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ORIGINAL ARTICLE

Yoshio Hirano · Ryuichiro Kondo · Kokki Sakai

5α-Reductase inhibitory tannin-related compounds isolated from *Shorea laeviforia*

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Abstract Five tannin-related compounds – gallic acid, flavogallonic acid dilactone, valoneic acid dilactone, gallagyldilactone, ellagic acid – were isolated from the heartwood of *Shorea laeviforia*, and the inhibitory activity of each against rat liver 5α-reductase was evaluated. Valoneic acid dilactone and gallagyldilactone exhibited positive inhibitory activity, but gallic acid and ellagic acid did not. Flavogallonic acid dilactone stimulated 5α-reductase activity, even though this compound is structurally similar to valoneic acid dilactone. The kinetic study of valoneic acid dilactone and gallagyldilactone indicated that the inhibitory behavior of 5α-reductase were not competitive against the substrate (testosterone) and were partially competitive against the cofactor (NADPH). Additionally, double inhibition analysis of valoneic acid dilactone and $NADP^+$ showed synergetic inhibition. These results suggested that neither valoneic acid dilactone nor gallagyldilactone can affect the binding of testosterone but that either compound could interact with an enzyme–$NADP^+$ complex to inhibit 5α-reductase.

Key words *Shorea laeviforia* · Tannin-related compounds · Valoneic acid dilactone · Gallagyldilactone · 5α-Reductase inhibitor

Introduction

Testosterone is converted to a potent androgen 5α-dihydrotestosterone (DHT) by reduced nicotinamide adenine dinucleotide phosphate (NADPH)-mediated reduction catalyzed by steroid 5α-reductase; excessive production of DHT is responsible for androgen-dependent diseases. A potent 5α-reductase inhibitor may be able to remedy or prevent diseases of this type, including male-pattern baldness, benign prostatic hyperplasia, acne, and female hirsutism. Two 5α-reductase isozymes are known, and many steroidal 5α-reductase inhibitors have been investigated. One of them, finasteride, a selective and competitive inhibitor of type 2 isozyme, has shown strong inhibitory activity[1] and has been commercialized for the treatment of benign prostatic hyperplasia and male-pattern baldness. This compound decreases the serum DHT concentration and increases the serum testosterone concentration,[2] but DHT levels do not reach zero because the type 1 isozyme produces DHT. On the other hand, some adverse effects of finasteride (e.g., loss of libido, loss of erection, ejaculatory dysfunction, hypersensitivity reactions, gynecomastia, and severe myopathy) have been reported.[3] Therefore, it is preferable to develop a therapeutic agent that can inhibit the type 1 isozyme without such serious adverse effects.

Since the development of the nonsteroidal inhibitor ONO-3805,[4] many nonsteroidal inhibitors have been investigated, and some have exhibited a manner of uncompetitive kinetics with the enzyme.[5] On the other hand, the kinetics of the steroidal inhibitor epristeride have also been reported as uncompetitive,[6] whereas many steroidal inhibitors have been reported as competitive inhibitors versus a substrate.[7] Uncompetitive inhibitors are thought to have advantages in vivo, as they cannot be affected directly by increasing the substrate concentration.[8,9]

We previously reported on rat liver 5α-reductase (rat type 1 isozyme) inhibitory compounds from *Shorea* species; two dimeric, four trimeric, and two tetrameric stilbenes were isolated as inhibitory compounds from the heartwood of two *Shorea* species.[10,11] Furthermore, dimeric stilbenes, (±)-resveratrol *trans*-dehydrodimer, synthesized by a horseradish peroxidase/H_2O_2 system, showed strong 5α-reductase inhibitory activity.[12]

We report here tannin-related compounds isolated from the heartwood of *Shorea laeviforia* (Dipterocarpaceae). We

Y. Hirano (✉)
Interior Design Research Institute, Fukuoka Industrial Technology Center, 405-3 Agemaki, Okawa, Fukuoka 831-0031, Japan
Tel. +81-944-86-3259; Fax +81-944-86-4744
e-mail: hirano@fitc.pref.fukuoka.jp

R. Kondo · K. Sakai
Department of Forest and Forest Products Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

evaluate each compound's 5α-reductase inhibitory activity and kinetic profile.

Materials and methods

Sample wood

A block of *Shorea laevifolia* was collected in Indonesia by Dr. Wasrin Syafii (Bogor Agricultural University, Indonesia). It was preserved at the herbarium of the Department of Forest and Forest Products Sciences, Kyushu University, Japan. The block was ground in a Wiley mill, and the meal was extracted with methanol for 24 h at room temperature.

Extraction and separation of extractives of *S. laevifolia*

The methanol extract of milled heartwood (500 g) of S. laeviforia was concentrated. After the same volume of water was added, the mixture was partitioned with *n*-hexane, diethylether, and *n*-BuOH. The *n*-BuOH-soluble fraction (64 g) was subjected to column chromatography on a Sephadex LH-20 using EtOH as the eluent. Further purification was performed by reverse-phase preparative high-performance liquid chromatography (HPLC). Gallic acid (**1**) 11 mg, flavogallonic acid dilactone (**2**) 39 mg, valoneic acid dilactone (**3**) 14 mg, gallagyldilactone (**4**) 18 mg, and ellagic acid (**5**) 27 mg were isolated by preparative HPLC (GL Sciences) (C-18 column Inertsil PREP-ODS: 20 mm i.d. $\times$ 250 mm) using an acetonitrile–0.1% TFA gradient (10%–50% acetonitrile) as eluent.

Compounds isolated from *S. laeviforia*

The ^{1}H and ^{13}C spectra were obtained with a JEOL JNM-AL400 spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C, respectively. Dimethylsulfoxide (DMSO)-*d*6 was used as a solvent for nuclear magnetic resonance (NMR) experiments.

Flavogallonic acid dilactone,[13] valoneic acid dilactone,[14] and gallagyldilactone[15] are known from the literature and were identified by fast atom bombardment – mass spectrometry (FAB-MS) data and by comparing their ^{1}H-NMR data. Gallic acid and ellagic acid were identified by comparing the spectroscopic data of commercial reagents (Sigma).

Flavogallonic acid dilactone (**2**)

Brown solid: FAB-MS (negative ion mode) *m/z*: 469($M-H)^-$, δ_H 7.11 (*s*), 7.49 (*s*); δ_C 107.3, 107.3 (C-1, C-1′), 109.5, 109.9 (C-6, C-6′), 111.3 (C-5), 112.5 (C-6″), 117.6, 120.0 (C-5′, C-2″), 125.8 (C-1″), 135.2, 136.2, 136.7, 137.8, 138.9, 143.1, 143.7, 145.8, 147.5 (C-2, C-3, C-4, C-2′, C-3′, C-4′, C-3″, C-4″, C-5″), 157.1, 158.9 (C-7, C-7′), 167.1 (C-7″).

Valoneic acid dilactone (**3**)

Brown solid: FAB-MS (negative ion mode) *m/z*: 469($M-H)^-$, δ_H 6.93 (*s*), 7.01 (*s*), 7.51 (*s*); δ_C 106.9 (C-6″), 108.0, 108.2, 108.4 (C-6, C-6′, C-1″), 110.5, 111.9 (C-1, C-1′), 113.8, 114.6 (C-5, C-5′), 135.1, 136.1, 136.5, 139.1, 139.4, 139.5, 140.4, 142.9, 148.4, 149.3 (C-2, C-3, C-4, C-2′, C-3′, C-4′, C-2″, C-3″, C-4″, C-5″), 159.0, 159.0 (C-7, C-7′), 165.6 (C-7″).

Gallagyldilactone (**4**)

Brown solid: FAB-MS (negative ion mode) *m/z*: 601($M-H)^-$, δ_H 7.50; δ_C 106.5, 107.4 (C-6, C-1′), 110.1 (C-6′), 112.0, 112.4, 122.7 (C-1, C-5, C-2′), 135.7, 136.1, 138.7, 139.1, 145.6, 147.7 (C-2, C-3, C-4, C-3′, C-4′, C-5′), 157.6, 158.8 (C-7, C-7′).

Preparation of rat liver microsomes

The livers of Sprague-Dawley female rats were removed and homogenized in medium A (0.32 M sucrose, 1 mM dithiothreitol, 20 mM sodium phosphate, (pH 6.5) as described in a previous report.[8,9] The homogenate was centrifuged at 10000 *g* for 10 min at 0°C. The resulting pellets were suspended in medium A and underwent centrifugation again. The microsomes were obtained as precipitates from further ultracentrifugation (105000 *g* for 1 h at 0°C) of the preceding supernatants. The washed microsomes were suspended in 10 ml of medium A, and the microsomes were dispersed using a syringe with 18-, 23-, and 26-gauge needles in succession. The microsome suspension was divided into small aliquots and stored at −80°C. The suspension was diluted with medium A just before use.

Determination of 5α-reductase inhibitory activity

The standard reaction mixture, in a total volume of 0.3 ml, contained the microsomes, 150 μM testosterone in 10 μl of ethanol containing 0.7 μM [4-^{14}C]testosterone, 167 μM NADPH, and medium A, with or without the indicated amount of a sample in 10 μl of DMSO. The amount of the enzyme fraction was adjusted so around 70% of the testosterone would be converted to DHT. Incubation was started by adding microsomes to the preheated reaction solution in a tube. After 10 min the incubation was terminated by adding 10 μl of 3 M NaOH.

The reaction mixture to determine the inhibitory behavior of 5α-reductase was the substrate that contained microsomes plus 150, 75, 50, or 37.5 μM testosterone in 10 μl of ethanol containing 0.70, 0.35, 0.23, or 0.18 μM [4-^{14}C]testosterone, respectively, plus 167 μM NADPH and medium A, with or without the indicated amount of a sample in 10 μl of DMSO, in a total volume of 0.3 ml. As with the standard mixture described above, the amount of the enzyme fraction in each mixture was adjusted so around 70% of the testosterone would be converted to DHT. Incubation was started by adding microsomes to the preheated

reaction solution in a tube. After 2 min the incubation was terminated by adding 10 μl of 3 M NaOH.

The reaction mixture to determine the inhibitory behavior of 5α-reductase at the cofactor contained microsomes, 150 μM testosterone in 10 μl of ethanol containing 0.7 μM [4-^{14}C]testosterone, plus 200, 150, 100, or 50 μM NADPH, and medium A, with or without the indicated amount of a sample in 10 μl of DMSO, in a final total volume of 0.3 ml. The amount of the enzyme fraction was adjusted so around 70% of the testosterone would be converted to DHT. Incubation was started by adding microsomes to the preheated reaction solution in a tube. After 2 min the incubation was terminated by adding 10 μl of 3 M NaOH.

The reaction mixture for the double inhibition analysis contained microsomes, 150 μM testosterone in 10 μl of ethanol containing 0.7 μM [4-^{14}C]testosterone, 167 μM NADPH, medium A, and 100, 75, 50, or 0 μM valoneic acid dilactone in 10 μl of DMSO plus 150, 75, and 0 μM $NADP^+$ in a final volume of 0.3 ml. The amount of the enzyme fraction was adjusted so around 70% of the testosterone would be converted to DHT. Incubation was started by adding microsomes to the preheated reaction solution in a tube. After 2 min the incubation was terminated by adding 10 μl of 3 M NaOH. To extract metabolites, 1 ml of diethyl ether was added, and the tubes were capped and shaken. The organic phase was applied to a silica plate (Kieselgel 60 F_{254}). The plate was developed in ethyl acetate-*n*-hexane (7:3) at room temperature. The radioactivity profile was determined with an imaging analyzer (Fuji Film). Inhibitory activity was calculated as $(1 - R_D / R_S) \times 100$, where R_D is the radioactivity of [4-^{14}C] DHT, and R_S is the radioactivity of total steroids: [4-^{14}C]testosterone and [4-^{14}C]DHT. The inhibitory potency was evaluated based on the 50% inhibitory concentration (IC_{50}).

Results and discussion

Five tannin-related compounds – gallic acid (**1**), flavogallonic acid dilactone (**2**), valoneic acid dilactone (**3**), gallagyldilactone (**4**), ellagic acid (**5**) – were isolated from the heartwood of *Shorea laeviforia.* The structures of these compounds are shown in Fig. 1. The rat liver 5α-reductase inhibitory activities of these compounds were evaluated using rat liver microsomes as a 5α-reductase crude enzyme. The inhibitory activities of compounds **3** and **4** were recognized with IC_{50} values of 129.1 and 91.2 μM, respectively, whereas neither **1** nor **5** exhibited any inhibitory activity. In contrast, stimulation of 5α-reductase activity was observed in **2** (Fig. 2). It is worth noting that different experimental results were observed for **2** and **3** despite their structural similarity. Thus, the inhibitory or stimulatory manner of these compounds might not be simply competitive behavior, as seen with analogues of a substrate. Oenotheins A and B, which are 5α-reductase inhibitory ellagitannins isolated from *Epilobium* species used as folk medicine for prostate disorders, were reported previously,[16,17] but their mechanisms of inhibition were not described. To determine these mechanisms, we determined the inhibitory behavior of compounds **3** and **4** at various inhibitory concentrations. Typical Lineweaver-Burk plots obtained by increasing the concentrations of **3** and **4** are shown in Fig. 3. The inhibitory behaviors of both compounds were uncompetitive versus the substrate (testosterone). The kinetic behaviors of many steroidal or nonsteroidal 5α-reductase inhibitors were also investigated to determine if the compounds interfere with binding of the substrate to the enzyme. The kinetic behavior of a cofactor as it binds to the enzyme was not investi-

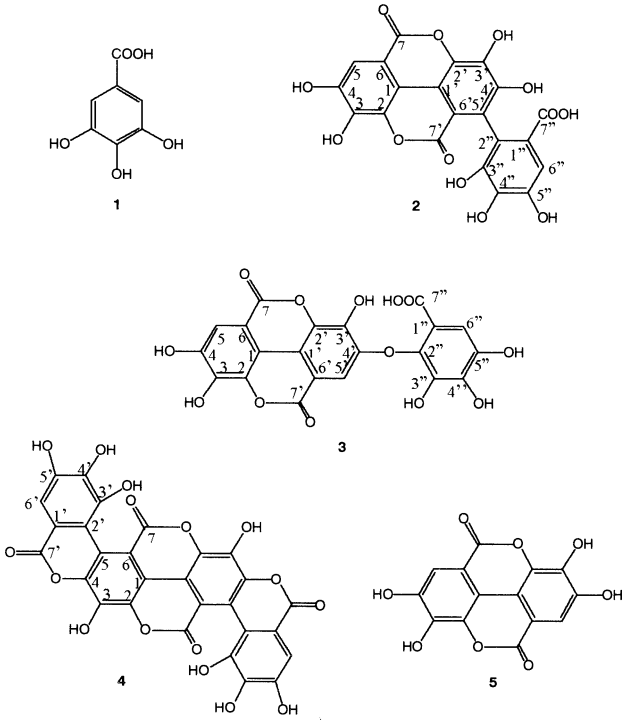

Fig. 1. Tannin-related compounds isolated from Shorea *laeviforia*

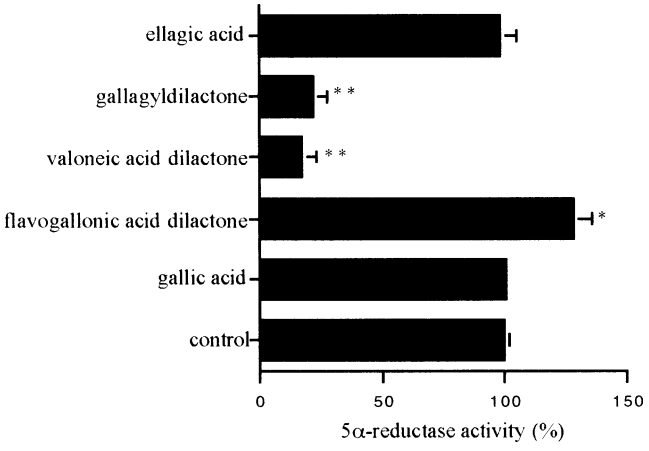

Fig. 2. Rat liver 5α-reductase inhibitory activity on tannin-related compounds isolated from the hearwood of *S. laeviforia*: compound concentration was 100 ppm in the assay system. *$P < 0.05$ ($n = 5$) versus control analyzed with Student's *t*-test. **$P < 0.001$ ($n = 5$) versus control analyzed with Student's *t*-test

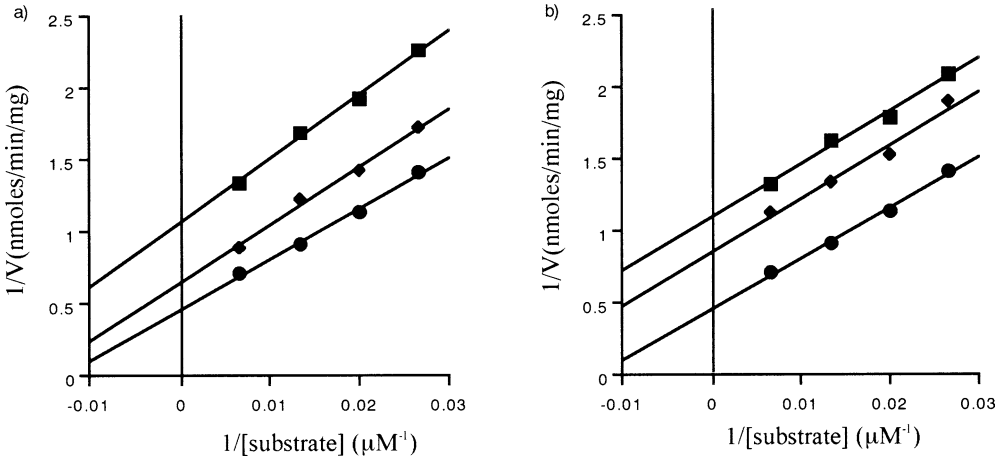

Fig. 3. Lineweaver-Burk plots with testosterone as the substrate. **a** Inhibitor: valoneic acid dilactone at concentrations of 100 μM (*squares*), 50 μM (*diamonds*), and 0 (*triangles*) in the assay system. **b** Inhibitor: gallagyldilactone at concentrations of 90 μM (*squares*), 60 μM (*diamonds*), and 0 (*triangles*) in the assay system

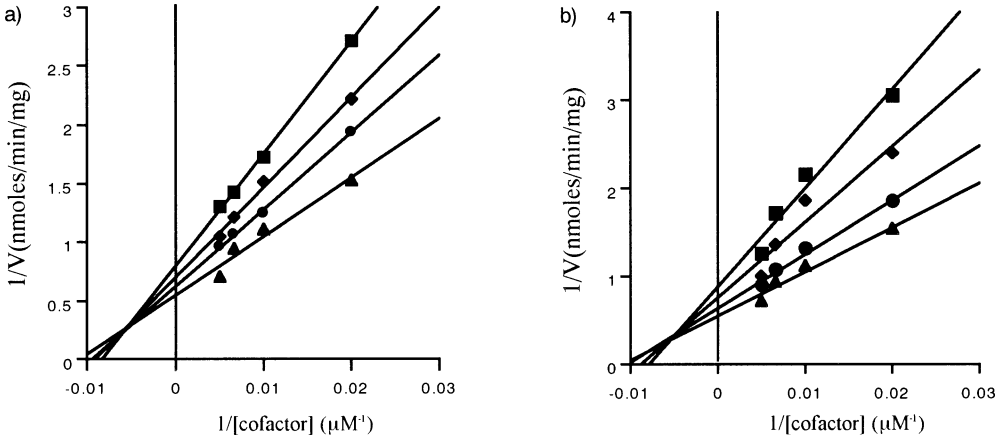

Fig. 4. Lineweaver-Burk plots with NADPH as the cofactor. **a** Inhibitor: valoneic acid dilacton at concentrations of 100 μM (*squares*), 75 μM (*diamonds*), 50 μM (*circles*), and 0 (*triangles*) in the assay system. **b** Inhibitor: gallagyldilacton at concentrations of 90 μM (*squares*), 75 μM (*diamonds*), 60 μM (*circles*), and 0 (*triangles*) in the assay system

gated. Suramin, developed as a trypanocidal agent, was recently used as an anticancer agent; and this type of kinetic study was reported.[18] According to that report, suramin showed competitive behavior versus the cofactor (NADPH). To determine if the inhibitors are associated with a cofactor, the inhibitory behaviors of compounds **3** and **4** versus the cofactor were determined at various inhibitor concentrations. Typical Lineweaver-Burk plots obtained by increasing the concentrations of **3** and **4** are shown in Fig. 4. Both compounds showed mixed-type inhibitory behavior versus the cofactor.

Uncompetitive steady-state kinetics versus the substrate have been reported for 3-carboxysteroid acrylate,[8,9,19] ONO3805,[4] 4,4′-difluorobenzhydryl indole derivative (KF18678),[20] and benzophenone derivatives,[5] including steroidal and nonsteroidal 5α-reductase inhibitors. It is thought that these uncompetitive inhibitors versus the substrate should interact with the enzyme–$NADP^+$ complex, preventing $NADP^+$ regeneration. Yonetani-Theorell plots of **3** and $NADP^+$ showed characteristics similar to those of KF18678 (Fig. 5).[20] These results suggested that inhibition of 5α-reductase by these tannin-related compounds are not due to an effect on the binding of the substrate but, instead, are due to interaction with the enzyme–$NADP^+$ complex.[20–22]

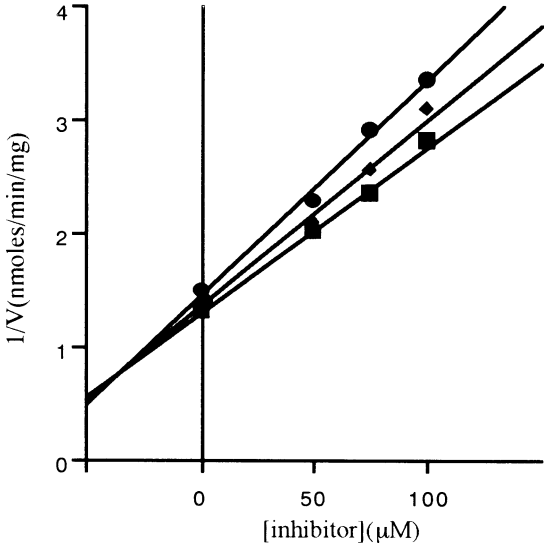

Fig. 5. Yonetani-Theorell plots with $NADP^+$ as the co-inhibitor. Inhibitor: valoneic acid dilacton at concentrations of 100, 75, 50, and 0 mM. $NADP^+$ concentrations of 150 mM (*circles*), 75 mM (*diamonds*), and 0 (*squares*) in the assay system

The adsorption of tannin on protein is well known, but it is difficult to understand how 5α-reductase is inhibited by the nonspecific adsorption of **3** or **4** because different behaviors on 5α-reductase (i.e., inhibition versus stimulation) were observed for the tannin-related compounds **2** and **3** despite their structural similarity. Perhaps these compounds could act with the enzyme–$NADP^+$ complex site specifically, and the complex could be deactivated by the binding of compound **3**, whereas the complex could stabilize or facilitate $NADP^+$ regeneration by the binding of compound **2**. Further data are needed to prove this hypothesis.

Conclusions

We demonstrated the isolation of 5α-reductase inhibitory or stimulatory activity of tannin-related compounds, the trimer of the galloyl group of flavogallonic acid dilactone (**2**) and of valoneic acid dilactone (**3**), and the tetramer of the galloyl group of gallagyldilactone (**4**). These three compounds have an ellagic acid portion in common, but this dimeric galloyl structure, ellagic acid (**5**), had no influence on 5α-reductase activity. Moreover, carbonic anhydrase inhibitory activity has been reported for compound **4**, whereas this enzyme was not inhibited or was only weakly inhibited by compound **5**.[23] Xanthine oxidase inhibitory effects have also been reported, in which compound **4** showed about four times the inhibitory activity of compound **5**.[14] Furthermore, according to the inhibitory or stimulatory action of **2** or **3**, the orientation of the galloyl moiety attached to ellagic acid could play an important role in stabilizing 5α-reductase. Thus, several enzymatic activities are affected by ellagitannin-related compounds and could be influenced by the number and orientation of the galloyl group.

We previously reported that strong 5α-reductase inhibitory activity was shown in the methanol extract of the heartwood of *S. laeviforia* and in stilbenoids from the heartwood of *S. laeviforia*.[10,11] The potency of the methanol extract of *S. laeviforia* heartwood can be attributed to the presence of two inhibitory compounds.

Tannin-related compounds, which we demonstrated here, were well known for their hydrolysis products of ellagitannins. Therefore, the plant kingdom has these compounds in abundance. They can be expected to become a source of chemicals for medical or cosmetic agents.

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