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ORIGINAL PAPER

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Constituents of *Laurus nobilis* L. inhibit recombinant human lanosterol synthase

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Abstract Extracts from 37 kinds of foods and foodstuffs were tested for inhibitory activity against recombinant human lanosterol synthase. Among them, extracts from five samples showed significant inhibition. Potent activity (55%) was found in 95% ethanol extract of *Laurus nobilis* L. Therefore, large-scale methanol extraction of the plant was carried out, and the constituents were separated by partition and fractionation by silica gel chromatography and HPLC. Four flavonoids, kaemperol 3-*O*-[2″,4″-*O*-di-*E*-*p*-coumaroyl-α-L-pyranorhamnoside] (**1**); 3,3′,4′,5,6,7,8-heptamethoxyflavone (**2**); 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin) (**3**); and 4′,5,6,7,8-pentamethoxyflavone (tangeretin) (**4**); and six sesquiterpens, eremanthine (**5**), dehydrocostus lactone (**6**), costunolide (**7**), zaluzanin C (**8**), zaluzanin D (**9**) and reynosin (**10**) were isolated. Eremanthine (**5**) showed the most potent activity, 70% inhibition, at the concentration of 500 μM.

Keywords Lanosterol synthase · Cholesterol biosynthesis · *Laurus nobilis* · Flavonoiod · Sesquiterpene · Bay leaf

Introduction

Hyperlipemia is a risk factor for arteriosclerosis. Control of the cholesterol level in the body is effective for prevention and improvement of hyperlipemia. Currently, HMG-CoA reductase inhibitors are used clinically for the treatment of hyperlipemia. The HMG-CoA reductase inhibitors, however, might cause simultaneous reduction of the physiologically essential non-steroidal isoprenoid metabolites such as dolichol, ubiquinone and prenylated proteins because HMG-CoA reductase is located in the upstream of the cholesterol biosynthetic pathway. Therefore, the long-term administration of the inhibitors of the enzyme may cause unexpected side effects. Lanosterol synthase is considered to be a more selective target for suppression of cholesterol biosynthesis, since it is located in the middle stage of the biosynthetic pathway of the cholesterol in mammals. Lanosterol synthase-inhibiting compounds were isolated from microbial cultures using in vitro assay with recombinant human enzyme [1, 2].

In recent years, a tertiary function of foods contributing to healthy life by modulating human homeostasis has been recognized in addition to their nutritional and organoleptic functions. From the viewpoint of this function, we have investigated a new function of foods and foodstuffs, and found that apple-condensed tannins had an antiallergic effect on type I allergic symptoms [3]; extracts of cabbage, red cabbage, tomato, and watercress showed antiallergic effects [4]; and some constituents in watercress inhibited histamine release from RBL-2H3 cells [5].

In order to find the foods which function to reduce the cholesterol level, 130 kinds of vegetable extracts were examined for inhibition of human lanosterol synthase in our previous study. Among them, the extract of *Colocasia esculenta* (taro) exhibited the most potent activity. Monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) were isolated as active components [6]. In this paper, we report inhibitory activities of another 37 kinds of foods and foodstuffs on human lanosterol synthase and isolation of the active compounds from the extract of *Laurus nobilis* L., which showed potent inhibitory activity among the tested samples.

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Materials and methods

Materials

Dried powder of laurel (*L. nobilis* L.) was purchased from Yasuma (Tokyo, Japan). Other plant materials for screening were gifts from San-Ei Gen F.F.I., Inc. (Osaka, Japan).

Screening of human lanosterol synthase-inhibiting food and foodstuff

Thirty-seven foods and foodstuffs, *Glycyrrhiza glabra* L. (roots), *Coriandrum sativum* L. (fruits), *Cistanche salsa* G. Beck (whole plant), *Cichorium intybus* L. (roots), *Dimocarpus longan* Lour (fruits), *Litchi chinensis* Sonn. (fruits), *Cymbopogon citratus* Stapf. (leaves), *Melissa officinalis* L. (leaves), *Uncaria gambir* Roxburgh (leaves), *Piper nigrum* L. (fruits), *L. nobilis* L. (leaves), *Pimpinella anisum* L. (fruits), *Acanthopanax senticosus* Harms (bark), *Diospyros kaki* Thunberg (leaves), *Urtica platyphylla* Wedd. (aerial part), *Elettaria cardamomum* Maton (seeds), *Citrus unshiu* Markovich (fruits), *Aloe arborescens* Mill. (leaves), *Lycium chinense* Mill. (fruits), *Syzygium aromaticum* (L.) Merr. et Perry (buds), *Gentiana lutea* L. (roots), *Jasminum grandiflorum* L. (flowers), *Illicium verum* Hook. fil. (fruits), *Mentha spicata* L. (aerial part), *Salvia officinalis* L. (leaves), *Curcuma longa* L. (rhizomes), *Myristica fragrans* Houttuyn (seeds), *Petroselium sativum* L. (aerial part), *Foeniculum vulgare* Mill. (fruits), *Brassica juncea* (L.) Czerniak (seeds), *Porphyra tenera* Kjellman. (whole plant), *Momordicae grosvenori* Swingle (fruits), *Achillea millefolium* L. (buds), *Zanthoxylum bungeanum* Maxim. (fruits), *Origanum majorana* L. (leaves), *Zizyphus jujuba* Mill. var. *inermis* Rehd. (fruits), and *Chrysanthemum morifolium* Ramat. (flowers) were extracted with 95% ethanol, 50% ethanol or water on a water bath at 80°C for 2 h and adjusted to 5 mg/ml with ethanol to afford 64 sample solutions. Sixty µl (300 µg) of each sample solution was tested for inhibition of human lanosterol synthase as described in previous papers [1, 2].

Isolation of components from *L. nobilis* L.

The laurel powder (800 g) was extracted by reflux with methanol (3.0 l×3). The solvent was removed under reduced pressure to yield 328.7 g of a methanol extract. The methanol extract (100 g) was suspended in water and partitioned with dichloromethane, ethyl acetate, and 1-butanol, successively, and resulted in three crude fractions, dichloromethane (8.6 g), ethyl acetate (16.1 g) and 1-butanol (20.1 g). The ethyl acetate soluble fraction was chromatographed over a silica gel column eluting with hexane and ethyl acetate, followed by MPLC and HPLC, to give kaemperol 3-*O*-[2″,4″-*O*-di-*E*-*p*-coumaroyl-α-L-pyranorhamnoside] (**1**; 13.3 mg) [7]. The dichloromethane soluble fraction was subjected to activated charcoal column chromatography (methanol, methanol-chloroform and chloroform) to afford four fractions (fractions 1–4). The third fraction was further separated by silica gel column chromatography using ethyl acetate-hexane, followed by HPLC, and yielded 3,3′,4′,5,6,7,8-heptamethoxyflavone (**2**, 24.3 mg) [8, 9]; 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin) (**3**, 2.9 mg) [8, 9]; 4′,5,6,7,8-pentamethoxyflavone (tangeretin) (**4**, 2.2 mg) [8, 9]. Fraction 1 was further separated by silica gel column chromatography using ether–hexane, followed by HPLC (80% MeOH) , and yielded eremanthine (**5**, 4.1 mg) [10], dehydrocostus lactone (**6**, 8.6 mg) [11] and costunolide (**7**, 8.1 mg) [12]. Fraction 2 was further separated by silica gel column chromatography using ether-hexane, followed by HPLC (80% MeOH), and yielded zaluzanin C (**8**, 31.7 mg) [13], zaluzanin D (**9**, 10.3 mg) [14], and reynosin (**10**, 5.1 mg) [15]. These compounds were identified by comparison of their spectral data with those published (Figs. 1, 2).

Results and discussion

Among 37 tested samples, five samples (*L. nobilis*, *A. millefolium*, *U. gambri*, *P. higrum* and *C. unshiu)* showed inhibitory activity (more than 5% inhibition) on human lanosterol synthase (Table 1). Among them, the active constituents of *L. nobilis* were investigated in this study. *L. nobilis* is an evergreen tree, family Lauraceae, native to Europe. The leaves of *L. nobilis*, bay leaf or laurel, are usually used as a spice and are known as a folk medicine for rheumatism in Europe [16, 17]. The constituents of *L. nobilis* have bioactivities such as nematocidal activity [18], ethanol-absorption inhibitory activity [19], trypanocidal activity [20] and inhibitory activity on nitric oxide production in lipopolysaccharide-activated mouse peritoneal macrophages [21].

The inhibitory activity of extracts of laurel with several solvents (95% aqueous ethanol, 50% aqueous ethanol and water) was 55, 22 and 0%, respectively, suggesting

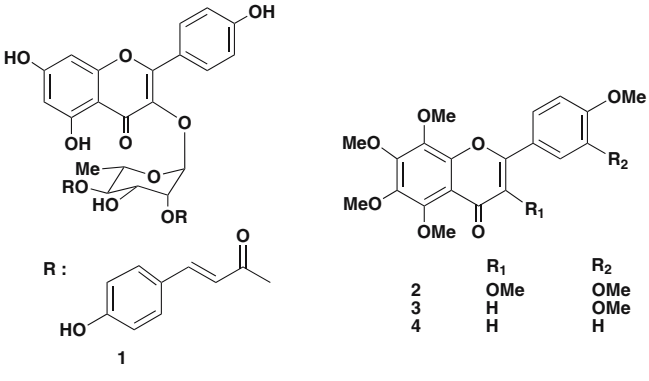

Fig. 1 Chemical structures of compounds **1**–**4**

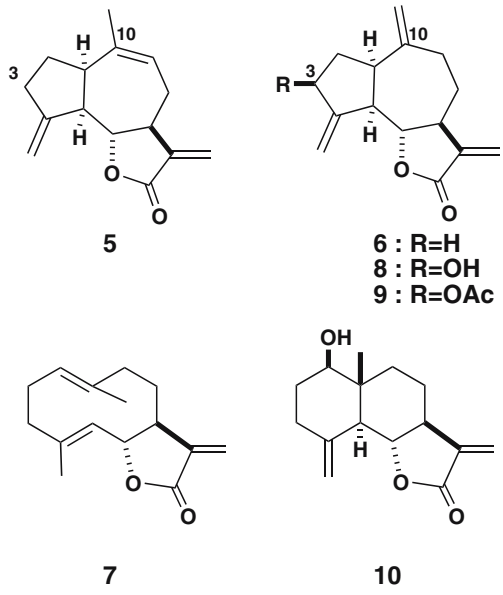

Fig. 2 Chemical structures of compounds **5**–**10**

that the active components of *L. nobilis* were hydrophobic. Therefore, laurel was first extracted with methanol, and the methanol extract was partitioned with water and organic solvents (dichloromethane, ethyl acetate and 1-butanol, successively), and then the active compounds in the dichloromethane and ethyl acetate layers were further investigated. From the dichloromethane layer, we isolated three flavonoids (**2**–**4**) and six sesquiterpenes (**5**–**10**) which were identified to be 3,3′,4′,5, 6,7,8-heptamethoxyflavone (**2**); 3′,4′,5,6,7,8-hexamethoxyflavone (nobiletin) (**3**); 4′,5,6,7,8-pentamethoxyflavone (tangeretin) (**4**); eremanthine (**5**), dehydrocostus lactone (**6**); costunolide (**7**); zaluzanin C (**8**); zaluzanin D (**9**) and reynosin (**10**). Kaemperol 3-*O*-[2″,4″-*O*-di-*E*-*p*-coumaroyl-α-L-pyranorhamnoside] (**1**) was isolated from the ethyl acetate layer. These isolated non-glycoside flavonoids (**2**–**4**) were not new compounds, but were isolated from *L. nobilis* for the first time.

The inhibitory activity of compounds **1**–**10** is shown in Table 2. Among them, **5** showed the most potent

Table 1 Foods and foodstuffs showing inhibitory activity on recombinant human lanosterol synthase

Foods and foodstuffs	Inhibition (% at 300 μg/ml)	
	95% EtOH extract	50% EtOH extract
Laurus nobilis L.	55	22
Achillea millefolium L.	42	ND
Uncaria gambir Roxburgh	ND	49
Piper higrum L.	ND	68
Citrus unshiu Markovich	50	8

Foods and foodstuffs, which showed more than 5% inhibition are listed. Others with no activity are described in experimental section.
ND Not determined

Table 2 Inhibitory activity of **1**–**10** on human lanosterol synthase

Compound	Inhibition (%)	
	at 250 μM	at 500 μM
1	–	–
2	–	23
3	–	42
4	3	39
5	12	70
6	22	63
7	–	–
8	4	16
9	12	26
10	2	16

– less than 5%

inhibitory activity (70% at 500 μM) followed by **6** (63% at 500 μM). These compounds are bicyclic, guaiane-type sesquiterpenes, and the difference is in the geometry of the double bond at C10. Inhibitory activity was reduced in compounds **8** and **9**, analogs of **6** with hydroxyl and acetoxyl at C3, suggesting that no substitution group at C3 is required for significant inhibitory activity. On the other hand, costunolide (**7**), a monocyclic, germacrane-type sesquiterpen, showed no enzyme inhibitory activity. Since the structure of **7** has a ten-membered ring, conformation of **7** is considerably flexible, compared to that of other sesquiterpenes listed in Fig. 2. These results suggested that a particular rigid conformation is a factor for high inhibitory activity.

Three flavonoids, **2**–**4**, were isolated as active compounds (20–40% inhibitory activity) in addition to the sequiterpenes. Compound **1**, a glycoside of flavonoid isolated from the ethyl acetate layer, however, showed no activity. The structures of **2**–**4** are characteristic in that all hydroxy groups are methylated. These results indicated that hydrophobicity of the molecule is indispensable for significant inhibitory activity. This is the first report to describe flavonoids as having lanosterol synthase inhibitory activity.

As shown in Table 1, the ethanol extract of *A. millefolium* L. and *C. unshiu* showed inhibitory activity. Since it has been reported that *A. millefolium* L. [22] and *C. unshiu* [23] contain non-glycosidic flavonoids, flavonoids are deduced to be the active components of *A. millefolium* L. and *C. unshiu*.

Five sesquiterpenes and three flavonoids were isolated from *L. nobilis* as lanosterol synthase inhibitors. The inhibitory activity of each compound was not as high as the known inhibitors, lauryldimethylamine *N*-oxide (LDAO), AMO 1618, lanopylins A_1, B_1, A_2 and B_2, and epohelmins A and B, for which the IC_{50} values were 0.84, 120, 15, 18, 33, 41, 10 and 6.0 μM, respectively, under the same assay conditions [1, 2]. However, the fact that laurel contains multiple compounds with moderate inhibitory activity and is ingested repeatedly may make laurel a functional food for reducing the amount of cholesterol in the human body by inhibition

of its de novo biosynthesis, even though the activity of each compound is not so high.

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