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# ANTIOXIDANT ACTIVITY OF PHENOLIC METABOLITES FROM MARINE SPONGES

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*The marine-sponge metabolites ilimaquinone (**1**), isospongiaquinone (**2**), puupenone (**3**), 15-methoxypuupenol (**4**), 2-methyl-2-pentaprenyl-6-hydroxychromene (**5**), (+)-curcuphenol (**6**), (+)-curcudiol (**7**), and semisynthetic sesquiterpenequinones (**8-11**) were investigated for ability to trap the free radical 1,1,-diphenyl-2-picrylhydrazyl (DPPH), to inhibit $Fe^{2+}$/ascorbate-induced oxidation of lipids from rat-brain homogenate, and to inhibit oxidation of linseed oil. It was shown that metabolites **3-5** were the most active antioxidants.*

**Key words:** marine metabolites, sesquiterpenequinones, terpenoid phenols, antioxidants, marine sponges.

Oxidation induced by active oxygen radicals can lead to peroxidation of lipids, damage to membrane proteins, and DNA mutations. It is one cause of various diseases including cancer, cardiovascular illness, and atherosclerosis. Antioxidants with antiradical activity play an important role in preventing development of these diseases and their treatment [1]. Phenolic compounds possess antioxidant activity [2]. During a search for new natural antioxidants, we investigated phenolic metabolites from marine invertebrates. Metabolites consisting of benzenoid (phenol or quinoid) and terpenoid parts are some of the most active marine metabolites. In particular, ilimaquinone (**1**) typically has anti-HIV [3], anti-inflammatory [4], antimicrobial, and antitumor activities [4]. It inhibits ricin cytotoxicity and diphtheria toxin [5], fragments selectively Golgi apparatus [6], and interacts with methylating enzymes [7]. Puupenone (**3**) and metabolites similar to it exhibit cytotoxic [8] and antitumor activities [9] and are potential lipoxygenase inhibitors [10].

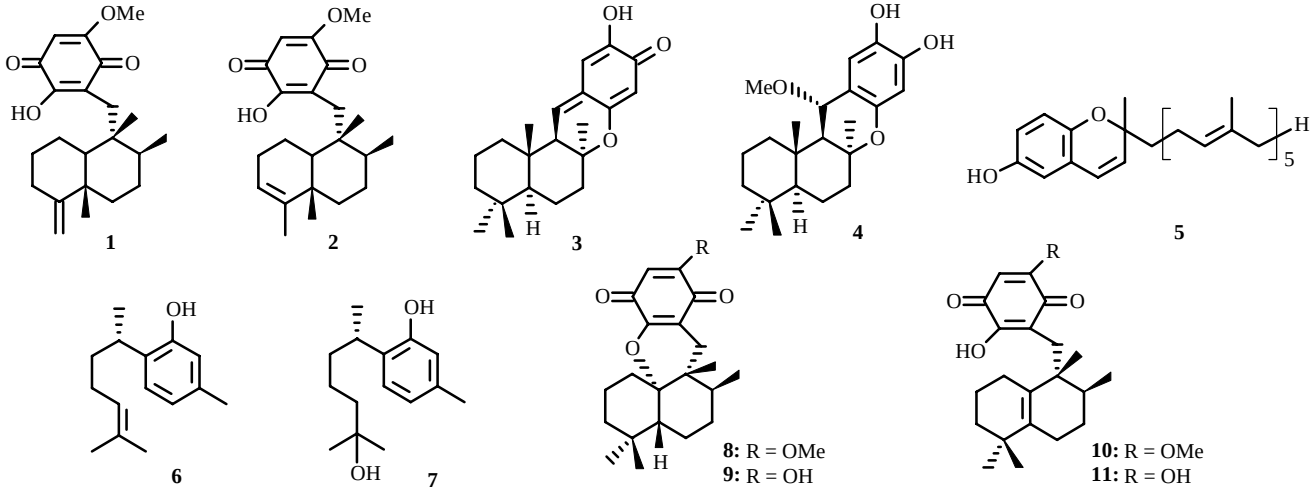


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TABLE 1. Antiradical Activity and Inhibitory Effect of **1-11** for $Fe^{2+}$/Ascorbate-Induced Oxidation of Rat-Brain Homogenate Lipids

| Compound | DPPH trapping, % | | | Oxidation inhibition | |
|---|---|---|---|---|---|
| | 10 min | 15 min | 45 min | at 400 μM, %** | $IC_{50}$, mM |
| **1** | 10.9 | 13.4 | 13.4 | 56.3 | 72 |
| **2** | 14.5 | 15.8 | 15.8 | 27.2 | - |
| **3** | 62.8 | 68.7 | 70.9 | 72.8 | 6.9 |
| **4** | 90.7 | 90.7 | 90.7 | 67 | 9.9 |
| **5** | 49 | 60.2 | 68.4 | 55 | 21 |
| **6** | 4 | 4.2 | 22.1 | 59.3 | 8.3 |
| **7** | 4 | 5.1 | 24.5 | 77 | 0.63 |
| **8** | 14 | 14.8 | 15.8 | 58 | 49 |
| **9** | 16.1 | 18.2 | 18.9 | 58.1 | 72 |
| **10** | 15.8 | 17.9 | 18.7 | 56.8 | 49 |
| **11** | 36.9 | 40.3 | 50 | 66 | 25 |
| BHT | 14.6 | 15.2 | 36.6 | 58.1 | 9.4 |
| α-T | 93.3 | 93.3 | 93.3 | - | - |

*Values of three independent measurements that did not differ by greater than 2-8%.
**Values of three independent experiments that did not differ by greater than 5-10%.

The antioxidant activity of marine phenolic metabolites is poorly studied [11-14].

Our goal was to investigate the antioxidant activity of terpenoid phenols and sesquiterpenequinones isolated from marine sponges and certain semisynthetic analogs of natural sesquiterpenequinones.

We used three methods to study the antioxidant properties of **1-11**: 1) bleaching alcoholic DPPH, 2) inhibition of rat-brain homogenate lipid peroxidation, and 3) inhibition of linseed-oil oxidation. The results from the DPPH test showed that natural compounds **3-5** are the best antiradical compounds among the tested substances (Table 1). The antiradical activity of **4** is comparable with that of α-tocopherol. The activities of **3** and **5** are greater than that of BHT. It is known that the antioxidant activity of phenolic compounds depends on the number and relative placement of hydroxyls. Compound **4**, with two *ortho*-hydroxyls in the aromatic ring, is the most active. It reacts quickly with DPPH, reaching a stable state after 10 min (Table 1). It was noted previously that **3** forms **4** upon reaction with $CH_3OH$ [13, 15]. A similar 1,6-addition of ethanol to **3** in alcoholic DPPH solution forms the ethyl analog of **4**, which increases the antiradical activity of quinonemethide **3**, but a stable state is not reached even after 45 min (Table 1). Compound **11** with two *para*-hydroxyls in the quinoid part of the molecule also exhibited high antiradical activity. Compounds **1**, **2**, and **8-10** were ineffective in trapping DPPH. Methylation of the hydroxyl in **9** and **11** reduces the activity of their derivatives **8** and **10**.

Compounds **3-5**, which exhibit high antiradical activity, also retarded the auto-oxidation of linseed oil (Fig. 1) whereas **1**, **2**, and **6-11** had no stabilizing action even at concentrations greater than 0.1% of the substrate mass. It is known that the stabilizing effect of many antioxidants is due to trapping of oxygen radicals that induce oxidation and/or hydroperoxyl radicals that propagate the lipid-oxidation chain reaction [1]. We checked the effect of **3-5** for linseed-oil oxidation induced by lipid hydroperoxides and $Fe^{2+}$. Figure 2 shows that **3-5** have no clear stabilizing effect whereas α-tocopherol significantly inhibits oxidation. Therefore, we propose that these antioxidants cannot trap lipid hydroperoxyl radicals that are formed during the oxidation and prevent the development of a chain reaction, like α-tocopherol.

Thus, the mechanism of the antioxidant action of **3-5** is probably connected with the trapping of free radicals during induction of lipid oxidation.

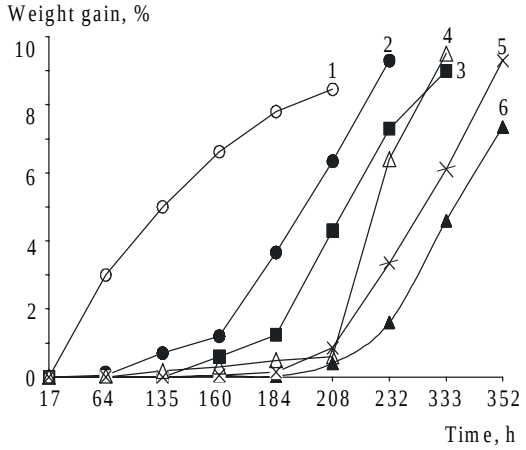


Fig. 1

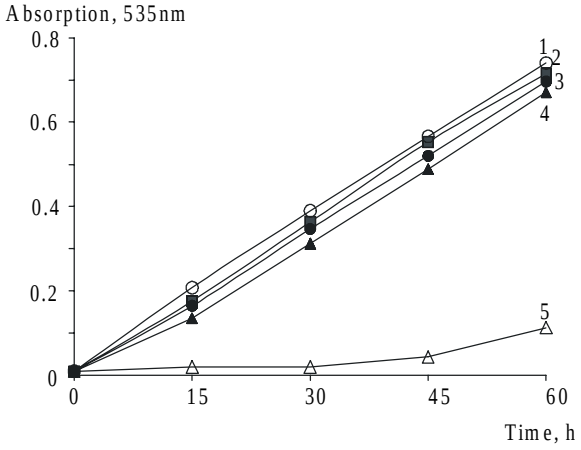


Fig. 2

Fig. 1. Auto-oxidation of linseed oil without antioxidants (1) and with **5** (2), **3** (3), $\alpha$-tocopherol (4), BHT (5), and **4** (6).
Fig. 2. Oxidation of linseed oil induced by lipid hydroperoxides without antioxidants (1) and with **3** (2), **5** (3), **4** (4), and $\alpha$-tocopherol (5).

The antioxidant activity of the compounds in a biological system was checked using rat-brain homogenate. The oxidation was initiated by $Fe^{2+}$ ions. All compounds, with the exception of **2**, exhibited an inhibitory effect (77-55%) at concentrations of 400 μM (Table 1). Compound **2** gave 50% inhibitory effect at a concentration greater than 400 μM. Compounds **3**-**7** and **11** were most active and had antiradical activity. The antioxidant activity of **5** is comparable with that of 2-methyl-2-prenyl-6-hydroxychromene isolated from *Amaroucium multiplicatum* ascides [16]. Nevertheless, phenols **6** and **7** have moderate antiradical activity (22.1 and 24.5%, respectively). They showed high antioxidant activity under heterogeneous conditions for oxidation of brain homogenate lipids. The activity of **7** is greater than that of **6**. This may be due to the presence of a hydroxyl in the side chain of **7**. A similar effect of a side-chain hydroxyl was observed previously during investigation of the antioxidant activity of prenylated hydroquinones [17].

Sesquiterpenequinones **1**, **2**, and **8**-**10**, which do not exhibit antiradical activity, showed a concentration-dependent inhibitory effect upon $Fe^{2+}$/ascorbate-induced oxidation of rat-brain homogenate lipids (Table 1). It is known that the most important reaction of quinones is their reversible reduction to the corresponding hydroquinones. This relatively facile chemical process can be carried out using $Fe^{2+}$ ion [18]. The $Fe^{2+}$ ions at physiologically low concentrations are the most widely distributed and effective pro-oxidants that initiate the radical-formation process [1]. The antioxidant activity of sesquiterpenequinones is probably due to their ability to oxidize $Fe^{2+}$ ions to $Fe^{3+}$ and thereby suppress radical formation initiated by $Fe^{2+}$.

Therefore, sesquiterpenequinones can play the role of preventive antioxidants. Of the studied sesquiterpenequinones, **3** has the highest antioxidant activity ($IC_{50}$ 6.9 μM). This may be due to the additional contribution from its high antiradical activity.

## EXPERIMENTAL

We used the following reagents: 1,1-diphenyl-2-picrylhydrazyl (DPPH, Fluka), *dl*-$\alpha$-tocopherol ($\alpha$-T, Sigma), 2-thiobarbituric acid (TBA), 2,6-di-*t*-butyl-4-methylphenol (BHT, Olaine), linseed oil (Oil & Lecithin Co., Russia); for column chromatography, Sephadex LH-20 (Pharmacia Fine Chemicals); for vacuum flash-chromatography, silica gel ICN (63-100, 60 Å, ICN Biomedicals). Absorption was measured on a UV mini spectrophotometer (Shimadzu). Compounds **1**-**4** were isolated from marine sponges [15, 19]. Compounds **8**-**11** were prepared by acid rearrangement of ilimaquinone (**1**) [20].

**Isolation of 2-Methyl-2-pentaprenyl-6-hydroxychromene (5).** Marine sponge *Sarcotragus spinulosus* (Order Dictyoceratida, family Irciniidae) sample 09-220 was collected from a depth of 470-570 m in the Fiji sea (28°13′ S, 175°23′4 E) in 1989 during the ninth scientific voyage of the NIS Academician Oparin. Lyophilized sponge (100 g) was exhaustively extracted by $CHCl_3$ at room temperature. The evaporated $CHCl_3$ extracts (6 g) were separated by vacuum flash-chromatography over a silica-gel column using hexane:ethylacetate (10:1). Fractions containing the phenolic compound were purified over a Sephadex LH-20 column using $CHCl_3$ to afford **5** (36 mg, 0.036%). The spectral and physical properties of **5** agreed with those published [21].

**Isolation of (+)-Curcuphenol (6) and (+)-Curcudiol (7).** The sponge *Didiscus aceratus* (Order Halichondrida, family Desmoxyidae) sample 012-216 was collected at a depth of 12 m on Scott reef in Australian waters (14°03′38 S, 121°46′67 E) in 1990 during the 12th scientific voyage of NIS Academician Oparin. Lyophilized sponge (25 g) was extracted with $CHCl_3$ at room temperature (2 × 100 mL). The combined $CHCl_3$ extracts (0.44 g) were separated by vacuum flash-chromatography over a silica-gel column using successively hexane, $CHCl_3$, and $CH_3OH$. Compound **6** was eluted by $CHCl_3$; **7**, $CH_3OH$. Fractions containing **6** and **7** were purified over a Sephadex LH-20 column using $CHCl_3$ to afford **6** (42 mg, 0.16%) and **7** (64 mg, 0.25%). Compounds **6** and **7** were identified by comparing their spectral and physical properties with those in the literature [22].

**Determination of Antiradical Activity.** Tested substances were dissolved in ethanol. A sample (10 μL, final concentration 50 μM) was added to DPPH in ethanol (1.5 mL, 0.1 mM). The change of absorbance at 517 nm was followed for 45 min [23]. The percent trapped DPPH was calculated using the formula: trapped (%) = 100 - ($A_{sample} \times 100/A_{control}$).

**$Fe^{2+}$/Ascorbate-Induced Oxidation of Rat-Brain Homogenate Lipids.** Brain was quickly excised from rat (Wistar, 180-200 g mass), washed with cold Tris-HCl buffer (20 mM, pH 7.4), homogenized in two portions of cold Tris-HCl buffer in a glass homogenizer, and centrifuged for 10 min at 3000 rpm. The supernatant was used as the lipid substrate. Peroxidation of brain homogenate lipids was initiated by adding $Fe^{2+}$. The degree of oxidation was estimated spectrophotometrically by the formation of substances reacting with TBA [24]. The reaction mixture consisted of brain homogenate (0.2 mL), $FeSO_4$ (0.2 mL, 10 μM), ascorbic acid (0.2 mL, 0.1 mM), and antioxidant (0.2 mL). The mixture was incubated at 37°C for 1 h. The reaction was stopped by adding trichloroacetic acid solution (0.2 mL, 20%) and TBA solution (0.2 mL, 0.7%) in aqueous acetic acid (50%). The mixture was heated at 80°C for 20 min and centrifuged for 10 min at 2000 rpm. The absorption of the supernatant at 535 nm was measured. The percent inhibition was calculated using the formula: inhibition (%) = 100 - $A_{sample} \times 100/A_{control}$. The concentration of substances that was necessary to inhibit oxidation by 50% of the control ($IC_{50}$) was found from a concentration calibration curve. BHT was used to evaluate the antioxidant effect of the substances.

**Auto-Oxidation of Linseed Oil.** Linseed oil was purified by column chromatography over $Al_2O_3$ in hexane under argon. The fatty-acid composition (%) of the linseed oil was as follows: linoleic 58, linolenic 14, oleic 20, palmitic 5, and stearic 3. Linseed oil (200 mg) in a beaker was treated with alcoholic antioxidant (10 μL, 20 mM). The control was ethanol (10 μL). Samples were incubated at 50°C in an oven. Beakers were weighed daily until the induction period was finished and 10% mass had been gained. The antioxidant activity of the substances was evaluated using BHT and $\alpha$-T.

**Oxidation of Linseed Oil Induced by Lipid Hydroperoxides.** Linseed oil (200 mg) was heated in an oven at 50°C for 72 h to produce lipid hydroperoxides according to the literature method [23]. The resulting preparation was dispersed without purification in phosphate buffer (1 mL, 0.5 mM, pH 7.4) and used as a source of hydroperoxides. The reaction mixture consisted of a hexane solution (20 μL, 20%) of fresh linseed oil, ethylenediaminetetraacetic acid (EDTA, 0.2 mL, 0.5 mM), $FeSO_4$ (0.2 mL, 1 mM), ethanolic antioxidant (20 μL, 20 mM, 20 μL ethanol as a control), hydroperoxide preparation (0.25 mL), and phosphate buffer to bring the final volume to 2 mL. The reaction was initiated at 37°C. The degree of oxidation was estimated for 1 h from the formation of substances reacting with TBA. $\alpha$-T was used to evaluate the antioxidant effect of the substances.

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