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Inhibition of lipid peroxidation by diterpenoid from *Podocarpus nagi*

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Abstract. A diterpenoid, totarol (**1**), from *Podocarpus nagi* was evaluated as an antioxidant. This diterpenoid inhibited autoxidation of linoleic acid. Mitochondrial and microsomal lipid peroxidation induced by Fe(III)-ADP/NADH or Fe(III)-ADP/NADPH were also inhibited. Nagilactone E (**2**), a norditerpene lactone isolated from the same source, had no antioxidative activity. Furthermore, totarol protected red cells against oxidative hemolysis. This diterpene was shown to be effective in protecting biological systems against oxidative stresses.
Key words. *Podocarpus nagi*; totarol; lipid peroxidation; oxidative stress.

In a previous paper, antimicrobial activity of a totarane diterpene, totarol (**1**) isolated in relatively high concentrations from the root bark of *Podocarpus nagi* (Podocarpaceae), was described[1]. Noticeably, this diterpene exhibited bactericidal activity against methicillin-resistant strains of *Staphylococcus aureus* (MRSA)[2]. The species of *Podocarpus* are a rich source of unique nor- and bisnor-diterpene dilactones known as nagilcatones[3], for example (**2**). Totarol is situated as a key precursor in the biosynthetic pathway of nagilactones[4]. In addition to their structural uniqueness, nagilactones are also known for their various biological activities[5–12].

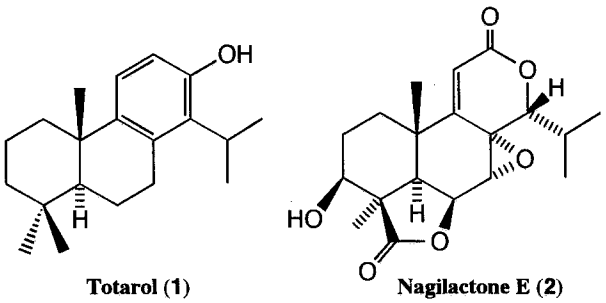
In our continuous search for bioactive substances from *P. nagi*, totarol was found to exhibit potent antioxidative activity. The oxidation of unsaturated fatty acids in biological membranes leads to a decrease in membrane fluidity and disruption of membrane structure and function[13,14]. Cellular damage due to lipid peroxidation causes serious derangements, such as ischemia-reperfusion injury[15], coronary arteriosclerosis[16], and diabetes mellitus[15], as well as being linked with aging and carcinogenesis[17]. A number of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been developed, but their use has begun to be restricted because of their toxicity[18–20]. As the result, there is considerable interest in the food industry and in preventive medicine in the characterization of natural antioxidants from botanical sources[21]. This report describes the antioxidative activity of totarol and its effects on biological systems.

Materials and methods

Chemicals. Totarol and nagilactone E were isolated from the root of *P. nagi*[22,23]. BHT, TBA, adenosine diphosphate (ADP) and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Reduced nicotinamide adenine dinucleotide (NADH) and reduced NAD phosphate (NADPH) were obtained from Oriental Yeast Co. (Toyko, Japan). Other chemical reagents were of commercial grade.

Assay of autoxidation. Oxidation of linoleic acid was measured by the modified method described by Osawa and Namiki[24]. Different amounts of samples dissolved in 120 µl of ethyl alcohol (EtOH) were added to a reaction mixture in a screw cap vial. Each reaction mixture consisted of 2.28 ml of 2.51% linoleic acid in EtOH and 9 ml of 40 mM phosphate buffer (pH 7.0). The vial was placed in an oven at 40 °C. At intervals during incubation, a 0.1-ml aliquot of the mixture was diluted with 9.7 ml of 75% EtOH, which was followed by adding 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured.

Preparation of mitochondria and microsomes. Mitochondria and microsomes were isolated from the livers of Wistar male rats weighing 100–150 g. The livers were removed quickly and dropped into ice-cold 3 mM Tris-



Totarol (1) **Nagilactone E (2)**

Figure 1. Structure of diterpenoids in *P. nagi*: Totarol (**1**)[23] and nagilactone E (**2**)[22].

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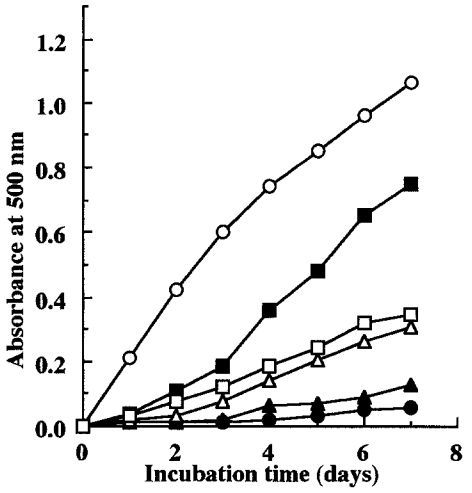

Figure 2. Antioxidative activity of totarol. The symbols show the final concentration of totarol added to the total reaction volume. ●: 30, ▲: 10, △: 3, ■: 1, ○: 0 μg/ml, □: α-tocopherol at 30 μg/ml.

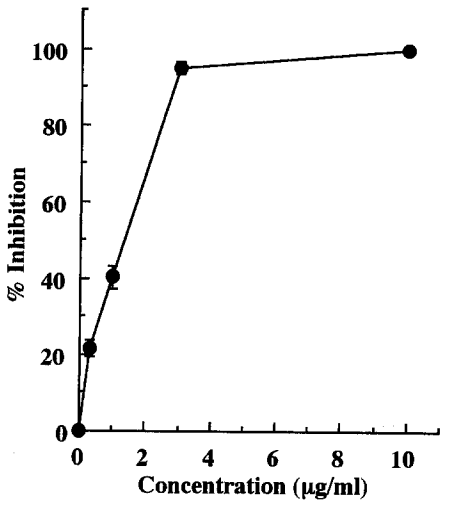

Figure 3. Inhibitory effect of totarol on microsomal lipid peroxidation. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar.

HCl buffer (pH 7.4) containing 0.24 M sucrose and 0.1 mM EDTA. Mitochondria were obtained as the resultant of centrifugation at 7000 × *g* according to the method of Hogeboom et al.[25]. Submitochondrial particles were prepared by sonication[26] for 1 min at 4 °C using a Branson Sonifier 450. Microsomes were obtained after centrifugation at 105,000 × *g* for 60 min[27]. Protein concentrations of the suspensions were determined by the method of Lowry et al.[28].

Measurement of lipid peroxidation. The NADPH-dependent peroxidation of microsomal lipid was assayed by the modified method described by Pederson et al.[29]. Rat liver microsomes (equivalent 0.2 mg of protein) were incubated at 37 °C in 1 ml of reaction mixture containing 0.05 M Tris-HCl (pH 7.5), 2 mM ADP, 0.12 mM $Fe(NO_3)_3$ and 0.1 mM NADPH. The reaction was initiated by the addition of NADPH. After 5 min, 2 ml of TCA-TBA-HCl reagent (15% w/v trichloroacetic acid; 0.375% thiobarbituric acid; 0.25 N HCl) and 90 μl of 2% BHT were added to the reaction mixture. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 × *g* for 10 min. The absorbance of the supernatant was determined at 535 nm[30].

Mitochondrial lipid peroxidation was assayed by the modified method described by Takayanagi et al.[31]. Rat liver submitochondrial particles (equivalent 0.3 mg of protein) were incubated at 37 °C in 1 ml of reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 2 mM ADP, 0.1 mM $FeCl_3$, 10 μM rotenone and 0.1 mM NADH. The reaction was initiated by the addition of NADH. After 5 min, the reaction was terminated and lipid peroxidation was determined by the same method mentioned in the case of microsomal peroxidation.

Preparation of erythrocyte and assay for hemolysis. Blood from healthy donors was collected in heparinized tubes. Erythrocytes were separated, by centrifugation, from plasma and buffy coat and were washed three times with saline. During the last washing, the cells were centrifuged at 2000 × *g* for 10 min to obtain a constantly packed cell preparation. A 10% suspension of erythrocytes in the solution containing 152 mM NaCl, 10 mM sodium phosphate buffer (pH 7.4) and a known concentration of totarol (**1**) was preincubated at 37 °C for 5 min before the addition of same volume of 100 mM 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) in the same buffer saline. The reaction mixture was gently shaken at 37 °C. At intervals during incubation, two samples were taken out from the mixture; one sample was diluted with 20 vol of 0.15 M NaCl and the other with distilled water to yield complete hemolysis. Both samples were centrifuged at 1000 × *g* for 10 min. The absorbance of the supernatants was determined at 540 nm. The percent of hemolysis was calculated according to the equation described by Miki et al.[32].

Results and discussion

Unsaturated fatty acids, especially linoleic acid, are the target of lipid peroxidation[33]. Effect of totarol on autoxidation of linoleic acid is shown in figure 2. In control reaction, the production of lipid peroxide increased almost linearly during 7 days of incubation. α-Tocopherol, a common natural antioxidant, inhibited the linoleic acid peroxidation almost 70% at 30 μg/ml. Totarol (**1**) was more effective in preventing lipid peroxidation; complete inhibition was observed at 10 μg/ml. Even at a concentration of 3 μg/ml, 70% inhibition was observed.

Membranal lipids are particularly susceptible to oxidation not only because of their high polyunsaturated fatty acid content but also because of their association

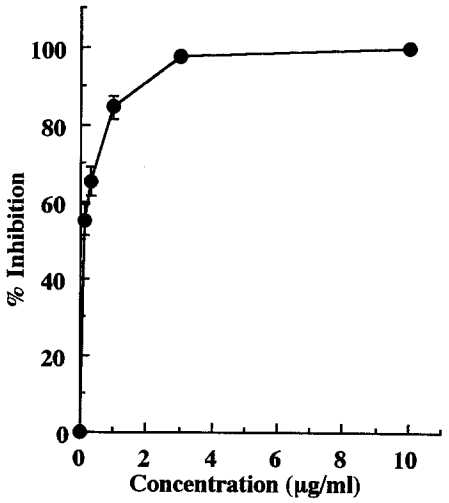

Figure 4. Inhibitory effect of totarol on mitochondrial lipid peroxidation. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar.

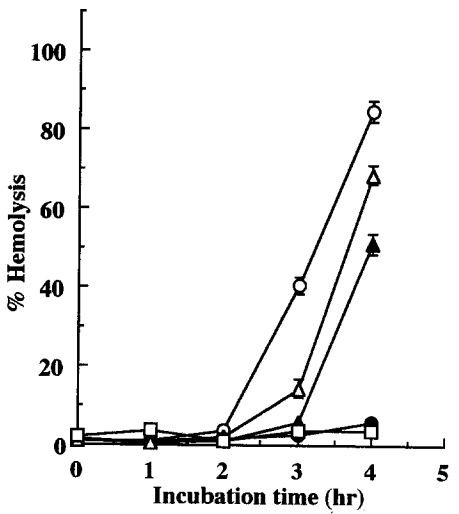

Figure 5. Inhibition of oxidative hemolysis by totarol. Each plot is the mean of triplicate determinations, with the standard deviation indicated by a vertical bar. ●: 3, ▲: 1, △: 0.3 μg/ml, ○: control, □: without AAPH.

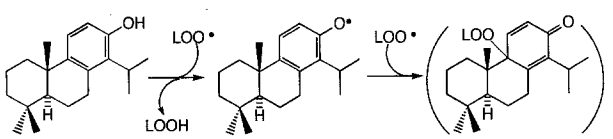

Figure 6. Proposed mechanism of antioxidative activity by totarol.

in the cell membrane with enzymic and nonenzymic systems capable of generating free radical species[34]. Microsomes, especially smooth-surfaced endoplasmic reticulum, produce lipid peroxides and are thought to supply peroxidation products to other tissues[35]. NADPH-P-450 reductase and cytochrome P-450 are involved in NADPH-induced microsomal lipid peroxidation. Lipid peroxidation, which can be measured by the TBA method, occurs when rat liver microsomes are incubated with Fe(III)-ADP/NADPH[29]. Totarol (**1**) inhibited the production of lipid peroxides induced by microsomal NADPH oxidation. As illustrated in figure 3, almost complete inhibition was observed at 3 μg/ml. Nagilactone E (**2**) had no effect on microsomal lipid peroxidation up to 30 μg/ml.

Redox reactions frequently occur in mitochondria, so mitochondria are constantly susceptible to oxidative stress[36]. Electrons at at least two sites of the mitochondrial electron transport system leak, and superoxide anions and hydrogen peroxides are produced[37]. Lipid peroxides produced by O_2^- and H_2O_2 affect mitochondrial function[38]. Lipid peroxidation by submitochondrial particles is supported by NADH or NADPH in the presence of ADP and Fe(III)[31]. As demonstrated in figure 4, totarol (**1**) completely inhibited mitochondrial lipid peroxidation at 3 μg/ml. Nagilactone E did not show any inhibition of this peroxidation up to 30 μg/ml.

The lipids in red cell membrane are highly unsaturated, and red cells are exposed to higher oxygen tension than are all other tissues. In addition, red cells are packed with hemoglobin, one of the most powerful catalysts for initiating lipid peroxidation[39]. When human erythrocytes were incubated in air at 37 °C, they were stable, and little hemolysis occurred within 4 h. The peroxy radicals generated by thermal decomposition of an azo-initiator AAPH induce free radical chain oxidation in erythrocyte membranes[32]. During incubation for 4 h with AAPH, 90% of erythrocytes were damaged. Totarol (**1**) inhibited the lysis of human red cells due to peroxy radical attack as pictured in figure 5. Complete inhibition was observed at 3 μg/ml.

Totarol inhibited the oxidation of linoleic acid and prevented mitochondrial and microsomal lipid peroxidation. Many secondary metabolites in plants exhibit antioxidative activity[40], and mechanisms of antioxidant action have been proposed[41–43]. In the case of totarol, phenoxy radical is stabilized not only by its aromatic character but also by the presence of an electron donating isopropyl group. Totarol may act as a hydrogen atom donor to peroxy radicals (LOO·) and may react with another peroxy radical (figure 6), resulting in termination of the chain radical reaction. Among the flavones, 4′-hydroxy-3′-methoxyflavone has been reported to have stronger antioxidative activity than corresponding 4′-hydroxyflavone[44]. The antiperoxidative activity of eugenol is also stronger than that of other phenylpropanoids[45]. Considering these findings, an electron donating group adjoined to a phenolic hydroxyl would have a substantial antioxidative property. However, a *p*-quinone-like structure has, generally, lower stability than a phenyl ring. Therefore, the hypothesized LOO-totarone shown in figure 6 should yield a more

stable final product. Chemical changes in totarol during antiperoxidative action are under investigation. Calculation of the sum of the resonance energy of totarol (**1**), peroxy radicals (LOO·) and the proposed final product would also be helpful for understanding the molecular mechanism. Present results from a model molecular system, subcellular systems and red blood cells showed that totarol was an effective bioantioxidant against various oxidative stresses. Antioxidative effects of totarol in vivo are under investigation.

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