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Radical-scavenging Activity and Antioxidative Effects of Olive Leaf Components Oleuropein and Hydroxytyrosol in Comparison with Homovanillic Alcohol

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Abstract: Olive leaf has great potential as a natural antioxidant, and one of its major phenolic components is oleuropein. In this study, the antioxidant activity of oleuropein against oxygen-centered radicals was measured by examining its sparing effects on the peroxyl radical-induced decay of fluorescein and pyrogallol red, in comparison with related compounds. The antioxidant capacity of oleuropein against lipid peroxidation was also assessed through its effect on the free radical-induced oxidation of methyl linoleate in a micelle system. On a molar basis, oleuropein and hydroxytyrosol inhibited the decay of fluorescein for longer than both homovanillic alcohol and the vitamin-E mimic 2-carboxy-2,5,7,8-tetramethyl-6-chromanol (Trolox), but did not suppress pyrogallol red decay in a concentration-dependent manner. Measurement of the fluorescein decay period revealed that the stoichiometric number of oleuropein and hydroxytyrosol against peroxyl radicals was twice that of Trolox, which is substantially higher than expectations based on chemical structure. Oleuropein and hydroxytyrosol were also more effective than Trolox and homovanillic alcohol at suppressing the oxidation of methyl linoleate in the micelle system. Thus, both oleuropein and hydroxytyrosol exhibit high antioxidative activity against lipid peroxidation induced by oxygen-centered radicals, but the high reactivity of phenolic/catecholic radicals makes their mechanism of action complex.

Key words: antioxidant, oleuropein, free radical, lipid peroxidation, olive leaf

1 INTRODUCTION

Oxidative stress is widely understood to be involved in the pathogenesis of various diseases and aging. Accordingly, antioxidants are extensively investigated for their ability to promote health and prevent disease. In folk medicine, olive leaf has been used for several thousand years, with numerous reports published on its beneficial properties, such as lowering blood pressure[1]. In olive leaf, the main phenolic compounds are oleuropein (OP) and hydroxytyrosol (HT) (Fig. 1). Their antioxidative properties have been studied extensively; previous work showing that OP and HT react with both hydroxyl and stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals[2], and are potent scavengers of peroxynitrite and superoxide anion radicals, but not of hypochlorous acid and hydrogen peroxide[3]. HT also inhibits hydrogen peroxide-induced kidney-cell injury by interacting with MAP kinase and PI3 kinase[4], inhibits lipid peroxidation in intestinal Caco-2 cells by scavenging peroxyl radicals[5], and induces heme oxygenase 1 gene expression in human keratinocytes[6]. Until now, the reactivity of OP and HT toward free radicals has not been measured systematically.

Antioxidant reactivity toward free radicals has been assessed by various methods[7]. Recently, we proposed a method to evaluate the radical-scavenging capacity of anti-

Abbreviations: APH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GSH, glutathione; HA, homovanillic alcohol; HT, hydroxytyrosol; OP, oleuropein; PGR, pyrogallol red; Trolox, 2-carboxy-2,5,7,8-tetramethyl-6-chromanol.

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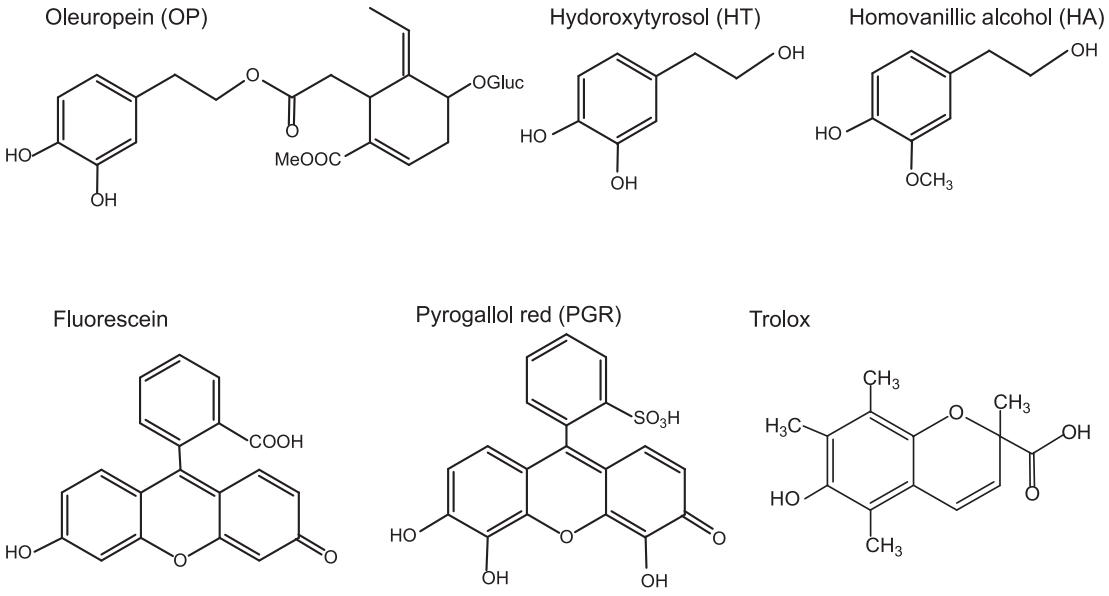


Fig. 1 The chemical structures used in this study.

oxidants by measuring the rate of free radical-induced decay of probes, such as fluorescein and pyrogallol red (PGR), in the presence and absence of the antioxidants[8, 9]. It should be noted that there is not always a correlation between a compound's ability to inhibit oxidation and its radical-scavenging capacity[9, 10]. Thus, in this study, we measured the reactivity of OP and HT toward free radicals and their antioxidant activity against lipid peroxidation in a micelle system, in direct comparison with their homologue, homovanillic alcohol (HA), which has only one phenolic alcohol.

2 EXPERIMENTAL

2.1 Materials

Purified OP and HT were supplied by Eisai Food & Chemical, Co. Ltd, Japan. Glutathione (GSH) was obtained from Nacalai Tesque Inc. (Kyoto, Japan), L-cysteine from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and Trolox from Cayman Chemical Company (Ann Arbor, MI, USA). Hydrophilic 2,2' -azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and used to generate free radicals at a constant and controlled rate. HA and pyrogallolsulfonephthalein (also called PGR) were purchased from Sigma Aldrich (St Louis, MO, USA), and fluorescein from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2 Spectrophotometric measurement of probe decay

AAPH, fluorescein, PGR, OP, HT, and HA are all water-soluble. HT was dissolved in acidic water (pH 3) and all others in phosphate-buffered saline (PBS). Stock solutions of OP, HT, and HA were prepared at concentrations around 0.5 mM. AAPH (0.5 M), fluorescein (1 mM), and PGR (1 mM) stock solutions were also prepared. Dye solutions (fluorescein and PGR) were added to the PBS solutions in quartz cells (4 mL). Reactions were initiated by addition of AAPH. The rates of reaction of fluorescein and PGR were monitored by measuring the decay of absorption (at 494 and 540 nm, respectively) using a UV-vis absorption spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) equipped with a thermostatted cell (maintained at 37℃). The lag phase was obtained graphically by extrapolating the slope of maximal probe decay to the intersection with the slope of minimal probe decay at the initial stage of the reaction (Fig. 2A). The rate of PGR consumption was measured from the slope of decay against time at the initial stage (Fig. 2B). Experiments were carried out in comparison with reference material Trolox, a water-soluble vitamin E mimic with available kinetic information.

2.3 Measurement of lipid peroxidation

The antioxidative action of the compounds was examined in the oxidation of methyl linoleate in neutral micelles formed by cholic acid and maintained at pH 7.4. Oxygen uptake in the reaction was measured. Micelles were prepared by vigorously vortexing methyl linoleate (0.2 vol.%) in PBS containing 0.1 M cholic acid for 2 min. The free radical-mediated oxidation of methyl linoleate proceeds via a straightforward mechanism to generate conjugated diene hydroperoxides with simultaneous oxygen consumption. The oxidation of methyl linoleate and its inhibition by antioxidants was followed by oxygen uptake measurement with an oxygen monitor equipped with a Clark-type oxygen electrode (YSI model 5300, Ohio) at 37℃. AAPH was used

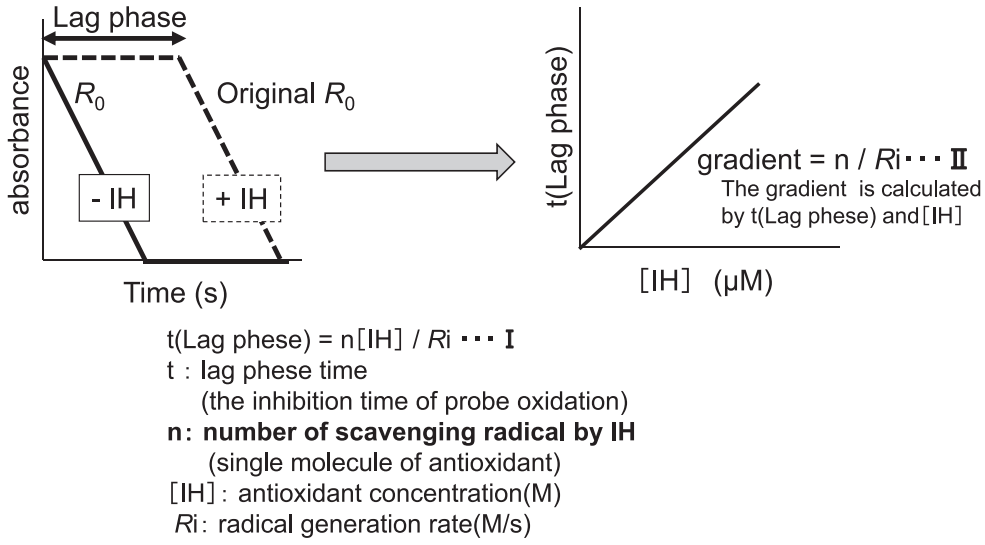


Fig. 2 The protocol of the assessment of radical-scavenging ability. (A) Fluorescein is used to determine the stoichiometric number of antioxidants against oxygen-cenered radicals.

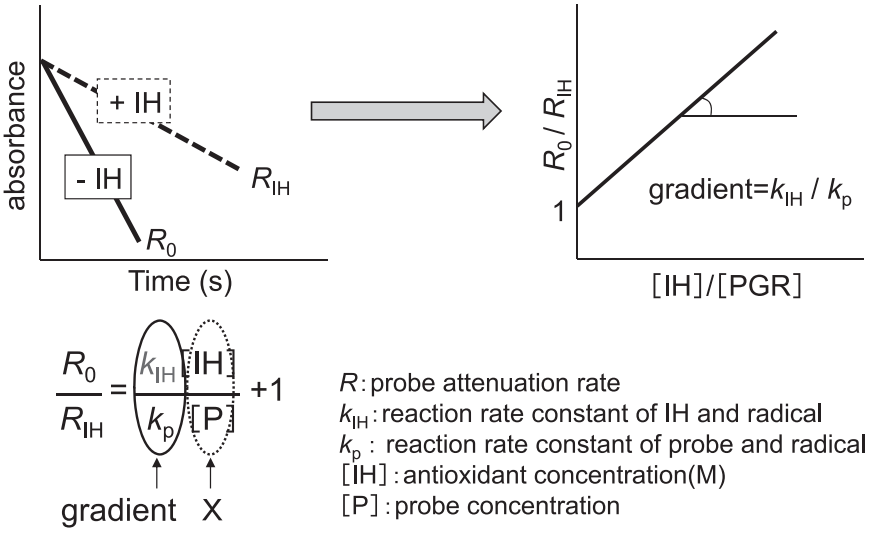


Fig. 2 (B) PGR is used to determine the ability of antioxidants to react with oxygen-centered radicals by a competitive reaction.

as an azo initiator.

2.4 Reproducibility

Experiments were conducted at least three times, with errors confirmed to be within 10%. The mean values calculated from these results are shown in the figures.

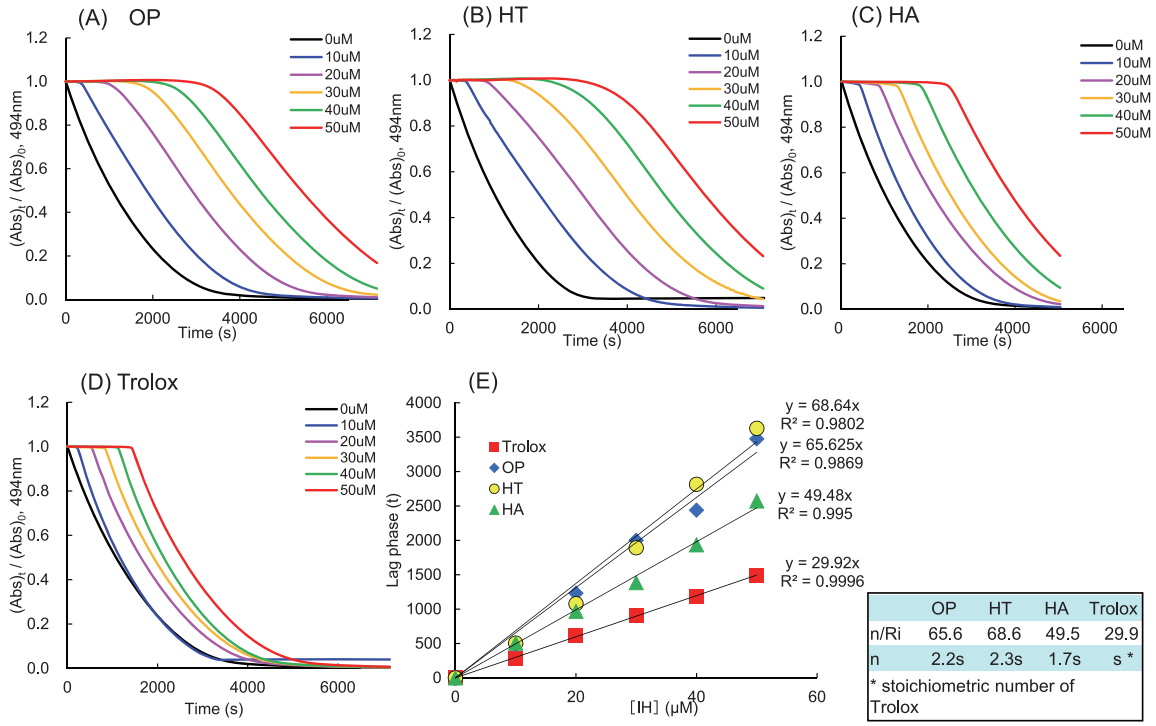
3 RESULTS

3.1 Capacity of olive leaf components for scavenging peroxyl radicals

The free radical-scavenging capacity of antioxidants can be assessed by measuring the decay of probes. Fluorescein is frequently used as a probe for such measurements. Figure 3 shows the effects of the tested compounds on the AAPH-induced decay of fluorescein. All of the compounds tested completely inhibited fluorescein decay and produced a clear lag phase proportional to their concentration (Fig. 3E). Lag phases produced by OP and HT were considerably longer than that produced by Trolox. The slopes of the plots for OP, HT, HA, and Trolox were calculated as 65.6, 68.6, 49.5, and 29.9, respectively. The lag phase is expressed as follows:

$$\text{Lag phase} = n[\text{IH}]/R\text{i} \quad (1)$$

where n is the stoichiometric number and Ri is the rate of radical flux. The stoichiometric number for Trolox is



	OP	HT	HA	Trolox
n/Ri	65.6	68.6	49.5	29.9
n	2.2s	2.3s	1.7s	s *

* stoichiometric number of Trolox

Fig. 3 Stoichiometric number of oleuropein (A), hydroxytyrosol (B), and homovanillic alcohol (C) against radicals induced by AAPH (50 mM) at 37℃. Fluorescein (10 μM) was used as a probe and the decay was followed at 494 nm. The stoichiometric number was calculated as shown in Fig. 1 (A) and compared with Trolox (D) as a reference.

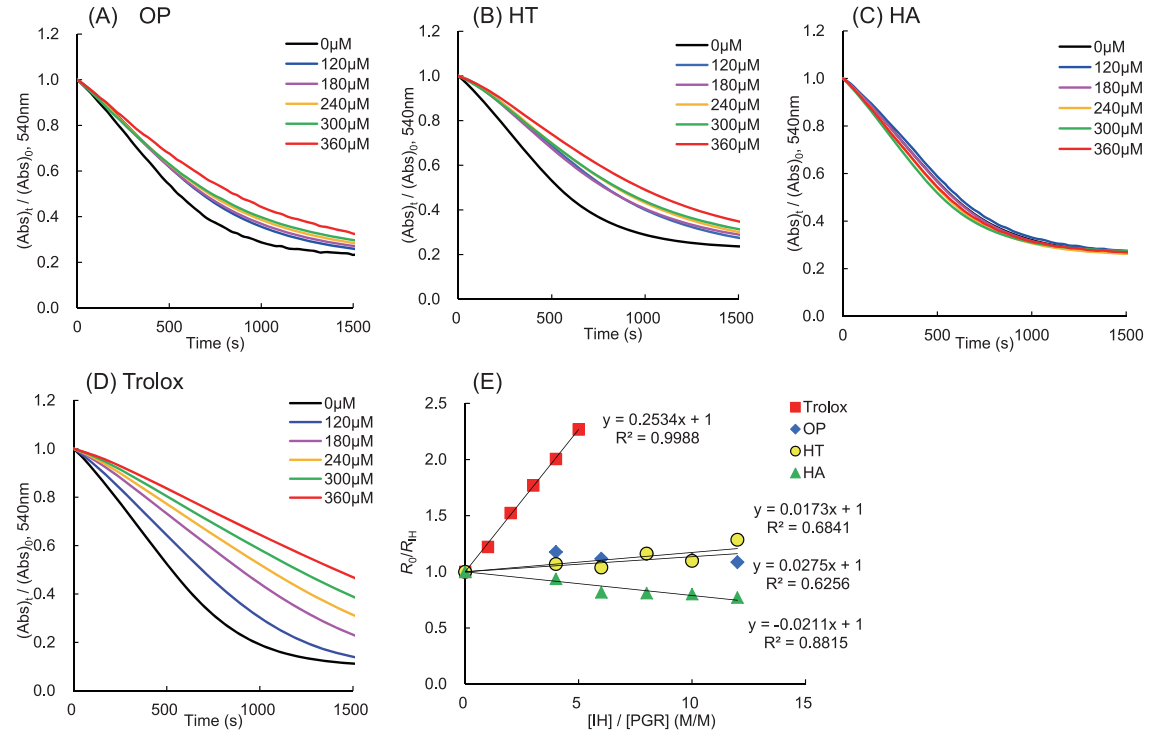


Fig. 4 Radical scavenging ability of oleuropein (A), hydroxytyrosol (B), homovanillic alcohol (C), and Trolox (D) against radicals induced by AAPH (50 mM) compared with PGR as a probe (10 μM) (Fig. 1 (B)) at 37℃. The decay of PRG was followed at the absorption of 540 nm.

known to be 2.0, which suggests that stoichiometric numbers for the other compounds are higher (further discussed in Section 4). The rate of radical flux (Ri) measured from 50 mM AAPH under the conditions used was calculated to be 6.67×10^{-8} M/s ($2/29.9 \times 10^{-6}$) using the Trolox data and equation (1).

PGR is more reactive towards free radicals than fluorescein, and thus it was used as a probe for assessing antioxidant reactivity toward free radicals. The effects of the tested compounds on AAPH-induced PGR decay in PBS are shown in Fig. 4. OP and HT inhibited PGR decay in a concentration-dependent manner, but inhibition was not directly proportional to concentration, while HA showed no inhibition. Trolox clearly suppressed PGR decay in a concentration-dependent manner (Fig. 4D). As discussed later, phenoxyl radicals formed simultaneously from their parent compounds are not stable and can undergo secondary reactions that affect the lag phase and rate of probe decay, thereby complicating quantification of the stoichiometric number and reactivity toward free radicals.

3.2 Antioxidant activity of olive leaf components against oxidation of methyl linoleate in micelles

Linoleic acid and its esters are convenient substrates for measuring lipid peroxidation due to the straightforward oxidization mechanism that generates conjugated diene hydroperoxides quantitatively. AAPH was used to initiate the oxidation, producing free radicals in the aqueous phase, and oxygen uptake increased linearly with time in an AAPH-concentration-dependent manner (data not shown). At a concentration of 1 μM, OP, HT, and HA suppressed oxidation much more efficiently than Trolox (Fig. 5A). OP, HT, and Trolox inhibited the oxidation in a concentration-dependent manner (Fig. 5B, C and E, respectively), while HA did not. However, OP and HT did not produce a clear lag phase at higher concentration (5 μM). In agreement with its reactivity toward free radicals (Fig. 4A and B), Trolox exerted a strong antioxidative effect early in the oxidation, demonstrated by a clear lag phase (Fig. 5E), while OP and HT had only moderate effects. In contrast, HA did not exert a concentration-dependent effect on the oxidation (Fig. 5D).

4 Discussion

This study has shown that olive leaf components such as OP, HT, and HA can scavenge free radicals, with reactivity in the order OP, HT > HA. In the fluorescein-decay test, the measured stoichiometric number of these compounds was higher than that of Trolox. HA addition accelerated PGR decay, suggesting that the phenoxyl radical, formed by oxygen radicals, acted as a chain carrier abstracting hydro-

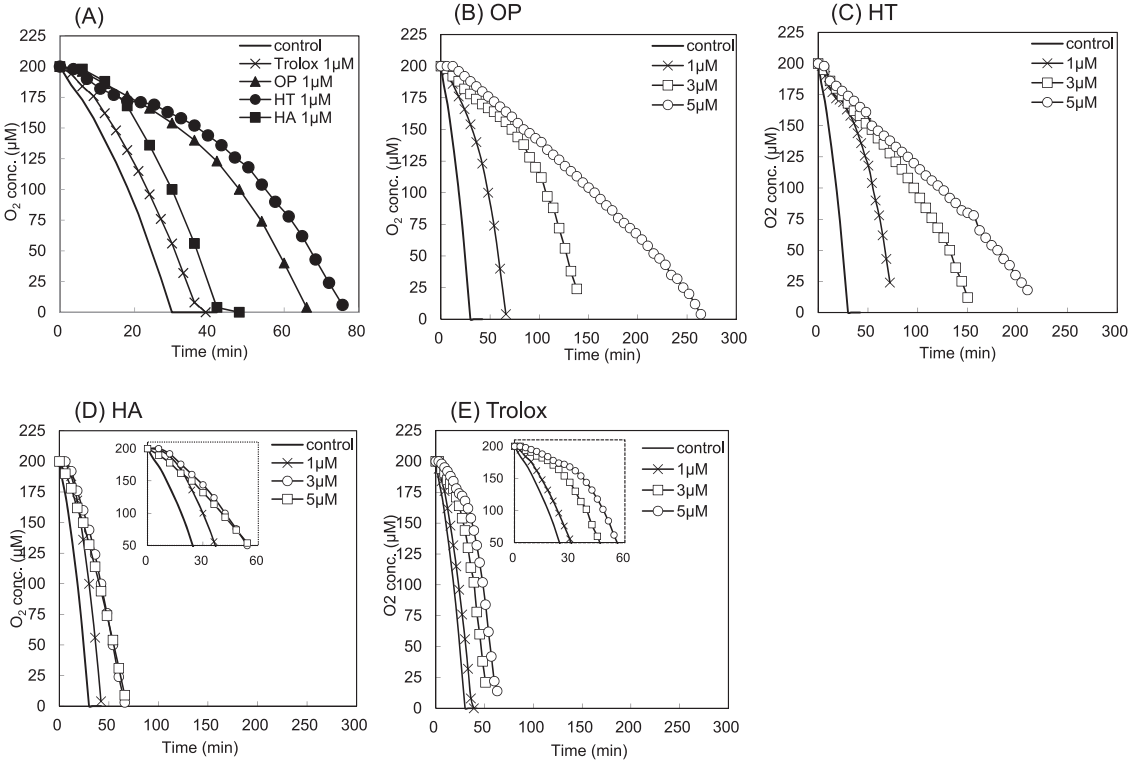


Fig. 5 The effects of oleuropein, hydroxytyrosol, and homovanillic alcohol as antioxidants against lipid peroxidation in methyl linoleates micelles at 37℃.

gen from PGR. In contrast, phenoxyl radicals formed from the catecholic compounds OP and HT were stabilized through structural conjugation. Fluorescein is a milder radical-scavenging material than PGR, as it has only one phenolic alcohol (Fig. 1), and is not considered to act as a chain carrier.

The antioxidant efficacy of phenolic compounds depends on the fate of the phenoxyl radical and rate of oxygen radical scavenging. To act as an efficient antioxidant, antioxidant-derived radicals must be stable and/or reduced rapidly. Among phenolic compounds, α-tocopherol has been studied in detail; its radical derivative, α-tocopheroxyl, is highly stable, with a rate constant for hydrogen abstraction from methyl linoleate reported to be in the order of 10^{-2} $M^{-1}s^{-1}$ [11]. The α-tocopheroxyl radical can scavenge other radicals, generating a stable adduct or, more importantly, be rapidly reduced by ascorbic acid or ubiquinol to regenerate α-tocopherol. The ascorbyl radical derived from ascorbic acid is oxidized to dehydroascorbate, which can be reduced to regenerate ascorbic acid. Thus, α-tocopherol and ascorbic acid inhibit lipid peroxidation synergistically[12]. Accordingly, in order to investigate the antioxidant effect *in vivo*, it is important to clarify the synergistic effect derived from the combination of antioxidants. This effect is discussed later.

In contrast to phenolic compounds, catecholic compounds are recognized to be potent antioxidants. For example, one molecule of quercetin, a water-soluble catecholic compound, reportedly scavenges 6.6 molecules of 1,1-diphenyl-2-picrylhydrazyl radical (DPPH)[13]. As shown in Fig. 6(A), catecholic compounds each scavenge two radicals to yield *o*-quinone (Reaction 1). They also participate in other reactions such as the coupling of phenoxyl radicals (Reaction 2) and the Diels-Alder reaction of *o*-quinone (Reaction 3). *o*-Quinone is reactive toward oxygen-centered radicals because it contains two α, β-unsaturated carbonyls. To our knowledge, the second-order rate constant of the reaction between *o*-quinone and peroxyl radicals remains undetermined. However, the rate constant for hydrogen abstraction is predicted to be higher than for

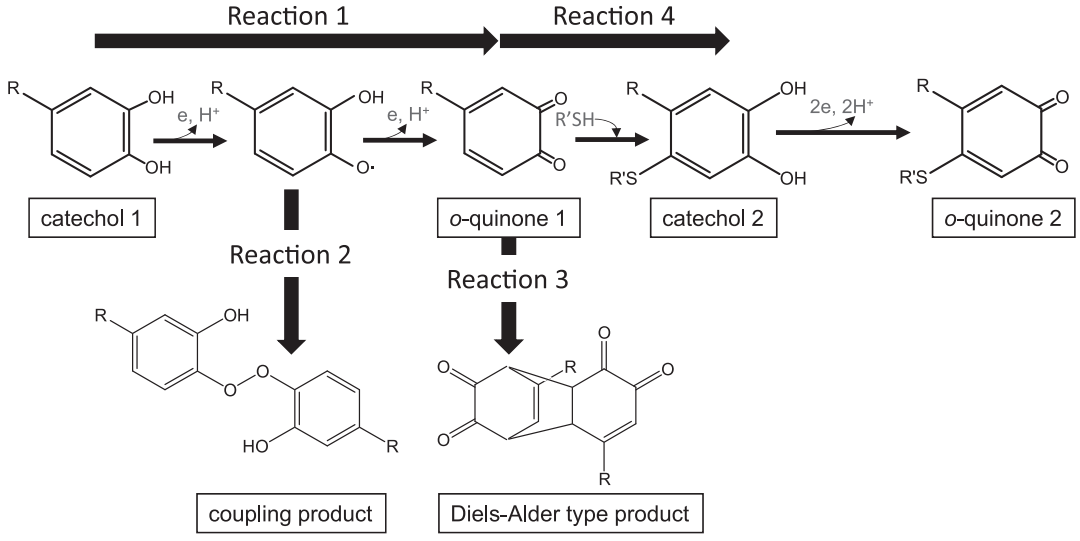


Fig. 6 (A) Proposed reaction mechanism of *o*-quinone with radicals

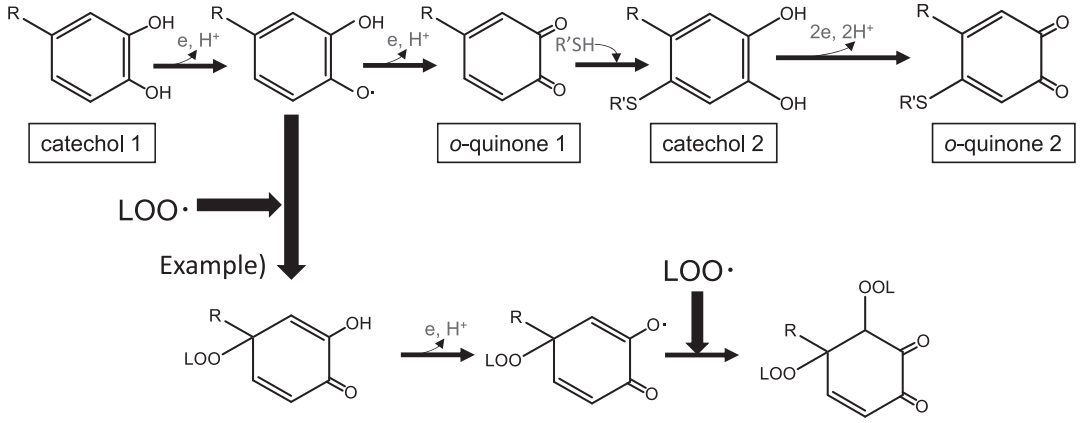


Fig. 6 (B) Proposed reaction mechanism of *o*-quinone with radicals

phenolic compounds (HA and fluorescein) and lower than catecholic compounds (OP, HT, and PGR). The fate of phenoxyl radicals in catecholic compounds is difficult to determine. As shown in Fig. 6(B), they may be targets for addition to peroxyl radicals, like 2,6-di-tert-butyl-4-methylphenol[14, 15], that react quickly yielding quinine compounds, such as *o*-quinone 1 and 2 (Fig. 6(A)), that may lead to physiological effects such as adaptation.

Another key *in vivo* reaction could be the nucleophilic addition of anionic compounds (Reaction 4 in Fig. 6(A)). When the coupling compound contains a catechol structure, it can scavenge more than two radicals. As nucleophiles, thiol compounds are one of the major anionic candidates[16]. They are abundant *in vivo*, with the thiol/disulfide balance of intracellular and extracellular compartments playing a crucial role in redox-system biology[2]. Thiol compounds are present in high concentrations in intracellular and extracellular fluids, and can act as hydrogen or electron donors, thereby scavenging active free radicals and repairing damaged molecules[17]. As described previously, combining antioxidants may lead to a synergistic effect and/or important physiological effect. We confirmed the synergistic effect by combining OP (10 μM) and various thiols (10 μM) using the protocol in Fig. 3. Notably, GSH (Fig. 7A) and cysteine (Fig. 7B) prolonged the OP-dependent inhibition time of fluorescein decay, but did not exert an inhibitory effect when alone. Given that intracellular GSH concentration is in the millimolar range, the radical-scavenging antioxidant activity of GSH might be of considerable importance in cells.

CONCLUSION

This study has shown that olive leaf components such as OP and HT act as scavengers of oxygen radicals, with their reactivity being mild compared with vitamin-E mimic Trolox. These catecholic compounds may play a critical role in inhibition of lipid peroxidation. Furthermore, their activity might be substantially increased by nucleophiles *in vivo*.

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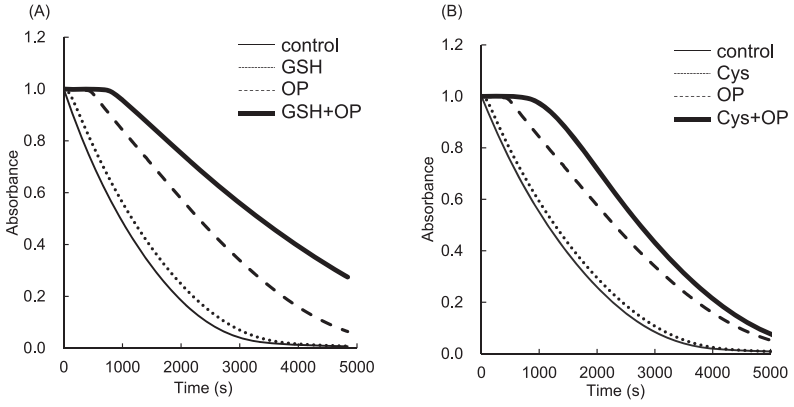

Fig. 7 The effects of glutathione (A) and cysteine (B) (10 μM) in the reaction of oleuropein (10 μM) with radicals induced by AAPH (50 mM). The decay of fluolescein was followed at the absorption of 494 nm.

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