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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

氏名：
Name Anastasia Wheni Indrianingsih

学位論文題目：
Title of Dissertation

Studies on Isolation and Characterization of Antioxidant and α-Glucosidase Inhibitory Compounds from Woody Plants and Endophytic Fungi
(木本植物およびその内生菌からの抗酸化物質および α-グルコシダ―ゼ阻害物質の単離と特性評価に関する研究)

学位論文要約：
Dissertation Summary

Antioxidants are important since it protect the human body from free radicals and preventing diseases. Plants such as medicinal herbs, vegetables and fruits have been used as medicines since ancient time. Several studies demonstrated that medicinal herbs and plants are a rich source of antioxidant compounds such as phenolics, alkaloids and vitamins. Meanwhile, several synthetic drugs have been used for diabetes treatment since diabetes mellitus is the major health problem worldwide. However, several side effects such as drug resistane and weight gain had been observed. Some traditional medicines was demonstrated potential for diabetes treatment with less side effects, thus an effort to search natural antidiabetic medicines is important. The objective of this research was to search for antioxidant and α-glucosidase inhibitors from several woody plants. The screening of antioxidant and α-glucosidase inhibitors was conducted and selected plants having the highest antioxidant and α-glucosidase inhibitory activity was then studied further for the isolation and characterization of the antioxidant and α-glucosidase inhibitor compounds. Endophytic fungi from selected plants that having the highest antioxidant and α-glucosidase inhibitory activity were also studied.

The antioxidant activity of the methanolic extracts of the 14 woody plants was determined using several assays: DPPH radical scavenging activity, H_2O_2 radical scavenging activity, β-carotene

bleaching assay, and reducing power. The scavenging capacity of extracts ranged from 25.0 to >500 µg/mL. Quercetin as a standard exhibited an IC_{50} of 8.4 µg/mL. Among the plant extracts, the highest DPPH scavenging capacity was shown by the extracts of *Q. phillyraeoides* A. Gray and *Q. gilva* Blume with an IC_{50} of 25.0 and 38.5 µg/mL, respectively. The scavenging capacity of extracts (IC_{50}) on hydrogen peroxide ranged from 104.2 to >300 µg/mL. *Q. phillyraeoides* A. Gray exhibited the highest scavenging activity with 104.2 µg/mL, while gallic acid had a scavenging activity of 52.0 µg/mL. The reducing power of the plant extracts ranged from 18.4 to 99.9 µg/mL. Overall, the results indicate that the subtropical plant *Q. phillyraeoides* A. Gray had the strongest reducing power of the extracts investigated, with 99.9 mg/g (gallic acid equivalent). *Q. phillyraeoides* A. Gray, *Q. gilva* Blume and *M. japonicus* had the highest ability in protecting β-carotene bleaching compared with other extracts with an activity of 52.1%, 52.4%, and 51.2%, respectively. These results were higher than the ascorbic acid as standard that has antioxidant ability of 25.2%. *M. japonicus* and *Q. phillyraeoides* A. Gray had the highest α-glucosidase inhibitory activity with an IC_{50} of 8.4 and 9.8 µg/mL, respectively. The difference in these values was not statistically significant with quercetin as standard, which had an α-glucosidase inhibitory activity (IC_{50}) of 4.2 µg/mL. The results of the present work showed that *Q. gilva* Blume, *Q. phillyraeoides*, and *M. japonicus* are potentially rich sources of natural antioxidants and anti-diabetes medicine.

Quercus gilva Blume of the family Fagaceae is a tall evergreen tree distributed in the lowland mountain regions of Jeju Island in Korea. Moreover, *Q. gilva* Blume as an oak species in warm temperate regions also grows in Japan, in which it is mainly distributed in the southern part of the country. In the present study, the antioxidant and α-glucosidase inhibitor activities of isolated compounds from the leaves of *Q. gilva* Blume were tested. An *in vitro* assay of α-glucosidase

inhibitory activity was conducted using α-glucosidase enzyme from *S. cerevisiae* yeast while an *in vitro* antioxidant activity assay was conducted using several methods: DPPH free radical scavenging, β-carotene bleaching assay, H_2O_2 radical scavenging assay, and reducing power assay.

The methanol soluble fraction in the methanolic extract of *Q. gilva* Blume was fractionated using silica gel chromatography and followed by recrystallization to give compounds **1**, **2**, and **3** (Fig. 3.2). The ^{1}H NMR and ^{13}C NMR spectral data of all isolated compounds were compared with reported data, and their structures were identified as catechin (**1**), epicatechin (**2**), and tiliroside (**3**).

The scavenging capacity of isolated compounds in DPPH free radical scavenging activity ranged between 22.55 and 160.24 µM. Of the isolated compounds, the highest DPPH scavenging capacity was shown by compound **2**, followed by compounds **1** and **3** with IC_{50} of 22.55, 40.86, and 160.24 µM, respectively. Quercetin and gallic acid were used as positive controls in this experiment and exhibited an IC_{50} of 28.08 and 20.01 µM, respectively. Compound **2** exhibited the highest scavenging activity on hydrogen peroxide assay of 116.71 µM, while gallic acid as a standard had scavenging activity of 308.24 µM. Compounds **1** and **3** had scavenging activities of 122.41 and 119.95 µM, respectively. The reducing power of isolated compounds ranged between 41.60 and 61.47 µg/mL in gallic acid equivalents and between 78.77 and 98.96 µg/mL in ascorbic acid equivalents. Overall, the results indicate that compound **2** had the strongest reducing power among the isolated compounds investigated, with 61.47 mg/g (gallic acid equivalent) and 98.96 mg/g (ascorbic acid equivalent). The antioxidant activities of compounds **1** to **3** from *Q. gilva* at 40 µg/mL in the β-carotene-linoleate model system resulted in compound **1** having the highest ability in protecting β-carotene bleaching followed by compound **2** and compound **3**, which still retained antioxidant activities of 14.93%, 10.44%, and 1.49%, respectively, after 60 min of the assay. These results were higher than that for ascorbic acid as

the standard, which had an antioxidant ability of 1.47%. Compound **3** had the highest α-glucosidase inhibitory activity (IC_{50}) of 28.36 μM, followed by compounds **1** and **2** with 168.6 and 920.6 μM, respectively. These results for α-glucosidase inhibitory activity (IC_{50}) were consistent with previous findings on catechin and epicatechin in green tea. Compound **1** and compound **2** exhibited an uncompetitive type of inhibition (Fig. 3.5), as shown by the straight parallel lines in the plot of 1/V versus 1/[S]. The *Ki* (inhibition constant) values of compounds **1** and **2** were determined to be 129.03 and 215.05 μM, respectively (Table 3.2). Compound **3** showed non-competitive inhibition, which indicated that it bound to a site other than the active site of the α-glucosidase enzyme (Fig. 3.6a) with a *Ki* value of 91.64 μM. In conclusion, three compounds were isolated from the leaves of *Q. gilva* Blume. Of the isolated compounds, catechin (**1**) and epicatechin (**2**) showed potent antioxidant activities, while tiliroside (**3**) and catechin (**1**) showed potent α-glucosidase inhibitory activities.

Quercus phillyraeoides A. Gray (*Q. phillyraeoides*) of the family *Fagaceae* is an evergreen tree that is distributed in the limestone mountains and acid bed rocks of East Asia (Korea, China and Japan). The leaves of *Quercus* species have been used in Korean folk medicine for dysentery, diarrhea, hemorrhage, dermatitis, and the exclusion of extravasated blood. Previous phytochemical studies on *Q. phillyraeoides* led to the identification of several tannins from the leaves of *Q. phillyraeoides*. However, the bioactivities of *Q. phillyraeoides* have not yet been examined. The present study was conducted in order to evaluate the antidiabetic and antioxidant potentials of constituents isolated from *Q. phillyraeoides*. Antidiabetic properties were assessed in terms of the ability to inhibit an intestinal carbohydrate-digesting enzyme, namely α-glucosidase.

The methanol-soluble fraction in the methanolic extract of *Q. phillyraeoides* was fractionated using silica gel column chromatography and followed by recrystallization to give compound **4**, while

Sephadex LH-20 column chromatography led to the isolation of condensed tannin fractions **5** to **9.** Compound **4** was identified as β-sitosterol-D-glucoside. The acid hydrolysis of **4** resulted in β-sitosterol and D-glucose. Fractions **5** to **9**: light brown powders. The acid-butanol assay of fractions **5** to **9** resulted in red colored anthocyanidin, which indicated that the fractions were condensed tannins (Schofield et al., 2001). The retention time of fractions **5** to **9** on GPC were 6.81, 12.78, 15.36, 10.55, and 15.87 min, respectively, therefore the molecular weights of fractions **5** to **9** were 5700, 4200, 3500, 4700 and 3400 g/mol, respectively, which were estimated using polystyrene as the standard. The condensed tannin fractions (**5** to **9**) had high α-glucosidase IC_{50} values in the range of 2.60 to 3.14 μg/mL, and no significant difference was observed from that of quercetin as the standard ($P < 0.05$) with an IC_{50} value of 4.81 μg/mL. Compound **4** showed moderate inhibitory activity against α-glucosidase with an IC_{50} value of 118.8 μg/mL, which was significantly lower than those of quercetin and the other isolated fractions. The α-glucosidase inhibitory activities of fractions **5** to **9** were higher than that of compound **4**, and this may have been because they consisted of more hydroxyl groups. The inhibitory mechanisms of the isolated constituents were analyzed further using Lineweaver-Burk plots. The results obtained showed that all of the isolated constituents and quercetin as the standard exhibited a mixed type of inhibition.

The antioxidant activities of the six isolated constituents were determined using several assays: DPPH free radical scavenging activity, hydrogen peroxide radical scavenging activity, reducing power, and β-carotene-linoleate model assays. The scavenging capacities of the isolated constituents in the DPPH free radical scavenging activity assay ranged between 9.34 and > 500 μg/mL. Of the isolated constituents, the highest DPPH scavenging capacity was exhibited by fraction **8**, followed by fractions **6**, **5**, **9**, and **7** with IC_{50} values of 9.34, 10.53, 10.84, 12.98, and 13.12 μg/mL, respectively. Quercetin

and gallic acid were used as positive controls in this experiment and had IC_{50} values of 8.45 and 3.41 μg/mL, respectively. The weakest activity was observed for compound **4** with an IC_{50} value of > 500 μg/mL in the DPPH free radical scavenging activity assay, which was significantly lower than those of the standard and other isolated fractions. The scavenging abilities of the isolated constituents on hydrogen peroxide were compared with gallic acid as the standard. Fraction **9** exhibited the highest scavenging activity (IC_{50}) of 84.05 μg/mL, followed by fractions **7**, **6**, **8**, **5**, and compound **4** with IC_{50} values of 84.51, 86.67, 86.94, 89.28, and > 500 μg/mL, while gallic acid as the standard had scavenging activity of 52.36 μg/mL. Based on a statistical analysis, no significant differences were observed in the IC_{50} values of fractions **5** to **9**. The reducing powers of the isolated constituents ranged between 19.34 and 54.35 μg/mL in gallic acid equivalents and between 56.16 and 91.72 μg/mL in ascorbic acid equivalents (Fig. 4.5a). The results indicated that fractions **5** to **9** actively reduced Fe^{3+} to Fe^{2+} with values of 52.59, 54.35, 51.72, 53.47, and 54.05 mg/g (gallic acid equivalent) and 89.94, 91.72, 89.05, 90.83, and 91.42 mg/g (ascorbic acid equivalent), respectively, while compound **4** showed the lowest activity at 19.34 mg/g (gallic acid equivalent) and 56.16 mg/g (ascorbic acid equivalent). These potential reducing powers may have been due to the presence of the dihydroxy type of benzene derivatives, catechin and epicatechin, which are integral parts of condensed tannins. The antioxidant activities of compound **4** and fractions **5-9** from *Q. phillyraeoides* at 40 μg/mL in the β-carotene linoleate model system resulted in fraction **9** having the strongest ability to protect against β-carotene bleaching, followed by fractions **6**, **8**, **7**, **5**, and compound **4**, which still retained antioxidant activities of 20.01%, 25.78%, 22.10%, 18.42%, 14.73%, and 1.05%, respectively, after 120 min of the assay. The results obtained for fractions **5** to **9** were higher than that of gallic acid as the standard, which had an antioxidant ability of 6.32%. In conclusion, β-Sitosterol-D-glucoside (**4**) and five

condensed tannin fractions (**5, 6, 7, 8**, and **9**) were isolated from the leaves of *Q. phillyraeoides*. Of the isolated constituents, condensed tannin fractions (**5** to **9**) showed potent α-glucosidase inhibitory activities and antioxidant activities, while β-sitosterol-D-glucoside (**4**) showed moderate inhibitory activity against α-glucosidase.

Several research indicate endophytic fungi have bioactive compounds that could potentially be applied in various applications such as antioxidant, antifungal, antiviral, antibacterial and cyotoxic. In our previous study, we found that several bioactive compounds were isolated from *Quercus gilva* Blume and *Quercus phillyraeoides* A. Gray therefore in this study we conducted screening of 27 endophytic fungi from *Quercus gilva* Blume and *Quercus phillyraeoides* A. Gray. Endophytic fungi QGS 01 from *Q. gilva* was found to have a strong α-glucosidase inhibitory activity. This QGS 01 fungus was identified as a *Xylariaceae* sp. In this study, the isolation of α-glucosidase inhibitors from the ethyl acetate extract of this fungus was reported.

The ethyl acetate extract of QGS 01 fungus mycelium was fractionated using silica gel chromatography to give constituents **10** and **11**. Their spectral data were compared with reported data and their structure were identified as isocoumarin derivative (**10**) and fatty acid fraction (**11**). Compound **10** was identified as 8-hydroxy-6,7-dimethoxy-3-methylisocoumarine. Constituents **11** (fatty acid fraction) was isolated as major active constituents of *Xylariaceae* sp and was the most active constituents on α-glucosidase inhibitory activity. Analysis using GC-MS and comparison with authentic standards showed that constituents **11** had two saturated fatty acids, palmitic acid (C16:0) and stearic acid (C18:0) and three unsaturated fatty acids, namely oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3). The α-glucosidase activity of compound **10** and constituents **11** (IC_{50}) 41.75 and 5.95 μg/mL, respectively. The fatty acids constituent (**11**) consists of 33.7% of linoleic acid,

29.8% of oleic acid, 24.1% of palmitic acid, 7.5% of stearic acid and 3.6% of linolenic acid. These results indicated that fatty acids with a double bond showed stronger inhibition than fatty acids without double bond. It is suggested that the existence of a double bond was important factor of inhibition α-glucosidase acitivity. In conclusion, endophytic fungi *Xylariaceae* sp QGS 01 was isolated from the stem of *Quercus gilva* Blume and showed the highest α-glucosidase inhibitory activity. Two active constituents, 8-hydroxy-6,7-dimethoxy-3-methylisocoumarine (**10**) and fatty acid fraction (**11**), were isolated from endophytic fungi *Xylariaceae* sp QGS 01. The results of the present work showed that *Q. gilva* Blume, *Q. phillyraeoides* A. Gray and endophytic fungi *Xylariaceae* sp QGS 01 from *Q. gilva* Blume are potentially a rich source as antidiabetic medicine.

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