that the peak at t_R 15 min is caffeic acid.

Biological Activity of Compounds. We chose HL-60 cells for evaluating the toxicity of the test compounds because this

A



B

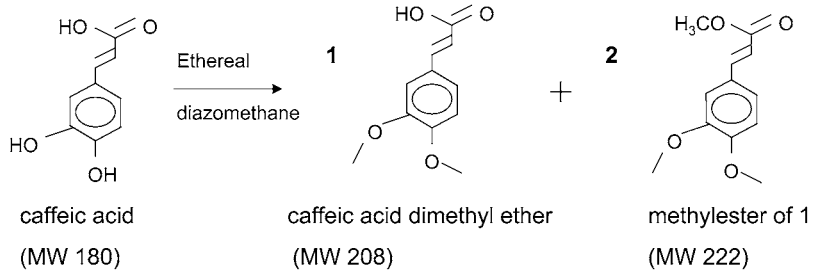


Figure 5. GC-MS identification of caffeic acid from stress-induced lavender cells. Authentic caffeic acid standard (not shown) and the peak at t_R 15 min were methylated with ethereal diazomethane and then subjected to GC-MS analysis. (**A**) Chromatogram and mass spectra of peak t_R 15 min after methylation: (lower panel) partial ion chromatogram of the methylated sample; (upper panels) mass spectra of respective peaks 1 and 2. (**B**) Structures and molecular masses of the two peaks after methylation with ethereal diazomethane. Peaks: 1, caffeic acid dimethyl ether; 2, methyl ester of compound **1**.

well-studied human myeloid cell line reacts sensitively to chemical or physical stress by altered cell cycle distribution and the initiation of apoptosis (*32*, *35*). Furthermore, the cell line is well suited for quantitative analysis by flow cytometry. In control cultures ~5% of the cells are characterized by lower (hypodiploid) DNA content, an indication of the apoptotic process. When HL-60 cells were exposed for 24 h to different concentrations of the test compounds or fractions (**Figure 6A**),

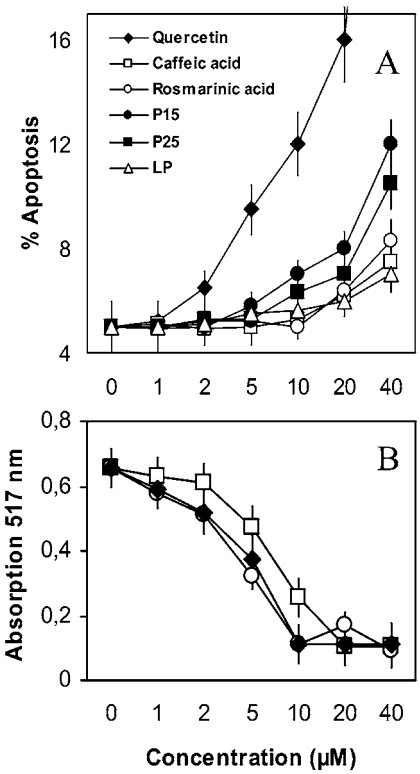


Figure 6. Biological activity of rosmarinic and caffeic acid. Mean values of independent experiments ± SD are given. (**A**) Dose dependence of apoptotic cell number after the treatment with rosmarinic acid (RA), caffeic acid (CA), total plant cell extract (LP) and purified 15 min (P15) and 25 min (P25) fractions and quercetin (Q) (1.0, 2.0, 5.0, 10.0, 20.0, and 40.0 μM). Because the stock solutions of all compounds were prepared in DMSO, all cultures were made 0.1% in DMSO including the control sample. After 24 h of treatment, cells were stained with PI and analyzed by DNA flow cytometry. Cell cycle distribution of 100000 cells was analyzed using FlowMax software (Partec, Germany). The data show the percentage of apoptotic cells with subdiploid amount of DNA (sub-G1 peak) from four independent experiments. (**B**) Antioxidative activity. The reduction of DPPH by caffeic acid and rosmarinic acid was compared with the reference compound quercetin. The data represent the arithmetic mean of six independent experiments. Symbols for single compounds are as in part **A**.

the percentage of apoptotic cells increased in a dose-dependent manner, but the level remained low and only at the highest concentration tested (40 μM) was the toxicity obvious ($p < 0.05$). However, compared to the reference flavonoid, quercetin, the toxicity of all test compounds was considerably lower, and the difference was statistically significant at 5 μM and higher concentrations ($p < 0.05$). None of the substances caused changes in the cell cycle distribution up to a concentration of 40 μM. However, the reference compound quercetin was effective at 10 μM concentration, and cell cycle arrest at the G_2 phase was observed. Whereas in untreated control cell cultures 10 ± 2% of HL-60 cells were in the G_2 phase, this fraction increased significantly in quercetin-exposed cultures and reached 20 ± 1% ($p < 0.05$).

The radical scavenging activity of caffeic acid and rosmarinic acid was quantified using the DPPH assay. Both compounds possess considerable antioxidative potency and showed a similar concentration-dependent increase in antioxidative activity compared to quercetin (**Figure 6B**). This pharmacologically beneficial property in combination with the low toxicity makes both caffeic acid and rosmarinic acid interesting compounds for further investigations concerning their biological effects.

DISCUSSION

The potential of plants to produce secondary metabolites with distinct biological activity has received renewed attention within recent years. In addition, many interesting plant species have been grown as cell cultures, which in turn produce increased amounts of desired compounds (*36*) or can be elicited to synthesize and secrete those substances (*37*, *38*). Such compounds can be identified by relatively easy screens using HPLC coupled to efficient testing of bioactivity (*1*). Cell cultures have the advantage of a controlled environment with little overall variation in their performance, and either medium or cells are easy to harvest for further processing.

The lavender cell cultures used in this study were previously selected to produce high amounts of rosmarinic acid (*27*). It has long been known that the presence of rosmarinic acid in medicinal plants, herbs, and spices has beneficial and health-promoting effects (reviewed in ref *22*). The main biological activities of rosmarinic acid include antioxidative, anti-inflammatory, antimutagen, and antibacterial/antiviral activities (*18*). We were able to show that rosmarinic acid has a low cytotoxic activity as determined for the low apoptotic rate measured with HL-60 cells. The pharmacologically beneficial property in combination with the low cytotoxicity makes rosmarinic acid an interesting compound for further investigations concerning the biological effects. The low toxicity was demonstrated in mice with an LD_{50} of 561 mg kg^{-1} after intravenous application (*18*).

In our study we were interested in the analysis of additional compounds with possible bioactive properties synthesized by lavender cells. It was previously shown that *L. vera* cells could be elicited by changes in nutrient supply to produce more rosmarinic acid (*34*, *39*). A suspension culture of *Agastache rugosa* produced more rosmarinic acid after treatment with either yeast extract or benzothiadiazole, an elicitor of plant systemic resistance (*40*). *L. officinalis* cells were elicited by low oxygen stress and jasmonic acid treatment (**Figure 3**). Although this did not result in an increase of rosmarinic acid, we observed novel metabolites that were also secreted into the medium under stress conditions. One major metabolite was identified as caffeic acid (**Figure 5**). Caffeic acid showed the same antioxidative potential as rosmarinic acid, again together with low cytotoxicity (**Figure 6**). This substance should be therefore further analyzed to exploit its use as a bioactive compound.

Caffeic acid is produced from *p*-coumaric acid, a branchpoint of the synthesis of rosmarinic acid (**Figure 7**; *24*). Cinnamic acid, a precursor of caffeic acid and rosmarinic acid, was also slightly induced after stress and JA treatment (**Figure 3**). Elicitation of the lavender cell cultures resulted in a specific increase in one branch of the biosynthetic pathway. This finding is interesting for the enrichment of additional bioactive compounds from these cultures. On the other hand, these findings should be also taken into account for the quality production of a single metabolite such as rosmarinic acid, where variations in the culture conditions should be avoided. The consequences are that the growth conditions have to be controlled very carefully to ensure that no other metabolites are synthesized. Variation in the extraction conditions could also result in the detection of additional metabolites. First, other extraction conditions for the isolation of secondary metabolites from the cells could be used such as acidified methanol or additional heating during the extraction procedure. Second, the changes in pH value during solvent partitioning resulted in additional metabolites in the HPLC profile and could thus be used for selective isolation of metabolites.

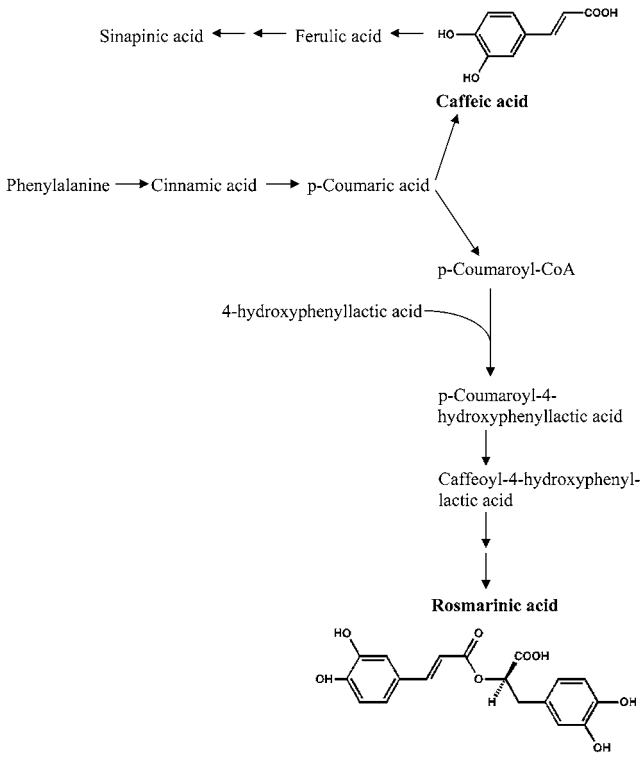


Figure 7. Scheme of caffeic acid and rosmarinic acid biosynthesis (adapted from ref *24*).

Compounds induced during stress in plants are highly likely to possess high biological activity. The increase in ROS released during stress has to be counterbalanced by plant metabolites with scavenging activity. Elicitation of metabolites by stress in plants is therefore a very promising approach to identify interesting biologically active substances in the future.

In conclusion, we have shown that caffeic acid synthesis can be elicited in lavender cells and that both rosmarinic acid and caffeic acid have a high potential as antioxidants because their cytotoxicity is very low. In addition, we have demonstrated that the culture conditions are important if a certain metabolite should be produced because other metabolites can be elicited under stress conditions. On the other hand, the solvent that is used for solvent partitioning and the respective pH value are also important, because different metabolites can be thus extracted. It will be possible to use this system in the future to elicit and identify other bioactive compounds from these cells, which have high potential in medicinal use.

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