Compounds from *Syzygium aromaticum* Possessing Growth Inhibitory Activity Against Oral Pathogens

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A crude MeOH extract of *Syzygium aromaticum* (clove) exhibited preferential growth-inhibitory activity against Gram-negative anaerobic periodontal oral pathogens, including *Porphyromonas gingivalis* and *Prevotella intermedia*. By means of bioassay-directed chromatographic fractionation, eight active compounds were isolated from this extract and were identified as 5,7-dihydroxy-2-methylchromone 8-*C*-*β*-D-glucopyranoside, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid, and oleanolic acid, based on spectroscopic evidence. The antibacterial activity of these pure compounds was determined against *Streptococcus mutans*, *Actinomyces viscosus*, *P. gingivalis*, and *P. intermedia*. The flavones, kaempferol and myricetin, demonstrated potent growth-inhibitory activity against the periodontal pathogens *P. gingivalis* and *P. intermedia*.

Dental plaque plays an important role in the development of caries and periodontal disease, which result in both tooth dysfunction and loss.[1−3] Extensive effort has been made to search out effective antiplaque agents from a variety of chemical and biological compounds to be incorporated into dental products, thereby reducing plaque-mediated diseases.[4,5] To date, only chlorhexidine and Listerine (a combination of "essential oils") have gained the approval of the American Dental Association Council on Dental Therapeutics. However, various adverse effects such as teeth staining and increased calculus formation are commonly observed with the currently used chemicals.[6,7] These drawbacks justify further research and development of alternative agents that are safe for the host while retaining antimicrobial specificity and efficacy.[5−7]

Higher plants have been shown to serve as sources of a number of diverse antimicrobial agents,[8,9] although fewer reports are available concerning their activity against oral pathogens.[10−13] Among these, the antimicrobial activity of plant extracts against cariogenic bacteria has been more frequently investigated, and rather less attention has been given to biological activity against common periodontal pathogens.[12−16] In our laboratory, efforts have been made to discover plant-derived antimicrobial compounds as plaque-control agents for the prevention of dental caries and periodontal disease.[17,18] We have previously demonstrated that clove oil, extracted from *Syzygium aromaticum* (L.) Merr. et Perry (Myrtaceae), commonly used in clinical dentistry in root canal therapy and temporary fillings, exhibits antimicrobial activity against oral bacteria that are commonly associated with dental caries and periodontal disease.[19] This study demonstrates that a crude MeOH extract of clove also possesses antimicrobial activity against oral pathogens with preferential activity against the Gram-negative anaerobic bacteria frequently associated with periodontitis. Antimicrobial components from a crude MeOH extract of clove were fractionated and isolated guided by bioassays, and the pure compounds obtained were identified by spectroscopic studies and compared with literature values. Their effects on growth of selected cariogenic and periodontal oral pathogenic bacteria were determined. These compounds may have potential for further development as natural antiplaque/antigingivitis agents.

A MeOH extract from the dried buds of *S. aromaticum* (clove) demonstrated preferential antimicrobial activity against the periodontal pathogens *Prevotella intermedia* and *Porphyromonas gingivalis* with MICs of 156 and 625 *μ*g/mL, respectively. No appreciable activity was noted when tested against the cariogenic oral bacteria *Streptococcus mutans* and *Actinomyces viscosus* (MIC values >2.5 mg/mL) (Table 1). Subsequent fractionation of the MeOH extract guided by antimicrobial assay afforded eight compounds—5,7-dihydroxy-2-methylchromone 8-*C*-*β*-D-glucopyranoside,[20] 5,7-dihydroxy-2-methylchromone 6-*C*-*β*-D-glucopyranoside (biflorin),[21] kaempferol,[22] rhamnocitrin,[23] myricetin,[24,25] gallic acid,[26] ellagic acid,[26] and oleanolic acid.[27]

As shown in Table 1, the eight compounds isolated and identified from the crude MeOH extract of *S. aromaticum* were evaluated for growth inhibitory activity against *P. gingivalis*, *P. intermedia*, *S. mutans*, and *A. viscosus*. As that observed with the crude MeOH extract, a majority of the purified compounds demonstrated less inhibitory activity against *S. mutans* and *A. viscosus*, with MIC values of 1.25 mg/mL or greater. However, the two periodontal pathogens, *P. gingivalis* and *P. intermedia*, were more susceptible to these compounds, with MIC values ranging from 20 *μ*g/mL to 1.25 mg/mL. Among these, two of the three flavones, kaempferol (3,5,7,4′-tetrahydroxyflavone) and myricetin (3,5,7,3′,4′,5′-hexahydroxyflavone), demonstrated the strongest inhibitory activity (MIC value of 20 *μ*g/mL) against the periodontal bacteria. A third flavone, rhamnocitrin (kaempferol 7-methyl ether) demonstrated a much lower level of antimicrobial activity (MIC value of 625 *μ*g/mL). This finding suggests that the free hydroxyl gruop at the C-7 position in ring A contributes significantly to the antimicrobial activity of these structurally related flavonoids.

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Table 1. MICs of Compounds Isolated from *Syzygium aromaticum* Against Oral Pathogens[a]

compound	*P. gingivalis*	*P. intermedia*	*S. mutans*	*A. viscosus*
MeOH extract	625	156	>2500	>2500
5,7-dihydroxy-2-methylchromone 8-*C*-β-glucopyranoside	312	156	>2500	>2500
biflorin	625	625	>2500	>2500
kaempferol	20	20	2500	1250
rhamnocitrin	625	625	1250	1250
myricetin	20	20	2500	1250
gallic acid	156	78	>2500	78
ellagic acid	1250	1250	1250	1250
oleanolic acid	625	1250	1250	625
sanguinarine (control)	2	2	15	15

[a] MIC data are expressed in μg/mL.

Osawa *et al.*[16] previously tested the antimicrobial activity of four groups of flavonoids against selected cariogenic oral streptococci. They reported that isoflavanones represented the only group that demonstrated potent growth-inhibitory activity, while flavones, isoflavones, and flavanones were not effective. It was proposed that the absence of a double bond at the C-2 position in ring C may be related to antimicrobial activity. Contrary to their findings, the two flavones kaempferol and myricetin, isolated from *S. aromaticum* in this study, demonstrated antimicrobial activity against *S. mutans* and *A. viscosus*. Structurally, these flavones are different from the three tested by Osawa *et al.* (flavone, apigenin, and diosmin) in that a hydroxyl group substituent is present at the C-3 position. We believe that this structural difference may have contributed to the difference in the observed antimicrobial activity.

On the basis of data obtained from this study, no appreciable antimicrobial activity was noted in the crude MeOH extract against the cariogenic bacteria *S. mutans* and *A. viscosus*. However, a preferential inhibition was noted against the two anaerobic periodontal pathogens *P. gingivalis* and *P. intermedia*, by both the crude MeOH extract and six of the pure compounds tested (excluding ellagic acid and oleanolic acid). Bioflorin, rhamnocitrin, ellagic acid, and oleanolic acid exhibited MIC values equal to or higher than that of the crude extract, suggesting that these compounds did not contribute significantly to the activity observed in the crude MeOH extract. Kaempferol, myricetin, and gallic acid represent the most active components present in the crude MeOH extract, capable of suppressing growth of the test oral pathogens.

Namba *et al.* have previously tested the antibacterial effect of crude MeOH extract of clove bud against *S. mutans* and *Streptococcus sobrinus* using the disk diffusion method on agar surfaces.[12] Growth inhibition was observed against only *S. sobrinus* but not against *S. mutans* (0.2 mg to 1.2 mg). Because the procedure employed by Namba *et al.* for antimicrobial-activity testing was different from the broth dilution method used in the present study, a comparison of data cannot be made. A subsequent study of Namba *et al.*[13] on an active MeOH extract of *Artemisia capillaris* Spica demonstrated that rhamnocitrin (along with other flavonoids such as kaempferol and myricetin) was capable of inhibiting the glucosyltransferase (GTF) activity and the adherence of *S. mutans* cells to glass surfaces. No information was given as to their antimicrobial activity against the test bacteria. Although not shown here, recent data obtained from our laboratory indicated that the crude MeOH extract of clove buds suppressed glucan synthesis from sucrose by the *S. mutans* GTF enzyme. Further investigation is underway.

In conclusion, in addition to clove oil, *S. aromaticum* contains additional antimicrobial compounds, especially flavones, that exhibit preferential inhibitory activity against periodontal pathogens. Although there have been many reports documenting the antifungal and antibacterial activities of flavonoids,[28−30] relatively little is known in regard to their biological activity against oral bacteria.[13,16] Selected flavonoids may have potential for further development as natural antiplaque/antigingivitis agents.

Experimental Section

General Experimental Procedures. Routine ^{1}H-NMR and ^{13}C-NMR spectra were recorded with TMS as the internal standard, employing a Bruker WM-360 instrument operating at 360.14 MHz, and 90.08 MHz, respectively. EIMS were recorded on a Finnigan MAT-90 instrument; UV spectra were recorded using a Perkin-Elmer Lambda 4B UV/vis spectrophotometer. IR spectra were determined on a Nicolet 205 FTIR spectrometer. Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium lamp operating at 589 nm. Mps were determined using a MEL-TEMP apparatus, and reported values are uncorrected. Column chromatography was performed using Si gel 60 (70−230 mesh and 230−400 mesh, E. Merck, Darmstadt, Germany) and Polyamide 11 (5−40 μm, E. Merck, Darmstadt, Germany). TLC was carried out on Merck aluminum-backed TLC sheets (Si gel F_{254}), and Baker-flex" polyamide 6-F TLC sheets, (J. T. Baker, Inc., Phillipsburg, NJ), $CHCl_3$−MeOH (9:1, 7:1, 4:1), $CHCl_3$−Me_2CO (9:1), EtOAc−HOAc (100:0.5), 80% MeOH, with visualization using 10% H_2SO_4−H_2O or NH_4OH−H_2O spraying reagent and under UV light.

Plant Material. Authentic flower buds of *S. aromaticum* were purchased from Frontier Plants Co., Norway, IA. A voucher sample of the plant was deposited at Dows Institute for Dental Research, College of Dentistry, University of Iowa.

Bioassays and Test Bacteria. The bacteria tested included *S. mutans* Ingbritt, *A. viscosus* W1053, *P. gingivalis* ATCC 33277, and *P. intermedia*. These were obtained from culture collection at the Dows Institute for Dental Research, University of Iowa, College of Dentistry. The growth media employed were: brain heart infusion broth (BHI, BBL Microbiology System, Cockkeyville, MO) for *S. mutans*; tryptic soy broth (3%) and yeast extract (0.5%) (Difco Laboratories, Detroit, MI) for *A. viscosus*; and trypticase soy broth−yeast extract medium supplemented with cysteine hydrochlo-

ride (0.05%), menadione (0.02 μg/mL), hemin (5 μg/mL), and potassium nitrate (0.02%) for *P. gingivalis* and *P. intermedia*. All cultures were incubated at 37 °C. *P. gingivalis* and *P. intermedia* cultures were incubated in an anaerobic growth chamber (Forma Scientific Inc., Marietta, OH) in 10% H_2, 5% CO_2, and 85% N_2.

For antimicrobial-activity screening during fractionation procedures, the disk diffusion in agar was used. Overnight cultures of *P. gingivalis* or *S. mutans* were inoculated onto respective agar media. Sterile paper disks (6.5-mm diameter, Difco Laboratories, Detroit, MI) containing reconstituted fractions in H_2O or DMSO were deposited onto the preinoculated agar surface. Where applicable, solvent controls were included, though no adverse effect had been noted at concentrations employed.

Determination of MIC Values. Sterile 96-well microtiter plates were used for this purpose. Each well contained 5×10^5 colony-forming units (CFU)/mL of test bacteria, serially diluted test compounds and respective growth medium. Triplicate samples were performed for each test concentration. The controls included inoculated growth medium without test compounds. Sample blanks contained uninoculated medium only. All plates were incubated at 37 °C under appropriate atmospheric condition, and growth was estimated spectrophotometrically (650 nm) after 48 h using a microtiter plate reader (Molecular Devices, Vmax Kinetics, Menlo Park, CA). The MIC for each test bacterium was defined as the minimum concentration of test compound limiting turbidity to < 0.05 absorbance at 650 nm. For positive antimicrobial controls, sanguinarine (Sigma Chemical Co., St. Louis, MO) was used.

Extraction and Isolation. The dried buds of *S. aromaticum* (700 g) were extracted three times overnight with MeOH (3 × 2 L) at room temperature. The resultant extracts were combined, concentrated under reduced pressure to 700 mL, and diluted with H_2O to afford an H_2O−MeOH solution (1:10). Then this solution was extracted exhaustively with petroleum ether to remove essential oil. The MeOH layer was concentrated to give an extract (73.4 g), which demonstrated preferential growth-inhibitory activity against periodontal pathogens including *P. gingivalis* and *P. intermedia*. The crude MeOH extract was chromatographed on a Si gel column and eluted with increasing percentages of MeOH in $CHCl_3$ (0%−20%). In all, 90 fractions were collected and combined into 16 pooled fractions based on TLC profiles. The active fractions were further separated as follows: the white precipitate of fraction 2 was purified by a Si gel flash column eluted with 2% Me_2CO in $CHCl_3$ to afford oleanolic acid (25 mg). Fraction 3 gave rhamnocitrin (34 mg) by a Si gel column chromatography eluted with 3% MeOH in $CHCl_3$. Fraction 6 yielded kaempferol (20 mg) through a Si gel column eluted with 9% MeOH in $CHCl_3$, followed by purification over a polyamide column eluted with 70% MeOH. Fraction 8 afforded myricetin (14 mg) by passing through a polyamide column and eluting with 70% MeOH. Fraction 10 was separated with repeated Si gel columns eluted with 10% MeOH in $CHCl_3$ and $CHCl_3$−MeOH−HOAc (350:50:1) successively to yield gallic acid (200 mg). The white amorphous powder separated from fraction 13 was chromatographed on a Si gel column and eluted with gradually increasing concentrations of MeOH (12.5% to 30%) in $CHCl_3$ to give 5,7-dihydroxy-2-methylchromone 8-*C*-β-D-glucoside (100 mg) and biflorin (50 mg). Ellagic acid as an amorphous solid (15 mg) was obtained from fraction 16 through a Si gel flash column eluted with 15−20% MeOH in $CHCl_3$ and was recrystallized from pyridine and H_2O.

5,7-Dihydroxy-2-methylchromone 8-*C*-β-D-glucoside: white amorphous solid; mp 182−183 °C; $[\alpha]^{25}_D$ + 71.1 (*c* 0.7 pyridine); {lit mp 183−184 °C, $[\alpha]^{21}_D$ + 74.2 (*c* 0.7 pyridine)},[20] exhibited spectral (UV, IR, ^{1}H NMR, ^{13}C NMR, and FABMS) data comparable to published values.[20]

5,7-Dihydroxy-2-methylchromone 6-C-β-D-glucoside (biflorin): white amorphous solid; mp 297−300 °C (dec); $[\alpha]^{25}_D$ +30.1° (*c* 0.7 pyridine); {lit mp 300−303 °C, (dec); $[\alpha]^{28}_D$ +24.4° (*c* 0.34 pyridine)};[21] ^{13}C NMR (DMSO-d_6) δ ppm 182.3 (C-4), 168.0 (C-2), 163.5 (C-7), 160.9 (C-5), 157.0 (C-8a), 108.9 (C-6), 108.1 (C-3), 103.4 (C-4a), 93.7 (C-8), 81.8 (C-5′), 79.2 (C-3′), 73.2 (C-1′), 70.8, 70.4 (C-2′, 4′), 61.7 (C-6′), 20.1 (CH_3); exhibited spectral (UV, IR, ^{1}H NMR, and FABMS) data comparable to published values.[21]

Kaempferol: yellow needles; mp 272−275 °C; (lit mp 276−278 °C);[31] structure assigned by comparison of UV and NMR data with those in the literature.[22,23,32]

Rhamnocitrin: yellow crystal; mp 256−260 °C; (lit mp 263−265 °C);[23] structure assigned by comparison of UV and NMR data with those in the literature.[23,32]

Myricetin: yellow needles; mp 350 °C (dec); (lit mp 357 °C);[31] structure assigned by comparison of UV and NMR data with those in the literature.[24,25,32]

Gallic acid: colorless needles; mp 230−232 °C (dec), (lit mp 235−240 °C),[31] exhibited spectral (UV, ^{1}H NMR, ^{13}C NMR, and EIMS) data comparable to an authentic standard compound and published values.[26]

Ellagic acid: cream-colored solid; mp 350−355 °C (dec), (lit mp above 360 °C);[31] exhibited spectral (UV, ^{1}H NMR, ^{13}C NMR, and EIMS) data comparable to an authentic standard compound and published values.[26]

Oleanolic acid: white powder; mp 303−306 °C; $[\alpha]^{25}_D$ +75.3 (*c* 0.7, $CHCl_3$); {lit mp 310 °C, $[\alpha]^{21}_D$ +83.3 (*c* 0.6, $CHCl_3$)},[31] exhibited spectral (IR, ^{1}H NMR, ^{13}C NMR, and EIMS) data comparable to published values.[27]

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