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Proanthocyanidin glycosides and related polyphenols from cacao liquor and their antioxidant effects

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Abstract

Purification of polar fractions from cacao liquor extracts gave 17 phenolics including four new compounds. The new compounds were characterized as a *C*-glycosidic flavan, an *O*-glycoside of a dimeric and two *O*-glycosides of trimeric A-linked proanthocyanidins, on the basis of spectroscopic data. Isolated polyphenols showed inhibitory effects on nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation in microsomes and on the autoxidation of linoleic acid. These effects were attributed to the radical-scavenging activity in the peroxidation chain reactions, based on the findings that the cacao polyphenols effectively scavenged the 1,1-diphenyl-2-picrylhydrazyl radical. © 2002 Published by Elsevier Science Ltd.

Keywords: Cacao liquor; *Theobroma cacao*; Sterculiaceae; Tannin; Polyphenol; Proanthocyanidin glycosides; Antioxidant; Lipid peroxidation; Radical scavenger

1. Introduction

Cacao liquor, which is produced by fermentation and roasting of cacao beans (*Theobroma cacao* L., Sterculiaceae), is rich in polyphenols. Flavan monomers and oligomers, flavonol glycosides and hydroxycinnamic acid derivatives have all been isolated from cacao beans or cacao liquor (Porter et al., 1991; Sanbongi et al., 1998). Antioxidant effects of the polyphenol fractions from cacao liquor have also been reported (Osakabe et al., 2000). We further investigated the constituents of cacao liquor, and isolated 13 polyphenolic constituents including four new compounds. The isolated polyphenols showed inhibitory effects on nicotinamide adenine dinucleotide phosphate (NADPH)-dependent lipid peroxidation in rat liver microsomes, and on the autoxidation of linoleic acid. They also showed activity to scavenge the l,1-diphenyl-2-picrylhydrazyl (DPPH) radical. This paper deals with the structures of the new compounds and the antioxidant effects of the polyphenols.

2. Results and discussion

2.1. Isolation of polyphenols from cacao liquor

Cacao liquor prepared from Ghana beans was defatted with *n*-hexane, and then homogenized in 70% aq. acetone. The concentrated filtrate from the homogenate was extracted with EtOAc and *n*-BuOH, successively, and the EtOAc and *n*-BuOH extracts were respectively subjected to Sephadex LH-20, MCI-gel CHP-20P, Diaion HP-20 and/or Toyopearl HW-40 chromatographies, to give four new compounds (**1**)–(**4**), together with 13 known compounds. The known compounds were identified as procyanidin B2 (**5**) (Porter et al., 1991), protocatechuic acid (**6**), (+)-catechin (**7**), (−)-epicatechin (**8**), 3T-*O*-β-D-galactopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin[1] (**9**) (Porter et al., 1991), procyani-

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[1] "T" and "ent" mean the top unit of the trimeric proanthocyanidin and enantiomer, respectively.

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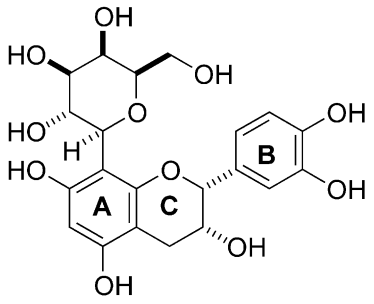

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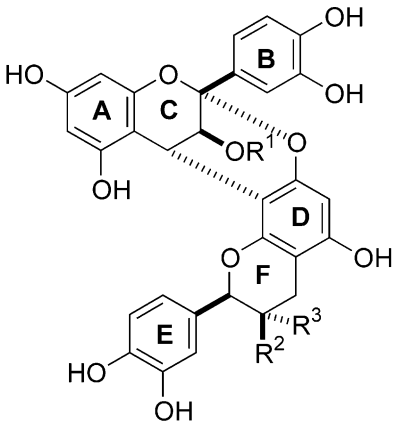
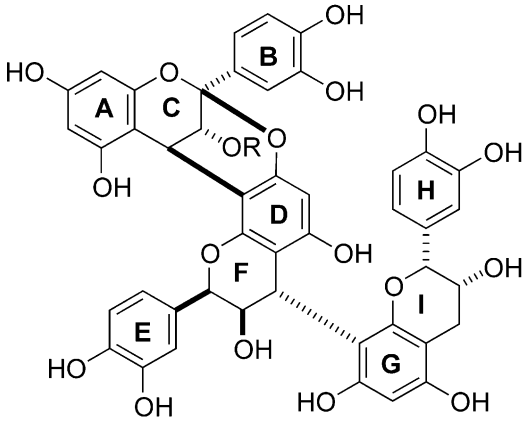
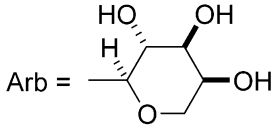
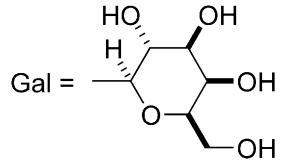


2: R^1= Arb, R^2 = H, R^3 = OH
9: R^1= Gal, R^2 = OH. R^3 = H
13: R^1= Arb, R^2 = OH. R^3 = H





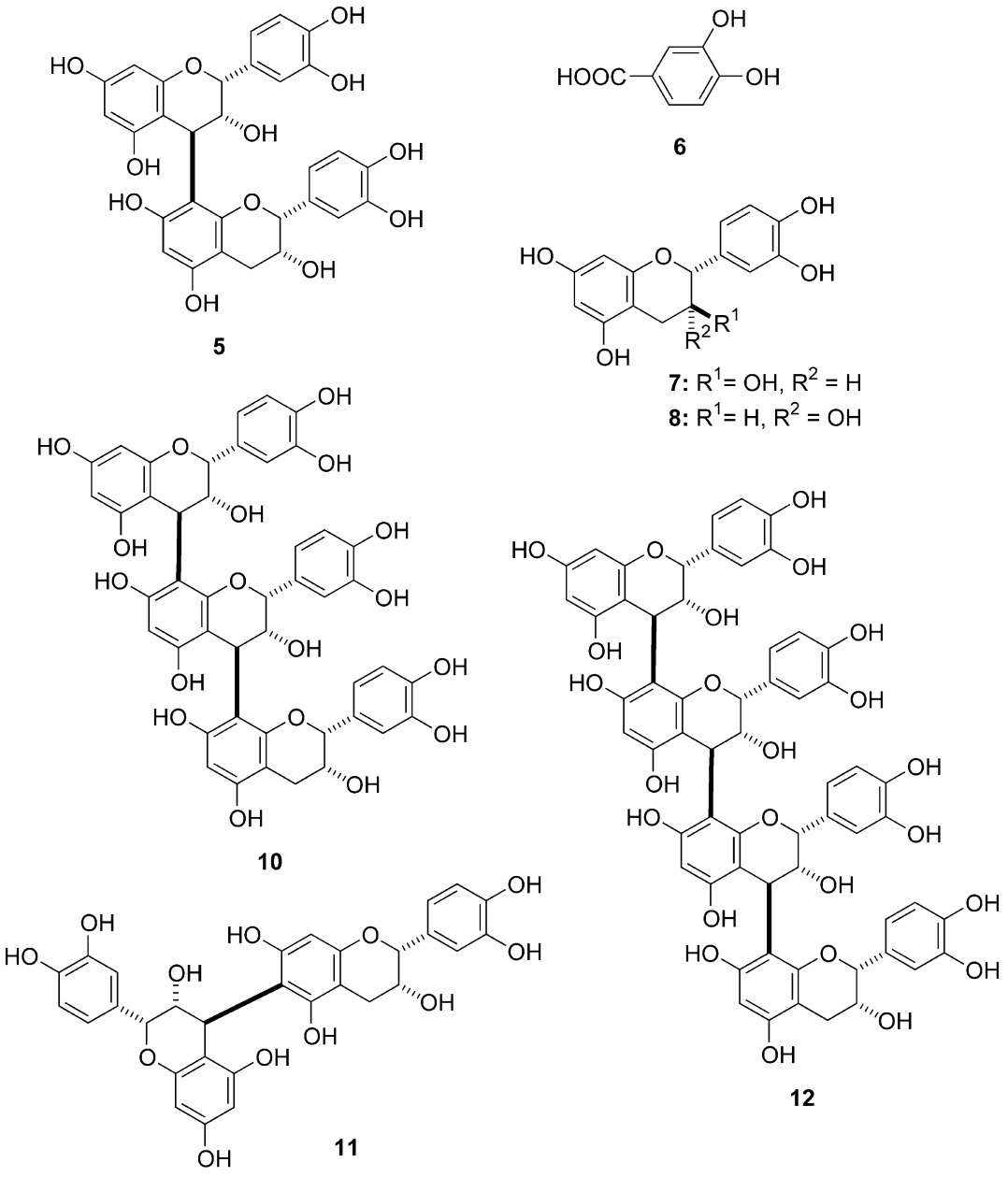


3: R = Arb
4: R = Gal
19: R = H

din C1 (**10**) (Porter et al., 1991), procyanidin B5 (**11**) (Thompson et al., 1972), cinnamtannin A_2 (**12**) (Morimoto et al., 1986), 3T-*O*-α-L-arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**13**) (Porter et al., 1991), proanthocyanidin A1 (**14**) (Nonaka et al., 1987), proanthocyanidin A2 (**15**) (Jacques et al., 1974), bis-8,8′-catechinylmethane (**16**) (Kiatgrajai et al., 1982), and quercetin 3-*O*-α-L-arabinopyranoside (**17**) (Sanbongi et al., 1998).

2.2. Structure of monomeric flavan C-glycoside *1*

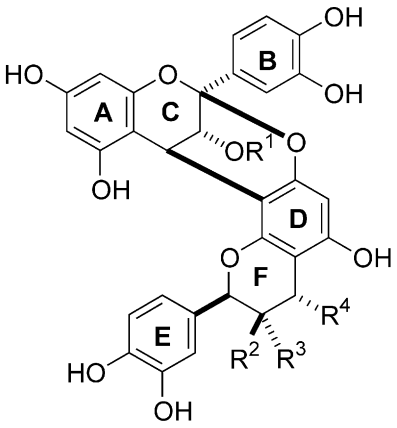
Compound **1** was obtained as a light-brown amorphous powder. Electrospray-ionization (ESI) MS of this compound showed an $[M+H]^+$ ion peak at *m*/*z* 453, and $[M+NH_4]^+$ ion peak at *m*/*z* 470. These peaks correspond to the molecular formula $C_{21}H_{24}O_{11}$, which was substantiated by high-resolution ESI MS. Based on this molecular formula and the following ^{1}H NMR spectral data, **1** was presumed to be a *C*-glycoside of a monomeric flavanol. The ^{1}H NMR spectrum of **1** (in MeOH-d_4, 40 °C) showed an aromatic singlet [δ 6.00 (1H; A-ring H)], three aromatic proton signals forming an ABX system [δ 6.77 (1H, *d*, *J* = 8 Hz; H-5′), 6.81 (1H, *dd*, *J* = 2, 8 Hz; H-6′) and 7.02 (1H, *d*, *J* = 2 Hz; H-2′)] and four aliphatic proton signals coupled with each other [δ 4.86 (1H, *br s*; H-2), 4.14 (1H, *br m*; H-3), 2.88 (1H, *dd*, *J* = 4, 17.5 Hz; H-4) and 2.83 (1H, *dd*, *J* = 2, 17.5 Hz; H-4)]. The spectrum also showed seven aliphatic proton signals of a sugar unit [δ 4.86 (1H, *d*, *J* = 9.5 Hz; H-1), 4.15 (1H, *t*, *J* = 9.5 Hz; H-2), 3.60 (1H, *dd*, *J* = 3, 9.5 Hz; H-3), 3.93 (1H, *br d*, *J* = 3 Hz; H-4), 3.57 (1H, *dd*, *J* = 5.5, 6.5 Hz; H-5), 3.74 (1H, *dd*, *J* = 6.5, 11.5 Hz; H-6), 3.68 (1H, *dd*, *J* = 5.5, 11.5 Hz; H-6)]. The coupling constants indicated that the sugar is β-galactopyranose, upon the assumption that it is of the D-form. The ^{13}C NMR spectrum of **1** confirmed that this molecule consists of epicatechin and galactose residues



(Experimental). The galactose residue on the epicatechin moiety was assigned to C-8, based on the rotating frame Overhauser effect (ROE) between H-1 of the galactose residue (δ 4.85) and B-ring H-2′ (δ 7.16) of the epicatechin residue in the ROESY experiment (in Me_2CO-$d_6 + D_2O$, 24 °C). The configurations at C-2 and C-3 of the epicatechin residue of **1** are both *R*, since **1** showed a CD spectrum similar to that of (−)-epicatechin (**8**). Based on these data, the structure of (−)-epicatechin 8-*C*-β-D-galactoside was assigned for **1**.

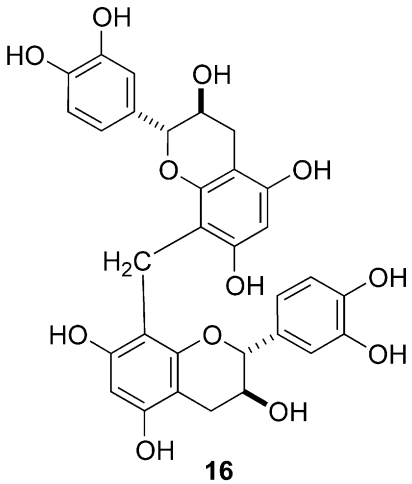
2.3. Structure of dimeric flavan O-glycoside 2

Compound **2** was obtained as a light-brown powder. Its molecular formula $C_{35}H_{32}O_{16}$ was derived by high-resolution ESI MS. The ^{1}H NMR spectrum (in Me_2CO-$d_6 + D_2O$, 40 °C) showed signals for three aromatic protons of A/D-rings [δ 5.95, 5.97 (1H each, *d*, $J = 2$ Hz; upper unit); δ 6.26 (1H, *s*; lower unit)], two sets of three aromatic protons of B/E-rings forming ABX systems [δ 6.99 (1H, *br s*; H-2′), 6.81 (2H, *s*; H-5′, H-6′) (lower unit); δ 7.19 (*d*, $J = 2.5$ Hz; H-2′), 6.85, (1H, *d*; $J = 8.5$ Hz, H-5′), 7.05 (1H, *dd*, $J = 2.5$, 8.5 Hz; H-6′) (upper unit)] and two series of aliphatic protons of C/F-rings [δ 4.28 (1H, *d*, $J = 3$ Hz; H-3), 4.60 (1H, *d*, $J = 3$ Hz; H-4) (C-ring); 4.72 (1H, *d*, $J = 8$ Hz, H-2), 4.14 (1H, *ddd*, $J = 5.5$, 8, 8.5 Hz; H-3), 2.94 (1H, *dd*, $J = 5.5$, 16 Hz, H-4), 2.58 (1H, *dd*, $J = 8.5$, 16.5 Hz; H-4) (F-ring)]. These signals and the absence of H-2 in the upper flavan unit indicated that this compound is a flavan dimer of



14: $R^1 = R^2 = R^4 = H$, $R^3 = OH$
15: $R^1 = R^3 = R^4 = H$, $R^2 = OH$
20: R^1 = Arb, $R^2 = OH$, $R^3 = H$, R^4 = SBz
21: $R^1 = R^3 = H$, $R^2 = OH$, R^4 = SBz

SBz = —S



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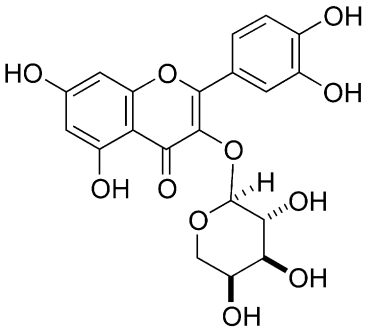

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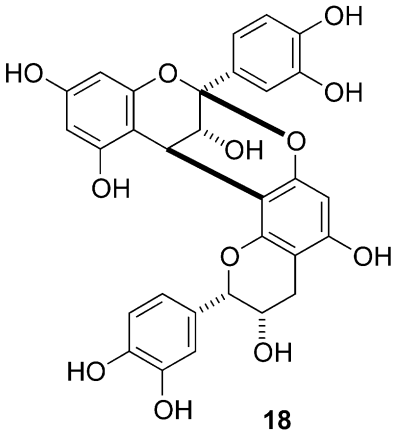

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proanthocyanidin A series. A 2,3-*trans* structure was identified in the lower unit from the coupling constants of the lower F-ring protons. The spectrum also showed six aliphatic protons ascribed to a sugar residue (δ, 4.46 (1H, *d*, $J = 4.5$ Hz; H-1), 3.31 (1H, *dd*, $J = 4.5$, 6.5 Hz; H-2), 3.45 (1H, *dd*, $J = 3.5$, 6.5 Hz; H-3), 3.66 (1H, *m*; H-4), 3.19 (1H, *dd*, $J = 3.5$, 12 Hz, H-5), 3.11 (1H, *dd*, $J = 6.5$, 12 Hz; H-5)]. The sugar in **2** was assigned as an arabinopyranose on the basis of ^{1}H and the ^{13}C signal patterns. This assignment was substantiated by acid hydrolysis of **2** followed by the analysis with HPLC, which showed the production of arabinose.

The location of the arabinose at O-3 of the upper flavan unit on the proanthocyanidin residue was further supported by the correlations, δ_H 4.46–δ_C 71.0 [arabinose H-1–aglycone C-3 (C-ring)] and δ_H 4.28–δ_C 98.9 [aglycone H-3 (C-ring)–arabinose C-1], in the HMBC spectrum of **2**. The HMBC spectrum also showed the connectivity of δ_H 4.72–δ_C 152.4–δ_H 4.60 [H-4 (C-ring)–C-8a (D-ring)-H-2 (F-ring)], indicating the presence of the C-4 (C-ring)–C-8 (D-ring) interflavan linkage.

The CD spectrum of compound **2** showed a negative Cotton effect in the short wavelength region ($[\theta]_{228} -6.9\times10^3$), indicating that the orientation of the interflavan bond at C-4 of the C-ring is α (Barrett et al., 1979; Botha et al., 1981; Kolodziej et al., 1991). The orientation of the phenyl group at C-2 of the C-ring, which is dependent on the C-4 orientation in the proanthocyanidin A-type structure, is therefore β. The β-orientation of the C-3 hydroxyl group of the C-ring was assumed tentatively, based on analogy to other natural

dimeric proanthocyanidins of the A-type with OH at C-5(A) (Balde et al., 1991). Although the coupling constant $J_{3,4}$ of the C-ring in **2** (3 Hz) was practically the same as the reported values for the 3,4-*trans* A-type dimers (3–4 Hz), the values are not indicative for the relative configuration C-3–C-4 (Cronje et al., 1990, 1993).

The orientation at C-2 of the F-ring was tentaviely assigned to be α, since the CD spectral pattern at around 270–290 nm ($[\theta]_{293}-2.3\times10^3$, $[\theta]_{271}+4.9\times10^3$), which reflects the orientation of the B/E-rings in the proanthocyanidins, was similar to that of proanthocyanidin **13** with a 2β-phenyl (C-ring)–2α-phenyl (F-ring) structure. Actually, proanthocyanidins with 2α-phenyl (C-ring)–2α-phenyl (F-ring) and 2α-phenyl (C-ring)–2β-phenyl (F-ring) structures showed different CD patterns in this region {$[\theta]_{287}-2.5\times10^3$, $[\theta]_{271}-1.8\times10^4$ for proanthocyanidin A-2 (**15**); $[\theta]_{297}+1.4\times10^4$, $[\theta]_{271}-1.3\times10^4$ for epicatechin-(2β→7, 4β→8)-*ent*-epicatechin (**18**)} (Lou et al., 1999). Based on these data, the structure of 3T-*O*-arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-catechin, which was the C-3 (F-ring) epimer of compound **13,** was assigned for compound **2**. The NOESY spectrum showed a correlation between H-1 and H-3 in the arabinopyranose residue. Therefore, the α-configuration at the anomeric center was assigned upon the assumption that this sugar is of the L-form.

The *ent*-epicatechin structure in the upper unit of **2** is probably derived from the catechin moiety in procyanidin B-3 upon the oxidative transformation to the A-type proanthocyanidin (Burger et al., 1990; Kondo et al., 2000), although procyanidin B-3 was not yet isolated from cacao beans or cacao liquor.

2.4. Structure of trimeric flavan O-glycosides 3 and 4

Compound **3** was obtained as a light-brown amorphous powder. High-resolution ESI MS indicated its molecular formula to be $C_{50}H_{44}O_{22}$. Although the ^{1}H NMR spectrum measured at 24 °C (in Me_2CO-d_6 + D_2O) showed some duplication of signals, measurement at an elevated temperature rectified this. The spectrum measured at 40 °C showed B/E/H-ring (δ 7.3–6.6), A/D/G-ring [δ 6.22 (1H, *s*), 5.97 (1H, *d*, *J* = 2 Hz), 5.95 (1H, *br s*), 5.90 (1H, *d*, *J* = 2 Hz)] and C/F/I-ring protons [δ 4.39 (1H, *d*, *J* = 3.5 Hz; C-ring H-3), 5.34 (1H, *br s*; F-ring H-2), 3.95 (1H, *br m*; F-ring H-3), 4.76 (2H, *br s*; C/F-ring H-4), 4.96 (1H, *br*, I-ring H-2), 4.30 (1H, *br*; I-ring H-3), 2.86 (1H, *br d*, *J* = 14 Hz, I-ring H-4), 2.71 (1H, *br*; I-ring H-4)] of the trimeric flavanoid skeleton. The absence of one of the H-2 signals indicated the presence of an interflavan linkage at C-2 (to O-7 or O-5), in addition to ordinary interflavan 4–8 or 4–6 bonds. The spectrum also showed aliphatic proton signals due to an arabinopyranose residue [δ 4.58 (1H, *d*, *J* = 5 Hz; H-1), 3.36 (3H, *m*; H-2, H-5×2), 3.50 (1H, *dd*, *J* = 3.5, 9 Hz; H-3), 3.72 (1H, *m*; H-4)]. The presence of arabinose in **3** was substantiated by its formation on the acid hydrolysis of **3.**

The ^{13}C NMR spectrum of **3** showed carbon signals of three flavan units and the arabinose residue, and the chemical shifts of the flavan units were similar to those reported for cinnamtannin B1 (**19**) (Nonaka et al., 1983), except for the C-ring carbons of the top flavan units [δ 104.2 (**19**)–99.3 (**3**) (C-2); 66.5 (**19**)–71.2 (**3**) (C-3); 30.0 (**19**)–24.9 (**3**) (C-4)]. These chemical shift changes are attributable to the glycosidation at O-3 of the top unit, suggesting the structure of 3-T-*O*-arabino sylcinnamtannin B1 for compound **3.** Duplication of the ^{1}H signals described above coincided with the 4–8 linkage in **3** between the middle and lower unit, rather than the less hindered 4–6 linkage. The positive Cotton effect at 225 nm ($[\theta]_{225}+4.5\times10^4$) in the CD spectrum of **3** satisfied a β-orientation of the interflavan linkage at C-4 in each of the top and middle flavan units.

The presence of the interflavan A-type linkage between the top and middle flavan units was verified by the acid-catalyzed degradation of **3** in the presence of toluene-α-thiol, which gave a benzylthioether (**20**) of a dimeric flavan, together with (−)-epicatechin. The ^{1}H NMR spectrum of **20** showed arabinose signals as discussed in experimental and the assignments of the other signals were based on comparisons with the data reported for the benzylthioether of proanthocyanidin A-2 (**21**) (Nonaka et al., 1983).

The arabinose residue in **3** was confirmed to be at O-3 on the top flavan unit, based on the correlations, δ_H 4.39 (C-ring H-3)–δ_C 99.5 (arabinose C-1) and δ_H 4.58 (arabinose H-1)–δ_C 71.2 (C-ring C-3) observed in the HMBC spectrum of **3**. The α-configuration at the anomeric center, upon the assumption that the arabinose is of the L-form, was shown by the NOE correlation between H-1 and H-3 of the arabinopyranose residue in the NOESY spectrum of **3.** The structural formula **3** was thus assigned for this compound. The aglycone of this compound was reported to be isolated from cinnamon (Nonaka et al., 1983) and cranberry (Foo et al., 2000).

Compound **4** was obtained as a light-brown amorphous powder. The molecular formula $C_{51}H_{47}O_{23}$ was derived by high-resolution ESI MS. Although the ^{1}H and ^{13}C NMR spectra of **4** were similar respectively to those of compound **3,** the spectra of **4** showed signals attributable to a β-galactopyranose residue [δ_H 4.40 (1H, *d*, *J* = 7.5 Hz; H-1), 3.28 (1H, *dd*, *J* = 7.5, 9.5 Hz; H-2), 3.44 (1H, *dd*, *J* = 3, 9.5 Hz; H-3), 3.77 (1H, *br d*, *J* = 3 Hz; H-4), 3.50 (1H, *t*, *J* = 6 Hz; H-5), 3.57 (2H, *d*, *J* = 6 Hz; H-6×2); δ_C 101.6 (C-1), 71.6 (C-2), 73.8 (C-3), 69.2 (C-4), 76.0 (C-5), 61.6 (C-6)] instead of the arabinopyranose residue in **3**. The presence of the galactose residue in **4** was verified by the formation of galactose upon the acid hydrolysis of **4**. The HMBC correlations, δ_H 4.48 (C-ring H-3)–δ_C 101.6 (galactose C-1) and δ_H 4.40 (galactose H-1)–δ_C 72.1 (C-ring C-3), indicated that

the galactose residue was at O-3 on the top flavan unit. The CD spectrum showed a positive Cotton effect at 234 nm ($[\theta]_{234} + 5.5\times10^4$), indicating the β-orientation of the two interflavan linkages at C-4 of the flavan units. Based on these data, the structural formula **4** was assigned for this compound.

2.5. Antioxidant activity of cacao polyphenols

Inhibitory effects of various tannins and related polyphenols on the lipid peroxidation in rat liver mitochondria induced by adenosine 5′-diphosphate (ADP) and ascorbic acid, and on that in liver microsomes induced by ADP and NADPH, have been reported (Okuda et al., 1983). The antioxidant effects of the polyphenols are attributable to their radical-scavenging properties, as shown by the effects on the diphenylpicryl hydrazyl (DPPH) radical as a model compound for lipid radicals or lipid peroxide radicals (Fujita et al., 1988a, b; Yoshida et al., 1989; Hatano et al., 1989). On the other hand, the polyphenolic constituents of cacao liquor have been reported to have antioxidant effects (Osakabe et al., 2000). Effects of the polyphenois isolated from cacao on the NADPH-dependent lipid peroxidation in rat liver microsomes, and on the autoxidation of linoleic acid were therefore examined. We also tested their effects on the DPPH radical.

The NADPH-dependent lipid peroxidation in rat liver microsomes was inhibited by almost all of the polyphenols tested as shown in Table 1. The polyphenols, except for (−)-epicatechin 8-*C*-galactoside (**1**), had inhibitory effects on the lipid peroxidation with IC_{50} values of 12–68 μM. Although these values are no better than that of *dl*-α-tocopherol, the results indicated that these compounds contribute to the antioxidant activity of the polyphenolic fraction of cacao.

The ranking of the polyphenols based on the strength of their inhibitory effects on the autoxidation of linoleic acid, which are shown in Table 2, was somewhat different from that observed for the NADPH-dependent lipid peroxidation, and the effects of almost all of the polyphenols on the oxidation of linoleic acid were stronger than the effect of *dl*-α-tocopherol. The difference may be attributed to the presence of other biomolecules in the microsomal solution, which interacted with the polyphenol.

Table 3 shows the scavenging activity of the test compounds for the DPPH radical. The effects were similar to or stronger than the effect of α-tocopherol. The ranking of the polyphenols in terms of scavenging activity was similar to that for the inhibitory effect on the autoxidation of linoleic acid.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were measured on a Varian VXR-500 (500 MHz for ^{1}H and 126 MHz for ^{13}C) in Me_2CO-d_6–D_2O at 40 °C unless mentioned otherwise,

Table 1
Inhibitory effects of cacao polyphenols on lipid peroxidation in rat liver microsomes

Compound	IC_{50} (μg/ml)
Procyanidin B2 (**5**)	12
Procyanidin B5 (**11**)	12
3T-*O*-β-D-Galactopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**9**)	18
3T-*O*-α-L-Arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**13**)	21
Cinnamtannin A_2 (**12**)	25
3T-*O*-α-L-Arabinopyranosylcinnamtannin B_1 (**3**)	28
(−)-Epicatechin (**8**)	29
3T-*O*-β-D-Galactopyranosylcinnamtannin B_1 (**4**)	36
Procyanidin C1 (**10**)	68
(−)-Epicatechin 8-*C*-galactopyranoside (**1**)	>100
dl-α-Tocopherol	5.6

Table 2
Inhibitory effects of cacao polyphenols on the autoxidation of linoleic acid initiated by the addition of V-70

Compound	IC_{50} (μg/ml)
(−)-Epicatechin (**8**)	0.62
3T-*O*-α-L-Arabinopyranosylcinnamtannin B_1 (**3**)	1.9
3T-*O*-β-D-Galactopyranosylcinnamtannin B_1 (**4**)	2.0
3T-*O*-α-L-Arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**13**)	2.2
Procyanidin B5 (**11**)	2.3
3T-*O*-β-D-Galactopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**9**)	2.5
Cinnamtannin A_2 (**12**)	2.7
Procyanidin C1 (**10**)	5.3
(−)-Epicatechin 8-*C*-galactopyranoside (**1**)	9.5
dl-α-Tocopherol	7.7

Table 3
Radical-scavenging effects of cacao polyphenols on the DPPH radical

Compound	EC_{50} (μM)
Procyanidin B2 (**5**)	1.4
Cinnamtannin A_2 (**12**)	2.1
(−)-Epicatechin (**8**)	2.1
3T-*O*-α-L-Arabinopyranosylcinnamtannin B_1 (3)	2.3
Procyanidin B5 (**11**)	2.3
3T-*O*-β-D-Galactopyranosylcinnamtannin B_1 (**4**)	2.4
Procyanidin C1 (**10**)	6.2
(−)-Epicatechin 8-*C*-galactopyranoside (**1**)	3.9
3T-*O*-β-D-Galactopyranosyl-*ent*-epicatechin-(2α→, 4α→8)-epicatechin (**9**)	3.9
3T-*O*-α-L-Arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**13**)	3.9
dl-α-Tocopherol	4.6

and chemical shifts are given in δ values (ppm), based on those of the solvent signals (δ_H 2.04 and δ_C 29.8 for Me_2CO-d_6, and δ_H 3.30 and δ_C 49.8 for MeOH-d_4).

3.2. Isolation of polyphenols from cacao liquor

The cacao liquor used in this study was prepared from fermented cacao beans imported from Ghana. A preparation of the liquor (420 g) defatted with *n*-hexane was homogenized in 70% aq. acetone (4.5 l), and the filtrate concentrated from the homogenate was extracted with $CHCl_3$, EtOAc and *n*-BuOH, successively. The EtOAc extract (4.5 g) was subjected to column chromatography on Toyopearl HW-40C (eluants, H_2O–30% EtOH–70% EtOH–70% acetone), to give procyanidin B2 (**5**) (132 mg) and other fractions rich in polyphenols. The fractions were further purified by column chromatography on MCI-gel CHP-20P (eluant, H_2O–MeOH), Sephadex LH-20 (EtOH) and/or YMC-gel ODS-AQ 120-S50 (H_2O–MeOH), to give protocatechuic acid (**6**) (28 mg), (+)-catechin (**7**) (15 mg), (−)-epicatechin (**8**) (27 mg), procyanidin B2 (**5**) (12 mg), 3T-*O*-arabinopyranosyl-*ent*-epicatechin-(2α–7, 4α–8)-catechin (**2**) (2 mg), 3T-*O*-β-D-galactopyranosyl-*ent*-epicatechin-(2α–7, 4α–8)-epicatechin (**9**) (5 mg), procyanidin Cl (**10**) (39 mg), procyanidin B5 (**11**) (48 mg) and cinnamtannin A_2 (**12**) (4 mg). The *n*-BuOH extract was subjected to Diaion HP-20 chromatography (eluant, H_2O–MeOH), and fractions were purified by column chromatography on Sephadex LH-20 (EtOH), YMC-gel ODS-AQ 120-S50 (H_2O–MeOH) and MCI-gel CHP-20P (H_20–MeOH), to give (−)-epicatechin 8-*C*-β-D-galactopyranoside (**1**) (8 mg), 3T-*O*-β-D-galactopyranosylcinnamtannin B_1 (**4**) (28 mg), cinnamtannin A_2 (**12**) (42 mg), procyanidin B2 (**5**) (9 mg), 3T-*O*-β-D-galactopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**9**) (23 mg), 3T-*O*-α-L-arabinopyranosyl-*ent*-epicatechin-(2α→7, 4α→8)-epicatechin (**13**) (6 mg), procyanidin C1 (**10**) (6 mg), procyanidin B5 (**11**) (10 mg) and 3T-*O*-α-L-arabinopyranosylcinnamtannin B_1 (**3**) (11 mg). Another portion of the EtOAc (14 g) extract was purified in an analogous way on columns of Diaion HP-20, Sephadex LH-20, MCI-gel CHP-20P and YMC-gel ODS-AQ 120-S50, to yield proanthocyanidin A1 (**14**) (5 mg), proanthocyanidin A2 (**15**) (4 mg), bis-8,8′-catechinylmethane (**16**) (5 mg) and quercetin 3-*O*-α-L-arabinopyranoside (**17**) (18 mg) in addition to the compounds described above.

3.3. Epicatechin 8-C-β-D-galactopyranoside (*1*)

A light-brown amorphous powder, $[\alpha]_D$, −25.8° ($c=0.9$, MeOH). ESI MS *m/z*: 453 ($[M+H]^+$), 470 ($[M+NH_4]^+$). High-resolution ESI MS *m/z*: 470.1649 ($(M+NH_4]^+$). Calc. for $C_{21}H_{24}O_{11}+NH_4$, 470.1662. UV λ_{max}^{MeOH} (log ε): 280 (3.32). CD (MeOH): $[\theta]_{207}-4.4\times10^4$, $[\theta]_{250}+1.8\times10^3$, $[\theta]_{279}-3.4\times10^3$. ^{1}H NMR (in Me_2CO-d_6 + D_2O, 24 °C) δ: 2.80 [1H, *dd*, $J=2.5$, 17 Hz, epicatechin (EC) H-4], 2.86 (1H, *dd*, $J=4$, 17 Hz, EC H-4), 3.62 [1H, *br dd*, $J=5$, 6.5 Hz, galactose (Gal) H-5], 3.66 (1H, *dd*, $J=3$, 9.5 Hz, Gal H-3), 3.70 (1H, *dd*, $J=5$, 11.5 Hz, Gal H-6), 3.73 (1H, *dd*, $J=6.5$ Hz, 11.5 Hz, Gal H-6), 4.03 (1H, *br d*, $J=3$ Hz, Gal H-4), 4.05 (1H, *t*, $J=9.5$ Hz, Gal H-2), 4.10 (1H, *m*, EC H-3), 4.85 (1H, *d*, $J=9.5$ Hz, Gal H-1), 4.92 (1H, *br s*, EC H-2), 5.98 (1H, *s*, EC H-6), 6.76 (1H, *d*, $J=8$ Hz, EC H-5′), 6.82 (1H, *dd*, $J=2$, 8 Hz, EC H–6′), 7.16 (1H, *d*, $J=2$ Hz, EC H-2′). ^{13}C NMR (in MeOH-d_4, 40 °C) δ: 30.5 (EC C-4), 63.8 (Gal C-6), 68.2 (EC C-3), 71.4 (Gal C-4), 73.1 (Gal C-2), 77.0 (Gal C-3), 77.7 (Gal C-1), 80.9 (EC C-2), 81.1 (Gal C-5), 98.2 (EC C-6), 100.5 (EC C-4a), 105.8 (EC C-8), 116.0 (EC C-2′), 116.9 (EC C-5′), 119.8 (EC C-6′), 133.2 (EC C-1′), 146.5 (EC C-4′), 146.8 (EC C-3′), 155.4 (EC C-8a), 157.9 (EC C-7), 158.9 (EC C-5).

3.4. 3T-O-Arabinopyranosyl-ent-epicatechin-(2α→7, 4α→8)-catechin (*2*)

A light-brown amorphous powder, $[\alpha]_D$ −16.4° ($c=1$, MeOH) ESI MS *m/z*: 709 ($[M+H]^+$). High-resolution ESI MS *m/z* 709.1713 ($[M+H]^+$). Calc. for $C_{35}H_{32}O_{16}+H$, 709.1769. UV λ_{max}^{MeOH} (log ε): 280 (4.17) CD (MeOH): $[\theta]_{283}-2.3\times10^3$, $[\theta]_{271}$, $+4.9\times10^3$, $[\theta]_{228}-6.9\times10^3$, $[\theta]_{211}+1.9\times10^4$. ^{13}C NMR δ: 24.8 [C-4 (C-ring)], 29.3 [C-4 (E)], 63.2 [arabinose (Arb) C-5], 66.2 (Arb C-4), 67.6 [C-3 (E)], 70.9 (Arb C-2), 71.0 [C-3 (C)], 72.5 (Arb C-3), 84.3 [C-2 (E)], 96.0 [C-8 (C)], 96.6 [C-6 (E)], 98.0 [C-6 (C)], 98.9 (Arb C-1), 99.2 [C-4a(E)], 103.2 [C-2(C), C-4a (A)], 105.2 [C-8 (D)], 115.4 (2C) [C-2′ (B), C-5′(B)], 115.9 (2C) [C-2′ (E), C-5′ (E)], 119.5 [C-6′ (B)], 120.5 [C-6′ (E)], 130.5 [C-1′ (E)], 131.6 [C-1′ (B)], 145.3 [C-4′ (B)], 145.7 [C-4′ (E)], 146.2 [C-3′ (E)], 146.5 [C-3′ (B)], 152.1 [C-7 (D)], 152.4 [C-8a (D)], 153.7 [C-8a (A)], 155.5, 155.6 [C-5 (A/D)], 158.6 [C-7 (A)].

3.5. 3T-O-α-L-Arabinopyranosylcinnamtannin B_1 (*3*)

A light-brown amorphous powder, $[\alpha]_D$ +12.6° ($c=1$, MeOH). ESI MS *m/z*: 997 ($[M+H]^+$). High-resolution ESI–MS *m/z*: 1014.2593 ($[M+NH_4]^+$). Calc. for $C_{50}H_{44}O_{22}+NH_4$, 1014.2668. UV λ_{max}^{MeOH} (log ε): 280 (4.20). CD (MeOH): $[\theta]_{283}-5.2\times10^3$, $[\theta]_{272}+3.1\times10^3$, $[\theta]_{257}+3.7\times10^3$, $[\theta]_{225}+4.5\times10^4$, $[\theta]_{209}+1.8\times10^4$, $[\theta]_{202}-9.5\times10^4$. ^{13}C NMR δ: 24.9 [C-4(C)], 36.4 [C-4 (F)], 64.3 (Arb C-5), 66.1 [C-3 (F)], 67.1 (Arb C-4), 71.0 (Arb C-2), 71.2 [C-3(C)], 72.5 (Arb C-3), 72.6 [C-3 (F)], 77.8 [C-2 (F)], 79.1 [C-2 (I)], 95.9 [C-8 (A)], 96.2 [C-6(D)], 96.9 [C-6 (G)], 97.9 [C-6 (A)], 99.3 [C-2 (C)], 99.5 (Arb C-1), 100.1 [C-4a (G)], 102.6 [C-8 (D)], 104.1 [C-4a (D)], 105.1 [C-4a (A)], 106.7 [C-8 (G)], 115.0,. 115.4, 115.6 (3C), 115.8 [C-2′ (B/E/H), C-5′ (B/E/H)], 118.9, 119.2, 119.5 [C-6′ (B/E/H)], 131.2, 131.3, 131.4 [C-1′ (B/

E/H)], 144.9–146.3 [C-3′ (B/S/H), C-4′ (B/E/H)], 152.2–158.3 [C-5 (A/D/G), C-7 (A/D/G), C-8a (A/D/G)].

3.6. 3T-O-β-D-Galactopyranosylcinnamtannin B_1 (**4**)

A light-brown amorphous powder, $[\alpha]_D$ +17.1° ($c=1$, MeOH) ESI MS m/z: 1044 ($[M+NH_4]^+$), 1027 ($[M+H]^+$), 865 ([M-galactose+H]$^+$). High-resolution ESI MS m/z: 1027.2531 ($[M+H]^+$). Calc. for $C_{51}H_{46}O_{23}+H$, 1027.2508. UV MeOH λ_{max} (log ε): 281 (5.02). CD (MeOH): $[\theta]_{204}-9.1\times10^3$, $[\theta]_{273}+4.0\times10^3$, $[\theta]_{258}+1.3\times10^3$, $[\theta]_{234}+5.5\times10^4$, $[\theta]_{209}+3.4\times10^4$, $[\theta]_{234}-5.6\times10^4$. ^{1}H NMR δ: 2.71 [1H, *br*, H-4 (I)], 2.84 [1H, *br d*, $J=15$ Hz, H-4 (I)], 3.95 [1H, *br s*, H-3 (F)], 4.31 [1H, *br*, H-3 (I)], 4.48 [1H, *d*, $J=3.5$ Hz, H-3 (C)], 4.73 [1H, *br s*, 11–4 (F)], 4.74 [1H, *br s*, H-4 (C)], 4.94 [1H, *br*, H-2 (I)], 5.35 [1H, *br s*, H-2 (F)], 5.88 [1H, *d*, $J=2$ Hz, H-6 (A)], 5.96 [2H, H-6 (G) and H-8 (A)], 6.22 [1H, *s*, H-6 (D)], 6.6–7.3 (9H, B-ring protons). Data for the galactose protons are shown in the text. ^{13}C NMR δ: 25.7 [C-4 (C)], 36.3 [C-4 (F)], 61.6 (Gal C-6), 66.0 [C-3 (I)], 69.2 (Gal C-4), 71.6 (Gal C-2), 72.1 [C-3 (C)], 72.7 [C-3 (F)], 73.7 (Gal C-3), 76.0 (Gal C-5), 77.7 [C-2 (F)], 79.0 [C-2 (I)], 95.9 [C-8 (A)], 96.3 [C-6 (D)], 96.9 [C-6 (G)], 97.8 [C-6 (A)], 99.4 [C-2 (C)], 100.1 [C-4a (G)], 101.0 (Gal C-1), 102.9 [C-8 (D)], 104.2 [C-4a (D)], 105.5 [C-4a (A)], 106.7 [C-8 (G)], 114.8, 115.0, 115.1, 115.6, 115.8, 116.2 [C-2′ (B/E/H), C-5′ (B/E/H)], 118.9, 119.0, 119.7 [C-6′ (B/E/H)], 131.2, 131.4, 131.5 [C-1′ (B/E/H)], 144.6–146.2 [C-3′ (B/E/H), C-4′ (B/E/H)], 152.3–158.1 [C-5(A/D/G), C-7 (A/D/G), C-8a (A/D/G)].

3.7. Acid hydrolysis of compounds **2**, **3** and **4**

A solution of **2** (1 mg) in 1% HCl (1 ml) in a sealed tube was heated in an oven set at 97 °C for 40 min. The resulting solution was extracted with EtOAc, and the remaining aqueous solution was treated with Diaion SA-20AP resin to neutralize the solution. Co-HPLC with the standard sample on a TSK-gel Amide-80 column [TOSOH], 4.6×250 mm; mobile phase, acetone–H_2O (1:1); detector, Shodex RI SE-51] showed the presence of arabinose in the solution. Compounds **3** and **4** were treated in analogous ways, and HPLC analyses of the reaction mixtures showed the presence of arabinose and galactose, respectively.

3.8. Degradation of **3** in the presence of toluene-α-thiol

An ethanol solution (0.75 ml) of compound **3** (3 mg) in a sealed tube was heated in an oven at 97 °C for 18 h. Then, the solvent was removed by evaporation, and the residue was subjected to column chromatogprahy on Sephadex LH-20 with EtOH as an eluant, to give 4β-benzylthioether (**20**) of 3T-*O*-β-L-arabinosyl-proanthocyanidin A2 and (−)-epicatechin (**8**). The latter compound was identified by HPLC on a Chiralcel OC column with *n*-hexane–isopropanol (7:13) containing 0.5% acetic acid at 40 °C in an oven.

3.8.1. 4β-Benzylthioether (**20**) of 3T-O-α-L-arabinosyl-proanthocyanidin A2

A light-brown amorphous powder. ESI MS m/z: 848 ($[M+NH_4]^+$), 831 ($[M+1]^+$), 707 ([M–$PhCH_2SH$+H]$^+$), 575 ((M–$PhCH_2SH$–arabinose+H]$^+$) ^{1}H NMR δ: 3.51 (1H, *dd*, $J=4.5$, 9 Hz, Arb H-3), 3.79 (1H, *m*, Arb H-4), 4.01 (2H, *s*, –CH_2S–), 4.19 [1H, *d*, $J=5$ Hz, H-4 (F)], 4.20 [1H, *d*, $J=5$ Hz, H-3 (F)], 4.35 [1H, *d*, $J=4.5$ Hz, H-3(C)], 4.59 (1H, *d*, $J=5$ Hz, Arb H-1), 4.79 [1H, *d*, $J=4.5$ Hz, H-4 (C)], 5.46 [1H, *br s*, H-2 (F)], 5.93 [1H, *d*, $J=2$ Hz, H-6 (A)], 5.95 [1H, *d*, $J=2$ Hz, H-8 (A)], 6.28 [^{1}H, *s*, H-6 (D)], 6.8–7.8 (11H, protons of B/E-rings and $C_6H_5CH_2$S–).

3.9. Inhibitory effects of cacao polyphenols on lipid peroxidation in rat liver microsomes

Spraque–Dawley rats (7 weeks old), which were obtained from Clea Japan Inc. (Tokyo, Japan), were kept in a controlled environment at 23 °C and 55% relative humidity under a 12 h dark–light cycle. Livers were excised from the rats which were fasted for 15 h prior to the excision, and were homogenized in 1.15% KC1 solution (4 fold volume of liver). A microsomal fraction was obtained as a pellet after centrifugation (78,000 *g* for 90 min at 0 °C) of the supernatant from centrifugation (10,000 *g* for 15 min at 0 °C) of a rat liver homogenate. A mixture of a suspension of the microsome (1.5 mg protein/ml) (50 μl), a solution of the test compound (50 μ1) and a solution (400 μl) containing Tris–HCl buffer (pH 7.5, 0.05 M), ADP (2 mM), $Fe(NO_3)_3$ (0.12 mM), $MgCl_2$ (7 mM), isocitric acid (2 mM), NADP (0.1 mM) and NADP-isocitrate dehydrogenase (0.05 U/ml) was incubated at 37 °C for 1 h. Then, a mixture of 0.8% thiobarbituric acid, 20% acetate buffer (pH 3.5) and 8.1% sodium dodecylsulfate (75:75:20) (2.0 ml) was added to the reaction mixture, and heated on a boiling-water bath for 1 h. After the solution was cooled in water for 5 min, *n*-butanol was added, and the mixture was agitated for 20 s. The fluorescence intensity (Ex 515 nm, Em 553 nm) of the upper layer obtained after centrifugation (3000 rpm, 10 min) was measured, and the amount of peroxides was estimated using tetramethoxypropane as a standard.

3.10. Inhibitory effects of cacao polyphenols on the V-70-induced autoxidation of linoleic acid

A solution of V-70 [2, 2′-azobis(4-methoxy-2, 4-dimethyl-valeronitrile)] (1.6 mM) (Wako) in 20% CH_3CN (0.1 ml) was added to a mixture of 1 mM linoleic acid (0.1 ml) in phosphate-buffered saline (PBS) solution

containing 10 mM sodium dodecyl sulfate and a PBS solution of the test compound (20 μl), and the mixture was incubated at 37 °C for 1 h. The amount of peroxide was estimated by measuring the absorbance at 234 nm, and the inhibitory effects of the test compounds were expressed as the IC_{50}, the concentration required for 50% inhibition of the formation of peroxides in the absence of the test compound.

3.11. Radical scavenging effects of cacao polyphenols on the DPPH radical

To a solution of DPPH radical in EtOH (60 μM, 0.5 ml), a solution of the test polyphenol in MeOH (0.5 ml) was added, and the reaction mixture was left to stand for 30 min at room temperature. The scavenging activity of each polyphenol at 1.25, 2.5, 5, 7.5 and 10 μg/ml was estimated by measuring the absorption of the mixture at 520 nm, which reflects the amount of DPPH radical remaining in the solution. The scavenging activity was expressed as the EC_{50}, the concentration of polyphenol required for scavenging 50% of DPPH radical in the solution.

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