INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disease characterized by persistent hyperglycemia resulting from either when the pancreas does not produce enough insulin (type 1) or when the body cannot effectively use the insulin it produces (type 2); type 2 accounts for 90–95% of all diabetic cases. Postprandial hyperglycemia (PPHG) is an independent risk factor for the development of macrovascular complications, which may lead into life-threatening complications such as cerebrovascular and cardiovascular diseases; thus, controlling PPHG is of vital importance to prevent diabetes and its complications. The rise of glucose levels in the blood, postprandially, is a result of carbohydrate hydrolysis, a process primarily catalyzed by the enzymes α-glucosidase and α-amylase. In the process, salivary α-amylase enzyme hydrolyzes carbohydrates to disaccharides and oligosaccharides, which are then hydrolyzed further by the α-glucosidase enzyme to monosaccharides such as glucose, which is then absorbed through the small intestines into the blood, and the remaining oligosaccharides are hydrolyzed by the pancreatic α-amylase to glucose and maltose. Therefore, inhibition of these enzymes results in a massive reduction of PPHG blood levels.

On the other hand, to date, all drugs used to inhibit the activities of carbohydrate hydrolyzing enzymes are synthetic. Their major drawbacks include adverse side effects such as gastrointestinal disturbances (flatulence, abdominal pain, and diarrhea), metabolic effect (such as weight gain), cost, and drug resistance. Hence, there is an urgency for the need to search for new antidiabetics that are effective, cheap, and have low toxicity. As a result, investigation of antidiabetics from natural sources has gained a lot of attention as they can offer all stated advantages. This is because they constitute secondary metabolites with a wide structural diversity that possess a broad spectrum of bioactivities including antidiabetic. Researchers have proven that some medicinal plants/consumption of functional foods effectively inhibit α-glucosidase and α-amylase and thus may prevent the complications.
development of diabetes. In the present study, we investigated the α-glucosidase and α-amylase enzyme inhibitors from olive mill wastes (OMW), with additional kinetics studies to help to clarify their underlying mechanisms of the enzymes’ inhibition.

The major by-product of olive oil extraction, OMW, has become a major environmental issue due to its high organic loads, which leads to serious environmental damage, especially the high phenolic metabolite contents, which are phytotoxic and have bactericidal effects on terrestrial microbes. Research suggests that new ways to utilize it could help to solve/reduce such damage. One of the most promising ways is through the recovery of valuable compounds, which can be utilized in different fields. In the present study, a total of seven compounds were isolated from OMW and were tested for their postprandial hyperglycemia lowering effects (α-glucosidase and α-lipase inhibitory activities). Also, enzyme inhibitory kinetics analysis of the most active compounds was performed to assist the possible characterization of the mechanisms of inhibition involved.

■ RESULTS

Identification of the Isolated Compounds. A total of seven compounds were isolated from OMW, including one novel, 3,4-dihydroxyphenyl-2-methoxyethanol 5 (a methoxy derivative of hydroxytyrosol). The details of the new compound have been discussed below, and chemical structures of all compounds are shown in Figure 1.

Compound 5 was obtained as a yellowish amorphous liquid with UV (EtOH) λmax 228, 280 nm. Its structure was elucidated by 1D- and 2D-NMR spectroscopy and HR-ESI-MS. The analysis of NMR data in comparison with that in the literature suggested that compound 5 has the main skeleton of the hydroxytyrosol, which was evident through both 1H NMR spectral peaks, whereby a doublet at δH 7.62 (d, 1H, J = 2.1 Hz, H-2) and a doublet-doublet at δH 6.53 (dd, 1H, J = 2.0 Hz, J = 8.0 Hz H-6) were observed, which entails the ABX system – meta- and para-aromatic ring substitution. The other two resonances corresponding to the protons H-1′ and H-2′ of the ethyl chain were observed resonating at δH 4.09 (dd, 2H, J = 3.8 Hz, H-1′) and δH 3.48 (dd, 2H, J = 3.8 Hz, J = 11.7 Hz H-2′), respectively. The first difference between 5 and hydroxytyrosol 7 was observed at H-1′, which was resonating at a more downfield region as compared to that of hydroxytyrosol (δH 2.67, t, 2H). Another main difference was observed through the 13C NMR spectrum at the region of aliphatic carbons. These signals were observed resonating at δC 84.7 and 66.4, which were later assigned to C-1′ and C-2′, respectively, through HSQC – both of them being more downfield as compared to the reported ones (δC 63.9 and 39.0). These data were suggestive of the possible attachment at C-1′.

The long-range HMBC correlations observed from δH 4.09 (H-1′) included the carbons resonating at δC 130.3 (C-1′), 118.3 (C-6), 66.4 (C-2′), and 55.2 (–OCH3), bearing in mind the ABX substitution in the aromatic ring and thus confirming our hypothesis of the presence of an oxygenated carbon attached to C-1′. Furthermore, the correlations between C-1′ and C-2′ and C-1′ and –OCH3 were clearly seen through the 1H-1 H COSY spectrum. The assignments of all 1H and 13C NMR signals were confirmed by HSQC and HMBC data and are summarized in Table 1 and Figure 1A. The HR-ESI-MS spectrum of compound 5 showed a molecular ion peak [M–H]+ at m/z 183.0639, which was agreeable with the suggested molecular formula of C9H11O4− (calculated m/z 183.0663). Some molecular peaks were observed at m/z 151.0391 and 167.078, corresponding to the fragments [M–CH3OH]+ and [M–OH]−, respectively.

The other compounds were identified as oleic acid, maslinic acid, 1-acetoxyresiniferol, hydroxytyrosol acetate, luteolin-7-O-β-D-glucoside, and hydroxytyrosol. The data used for the identification of these compounds is attached in the Supporting Information (pages S5–S8).

UPLC/qTOF-MS Analysis. Compounds isolated in the present study belong to phenolic compounds and triterpene...
Inhibitory activity on α-glucosidase was higher than that of acarbose (p < 0.05), while in the case of α-amylase, acarbose had higher activity than them (p < 0.05).

In general, our results show that, even though both fractions had good activity, the inhibitory activity of individual compounds from the respective fractions varies widely (from highly active to poorly/not active). For instance, hydroxytyrosol acetate 4, 3,4-dihydroxyphenyl-2-methoxyethanol 5, and hydroxytyrosol 7, which were isolated from the active EtOAc fraction, showed inhibitory activity of less than 10% at highest tested concentrations (1000 and 500 μM for α-glucosidase and α-amylase, respectively). This may suggest that there is a possibility of either an additive or synergism effect for the overall activity of the fraction(s) or some minor compounds, which were not isolated, may have contributed to the overall higher activity of the fractions. The synergism effect of plant extracts/fractions has been widely known in phytochemistry.25,26

Finally, we compared their total activities (Table 3). Generally, the total activities for both α-glucosidase and α-amylase enzymes were highest for oleanolic acid 1 and maslinic acid 2 when compared to other active compounds (3 and 6). This is because, apart from having high activity and low IC50 values, they are also more abundant than compounds 3 and 6 in OMW. Another instance is observed in 1-acetoxypinoresinol 3; regardless of its highest α-amylase inhibitory activity, its total activity for α-amylase was lower than those of 1 and 2.

**Kinetics Study of the Active Compounds.** Due to their interestingly higher inhibitory activity against the α-glucosidase enzyme, we investigated further the possible underlying mechanisms of inhibition by oleanolic acid 1, 1-acetoxypinoresinol 3, and luteolin-7-O-β-D-glucoside 6 for inhibition of the enzyme. In the experiment, α-glucosidase was treated with these compounds, individually, using sucrose as the substrate enzyme. In the experiment, α-glucosidase was treated with these compounds, individually, using sucrose as the substrate enzyme. In the experiment, α-glucosidase was treated with these compounds, individually, using sucrose as the substrate enzyme. In the experiment, α-glucosidase was treated with these compounds, individually, using sucrose as the substrate enzyme. In the experiment, α-glucosidase was treated with these compounds, individually, using sucrose as the substrate enzyme.

After analysis of the Lineweaver–Burk plots and based on the best fit results and goodness to fit analysis, we concluded that oleanolic acid 1 is a partial mixed inhibitor,27 luteolin-7-O-β-D-glucoside 6 is a noncompetitive inhibitor, while 1-acetoxypinoresinol 3 is a partial competitive inhibitor to the α-glucosidase enzyme.28 The Lineweaver–Burk plots of the reaction of α-glucosidase for the active compounds are shown in Figure 4.

The mixed-type inhibition by oleanolic acid 1 is similar to noncompetitive inhibition, as of luteolin-7-O-β-D-glucoside 6,

### Table 1. 1H and 13C NMR Data for Compound 5 and the Reference (Hydroxytyrosol 7) *

| compound 5 | reference (hydroxytyrosol 7) |
|------------|------------------------------|
| no.        | 1H (J in Hz) | 13C                     | 1H (J in Hz) | 13C                     |
| 1          | 131.3        | 130.3                   | 1            |                          |
| 2          | 6.72 d (2.0) | 115.7                   | 6.72 d (2.1) | 113.5                   |
| 3          | 145.2        | 145.0                   | 4            | 143.9                   | 144.7                 |
| 5          | 6.68 d (8.0) | 116.4                   | 6.67 d (8.0) | 113.4                   |
| 6          | 6.53 dd (2.0, 8.0) | 120.6 | 6.61 dd (2.0, 8.0) | 118.3 |
| 1'         | 4.09 dd (3.8, 8.2) | 84.7 | 2.67 t (7.2) | 39.0 |
| 2'         | 3.48 dd (3.8, 8.2) | 66.4 | 3.68 t (7.2) | 63.9 |
| OCH3       | 3.21 s       | 55.2                    |                          |                     |

*The values of chemical shifts are expressed in ppm; for 5, 1H and 13C NMR at 600 and 150 MHz, respectively. In both cases, the compounds were dissolved in methanol-d4 (CD3OD).

### Table 2. Characterization of the Isolated Compounds (and (+)-Pinoresinol) from the OMW Extract by UPLC/qTOF-MS

| peak no. | retention time, tR (min) | formula | [M−H]+ (m/z exp) | [M−H]+ (m/z theo) | error (ppm) | identification |
|----------|--------------------------|---------|------------------|-------------------|-------------|---------------|
| 1        | 15.295                   | C29H48O3 | 453.3534         | 453.3530          | 0.8784      | oleanolic acid |
| 2        | 12.829                   | C29H48O4 | 471.3487         | 471.3480          | 1.4851      | maslinic acid |
| 3        | 8.313                    | C22H24O8 | 415.1390         | 415.1398          | −1.9271     | 1-acetoxypinoresinol |
| 4        | 6.674                    | C21H24O3 | 195.0674         | 195.0663          | 0.6391      | hydroxytyrosol acetate |
| 5        | 3.381                    | C18H16O5 | 183.0663         | 183.0663          | 0.0000      | 3,4-dihydroxyphenyl-2-methoxyethanol |
| 6        | 5.123                    | C21H20O11| 447.0933         | 447.0933          | 0.0000      | luteolin-7-O-β-D-glucoside |
| 7        | 3.268                    | C29H24O3 | 153.0577         | 153.0577          | 0.0000      | hydroxytyrosol |
| (+)-pinoresinol | 7.43                   | C20H22O6 | 357.1331         | 357.1344          | −3.6401     | (+)-pinoresinol |

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except that binding of the substrate or the inhibitor affects the enzyme’s binding affinity for the other. The findings of oleanolic acid 1 and luteolin-7-O-β-D-glucoside 6 inhibition types are in line with the previous report on oleanane-type pentacyclic triterpene acids and flavonoid luteolin, respectively. On the other hand, this is the first report on lignan 1-acetoxypinoresinol 3. As an uncompetitive inhibitor, 1-acetoxypinoresinol 3 binds to the enzyme at the same time as the enzyme’s substrate; that is, it binds to the enzyme–substrate complex (it neither binds to the enzyme itself nor to the substrate), and this type of inhibition cannot be overcome but can only be reduced by increasing the substrate concentration. Thus, for all inhibition types, mixed-type, noncompetitive, and uncompetitive, the kinetic constant, which measures the affinity of the inhibitor for an enzyme, is usually greater than 1 ($K_i > 1$) for oleanolic acid 1, $K_i = 8.4$; luteolin-7-O-β-D-glucoside 6, $K_i = 12.7$; and 1-acetoxypinoresinol, $K_i = 61.1$. Moreover, this shows the fact that all these
Table 3. Enzymatic Inhibitory Activities (IC_{50}), the Total Amounts in Dry Weight, and the Total Activities of the Isolated Compounds from OMW

| compound                        | IC_{50} (µM) [µg/mL] | amount (mg/kg dried OMW) | total activity^c | α-glucosidase | α-amylase |
|---------------------------------|-----------------------|---------------------------|------------------|---------------|-----------|
| oleanolic acid 1                | 33.5 ± 0.6 [15.3]     | 81.3 ± 2.7 [37.1]         | 82.1             | 5359.5        | 2212.9    |
| maslinic acid 2                 | 34.5 ± 1.4 [16.3]     | 67.0 ± 5.4 [31.7]         | 180.0            | ND            | ND        |
| 1-acetoxyproinosresinol 3       | 313.1 ± 12.5 [130.4]  | 13.9 ± 3.9 [5.5]          | 1.13             | 8.7           | 205.5     |
| hydroxytyrosol acetate 4        | >1000 [>200]          | >500 [>100]               | 21.46            | ND            | ND        |
| 3,4-dihydroxyphenyl-2-methoxethanol 5 | >1000 [>200]          | >500 [>100]               | 128.79           | ND            | ND        |
| luteolin-7-O-β-D-glucoside 6    | 26.6 ± 0.8 [11.9]     | 404.7 ± 0.0 [181.5]       | 46.71            | 3925.2        | 256.6     |
| hydroxytyrosol 7                | >1000 [>200]          | >500 [>100]               | 303.03           | ND            | ND        |
| (+)-pinoresinol                 | >1000 [>400]          | >500 [>200]               | ND               | ND            | ND        |
| acarbose^b                      | 323.4 ± 4.2 [208.8]   | 15.0 ± 1.3 [9.7]          |                  |               |           |

^a The results are expressed as mean ± SD (n = 3). ^b Standard drug and ND could not be determined since the exact value of IC_{50} could not be calculated (beyond the maximum tested concentration). ^c Total activity of each compound was estimated mathematically by using the following formula: total activity = amount (mg/kg)/IC_{50} (mg/mL).

compounds have a quite different structure from the substrate for the enzyme, and that is why they cannot inhibit the enzyme competitively.

**DISCUSSION**

The increase of postprandial glucose levels in plasma is a result of the hydrolysis of carbohydrates, such as starch and sucrose — a process that is primarily catalyzed by two hydrolase enzymes: α-glucosidase and α-amylase. While the pancreatic α-amylases specifically hydrolyze the internal α-1,4-glycosidic bond of starch to yield maltose and glucose, α-glucosidases, which are a series of enzymes found in the brush border of enterocytes, hydrolyze both starch and sucrose down to glycogen levels and glycogenic enzymes in rats. For maslinic acid, it was reported to reduce blood glucose levels by inhibiting glycogen phosphorylase. Our results suggest that the oleane skeleton may be responsible for their activity. This is not uncommon as previous research showed that other oleane-type triterpenes, apart from oleanolic and maslinic acids, were also active. For instance, corosoric acid showed strong inhibitory activity not only in vitro against both α-glucosidase and α-amylase enzymes but also reported having a significant effect on lowering post-challenge plasma glucose levels in vivo. Considering the higher abundances of oleanolic 1 and maslinic acids 2 in OMW and their total activities for both α-glucosidase and α-amylase, these data attract more attention to OMW as a potential source of PPHG-lowering agents.

Furthermore, two phenolic compounds (1-acetoxyproinosresinol 3 and luteolin-7-O-β-D-glucoside 6) showed higher activity, while the rest, three of them (4, 7, and (+)-pinoresinol), had very weak activities against both α-glucosidase and α-amylase enzymes. 1-Acetoxyproinosresinol 3 showed a very strong inhibitory activity against both enzymes (with the highest activity for α-amylase, IC_{50} = 13.9 vs 15.0 µM acarbose, while for α-glucosidase, IC_{50} = 313.1 vs 323.4 µM). Regardless of its highest activity against α-amylase, its total activity was lower than those of oleanolic acid 1 and maslinic acid 2. This is accounted for by its low abundance in OMW, as opposed to 1 and 2, which were more abundant by several folds compared to 3 (Table 3). On the other hand, the inhibitory activity of (+)-pinoresinol, a compound with a closely related chemical structure to 3, was very weak (IC_{50} > 500 µg/mL vs both enzymes). Taking into account the differences in their inhibitory activities, between 1-acetoxyproinosresinol 3 vs (+)-pinoresinol, it is arguable that esterification of the furofuran ring at position one (C-1) of 1-acetoxyproinosresinol 3 enhances its α-glucosidase and α-amylase enzymatic inhibitory activities. While the inhibitory activities of 1 and 2 were consistent with those reported elsewhere, this is the first report on antidiabetic activity for 1-acetoxyproinosresinol 3 and (+)-pinoresinol. Luteolin-7-O-β-D-glucoside 6 was more active against α-glucosidase and relatively poor activity against α-amylase. Our findings are in line with the ones previously reported about flavonoid glycosides.

The least active phenolic compounds, hydroxytyrosol 7, hydroxytyrosol acetate 4, and 3,4-dihydroxyphenyl-2-methoxethanol 5, share a common structural feature — the presence...
of a 3,4-dihydroxyphenylethyl (3,4-DHPE) moiety. All of them had weak inhibitory activity against both $\alpha$-glucosidase and $\alpha$-amylase. These findings show, even with several modifications, for instance, esterification of hydroxytyrosol at the ethyl $\text{--OH}$ (to form hydroxytyrosol acetate) or alkylation at the ethyl C-2 (to form the new compound, 3,4-dihydroxyphenyl-2-methoxyethanol). Again, this is the first report on their inhibitory effect on hydrolase enzymes $\alpha$-glucosidase and $\alpha$-amylase. Previously, only the inhibitory activity of a phenolic extract from virgin olive oil (VOO) was reported to have an inhibitory effect on these enzymes; it was more active against $\alpha$-glucosidase than it was for $\alpha$-amylase. However, the researchers did not go further to test the activity of individual phenolic compounds.53

To the best of our knowledge, most studies that have reported about antidiabetic properties of olives and virgin olive oil are based on epidemiological facts, whereby they correlated olive fatty acids, especially oleic acid, and antidiabetic effect of virgin olive oil.40–43 However, our findings clearly showed that the $n$-hexane fraction, which contains all fatty acids including up to 75% oleic acid,44 has a poor activity. Therefore, our results give evidence that not only fatty acid constituents but also other metabolites, such as triterpenes and phenolic compounds, contribute to the antidiabetic properties. Our result is consistent with one study whereby some researchers reported about the effectiveness of olive phenolic acids on $\alpha$-glucosidase and $\alpha$-amylase inhibitory activities.53

In general, we confirmed that some metabolites in OMW are potential inhibitors of the hydrolase enzymes involved in type 2 diabetes — olive lignan (1-acetoxypinoresinol 3), pentacyclic triterpenes (oleanolic acid 1 and maslinic acid 2), and luteolin-7-O-$\beta$-D-glucoside 6. This is the first study to address the $\alpha$-glucosidase and $\alpha$-amylase inhibitory activities of olive lignans ((+)-pinoresinol and 1-acetoxypinoresinol), as well as hydroxytyrosol derivatives including the new compound, 3,4-dihydroxyphenyl-2-methoxyethanol, and hydroxytyrosol acetate. While the other compounds (1, 2, and 6) have been reported in the previous studies, this is the first study that quantifies their contribution (of individual metabolite) in the OMW dry mass to the total inhibitory activity against $\alpha$-glucosidase and $\alpha$-amylase enzymes (antidiabetic).

## CONCLUSIONS

In summary, the results from this study clearly show that some compounds from OMW are potential inhibitors of $\alpha$-glucosidase and $\alpha$-amylase enzymes, which are involved in the hydrolysis of carbohydrates to generate glucose (PPHG) — one of the very first steps in the pathogenesis of diabetes. We demonstrated the mechanism of $\alpha$-glucosidase inhibition by active compounds, one of them being an olive lignan, 1-acetoxypinoresinol, which has been reported for the first time. Our results potentiate OMW as an important source of lead compounds for antidiabetic formulations, food supplements, or for modification to develop new antidiabetic drugs. It is, therefore, worth mentioning that OMW (and its metabolites) will be valuable for further studies, prevention, and/or treatment of type 2 diabetes.

## MATERIAL AND METHODS

### Chemicals and Reagents.

$\alpha$-Glucosidase (from *Saccharomyces cerevisiae*) was purchased from Oriental Yeast Co.
(Tokyo, Japan), while α-amylase (from the porcine pancreas) was from Sigma-Aldrich (St. Louis, MO, USA), and acarbose was obtained from Wako Pure Chemical Industries (Osaka, Japan). Deionized water (18 MΩ cm) was obtained by using a Milli-Q purification system from Millipore (Bedford, MA, USA). LC–MS-grade acetonitrile and formic acid were used to prepare chromatographic mobile phases. Commercial pure standard of (α)–pinosanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Silica gel and solvents used for extraction and open column chromatography were purchased from Wako Pure Chemical Industries (Osaka, Japan), while TLC silica gel 60 F254 plates were from Merck (Darmstadt, Germany), and chloroform–d (CDCl3) and methanol–d4 (CD3OD) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

**Extraction and Isolation.** Dried OMW was collected from an olive farm in Ukiha (Kyushu Island) in December 2018. The freeze-dried OMW (350.48 g) was extracted by maceration at 200 rpm with 3.6 L of MeOH at rt for 72 h – this process was repeated twice for exhaustive extraction. After evaporation under reduced pressure at 45 °C, 27.15 g of extract was obtained. Following the screening with α-glucosidase inhibitory activity, the extract was shown to be active, and hence, it was subjected to LLE to obtain n-hexane (7645.9 mg), dichloromethane, DCM (899.11 mg), ethyl acetate, EtOAc (1680.20 mg), and aqueous (12927.29 mg) fractions. The α-glucosidase inhibitory activity showed that only DCM and EtOAc fractions were most active, while the n-hexane and aqueous fractions were the least active (see Scheme S1 in the Supporting Information). Therefore, fractionation and isolation proceeded with the most active fractions (DCM and EtOAc Fr). Each of these fractions was separately subjected to a medium-pressure liquid chromatography (MPLC) system (BUCHI, Reveveris Prep Purification System, Switzerland), and further purification, when necessary, was achieved by a preparative TLC or MPLC system again. In both cases, elutes were monitored with TLC analysis. To begin with the DCM fraction; it was purified by the BUCHI MPLC system connected with a C-18 column (particle size 35–45 μm, 40 g) flushed with the water-MeOH system (95:5 → 100) resulting into 95 collected tubes, each containing 25 mL of elute. After TLC analysis of the elutes, flasks numbers 83–95, eluted with water-MeOH (95:5 → 100), were confirmed to contain a pure compound 1 (37 mg). Also, while elutes in the flask number 77, eluted with water-MeOH (15:85), came out as pure compound 2 (39.7 mg), other elutes from flask number 81, which was eluted with water-MeOH (10:90) by MPLC in the first purification step, were further purified in the second step by preparative TLC using n-hex-EtOAc (30:70) to afford pure compound 2 (4.0 mg) – making a total of 43.7 mg for compound 2. Then, for the EtOAc fraction, its first purification step was done with the BUCHI MPLC system connected with the C-18 column (particle size 35–45 μm, 3.0 × 16.5 cm, 40 g), flushed with the water-MeOH system (95:5 → 100) to afford 120 collected tubes (each with 25 mL of elutes). Subfractions from flask numbers 77 to 120 (187.4 mg), eluted with water-MeOH (20:80 → 100), were further purified using a Yamazen MPLC system (EPCLC, Yamazen, Osaka, Japan) connected with the silica gel column (particle size 50 μm, 3.0 × 16.5 cm, 40 g) flushed with a CHCl3–MeOH gradient (90:10 → 100) and then by preparative TLC eluted with n-hex-EtOAc (30:70) and (20:80) to afford compounds 3 (1.6 mg) and 4 (8.0 mg), respectively. The subfractions from flask numbers 1 to 7 (23.3 mg) eluted with water-MeOH (95:5) were further purified using the BUCHI MPLC system connected with the C-18 column flushed with a water-MeOH gradient (95:5 → 85:15) to afford compound 7 (4.0 mg). Elutes from flasks 13 to 60 (680.3 mg) eluted with water-MeOH (90:10 → 30:70) were further purified using a Yamazen MPLC system connected with the silica gel column flushed with a n-hex-EtOAc gradient (30:70 → 0:100) and lastly washed with MeOH to afford four subfractions. The first, most polar, subfraction was purified by preparative TLC ran with EtOAc:MeOH:Hex = 9:1:1 to afford compounds 5 (3.0 mg) and 6 (4.5 mg). A schematic presentation of the fractionation and isolation is attached in the Supporting Information (Scheme S1, pages S3 and S4).

**Identification of the Isolated Compounds.** The NMR spectra of the compounds were recorded using a Bruker DRX-600 spectrometer (Bruker Daltonics, Billerica MA, USA) at room temperature with trimethylsilane (TMS) as the internal standard of the chemical shifts, CD3OD or CDCl3 as dissolving solvent, and the chemical shifts were expressed as δ values. HR-ESI-MS and HR-ESI-MS were performed using a quadrupole time-of-flight (qTOF) mass spectrometer (Agilent Technologies, USA) and JMS 700 MStation mass spectrometer (JEOL, Tokyo, Japan).

**Analysis of the Extract by UPLC/qTOF-MS.** The powdered OMW (100 mg) was extracted by sonication with 10 mL of MeOH for 45 min at 30 °C. The extracted solution was then filtered, and this process was repeated twice for exhaustive extraction. Then, the combined filtrate was evaporated to dryness at reduced pressure by a rotary evaporator at 45 °C. The residue was finally reconstituted in MeOH to a final concentration of 200 μg/mL. Before injection for chromatographic separation, it was filtered twice through Millipore 0.20 μm PTFE filters (Milllex-LG, Japan).

An Agilent 1290 Series UPLC system equipped with a 1290 photodiode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 6545 q-TOF hybrid mass spectrometer (MS) with a dual electrospray ionization (ESI) source for simultaneous spraying of a mass reference solution that enabled continuous calibration of detected m/z ratios was used for the analysis of the samples. An injection volume of 2 μL and a flow rate of 0.2 mL/min were used. The mobile phases were 0.1% (v/v) formic acid aqueous solution (phase A) and 0.1% (v/v) formic acid acetonitrile (phase B). Separation of the analytes was achieved through an Agilent Poroshell 120 EC C-18 column (100 × 2.1 mm inner diameter, 2.7 μm particle size, Agilent Technologies, Santa Clara, CA, USA) at 40 °C. The gradient method was as follows: 4% B to 100% B in 15 min and 100% A for 2.5 min. At last, re-equilibration was done for 3.5 min. The dual ESI source was operated in negative ionization using the following conditions: nebulizer gas at 35 psi and drying gas flow rate and temperature at 10 L/min and 325 °C, respectively. The capillary voltage was set at 3200 V, while the fragmentor, skimmer, and octapole voltages were fixed at 60, 55, and 750 V, respectively. The data were acquired in centroid mode in the extended dynamic range (2 GHz). The full scan was operated at 1.5 spectra per second within the m/z range of 100–1700. A continuous internal calibration was performed during analyses to ensure the desired mass accuracy of recorded ions with the use of signals at m/z 112.9855 (ammonium-abscrated TFA/TFA anion) and m/z 1033.9881 (trifluoroformate adduct of hexakis[1H,1H,3H-
tetrafluoropropoxyporphazine). For data acquisition and monitoring the hardware, Agilent MassHunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used. The obtained UPLC/qTOF-MS raw data were further processed by Agilent MassHunter Qualitative Analysis software (version B.10.00, Agilent, USA). The extracted ion chromatograms (EICs) algorithm was applied to extract and identify all metabolites from the total ion chromatograms (TICs) of the extract according to their metabolic features including m/z, retention time, and ion intensities, whereby reference was made from a standard mix. After identification of all metabolites by Agilent MassHunter Qualitative Analysis software, the raw data were then transferred to Agilent MassHunter Quantitative Analysis software (version B.10.00, Agilent, USA) for making the calibration curve and quantification.

**Assay for α-Glucosidase Inhibitory Activity.** The method used to assay the α-glucosidase inhibitory activity of the compound was adopted as described elsewhere. Briefly, 100 μL of an appropriate solvent such as DMSO, water, or a mixture of them with or without (control) samples and 100 μL of the enzyme (α-glucosidase, 5 U/mL in 0.15 M HEPES buffer) were added to 100 μL of the substrate (0.1 M sucrose solution, made by dissolving into 0.15 M HEPES buffer), and the mixture was homogenized by vortexing for 5 s and then incubated at 37 °C for 30 min to allow enzymatic reaction. After incubation, the reaction was stopped by heating at 100 °C for 10 min in a block incubator. The formation of glucose was determined by means of the glucose oxidase method using a BFF-SS Biosensor (Oji Scientific Instruments, Hyogo, Japan).

Mathematically, the α-glucosidase inhibitory activity of each sample was calculated using the following equation

\[
\text{inhibition (\%)} = \frac{[A_c - A_s] / A_c \times 100}
\]

where \(A_c\) is the average value of control and \(A_s\) is the average value of the sample \((n = 3)\).

**Assay for α-Amylase Inhibitory Activity.** The α-amylase inhibitory effect was assayed according to the procedure as described by previous researchers, with some minor modifications. Briefly, 0.2% (w/v) potato starch solution and phosphate buffer with or without (control) samples were mixed with PPA in phosphate buffer to start the enzyme reaction. After incubation at 37 °C for 30 min, the reaction was stopped by the addition of 0.4 M HCl. Then, 100 μL of 5 mM KI was added, and absorbance was read at 660 nm (Corona Electric Co, Japan). All samples were assayed in triplicate \((n = 3)\). The percent inhibitory activity (\%) was calculated mathematically as follows

\[
\text{inhibition (\%)} = \left\{1 - \frac{(A_1 - A_2)}{(A_4 - A_3)} \times 100\right\}
\]

where \(A_1\) is the average absorbance of the incubated solution containing the sample, starch, and α-amylase; \(A_2\) is the average absorbance of incubated solution containing the sample and starch; \(A_3\) is the average absorbance of incubated solution containing starch and α-amylase; and \(A_4\) is the average absorbance of incubated solution containing starch only \((n = 3)\).

**Inhibitory Kinetics Analysis against the α-Glucosidase Enzyme.** Inhibitory kinetics analysis was performed on the most active compounds. In order to evaluate the inhibition type of the active compounds against α-glucosidase, the enzyme activity was quantified at increasing concentrations of the substrate (sucrose) at constant enzyme concentration in the absence or presence of the inhibitors (active compounds) at different concentrations — whereby three different concentrations were used (ranging from 0 μM to the one near their respective IC_{50} values). Briefly, enzyme (5 U/mL), dissolved in 100 mM HEPES buffer, was preincubated at 37 °C with the inhibitor (most active compound, individually) for 5 min. The substrate (sucrose) at different concentrations was added to the preincubated mixture of enzyme—inhibitor and incubated at 37 °C in the reaction mixture for 30 min. The inhibition type (competitive, uncompetitive, noncompetitive, or mixed), were determined by Lineweaver–Burk plot analysis of the data, which was calculated from the result according to the Michaelis–Menten kinetics, whereby the plots of 1/V versus 1/S were constructed, where \(S\) is the substrate concentration and \(V\) is the reaction velocity. The types of inhibition parameters (to determine the values of \(K_m\), \(K_i\), and \(V_{max}\) were calculated by SigmaPlot 12.3 software (Systat Software Inc., CA, USA).

**Data Analysis.** The data values in the present study were expressed as mean ± standard deviation (SD) of at least three independent experiments \((n = 3)\). The significant differences between each tested group and the control group were determined using Dunnett’s multiple post hoc test. The p-value cutoff was set at 95% (where \(p < 0.05\)) when the one-way analysis of variance (ANOVA) was statistically significant. The inhibitory bar graphs of the isolated compounds (and fractions) were drawn by Microsoft Excel 2016.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01622.

NMR and HR-ESI-MS data for the novel compound 5 are shown in Figures S1 and S2, respectively. The schematic procedure for extraction and isolation of the compounds is shown in Scheme S1, while NMR data used for the identification of other compounds (1, 2, 3, 4, 6, and 7) are attached in Supplementary S1 (PDF).

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Notes
The authors declare no competing financial interest.
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