Phytochemistry, Vol 27, No 5, pp 1315 1319, 1988
Printed in Great Britain

0031-9422/88 $3 00+0 00
Pergamon Press plc

TANNIN ANTIOXIDANTS FROM *OSBECKIA CHINENSIS*

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(*Revised received* 1 *September* 1987)

Key Word Index—*Osbeckia chinensis*, Melastomataceae; hydrolysable tannins, casuarinin, casuarin, antioxidative activities; *in vitro* lipid peroxidation

Abstract—Six hydrolysable tannins, casuarinin (**1**), casuariin (**2**), punicacortein A (**3**), degalloyl-punicacortein A (**4**), 2,3-[(*S*)-4,4′,5,5′,6,6′-hexahydroxydiphenoyl]-D-glucopyranoside (**5**), and 4,6-[(*S*)-4,4′,5,5′,6,6′-hexahydroxydiphenoyl]-D-glucopyranoside (**6**), and related compounds, gallic acid, methyl gallate and ellagic acid, were isolated from the methanol extract of *Osbeckia chinensis*. In order to confirm the structure–activity relationship, the antioxidant activities of these compounds were examined both in the food model and *in vitro* systems. The antioxidative efficiency, using the thiocyanate and TBA methods, increased in the order of gallic acid < α-tocopherol < methyl gallate < (**4**) < (**3**) < (**5**) = (**6**) < (**2**) < (**1**) = BHA < ellagic acid The *in vitro* antioxidant order is α-tocopherol < gallic acid < methyl gallate < (**4**) < (**3**) = (**5**) = (**6**) = ellagic acid < (**2**) < (**1**) in the rabbit erythrocyte membrane ghost system, and ellagic acid < (**4**) < gallic acid < (**3**) = (**5**) = (**6**) < methyl gallate < (**2**) < (**1**) = α-tocopherol in the rat liver microsome system

INTRODUCTION

Antioxidant activities of crude drugs have been already described by many investigators [1–3]. Most antioxidant activity has been attributed to polyphenols, such as gallate esters, ellagic acid, hydrolysable tannins and flavonoids [4–8]. The isolation of five flavonoids, from *Osbeckia chinensis* L (Melastomataceae), which had the strongest antioxidant activity among the 195 crude drugs screened [3], was reported in a recent paper [8] During the isolation procedure, a large amount of ellagic acid was also obtained together with many hydrolysable tannins The present study has been undertaken in order to isolate and identify the hydrolysable tannins of *O. chinensis* L and investigate their antioxidant activities.

RESULTS AND DISCUSSION

Identification of hydrolysable tannins and related compounds.

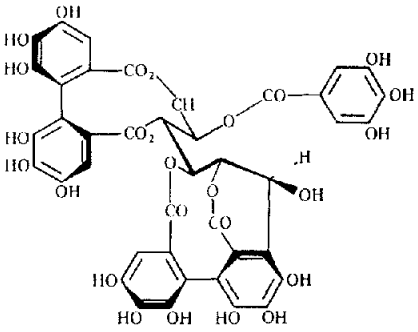
The methanol extract of *O. chinensis* L. was chromatographed on Amberlite XAD-2 column to give five fractions. Fraction (II) was purified on Toyo Pearl HW-40 F column chromatography and preparative HPLC to give (**1**), methyl gallate, ellagic acid, and flavonoids [8] Fraction (I) was repeatedly charged on preparative HPLC to afford **2**–**6** and gallic acid. Compounds **1**–**6** gave the characteristic colour of ellagitannins with the sodium nitrite–acetic acid reagent [9]. Compound **1**, FABMS *m/z* 937 $[M-H]^+$, $[\alpha]_D$ +43.6° (MeOH), the main tannin of *O. chinensis* L. was found to be identical with an authentic sample of casuarinin, isolated from *Casuarina stricta* [11]. Compounds **2**–**6** were identified as casuariin, punicacortein A, degalloyl-punicacortein A, 2,3-*O*-[(*s*)-4,4′5,5′6,6′-hexahydroxydiphenoyl]-D-glucopyranoside and 4,6-*O*-[(*s*)-4,4′5,5′6,6′-hexahydroxydiphenoyl]-D-glucopyranoside, by comparison with the published data on $[\alpha]_D$, UV, IR, ^{1}H NMR, and ^{13}C NMR spectra [11–14].

Effect on autoxidation of linoleic acid in alcohol–water system

The inhibitory effect of the isolated tannins and related compounds was examined in the alcohol–water model system with the thiocyanate and TBA methods [15, 16] Sixteen μM offeach sample, α-tocopherol, and butyl hydroxyanisole (BHA) was used for the assays. As shown in Fig. 1(a, b), ellagic acid markedly inhibited the formation of linoleic acid hydroperoxides or TBA-reacting substances than that of BHA. All hydrolysable tannins and methyl gallate exhibited stronger activities than that of α-tocopherol The antioxidative efficiency increased in the order of gallic acid < α-tocopherol < methyl gallate < (**4**) < (**3**) < (**5**) = (**6**) < (**2**) < (**1**) = BHA < ellagic acid.

Effect of isolated tannnins and related compounds on lipid peroxidation of rabbit erythrocyte membrane ghost induced by t-BuOOH

Like many other biological membranes, red blood cell membranes are prone to be oxidized because of their high polyunsaturated lipid content. Hence, *in vitro* evaluation of the antioxidant activity of tannins and related compounds using the rabbit erythrocyte membrane ghost system was undertaken [17–19]. Ten or 25 μM of each sample, α-tocopherol as the standard antioxidant was used for the assay (Fig 2) The antioxidant efficiency, at the concentration of 10 μM, increased in the order of α-tocopherol < gallic acid < methyl gallate < (**4**) < (**3**) = (**5**) = (**6**) = ellagic acid < (**2**) < (**1**) At the concentration of



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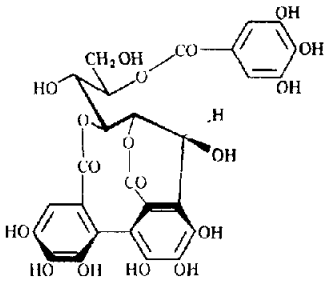

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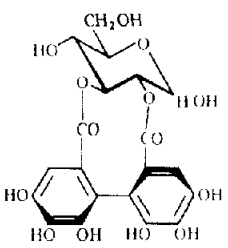

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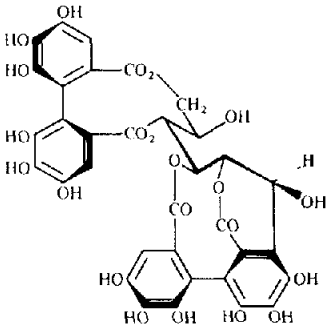

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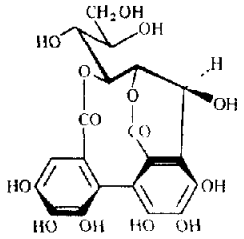

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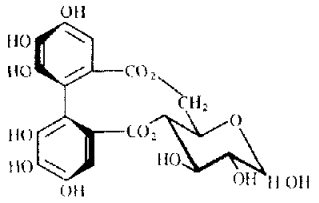

6

25 μM, the isolated tannins showed a maximal inhibition (about 90%), and (1) exhibited complete inhibition (over 95%)

Effect of isolated tannins and related compounds on lipid peroxidation of rat liver microsome induced by ADP-Fe^{3+}–EDTA-Fe^{3+}–NADPH

Numerous enzymatic lipid peroxidation systems have been reported that addition of iron or iron-chelate complex is required for promotion of peroxidation [20]. This requirement has been well studied in NADPH-dependent microsomal lipid peroxidation, especially, by addition of ADP-Fe^{2+} or by ADP-Fe^{3+}–EDTA-Fe^{3+} [21] Enhancement of lipid peroxidation by ADP-Fe^{2+} complex and NADPH has been considered to correlate with the perferryl ion-dependent initiation step [22–24] Addition of EDTA has been suggested to increase the hydroperoxide-dependent initiation step of microsomal lipid peroxidation [23–25] The effects of isolated tannins and related compounds on ADP-Fe^{3+}–EDTA-Fe^{3+}–NADPH-induced lipid peroxidation in rat liver microsome were also determined Figure 3 shows the inhibition of microsomal lipid peroxidation with the addition of hydrolysable tannins and related compounds at the concentration of 25 or 50 μM. At low concentration (25 μM), besides methyl gallate, (1) and (2), the other compounds showed no inhibitory effect At the concentration of 50 μM, the antioxidative order was ellagic acid < (4) < gallic acid < (3) = (5) = (6) < methyl gallate < (2) < (1) = α-tocopherol

Recent studies have shown that ellagic acid inhibits the mutagenicity of diol-epoxides of several polycyclic aromatic hydrocarbons (PAH) in the Ames test using *Salmonella typhumurium* [26], epidermal metabolism and DNA-binding of benzo[a]pyrene [27]. PAH-induced skin carcinogenesis in BALB/c mice by external or oral treatment [28, 29] It is well known that ellagic acid is easily produced from hydrolysable tannins in plants During the last 10 years, Nishioka *et al* [30] and Okuda *et al* [31] have reported the isolation, identification, and investigation of physiological activities of several hundred tannins Of these, 25 tannins and related compounds were examined for the inhibitory effects on lipid peroxidation using the rat liver mitochondria and microsome systems when hydrolysable tannins were found to show stronger inhibition than condensed tannins in both systems [32] In our previous report, ellagic acid was reported to have strong inhibitory effect on peroxidation of linoleic acid in alcohol-water system Ellagic acid exhibited a strong inhibitory effect on lipid peroxidation induced by adriamycin, but, showed only a weak antioxidant activity in the other *in vitro* systems [7, 32] On the other hand, casuarinin exhibited exceedingly strong inhibitory activities both on *t*-BuOOH-induced lipid peroxidation system using the rabbit erythrocyte membrane ghost and ADP-Fe^{3+} EDTA-Fe^{3+}–NADPH-induced lipid peroxidation system using rat liver microsome, and it had almost the same activity like BHA in the food model system Antioxidant activity of (2) was not as strong as (1) in both the food model and *in vitro* systems, but, stronger than that of (3) or (4) This fact indicates that the hexahydroxydiphenoyl group (HHDP) contributes to antioxidant activity while galloyl group does not

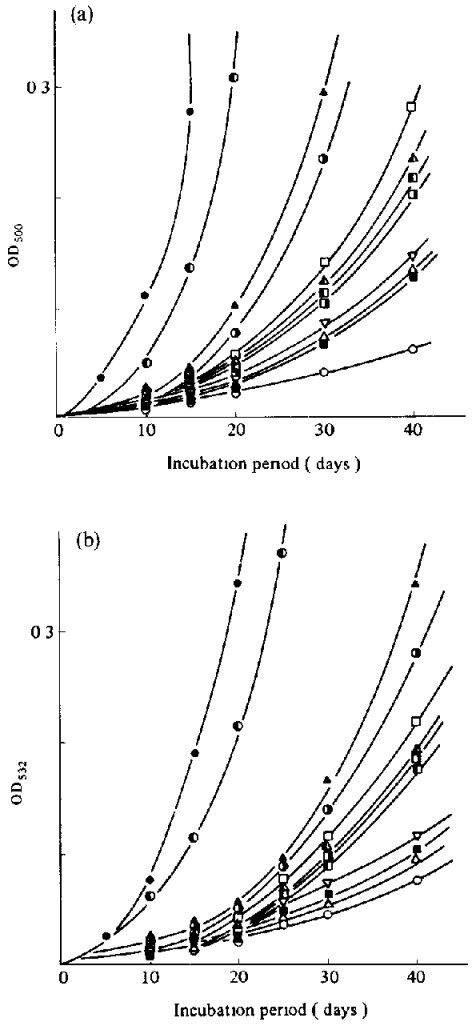

Fig 1 Effect of isolated tannins and related compounds on autoxidation of linoleic acid in the water/alcohol system (a) Thiocyanate method. (b) TBA method. Sixteen μM of each sample, α-tocopherol, and BHA was used for these experiments Results are averages of three replicates -△ (1), -△- (2), -▲-(3), -□- (4), -◧- (5), -◨-(6), -◐- gallic acid, -◑- methyl gallate, -○- ellagic acid, -▲-α-tocopherol, -■-BHA, and -●-control

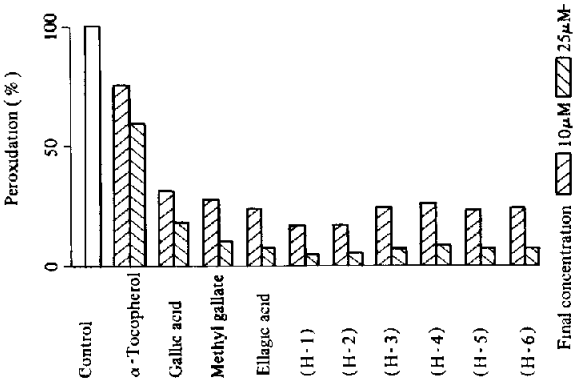

Fig 2. Effect of isolated tannins and related compounds on lipid peroxidation of rabbit erythrocyte membrane ghost induced by *t*-BuOOH. Ten or 25 μM of each sample, α-tocopherol was used for this experiment The values obtained without samples were taken for 100% lipid peroxidation

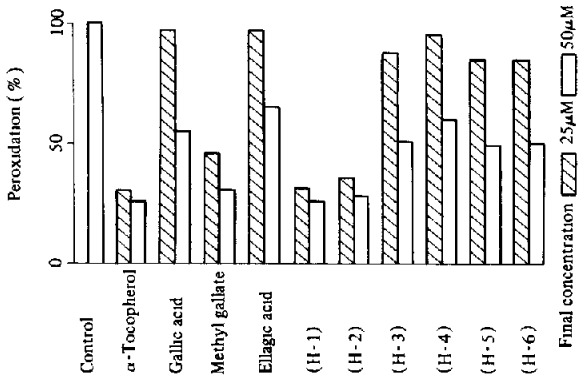

Fig 3 Effect of isolated tannins and related compounds on lipid peroxidation of rat liver microsome induced by ADP-Fe^{3+}-EDTA-Fe^{3+}/NADPH Twenty-five or 50 μM of each sample, α-tocopherol was used for this experiment. The values obtained without samples were taken for 100% lipid peroxidation.

In a previous paper, quercetin 3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside isolated from *O. chinensis* L, and rutin, quercetin-disaccharide, were shown to have very strong antioxidant activity [8]. The inhibitory effects of tannins were much stronger than the flavonoids on *t*-BuOOH-induced lipid peroxidation of membrane ghost (data not shown). Nevertheless, antioxidant activity of tannins was not so strong comparing with the flavonoids on ADP-Fe^{3+}-EDTA-Fe^{3+}-NADPH-induced lipid peroxidation of liver microsome (data not shown). This reverse relationship cannot be explained at the present stage, more extensive studies need to be carried out.

Tannins and flavonoids are widely distributed in the plant kingdom [33] The human diet contains a complex mixture of these plant phenols, and it has been estimated that some individuals consume as much as 1 g of plant phenols per day in their diet [33]. Our data showing inhibition of enzymatic and non-enzymatic lipid peroxidation by plant phenols suggest that the use of certain plant phenols in cosmetic products and pharmaceutical preparation may play a therapeutic role via lipid peroxidation.

EXPERIMENTAL

EIMS were measured with a JEOL JMS D-100, while FABMS were recorded on a JEOL JMS DX-300 instrument ^{1}H NMR (200 MHz) and ^{13}C NMR (50 MHz) spectra were measured on a JEOL JNM-FX 200 instrument The dried plant material of *O. chinensis* L. was collected and identified by Dr T-S Wu in June 1983 A voucher specimen is deposited in the

Faculty of Pharmaceutical Science, Chinese Medicine College, Taichung, Taiwan, R O C

The air-dried plant materials (4 kg) of *O chinensis* L were repeatedly extracted with methanol The methanol extract was chromatographed on a packed column of Amberlite XAD-2 using MeOH–H_2O (0–100%) to give five fractions (I–V) Fraction (II) (elution of 25% MeOH) was separated on Toyo Pearl HW-40 F with MeOH as eluent, and purified by HPLC using a Develosil ODS-10 to give (**1**), methyl gallate and ellagic acid Fraction (I) (elution of 100% H_2O) was repeatedly separated on HPLC using a Develosil ODS-10 to yield (**2–6**) and gallic acid Total yield of each tannin and related compound (**1**) 0 28%, (**2**) 0 07%, (**3**) 0 188%, (**4**) 0 011%, (**5**) 0 012%, (**6**) 0 012%, ellagic acid 0 25%, gallic acid 0 188 and methyl gallate 0 025%

1 This compound was obtained as a pale yellow, amorphous powder, $[\alpha]_D^{25}$ +43 6° (MeOH, *c* 1 0), FABMS *m/z* 937 $[M+H]^+$, IR ν_{max}^{KBr} cm^{-1} 3400, 1720, 1610, 1500, 1445, 1355–1300, 1175, and 1085, UV λ_{max}^{MeOH} nm 221 and 267, ^1H NMR ($CD_3OD + D_2O$), δ 7 12 (2H, s), 6 90, 6 64, 6 52 (each 1H, s), 5 64 (1H, *d*, *J* = 5 Hz) 5 38 (3H, *br* s), 4 68 (1H, *dd*, *J* = 2 and 5 Hz), 4 18 (1H, *dd*, *J* = 3 and 13 Hz), and 4 06 (1H, *d*, *J* = 13 Hz), ^{13}C NMR ($CD_3OD + D_2O$), δ 170 6, 170 2, 169 3, 167 0, 166 8, 146 1, 145 9, 145 6 (3), 145 4 (2), 144 5, 143 9, 143 7, 139 8, 139 4, 137 3, 136 2, 135 2, 124 4, 126 8, 126 4, 120 2, 119 7, 117 6, 116 1, 116 0, 115 1, 119 7 109 0, 107 5, 105 3 (2), 110 5, glucose moiety 77 4, 74 4, 71 1, 70 2, 67 0, 65 2

2 This compound was obtained as a pale yellow, amorphous powder, $[\alpha]_D^{25}$ +162° (MeOH, *c* 0 5), FABMS *m/z* 785 $[M-H]^+$, IR ν_{max}^{KBr} cm^{-1} 3400, 1720, 1610, 1450, and 1360–1295, UV λ_{max}^{MeOH} nm 213, 233, 257, ^1H NMR ($CD_3OD + D_2O$) δ 6 79, 6 62, 6 46, 5 66 (1H, *d*, *J* = 5 Hz) 5 43 (1H, *t*, *J* = 3 Hz), 5 02 (1H, *dd*, *J* = 3 and 9 Hz), 4 74 (1H, *dd*, *J* = 3 and 5 Hz), 4 64 (1H, *dd*, *J* = 3 and 13 Hz), 4 15 (1H, *dd*, *J* = 3 and 9 Hz), 3 84 (1H, *d*, *J* = 13 Hz), ^{13}C NMR (50 MHz, $CD_3OD + D_2O$), δ 171 6, 170 6, 169 9, 167 5, 146 2, 146 0, 145 8 (2), 145 1 (2), 144 1, 143 9, 140 2, 137 6, 136 4, 135 6, 126 8, 126 5, 123 3, 119 8, 118 0, 116 3 (2), 115 2, 119 8, 108 9, 107 7, 105 5, glucose moiety 77 6, 74 4, 70 8, 68 5, 67 2, 66 7

3 This compound was obtained as a tan amorphous powder, $[\alpha]_D^{25}$ −71 8° (MeOH, *c* 1 0), FABMS *m/z* 635 $[M-H]^+$, UV λ_{max}^{MeOH} nm 213 233, 272, ^1H NMR ($CD_3OD + D_2O$) δ 7 08 (2H, s), 6 41 (1H *s*), 5 65 (1H, *J* = 5 Hz), 5 38 (1H, *br s*), 5 00 (1H, *m*), 4 86 (1H, *dd*, *J* = 3 and 5 Hz), 4 16 (1H, *dd*, *J* = 3 and 9 Hz), and 3 82 (2H, s), ^{13}C NMR ($CD_3OD + D_2O$) δ 171 4, 168 3, 168 0, 146 3, 146 0, 145 5 (2), 144 4, 144 0, 139 8, 139 4, 135 1, 127 7, 121 8, 120 2, 116 4, 116 2, 118 8, 105 9, 111 2 (2), glucose moiety 77 3, 74 2, 72 6, 71 6, 68 2, 61 3

4 This compound was obtained as white amorphous powder, FABMS *m/z* 483 $[M-H]^+$, $[\alpha]_D^{25}$ +55 2° (MeOH, *c* 0 5), UV λ_{max}^{MeOH} nm 213, 233, 259, ^1H NMR (D_2O) δ 6 52 (1H, *s*), 5 64 (1H, *d*, *J* = 5 Hz), 5 26 (1H, *br* s) 4 81 (1H, *dd*, *J* = 2 and 5 Hz), 3 74 (2H, *m*), 3 61 (2H, s), ^{13}C NMR (D_2O) δ 172 3, 168 2, 146 2, 146 0, 144 4, 144 2, 139 2, 135 5, 121 8, 120 0, 116 2 (2), 119 2, 105 7, glucose moiety 77 5, 74 5, 72 6, 71 6, 68 0, 63 8

5 A tan amorphous powder, $[\alpha]_D^{25}$ +63 8° (H_2O, *c* 1 0), FABMS *m/z* 483 $[M-H]^+$, ^1H NMR (D_2O) δ 6 74, 6 64 (each 1H, s), 5 43 (1H, *d*, *J* = 4 Hz), 5 38 (1H, *t*, *J* = 9 Hz), ^{13}C NMR (D_2O), δ 171 2, 169 8, 146 6 (2), 145 7 (2), 136 8, 136 5, 127 2, 126 8, 116 9, 116 7, 109 7 (2), glucose moiety, α 93 4, 75 9, 73 9, 73 4, 70 9, 63 9, β 97 8, 76 9, 75 9, 73 4, 72 2, 63 9

6 A tan amorphous powder, $[\alpha]_D^{25}$ +43 7° (H_2O, *c* 1 0), FABMS *m/z* 483 $[M-H]^+$, ^1H NMR (D_2O) δ 6 73, 6 63 (each 1H, s), 5 19 (1H, *d*, *J* = 4 Hz), ^{13}C NMR (D_2O) δ 172 2, 170 0, 147 2 (2), 146 2 (2), 136 9, 136 7, 127 5, 127 3, 116 9, 116 8, 109 9 (2), glucose moiety, α 95 5, 75 4, 74 5, 74 0, 69 0, 66 6, β 99 5, 77 2, 76 1, 75 3, 73 3, 66 6

Autoxidation of linoleic acid in H_2O*–alcohol system* Autoxidation of linoleic acid in the water/alcohol solution has been assayed by the thiocyanate and TBA methods [15, 16] Each sample was dissolved in DMSO to obtain a concentration of 16 μM, and added to a solution mixture of linoleic acid–EtOH 0 2 M Pi buffer (pH 7 0) The mixture was incubated at 40° and the peroxide value was determined at 500 nm after a colour reaction with $FeCl_3$ and ammonium thiocyanate The formation of TBA-reacting substances was measured at 532 nm after the reaction with thiobarbituric acid

Lipid peroxidation of rabbit erythrocyte membrane ghost Commercially available rabbit blood (50 ml) was obtained from Japan Biotest Institute Co Ltd and diluted with 150 ml of isotonic buffer solution (10 mM phosphate–152 mM NaCl) After centrifugation (1500 × g, 10 min), the blood was washed × 3 with 10 ml of isotonic buffer solution and lysed in 10 mM Pi buffer, pH 7 4 Erythrocyte membrane ghosts were pelleted by centrifugation 20 000 g, 40 min) and the precipitate was diluted to give a suspension (1 5 mg protein/ml) as determined by the method of ref [17] Peroxidation of the erythrocyte membrane ghosts induced by *t*-butylhydroperoxide was carried by the method of ref. [18]. Isolated hydrolysable tannins and related compounds were prepared by dissolving them in dimethyl sulphoxide to make final concentration at 10 or 25 μM. After incubation for 30 min., 1 ml of 2.0 M trichloride acetate–1.7 M HCl and 2 ml of 0 67% TBA–NaOH solution was added to stop the reaction The quantity of TBA-reacting substances was determined at 532 nm after coloration with thiobarbituric acid [19]

Lipid peroxidation of rat liver microsome Wister rats (8 weeks, 180–200 g) were killed and the liver was removed and homogenized Microsomes were prepared by differential centrifugation with the method of ref [34] Fresh solutions, in 50 mM Tris–HCl buffer, were prepared each time at the concentration of 1 mg/ml microsomal protein as determined by the Lowry method Test compounds were dissolved in DMSO and added to the microsomal incubation (final concentration at 25 or 50 μM). The following addition were made and incubated at 37 for 30 min 2 5 mM adenosine-5'-diphosphate (ADP), 0 1 mM ethylenediaminetetraacetic acid (EDTA) and 0 1 mM ferric nitrate (ADP-Fe^{3+} EDTA-Fe^{3+} system) and 5 mM nicotinamide-adenine dinucleotide phosphate reduced form (β-NADPH) in 50 mM Tris–HCl buffer solution After incubation, formation of TBA-reacting substances was measured as described above

Acknowledgements—The authors thank Dr T Okuda, Faculty of Pharmaceutical Sciences, Okayama University, for supplying the authentic sample of casuarinin Thanks are also due to Mr S Kumazawa for the measurement of FABMS This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan and the Research Foundation for Oriental Medicine

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