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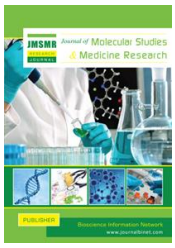

Inhibitory effects of Japanese plant leaf extracts on α-glucosidase activity

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ABSTRACT

In this study, methanol extracts of the leaves of nineteen Japanese plant species for which there have been no previous reports regarding in vitro α-glucosidase activities, were tested for their potential α-glucosidase inhibitory activities. Five plants, Quercus phillyraeoides, Mallotus japonicus, Sapium sebiferum, Elaeocarpus sylvestris var. ellipticus, and Myrica rubra showed high inhibitory activity against α-glucosidase with IC_{50} values in the range of 22 – 92 µg/ml. The methanol extracts of these five plants at 1 mg/ml showed significant inhibitory activity against rat intestinal sucrase. The hexane, ethyl acetate, butanol, and water extracts from the five plants were also screened for yeast α-glucosidase and rat intestinal sucrase inhibitory activity. The extracts of Q. phillyraeoides leaves (ethyl acetate and butanol extracts), M. japonicas leaves (ethyl acetate and butanol extracts), and E. sylvestris var. ellipticus leaves (ethyl acetate and butanol extracts) showed excellent inhibitory effects against yeast α-glucosidase. Methanol, ethyl acetate, and butanol extracts of the five plants also exhibited potent inhibitory effects against rat intestinal sucrase. Due to their inhibitory effect, the leaves of these five plant species were selected for further investigation to isolate and identify the active constituent(s) responsible for the potential antidiabetic activity.

Key Words: *Diabetes, α-glucosidase inhibitor and Diabetes mellitus*

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I. Introduction

Diabetes mellitus is one of the most serious chronic metabolic diseases. The number of diabetes patients is increasing in the world not only in developed countries but also in developing countries due to changes in lifestyle and food habits. The total diabetic population is predicted to increase from 171 million in 2000 to 366 million in 2030 (Wild *et al.* 2004). In Japan, the prevalence of type 2 diabetes is

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increasing in both adults and children; about 13.5% of the Japanese population had type 2 diabetes in 2009 (Neville *et al.* 2009). Therefore, it is clear that diabetes is a very serious concern. Blood glucose levels in the human body are increased due to diabetes, often leading to various complications, such as cardiovascular disease, eye problems, kidney disease, cerebrovascular disease, and limb amputation. Therefore, control of postprandial blood glucose levels is important for the treatment of diabetes. Diet and exercise are recommended to control blood glucose levels. Although diet and exercise are recommended for diabetes management. In most cases, medications are needed for the treatment of diabetes. Neville *et al.* (2009) reported that 51.4% of Japanese people with diabetes take oral antidiabetic drugs, while only 25.4% control diabetes by diet. Acarbose and voglibose are common α-glucosidase inhibitors used in the treatment of diabetes (Playford *et al.* 2013; Saito *et al.* 1998). However, they often cause severe gastrointestinal side effects. Therefore, an investigation of new α-glucosidase inhibitors from natural resources has become an attractive approach for the treatment of diabetes.

A wide variety of active components of plants has been identified that have effects on human health. From ancient times, plants have fulfilled our dietary requirements and have also been used for various medical purposes (Kim and Kwon, 2011). Medicinally active compounds, including carbohydrate digestive enzyme (α-glucosidase and sucrase) inhibitors, are widely distributed in plants (Mai *et al.* 2007). Some plants have been shown to contain α-glucosidase inhibitors (Wu *et al.* 2012; Alagesan *et al.* 2012; Chu *et al.* 2014). In this study, the leaves of Japanese plant species were examined for α-glucosidase inhibitory activity. There have been no previous reports regarding such components of these plant species. Chowdhury *et al.* (2009) reported that among plant parts, the leaves are used most commonly against diseases (37%). Chowdhury *et al.* (2009) also mentioned that most researchers use the leaves of plants as test materials to screen for active components. Halim *et al.* (2007) suggested that the common use of leaves was due to the ease of harvesting from plants. The present study was designed to investigate the antidiabetic effects of different extracts of the leaves of nineteen Japanese woody plant species on yeast α-glucosidase (maltase) and rat intestinal sucrase.

II. Materials and Methods

Plant collection and extraction: The leaves of the nineteen plants species (scientific and family names along with extract yields are listed in Table 01) were collected from Kochi University, Monobe campus, Japan.

Yeast α-glucosidase inhibitory assay: The inhibitory activity of yeast α-glucosidase (Wako Pure Chemical Industries, Ltd. Osaka, Japan) was determined by the method of Babu *et al.* (2004) with slightly modifications. Aliquots of 20 µl of the test samples dissolved in methanol at 10 mg/ml were serially diluted in 96-well microtiter plates. The plant extracts diluted in10 µl of phosphate buffer 0.1 mol/l (pH 6.8) and 150 µl (5 mmol/ml) of *p*-nitrophenyl-α-D-glucopyranoside (PNPG) were added to each well. The reaction was started by addition of 20 µl (5 µg/ml) of the enzyme to the reaction mixture in the 96-well plates. Individual blanks where the substrate was replaced with 40 µl of phosphate buffer were prepared to correct for background absorbance. The controls contained 20 µl of phosphate buffer in place of the test sample, while the leaf extract was replaced with acarbose in positive controls. All determinations were performed in triplicate. The changes in absorbance at 405 nm (A_{405}) were recorded at 1-minute intervals for 10 minutes, and the percentage inhibition was estimated from the slope using the following equation:

$$\text{Inhibition \%} = (1 - \text{slope of test sample}/\text{slope of control}) \times 100.$$

The concentration that gave the half-maximal response (IC_{50}) was determined from the sample concentration vs. percentage inhibition rate. α-Glucosidase inhibitory activity was expressed as inhibition % and IC_{50} value, with a lower IC_{50} value was indicating higher inhibitory activity.

Rat intestinal α-glucosidase (sucrase) inhibitory assay: Rat intestinal α-glucosidase inhibitory activity was determined by a modification of the method of Babu *et al.* (2004). Aliquots of 20 µl of plant extract test samples dissolved in methanol at 10 mg/ml were added to each well of 96-well plates. Then,

150 µl of 5 mg/ml saccharose was added to each well. Rat intestinal acetone powder (Sigma-Aldrich, St. Louis, MO) was added at 100 mg/ml to phosphate buffer 0.1 mol/l (pH 6.8) and sonicated for 5 minutes. The suspension was centrifuged at 2500 rpm for 5 minutes to remove particulate matter, and the resulting supernatant was used as the enzyme solution. The reaction was initiated by addition of 30 µl of the enzyme to the reaction mixture in 96-well plates. The reaction mixture was incubated at 37ºC for 30 minutes followed by 70ºC for 3 minutes on a heating block to stop the reaction. The reaction mixture was cooled to room temperature for 10 minutes and aliquots of 20 µl were transferred to another plate. The reaction was started by addition of 150 µl of reagent (Glucose C2; Wako) and cooled to 25ºC for 15 minutes. Individual blanks where the substrate was replaced with 50 µl of phosphate buffer were prepared to correct for background absorbance. The controls contained 20 µl of phosphate buffer in place of the test sample, while the leaf extract was replaced with acarbose in positive controls. All determinations were performed in triplicate. The changes in absorbance at 492 nm (A_{492}) were recorded at 1-minute. The percentage inhibition was estimated using the following equation:

Inhibition % = (1 - (absorbance of test sample-absorbance of blank)/ (absorbance of control-absorbance of blank)) ×100.

III. Results and Discussion

The methanol extracts of nineteen plants were investigated for their α-glucosidase activities. Extract concentrations of 200 µg/ml and 50 µg/ml were used for preliminary investigations (Table 02). Five plants (*E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum*) showed high levels of inhibitory activity against α-glucosidase (Table 2.2). Therefore, these five plants were analyzed comparatively with regard to their α-glucosidase inhibitory activities. The methanol extracts of the five plants showed significant in *vitro* α-glucosidase inhibitory activity compared with acarbose (13 mg/ml). Among the five plants, *Q. phillyraeoides* and *E. sylvestris* var. *ellipticus* showed the same inhibitory effects (IC_{50} = 22 µg/ml and 22 µg/ml, respectively), followed by *S. sebiferum* (IC_{50} = 42 µg/ml), *M. japonicus* (IC_{50} = 52 µg/ml), and *M. rubra* (IC_{50} = 92 µg/ml) (Table 03).

The hexane and water fractions of *Q. phillyraeoides* leaves had little activity, while ethyl acetate and butanol fractions showed stronger α-glucosidase inhibitory activities. The *Q. phillyraeoides* ethyl acetate fraction showed a strong inhibitory effect with an IC_{50} value of 4 µg/ml. The hexane and water fractions of *M. japonicus* leaves showed no inhibitory activity against α-glucosidase (Table 2.4). The ethyl acetate and butanol fractions of *M. japonicus* leaves showed significant inhibitory activities, with IC_{50} values of 10 µg/ml and 25 µg/ml, respectively. The hexane and water fractions of *S. sebiferum* leaves were also less active than the ethyl acetate and butanol fractions, the latter of which had IC_{50} values of 14 µg/ml and 16 µg/ml, respectively (Table 2.4). The hexane fraction of *E. sylvestris* var. *ellipticus* leaves was inactive, while the ethyl acetate, butanol, and water fractions showed α-glucosidase inhibitory activities, with IC_{50} values of 6 µg/ml, 7 µg/ml, and 9 µg/ml, respectively. The hexane fraction of *M. rubra* leaves was inactive. The ethyl acetate fraction of *M. rubra* was less active than the butanol and water fractions, the latter of which had IC_{50} values of 15 µg/ml and 8 µg/ml, respectively (Table 04).

In this study, the inhibitory effects of leaf extracts from the five plant species against sucrase activity were determined and compared with that of acarbose (91%, Supplemental Data). Methanol extracts of *E. sylvestris* var. *ellipticus*, *M. japonicus*, *M. rubra*, *Q. phillyraeoides*, and *S. sebiferum* leaves at 1 mg/ml in the reaction mixtures showed strong inhibitory effects against sucrase activity (Supplemental Data, Table 5), with that of *Q. phillyraeoides* showing the greatest effect (90%).

The hexane and water extracts of five plant species showed weak inhibitory effects on rat intestinal sucrase activity (Supplemental Data). The ethyl acetate and butanol extracts of *Q. phillyraeoides* were shown to inhibit the enzyme activity by 63% and 57%, respectively (Supplemental Data). However, the hexane and water extracts of *Q. phillyraeoides* leaves showed low levels of inhibition on sucrase activity, while acarbose (positive control) showed 91% inhibition of the enzyme activity at a concentration of 1 mg/ml (Supplemental Data, Table 05).

The ethyl acetate and butanol extracts of *M. japonicus* leaves showed the greatest inhibitory effects (57% and 67%, respectively), and the weakest effects were observed for hexane and water extracts of *M.*

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japonicus (35% and 50%, respectively). The ethyl acetate and butanol extracts of *E. sylvestris* var. *ellipticus* leaves showed the greatest inhibitory effects (70% and 62%, respectively) against sucrase activity. The butanol extract of *M. rubra* leaves showed an inhibitory effect of 48%, while the hexane, ethyl acetate, and water extracts showed lower inhibitory activities of 30%, 41%, and 28%, respectively. The hexane, ethyl acetate, butanol, and water extracts of *S. sebiferum* showed high inhibitory activities of 51%, 63%, 59%, and 56%, respectively (Supplemental Data, Table 05).

Table 01. Efficiency of methanol extraction from the leaves of nineteen Japanese plants

Scientific Name	Family Name	Leaf weight (g)	Quantity of methanol extract (g)	Extract yield (%, w/w)
Robinia pseudoacacia	Fabaceae	10	2.44	24.4
Morus bombycis	Moraceae	10	1.68	16.8
Rubus hirsutus	Rosaceae	20	4.22	21.1
Broussonetia kazinoki	Moraceae	20	4.76	23.8
Celtis sinensis var. *japonica*	Cannabaceae	15	2.46	16.4
Sapium sebiferum	Euphorbiaceae	5	1.86	37.2
Prunus jamasakura	Rosaceae	20	3.39	16.95
Prunus × *yedoensis*	Rosaceae	10	2.13	21.3
Cinnamomum camphora	Lauraceae	15	2.57	17.1
Elaeocarpus sylvestris var. *ellipticus*	Elaeocarpaceae	30	6.72	22.4
Zelkova serrata	Ulmaceae	10	1.83	18.3
Melia azedarach var. *subtripinnata*	Meliaceae	20	4.62	23.1
Mallotus japonicus	Euphorbiaceae	15	4.84	32.3
Hedera rhombea	Araliaceae	10	2.37	23.7
Quercus phillyraeoides	Fagaceae	20	3.60	36
Lonicera japonica	Caprifoliaceae	15	1.92	12.8
Myrica rubra	Myricaceae	20	2.97	14.9
Aphananthe aspera	Cannabaceae	10	1.76	17.6
Hibiscus syriacus	Malvaceae	10	2.20	22

Percentage extract yield (w/w) was calculated as (dry extract weight/dry starting material weight) × 10

Table 02. Effects of methanol extracts of the leaves of nineteen plants against yeast α-glucosidas

Scientific Name	Inhibition %		Scientific name	Inhibition %	
	200 µg/ml (%)	50 µg/ml (%)		200 µg/ml (%)	50 µg/ml (%)
Robinia pseudoacacia	93 ± 1	15 ± 3	*Hedera rhombea*	6 ± 12	7 ± 1
Morus bombycis	18 ± 2	1 ± 2	*Quercus phillyraeoides*	99 ± 1	91 ± 2
Rubus hirsutus	85 ± 1	20 ± 3	*Lonicera japonica*	7 ± 9	3 ± 9
Broussonetia kazinoki	5 ± 8	1 ± 8	*Myrica rubra*	82 ± 0	27 ± 0
Celtis sinensis var. *japonica*	75 ± 4	4 ± 8	*Aphananthe aspera*	30 ± 7	0 ± 1
Sapium sebiferum	62 ± 1	53 ± 3	*Hibiscus syriacus*	21 ± 2	0 ± 1
Prunus jamasakura	12 ± 2	6 ± 9	*Zelkova serrata*	95 ± 6	11 ± 2
Prunus × yedoensis	17 ± 8	11 ± 2	*Melia azedarach* var. *subtripinnata*	18 ± 2	1 ± 9
Cinnamomum camphora	79 ± 2	6 ± 3	*Mallotus japonicus*	86 ± 1	48 ± 2
Elaeocarpus sylvestris var. *ellipticus*	65 ± 2	60 ± 5			

Table 03. Inhibitory effects of fractionated extracts of five medicinal plants on α-glucosidase activity

Plant Name	IC_{50} µg/ml (Methanol extract)
Quercus phillyraeoides	22
Mallotus japonicus	52
Sapium sebiferum	42
Elaeocarpus sylvestris var. *ellipticus*	22
Myrica rubra	92
Acarbose (positive control)	13 mg/ml

Acarbose was used as a positive control; IC_{50}: Concentration of the antagonist that inhibited the enzyme activity by 50%.

Table 04. Inhibitory effects of fractionated extracts of five medicinal plants on yeast α-glucosidase activity

Plant Name	IC_{50} µg/ml			
	Hexane extract	Ethyl acetate extract	Butanol extract	Water extract
Quercus phillyraeoides	86	4	6	83
Mallotus japonicus	NA	10	25	NA
Sapium sebiferum	65	14	16	135
Elaeocarpus sylvestris var. *ellipticus*	NA	6	7	9
Myrica rubra	NA	84	15	8
Acarbose (positive control)	13 mg/ml			

Acarbose was used as a positive control; IC_{50}: Concentration of the antagonist that inhibited the enzyme activity by 50%; NA: No activity.

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IV. Supplemental data of the study

Table 05. Inhibitory effects of extracts of the leaves of five plants on rat intestinal sucrase and yeast α-glucosidase

Plant Name	Extract type	% of rat intestinal sucrase inhibitory effect	% of yeast α-glucosidase inhibitory effect
		1mg/ml	200 µg/ml
Quercus phillyraeoides	MeOH	90 ± 4	99 ± 0.6
	Hexane	43 ± 21	69 ± 6
	EtOAc	63 ± 6	100 ± 0
	BuOH	57 ± 18	99 ± 0
	Water	41 ± 7	81 ± 8
Mallotus japonicus	MeOH	73 ± 9	86 ± 2
	Hexane	35 ± 2	20 ± 3
	EtOAc	57 ± 15	99 ± 0
	BuOH	67 ± 11	86 ± 1
	Water	50 ± 5	40 ± 1
Elaeocarpus sylvestris var. *ellipticus*	MeOH	77 ± 1	65 ± 3
	Hexane	13 ± 5	12 ± 3
	EtOAc	70 ± 5	91 ± 2
	BuOH	62 ± 2	83 ± 6
	Water	27 ± 10	94 ± 2
Myrica rubra	MeOH	76 ± 0	82 ± 0
	Hexane	30 ± 6	28 ± 6
	EtOAc	41 ± 8	73 ± 2
	BuOH	48 ± 4	94 ± 1
	Water	28 ± 9	99 ± 0
Sapium sebiferum	MeOH	69 ± 8	62 ± 4
	Hexane	51 ± 18	81 ± 0.4
	EtOAc	63 ± 17	92 ± 1
	BuOH	76 ± 6	99 ± 2
	Water	56 ± 17	58 ± 1
Acarbose	91 ± 3		5 ± 4

In this study, we used acarbose as a positive control for both yeast α-glucosidase and rat intestinal α-glucosidase. Acarbose did not show a stronger inhibitory effect against yeast α-glucosidase, whereas it showed the greatest inhibitory effect against rat intestinal α-glucosidase. The differences in the inhibitory activities against these two enzymes may have been due to their structural differences (Chiba, 1997). It was confirmed that the experimental protocols were identical because acarbose was used as a positive control for both enzymes. It is important to note that acarbose has been used clinically to treat diabetes mellitus. Therefore, several groups have used acarbose as a positive control to identify or screen for suitable natural antidiabetic compounds (Zhang *et al.* 2014; Ghadyale *et al.* 2011; Wu *et al.* 2012).

Although acarbose has been clinically approved for use as an antidiabetic medication, Shai *et al.* (2010) reported that acarbose showed a weak inhibitory effect against yeast α-glucosidase. This result was compatible with the present study, in which acarbose showed only a weak inhibitory effect against yeast α-glucosidase with an IC_{50} value of 13 mg/ml, while the five plant extracts showed strong inhibitory effects against the activity of this enzyme (Table 04).

The methanol, ethyl acetate, and butanol extracts of the leaves from the five plant species, *Q. phillyraeoides*, *M. japonicus*, *S. sebiferum*, *E. sylvestris* var. *ellipticus*, and *M. rubra*, showed stronger inhibitory effects against yeast α-glucosidase than rat intestinal sucrase (Supplemental Data, Table 05). At 200 µg/ml, methanol, ethyl acetate, and butanol extracts of *Q. phillyraeoides* inhibited yeast α-

glucosidase activity by 99%, 100%, and 99%, respectively, while at 1 mg/ml, the inhibitory effects of these extracts on rat intestinal sucrase were lower at 90%, 63%, and 57%, respectively. This result was similar by those of Babu *et al*. (2004), who reported that methanol extracts of various plants had greater inhibitory effects against yeast α-glucosidase than mammalian α-glucosidase.

The ethyl acetate and butanol fractions of *Q*. *phillyraeoides* had IC_{50} values for yeast α-glucosidase of 4 µg/ml and 6 µg/ml, respectively (Table 04). Dewi *et al*. (2007) reported that the ethyl acetate extract of *koji Aspergillus terreus* exhibited a strong inhibitory effect against α-glucosidase with an IC_{50} value of 8.6 µg/ml. These findings indicated that α-glucosidase inhibitory activity of *Q*. *phillyraeoides* was stronger than that of *koji Aspergillus terreus*, which is known as a potent α-glucosidase inhibitor.

These results of the present study indicated that the methanol extract of *Q*. *phillyraeoides* leaves showed the strongest inhibitory effect against rat intestinal sucrase activity (91%), followed by those of *E*. *sylvestris* var. *ellipticus* (77%), *M*. *rubra* (76%), *M*. *japonicus* (73%), and *S*. *sebiferum* (69%) (Supplemental Data, Table 5). On the other hand, Kajaria *et al*. (2013) reported that ethanolic extract of *Shirishadi* showed a strong inhibitory effect of 45% (1 mg/ml) against α-glucosidase. The results suggested that α-glucosidase inhibitory activity of *Q*. *phillyraeoides* was higher than that of ethanolic extract of *Shirishadi*, which has been identified as a potent α-glucosidase inhibitor.

IV. Conclusion

The results of the present study indicated that different extracts of five plants (*E*. *sylvestris* var. *ellipticus*, *M*. *japonicus*, *M*. *rubra*, *Q*. *phillyraeoides*, and *S*. *sebiferum*) showed strong inhibitory effects against yeast α-glucosidase activities. *Q*. *phillyraeoides* (ethyl acetate) leaf extract showed the greatest inhibitory effect against yeast α-glucosidase. Methanol, ethyl acetate, and butanol extracts of *E*. *sylvestris* var. *ellipticus*, *M*. *japonicus*, *M*. *rubra*, *Q*. *phillyraeoides*, and *S*. *sebiferum* showed strong inhibitory effects against sucrase activity. The leaves of these five plant species should be investigated further to purify and identify the specific compound(s) responsible for the observed potential antidiabetic activities.

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