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

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学位論文題目：
Title of Dissertation

Isolation and Evaluation of Bioactive Compounds from Temperate Woody Plants and Endophytic Fungi
(温帯産木本植物およびその内生菌からの生理活性物質の単離と評価)

学位論文要約：
Dissertation Summary

Many natural bioresources in the world have not been evaluated for biological activities. Meanwhile, in treating many human diseases, there is increasing focus on products extracted from nature. Many medicinal products are derived from nature, so intense research on such products is essential and gaining much attention. Medicinal natural products can be isolated from plants and fungi including endophytic fungi. In addition, producing medicinal products can be enhanced using tissue culture techniques. Therefore, the objectives of this study were to isolate and evaluate biologically active compounds from temperate woody plants and endophytic fungi for potential use in medicines and to enhance the production of biologically active compounds using plant tissue cultures.

Screening of Potential Antioxidant and α-Glucosidase Inhibitor Plants

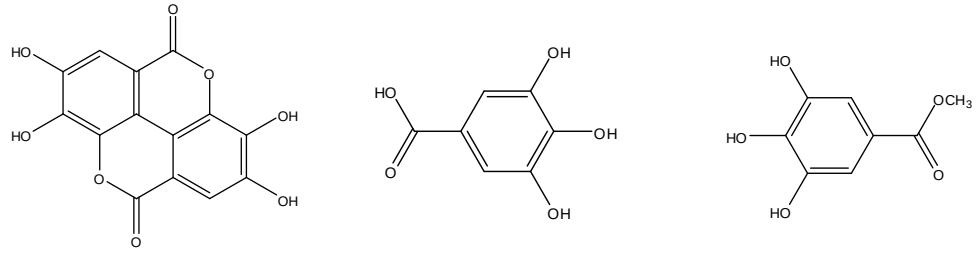
This study started with the screening of fourteen temperate woody plants (*Buxus microphylla, Osmanthus fragrans, Cedrus deodora, Pinus thunbergii, Xylosma congestum, Pinus palustris, Liquidambar styraciflua, Ternstroemia gymnanthera, Metasequioia glypteostrobides, Magnolia grandiflora, Elaeocarpus sylvestris, Acer mono Maxim, Distylum racemosum,* and *Cryptomeria japonica)* for their antioxidant and α-glucosidase inhibitory activities. Antioxidant activities were evaluated based on the 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay, reducing power assay, hydrogen peroxide assay, and β-carotene bleaching assay. The α-glucosidase inhibitory activity and total phenolic content of the extracts were also investigated. Of those fourteen plants, the *E. sylvestris* extract had the highest activities in the DPPH radical scavenging activity assay (IC_{50} 12.7±0.5 μg/mL), reducing power assay (491.1±6.3 mg quercetin equivalent/g dry extract), hydrogen peroxide assay (IC_{50} 65.6±0.4 μg/mL), and β-carotene bleaching assay (IC_{50} 5.1±1.9 μg/mL), and also the highest total phenolic content (353.8±28.6 mg GAE/g dry extract); 294.9±24.5 mg Q

equivalent/g dry extract; 663.0±52.3 mg R equivalent/g dry extract). On the other hand, the α-glucosidase inhibition assay revealed that *D. racemosum* had the highest activity with IC_{50} 22.6±1.9 μg/mL.

The strong activities of the *E. silvestris* extract in both the antioxidant and α-glucosidase inhibition assays can be considered to result from the contribution of its high phenolic content. This study also suggested the potential of *E. sylvestris*, *D. racemosum*, *A. mono Maxim*, and *L. styraciflua* as alternative sources of antioxidants and α-glucosidase inhibitors.

Isolation of Antioxidant Compounds from *E. sylvestris*

Antioxidant compounds were isolated from the leaves of *E. sylvestris*, which had the highest antioxidant potential of the screened plants. Three antioxidant active compounds (**1**–**3**) from the methanolic extract of *E. sylvestris* leaves were isolated using repeated column chromatography and preparative TLC and identified as ellagic acid, gallic acid, and methyl gallate (Figure 1). The evaluation of biological activities showed all three isolated compounds had good antioxidant activity in the scavenging DPPH radicals, reducing power, and β-carotene bleaching assays. However, the tetraacetate of ellagic acid showed no activity in the DPPH radicals scavenging assay indicating the absence of phenolic hydroxyl groups or the conversion of hydroxyl groups to the acetyl form decreased the free radical scavenging activity. Therefore, it was considered that the hydroxyl groups in the isolated compounds stabilized the DPPH radicals.



(**1**) (**2**) (**3**)

Figure 1. Chemical structure of the following isolated compounds: (**1**) ellagic acid; (**2**) gallic acid; (**3**) methyl gallate

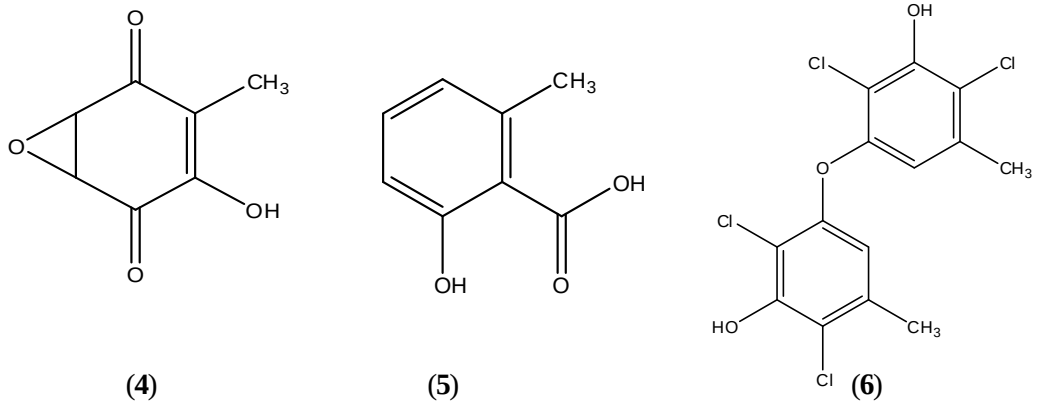
To gain further information on the isolated compounds, each with a gallic acid backbone, the relationship between the structures and antioxidant activity of the isolated compounds and fourteen benzoic acid derivatives was further analyzed. The purpose of the analysis was to investigate the importance of hydroxyl groups, the effect of the number of phenolic hydroxyl groups (mono-, di-, and trihydroxylbenzoic acid), the effect of the position of the phenolic hydroxyl groups (*ortho*, *meta*, and *para* positions), and the effect of

replacing the hydroxyl groups with methoxy groups (mono-, di-, and trimethoxybenzoic acid). The result showed that the phenolic hydroxyl groups are more important for antioxidant activity compared with the carboxyl group. Moreover, the number and position of the phenolic hydroxyl groups are important in the antioxidant activity, particularly in the *ortho* and *para* positions. Furthermore, the results also showed that the substitution of all the phenolic hydroxyl groups with methoxy groups (3,4,5-trimethoxybenzoic acid) resulted in no antioxidant activity.

Isolation of Antioxidant Compounds from Endophytic Fungi Isolated from *E. sylvestris*

In further study of *E. sylvestris*, we investigated the isolation of endophytic fungi from the plant and the isolation of antioxidant compounds from the potential endophyte. Seven endophytic fungi were isolated from the stem and leaves of the plant. DNA analysis identified the fungi as *Pestalotiopsis* sp. EST 01, *Pestalotiopsis* sp. EST 02, *Diaporthales* sp. EST 03, *Meyerozema* sp. EST 04, *Diaporthales* sp. EST 05, *Pestalotiopsis* sp. ESL 01, and *Pseudocercospora* sp. ESL 02. Of those seven fungi, the filtrate extract of *Pseudocercospora* sp. ESL 02 obtained from a shaking condition had the highest antioxidant activity (IC_{50} 30.54±0.88 µg/mL).

Three biologically active compounds from *Pseudocercospora* sp. ESL 02 were isolated and identified as terreic acid, 6-methylsalycilyc acid, and 3,3'-oxybis(2,6-dichloro-5-methylphenol) (Figure 2), with terreic acid having the strongest antioxidant activity with an IC_{50} in the DPPH radical scavenging assay of 0.22±0.02 mM. Notably, terreic acid is considered derived from 6-methylsalicylic acid, a bio-metabolism process from a weaker antioxidant active compound to a stronger antioxidant active compound. The results indicate the potential of *Pseudocercospora* sp. ESL 02 as a novel source of terreic acid. Moreover, the result also complements the study of antioxidant potency of *E. sylvestris* as the host plant of the fungus.



(**4**) (**5**) (**6**)

Figure 2. Chemical structure of the following isolated compounds: (**4**) terreic acid; (**5**) 6-methylsalicylic acid; (**6**) 3,3'-Oxybis(2,6-dichloro-5-methylphenol).

Isolation of Biologically Active Compounds from *D. racemosum*

The previous screening of potential antioxidant and α-glucosidase inhibitor plants resulted in *E. sylvestris* and *D. racemosum* having the most potential as source of antioxidant and α-glucosidase inhibitors, respectively. The result showed that *D. racemosum* not only had α-glucosidase inhibitory activity, but its methanol extract also had high antioxidant activity.

The isolation of bioactive compounds from *D. racemosum* resulted in two compounds (**3** and **7**) identified as methyl gallate and 2,4-dihydroxy-6-methoxyacetophenone. Methyl gallate showed very strong antioxidant activity (IC_{50} 0.03±0.01 mM) and no activity against the α-glucosidase enzyme, whereas compound **7** showed no antioxidant activity with a moderate α-glucosidase inhibitory activity (IC_{50} 0.88±0.01 mM) as shown in Table 1.

Table 1. DPPH radical scavenging activity and α-glucosidase inhibitory activity

Compound	IC_{50} on scavenging DPPH radical (mM)	IC_{50} on α-glucosidase inhibition (mM)
Quercetin	0.03±0.02	0.01±0.01
Methyl gallate	0.03±0.01	N.A.
2,4-dihydroxy-6-methoxyacetophenone	N.A.	0.88±0.01

N.A.= no activity

Production of Antioxidant Compounds from a Tissue Culture of *Artemisia annua*

A tissue culture of *Artemisia annua* was also investigated. Four combinations of plant hormones (NAA, BA, kinetin, and 2,4-dichlorophenoxyacetic acid) were used, with the combination of NAA 0.5 mg/L + BA 0.5 mg/L as treatment 1 showing the highest DPPH radical scavenging activity and total phenolic content. Moreover, the callus in treatment 1 also had potency as an α-glucosidase inhibitor. Caffeic acid, a well-known antioxidant compound, was detected in the callus extracts, particularly in treatments 1 and 2 (NAA 0.5 mg/L + kinetin 0.5 mg/L). Quantifying the caffeic acid content in the callus treatment showed that treatment 1 had the highest caffeic acid content. Furthermore, it is considered that caffeic acid plays a role in the antioxidant activity of the extract.

The caffeic acid content from the leaf extract of *A. annua* was also investigated. Caffeic acid was detected in the leaf extract, but the amount of caffeic acid and the bioactivity of leaf extract were lower than the

callus extracts, particularly in treatment 1. Therefore, compared with the leaf extract, treatment 1 provided the best plant hormone combination studied, increasing both the biological activities and caffeic acid content.

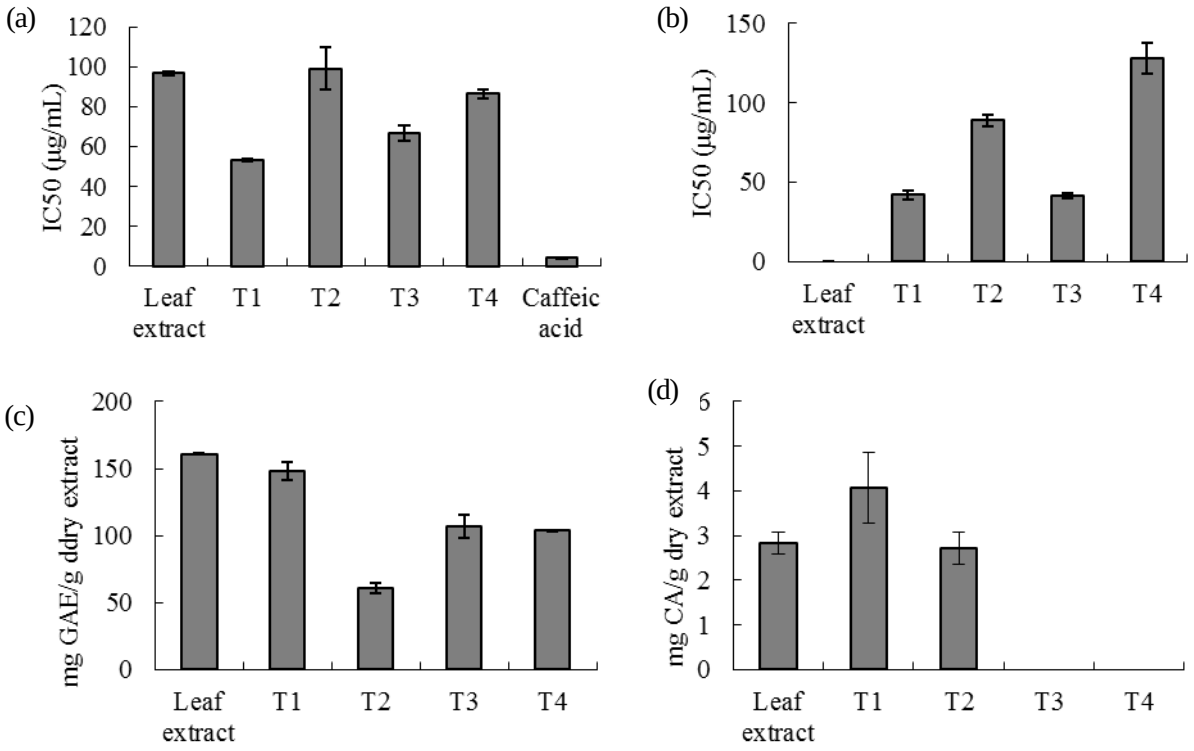


Figure 3. Comparison between leaf extract and callus extracts for DPPH radical scavenging activity (a), α-glucosidase inhibitory activity (b), total phenolic content (c), and quantification of caffeic acid content (d)

Conclusion

A comprehensive study of bioactive compounds was conducted, particularly from *E. sylvestris*. Three antioxidant compounds were isolated from the leaf extract, whereas seven endophytic fungi were isolated from the leaves and stems, and three compounds were isolated from *Pseudocercospora* sp. ESL 02, an endophytic fungus from the plant that is potential of antioxidant. Furthermore, bioactive compounds were also isolated and studied from *D. racemosum*, a screened plant having high antioxidant and α-glucosidase inhibitory activities. This study also investigated the production of a bioactive compound, caffeic acid, from a tissue culture of *A. annua*, with the best combination of plant hormones being NAA 0.5 mg/L + BA 0.5 mg/L, which increased both the biological activities and caffeic acid content.

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