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Antioxidant activity of heartwood extracts of Papua New Guinean woods

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Abstract Antioxidant effects of methanol extracts from the heartwood of 23 Papua New Guinea (PNG) wood species were examined. The extract of *Amoora* sp. (Meliaceae) showed the strongest antioxidant activity against lipid peroxidation in rabbit erythrocyte membrane and linoleic acid autoxidation. Also, the extract of *Amoora* sp. showed potent 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity. These results showed that the heartwood of *Amoora* sp. is a possible source of antioxidative agents. The result of antioxidant activity-guided fractionation suggested that gallic acid, protocatechuic acid, and hydrolyzable tannins in the extract of *Amoora* sp. caused the potent antioxidant activity.

Key words *Amoora* sp. · Heartwood · Extractives · Antioxidant · Meliaceae

Introduction

Tropical forest area occupies 40%–50% of all forest areas of the world. The Papua New Guinea (PNG) mainland and surrounding islands have an extraordinarily rich flora and great diversity of vegetation types that parallel the diverse physiography of the land. A high proportion of the land area has a forest cover, most of it evergreen rain forest. The great diversity of tropical woods appears in the qualitative and quantitative diversity of extractives of the woods or, from a chemical point of view, the wood components. We are interested in the biological activities of the extract of heartwood of PNG woods for effective, useful utilization as a biomass resource. Recently, we reported antifungal components,[1] melanin biosynthesis inhibitory compounds[2] and 5α-reductase inhibitory compounds,[3] from PNG wood species. Thus, these tropical woods give us a good chance to find bioactive materials.

Interest in active oxygen species has grown with experimental evidence that free radicals play important roles in a variety of pathological conditions such as ischemia-reperfusion, autoimmune diseases, cardiovascular diseases, cancer initiation, and aging processes.[4–6] As a consequence, antioxidative materials are now thought to be prospective protective agents against these diseases.

Moreover, antioxidants are major ingredients that protect the oil from peroxidation. Because synthetic antioxidants such as butylhydroxyanisole (BHA) have possible activity as promoters of carcinogenesis,[7] utilization of natural antioxidants is desirable. Although natural antioxidants such as α-tocopherols and L-ascorbic acid are widely used, investigations are being carried out to discover more potent, safer antioxidants. We report here the antioxidant activities of the methanol extracts from the heartwood of PNG woods and antioxidative components of *Amoora* sp. (Meliaceae), which showed the strongest antioxidant activity among the 23 PNG wood samples we studied.

Materials and methods

Sample woods

Heartwood from the following 23 PNG wood species were obtained from the PNG Forest Research Institute: *Albizia falcataria* (Mimosaceae), *Alstonia scholaris* (Apocynaceae), *Amoora* sp. (Meliaceae), *Anthocephalus chinensis* (Rubiaceae), *Artocarpus incisus* (Moraceae), *Buchanania* sp. (Anacardiaceae), *Calophyllum* sp. (Guttiferae), *Cananga adorata* (Annonaceae), *Canarium indicum* (Burseraceae), *Canarium oleoseum* (Burseraceae), *Dracontomelon dao* (Anacardiaceae), *Dysosylum pettigrewianum*

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(Meliaceae), *Eucalyptus deglupta* (Myrtaceae), *Garcinia latissima* (Guttiferae), *Hibiscus ellipticifolius* (Malvaceae), *Intsia bijuga* (Leguminosae), *Neonuaclea acuminate* (Rubiaceae), *Octomeles sumatrana* (Datiscaceae), *Palaquium galactoxylum* (Sapotaceae), *Pterocarpus indicus* (Leguminosae), *Terminalia* sp. (Combretaceae), *Toona surenii* (Meliaceae), and *Xanthophyllum papuanum* (Polygalaceae). All voucher specimens are preserved at the herbarium of the Department of Forest Products, Kyushu University, Japan.

Extraction of heartwood meals of 23 PNG species

The heartwood portion was chipped and milled to pass a no. 40 screen. Air-dried milled heartwoods were Soxhlet-extracted with methanol (MeOH) for 10 h.

Separation of extractives of *Amoora* sp.

Air-dried milled heartwood of *Amoora* sp. (108 g) was Soxhlet-extracted with methanol for 10 h. The extract was concentrated in vacuo. The residue (6.6 g) was suspended in water (0.5 l) and partitioned with *n*-hexane (0.5 l), diethyl ether (0.5 l), and ethyl acetate (EtOAc) (0.5 l) three times, respectively, successively to give an *n*-hexane-soluble portion (0.085 g, 1.6% of the methanol extract), a diethyl ether-soluble portion (0.97 g, 18.4% of the methanol extract), an ethyl acetate-soluble portion (0.69 g, 13.1% of the methanol extract), and an aqueous portion (3.52 g, 66.9% of the methanol extract). The diethyl ether-soluble portion (0.90 g) was fractionated into eight fractions (A–H) by column chromatography (CC) on silica gel 200 g (Wakogel C-200; Wako, Osaka, Japan) eluting with ethyl acetate-*n*-hexane gradient. Of these fractions, fraction E [13 mg, thin-layer chromatography (TLC), silica gel, EtOAc/*n*-hexane = 1/3, $R_f < 0.2$] and fraction F (87 mg, TLC, silica gel, EtOAc/*n*-hexane = 1/3, $R_f < 0.1$) were obtained as the antioxidative fractions evaluated by the rabbit erythrocyte membrane ghost method. Protocatechuic acid (10 mg from fraction E and 4 mg from fraction F, respectively) and gallic acid (20 mg from fraction F), the active constituents from fractions E and F were isolated by preparative high-performance liquid chromatography (HPLC) (Inertsil PREP-ODS: 20 mm i.d. ×250 mm) using 0.1% TFA/MeOH (70/30), 12 ml/min. The ethyl acetate-soluble portion (0.69 g) was fractionated into four fractions (a–d) by CC on silica gel (100 g) eluting with ethyl acetate-*n*-hexane gradient. Of these, fraction c (237 mg, TLC, silica gel, EtOAc/*n*-hexane = 1/3, $R_f < 0.1$) was obtained as the main fraction. Gallic acid (87.3 mg) was isolated as the main active constituent from fraction c by preparative HPLC (Inertsil PREP-ODS) using 0.1% TFA/MeOH (70:30), 12 ml/min.

Acid hydrolysis of aqueous portion of *Amoora* sp.

A solution of the aqueous portion (50 mg) in 5% H_2SO_4 (50 ml) was heated in a boiling waterbath for 6 h. After cooling, the reaction mixture was extracted with EtOAc. The EtOAc extract was analyzed by HPLC [(Inertsil ODS: 4.6 mm i.d. ×150 mm) using 0.1% TFA/MeOH (90:10), 1 ml/min] to show some peaks identical to those of authentic gallic acid (t_R 6.46 min) and protocatechuic acid (t_R 14.53 min).

Antioxidant activity assays

Methanol extracts of heartwood of PNG woods were used. Butylhydroxytoluene (BHT), butylhydroxyanisole (BHA), and α-tocopherol were used as positive controls.

Rabbit erythrocyte membrane ghost method

The rabbit erythrocyte membrane ghost method was performed as previously described.[8] For the ghost method, rabbit blood (40 ml) was diluted with 120 ml isotonic buffer (10 mM phosphate/152 mM NaCl, pH 7.4) and centrifuged (1500 *g*, 10 min). The precipitated blood was washed three times with 24 ml isotonic buffer, lysed by suspending it in 10 mM phosphate buffer (pH 7.4), and centrifuged (20000 *g*, 40 min) to precipitate erythrocyte membrane ghosts, which were then diluted to give a suspension (205 mg protein/ml). The protein concentration was determined by the Bradford method. Peroxidation of the erythrocyte membrane ghosts was induced by the addition of *t*-butyl hydroperoxide. A mixture of 0.9 ml of the membrane ghosts suspension and 0.05 ml of the test solution in dimethylsulfoxide was incubated for 30 min at 37°C. After the incubation, 1 ml of 2.0 M trichloroacetic acid/1.7 M HCl was added to stop the reaction. The mixture was colored by heating with 2 ml of 0.67% thiobarbituric acid (TBA)/1.0 N NaOH solution for 10 min in boiling water. The quantity of the TBA-reacting substance was estimated from the absorbance at 532 nm with a spectrophotometer. Lipid peroxidation was calculated as follows.

$$\text{Lipid peroxidation}\,(\%) = 100\,(A - C)/(B - C)$$

where A, B, and C denote absorbances at 532 nm after incubation with a sample, after incubation without a sample, and before incubation without *t*-butyl hydroperoxide or a sample, respectively.

Linoleic acid autoxidation method

The linoleic acid autoxidation method was performed as previously described.[8] A 5-ml aliquot of 0.05 M phosphate buffer (pH 7.0), 5.0 ml of linoleic acid solution (1.4% v/v in ethanol), 0.1 ml of antioxidant sample solution in methanol, 0.1 ml of 1.87 mM $FeCl_2$ aqueous solution, and 0.1 ml of 12.5 mM ascorbate aqueous solution were added to 2.2 ml of water in a test tube; the mixture was kept at 50°C. After every 48 h, 0.2 ml of the reaction mixture was removed and colored by vigorously mixing and incubating with a solution containing 9.4 ml of 75% aqueous ethanol, 0.2 ml 30% ammonium thiocyanate, and 0.2 ml 0.02 M $FeCl_2$/3.5% HCl for

exactly 3 min at room temperature. The absorbance of the colored solution was measured at 500 nm with reference to a solution incubated without linoleic acid or an antioxidant sample. A blank test was performed with no antioxidant sample.

Scavenging of 1,1-diphenyl-2-picrylhydrazyl

Free-radical scavenging activity of each compound was assayed using a stable free-radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH), according to a method previously reported[9] with modification. Thus, the reaction mixture contained 0.5 ml of 60 μM DPPH in ethanol and 0.5 ml of sample solution in ethanol. After the reaction was carried out at room temperature for 20 min, the free-radical scavenging activity of each sample was quantified by the decolorization of DPPH at 514 nm.

Results and discussion

The meals of heartwood from 23 PNG wood species were Soxhlet-extracted with methanol for about 10 h. Each of the methanol extracts was examined for antioxidant activity test. During the preliminary screening of 23 PNG woods by the linoleic acid autoxidation method the extracts of *Amoora* sp., *G. latissima*, *I. bijuga*, *Terminalia* sp., *D. dao*, *A. falcataria*, and *N. acuminate* showed potent antioxidant activity. Among them, the *Amoora* sp. extract showed the strongest antioxidant activity (Fig. 1). Its extract showed the strongest antioxidant activity also by the rabbit erythrocyte membrane ghost method (Table 1). As a result, the methanol extract of *Amoora* sp., which showed the strongest antioxidant activity among 23 PNG species by both methods, was selected for further investigation.

The methanol extract of *Amoora* sp. was partitioned by *n*-hexane, diethyl ether, and ethyl acetate. The lipid peroxidations with the *n*-hexane-soluble, diethyl ether-soluble, ethyl acetate-soluble, and aqueous portions were 90.4%, 38.2%, 58.5%, and 76.8% respectively, at a concentration of 200 μg/ml by the rabbit erythrocyte membrane ghost method. The lipid peroxidations with the same series of extracts were 98.3%, 24.5%, 17.6%, and 19.3% at a concentration of 40 μg/ml for 48 h by the linoleic acid autoxidation method (Table 2). Thus, the compounds with antioxidant activity were dispersed. These results showed that several compounds in the ether-soluble, ethyl acetate-soluble, and aqueous portions caused the potent antioxidant effects of the methanol extract of *Amoora* sp. heartwood.

Membrane lipids are abundant in unsaturated fatty acids. These unsaturated molecules are most susceptible to the oxidative process, particularly linoleic acid. Lipid peroxidation is a typical free-radical oxidation and proceeds via a cyclic chain reaction. Therefore, their free-radical scavenging activities were determined by the use of a stable free radical, DPPH. The reducing activity of the DPPH

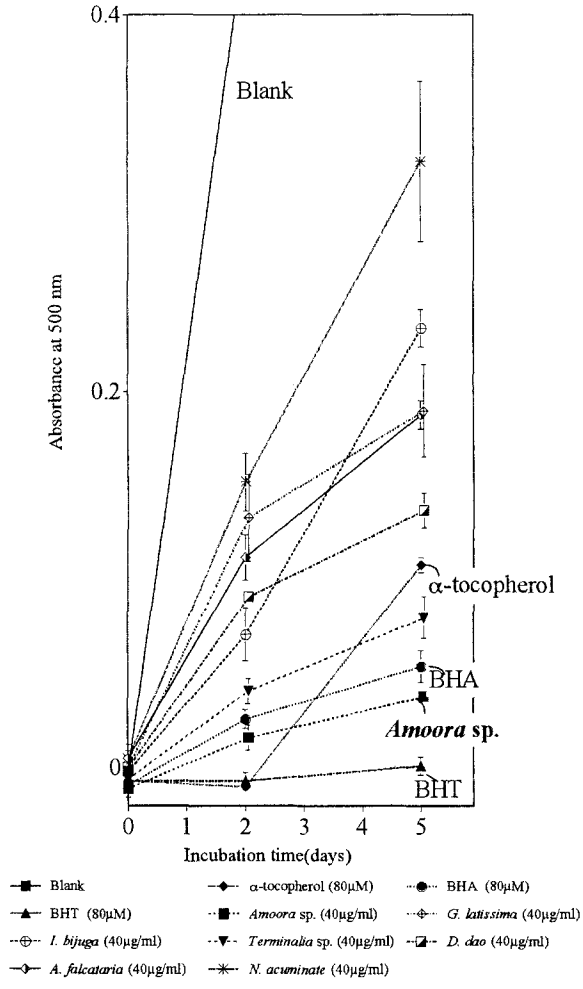

Fig. 1. Antioxidant activity of the methanol extracts of Papua, New Guinea (PNG) woods by the linoleic acid autoxidation method. Small absorbance denotes strong antioxidant activity. Results are means ± SD, $n = 3$

radical was determined by spectrophotometry; the 50% inhibitory concentrations (IC_{50}) of the *n*-hexane-soluble, ether-soluble, ethyl acetate-soluble, and aqueous portions were 34.6, 2.52, 1.00, and 1.16 μg/ml, respectively (Table 2). The DPPH radical-scavenging activities of the ether-soluble, ethyl acetate-soluble, and aqueous portions were more potent than those of BHT and BHA. These results suggested that most of the antioxidant activities of the ether-soluble, ethyl acetate-soluble, and aqueous portions are caused by their free-radical scavenging activities, and the tendency of their activities to differ slightly between the two methods may be due to the affinity of the extract constituents toward the site of the oxidation reaction, and the redox-combination (interaction) of the constituents.

Antioxidative activity-guided fractionation using the rabbit erythrocyte membrane ghost method led to the active fractions (E and F) from the diethyl ether-soluble portion. Protocatechuic acid and gallic acid were isolated as the main compounds in the active fractions. Gallic acid was isolated as the main constituent from the ethyl acetate-soluble portion (Fig. 2). It should be noted that several compounds with ultraviolet-visible spectra similar to those

of gallic acid and protocatechuic acid (data not shown) were detected in the diethyl ether-soluble and ethyl acetate-soluble portions but were not isolated because of the low concentrations. The isolated compounds are well known as potent antioxidants.[10,11] In addition, as shown in Table 2,

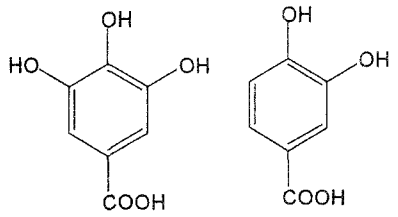

Fig. 2. Chemical structures of antioxidants isolated from ether-soluble and ethyl acetate-soluble fractions of *Amoora* sp.

Table 1. Antioxidant activity of methanol extracts from heartwood of PNG trees determined by the ghost method

Sample	Lipid peroxidation (%)
α-Tocopherol (400μM)	83.9 ± 3.9
BHA (400μM)	48.9 ± 8.0
BHT (400μM)	32.9 ± 13.1
Albizia falcataria	94.1 ± 11.7
Alstonia scholaris	92.8 ± 8.9
Amoora sp.	48.0 ± 3.1
Anthocephalus chinensis	97.5 ± 2.0
Artocarpus incisus	85.4 ± 6.4
Buchanania sp.	93.2 ± 7.5
Calophyllum sp.	55.3 ± 15.3
Cananga adorata	102.1 ± 10.8
Canarium indicum	108.3 ± 17.5
Canarium oleoseum	104.3 ± 11.6
Dracontomelon dao	75.2 ± 8.5
Dysosylum pettigrewianum	72.9 ± 3.4
Eucalyptus deglupta	75.1 ± 11.1
Garcinia latissima	96.8 ± 8.7
Hibiscus ellipticifolius	100.1 ± 10.4
Intsia bijuga	61.9 ± 4.0
Neonuaclea acuminate	107.3 ± 11.3
Octomeles sumatrana	103.1 ± 10.1
Palaquium galactoxylum	96.6 ± 8.3
Pterocarpus indicus	70.1 ± 10.9
Terminalia sp.	76.2 ± 12.4
Toona surenii	60.5 ± 5.6
Xanthophyllum papuanum	97.7 ± 17.5

The smaller lipid peroxidation denotes stronger antioxidant activity (mean ± SD, $n = 3$, sample concentration 200μg/ml)
PNG, Papua New Guinean; BHA, butylhydroxyanisole; BHT, butylhydroxytoluene

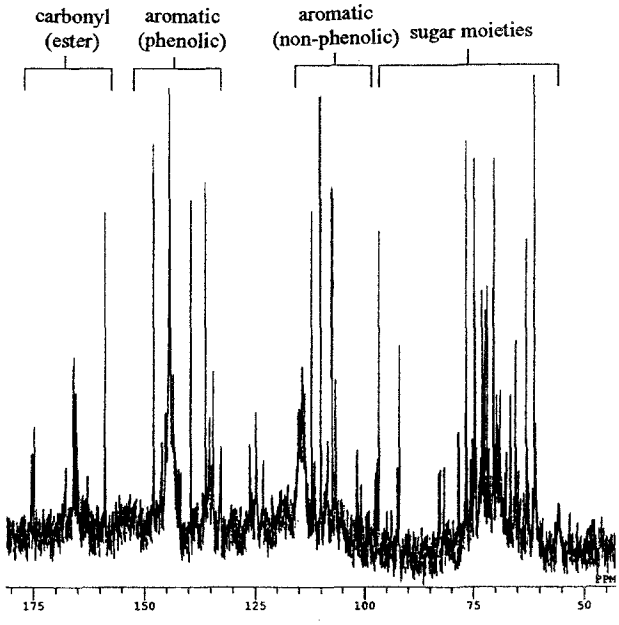

Fig. 3. Carbon-13 nuclear magnetic resonance (^{13}C NMR) of the aqueous portion of *Amoora* sp. (DMSO-*d6*)

Table 2. Antioxidant activities of methanol extract of *Amoora* sp., its fractionated portions, and the known antioxidants

Sample	Lipid peroxidation (%)[a]		IC_{50}[b] (μg/ml) (DPPH method)
	Ghost method	Linoleic acid method	
MeOH extract	48.0	11.5	2.16
n-Hexane-soluble	90.4	98.3	34.6
Diethyl ether-soluble	38.2	24.5	2.52
Ethyl acetate-soluble	58.5	17.6	1.00
Aqueous portion (residue)	76.8	19.3	1.16
BHT	32.9 (400μM)	4.28 (80μM)	22.0 (99.8μM)
BHA	48.9 (400μM)	19.8 (80μM)	14.1 (78.5μM)
α-Tocopherol	83.9 (400μM)	0.87 (80μM)	0.966 (2.24μM)
Gallic acid[c]	ND	ND	0.206 (1.10μM)
Protocatechuic acid[c]	ND	ND	0.462 (3.00μM)

ND, not determined; DPPH, 1,1-diphenyl-2-picrylhydrazyl
[a] Lipid peroxidation (%) added with MeOH extract and *n*-hexane-soluble, diethyl ether-soluble, ethyl acetate-soluble, and aqueous portions was measured at a concentration of 200μg/ml by the rabbit erythrocyte membrane ghost method and at a concentration of 40μg/ml for 48h by the linoleic acid autoxidation method
[b] The IC_{50} values were calculated from regression lines where the abscissa represented the concentration of the tested compound, and the ordinate represented the average percent reduction of DPPH radical from three tests
[c] Ethyl acetate-soluble agents contain gallic acid, and diethyl ether-soluble agents contain gallic acid and protocatechuic acid

DPPH radical-scavenging activities of gallic acid and protocatechuic acid were more potent than those of BHT, BHA, and α-tocopherol.

As shown in Fig. 3, ^{13}C nuclear magnetic resonance (NMR) of the aqueous portion of *Amoora* sp. showed a carbonyl group (155–170 ppm), phenolic carbon (130–150 ppm), aromatic carbon (100–120 ppm), and sugar moieties (60–100 ppm), which are characteristic signals of hydrolyzable tannin.[12–14] Furthermore, acid hydrolysis of the aqueous portion gave mainly gallic acid and a small amount of protocatechuic acid. Hydrolyzable tannins are known to exhibit potent antioxidant activity and their activity depends on the number of phenolic hydroxyl groups on a phenyl ring and in a molecule.[11] These results suggested that the potent antioxidant activity of the aqueous portion of *Amoora* sp. was caused by hydrolyzable tannins in it.

These results are comparable study of antioxidant effects of the extract from the heartwoods of 23 PNG woods. The *Amoora* sp. extract showed the strongest antioxidant effect. These results indicate that an extract of *Amoora* sp. heartwood is a possible source of antioxidative agents. The results of antioxidant activity-guided fractionation suggest that gallic acid, protocatechuic acid, and hydrolyzable tannins in the extract of *Amoora* sp. cause potent antioxidant activity.

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