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NOTE

Investigating glucosyltransferase inhibitory activities of polyphenols from kapur (*Dryobalanops* sp.) heartwood extracts

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Abstract The inhibitory effects of 50% aqueous ethanol extracts obtained from 36 tropical woody plants species on glucosyltransferase (GTase) activity were studied. Out of the 36 species examined, those obtained from kapur (*Dryobalanops* sp.), a species growing in Kalimantan (Indonesia), showed the highest level of GTase inhibition. Kapur extracts were further subjected to fractionation using column chromatography (LH-20 gel, cellulose and C-18 silica gel column). LH-20 gel provided the most successful method of fractionation. The separated fractions showed positive with Folin-Ciocalteau's reagent and negative with vanillin-HCl reagent, indicating that the main constituents of the active fractions were polyphenols but not proanthocyanidin (condensed tannins). Results of the assay for protein precipitating ability with bovine serum albumin (BSA) solution suggested these polyphenols have strong protein-precipitating ability. The predominant compound produced after acid hydrolysis was ellagic acid, indicating that the GTase-inhibitory components were mainly ellagitannins. Two polyphenolic compounds referred to as compounds **1** and **2** were isolated from the water eluate fraction with LH-20 gel column, and these compounds showed comparatively strong GTase-inhibitory activities and relatively low molecular weight. Using a combination of two-dimensional, ^{1}H and ^{13}C nuclear magnetic resonance analysis, compound **1** was identified as 4-methoxy-2-[tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)pyran-2-yl]-α-resorcylic acid δ–lactone (bergenin), and **2** was identified as 4-*O*-(α-rhamnopyranosyl) ellagic acid (eschweilenol C). Bergenin has been previously isolated from the roots of *Bergenia crassifolia*, and eschweilenol C has been isolated from the bark of *Eschweilera coriacea*. Both compounds were found in kapur for the first time.

Keywords Glucosyltransferase · *Dryobalanops* sp. · Hydrolyzable tannin · Ellagitannin · Bergenin · Eschweilenol C

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Introduction

Tropical woody species have long been viewed as important sources of natural remedies in traditional medicine. They produce a diverse range of secondary metabolites, such as tannins, and in general are considered to have a variety of biological roles including as plant chemical defenses against pathogens and herbivores (from bacteria and fungi to insects and mammals) [1]. Secondary metabolites derived from plants are reported to demonstrate potentially significant pharmaceutical activity including antiviral [2], antimicrobial [3], antioxidant [4] and enzyme inhibiting [5]. Therefore, investigating extractives isolated from tropical woody species offers a valuable opportunity for the utilization of forest products.

Dental caries is one of the serious infectious diseases caused largely by mutans *Streptococci* bacteria, which erodes hard dental enamel and results in tooth decay. More than 90% of Japanese people are affected by dental caries [6]. Recently, condensed tannins of bark [7] and polyphenols of both Japanese green tea [8] and oolong tea [9] have been shown to have inhibitory activity for GTase, an

enzyme that synthesizes glucans from sucrose and promotes the binding of cariogenic bacteria on the teeth. In this study, we elucidated that heartwood extractives of kapur (*Dryobalanops* sp.) exhibited strong inhibitory activity for GTase.

Kapur is a tropical woody species growing in Kalimantan, Indonesia. To date, studies of kapur heartwood extractives have been limited to the discovery of several terpenoids and low-molecular-weight phenolic compounds [10, 11]. Recently, the effect of aqueous extracts of kapur as natural medicine on catecholamine secretion has been reported [12]. However, the biological function of the heartwood extractives has not yet been investigated. In this paper, kapur extracts that showed strong GTase inhibition were fractionated in order to identify the active compounds responsible for GTase inhibition.

Materials and methods

Plant materials

Fifty-percent aqueous ethanol extracts of the heartwood of 36 species provided by the Department of Forest Product Technology in Mulawarman University were used in the screening test for GTase inhibition. A voucher specimen of *Dryobalanops* sp. (FHT.LA.4.4s) was deposited at the Wood Anatomy Laboratory of Forest Product Technology Department, Mulawarman University, Indonesia.

Fractionation of kapur heartwood extracts

Kapur heartwood meal was prepared by grinding a chip with Willay mill. Wood meal (300 g) was extracted with 3 l of 50% aqueous ethanol for 1 week in the dark. The 50% aqueous ethanol extract was evaporated and subjected to fractionation with Sephadex LH-20 gel column (Amersham Biosciences, Sweden) using ethanol, methanol and 70% aqueous acetone as eluents (method 1). These fractions were termed KP-E, KP-M and KP-A, respectively. A second method similar to method 1 was developed using water, methanol/water (volume ratio 2/8, 4/6, 6/4 and 8/2), and 70% aqueous acetone as eluents (method 2). These fractions were termed Fr-I through Fr-VI, respectively. The Fr-IV was further fractionated by cellulose column chromatography (cellulose powder CC31, Whatman, USA) using 70% aqueous 2-butanol containing 5% acetic acid and 6% acetic acid aqueous solutions as eluents. These fractions were termed Fr-IV-A and Fr-IV-B, respectively. Furthermore, Fr-IV-A was separated by preparative high performance liquid chromatography (HPLC) with reversed phase column Develosil ODS-10 (20 mm i.d. × 250 mm, Nomura Chemical, Japan) monitored at 280 nm. The solvent system used was as follows: a stepwise gradient of 25, 35, 45, 55, 65 and 100% volume of solvent B (100% methanol) in solvent A (0.01% trifluoroacetic acid in H_2O) at a flow rate of 10 ml/min. The obtained fractions were named IV-A-1 through IV-A-6, respectively.

Assay for GTase-inhibitory activity

Preparation of GTase and assay for GTase-inhibitory activity followed the methods as previously described [7]: *Streptococcus sobrinus* 6715 was grown at 37°C for 24 h on Todd-Hewitt (TH) broth-agar. The *S. sobrinus* was transferred into TH broth (40 ml), and after incubation at 37°C for 24 h, it was transferred into 4 l of TH broth. After incubation at 37°C for 20 h, it was centrifuged (1,300 *g*) at 4°C for 10 min. The precipitates from the broth were extracted with 8 M urea solutions for 1 h and dialyzed against 10 mM sodium phosphate buffer (pH 6.0).

Substrate solution containing 0.5% sucrose, sample solution and a fixed volume of crude GTase solution was mixed and incubated at 37°C for 3 h. Absorbance at 550 nm of the solution was measured turbidimetrically.

Qualitative analysis for polyphenols and tannins

Total phenol and flavanol contents were obtained by Folin-Ciocalteau's method [13] and vanillin-HCl method [7]. Protein adsorption using bovine serum albumin (BSA) was determined by ninhydrin method as follows. BSA solution (200 µl at 1 mg/ml) and 200 µl of sample solution (5 or 0.5 mg/ml) were mixed and adsorbed at room temperature for 1 h. After centrifugation (2,000 *g*, 2 min), 200 µl of the supernatant solution and 100 mg of barium hydroxide were mixed and heated at 120°C for 10 min. After cooling, 2 ml of sodium acetate buffer, containing 1 mM NaCN and 3 ml of 0.5% ninhydrin-2-methoxyethanol solution, was added to the above solution and heated at 108°C for 5 min. Absorbance of the solution was measured at 570 nm.

Determination of reducing sugar and aglycones

Sample (5 mg) was treated with 5 ml of 1.5 M H_2SO_4 at 110°C for 5 h. After cooling, 2 ml of 6 M NaOH was added to the solution, and the solution was adjusted to 8 ml with deionized water. Fifty microliters of the solution was analyzed by HPLC with reversed-phase column Develosil ODS HG-5 (4.6 mm i.d. × 250 mm, Nomura Chemical, Japan). The solvent system used was as follows: a linear gradient elution for 45 min from 5 to 90% solvent B (100% methanol) in solvent A (0.01% trifluoroacetic acid in H_2O) at a flow rate of 10 ml/min. A fixed volume of lead acetate was added to the remaining solution. After centrifugation (3,500 *g*, 15 min), the supernatant solution was adjusted to

pH 8. Determination of reducing sugar was achieved by Somogyi-Nelson's method [14, 15].

Isolation and identification of compounds 1 and 2

Fraction I was fractionated by LH-20 gel column chromatography using water. Compound **1** was obtained as pure compound. The fraction containing **2** was purified by preparative HPLC with a reserved-phase column Develosil ODS-10 (20 mm i.d. × 250 mm, Nomura Chemical, Japan) monitored at 280 nm. The solvent system used was as follows: 50% solvent B (100% methanol) in solvent A (0.01% trifluoroacetic acid in H_2O) at a flow rate of 10 ml/min. The nuclear magnetic resonance (NMR) spectra were recorded on JNM Alpha-500 spectrometer (JEOL, Japan). The secondary ion mass spectrometry (SIMS) and electrospray ionization mass spectrometry (ESI-MS) spectra were obtained by using Hitachi M-80B (Hitachi, Japan) and Finnigan LCQ (San Jose, CA, USA), respectively.

Results and discussion

GTase-inhibitory activity of kapur extracts

Castanopsis sp. and *Dryobalanops* sp. showed comparatively strong GTase-inhibitory activity among the 36 species extracts tested in this study (Table 1). Kapur, a local name of *Dryobalanops* sp., is a very important wood commercially and industrially in Indonesia, so we chose to investigate kapur extracts for active GTase-inhibitory compounds. The inhibitory components of kapur extracts were found to be widely distributed in methanol and 70% aqueous acetone fractions (KP-M and KP-A, respectively) by Sephadex LH-20 column chromatography (Table 2).

In our previous paper, condensed tannins from larch (*Larix leptolepis*) bark showed strong GTase-inhibitory activity [7]. In order to confirm the existence of condensed tannin, qualitative and quantitative analyses were carried out for each fraction separated by LH-20 column chromatography (KP-E, KP-M and KP-A). In general, Folin-Ciocalteau's and vanillin-HCl methods were used to determine total phenol and flavanol contents, respectively. As shown in Fig. 1, results from the Folin-Ciocalteau's and vanillin-HCl tests indicated that the active GTase-inhibitory fractions were largely composed of phenolic compounds rather than flavanols. The results suggested that the GTase-inhibitory fractions would not contain condensed tannin. Furthermore, adsorptive activities for BSA of these fractions were quite high (Fig. 1). Tannins are known to form precipitates with proteins [16]. Therefore it was suggested that these fractions might contain a series of hydrolyzable tannins.

To confirm this, each fraction was hydrolyzed with sulfuric acid. The optimum acid hydrolysis condition for kapur extracts was investigated because hydrolyzable tannins have different reactivities against acid hydrolysis due to different structures, e.g., gallotannin and ellagitannin [17]. The largest amounts of aglycones were detected when treated with 1.5 M H_2SO_4 at 110°C for 5 h (data not shown). The amount of reducing sugar was decreased as the treatment time of hydrolysis was increased, however the calibration line that showed the relationship between

Table 1 GTase-inhibitory activity of 50% aqueous ethanol extracts of 36 tropical woody plants

No.	Scientific name	Inhibition (%)[a]
1	*Acacia mangium*	ND
2	*Aleurites moluccana*	ND
3	*Anthocepallus cadamba*	4.5
4	*Artocarpus integra*	33.4
5	*Astronium* sp.	22.4
6	*Callophyllum* sp.	8.7
7	*Castanopsis* sp.	96.3
8	*Casuarina* sp.	25.1
9	*Ceiba petandra*	14.7
10	*Clarissia racemosa* R.& P.	11.2
11	*Coccus nucifera*	15.0
12	*Curculigo capitulata*	15.0
13	*Diospyros* sp.	ND
14	*Dipterocarpus* sp.	4.2
15	*Dryobalanops* sp.	85.5
16	*Durio zibetinus*	12.7
17	*Dyera costulata*	3.0
18	*Goniorrachis* sp.	6.7
19	*Gonystylus bancanus*	5.0
20	*Knema* sp.	4.7
21	*Manilkara* sp.	9.4
22	*Mycroxylon* sp.	4.8
23	*Octomeles sumatrana*	4.5
24	*Palaqium* sp.	4.7
25	*Parashorea* sp.	17.2
26	*Peronema canescens*	6.7
27	*Populus nigra* L.	ND
28	*Shorea hemsleyana*	18.8
29	*Shorea leprosula*	13.2
30	*Shorea (Rubroshorea)* sp.	38.4
31	*Shorea* spp.	13.3
32	*Shorea squamata*	9.7
33	*Sindora walichii*	6.4
34	*Tamarindus indica*	6.1
35	*Vachysia* sp.	ND
36	*Vatairea* sp.	9.6

[a] GTase inhibition at 15 μg/ml of sample concentration

Table 2 GTase-inhibitory activities of aqueous acetone kapur extract fractions separated by LH-20 gel column chromatography

Sample[a]	GTase inhibition (%)		IC_{50}[c] (μg/ml)
	15 μg/ml[b]	5 μg/ml	
KP-E	3.5	2.1	ND
KP-M	92.3	18.5	9.3
KP-A	86.3	27.4	8.7

[a] *KP-E, KP-M*, and *KP-A* Fractions eluted by ethanol, methanol, and 70% aqueous acetone on Sephadex LH-20 chromatography, respectively

[b] Final concentration of samples

[c] Sample concentration given a 50% inhibition of GTase

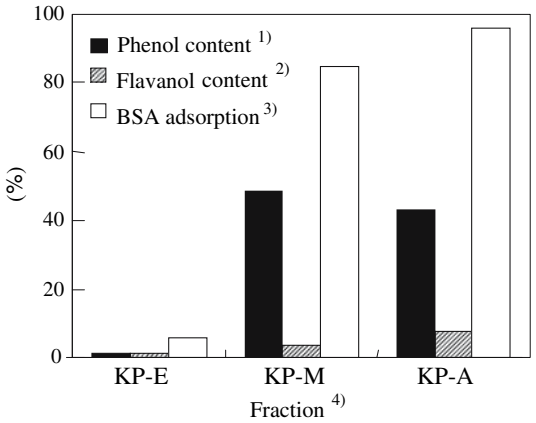

Fig. 1 Total phenol and flavanol content and BSA adsorptive activity of the fractions from kapur 70% aqueous acetone extracts. *Black columns* Total phenol content, analyzed by Folin-Ciocalteau's method. *Striped columns* Total flavanol content, analyzed by vanillin-hydrochloric acid method. *White columns* Bovine serum albumin (*BSA*) adsorption rate, calculated as the amount that BSA decreased when a sample solution (5 mg/ml) was added to BSA solution. See Table 2 for fraction abbreviations

the amount of glucose in the same hydrolysis condition and the absorbance at 660 nm by Somogyi-Nelson's method had a high regression coefficient (R^2 = 0.993).

The detection of the resulting aglycones as well as large amounts of ellagic acid and some minor amounts of gallic acid in the hydrolysate by HPLC confirmed that these fractions mainly contained ellagitannins and to a lesser extent gallotannins. The reducing sugar contents were extremely low compared to the aglycone contents (0–0.04 μmol/5 mg). It was suggested that hydrolyzable tannins in kapur might not be simple ellagitannins, which only have an ester linkage between sugar and aglycone, because it is assumed that C-glycosyl-type ellagitannins, such as castalagin and vescalagin [18], could not be hydrolyzed by acid treatment.

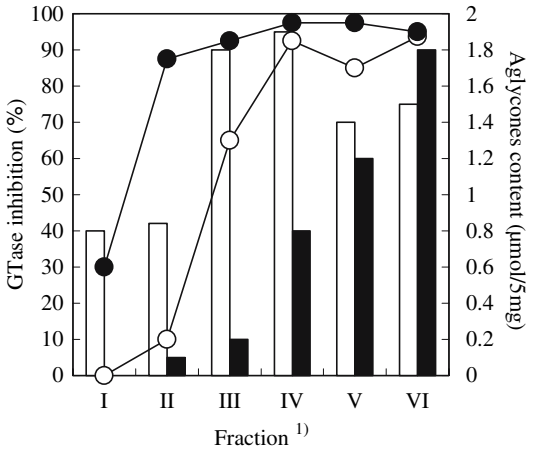

Fig. 2 Relationship of GTase-inhibitory activity of the fractions separated by LH-20 and the concentration of aglycones as determined by hydrolysis. *Fractions I-VI* were those eluated by water, 20, 40, 60 and 80 aqueous methanol and 70% aqueous acetone on Sephadex LH-20 column chromatography, respectively. *Open bars* and *filled bars* indicate content of released ellagic acid and gallic acid, respectively; *filled circles* and *open circles* indicate GTase inhibition at final concentrations of 15 and 5 μg/ml, respectively

Figure 2 shows GTase inhibitory activity of each fraction obtained by method 2. Fractions III–VI showed high inhibitory activity for GTase. The active fractions showed a broad peak of HPLC profile compared to fractions I and II. The retention time of the active fractions increased with the larger fraction numbers. Generally, compounds of a low polarity or high molecular weight were retained for a comparatively longer time in an ODS column. Since the active fractions are soluble in aqueous methanol, they would consist of high-molecular-weight compounds.

As shown in Fig. 2, the fractions with high amounts of gallic acid also showed higher GTase-inhibitory activity. In other words, hydrolyzable tannins possessing more galloyl groups might show stronger GTase-inhibitory activity than hydrolyzable tannins without galloyl groups. This observation is similar to that reported by Kakiuchi et al. [19]. They also demonstrated that ellagitannin had no effect on GTase inhibition. However, since the amount of gallic acid released from the active fractions after 10 h of hydrolysis treatment was not greater than after 5 h of hydrolysis treatment, the hydrolyzable tannins isolated from kapur extracts would most likely belong to the ellagitannin type. Generally, gallotannin needs a longer hydrolysis time than ellagitannin [17]. In the study of hydrolysis using tannic acid that consisted of gallotannin, the amount of gallic acid after 10 h of hydrolysis was five times more than after 5 h of treatment. Therefore, our results suggest that some GTase-inhibitory compounds in kapur extracts may be ellagitannins.

Table 3 GTase inhibiton of kapur extracts separated by cellulose and ODS column chromatography

Fraction	GTase inhibition (%)			IC_{50}[b](μg/ml)
	15 μg/ml[a]	5 μg/ml	1 μg/ml	
Fr-IV-A[c]	96.5	86.3	1.8	3.3
Fr-IV-B	10.6	–	–	ND
IV-A-1[d]	96.8	89.2	4.7	3.1
IV-A-2	97.4	95.1	8.1	2.9
IV-A-3	97.4	95.0	3	3.0
IV-A-4	96.9	90.7	1.7	3.2
IV-A-5	96.6	91.5	4.9	3.1
IV-A-6	96.4	87.5	1.3	3.3

[a] Final concentration of samples

[b] Sample concentration given a 50% inhibition of GTase

[c] The fractions eluted by 6% aqueous acetic acid and butanol-acetic acid on cellulose column chromatography from Fr-IV obtained by Sephadex LH-20 gel column were named Fr-IV-A and B, respectively

[d] The fractions separated by ODS column from Fr-IV-A were named IV-A-1 through 6

Fraction IV, which showed high inhibition of GTase, was fractionated with cellulose column to obtain Fr-IV-A and Fr-IV-B. The Fr-IV-A eluated with 6% aqueous acetic acid showed high inhibitory activity but Fr-IV-B did not. The Fr-IV-A released ca. 7% ellagic acid and 2% gallic acid under hydrolysis. In contrast, Fr-IV-B released no aglycones. Fr-IV-A was separated into six fractions by ODS-preparative HPLC, and these fractions were examined for GTase-inhibitory activity. All fractions showed strong activity (Table 3). The UV-vis spectra and HPLC profiles of these fractions were very similar to each other. Therefore, the GTase-inhibitory compounds in these fractions might have very similar structures.

Identification of phenolic compounds from kapur and GTase-inhibitory activities

Two phenolic compounds **1** and **2** were isolated from the water eluate fraction by method 2. Compound **1** was identified as bergenin (Fig. 3) by 2D ^{1}H and ^{13}C NMR. This compound has been previously isolated from roots of *Bergenia crassifolia* [20]. Compound **2** showed a quasi-molecular ion at *m/z* $[M-H]^+$ 448 and MS-MS products (*m/z* 301, 257, 229 and 185) that correspond to ellagic acid in its ESI-MS. Thus it was assumed that compound **2** was an ellagic acid glycoside. In the ^{1}H NMR of compound **2**, signals that indicated rhamnoside were observed. Furthermore, the ^{1}H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC) and nuclear overhauser and exchange spectroscopy (NOESY) suggested the presence of an ether linkage between the 4 position of

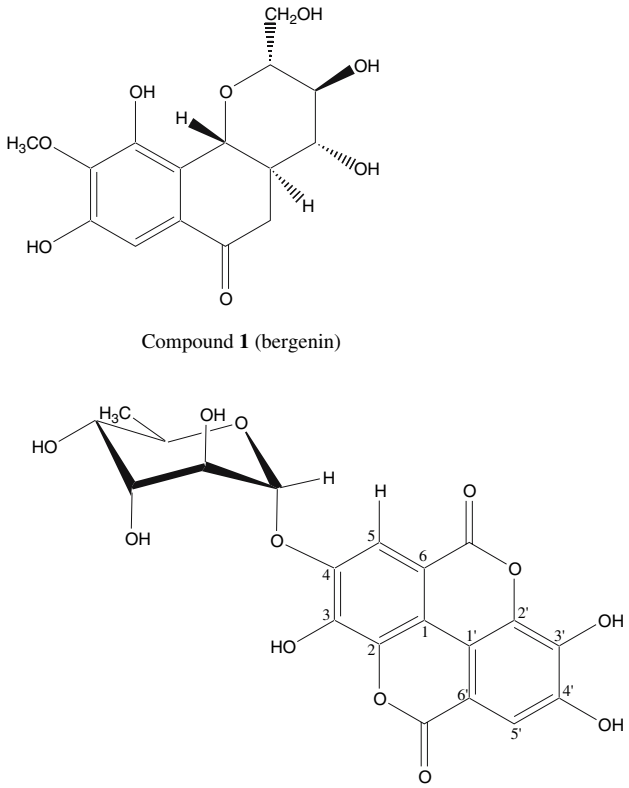

Fig. 3 Structures of compounds **1** and **2**

ellagic acid and the anomeric carbon of rhamnoside. Therefore **2** was identified as 4-*O*-(α-rhamnopyranosyl) ellagic acid (eschweilenol C, Fig. 3). This compound has been isolated from the bark of *Eschweilera coriacea* [21] and from the heartwood of *Punica granatum* [22]. Our study is the first report to find both compounds in kapur.

The GTase-inhibitory activities of compounds **1** and **2** were investigated, and the IC_{50} are shown in Table 4 together with (–)-epigallocatechin 3-*O*-gallate (EGCG) and pentagalloylglucose (PGG) as positive controls. EGCG is one of the major catechins contained in green tea, and PGG is a representative of the gallotannins,such as tannic acid, found widely in natural products. These compounds have been shown to inhibit GTase activity in previous research, and also have been utilized as anti-caries agents in chewing gum, beverages, and dentifrices.

In research on the inhibitory effects of polyphenols on glucosyltransferase, the inhibitory effect increases with an increase in molecular weight of the polyphenols. For instance, monomeric and dimeric proanthocyanidins have no effectiveness, but those beyond trimer gradually begin to show the inhibitory effect [7]. In gallotannins as well, increasing the number of galloyl groups enhances the inhibitory effects. However compounds **1** and **2** showed comparatively high GTase-inhibitory activity in spite of

Table 4 GTase-inhibitory activities of compounds 1 and 2 together with positive controls

	Molecule weight	IC_{50} (μM)	IC_{50} (μg/ml)[a]
Compound 1	328	36.59	12
Compound 2	448	17.86	8
EGCG	458	115.47	53
PGG	940	5.32	5

[a] Refer to Table 2

EGCG (–)-Epigallocatechin 3-*O*-gallate, *PGG* Pentagalloylglucose

lower molecular weight. Additionally these compounds are a relatively light-yellowish color compared to tannins, which are a tan color, which indicates the possibility that these compounds can be used for food ingredients and cosmetics.

Conclusion

Kapur extracts showed a strong inhibitory activity for GTase among 36 extracts of tropical woody species. The results of acid hydrolysis suggested that active GTase-inhibitory compounds of kapur heartwood extracts were hydrolyzable tannins, mainly ellagitannins. Bergenin and eschweilenol C were isolated from the water eluate fraction of LH-20 gel column chromatography of 50% aqueous ethanol extracts, which is the first isolation from kapur. These compounds showed comparatively strong inhibitory activities on GTase despite their relatively low molecular weight.

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