Identification of Kaempferol from the Leaves of Diospyros kaki and its Antimicrobial Activity against Streptococcus mutans

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This paper describes the isolation and identification of an antimicrobial compound against Streptococcus mutans from the leaves of Diospyros kaki. After repeated chromatography of the weakly acidic fraction of acetone extracts, an antimicrobial compound against S. mutans was isolated. The chemical structure of the active compound was elucidated by spectroscopic methods, and identified as kaempferol. Kaempferol is an aglycone of astragalin (kaempferol 3-O-β-D-glucopyranoside) which has been isolated from the leaves of D. kaki. The minimum inhibitory concentrations of kaempferol against Streptococcus mutans, Escherichia coli, Bacillus subtilis, and Staphylococcus aureus were estimated.

Key words : Antimicrobial activity/Kaempferol/Diospyros kaki/Streptococcus mutans/Dental caries.

In the course of searching for natural antimicrobial compounds from plants, we attempted to isolate growth inhibitory compounds against Streptococcus mutans which is one of the primary causative organisms of dental caries. We screened several plants which have traditionally been used in order to wrap foods, and found a growth inhibitory effect of the acetone extracts from the leaves of kaki (Diospyros kaki). There are no reports on the antimicrobial components contained in leaves of D. kaki although some pharmaceutical and pharmacological studies have been done on their use in the treatment of hypertension (Funayama and Hikino, 1979).

The leaves of kaki have been utilized for wrapping a kind of sushi called "kakinoha-sushi" which is a traditional food in Yoshino, Nara, Japan. In the present paper, the isolation and identification of an antimicrobial compound from the commercially available leaves of D. kaki against S. mutans will be described.

During summer in Nara prefecture, the soft, young leaves of D. kaki are customarily collected in order to prepare the material for wrapping kakinoha-sushi. The leaves are soaked in an aqueous solution of sodium chloride (approximately 20%, w/v, NaCl) and stored in a refrigerator, and then washed and dried for the purpose of wrapping kakinoha-sushi. In our study, the commercially prepared leaves of D. kaki were extracted with acetone three times. The combined acetone solution was concentrated under reduced pressure to yield the extracts. The ethyl acetate soluble portion from the acetone extracts was fractionated into strongly acidic (SA), weakly acidic (WA), and neutral (N) fractions as shown in FIG.1. Yields were SA 0.93%, WA 0.14%, and N 1.54% on the basis of the plant material. Antimicrobial activity of SA, WA, and N fractions against S. mutans was examined by the agar dilution method under anaerobic conditions (Nakatani and Yamada, 1996). Each test sample (2
FIG. 1. Extraction and fractionation procedure of antimicrobial compounds from the leaves of *D. kaki*. Abbreviations: Ac, acetone; EtOAc, ethyl acetate; SA Fr., strongly acidic fraction; WA Fr., weakly acidic fraction; N Fr., neutral fraction.

mg) was dissolved in dimethylsulfoxide (0.4 ml) and diluted with dimethylsulfoxide for a series of two-fold dilutions. The dilution (0.1 ml) was added into agar medium (4.9 ml, 55°C) prepared with Brain Heart Infusion (BHI) broth, and mixed thoroughly in a short time. In this way, prepared concentrations were 100, 50, 25, 12.5 μg/ml. Each of the three strains of *Streptococcus mutans* (K1, HS-6, IFO 13955) was inoculated on BHI agar medium containing a test sample. After incubation for 24 h at 37°C under anaerobic conditions, minimal inhibitory concentrations (MICs) were determined. Antimicrobial activities against some general bacteria (*Escherichia coli* B, *E. coli* K12, *Bacillus subtilis*, *Staphylococcus aureus*) were also examined in the similar manner mentioned above, using bouillon agar medium under aerobic conditions.

The WA part of the three fractions exhibited antimicrobial activity against *S. mutans*. The WA part was chromatographed on silica gel with hexane-ethylacetate gradiently. Each fraction was monitored for its antimicrobial effects. The eluted fraction with hexane-ethylacetate (6 : 4, v/v) gave an antimicrobial fraction, which was further purified by a silica gel column with dichloromethane-methanol (9 : 1, v/v), and a sephadex LH-20 column with methanol subse-

FIG. 2. Chemical structures of kaempferol and kaempferol tetraacetate.
KAEMPFEROL AS AN ANTIMICROBIAL AGAINST S. MUTANS

quently, and the growth inhibitory effect against S. mutans was monitored. After chromatography, an antimicrobial compound against S. mutans was isolated from the leaves of D. kaki. The chemical structure of the active compound was elucidated by spectroscopic methods. The physicochemical data of the compound were as follows. M.p. 271-274°C, UV λ max (MeOH) nm (log ε) : 206 (4.4), 218 sh, 267 (4.2), 366 (4.3), IR ν max (KBr) cm⁻¹ : 3340, 1660, 1615, 1570, 1505, 1445, 1380, 1305, 1255, 1225, 1175, 1130, 1090, 1010, 975, 895, 850, 835, 820, 795, 720, 700. MS m/z (%) : 285.9 (M⁺, 100), 256.9 (8), 228.9 (6), 212.9 (5), 166.9 (8), 143.0 (5), 120.9 (45), 104.9 (9), 82.8 (12), 65.0 (13), 43.9 (80), 29.0 (43). ¹H-NMR (CD₃OD) δ : 6.16 (1H, d, J=2.0Hz), 6.38 (1H, d, J=2.0Hz), 6.89 (2H, d, J=9.0Hz), 8.07 (2H, d, J=9.0Hz), 13C-NMR (CD₃OD) δ : 94.4, 99.2, 104.5, 116.3, 123.7, 130.6, 137.1, 148.0, 158.2, 160.5, 162.5, 165.5, 177.3 (Wagner et al., 1976). The structure of the antimicrobial compound was identified as kaempferol (C₁₅H₁₀O₆) (FIG.2), and these spectral data accorded with those of an authentic sample. The flavonol was acetylated with acetic anhydride in pyridine to give kaempferol tetraacetate. The data was as follows. M.p. 180-185 ºC, UV λ max (CHCl₃) nm (log ε) : 251 (7.3), 299 (7.3), IR ν max (KBr) cm⁻¹ : 3075, 2950, 1780, 1715, 1650, 1630, 1505, 1480, 1430, 1370, 1270, 1200, 1125, 1080, 1020, 965, 910, 850, 795, 700. MS m/z (%) : 412.1 (M⁺–42, 63), 370.0 (64), 328.1 (91), 286.1 (100), 257.0 (41), 229.0 (8), 193.1 (6), 120.9 (37), 93.0 (11), 68.9 (20), 41.0 (27), 14.7 (34). ¹H-NMR (CDCl₃) δ : 2.32 (3H, s), 2.35 (6H, brs), 2.44 (3H, s), 6.87 (1H, d, J=2.0Hz), 7.26 (2H, d, J=9.0Hz), 7.33 (1H, d, J=2.0Hz), 7.85 (2H, d, J=9.0Hz), ¹³C-NMR (CDCl₃) δ : 20.6, 21.1, 21.2, 109.0, 113.8, 114.8, 122.1, 127.0, 129.6, 133.9, 150.4, 152.9, 154.2, 154.8, 156.9, 167.8, 167.9, 168.9, 169.3, 170.1. In previous reports, ursolic acid, betulinic acid, oleanolic acid, ascorbic acid (Nanba, 1980), tannin, carotene, cryptoxanthine, rutin, quercetin, astragalin (kaempferol 3-β-glucoside), and isoquercitrin (quercetin 3-β-glucoside) (Funayama and Hikino, 1979) were isolated from the Japanese traditional crude drug "kaki-yo", which is prepared from the leaves of D. kaki and used as a remedy for hypertension. Kaempferol has been found to be contained in the leaves of kaki, although kaempferol has already been identified from calyx of the same plant (Matsuura and Linuma, 1977).

Hitherto, there have been only a few reports on the antimicrobial activity of kaempferol and its glycoside. Kaempferol showed antimicrobial activity against Bacillus cereus, B. subtilis, Staphylococcus aureus, Fusarium oxysporum, Macrophomina phaseoli, Diplodia oryzae, Rhizoctonia solani, Helminthosporium turcicum, Aspergillus carneus, except B. mycoides, Sarcina lutea, Escherichia coli, and Candida albicans by the paper disk method (El-Gammal and Mansour, 1986). Glycoside, kaempferol 3-(2,4-di-E-p-coumaroylrhamnoside), has been reported to show a growth inhibitory activity against MRSA (methicillin resistant Staphylococcus aureus) (Bloor, 1995). In the present investigation, the inhibitory effects of kaempferol and kaempferol tetraacetate on the growth of three strains of Streptococcus mutans (K1, HS-6, IFO 13955) as primary causative organisms of dental caries, and four other strains of bacteria were examined and are summarized in TABLE 1. The MIC (minimum inhibitory concentration) of kaempferol was 25.0, 100.0, and 50.0 µg/ml against Streptococcus mutans K1, HS-6, and IFO 13955 respectively, and 25.0 µg/ml against Escherichia coli B (IFO 13168), E. coli K-12 (IFO 3301), Bacillus subtilis (IFO 12210), and Staphylococcus aureus (IFO 3060). However, the acetylation of kaempferol suppressed the antimicrobial activity against all the strains tested. These observations supported the conjecture that phenolic hydroxyl groups on the molecule of kaempferol play important roles in antimicrobial activity.

Kaempferol contained in the commercially prepared

<table>
<thead>
<tr>
<th>Strain</th>
<th>Kaempferol</th>
<th>Kaempferol tetraacetate</th>
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<tbody>
<tr>
<td>Streptococcus mutans K1</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Streptococcus mutans HS-6</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Streptococcus mutans (IFO 13955)</td>
<td>50.0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Escherichia coli B (IFO 13168)</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Escherichia coli K-12 (IFO 3301)</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Bacillus subtilis (IFO 12210)</td>
<td>25.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Staphylococcus aureus (IFO 3060)</td>
<td>25.0</td>
<td>50.0</td>
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*The serial agar dilution method was applied to the assay.
leaves of kaki (D. kaki) showed that it possibly contributed to the preservation of sushi together with vinegar and salt. Furthermore, kaempferol might be useful in the prevention of dental caries.

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REFERENCES


