Studies on the Radical Scavenging Ability and Antimicrobial Activity of Ferulic Acid and Glycoside

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ABSTRACT

The objective was to prepare highly water soluble ferulic acid without losing the antioxidant activity and anti-microbial activity possessed by naturally available ferulic acid. Evaluation of antioxidant activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, and evaluation of antimicrobial activity by paper disk method were carried out by using ferulic acid analogues to study the structure-activity relationship. Based on the findings, highly water soluble saccharide was introduced into the carboxyl group of ferulic acid. The solubility was estimated by q NMR measurement. The result indicated that glycoside 11 was about thirteen times more soluble than ferulic acid 1 in water.

Keyword: Glycoside; radical scavenging; antimicrobial activity; glycoside

INTRODUCTION

Ferulic acid is a natural organic compound found in major components of human and animal diets like rice and vegetables. Ferulic acids have gained attention for its potential role in several free radical-induced therapy because of its ability to scavenge radicals and activate cell stress response. Recently, consecutive evidences have revealed that the bioactivity of ferulic acid could be attributable to suppression of cell cycle progression related to colon cancer cells [1]. In vivo evaluation, ferulic acid demonstrates to counteract lipid peroxidation in the organ of the guinea pigs [2]. Further states that alkyl esters of ferulic acid inhibited growth of gram positive bacteria [3]. However, attempts of unraveling the bioavailability of ferulic acid upon ingestion or oral
administration are still ongoing due to its limitation of sparing water-soluble.
The aim of this study is to produce highly water soluble ferulic acid molecules without affecting its bioactivity effects. To allow this modification, structure activity relationships of ferulic acid was investigated using DPPH assay and paper disk method. The DPPH assay was used to evaluate the free radical scavenging activity [4]. The paper disk method was used to determine antimicrobial activity. Then, based on the results, the moderate kinetic solubility of ferulic acid was developed by introducing monosaccharide into the exact functional group. The quantitative analysis of aqueous solubility of ferulic acid and its synthesized glycoside was examined by powerful method, $^1$H qNMR analysis.

**MATERIALS & METHODS**

**Determination of free radical – Scavenging ability with DPPH test**
The free radical-scavenging capacity of the ferulic acid and its glycoside were determined by using DPPH according to the previously described procedure [4]. Antioxidant solution to be tested was added in a final concentration of 1 – 60 μM to the reaction mixture. Absorbance of the DPPH-solution (0.45 mg/L in ethanol) was recorded at 517 nm using a spectrometer (JASCO V-630).

**Determination of antimicrobial activity assay**

**In vitro antimicrobial activity**
All cultures were stored as frozen stock. For the antimicrobial activity determinations, a broth microdilution method was used to quantitate antibacterial activity for these compounds. The antibacterial activity was determined by agar dilution assay using a multipoint inoculator. The test compounds were dissolved and incorporated by the twofold dilution method in the agar medium. Bacterial inocula, coming from overnight broth and containing $10^7$ colony-forming units per point, were inoculated by multipoint inoculator. Bacterial growth was observed after 24 h, of incubation at 37°C. The growth-inhibitory ring of tested compounds was observed [5]. The antimicrobial activity was evaluated by measuring diameter of the inhibitory ring.

**Experimental Section**

**General methods**
$^1$H and $^{13}$C NMR spectra were recorded, respectively, at 400 and 100 MHz (Bruker AVANCE400). Chemical shifts are reported in ppm relative to Me$_4$Si and CDCl$_3$ with CHCl$_3$ as the internal reference (7.26 ppm for $^1$H NMR and 77.0 ppm for $^{13}$C NMR). Coupling constants are reported in hertz (Hz) and determined directly from $^1$H NMR spectra. Spectral splitting patterns were designated as s (singlet), d (doublet), t (triplet), m (multiplet), and br (broad). Mass spectra were obtained on JEOL JMS-700T or JEOL JMS-AX500 spectrometers. Infrared spectra were taken on a Jasco A-100 spectrometer or a Hitachi 270-30 spectrophotometer. Elemental analyses were carried out at Analytical Center of Osaka City University.

All air- and moisture-sensitive reactions were carried out in flame-dried, argon-flushed, two-necked flask sealed with rubber septum, and dry solvents and reagents were introduced with a syringe. THF was freshly distilled from sodium benzophenone ketyl. CH$_2$Cl$_2$ was distilled from P$_2$O$_5$ and stored over 4-Å molecular sieves. Pyridine was dried over KOH and stored over 4-Å molecular sieves. TLC was performed on Merck precoated silica gel (#5715) or RP-18 plates (#15685), and the TLC spots were visualized under 254-nm UV light and/or by charring after dropping the plate into vanillin solution in 5% sulfuric acid/methanol. Purification of products was accomplished via Merck silica gel (#7734 and #9385) flash column chromatography, or ODS (Wakogel® 50C18) column chromatography for compounds 11.
Hexane and ethyl acetate were distilled and used for column chromatography.

RESULTS AND DISCUSSION

Investigation of structure-activity relationship

To covalently link monosaccharide, glucose with ferulic acid without attenuation of antioxidant and antimicrobial activities, structure-activity relationships (SAR) was investigated. The SAR represents an important procedure for determining which functional groups are responsible for endowing with bioactivity effects. The analysis of SAR involves the classic methods of investigating the antioxidant and antimicrobial activities of ferulic acid and its four analogue (Figure 1).

![Fig. 1 Ferulic acid and its analogues](image)

Ferulic acid 1; \( R_1 = \text{OCH}_3, R_2 = \text{H}, R_3 = \text{OH} \)
Ethyl ferulate 2; \( R_1 = \text{OCH}_3, R_2 = \text{CH}_2\text{CH}_3, R_3 = \text{OH} \)
Caffeic acid 3; \( R_1 = \text{OH}, R_2 = \text{H}, R_3 = \text{OH} \)
p-Coumaric acid 4; \( R_1 = \text{H}, R_2 = \text{H}, R_3 = \text{OH} \)
Cinnamic acid 5; \( R_1 = \text{H}, R_2 = \text{H}, R_3 = \text{H} \)

Antioxidant activity of ferulic acid and its analogue were evaluated by using the DPPH method. The reaction was performed in ethanol containing prepared 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH). The reaction mixtures were protected from light and incubated for 90 min at room temperature, after which the absorbance of the remaining DPPH was calorimetrically determined at 517 nm. The scavenging activities of formulated compounds were expressed as percentage of decrease in absorbance against absorbance of control.

Paper disk method was used for the evaluation of antimicrobial activity. A paper disk impregnated with the sample was placed on a Petri dish inoculated with the microorganism under test and kept for 24 hours. Then the antimicrobial activity was evaluated from the size of the inhibiting ring occurring along the periphery of the disk. Bacillus cereus, which causes food poisoning, was used as the test microorganism.

Antioxidant activity assay

The radical scavenging rate of the evaluated compounds was computed from equation 1 (Figure 2).

### Table 1: Radical scavenging rate of ferulic acid and its analogues

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration (mM)</th>
<th>0.5</th>
<th>1</th>
<th>3</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
<td>mM</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>77.8</td>
<td>83.1</td>
<td>84.1</td>
<td>85.4</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>70.8</td>
<td>77.0</td>
<td>85.7</td>
<td>86.0</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>81.6</td>
<td>82.5</td>
<td>83.8</td>
<td>84.5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>12.4</td>
<td>17.0</td>
<td>35.0</td>
<td>42.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
DPPH radical scavenging activity (%) = \( \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{control}}} \times 100 \) (Equation 1), where \( \text{Abs}_{\text{control}} \) is the absorbance of DPPH radical in ethanol, \( \text{Abs}_{\text{sample}} \) is the absorbance of DPPH radical solution mixed with sample. All determination were performed in triplicate (n = 3) and evaluated by JASCO V-630 spectrophotometer.

From the measurement results, ferulic acid 1, ethylferulate 2 and caffeic acid 3 demonstrated good radical scavenging rate of approximately 80% while p-coumaric acid 4 showed a comparatively low radical scavenging rate. Ferulic acid 1 and its ester, ethylferulate 2 were supposed to have lower radical scavenging rate compared to caffeic acid 3 whereas not much difference was observed in this assay [4]. The dissimilar result is presumably due to relatively high concentration of tested compounds as at 0.5 mM, this result is found to be congruent to that reported in the literature. Figure 2 is also shown that a complete absence of antiradical capability of cinnamic acid 5. Therefore, presence of phenolic hydroxyl group is essential for exhibiting radical scavenging activity. Further, unlike ferulic acid 1 and caffeic acid 3, p-coumaric acid 4 which has no linkage of electronegative contributing functional group in meta position showed a poor ability to scavenge radical. It can be therefore concluded that having electron donating substituent at meta position results in a significant role in antiradical capability. This result is in accordance to the finding provided by Gasper et al. revealing that presence of electron donating substituent stabilizes the phenoxy radical and increases the efficiency of scavenging radical [6]. Radical scavenging rate of ethylferulic acid 2 at 3-5 mM concentration was almost equal to that of ferulic acid 1, which indicates that contribution of carboxylic group to radical scavenging ability is trivial. The present findings highlight that introducing glucose to carboxylic group of ferulic acid would be the best molecular design of glycoside and prefect modification to enhance the aqueous solubility of ferulic acid 1.
Antimicrobial activity assay

Table 2: Mean of inhibition growth diameter obtained by paper disk method using different concentration of different natural product against B. Cereus

<table>
<thead>
<tr>
<th>No.</th>
<th>Natural product</th>
<th>Concentration (µg/disk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1000</td>
</tr>
<tr>
<td>1</td>
<td>Ferulic acid</td>
<td>SI</td>
</tr>
<tr>
<td>2</td>
<td>Ethyl ferulate</td>
<td>11.00</td>
</tr>
<tr>
<td>4</td>
<td>p-Coumaric acid</td>
<td>8.79</td>
</tr>
<tr>
<td>5</td>
<td>Cinnamic acid</td>
<td>NI</td>
</tr>
</tbody>
</table>

*Values shown are mean of triplicate (n=3) and represent inhibition ring in millimetres (mm), including disc diameter of 5 mm, NI=No Inhibition; SI=Slight Inhibition.

Table 2 details the antimicrobial activity test for ferulic acid 1 and its analogue in case of B. cereus. A clear inhibition ring was detected for ethyl ferulate 2, which implied that lengthening side chain of ester activates antimicrobial activity. This result is in agreement with those reported by Taniguchi et al., that longer the side-chain of phenol, stronger the antimicrobial activity and by Ishihara et al., that ferulic acid glycoside (feruloyl oligosaccharide ester) extracted from pineapple shows strong antimicrobial effect against E. Coli [7-8]. Hence, these consequence evidences confirm the suggestion of introducing glucose to the carboxylic group of ferulic acid 1 would not affect the bioactivity. Ferulic acid 1 and p-coumaric acid 4 showed slight inhibition of microbial growth and diameter of the inhibition ring could not be determined due to the ambiguous inhibition ring and therefore, presence of its activity was indicated as slight inhibition (SI). For caffeic acid 3, the color of disk circumference changed to red, however the causing factor is not clear. Coumaric acid 5 did not show any antimicrobial activity and this outcome established that phenoxy group is a prerequisite for inhibition of microorganisms growth. To sum up, phenoxy group and electronegative contributing functional group at meta position are essential for evoking the bioactivity effects and the adjacent double bond is responsible for electron delocalization from phenoxy ring and augments the biological activity of radical scavenging activity. In contrast, the carboxylic group of ferulic acid 1 shows no mutual interaction with both antiradical nor antimicrobial activities because of the slight difference in rate of radical scavenging ability between ferulic acid 1 and its ester analogue, ethylferulate 2 and promising antimicrobial activity of ethylferulate 2 in vivo experiment. The evidence from Antioxidant activity assay and Antimicrobial activity assay intimates that glucose is best to be linked to carboxylic group of ferulic acid 1.

**Synthesis of ferulic acid glycoside**

As mentioned above, in order to design a highly water soluble molecule of ferulic acid 1, the simplest source of energy in our body, glucose was selected as precursor and decided to be introduced to carboxylic group of ferulic acid 1. The formation of glycoside of ferulic acid was prepared from the coupling reaction between compound 8 and 9. So far, the synthesis of glycoside was achieved by the bonding between acid chloride of ferulic acid 9 and acetyl protected of glucose [8]. Yet, the product yield of compound 10 was low. We have, therefore, studied an improved method of synthesis of glycoside by converting the anomeric position of glucose into highly reactive hydroxyl group followed by esterification with 9 [9].
First, reductive amination of anomeric position was carried out by using N,O-dimethylhydroxylamine hydrochloride and then accompanied by acetylation of the remaining hydroxyl groups to obtain glucose protector 7. The anomeric protected group was selectively cleaved by using N-chlorosuccinimide and it was converted into hydroxyl group by the hydrolysis of water, to achieve glucose protector 8. Finally, highly reactive acid chloride 9, which synthesized from acetyl protected ferulic acid was converted into glucose protected compound under the existence of pyridine as the catalyst and subsequent deprotection with lithium hydroxide to deliver ferulic acid glycoside 11.

**Evaluation of water solubility**

To date, Shake-flask method, GC analysis and cryoscopy are the methods use in acquiring solubility information of drug in various field. Currently, evaluation method using $^1$H qNMR spectrum (Quantitative Nuclear Magnetic Resonance; Q-NMR) was reported [10]. As most $^1$H NMR spectra are traced and detected in deuterated solvents with purpose of avoiding domination of solvent signal, deuterium oxide (D$_2$O), physically and chemically similar to water was used as solvent in qNMR to quantify the solubility of ferulic acid 1 and its glycosides 11. One can use H$_2$O solutions, yet molecular sieves are necessary to suppress water signals and loading appropriate parameters are also important in NMR data acquisition. However, using D$_2$O in the sample preparation contributes an easy experimental setup and the process can be automated. To evaluate and compare the solubility between ferulic acid 1 and its glycoside 11, aromatic protons and unsaturated protons in trans-position derived from ferulic acid 1 were selected because of their clear signal separation. Methanol and ethanol in D$_2$O were determined in $^1$H NMR respectively to characterize the perfect candidate for internal reference. Figure 3 shows that proton NMR spectra of methanol contained overlapping signals while ethanol has independent signals with ferulic acid 1 and glycoside 11 in both aliphatic and aromatic region which gives better accuracy in extracting the solubility information. Thus, the triplet at $\delta$ 1.03 ppm (t, 2H, $J = 6.8$ Hz) belonging to the methyl of ethanol in the NMR spectrum was selected as the analytical signal for quantitative purposes.
Fig. 3 $^1$H NMR spectra of mixture of methanol and ethanol in D$_2$O

Fig. 4 $^1$H NMR spectra of mixture ferulic acid 1 and ethanol in D$_2$O

Table 3: Computation of number of moles of ferulic acid 1 in 0.6 mL D$_2$O from $^1$H NMR assignment of ferulic acid molecule

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>$\delta$ ppm</th>
<th>Proton</th>
<th>Integral</th>
<th>$\text{Integral}/1H$</th>
<th>Average of 1H Integral</th>
<th>mol (%)</th>
<th>mol (µmol)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ethanol</td>
<td>1.010</td>
<td>3</td>
<td>1.00</td>
<td>0.33</td>
<td>0.33</td>
<td>94.3</td>
<td>34.25</td>
</tr>
<tr>
<td></td>
<td>Ferulic acid</td>
<td>7.464</td>
<td>1</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>5.70</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.147</td>
<td>1</td>
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<tr>
<td></td>
<td></td>
<td>6.802</td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>6.258</td>
<td>1</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>0.35</td>
<td></td>
<td>100.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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**Fig. 5**: $^1$H NMR spectra of mixture ferulic acid glycoside 11 and ethanol in D$_2$O

Table 4: Computation of number of moles of glycosides 11 in 0.6 mL D$_2$O from $^1$H NMR assignment of glycoside molecule

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>δ  (ppm)</th>
<th>Proton</th>
<th>Integral</th>
<th>Integral/1H</th>
<th>Average of 1H Integral</th>
<th>mol (%)</th>
<th>mol (µmol)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Glyceride</td>
<td>7.147</td>
<td>1</td>
<td>0.25</td>
<td>0.27</td>
<td>0.26</td>
<td>44.1</td>
<td>27.02</td>
</tr>
<tr>
<td></td>
<td>Glycoside</td>
<td>6.978</td>
<td>1</td>
<td>0.24</td>
<td>0.27</td>
<td>0.26</td>
<td>44.1</td>
<td>27.02</td>
</tr>
<tr>
<td></td>
<td>Glycoside</td>
<td>6.884</td>
<td>1</td>
<td>0.24</td>
<td>0.27</td>
<td>0.26</td>
<td>44.1</td>
<td>27.02</td>
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<tr>
<td></td>
<td>Glycoside</td>
<td>6.463</td>
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<td>0.25</td>
<td>0.26</td>
<td>0.26</td>
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<td>Glycoside</td>
<td>6.067</td>
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<td>0.26</td>
<td>0.26</td>
<td>44.1</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
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<td></td>
<td></td>
<td>0.59</td>
<td>100.0</td>
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</table>

In the preparation of the $^1$H qNMR measurements, both the solute of 1 and 11 were prepared at similar concentration in 0.6 mL of D$_2$O. Also, the concentration of internal standard was kept constant in formulation to improve the quality of solubility assignment. Figure 4 and 5 show $^1$H NMR spectrum of ferulic acid 1 and glycosides 11 in D$_2$O with ethanol as internal standard. By working with Bruker Advance NMR Spectrometer (400 MHz), both compounds exhibited clear separation and resolution of three aromatic protons and vicinal protons. The actual mole of compound dissolved in D$_2$O was computed from the following equation 2:

$$n_s = n_e \times \frac{P_s}{P_e} \quad \text{(Equation 2)}$$

$n_s$ is the mole of sample and $n_e$ is the mole of internal reference respectively in the deuterated solution. Mole percent of sample, $P_s$, is calculated from ratio between the integrals of the ferulic acid measurement peaks and the total analytic peaks. Similarly, mole percent of ethanol, $P_e$, is calculated from ratio between the integral of reference peak of ethanol at 51.010 ppm and the total analytic peaks. The qNMR measurement of the two compounds indicates...
that glycoside 11 (27.02 µmol) was about thirteen times more soluble than ferulic acid 1 (2.07 µmol) in water.

CONCLUSION
This paper has investigated a synthesis of ferulic acid glycoside based on the structure-activity relationship. We provided further evidence that the synthetic method utilizing hydroxyl carbohydrate as an intermediate prepares efficiently glycoside. Obtained glycoside observed high water solubility than native ferulic acid, on the other hand its glycoside also observed decrease in antioxidant ability. This study makes an impact on the efficient synthesis of glycoside and determination of solubility using 1H qNMR methods.

ACKNOWLEDGEMENTS
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CONFLICT OF INTEREST STATEMENT
The authors declare that they have no competing interests.

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