# Chemical and Pharmacological Investigations of *Metaxya rostrata*

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In a bioassay-guided approach the chemical composition of rhizomes of *Metaxya rostrata* (Kunth C. Presl) was studied for the first time. Investigations of the cytotoxicity of extracts and fractions on SW480 colorectal carcinoma cells resulted in the isolation of two polyphenols – cinnamtannin B-1 and aesculitannin B. The structures of the compounds were elucidated by different NMR experiments. Additionally, sugars, common sterols, such as sitosterol, stigmasterol and campesterol, as well as chlorogenic acid and caffeic acid were identified in *Metaxya rostrata*.

*Key words: Metaxya rostrata*, Polyphenols, Cytotoxic Effects

## Introduction

*Metaxya rostrata* is a tree fern widespread in lowland rain forests of Central America and the north-western parts of South America. Within the Metaxyaceae family only two species – *Metaxya rostrata* (Kunth C. Presl) and *Metaxya lanosa* A. R. Sm. & H. Tuomisto – have been described until now (Smith *et al.*, 2001). The chemical composition of both species has not been investigated before.

From physicians and traditional healers in the region of Corcovado Parque National in Costa Rica informations were obtained that suspensions of the dried rhizome of *Metaxya rostrata* in water are administered orally in Costa Rican ethnic medicine in intestinal diseases such as ulcers or tumours. As colorectal carcinomas are among the most common cancers causing a large part of cancer deaths in western industrialized countries and are characterized by high inherent resistance and low response to therapy (Ragnhammar *et al.*, 2001), the development of new therapeutics for colorectal cancer is an important ongoing task (Kinghorn *et al.*, 2003; Cragg and Newman, 2005). Tropical plants used in ethnomedicine provide a rich source for new cytotoxic compounds (Kim and Park, 2002). Thus, a bioassay-guided chemical investigation based on the antiproliferative activity, the capacity to alter the cell cycle, and the induction of apoptosis in SW480 colorectal carcinoma cells by extracts from the rhizome of *Metaxya rostrata* seemed of interest.

## Materials and Methods

### *General*

ESI-mass spectra were recorded on a PE Sciex API 150 EX single quadrupole instrument (Applied Biosystems, Foster City, CA, USA) configurated for negative ionization. Full scan spectra were acquired over the range *m/z* 150–2000; scan time was 2 s. NMR spectra were recorded on a Bruker Avance DRX 600 NMR spectrometer using either a 5 mm triple resonance probe (TBI, $^{1}$H, $^{13}$C, broad band) with triple axis gradients for all $^{1}$H-detected experiments, or a 5 mm switchable quadruple probe (QNP, $^{1}$H, $^{13}$C, $^{19}$F, $^{31}$P) with z-axis gradients and automatic tuning and matching accessory for direct detected carbon experiments. The resonance frequency for $^{1}$H NMR was 600.13 MHz, for $^{13}$C NMR 150.92 MHz. All measurements were performed for a solution in fully deuterated methanol at 300 K. Standard 1D and gradient-enhanced (ge) 2D experiments, like double quantum filtered (DQF) COSY, TOCSY, NOESY, ROESY, HSQC, and HMBC, were used as supplied by the manufacturer. The setup for the diffu-

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sion difference experiments was chosen as published (Kählig *et al.,* 2002).

TLC was performed on silica gel plates (Merck, Germany; 0.25 mm); mobile phases for the identification of phenolic acids: (A) EtOAc/HOAc/$HCO_2H$/$H_2O$ (100:11:11:26 v/v/v/v); (B) EtOAc/butanone/HCOOH/$H_2O$ (50:30:10:10); detection: Naturstoffreagens A/PEG 400 under $UV_{366}$ (Wagner and Bladt, 1996); mobile phases for the identification of sugars: (C) *n*-propanol/acetone/lactic acid (40:40:20); (D) *n*-butanol/acetic acid/diethyl ether/$H_2O$ (9:6:1:1); naphthoresorcinol/phosphoric acid (Merck, 1970).

*Plant material and extraction*

Rhizomes of *Metaxya rostrata* were collected in the surroundings of La Gamba, Costa Rica, in February 2003 (Guaymi Reservation, Cerro Rincon, La Gamba) and authenticated in the Herbarium of the Museo National in San Jose. The roots were sundried for 2 h and exported with permission from Ministerio de Ambiente y Energia (MinAE) and INBio of Costa Rica. Voucher specimens are deposited in the Herbarium of the Department of Pharmacognosy (University of Vienna, Vienna, Austria).

800 g dried roots were pulverized, and portions of 100 g were extracted by sonification with 1 l water at 50 °C for 1 h, each. Each extraction was repeated 5 times. After lyophilization the yield of the combined water (W) extract was 90 g. Compounds of medium polarity were extracted under the same conditions with methanol to yield 20 g of MeOH (M) extract. The apolar components were extracted from 100 g portions of the drug with 650 ml of chloroform (C), each. These extractions were repeated twice and resulted in 2.88 g of extract C.

*Fractionation*

80 g of extract W were extracted sequentially with ethylacetate, butanol and methanol to yield 0.75 g of fraction WE, 26.35 g of fraction WB and 18.7 g of fraction WM as well as 33 g residue WW after evaporation. 15 g of extract M were partitioned between ethylacetate and water as well as between butanol and water. The solvents were evaporated or lyophilized to yield fractions ME (4 g), MB (9 g) and MW (1.5 g). The cytotoxic fractions WE, WB, WM, ME and MB were subjected to vacuum liquid chromatography (VLC) on silica (15–40 $\mu$m, Merck) using EtOAc/MeOH/$H_2O$ mixtures of increasing polarity as mobile phases. 15 subfractions were obtained. The most active fraction 11 was subjected to column chromatography on Sephadex® LH-20 by gradient elution with MeOH/$H_2O$ mixtures (70% to 100% MeOH). Of 16 subfractions collected fraction Sx11 (220 mg) was submitted to another separation step under similar conditions, and from the resulting fractions Sy3 (25 mg) and Sy4 (81 mg) compound Rx (15 mg) was crystallized.

Extract C (2.6 g) was extracted with hexane resulting in 1.46 g hexane fraction and 0.84 g chloroform residue. By threefold VLC on silica using methanol/chloroform and hexane/ethylacetate mixtures as mobile phases, sitosterol (9 mg), stigmasterol (3 mg) and campesterol (1.3 mg) were isolated (Rigler, 2005; Metnitzer, 2007).

*Determination of cytostatic and cytotoxic activity*

SW480 carcinoma cells obtained from the American Type Culture Collection and cultivated under standard conditions [minimal essential medium (MEM) containing 10% fetal calf serum (FCS)] were used to assess the cytotoxic capacity of bioactive fractions as described by Hausott *et al.* (2004). In short, cells were exposed to increasing concentrations of bioactive compounds diluted in MEM containing 1 mg/ml bovine serum albumin 48 h after plating.

Another 48 h later cell viability was determined by neutral red uptake. The presence of apoptotic structures in the culture was detected by staining with Hoechst 33258 (800 ng/ml) to visualize the morpholgy of nuclei. Apoptosis was indicated by condensation or fragmentation of chromatin. The incidence of such structures was counted from 1000 cells each in triplicate cultures.

Unspecific cytotoxicity and cell lysis were determined with the Cytotoxicity Detection Kit from Roche Diagnostics (Vienna, Austria), which quantifies the release of lactat dehydrogenase (LDH) into the culture medium.

Cell cycle analysis was performed on nuclei isolated from cultures treated with plant extracts (0.5 mg/ml serum-free medium) and fractions at different concentrations as described before (Hausott *et al.*, 2004)

After identification of apoptotic structures by staining with Hoechst 33258 mitochondrial polarization was assessed by incubation of the harvested cells with the mitochondrial tracking dye JC1 followed by FACS analysis (Hausott *et al.*, 2004).

## Results and Discussion

Preliminary phytochemical analyses of the rhizome of *Metaxya rostrata* showed the presence of sterols, phenolic compounds and saccharides. Additionally the independence of the composition of secondary metabolites of environmental factors was verified by chromatographic comparison of samples from three different collection sites, at Cerro Rincon, La Gamba and in Guaymi Reservation.

The initial extraction of the material copied, as far as possible, the preparation for the use in therapy, which is one promising strategy in medicinal plant research (Hostettmann *et al.*, 1998). Thus, the material was extracted by sonification with hot water. For the isolation of less polar compounds additional extraction with methanol and chloroform followed.

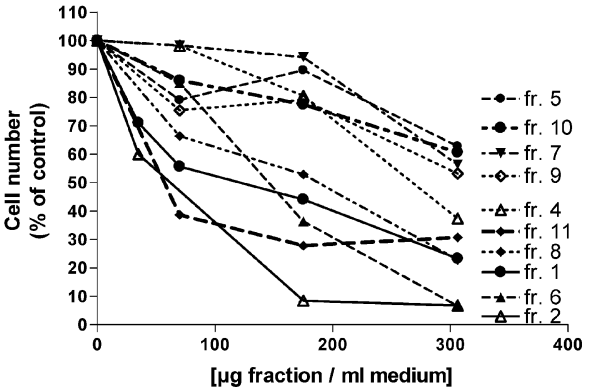


Fig. 1. Cytotoxicity of frs. 1, 2, 4–11 determined by neutral red uptake from serum-free DMEM. Cultures were exposed to the fractions obtained by vaccum liquid chromatography diluted in serum-free medium, and viability was determined 48 h later by neutral red uptake.

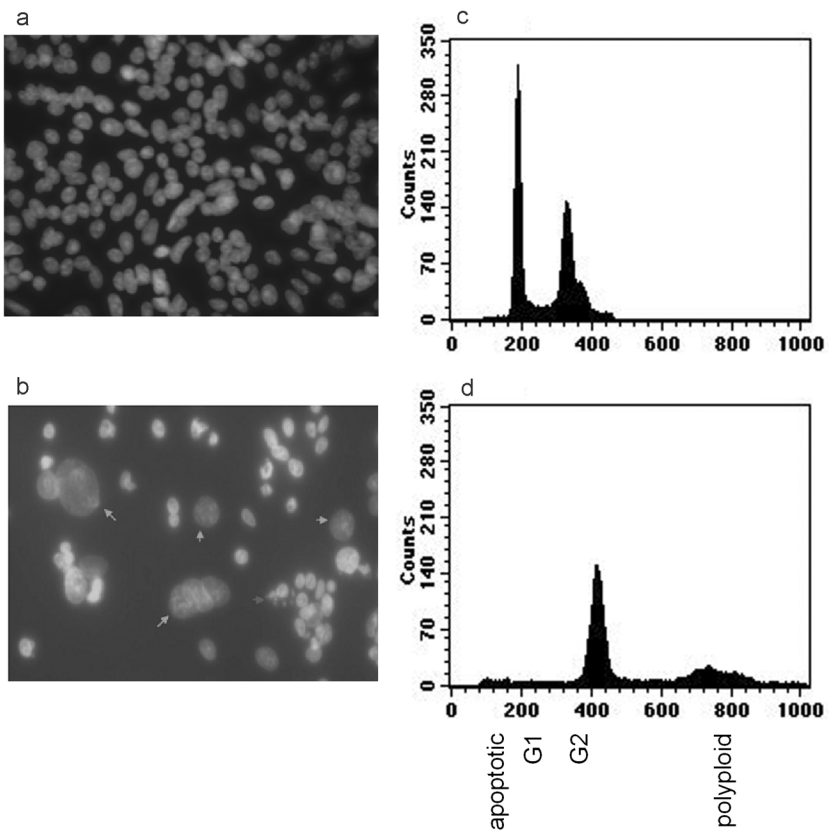


Fig. 2. Growth effects of fr. 11. (a, b) Cells were exposed to 70 $\mu$g/ml of fr. 11 in serum-free medium for 48 h before viability was determined by neutral red uptake to show a loss of viability of about 50% as compared to control. After removal of the neutral red solution cultures were washed with PBS and stained with Hoechst 33258 to visualize the nuclei. (c, d) From parallel cultures nuclei were isolated and stained with propidium iodide for cell cycle analysis by fluorescence-activated cell sorting. (a, c) Control; (b, d) fr. 11.

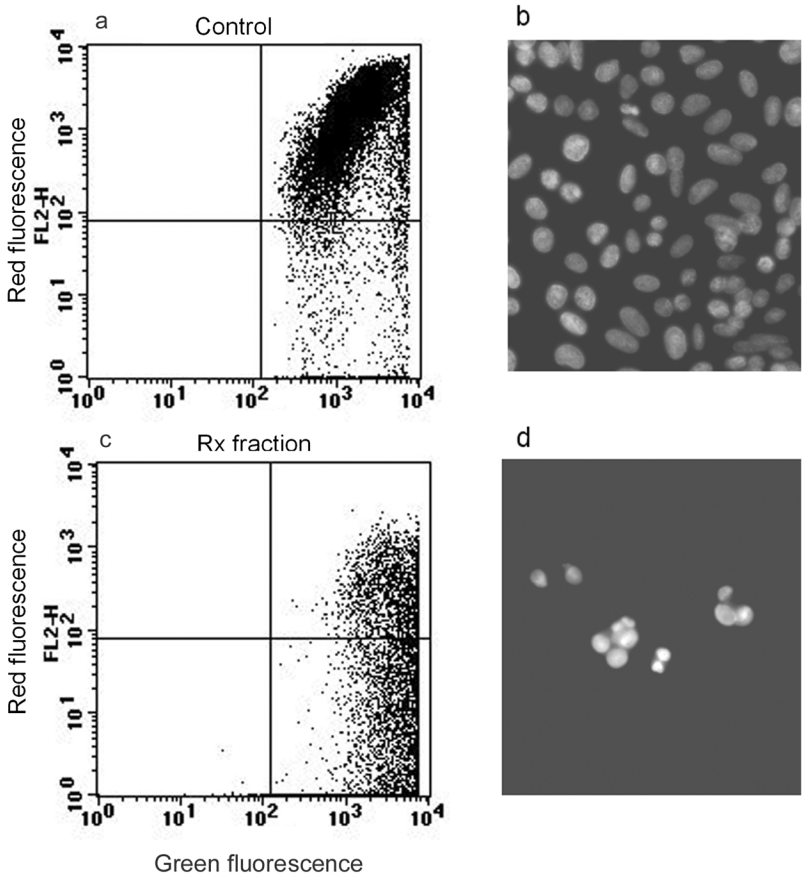


Fig. 3. Biological activity of cinnamtannin B-1 and aesculitannin B. (b, d) Cultures were exposed to the purified Rx fraction at the $IC_{50}$ concentration (46 $\mu$M), fixed with ethanol/acetic acid after 24 h to visualize the nuclei. (a, c) As the majority of nuclei presented an apoptotic morphology, parallel cultures were harvested and loss of mitochondrial membrane polarity (as an early apoptotic marker) was determined by incubation with the tracking dye JC1 and FACS analysis. The decrease in red fluorescence indicates apoptosis. (a, b) Control; (c, d) fr. 11.

To cultures of SW480 colorectal carcinoma cells the aqueous and methanolic extracts were cytotoxic in a concentration-dependent manner with cell losses between 60 and 100% at concentrations from 0.3 to 3.5 mg/ml. Cell loss was caused by apoptosis as shown by the characteristic morphology of the nuclei. In addition, FACS analysis using JC1 as mitochondrial tracker showed loss of mitochondrial membrane polarization in about 25% of the population exposed to the extracts at a concentration of 500 $\mu$g/ml. Nuclei with increased size suggested a cell cycle block in G2 and/or polyploid cells, which was also shown by FACS analysis.

Fractionation of the extracts by partition and vacuum liquid chromatography resulted in 15 subfractions. Due to the very low amount of subfraction 3 this fraction was not included in the assay. The highest cytotoxicity was determined for subfractions (frs.) 1, 2, 6 and 11 (see Fig. 1).

Fractions 1, 2 and 6 induced apoptosis as proven by Hoechst staining and JC1 FACS analyses. For fr. 6 a loss of mitochondrial membrane polarization occurred in 4% of the population within only 3 h. Unfortunately, only low amounts were available of frs. 1, 2 and 6. Fr. 11 showed the highest activity at concentrations below 100 $\mu$g/ml. Thus, cultures were exposed to 70 $\mu$g/ml of fr. 11 for a more detailed analysis. Apoptosis was identified by staining the nuclei with Hoechst dye and cell cycle distribution determined by FACS analysis 24 h later. Results in Fig. 2 showed the appearance of both apoptotic structures and very large nuclei

Table I. $^{1}H$ and $^{13}C$ NMR data of trimeric procyanidins (methanol-$d_4$; 300 K); $\delta_H$ in ppm relative to the residual solvent signal ($\delta$ = 3.31 ppm), coupling constants in Hz in parentheses, $\delta_C$ relative to the solvent signal ($\delta$ = 49.00 ppm).

| | Cinnamtannin B-1 | | Rotamer B-1 | | Aesculitannin B | |
|---|---|---|---|---|---|---|
| No. | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| Upper unit | | | | | | |
| C-2 | 99.70 | – | n.a. | – | n.a. | – |
| C-3 | 67.17 | 3.285 (3.5) | 68.29 | 4.097 (3.3) | 67.02 | 3.313 |
| C-4 | 28.85 | 4.146 (3.5) | 29.53 | 4.452 (3.3) | 28.93 | 3.944 (3.2) |
| C-5 | 156.68 | – | n.a. | – | n.a. | – |
| C-6 | 98.32 | 5.965 (2.4) | 98.26 | 6.023 | 97.82 | 5.848 (2.4) |
| C-7 | 157.67 | – | n.a. | – | n.a. | – |
| C-8 | 96.58 | 6.015 (2.4) | 96.60 | 6.082 | 96.50 | 5.998 (2.4) |
| C-9 | 154.02 | – | n.a. | – | n.a. | – |
| C-10 | 104.95 | – | n.a. | – | n.a. | – |
| C-1′ | 132.46 | – | n.a. | – | n.a. | – |
| C-2′ | 115.76 | 7.030 (2.0) | 115.73 | 7.166 (2.2) | 115.70 | 7.013 (2.1) |
| C-3′ | n.a. | – | n.a. | – | n.a. | – |
| C-4′ | n.a. | – | n.a. | – | n.a. | – |
| C-5′ | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| C-6′ | 119.87 | 6.850 | 119.84 | 7.040 | 119.24 | 6.880 |
| Middle unit | | | | | | |
| C-2 | 78.88 | 5.699 | 78.93 | 5.251 | 84.50 | 4.615 (9.2) |
| C-3 | 72.57 | 4.121 (1.4) | 73.14 | 3.945 | 73.89 | 4.562 (9.2/9.2) |
| C-4 | 38.28 | 4.553 | 38.07 | 4.678 | 39.04 | 4.509 (9.2) |
| C-5 | 155.61 | – | n.a. | – | n.a. | – |
| C-6 | 96.03 | 5.802 | 96.47 | 6.108 | 97.15 | 5.796 |
| C-7 | n.a. | – | n.a. | – | n.a. | – |
| C-8 | n.a. | – | n.a. | – | n.a. | – |
| C-9 | n.a. | – | n.a. | – | n.a. | – |
| C-10 | 106.40 | – | n.a. | – | 108.72 | – |
| C-1′ | 131.78 | – | n.a. | – | n.a. | – |
| C-2′ | 116.74 | 7.312 (2.0) | 116.01 | 7.092 (1.5) | 116.44 | 7.194 |
| C-3′ | n.a. | – | n.a. | – | n.a. | – |
| C-4′ | n.a. | – | n.a. | – | n.a. | – |
| C-5′ | n.a. | n.a. | n.a. | n.a. | n.a. | n.a. |
| C-6′ | 121.39 | 7.185 (2.0/8.4) | 120.57 | 6.926 (1.5/7.9) | 121.17 | 7.140 (1.8/8.3) |
| Lower unit | | | | | | |
| C-2 | 80.28 | 4.384 | 79.80 | 4.963 | 79.64 | 4.366 |
| C-3 | 67.52 | 3.864 | 67.06 | 4.273 | 67.64 | 4.070 |
| C-4 | 29.89 | 2.832 | 29.76 | 2.932 (4.8/17.2) | 29.96 | 2.875 (17.0/4.9) |
| | | – | | 2.806 | | n.a. |
| C-5 | 155.81 | – | n.a. | – | n.a. | – |
| C-6 | 96.47 | 6.125 | 97.42 | 5.903 | 96.58 | 6.077 |
| C-7 | n.a. | – | n.a. | – | n.a. | – |
| C-8 | 108.50 | – | n.a. | – | n.a. | – |
| C-9 | n.a. | – | n.a. | – | n.a. | – |
| C-10 | 99.80 | – | n.a. | – | n.a. | – |
| C-1′ | 133.17 | – | n.a. | – | n.a. | – |
| C-2′ | n.a. | n.a. | n.a. | n.a. | 115.17 | 6.981 (1.8) |
| C-3′ | n.a. | – | n.a. | – | n.a. | – |
| C-4′ | n.a. | – | n.a. | – | n.a. | – |
| C-5′ | 116.01 | 6.753 (8.2) | n.a. | n.a. | n.a. | n.a. |
| C-6′ | 119.43 | 6.723 (1.8/8.2) | n.a. | n.a. | n.a. | n.a. |

n.a., not assignable due to overlay or low intensity.

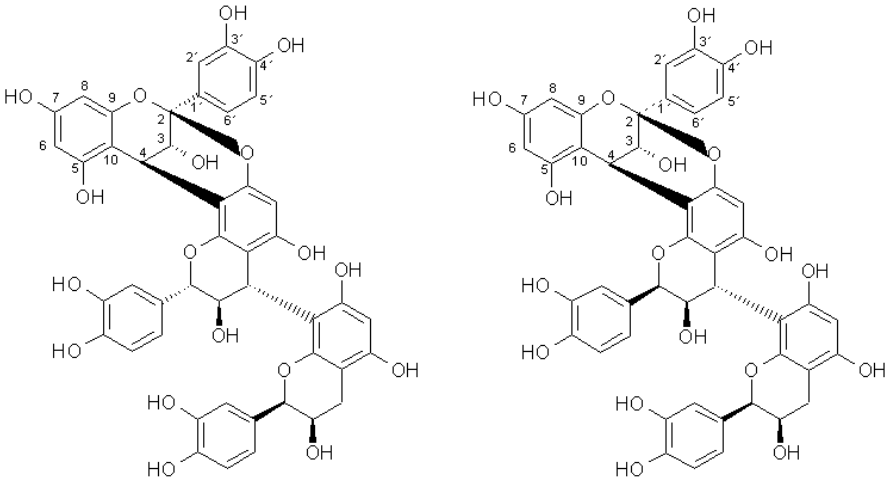


Fig. 4. Chemical structures of aesculitannin B and cinnamtannin B-1.

indicating the accumulation of polyploid cells in cultures exposed (Fig. 2, left panels). FACS analysis confirmed a loss of G1 cells from the culture and the appearance of a polyploid cell fraction (Fig. 2, right panels)

After repeated gel permeation chromatography of fr. 11 on Sephadex®LH 20 two main fractions showed cytostatic activity causing a cell cycle block and apoptosis. From those the procyanidin fraction Rx was isolated, showing cytotoxicity in the neutral red assay with an $IC_{50}$ value of 46 $\mu$M. This concentration induced apoptosis in SW480 cultures within 24 h as shown by both the appearance of apoptotic nuclei and the loss of mitochondrial membrane potential (Fig. 3).

Two trimeric proanthocyanidins were characterized by mass spectrometry, $^{1}H$, $^{13}C$ NMR spectroscopy as well as by 2D NMR techniques. The molecular weight of both compounds was 864 Dalton pointing to a molecular formula of $C_{45}H_{36}O_{18}$. The fragment at *m/z* 711 ([M-H-152]$^-$) was generated by the retro-Diels-Alder product of ring B in a catechin/epi-catechin, the one at *m/z* 573 ([M-H-290]$^-$ or [M-H-152-138]$^-$) by the cleavage of a monomeric catechin/epi-catechin and the retro-Diels-Alder reaction of this monomer to 4-(2-hydroxyethenyl)-1,2-benzenediol and 2,4,6-trihydroxybenzylium ion, respectively (Rohr *et al.*, 2000; Hayasaka *et al.*, 2003). By detailed NMR experiments via 1D- ($^{1}H$, $^{13}C$-ATP) und 2D-NMR (ge-DQF-COSY, ge-HSQC, ge-HMBC, NOESY) (see Table I) and comparison with published data aesculitannin B [epicatechin-(2$\beta\rightarrow O\rightarrow$7, 4$\beta\rightarrow$8)-*ent*-chatechin-(4$\beta\rightarrow$8)-epichatechin] and cinnamtannin B-1 [epicatechin-(2$\beta\rightarrow O\rightarrow$7, 4$\beta\rightarrow$8)-epichatechin-(4$\beta\rightarrow$8)-epichatechin] were identified (Kamiya *et al.*, 2001) (see Fig. 4). In addition all spectra showed small signals belonging to a third component. These resonances gave strong exchange crosspeaks with the corresponding signals of cinnamtannin B-1 in the 2D-NOESY and 2D-ROESY spectra, respectively. NMR self-diffusion measurements performed as diffusion difference experiments exhibited the same hydrodynamic radius for all proanthocyanidins. As a result of all NMR investigations cinnamtannin B-1 occurs as two rotamers in methanolic solution with a ratio of 3:1. The minor rotamer was to our knowledge up to now not characterized in the literature.

Cinnamtannin B-1 has been described in the bark of *Cinnamomum ceylanicum* (Nonaka *et al.*, 1983) for the first time and aesculitannin B from the episperm of *Aesculus hippocastanum* (Morimoto *et al.*, 1986). Interestingly, the occurrence of both procyanidines has been proven in *Parameria laevigata,* a plant used in traditional Indonesian medicine against ulcers (Kamiya *et al.*, 2001). Thus, the obtained results underlined the biological activity of these trimeric procyanidins.

In addition to the procyanidins several ubiquitous compounds such as glucose, fructose, chlorogenic acid and caffeic acid were identified in the

root extracts by TLC (Csontala, 2004; Rigler, 2005; Metnitzer, 2007). The identification of the widespread sterols sitosterol, stigmasterol and campesterol was performed by $^1$H NMR spectroscopy.

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