Pergamon

0031-9422(93)E066-N

Phytochemistry, Vol. 35, No. 6, pp. 1559–1565, 1994

0031-9422/94 $6.00+0.00

PHENOLIC METABOLITES FROM ROOIBOS TEA (*ASPALATHUS LINEARIS*)

CHARLENE RABE, JACOBUS A. STEENKAMP,* ELIZABETH JOUBERT,† JOHANN F. W. BURGER and DANEEL FERREIRA*

Department of Chemistry, University of the Orange Free State, P.O. Box 339, Bloemfontein, 9300 South Africa; †Infruitec, Private Bag X5013, Stellenbosch, 7599 South Africa

(*Received in revised form* 11 *October* 1993)

Key Word Index—*Aspalathus linearis*; Leguminosae; rooibos tea; phenolic metabolites; non-nutritive sweeteners; anti-oxidants.

Abstract—The processed leaves and stems of *Aspalathus linearis* contain hydroxylated benzoic and cinnamic acids, luteolin, chrysoeriol, quercetin, isoquercitrin, the C–C linked β-D-glucopyranosides based on four flavones and the dihydrochalcone aspalathin.

INTRODUCTION

Aspalathus linearis is a leguminous shrub indigenous to the mountainous areas of the north-western Cape in South Africa [1]. Its leaves and stems are used for the manufacture of rooibos tea, and it is increasingly recognized as one of the relatively few economic plants that has made the transition from a local wild resource to a cultivated crop in the 20th century. Rooibos tea is a unique beverage that is rich in volatile components, minerals and ascorbic acid, is caffeine-free, and is claimed to have a low tannin content (as gallic acid) [1, 2]. Owing to the absence of deleterious effects of the beverage on human health [1], rooibos tea is now exported to an increasing number of countries around the globe. The demonstration of the presence of the flavonol, quercetin, the flavone, luteolin, with their known antispasmodic properties [3, 4], and five additional flavonoid glycosides, the dihydrochalcone, aspalathin [5], the flavones, orientin and iso-orientin [6], and the flavonols, isoquercitrin and rutin [7], when considered in conjuction with the interest to utilize the scavenging effects of rooibos tea on active oxygen species [8], prompted re-investigation of the phenolic metabolites of this beverage.

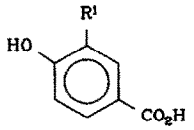
RESULTS AND DISCUSSION

To simulate the composition of the mixture that is reminiscent of a 'cup of rooibos tea', the aqueous extract of the commercial product, kindly supplied by the 'Rooibos Tea Control Board', Clanwilliam, was selected for the current investigation. The material was initially extracted with chloroform to remove chlorophyll and subsequently with boiling water to give an aqueous mixture which was successively extracted with hexane (to remove waxy materials), diethyl ether and ethyl acetate. The residual plant material was finally extracted exhaustively with acetone at ambient temperatures. Owing to the complexity of the various extracts, certain fractions had to be derivatized to attain an acceptable level of purity. A conspicuous feature of some of the *O*-acetyl derivatives was their exceptional solubility in water, which required replacement of the aqueous quench of the work-up procedure of the acetylation reaction with acetic anhydride–pyridine (cf. Experimental).

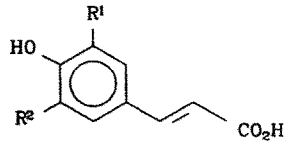
The ether extract afforded a mixture comprising a series of carboxylic acids, flavones, a flavonol and a flavonol glucoside. Comparison of the ^{1}H NMR spectra of the phenolic carboxylic acids, 4-hydroxybenzoic acid (**1**), protocatechuic acid (**2**) and vanillic acid (**3**), and the hydroxycinnamic acids, 4-coumaric acid (**4**), caffeic acid (**5**) and ferulic acid (**6**), with those of commercially available reference compounds, established their identity. In *A. linearis*, the hydroxycinnamic acids (**4**–**6**) co-exist with the 3,4,5-trihydroxy analogue (**7**) [*vide infra*], this natural source hence giving credence to the central position of activated hydroxycinnamic acids, i.e. as CoA esters, in the biosynthesis of various phenylpropanoid metabolites [9]. The anti-microbial properties of hydroxybenzoic acids have been firmly established [10–12]; thus, **1**–**3** may function as natural preservatives in rooibos tea. These simple hydroxylated benzoic and cinnamic acids were previously unknown in this natural source.

In addition to the aforementioned carboxylic acids, the ether extract also afforded 5,7,4′-trihydroxy-3′-methoxyflavone (**8**) {chrysoeriol [13], obtained from rooibos tea for the first time}, the 3′-*O*-demethyl analogue, luteolin (**10**) [4], the flavonol, quercetin (**13**) [4], and its 3-*O*-β-D-glucopyranoside derivative (**15**, isoquercitrin [7]). The ^{1}H NMR spectrum of chrysoeriol (**8**) displayed the anticipated H-3 singlet (δ6.69), an aromatic *O*-methyl resonance at δ3.99, the chelated hydroxylic 5-OH proton (δ8.44), and the aromatic AB- and ABX-spin systems for the A- and B-rings, respectively. The location of the *O*-

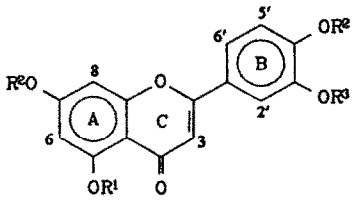
*Authors to whom correspondence should be addressed.



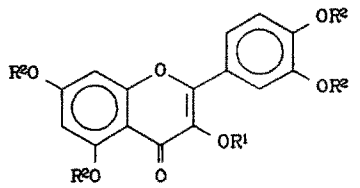
1 R^1=H
2 R^1=OH
3 R^1=OMe



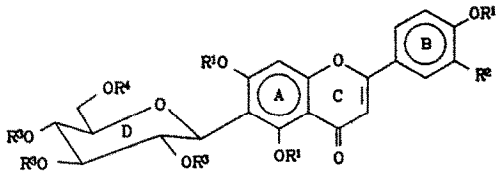
4 R^1=R^2=H
5 R^1=OH, R^2=H
6 R^1=OMe, R^2=H
7 R^1=R^2=OH



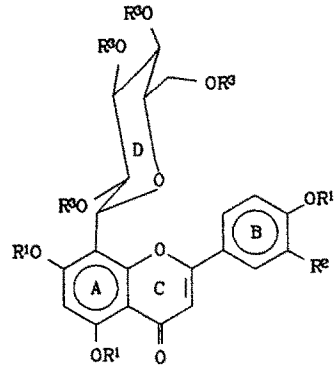
8 R^1=R^2=H, R^3=Me
9 R^1=H, R^2=Ac, R^3=Me
10 R^1=R^2=R^3=H
11 R^1=R^2=R^3=Me
12 R^1=Ac, R^2=R^3=Me



13 R^1=R^2=H
14 R^1=R^2=Ac
15 R^1=β-D-glucopyranosyl, R^2=H
16 R^1=tetra-*O*-acetyl-β-D-glucopyranosyl, R^2=Ac



17 R^1=R^2=R^3=R^4=H
18 R^1=R^4=Me, R^2=H, R^3=Ac
19 R^1=Me, R^2=H, R^3=R^4=Ac
20 R^1=R^3=R^4=H, R^2=OH



21 R^1=R^1=R^3=H
22 R^1=Me, R^2=H, R^3=Ac
23 R^1=R^3=H, R^2=OH

methyl group was confirmed by NOE experiment, which indicated selective association of these protons with H-2′ (δ7.37, 9.8%) in the di-*O*-acetyl derivative (**9**). Structural elucidation of luteolin (**10**), quercetin (**13**) and isoquercitrin (**15**) was performed by comparison of the ^{1}H NMR data of the underivatized compounds, the 3′,4′,7-tri-*O*-methyl ether (**11**) and 5′-*O*-acetyl-3′,4′,7-tri-*O*-methyl ether (**12**) of luteolin, and the peracetates (**14**) and (**16**) of quercetin and isoquercitrin, respectively, with those of the same derivatives of authentic specimens.‡

Besides the flavone, luteolin (**10**), the ethyl acetate fraction also afforded 3,4,5-trihydroxycinnamic acid (**7**) and a series of C–C linked β-D-glucopyranosides based on flavones, and a dihydrochalcone. During PLC separation the trihydroxycinnamic acid (**7**) migrated in a band (4.5 mg) containing also protocatechuic acid (**2**) and caffeic acid (**5**), a mixture which was inseparable even after methylation. Identification of the methyl (3,4,5-trimethoxy) cinnamate was thus effected by comparison of the ^{1}H NMR data of a synthetic sample of this compound obtained via a Perkin reaction using 3,4,5-trimethoxybenzaldehyde in acetic anhydride–triethylamine–potassium acetate, and those of authentic specimens of the methyl ether esters of protocatechuic and caffeic acid. Notably, NOE experiment on the methyl ether ester of **7** indicated association of the two-proton aromatic singlet (δ6.73) with two equivalent methoxy resonances (δ3.87, 1.4%), and with both protons of the vinylic system (δ 6.33, 11.5%; δ7.59, 13.2%, $^3J_{\alpha,\beta}$ = 16.0 Hz).

‡One of us (E.J.) has access to the collection of samples from unprocessed rooibos tea of the late Dr B. H. Koeppen.

Table 1. ^{1}H NMR peaks (ppm) of flavone glucosides (**18–20**, **22** and **23**) at 300 MHz. Splitting patterns and J-values (Hz) are given in parentheses

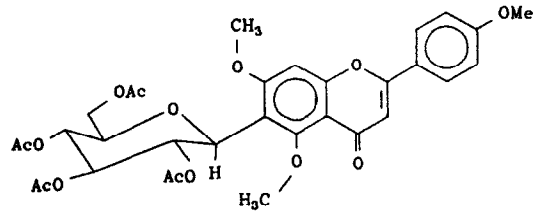
Ring	H	**18** ($CDCl_3$, 296 K)	**19** ($CDCl_3$, 296 K)	**20** (Me_2CO-d_6 D_2O, 296 K)	**22** ($CDCl_3$, 296 K)	**23** (DMSO-d_6, 393 K)
A	6	—	—	—	6.38, 6.33 (each *s*)	6.50 (*s*)
	8	6.73 (*s*)	6.76 (*s*)	6.54 (*s*)	—	—
B	2 (2/6)	7.80 (*d*, 9.0)	7.80 (*d*, 9.0)	7.47 (*d*, 2.2)	7.98, 7.75 (each *d*, 8.9)	7.45*
	5 (3/5)	6.99 (*d*, 9.0)	6.99 (*d*, 9.0)	6.97 (*d*, 8.1)	7.12, 7.07 (each *d*, 8.9)	6.90 (*d*, 8.9)
	6	—	—	7.43 (*dd*, 2.2, 8.1)	—	—
C	3	6.56 (*s*)	6.57 (*s*)	6.59 (*s*)	6.59, 6.55 (each *s*)	6.24 (*s*)
D	1	5.07 (*d*, 10.1)	5.10 (*d*, 10.1)	4.91 (*d*, 10.0)	5.22, 5.24 (each *d*, 10.1)	8.45 (*d*, 9.8)
	2	5.97 (*dd*, 9.4, 10.1)	6.01 (*dd*, 9.4, 10.1)	3.91 (*dd*, 9.0, 10.0)	5.99, 5.87 (each *dd*, 9.2, 10.1)	3.89 (*dd*, 8.7, 9.8)
	3	5.31 (*t*, 9.4)	5.32 (*t*, 9.4)	3.59 (*t*, 9.0)	5.39, 5.36 (each *t*, 9.2)	3.47 (*dd*, 8.0, 8.7)
	4	5.18 (*dd*, 9.4, 10.1)	5.18 (*dd*, 9.4, 10.2)	3.54 (*t*, 9.0)	5.51, 5.19 (*dd*, 9.2, 10.0)	3.43 (*dd*, 8.0, 9.0)
	5	3.78 (*m*)	3.83 (*m*)	3.45 (*m*)	3.85 (*m*)	3.37 (*m*)
	6	3.50 (*dd*, 5.8, 11.1)	4.20 (*dd*, 5.5, 12.8)	3.79 (*m*, 2 × H)	4.12–4.33 (*m*)	3.79 (*dd*, 2.7, 11.5)
		3.44 (*dd*, 2.8, 11.1)	4.12 (*dd*, 2.8, 12.8)			3.65 (*dd*, 5.0, 11.5)
	OMe	3.97 (7-A), 3.88 (5-A), 3.87 (4-B), 3.30 (6-D) (each *s*)	3.99 (7-A), 3.89 (5-A), 3.85 (4-B) (each *s*)		4.00, 3.98 (2 ×), 3.95, 3.90, 3.89 (each *s*)	
	OAc	1.74, 2.51, 2.01 (each *s*)	2.51, 2.48, 2.01, 1.75 (each *s*)		2.08 (2 × OAc), 2.03, 2.03, 1.96, 1.91, 1.70, 1.68 (each *s*)	

*Second order.

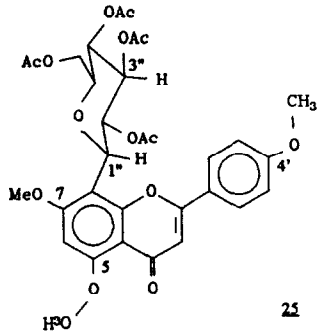
Although the 5,7,4′-trihydroxy-6-C-β-D-glucopyranosylflavone, isovitexin (**17**), and its C-8 isomer, vitexin (**21**), and their glycosidic derivatives exhibit a wide taxonomic distribution [14], their presence in rooibos tea is now demonstrated for the first time. Successive methylation and acetylation of the fraction containing isovitexin (**17**) and vitexin (**21**) with a view to purifying these compounds, afforded the tetra-*O*-methyl-tri-*O*-acetylisovitexin (**18**), the phenolic trimethyl ether tetra-*O*-acetyl derivative (**19**), and the tri-*O*-methyl-tetra-*O*-acetylvitexin (**22**). The structure of tri-*O*-methyl-tetra-*O*-acetylisovitexin (**19**) was evident by comparison of its ^{1}H NMR data (Table 1) at 300 MHz in $CDCl_3$ with those in the literature [15, 16]. Several novel features, however, emerged from this investigation at high magnetic field strength: (i) The chemical shifts of H-3 and H-8 (δ6.57, 6.76, respectively) were unambiguously differentiated via a NOE experiment, which showed selective association of H-3 with H-2′ and H-6′ (δ7.80, *d*, J = 9.0 Hz, 7.8%) and of H-8 with 7-OMe (δ3.99, 2.8%); (ii) In contrast to previous observations [17], the spectrum displayed duplication of signals at 23° reminiscent of the effects of dynamic rotational isomers and culminating in a *ca* 4:1 ratio of rotamer populations. In the main rotamer, the anomeric proton (δ5.10, *d*, J = 10.1 Hz) exhibited NOE association with 5-OMe (δ3.89, 1.26%), but not with 7-OMe (δ3.99). Such a selectivity presumably reflects a preferred conformation (**24**), in which the 5-OMe function is rotated along the O-Me bond axis to minimize 1,3-allylic strain [18] with the rigid and sterically demanding 4-carbonyl group. The 7-OMe is probably rotated similarly in order to alleviate steric strain with the 6-C-β-D-glucopyranosyl moiety; (iii) Despite the fact that isovitexin was first identified in the early sixties [15], we could not find published data for the chemical shifts of the glucosidic protons, except for the anomeric proton, and are thus presenting full ^{1}H NMR data for both derivatives **18** and **19** in Table 1.

Notable also is the formation of a tetra-*O*-methyl ether (**18**) involving methylation of the primary 6″-OH function of the sugar moiety with diazomethane. The ^{1}H NMR spectrum (Table 1) of this derivative is very similar to that of the tri-*O*-methyl ether (**19**), but for replacement of an acetoxy resonance (δ2.48) in the latter compound with a methoxy signal (δ3.30) in the former. Involvement of the glucosidic 6″-OH group in the methylation process, implicated by the chemical shift of the 6″-methylene protons, was confirmed by the NOE association of this methoxy group with both H-5″ (δ3.78, *m*, 4.1%) and one of the 6-CH_2 protons (δ3.44, 2.5%) of the sugar moiety. The preferential methylation of 6″-OH in the presence of the three secondary glucosidic hydroxyl functions is presumably explicable in terms of a prototropic equilibrium between the four possible oxy-anionic species which are stabilized by intramolecular hydrogen bonding [19].

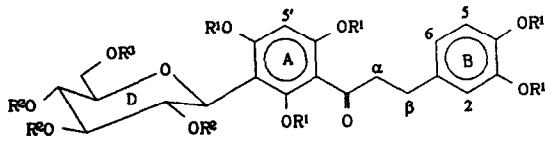
The ^{1}H NMR spectrum of tri-*O*-methyl-tetra-*O*-acetylvitexin (**22**) at 23° displayed the typical duplication of signals that characterizes the spectra of derivatized C-8 carbon–carbon linked glycoflavones [16, 17, 20] and flavonols [21]. Full assignment of signals (Table 1) of both rotamers (*ca* 3:2 ratio) was again facilitated by the utilization of extensive homonuclear NOE experiments and spin decouplings. Such an approach also provided additional proof for the position of the β-D-glucopyranosyl moiety (δ5.24, 5.25, both *d*, J = 10.1 Hz, anomeric



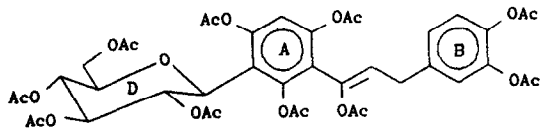
24



25



26 $R^1=R^2=R^3=H$
27 $R^1=R^2=R^3=Ac$
28 $R^1=Me$, $R^2=R^3=Ac$
29 $R^1=R^3=Me$, $R^2=Ac$



30

proton) at C-8 and presumably also preferred conformation **25** for the main rotamer via the mutual NOE effects of 4'-OMe (δ3.89) and H-1'' (δ5.24, 5.25), H-3'' (δ5.39, 5.36), and H-4'' (δ5.51, 5.19) of the glucosyl unit. These 'long-distance' NOE associations of B-ring and C-8 glucosyl protons have previously been observed by us [22] for derivatized regio-isomeric glycoflavonols.

In contrast with the duplication of signals in the ^{1}H NMR spectra of the isovitexin and vitexin derivatives (**18**, **19**, and **22**), the 300 MHz spectrum (Table 1) of isoorientin (**20**) showed no evidence of rotational isomerism at 23° in $(CD_3)_2CO$–D_2O. The severe line-broadening in the spectrum of orientin (**23**) necessitated investigation at elevated temperature (120°) in DMSO-d_6 where assignment of all signals became possible. These isomeric compounds were identified and differentiated by comparison of their ^{1}H NMR data (Table 1) with those of authentic specimens previously isolated from *A. linearis* [6].

Following demonstration of the presence of the 2',3,4,4',6'-pentahydroxy-3-C-β-D-glucopyranosyldihydrochalcone, aspalathin (**26**), hithertho unique to *A. linearis* [5, 7, 23], in the acetone extract of the processed leaves and stems of this natural source, we focused on full analysis of the ^{1}H NMR data at 300 MHz (Table 2) of authentic aspalathin (**26**) and its derivatives (**27**–**30**) from the unprocessed rooibos, since only data at 60 MHz have thus far been published [5, 23]. Allocation of signals was either straightforward or required a few well-selected decouplings at ambient conditions or at elevated temperatures to eliminate the effects of rotational isomerism (cf. Table 2). Notable is the formation of an inseparable mixture of both the nona-*O*-acetyl derivative (**27**) and the enol acetate (**30**) when aspalathin (**26**) was acetylated with acetic anhydride–pyridine. These compounds were, however, separately accessable via acetylation with, respectively, acetic anhydride–70% perchloric acid in anhydrous ethyl acetate and acetic anhydride in pyridine–triethylamine. The main difference in the ^{1}H NMR spectra of the peracetate (**27**) of aspalathin and the enol acetate (**30**) in $CDCl_3$ is the replacement of the A_2B_2-system (α-CH_2: δ3.05, *m*; β-CH_2: δ2.93, *m*) of the 1,3-diarylpropanone moiety in **27** by an AM_2-system (vinylic H: δ5.53, *t*, $J=8.0$ Hz; β-CH_2: δ3.24, *d*, $J=8.0$ Hz) in the enolate (**30**).

Formation of the enol acetate (**30**) under mild conditions represents, as far as may be established, the first example of the ready *in vitro* formation of a keto–enol equilibrium in the chemistry of the $C_6.C_3.C_6$-metabolites. Such an equilibrium may represent the first step of the biosynthetic oxygenation sequence leading to the equivalent of C-3 hydroxylation, hence giving additional credence to the presumed central role of the α-hydroxychalcone-α-hydroxydihydrochalcone pair in flavonoid biosynthesis [24–26]. We are currently investigating the possible role of the glucosyl unit in the establishment of the keto–enol equilibrium in dihydrochalcones.

Methylation of aspalathin (**26**) with diazomethane and subsequent acetylation afforded the penta-*O*-methyl-tetra-*O*-acetyl and hexa-*O*-methyl-tri-*O*-acetyl derivatives (**28**) and (**29**). The unexpected methylation of the 6-OH function of the glucosyl moiety is presumably explicable in terms of the same phenomenon that was advanced above to verify a similar observation during methylation of isovitexin (**17**).

In contrast to the bitter taste of black tea, rooibos tea possesses a natural sweet taste, which substantially contributes towards its appeal as a health beverage. This sweetness may well in part result from the presence of the dihydrochalcone, aspalathin (**26**), following demonstration of the non-nutritive sweet characteristics of this and related structural types [27–29]. Our current efforts aimed at unravelling the phenolic profile in the complex metabolic pool of *A. linearis* thus also focus on the phenomenon of the natural sweetness of rooibos tea, as well as the potential of the various phenolic compounds

Table 2. ^{1}H NMR peaks (ppm) of aspalathin (**26**) and derivatives (**27**–**30**) at 300 MHz. Splitting patterns and *J*-values (Hz) are given in parentheses

Ring	H	**26** (Me_2CO-d_6, 296 K)	**27** ($CDCl_3$, 353 K)	**28** ($CDCl_3$, 296 K)	**29** ($CDCl_3$, 296 K)	**30** (C_6D_6, 353 K)
A	5	5.91 (*s*)		6.21 (*s*)	6.19 (*s*)	7.05 (*s*)
B	2	6.74 (*d*, 2.1)	7.01–7.07*	6.72–6.81*	6.66*	7.07 (*d*, 2.0)
	5	6.71 (*d*, 8.0)				7.01 (*d*, 8.5)
	6	6.57 (*dd*, 2.1, 8.0)				6.87 (*dd*, 2.0, 8.5)
	α	3.31 (*t*, 8.0)	3.05 (*m*)	3.01 (*t*, 7.2)	3.00 (*t*, 7.2)	5.53 (*t*, 8.0)
	β	2.81 (*t*, 8.0)	2.93 (*m*)	2.91 (*m*)	2.91 (*m*)	3.24 (*d*, 8.0)
D	1	4.92 (*d*, 10.0)	4.67 (*d*, 10.0)	4.75, 4.96 (*d*, 10.2)	4.72 (*d*, 10.2)	4.79 (*d*, 10.0)
	2	3.64 (*dd*, 8.9, 10.0)	5.59 (*dd*, 9.0, 10.0)	6.00, 5.81 (*dd*, 9.0, 10.2)	5.60 (*dd*, 9.0, 10.2)	6.01 (*dd*, 9.0, 10.0)
	3	3.53 (*t*, 8.9)	5.22 (*dd*, 9.0, 9.2)	5.25, 5.26 (*t*, 9.0)	5.25 (*t*, 9.0)	5.45 (*t*, 9.0)
	4	3.63 (*dd*, 8.9, 9.8)	5.10 (*dd*, 9.2, 9.8)	5.17 (*dd*, 9.0, 10.0)	5.16, 5.14 (*dd*, 9.0, 10.2)	5.30 (*dd*, 9.0, 10.5)
	5	3.47 (*m*)	3.72 (*m*)	3.75 (*m*)	3.45 (*m*)	3.50 (*m*)
	6	3.84 (*m*)	4.32 (*dd*, 5.6, 13.0)	4.21 (*dd*, 5.0, 12.5)	4.20 (*m*)	4.40 (*dd*, 4.9, 12.5)
			4.01 (*dd*, 2.8, 13.0)	4.10 (*dd*, 2.3, 12.5)	4.15 (*m*)	3.88 (*dd*, 2.0, 12.5)
	OMe			3.63, 3.75, 3.83 (H-6), 3.85, 3.88 (each *s*)	3.30 (6-D), 3.87, 3.86, 3.84, 3.74, 3.62 (each *s*)	
	OAc		1.77, 1.97, 2.00, 2.03, 2.10, 2.17, 2.23 (×2), 2.34 (each *s*)	2.04, 2.02, 2.01, 2.00 (each *s*)	2.16, 2.04, 2.00 (each *s*)	2.07, 2.04, 1.86, 1.85, 1.82, 1.73, 1.72 (×2), 1.70, 1.62 (each *s*)

*Second order.

to exercise a scavenging effect on active oxygen species [8]. These results will be discussed in an impending manuscript.

EXPERIMENTAL

^{1}H NMR spectra were recorded in various solvents with TMS as int. standard. TLC was performed on precoated Merck plastic sheets (silica gel 60 F_{254}, 0.25 mm) and the plates sprayed with H_2SO_4–HCHO (40:1) after development. Prep. plates (PLC), 20 × 20 cm, Kieselgel PF_{254} (1.0 mm) were air-dried and used without prior activation. Small scale prep. sepns were on Merck precoated plates (silica gel PF_{254}, 0.25 mm). Compounds were recovered from the adsorbent with Me_2CO. Two-dimensional paper chromatograms were on Whatman No. 1 paper in H_2O-satd 2-BuOH and 2% HOAc soln. CC was on Sephadex LH-20, silica (Flash CC), or cellulose. Methylations were performed with an excess of CH_2N_2 in MeOH–Et_2O at −15° for 48 hr, while acetylations were in Ac_2O–pyridine at ambient temps. Evapns were done under red. pres. at 45° in a rotary evaporator and freeze-drying of aq. solns on a Virtis 12 SL freezemobile.

Extraction and fractionation. The commercial form of *A. linearis* (15 × 200 g) was extracted with $CHCl_3$ (15 × 2.5 l) in a Soxhlet extractor for 15 hr. The dry residual material (2 × 1 kg) was boiled in H_2O (2 × 4 l) for 15 min, the mixt. left at room temp. for 24 hr, and decanted. This process was repeated twice, and the aq. extracts were combined and extracted with hexane (2 × 675 ml) to remove waxy materials. The residual H_2O phase was successively extracted with Et_2O (7 × 500 ml) and EtOAc (7 × 500 ml), the resulting extracts separately dried (Na_2SO_4) and evapd to dryness to give yellow solids, 6.28 g (from Et_2O) and 10.95 g (from EtOAc). The dried plant material was subsequently extracted with Me_2CO (3 × 2.5 l, 24 hr each) at room temp., and the solvent evapd to give a dark-green solid (13 g).

Metabolites from the ether extract. The ether extract was treated with EtOH (125 ml), the mixt. filtered, and the EtOH evapd to give a solid (5.93 g) which was subjected to CC on Sephadex LH-20 (2 × 65 cm column, 11.0 ml frs) in a gradient solvent system [EtOH (1.82 l), 5% MeOH in EtOH (520 ml), 10%MeOH in EtOH (275 ml), and 30% MeOH in EtOH (88 ml)] to give 15 frs, A_1 (tubes 35–38, 110 mg), A_2 (39–46, 220 mg), A_3 (47–58, 140 mg), A_4 (59–63, 30 mg), A_5 (64–73, 90 mg), A_6 (74–78, 30 mg), A_7 (79–92, 70 mg), A_8 (93–96, 10 mg), A_9 (97–110, 180 mg), A_{10} (111–124, 50 mg), A_{11} (125–132, 20 mg), A_{12} (133–150, 70 mg), A_{13} (151–166, 40 mg), A_{14} (167–210, 20 mg) and A_{15} (211–230, 10 mg). Owing to their complexity and/or the limited sample quantities, frs A_4, A_6, A_8 and A_{10}–A_{12} were not further investigated.

Fr. A_1 was further resolved by PLC in C_6H_6§–Me_2CO–MeOH (7:2:1) to give 2 bands at R_f 0.63 (40 mg) and 0.45 (80 mg). Purification of the former band by PLC in C_6H_6–Me_2CO (9:1) gave vanillic acid (**3**, R_f 0.36, 2.3 mg) and of the latter band in the same system, 4-hydroxybenzoic acid (**1**, R_f 0.31, 1.0 mg).

Prep. TLC of fr. A_2 in C_6H_6–Me_2CO–MeOH (7:2:1) gave a band at R_f 0.40 (100 mg). This was subjected to PLC in C_6H_6–Me_2CO (9:1) to give ferulic

§Owing to its adverse physiological effects benzene should be used with the necessary care.

acid (**6**, R_f 0.45, 2.0 mg) and 4-coumaric acid (**4**, R_f 0.44, 3.0 mg) and PLC of fr. A_3 in C_6H_6–Me_2CO–MeOH (7:2:1) afforded protocatechuic acid (**2**, R_f 0.23, 8.0 mg) and caffeic acid (**5**, R_f 0.68, 2.0 mg).

Fr. A_5 was resolved by PLC in C_6H_6–Me_2CO–MeOH (7:2:1) to give chrysoeriol (**8**, R_f 0.48, 20 mg), which gave the 4′,7-di-*O*-acetyl derivative (**9**) after acetylation. PLC of fr. A_7 in C_6H_6–Me_2CO–MeOH (7:2:1, ×2) afforded luteolin (**10**, R_f 0.68, 2 mg).

Acetylation of fr. A_9 followed by PLC in hexane–EtOAc–Me_2CO (60:25:25) gave octa-*O*-acetylisoquercitrin (**16**, R_f 0.27, 3 mg). Fr. A_{13} afforded quercetin (**13**, R_f 0.68, 4.1 mg) following PLC in C_6H_6–Me_2CO–MeOH (7:2:1, ×2).

Metabolites from the EtOAc *extract.* The EtOAc extract (10.95 g) was sepd by CC on Sephadex LH-20 (7 × 90 cm column, 13.5 ml frs) in gradient solvent system [EtOH (2.7 l), 5% MeOH in EtOH (556 ml), 10% MeOH in EtOH (440 ml) and 15% MeOH in EtOH (2.4 l) to give 17 frs, B_1 (tubes 1–34, 140 mg), B_2 (35–53, 310 mg), B_3 (54–64, 370 mg), B_4 (65–80, 650 mg), B_5 (81–102, 1.59 g), B_6 (103–114, 687 mg), B_7 (115–124, 248 mg), B_8 (125–131, 135 mg), B_9 (132–140, 186 mg), B_{10} (141–149, 240 mg), B_{11} (150–164, 370 mg), B_{12} (165–200, 1.54 g), B_{13} (201–220, 401 mg), B_{14} (221–236, 416 mg), B_{15} (237–268, 352 mg), B_{16} (269–320, 179 mg), and B_{17} (321–457, 295 mg). Only the frs (B_{11} and B_{15}) that showed a reasonable degree of simplification by 2D paper chromatograms and that did not coincide with frs from the ether extract, were further investigated.

A portion (270 mg) of fr. B_{11} was methylated with CH_2N_2 and part of the mixt. (130 mg) sepd by prep. TLC in C_6H_6–Me_2CO–MeOH (4:5:1, ×2) to give 11 bands, each of which was acetylated directly, $B_{1.1}$ (R_f 0.85, yield of methyl ether acetate—13.6 mg), $B_{1.2}$ (R_f 0.77, 6.5 mg), $B_{1.3}$ (R_f 0.71, 5.4 mg), $B_{1.4}$ (R_f 0.59, 11.4 mg), $B_{1.5}$ (R_f 0.53, 5.7 mg), $B_{1.6}$ (R_f 0.46, 0.9 mg), $B_{1.7}$ (R_f 0.33, 4.2 mg), $B_{1.8}$ (R_f 0.25, 13.9 mg), $B_{1.9}$ (R_f 0.19, 7.6 mg), $B_{1.10}$ (R_f 0.09, 5.1 mg), $B_{1.11}$ (R_f 0.04, 4.8 mg). The derivative of $B_{1.1}$ (13.6 mg) was purified by PLC in hexane–EtOAc–Me_2CO (60:25:25) to give 5-*O*-acetyl-3′,4′,7-tri-*O*-methylluteclin (**12**, R_f 0.47, 5 mg). Bands $B_{1.2}$ and $B_{1.3}$ were combined and purified by PLC in hexane–EtOAc–Me_2CO (12:5:5) to give tetra-*O*-methylluteolin (**11**, R_f 0.09, 3.0 mg). The derivatives of bands $B_{1.5}$ and $B_{1.6}$ were combined (6.6 mg) and purified by PLC in hexane–EtOAc–Me_2CO (60:25:25) to give 2″,3″,4″-tri-*O*-acetyl-4′,5,6″,7-tetra-*O*-methylisovitexin (**18**) as a solid (R_f 0.31, 1.5 mg), ^{1}H NMR data (Table 1). Similar combination of the derivatives of bands $B_{1.8}$ and $B_{1.9}$ (21.5 mg) and purification by PLC in $CHCl_3$–Me_2CO (4:1) afforded 2″,3″,4″,6″-tetra-*O*-acetyl-4′,5,7-tri-*O*-methylisovitexin (**19**, R_f 0.38, 4.9 mg) and 2″,3″,4″6″-tetra-*O*-acetyl-4′,5,7-tri-*O*-methylvitexin (**22**, R_f 0.01, 2.3 mg), both as solids, ^{1}H NMR data (Table 1). The derivative of band $B_{1.11}$ comprised of an inseparable mixt. of methyl-(3,4-dimethoxy)-benzoate,-(3,4-dimethoxy)cinnamate, and -(3,4,5-trimethoxy) cinnamate.

Fr. B_{15} (352 mg) was resolved by CC on silica under flash conditions into 2 sub-frs at R_f 0.67 (5.4 mg) and 0.12 (83.9 mg), according to TLC in C_6H_6–Me_2CO–MeOH (6:3:1). Acetylation of the R_f 0.67 band followed by PLC in hexane–EtOAc–Me_2CO (60:25:25) afforded penta-*O*-acetylquercetin (**14**) as a tan solid (R_f 0.37, 0.6 mg). The R_f 0.12 band will be dealt with elsewhere.

Metabolites from the acetone extract. The acetone extract (13 g) was sepd by Medium Pressure Liquid Chromatography (MPLC) on Sephadex LH-20 (5 × 125 cm column, 20 ml frs) in a gradient solvent system [15% MeOH in Me_2CO (4.8 l), 20% MeOH in Me_2CO (1.67 l)] to give 11 frs, C_1 (tubes 1–62, 3.02 g), C_2 (63–87, 900 mg), C_3 (88–101, 732 mg), C_4 (102–127, 1.40 g), C_5 (128–133, 211 mg), C_6 (134–141, 262 mg), C_7 (142–152, 660 mg), C_8 (153–167, 2.64 g), C_9 (168–189, 1.60 g), C_{10} (190–221, 1.01 g) and C_{11} (222–310, 684 mg). Only fr. C_{10} differed sufficiently from the EtOAc frs and showed reasonable simplification by 2D paper chromatograms to merit further investigation. A portion (270 mg) of this fr. was subjected to CC on cellulose MN 300 [Macherey, Nagel and Co.] (2 × 40 cm column, 13 ml frs) in H_2O to give an additional sample of the R_f 0.12 band of the preceding paragraph, details of which will be published separately.

Purification of a further portion (200 mg) of fr. C_{10} by PLC in C_6H_6–Me_2CO–MeOH (5:3:2) gave aspalathin (**26**) as a light yellow solid (51 mg, R_f 0.29), ^{1}H NMR data (Table 2). Acetylation of aspalathin (50 mg) with acetic anhydride–pyridine followed by PLC in C_6H_6–Me_2CO (8:2) gave a mixt. of the nona-*O*-acetylaspalathin (**27**) and the enol acetate (**30**) as a solid (4.0 mg, R_f 0.81). The nona-*O*-acetyl derivative (**27**) was prepd by treating aspalathin (50 mg) with 5 ml of a mixt. of 1 M acetic anhydride and 10^{-3} M $HClO_4$ in anhydrous EtOAc at 0° under N_2 for 5 min. The EtOAc layer was extracted with satd $NaHCO_3$ soln (15 ml) and the aq. layer extracted with EtOAc (3 × 30 ml). The combined extract was dried (Na_2SO_4) and evapd to dryness. Purification by PLC in C_6H_6–hexane–Me_2CO (4:4:2, ×2) afforded nona-*O*-acetylaspalathin (**27**) as a solid (4.2 mg, R_f 0.34), ^{1}H NMR data (Table 2). The enol acetate (**30**) was prepd as a yellow solid (13.9 mg) by dissolving aspalathin (10 mg) in pyridine (1 ml) and triethylamine (0.05 ml), adding acetic anhydride (1 ml), stirring the mixt. for 24 hr at 30°, and removing the solvent with N_2 current at room temp. (found: M^+, 872.2369; $C_{41}H_{44}O_{21}$ requires: M, 872.2375), ^{1}H NMR data (Table 2).

Methylation of aspalathin (100 mg) followed by PLC in C_6H_6–Me_2CO–MeOH (6:3:1, ×4) gave 2 bands at R_f 0.54 [C_6H_6–Me_2CO–MeOH (6:3:1, ×2)] (1.7 mg) and 0.37 [C_6H_6–Me_2CO–MeOH (6:3:1, ×2)] (4.6 mg). Acetylation of these bands afforded the tri-*O*-acetyl-hexa-*O*-methylaspalathin (**29**) as a solid (3.1 mg), ^{1}H NMR data (Table 2), and the tetra-*O*-acetyl-penta-*O*-methyl-aspalathin (**28**) as a solid (3.5 mg), ^{1}H NMR data (Table 2).

Acknowledgements—Support by the Sentrale Navorsingsfonds of this University, the Foundation for Research Development, Pretoria and by the Rooibos Tea Control Board, Clanwilliam, is acknowledged.

REFERENCES

1. Morton, J. F. (1983) *Econ. Bot.* **37**, 164.
2. Habu, T., Flath, R. A., Mon, T. R. and Morton, J. F. (1985) *J. Agric. Food. Chem.* **33**, 249.
2. Shibata, S., Harada, M. and Budidarmo, W. (1960) *Yakugaku Zasshi* **80**, 620.
4. Snyckers, F. O. and Salemi, G. (1974) *J. South African Chem. Inst.* **27**, 5.
5. Koeppen, B. H. and Roux, D. G. (1966) *Biochem. J.* **99**, 604.
6. Koeppen, B. H. and Roux, D. G. (1965) *Biochem. J.* **97**, 444.
7. Koeppen, B. H. (1959) M.Sc. Thesis, University of Stellenbosch, South Africa.
8. Yoshikawa, T., Naito, Y., Oyamada, H., Ueda, S., Tanigawa, S., Takemura, T., Sugino, S. and Kondo, M. (1990) in *Antioxidants in Therapy and Preventive Medicine* (Emeril, I., ed.), p. 171. Plenum Press, New York.
9. Heller, W. and Forkmann, G. (1988) in *The Flavonoids—Advances in Research since* 1980 (Harborne, J. B., ed.), p. 399. Chapman and Hall, London.
10. Eklund, T. (1985) *Int. J. Food Microbiol.* **2**, 159.
11. Bui, L. V. and Cooper, C. (1987) *J. Assoc. Off. Analyt. Chem.* **70**, 892.
12. Kohno, M., Yamada, M., Mitsuta, K., Mizuta, Y. and Yoshikawa, T. (1991) *Bull. Chem. Soc. Jpn* **64**, 1447.
13. Brieskorn, C. H. and Riedel, W. (1977) *Planta Med.* **31**, 308.
14. Chopin, J., Bouillant, M. L. and Besson, E. (1982) in *The Flavonoids—Advances in Research* (Harborne, J. B. and Mabry, T. J., eds), p. 449. Chapman and Hall, London.
15. Horowitz, R. M. and Gentili, B. (1964) *Chem. Ind.* **1964**, 498.
16. Gentili, B. and Horowitz, R. M. (1968) *J. Org. Chem.* **33**, 1571.
17. Eade, R. A., Hillis, W. E., Horn, D. H. S. and Simes, J. J. H. (1965) *Aust. J. Chem.* **18**, 715.
18. Johnson, R. (1968) *Chem. Rev.* **68**, 375.
19. Baczko, K. and Plusquellec, D. (1991) *Tetrahedron* **47**, 3817.
20. Hillis, W. E. and Horn, D. H. S. (1965) *Aust. J. Chem.* **18**, 531.
21. Bezuidenhoudt, B. C. B., Brandt, E. V. and Ferreira, D. (1987) *Phytochemistry* **26**, 531.
22. Steynberg, J. P., Brandt, E. V., Burger, J. F. W., Bezuidenhoudt, B. C. B. and Ferreira, D. (1988) *J. Chem. Soc., Perkin Trans I* 37.
23. Koeppen, B. H. and Roux, D. G. (1965) *Tetrahedron Letters* 3497.
24. Ferreira, D. and Roux, D. G. (1974) *Phytochemistry* **13**, 2039.
25. Bezuidenhoudt, B. C. B., Brandt, E. V. and Roux, D. G. (1981) *J. Chem. Soc., Perkin Trans I* 263.
26. Bezuidenhout, S. C., Bezuidenhoudt, B. C. B. and Ferreira, D. (1988) *Phytochemistry* **27**, 2329.
27. DuBois, G. E., Crosby, G. A. and Stephenson, R. A. (1981) *J. Med. Chem.* **24**, 408.
28. Chinn, L. J., Walters, D. E., Williams, K. and Mazur, R. H. (1987) *J. Agric. Food. Chem.* **35**, 409.
29. Arnoldi, A., Bassoli, A., Merlini, L. and Ragg, E. (1991) *J. Chem. Soc., Perkin Trans II* 1399.