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ARTICLE

In Vitro Hepatic Biotransformation of Aspalathin and Nothofagin, Dihydrochalcones of Rooibos (*Aspalathus linearis*), and Assessment of Metabolite Antioxidant Activity

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Aspalathin (2′,3,4,4′,6′-pentahydroxy-3′-*C*-*β*-D-glucopyranosyldihydrochalcone) is the major flavonoid present in the South African herbal tea rooibos. In vitro metabolism of aspalathin and a structural analogue nothofagin, lacking the A ring catechol group, was investigated by monitoring the formation of glucuronyl and sulfate conjugates using Aroclor 1254 induced and uninduced rat liver microsomal and cytosolic subcellular fractions. Following glucuronidation of both aspalathin and nothofagin, HPLC-DAD, LC-MS, and LC-MS/MS analyses indicated the presence of two metabolites: one major and one minor. Only one aspalathin metabolite was obtained after sulfation, while no metabolites were observed for nothofagin. Two likely sites of conjugation for aspalathin are 4-OH or 3-OH on the A-ring. For nothofagin, the 4-OH (A-ring) and 6′-OH (B-ring) seem to be involved. The glucuronyl conjugates of aspalathin lack any radical scavenging properties in online postcolumn DPPH radical and ABTS radical cation assays. Deconjugation assays utilizing glucuronidase and sulfatase resulted in the disappearance of the metabolites, with the concomitant formation of the unconjugated form in the case of the glucuronidated product. The balance between conjugated and unconjugated forms of aspalathin could have important implications regarding its role in affecting oxidative status in intra- and extracellular environments in vivo.

KEYWORDS: Aspalathin; nothofagin; metabolism; biotransformation; rooibos; *Aspalathus linearis*; bioavailability; online antioxidant activity

INTRODUCTION

The biological properties of polyphenols have been the topic of many research investigations. Assessing biological activity in vitro does not give a true reflection of their activity in vivo, since polyphenols are known to be metabolized, especially by phase I and phase II enzymes in the liver, leading to high levels of conjugates in the plasma and urine (*1*). Studies in animals and humans indicated that these conjugation reactions also occur in the kidneys (*2*). This could explain why certain metabolites are not detected in the blood but high levels are present in urine. Metabolic alterations possibly reduce or abolish the biological properties associated with the parent compound in vitro (*3*). However, conjugation/deconjugation reactions in the liver related to sulfate and glucuronide metabolites play an important role in the physiological regulation of xenobiotics (*4*).

The health promoting properties of rooibos (*Aspalathus linearis*) have been reported in different in vitro and in vivo assays, stimulating studies on the bioactivity of its polyphenolic constituents, as they relate to their bioavailability and metabolism (*5*). Aspalathin and nothofagin (**Figure 1**), two *C*-linked dihydrochalcone glucosides, are the major flavonoid constituents in green rooibos (*6*), with nothofagin demonstrating slightly less potent antioxidant activity in an aqueous environment when using in vitro radical scavenging assays (*7*, *8*). Furthermore, nothofagin exhibits a far less protecting effect against Fe(II)-induced lipid peroxidation in a lipid environment when using a hydrophobic biomembrane assay system (*8*). Conformational differences and the absence of the catechol moiety in the nothofagin molecular structure were postulated to explain differences in their antioxidant effects in hydrophobic/hydrophilic environments (*8*).

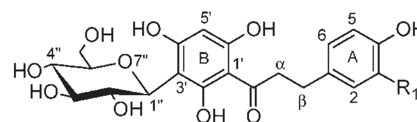

Figure 1. Structures of the *C*-*β*-D-glucopyranosyl dihydrochalcone aspalathin (R_1 = OH) and nothofagin (R_1 = H).

Recent studies on the in vivo metabolism of aspalathin showed that its deglycosylation is not a prerequisite for its

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absorption. *O*-linked methyl, sulfate, glucuronide, and *O*-methyl-*O*-glucuronide aspalathin metabolites were found to occur in the urine of human subjects after ingesting rooibos tea (*9*, *10*). No nothofagin, nor any of its metabolites, could be detected in urine in spite of a very low detection limit (*9*). Ingestion of an aspalathin-enriched green rooibos extract over a two week period showed no increase in the plasma antioxidant status of humans (*11*), probably due to either poor bioavailability and/or loss of potency as a result of aspalathin metabolism. A feeding study with pigs conducted with a similar green rooibos extract showed that aspalathin could not be detected in the plasma, except in trace quantities, when a high dose (ca. 480 mg/kg of body weight) was ingested (*12*). In contrast to the results of Stalmach et al. (*9*), Laue et al. (*13*) found unmetabolized aspalathin in the plasma samples of humans.

The aim of the present study was to investigate in vitro metabolism of aspalathin by subcellular rat liver fractions obtained from induced (Aroclor 1254) and uninduced (control) male Fischer rats. The metabolism of nothofagin was also investigated to provide information on the possible site of conjugation, since it lacks the catechol moiety. The effect of the in vitro biotransformation on the antioxidant activity of aspalathin was investigated with two online HPLC radical scavenging assays. Both $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ were used, as they display different scavenging mechanisms.

MATERIALS AND METHODS

Chemicals and Water Purification. Tris(hydroxymethyl)aminomethane (TRIZMA base) (≥99.9%), bovine serum albumin (BSA), Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid, 97%), citric acid (anhydrous), 2,2′-diphenyl-1-picrylhydrazyl (DPPH), uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), β-glucuronidase type IX-A from *E. coli* (1 134 600 units/g solids), sulfatase from *Helix pomatia* (15 000 units/g of solids), β-nicotinamide adenine dinucleotide phosphate (NADPH), and Sepharose 2B were obtained from Sigma Chemicals Co. (St. Louis, MO). Sodium dithionite (≤85%) (sodium hydrosulfite; $Na_2S_2O_4 + H_2O$; assay idiometric) and Triton-X-100 were supplied by BDH Chemicals Ltd. (Poole, England). Carbon monoxide (≥99.3%) was supplied by AFROX, BOC Special Products (Cape Town, South Africa). Dimethyl sulfoxide (DMSO for UV-spectroscopy, ≥99.8%) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were supplied by Fluka (Steinheim, Germany). BCA protein assay reagent A, containing sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 N sodium hydroxide, and BCA protein assay reagent B were purchased from Separations (Cape Town, South Africa). Aroclor 1254 was obtained from Monsanto (St. Louis, MO). Methylene chloride (99%) was supplied by Merck, Darmstadt, Germany. Aspalathin and nothofagin (purity of both ≥95% as determined by HPLC and LC-MS) were isolated from green rooibos and supplied by the PROMEC (Programme on Mycotoxins and Experimental Carcinogenesis) Unit of the Medical Research Council (MRC, Bellville, South Africa). Deionized water was prepared using a Modulab Water Purification System (Separations, Cape Town, South Africa). For HPLC eluant and sample preparation, deionized water was further purified by means of a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, MA). The solvents required for HPLC analysis were HPLC grade acetonitrile (Merck, Darmstadt, Germany) and formic acid (98%) supplied by BDH Chemicals Ltd. (Poole, England). Solvents for LC-MS and LC-MS/MS analyses were HPLC grade acetonitrile from Romil (Cambridge, U.K.) and formic acid from Sigma Chemicals Co. (St. Louis, MO).

Preparation of Rat Liver Microsomes and Cytosolic Fractions. Induced and uninduced livers were obtained from male Fischer rats. The induction of drug metabolizing enzymes was conducted according to the method described by Czygan et al. (*14*). The induction procedure involved dilution of Aroclor 1254 in sunflower oil to a concentration of 200 mg/mL and administration of a single intraperitoneal injection of 500 mg/kg to each Fischer rat (ca. 200 g) 5 days before sacrifice. Freshly excised livers were weighed and washed several times in chilled KCl before being homogenized in 0.15 M KCl (3:1 w/v) for 1 min, using a Thomas homogenizer (*15*). The homogenate was filtered through double-layered cheesecloth and homogenized using 10 strokes in a glass tissue grinder with a tight plunger. The homogenate was centrifuged at 9000*g* for 10 min, and aliquots of the supernatant (S9 fraction) were stored in glass vials at −80 °C until use.

Microsomal and cytosolic fractions were prepared by applying the S9 fraction to a Sepharose 2B column (2.8 cm × 30 cm, 1.7 mL/min flow rate) and eluting with a 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM KCl. The microsomal and cytosolic fractions were collected based on visual separation and stored at −80 °C. The fractions were thawed at room temperature on the day of the assay and diluted to the required protein concentration using ice-cold Tris-HCl buffer. The protein concentration of the microsomes and cytosol was determined according to the method described by Kaushal and Barnes (*16*) using BSA as standard. The use of experimental animals was approved by the Ethics Committee for Research on Animals of the Medical Research Council.

Phase II Conjugation. The phase II conjugation of aspalathin and nothofagin was investigated using microsomal and cytosolic fractions as the respective sources of glucuronosyltransferase and sulfotransferase. Cofactors comprised UDPGA and PAPS. The optimum protein concentration and incubation period for biotransformation were determined with aspalathin in preliminary experiments. Microsomal protein concentrations of 0.5, 1, and 2 mg/mL and cytosolic protein concentrations of 1, 2, and 4 mg/mL were used, while the incubation times investigated were 30, 60, 90, 120, and 180 min. Different cofactor and aspalathin (50−200 μM) concentrations were also employed to optimize glucuronidation. Solvents for reconstitution of samples were evaluated for optimal recovery prior to HPLC analyses and included 50% acetonitrile−water, DMSO, methanol, and deionized water.

To estimate extraction recoveries of aspalathin, a spiked sample with a known concentration of aspalathin added prior to incubation and cleanup was included in every experiment. Controls were incubated in the absence of the cofactor, while sample blanks, which did not contain the polyphenol or cofactor, were used to screen for interfering substances in the matrix during HPLC and LC-MS analyses.

The stabilities of aspalathin, nothofagin, and their metabolites in the reconstituted samples were investigated at room temperature by repeated HPLC analysis every 4 h over a period of 24 h. The stabilities of aspalathin and its metabolites during storage at −20 °C were monitored by analyzing two samples at day 0 and again after 21 days.

Glucuronidation. A typical reaction mixture (490 μL) for glucuronidation, consisting of 100 μM nothofagin or 200 μM aspalathin, 2 mg/mL microsomal protein, 2 mM NADPH, 0.02% Triton-X-100, and 50 mM Tris-HCl buffer (pH 7.4) was equilibrated for 3 min at 37 °C. The reaction was initiated by adding 10 μL of a 100 mM UDPGA stock solution to obtain 2 mM in the final reaction volume (500 μL). Incubations were carried out at 37 °C for 120 min in a shaking water bath. Each reaction was terminated by addition of an equal volume of ice-cold methanol to precipitate proteins, followed by centrifugation at 10 000*g* for 10 min at 4 °C. The supernatant was extracted with 500 μL of methylene chloride to remove lipids and Triton-X-100, followed by centrifugation at 10 000*g* for another 10 min at 4 °C. The supernatant was evaporated to dryness under a stream of nitrogen gas and stored at −20 °C. The dried samples were reconstituted in purified water (200 μL) and vortexed prior to HPLC analysis.

Sulfation. The final reaction mixture (500 μL) for sulfation contained either 100 μM nothofagin or 200 μM aspalathin, 25 mM $MgCl_2$, 50 μM PAPS, and cytosol (4 mg/mL in 50 mM Tris-HCl buffer, pH 7.4). The reaction was initiated by adding 10 μL of a 2.5 mM PAPS stock solution to obtain 50 μM PAPS in the final reaction volume. Samples were incubated for 60 min. Termination and subsequent sample preparation were conducted as described above.

Enzymatic Hydrolysis. Glucuronidation and sulfation of aspalathin were performed as described above. Following the respective incubation periods, the conjugation reaction was terminated by centrifugation at 16 000*g* at 4 °C for 30 min. Aliquots (200 μL) of the respective supernatants were incubated for 120 min at 37 °C with 20 μL of β-glucuronidase (5000 U/mL) or 20 μL of sulfatase (125 U/mL). This reaction was terminated by adding an equal volume of ice-cold

methanol and further prepared for HPLC analysis as described above. The control sample contained a buffer instead of β-glucuronidase or sulfatase.

HPLC Analysis. HPLC separations were conducted using an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) consisting of a quaternary pump, autosampler, in-line degasser, column thermostat, diode-array detector, and Chemstation software for LC 3D systems (Rev. B.02.01). The column used was a 150 mm × 4.6 mm i.d., 3 μm, Phenomenex Luna Phenyl-Hexyl, with a RP/C_{18} 5 μm Jour Guard column (Separations, Johannesburg, South Africa). Column temperature was maintained at 30 °C, and the following solvent gradient with acetonitrile (eluent A) and 1% formic acid (eluent B) at a flow rate of 1 mL/min was applied: 12% A (0−6 min); 12−18% A (6−7 min); 18−25% (7−14 min); 25−40% A (14−19 min); 40−90% A (19−24 min); 90% A (24−29 min); 90−12% A (29−35 min); 12% A (35−40 min). Samples were filtered through Millex-HV hydrophilic PVDF syringe filter units (0.45 μm, 4 mm, Millipore) directly into HPLC sample vials for duplicate injection (10 μL). Tentative identification was made on the basis of the UV/vis spectra and retention times of the aspalathin and nothofagin standards. Quantification of compounds at 288 nm for calculation of recoveries was based on peak area, obtained with valley-to-valley integration. The concentration range of the respective calibration series used for quantification was based on the expected concentration of the compounds in the spiked samples.

Liquid Chromatography−Mass Spectrometry (LC-MS) Analysis. LC-MS analysis of glucuronidated and sulfated samples was performed using a Waters API Quattro Micro triple quadropole mass spectrometer connected to a Waters Alliance 2690 quaternary HPLC pump with a 966 diode-array detector (Waters, Milford, MA). Sample preparation and separation conditions were the same as described for HPLC analysis. Electrospray ionization in the negative mode was carried out under the following conditions: desolvation temperature, 370 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.5 kV; and cone voltage, 18 V.

LC-MS/MS Analysis. For further confirmation of aspalathin conjugation, LC-MS/MS analysis was performed using a Waters API QTOF Ultima apparatus with a Waters UPLC system. Sample preparation and separation conditions were the same as described for HPLC analysis. Electrospray ionization in the negative mode was carried out under the following conditions: desolvation temperature, 350 °C; nitrogen flow rate, 350 L/h; source temperature, 100 °C; capillary voltage, 3.7 kV; cone voltage, 35 V; and collision energy, 20.

Qualitative Assessment of Radical Scavenging Ability of Aspalathin Metabolites. The sample mixture containing aspalathin and its glucuronidated metabolites was subjected to online HPLC-DPPH$^{\bullet}$ and HPLC-ABTS$^{\bullet+}$ analyses. The online radical scavenging methods were set up as described by Dapkevicius et al. (*17*) and Pellegrini et al. (*18*), respectively, with some modifications to improve radical intensity and prevent precipitation. The online system consisted of an LKB Bromma 2150 HPLC pump (Bromma, Sweden), an in-line Phenomenex Degasser Model DG-4400, and an LKB Bromma 2151 UV/vis variable wavelength detector. Mixing of the HPLC effluent and the radical solution, at a flow rate of 0.5 mL/min, was achieved with a high pressure static mixing tee (Upchurch, Anatech, Cape Town, South Africa). The reaction coil was made of 15.24 m PEEK tubing (0.25 mm i.d.) to give a reaction time of 0.5 min. Data were captured using DataApex Clarity v2.4.1.91 software (DataApex, Prague, Czech Republic). Scavenging of the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ was detected as negative peaks at 515 and 430 nm, respectively. An injection volume of 30 μL was used.

The DPPH$^{\bullet}$ stock solution (58 mg/L) was freshly prepared in acetonitrile on the day of analysis and kept in a flask protected from light. The working solution, containing 250 mL of a 0.068 mM citric acid phosphate buffer (6.8 mL of 0.01 M citric acid added to 93.2 mL of 0.02 M Na_2HPO_4 and made up to 1000 mL with deionized water) and 750 mL of DPPH$^{\bullet}$ in acetonitrile, was filtered (0.45 μm PVDF-filter, Milipore) before use.

The ABTS$^{\bullet+}$ stock solution (7 mM) was prepared as described by Pelligrini et al. (*18*). A working solution was prepared on the day of the analysis and contained 250 mL of citric acid phosphate buffer (6.8 mL of 0.01 M citric acid and 93.2 mL of 0.02 M Na_2HPO_4 made up to 1000 mL with deionized water), 25 mL of ABTS$^{\bullet+}$ stock solution, and 750 mL of acetonitrile. The temperature of the ABTS$^{\bullet+}$ solution was maintained at 4 °C using a thermostatically controlled cooling bath.

The antioxidant activity of the sulfated aspalathin and glucuronidated nothofagin was not investigated, since only a low level of biotransformation was achieved. It would not have been likely to observe activity of the metabolites at such a low concentration in the online system.

RESULTS

Optimization of Phase II Conjugation. The solvent used for reconstitution of the samples had a significant effect on the compound separation and repeatability of the HPLC chromatograms. Deionized water proved to be the optimum solvent for reconstitution (data not shown). Incubation for 120 min was sufficient to obtain a relatively high level of glucuronidated metabolites, while 60 min was adequate for sulfation. Aspalathin was stable in the reconstituted samples at room temperature over 24 h and when stored at −20 °C for 21 days, with its extraction recoveries varying from 90 to 100%. In contrast, nothofagin and its metabolites were rapidly degraded over time. Samples were therefore reconstituted less than 10 min before injection. No matrix peaks were observed in sample blanks in either microsomal or cytosolic fractions with HPLC and LC-MS analyses.

Glucuronidation. No metabolism was observed when incubating with the microsomal fraction (induced and uninduced) in the absence of the cofactor UDPGA (control samples) (**Figure 2A** and **C**). Glucuronidation of aspalathin with induced microsomes resulted in two conjugates, represented in the HPLC chromatogram (**Figure 2B**) by one minor peak (AG1) and one major peak (AG2). The retention times of AG1 and AG2 (10.65 and 11.54 min, respectively) corresponded to molecular species more polar than aspalathin (t_r = 11.95). Incubation with uninduced microsomes resulted in only one peak with the same retention time as that for AG2. In this case, a substantially smaller metabolite peak was obtained (data not shown). The LC-MS analysis confirmed the presence of a pseudomolecular ion ($[M - H]^-$) with m/z 627 (**Table 1**) for both metabolite peaks, indicating two regioisomers of monoglucuronidated aspalathin. Treatment with β-glucuronidase resulted in the disappearance of both peaks (AG1 and AG2), with concomitant formation of aspalathin, observed as an increase in the aspalathin peak (data not shown). LC-MS/MS analysis resulted in the characteristic *C*-linked glycoside losses of 90 and 120 amu, giving the respective fragment ions m/z 361 and 331 for aspalathin and m/z 537 and 507 for both aspalathin glucuronides, AG1 and AG2. Although the same fragment ions were produced for these metabolites, their relative abundances differed (**Table 2**). The neutral loss of 176 amu to give a fragment ion with m/z 451 is due to the cleavage of the glucuronide moiety.

Glucuronidation of nothofagin with induced microsomes also resulted in two metabolites: one major (NG1) and one minor peak (NG2) (**Figure 2D**). LC-MS analysis of the two metabolites gave $[M - H]^-$ ions at m/z 611 (**Table 1**), indicating that NG1 and NG2 correspond to two regioisomers of monoglucuronidated nothofagin. Incubation with uninduced microsomes only resulted in the presence of the major metabolite (NG1) (**Table 1**), and the extent of biotransformation was less than that with induced microsomes (data not shown).

Sulfation. No metabolism was observed when incubating the compounds with the cytosolic fraction (induced and uninduced) in the absence of the cofactor PAPS (control samples) (**Figure 2E**). Sulfation of aspalathin with the Aroclor-induced cytosolic rat liver fraction resulted in one metabolite (AS) (**Figure 2F**). A metabolite eluting at the same retention time was present when incubating aspalathin with uninduced cytosol. The retention time of AS (16.15 min) corresponds to a molecular species less polar than aspalathin (t_r = 11.71). LC-MS analysis of the sulfated samples of both induced and uninduced cytosolic

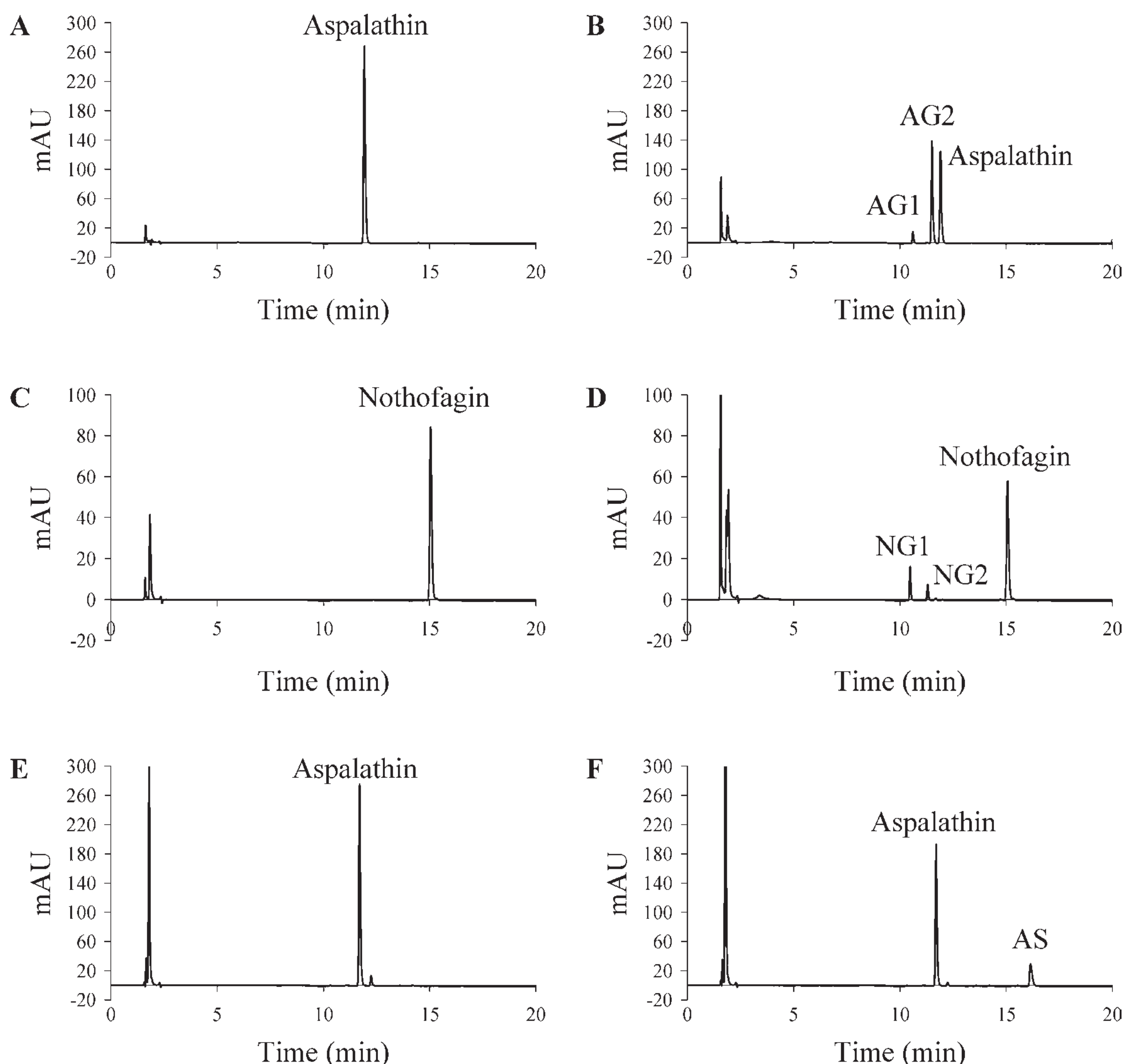

Figure 2. Representative HPLC chromatograms showing a control (absence of UDPGA) sample spiked with aspalathin (**A**); a sample containing aspalathin glucuronides (AG1 and AG2) (**B**); a control sample spiked with nothofagin (absence of UDPGA) (**C**); a sample containing nothofagin glucuronides (NG1 and NG2) (**D**); a control (absence of PAPS) sample spiked with aspalathin (**E**); and a sample containing sulfated aspalathin AS (**F**).

Table 1. LC-MS of Aspalathin, Nothofagin, and Glucuronidated and Sulfated Metabolites

liver fraction	compound	M_r	LC-MS ions (*m/z*)
	aspalathin	452	451
induced microsomes	aspalathin monoglucuronide (AG1)	628	627
induced microsomes	aspalathin monoglucuronide (AG2)	628	627
uninduced microsomes	aspalathin monoglucuronide (AG2)	628	627
induced cytosol	aspalathin monosulfate (AS)	532	531
uninduced cytosol	aspalathin monosulfate (AS)	532	531
	nothofagin	436	435
induced microsomes	nothofagin monoglucuronide (NG1)	612	611
induced microsomes	nothofagin monoglucuronide (NG2)	612	611
uninduced microsomes	nothofagin monoglucuronide (NG1)	612	611
induced cytosol	nothofagin sulfate	nd[a]	nd[a]

[a] nd = not detected.

fractions gave a pseudomolecular ion $[M-H]^-$ with *m*/*z* 531 (**Table 1**). Treatment with sulfatase resulted in disappearance of AS but also decreased the concentration of aspalathin. The sulfated metabolite (*m*/*z* 531) produced a fragment ion with *m*/*z* 451 (aspalathin) upon loss of 80 amu (sulfate moiety). The other two fragment ions present were *m*/*z* 361 and 331, characteristic of aspalathin. No sulfation of nothofagin was observed under the experimental conditions. HPLC analysis did not show any additional peaks to those present in the controls after incubation.

Table 2. LC-MS/MS of Aspalathin and Glucuronidated and Sulfated Metabolites

compound	precursor ion (*m/z*)	LC-MS/MS product ions (*m/z*) (% relative abundance)
aspalathin	451	361 (13); 331 (100); 209 (52); 167 (35)
aspalathin monoglucuronide (AG1)	627	627 (7); 537 (100); 507 (84); 361 (48); 331 (43)
aspalathin monoglucuronide (AG2)	627	627 (13); 537 (74); 507 (100); 361 (26); 331 (33)
aspalathin monosulfate	531	531 (14); 451 (42); 361 (100); 331 (62)

Qualitative Assessment of Radical Scavenging Ability of Metabolites. The combined HPLC and DPPH$^•$ quenching chromatograms of aspalathin and its glucuronidated metabolites (AG1 and 2) are shown in **Figure 3A**. Aspalathin scavenged DPPH$^•$, as manifested by the decrease in absorbance, while no activity was observed for the conjugates, even considering that AG2 was present in a slightly higher concentration than aspalathin. This

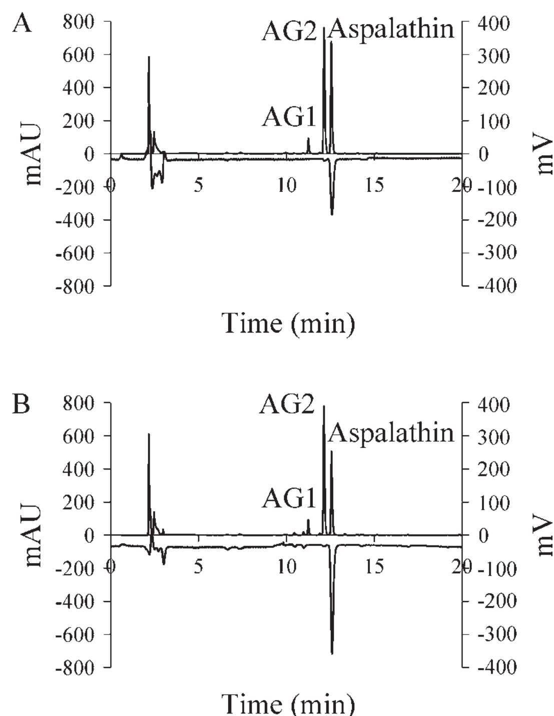

Figure 3. Combined UV and radical quenching chromatograms of aspalathin and glucuronidated metabolites. DPPH$^•$ (**A**); ABTS$^{•+}$ (**B**).

difference in peak height between AG2 and aspalathin was even more prominent in the sample used for testing the ABTS$^{•+}$ scavenging ability of the metabolites (**Figure 3B**). In spite of this, and the greater sensitivity of the ABTS$^{•+}$ scavenging assay, as implicated by the positive/negative peak ratios for aspalathin in the respective assays, no activity could be demonstrated for the metabolites.

DISCUSSION

Conjugation of catechols is one of the major metabolic reactions that occur in biological systems and allows for their rapid excretion into the bile and urine (*19*). Conjugation reactions are mainly catalyzed by UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), and the catechol *O*-methyl transferases (COMT) (*20*). Competition between the different conjugation reactions for the same substrate depends on various factors. These include the dosage of the polyphenol and the hydrophobic/hydrophilic nature of the subcellular environment (*19*, *21*). An increased dose will result in a shift from sulfation toward glucuronidation, since sulfation is generally a higher-affinity, lower-capacity pathway than glucuronidation (*21*). Methylation by COMT also results in a shift of conjugation of polyphenols from sulfation to glucuronidation (*22*).

Aspalathin, due to its prominence and radical scavenging activities, has been implicated in many of the antioxidant properties of rooibos (*5*). Nothofagin is also a good ABTS$^{•+}$ scavenger (*8*), and is of particular interest for the present study due to the absence of the catechol group, from which insight might be gained on the relative position of conjugation of dihydrochalcone *C*-glycosides. Aroclor 1254 was selected as an enzyme inducer, since it has been shown to enhance the glucuronidation rate of catechols up to 15-fold in rat liver microsomes (*23*). One major and one minor glucuronidated product of both aspalathin and nothofagin were observed after incubation with Aroclor 1254-induced microsomes. The presence of the aspalathin glucuronides was confirmed by LC-MS as indicated by a neutral loss of *m*/*z* 176, representing the glucuronide moiety. The fragmentation pattern of aspalathin and aspalathin-*O*-glucuronide, which is characteristic of *C*-linked glycosides (*24*), was in accordance with that previously reported (*9*, *12*).

Only one minor product was noticed for aspalathin and nothofagin when incubated with uninduced microsomes. PCB inducers such as Aroclor 1254 have been found to induce several UGT activities and, in particular, high-affinity enzyme isoforms (*23*), which explains the higher level of biotransformation and additional metabolite observed when using induced microsomes. This study indicates that the major glucuronidated product of aspalathin and nothofagin results from conjugation at the 4-OH, since nothofagin lacks the 3-OH on the A-ring. Glucuronidation studies of quercetin with human liver microsomes indicated four products, of which the catechol (4′-OH) also represents the major conjugate, followed by the 3′-OH (*3*). The minor glucuronidated product of aspalathin may result from conjugation of the 3-OH on the catechol group or one of the OH groups on the B-ring, presumably the 6′-OH, due to the steric hindrance of the *C*-glycoside involving the 2′- and 4′-OH groups. This became evident, as it is known that the conformational distribution of these dihydrochalcone exists due to hydrogen bonding between the C2′ and C4′-OH groups and the C2′-OH and the ethereal oxygen of the glucopyranosyl moiety (*8*). The minor glucuronidated metabolite of nothofagin will therefore most likely result from conjugation on the 6′-OH.

Sulfation yielded one minor conjugated product for aspalathin with both induced and uninduced rat liver cytosolic fractions. The MS fragmentation pattern obtained after sulfation was analogous to that reported for an aspalathin-*O*-sulfate (*9*). Sulfation seems to be one of the major pathways in the conjugation of aspalathin, since aspalathin-*O*-sulfate in addition to *O*-methyl glucuronide and *O*-methylsulfate conjugates has been identified in human urine (*9*). Nothofagin was not sulfated, which implies that the catechol is required for sulfation or that the sulfated product is unstable under in vitro and in vivo conditions.

The relative contribution of the conjugation reactions in the metabolism of aspalathin will depend on the distribution of aspalathin between the aqueous and lipid phases within the cell. At the physiological pH of 7.4, most polyphenols are associated with the polar head groups of the membrane phospholipids. This makes the OH groups more accessible to glucuronidation (*25*). However, more polar metabolites will largely favor the aqueous compartment phase, making them more accessible to cytosolic conjugation, such as methylation and sulfation. As the major urinary metabolite of aspalathin in humans is *O*-methyl glucuronide (*9*), it would appear that methylation in the cytosol and the subsequent glucuronidation in the endoplasmic reticulum are the major conjugating pathways involved. This agrees with the notion that methylation diverts the conjugation reaction away from sulfation (*22*).

Although conjugation of polyphenols has been recognized for many years, most biological studies have been carried out with the unmetabolized, naturally occurring, plant polyphenols. Glucuronidated products are one of the predominant forms of flavonoids in blood circulation, and their biological activities have been of interest (*26*). However, very little is known about the biological properties of conjugated polyphenolic derivatives due to the lack of commercial standards (*27*). Conjugation renders them more hydrophilic than the parent compounds, which may affect their site of action and their interaction with antioxidants in the cell if they retain their antioxidant properties. In the present study, the online HPLC antioxidant assays, utilizing DPPH$^•$ and ABTS$^{•+}$, showed that glucuronidation of aspalathin eradicated its antioxidant properties under the assay conditions. The assay

conditions, however, do not allow detection of slow radical scavengers due to the short reaction time (*17*).

Although aspalathin metabolites apparently lost their radical scavenging properties, the conjugated products are likely, as in the case of quercetin conjugates, to exhibit some other biological properties (*3*, *26*). Quercetin conjugates retain the differential inhibitory function of xanthine oxidase and lipooxygenase, with the 4′-*O*-glucuronidated product exhibiting an inhibitory effect relative to the parent compound with respect to inhibition of the xanthine oxidase (*3*). Glucuronides of quercetin and epicatechin also possess biological activity including antioxidant activity and inhibition of the growth of human lung cancer cells (*26*). The antioxidant activity of the nothofagin metabolite was not tested in the present study, but based on its structure and relative antioxidant activity in the $ABTS^{\bullet+}$ assay (*8*), it may be assumed that these metabolites would also display no activity. The antioxidant activity of the sulfated aspalathin was not investigated, since only a low level of biotransformation was achieved and it would not have been likely to observe activity of the metabolite at such a low concentration in the online system.

Unconjugated polyphenols and some of their conjugates may accumulate in the body to produce pharmacological activities (*26*). Therefore, despite the poor bioavailability of aspalathin and nothofagin (*5*, *9*), they are likely to accumulate in tissue and body fluids and still exert effects due to being available in both conjugated and unconjugated forms. In this regard, unmetabolized aspalathin has been detected in human plasma (*13*). In addition to the liver, glucuronidation could also occur in the kidneys and contribute to the presence of the conjugated products of aspalathin found in the urine of humans after consumption of rooibos (*9*, *10*). Some isoforms such as UGT1A8/9 that are highly expressed in the human kidney (*2*) exhibit a broad spectrum of substrate specificity and could be involved in the metabolism of aspalathin. Low concentrations of nothofagin in rooibos and/or the poor stability of the parent compound and metabolites could be reasons why they were not detected in urine. In this regard, phase II metabolism and subsequent bile excretion coupled to microbial metabolism are likely to lead to the enterohepatic recycling of conjugated and deconjugated forms. As the deconjugating enzyme β-glucuronidase is present in the blood, liver, and kidneys, it will result in sustained hepatic and/or blood circulation of polyphenols and/or their metabolites (*4*). Lysosomal β-glucuronidase released from neutrophils can deconjugate flavonoid glucuronides to the aglycone when injury such as inflammation occurs (*4*). The turnover rate of the conjugated and unconjugated forms of aspalathin will be important to evaluate its biological properties in vivo.

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