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Antibacterial Effect of Amentoflavone and Its Synergistic Effect with Antibiotics

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Selaginella tamariscina is a traditional herb used in medicine. Phytochemical amentoflavone, a biflavonoid class of flavonoids, was isolated from the plant of *Selaginella tamariscina*. In this study, the antibacterial effects and combination effects of amentoflavone and conventional antibiotics such as ampicillin, cefotaxime, and chloramphenicol were investigated. These results showed that amentoflavone had a considerable antibacterial effect and synergistic interaction with antibiotics against various bacterial strains (fractional inhibitory concentration index ≤ 0.5), except for *Streptococcus mutans*. To study the mechanism(s) involved in the synergistic activities between amentoflavone and antibiotics, we detected hydroxyl radical formation using 3'-(*p*-hydroxyphenyl) fluorescein and measured the NAD^+/NADH ratio by NAD^+ cycling assay. The results indicated that the formation of hydroxyl radical would be a cause of the synergistic effect and that this oxidative stress originated from a transient NADH depletion. This study suggests that amentoflavone synergizes with antibiotics and has potential as a therapeutic agent for antimicrobial chemotherapy.

Keywords: Amentoflavone, phytochemical, *Selaginella tamariscina*, synergistic effect, hydroxyl radicals

Introduction

Infectious disease caused by pathogenic microorganisms affects millions of people worldwide. An increase in the antibiotic resistance of these pathogenic microbes has driven focus on the development of alternative ways to cope with this problem and new antimicrobial substances from any sources, including plants [19]. Natural phytochemicals with bioactive properties have less cytotoxicity and are more cost-effective. They have been proven to have antimicrobial properties by acting as protective agents against external stress and pathogenic stress [7]. About 61% of the new drugs developed between 1981 and 2002 were based on natural products, and they have been very successful, especially in the areas of infectious disease [3]. Several studies have reported that combinatorial therapy with natural compounds and antibiotics is a new strategy for treating bacterial infections [13]. Moreover, plant antimicrobials have been found to be synergistic enhancers when taken concurrently with conventional antibiotics [11].

Flavonoids are ubiquitous polyphenolic compounds and are one of the phytochemical derivatives that are extracted from edible plants, such as fruits and vegetables. They are important constituents of human dietary sources and are essential for the growth, development, and defense of plants. Amentoflavone, which belongs to the biflavonoid class of flavonoids, is abundant in *Selaginella tamariscina* and exhibits anti-inflammatory and anti-ulcerogenic activities in various animal models [8], cytotoxicity against a human tumor cell line [14], and anti fungal activity [10].

In this study, we investigated the antimicrobial activities of amentoflavone alone or in combination with conventional antibiotics (ampicillin, cefotaxime, and chloramphenicol) against different bacterial strains. Additionally, we conducted the hydroxyl radical assay and investigated changes in the NAD^+/NADH ratio by NAD^+ cycling assay.

Materials and Methods

Extraction and Isolation of Amentoflavone

The whole plant of *Selaginella tamariscina* (600 g) was extracted with methanol at room temperature, yielding 50.54 g of residue. The methanol (MeOH) extract was resuspended in water and sequentially partitioned using dichloromethane, ethyl acetate, and *n*-butanol. The ethyl acetate fraction (3.0 g) was placed in a silica gel (300 g, 4.8 × 45 cm) column and eluted using a $CHCl_3$-MeOH-H_2O (12:1:0.1→8:1:0.1→5:1:0.1→2:1:0.1→1:1:0.1→MeOH only) gradient system. Based on their TLC patterns, the fractions were combined to yield subfractions, which were designated as E1–E10. Subfraction E7 (296.33 mg) was finally purified using repeated column chromatography over a silica gel, RP-18, and Sephadex LH 20, yielding amentoflavone (82.23 mg). The UV, IR, ^{1}H-, and ^{13}C-NMR data for amentoflavone were identical to those reported in other literature [10].

Bacterial Strains and Culture Medium

Enterococcus faecium (ATCC 19434), *Staphylococcus aureus* (ATCC 25923), *Streptococcus mutans* (ATCC 3065), *Escherichia coli* O-157 (ATCC 43895), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the American Type Culture Collection (ATCC) (USA). Before use, all bacteria were stored in 30% glycerol and frozen at −70°C. The bacteria were cultured in Mueller–Hinton broth (MHB) and Mueller–Hinton agar (MHA) (Difco Laboratories, USA) and incubated at 37°C. Cell growth was monitored by measuring the optical density at 620 nm.

Antibiotics

The following antibiotics were all purchased from Sigma Aldrich Co. (USA): ampicillin, cefotaxime, chloramphenicol.

Antimicrobial Susceptibility Assay

Bacterial strains were cultured in MHB and the cell suspensions were adjusted to obtain standardized populations by measuring their turbidity with a spectrophotometer (DU530; Beckman, USA). The bacterial strains at mid-log phase (1×10^6/ml) were inoculated into MHB and 0.1 ml was dispensed per well into 96-well microtiter plates. Susceptibility tests were performed by a 2-fold standard broth microdilution of the test compounds, including amentoflavone, ampicillin, cefotaxime, and chloramphenicol, following the Clinical and Laboratory Standards Institute (CLSI) guideline [2]. After 18 h of incubation at 37°C, the minimum inhibitory concentration (MIC) required to prevent the growth of a given test organism was determined. The growth was assayed with a microtiter ELISA reader (Molecular Devices Emax, USA) by monitoring the optical density at 620 nm.

Combination Assay

The MICs of each antibiotic alone or in combination with amentoflavone were determined by the broth microdilution method in accordance with the National Committee for Clinical Laboratory Standards by using a cation-adjusted MHB modified for a broth microdilution checkerboard procedure [6, 15]. For the double treatment, a two-dimensional checkerboard with 2-fold dilutions of each drug was used for the study. A checkerboard with 2-fold dilutions of amentoflavone and the antibiotics (ampicillin, cefotaxime, and chloramphenicol) was set up as described above for the combined treatment. Control wells containing medium were included in each plate. Each test was performed in triplicate. For the first clear well in each row of the microtiter plate containing all antimicrobial agents, the fractional inhibitory concentration (FIC) was calculated as follows: FIC of drug A (FIC A) = MIC of drug A in combination/MIC of drug A alone, and FIC of drug B (FIC B) = MIC of drug B in combination/MIC of drug B alone [17]. The FIC Index (FICI), calculated as the sum of each FIC, was interpreted as follows: FICI ≤ 0.5, synergy; 0.5 < FICI ≤ 4, no interaction; 4 < FICI, antagonism [16].

Hydroxyl Radical Formation Assay

Bacterial cells (1×10^6/ml) were treated with each substance or combination so that the final concentration was the same for each of the MICs or FICs. Samples were incubated for 2 h at 37°C. To detect hydroxyl radical formation, we used the fluorescent reporter dye 3′-(*p*-hydroxyl-phenyl) fluorescein (HPF) (Invitrogen, USA) at a concentration of 5 μM and measured using a spectrofluorophotometer (Shimadzu RF-5301PC; Shimadzu, Japan) at 490 nm excitation and 515 nm emission wavelengths [18]. The percentage of hydroxyl radical increase was calculated with the following equation: [(OD_{490} of cells treated with amentoflavone – OD_{490} of non-treated control) / A_{490} of non-treated control)] × 100. Experiments were performed in triplicate, the data were averaged, and the SD was calculated.

NAD^+, NADH Extraction

The dinucleotide extraction and cycling assay were performed according to protocols described previously [4]. The bacterial cells at mid-log phase (1×10^6/ml) were centrifuged at 13,000 rpm for 5 min and resuspended in 1 ml of Luria–Bertani (LB) medium. The samples were collected, each for NAD^+ and NADH extraction, by centrifugation at 13,000 rpm for 1 min from *S. aureus* and *E. coli* cultures at every half hour between 0 and 2 h after adding amentoflavone and ampicillin (at the MIC). The supernatant was removed and the pellets were frozen immediately in a dry ice-ethanol bath, and the pellets were stored at −80°C until we finished collecting all the time-points. Then 75 μl of 0.2 M NaOH (for NADH extraction) or 75 μl of 0.2 M HCl (for NAD^+ extraction) was added to the ice-cold pellets. The samples were heated at 100°C for 10 min and subsequently centrifuged at 10,000 rpm for 5 min. The NAD^+/NADH-containing supernatants were transferred to fresh tubes and stored in the dark on ice until use.

We performed the NAD^+ cycling assay in 96-well plates. The reaction mixture contained 16 μl of 1.0 M bicine (pH 8.0) (Sigma-Aldrich, USA), 40 μl of sample extract, 40 μl of neutralizing buffer (0.1 M HCl for NADH, or 0.1 M NaOH for NAD^+), 16 μl of

phenazine ethosulfate (PES; Sigma-Aldrich), 16 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich), 16 µl of 100% ethanol, and 30 µl of 40 mM EDTA (pH 8.0). We finally added PES and MTT to the 96-well plates and incubated the mixture for 3 min at 30°C. Then 3.2 µl of alcohol dehydrogenase (500 U/ml; Sigma-Aldrich) in bicine buffer (pH 8.0) was added to the reaction mixture to begin the assay. The increase in optical density at 570 nm was recorded within 10 min. The rate of MTT reduction is proportional to the concentration of NAD^+ or NADH in the sample. NAD^+ and NADH standards (Sigma-Aldrich) were used between 0.0375 and 0.75 nM to calibrate the assay. Experiments were performed in triplicate for a plate, by 3 independent assays, and the results were expressed as the mean ± SD.

Results and Discussion

Phytochemicals, an enormous array of secondary metabolites in plants, have been considered as novel antibiotic agents [5]. According to previously reported studies, amentoflavone exhibited potent antifungal effects [9, 10]. For this reason, we assumed that amentoflavone also has antibacterial activity and synergistic effects with antibiotics on diverse pathogenic bacterial strains.

To identify the antibacterial effects of amentoflavone, ampicillin, cefotaxime, and chloramphenicol, the antimicrobial susceptibility testing was conducted against bacterial strains including *E. faecium*, *S. aureus*, *S. mutans*, *E. coli* O-157, *E. coli*, and *P. aeruginosa*, using the CLSI method. The results showed that amentoflavone, with MIC values of 4–32 µg/ml, had remarkable antibacterial activity against Gram-positive and Gram-negative bacteria. The bacterial strains showed MIC values ranging from 2–8 µg/ml for ampicillin and cefotaxime, or 2–16 µg/ml for chloramphenicol (Table 1). Amentoflavone and the antibiotics showed varying antibacterial activities against different bacterial species. Amentoflavone and all of the antibiotics were less effective against *S. mutans* than other bacterial strains. The Gram-positive bacterium *E. faecium*, and the Gram-negative bacteria *E. coli* O-157 and *P. aeruginosa* were significantly sensitive to cefotaxime. Amentoflavone had equal MIC levels for *E. faecium*, *E. coli* O-157, and *P. aeruginosa*. These results suggested that amentoflavone had potent and similar antibacterial activity as the antibiotics (ampicillin, cefotaxime, and chloramphenicol).

Recently, several studies have reported that phytochemicals produced synergistic effects in combination with antibiotics [1]. Combined antibiotic therapy has been shown to prevent the emergence of bacterial resistance to antimicrobial agents and to lead to effective therapy along with a reduction in therapeutic doses. The drug combinations were classified as synergistic, antagonistic, or without influence on antimicrobial activity if the effect of the combined drugs was better than, worse than, or the same as that of the individual drug activities, respectively. We investigated the interaction between amentoflavone and the conventional antibiotics ampicillin, cefotaxime, and chloramphenicol to check the synergistic effects using the checkerboard assay. The results of the combination assay are presented in Table 2. Synergic interactions between AME-AMP, AME-CEF, and AME-CHL were observed against *E. coli* and *P. aeruginosa*, whereas all combinations against *S. mutans* exhibited no interactions. The combination of AME-CHL had a synergistic effect against all bacterial strains except for *S. mutans*. The combination of AME-CEF had no interaction effect against the Gram-positive bacterial strains, but had a synergistic effect against the Gram-negative bacterial strains.

Antibacterial drug-target interactions are well studied and fall predominantly into three classes: inhibition of protein synthesis, inhibition of DNA replication, and repair and inhibition of cell-wall turnover. Recently, Kohanski *et al*. [12] reported that the bactericidal antibiotics induced the production of hydroxyl radicals. The bactericidal antibiotics disrupted normal cellular metabolism, such as the tricarboxylic

Table 1. Antibacterial activity of amentoflavone against human pathogenic bacteria.

Species	MIC (µg/ml)			
	Amentoflavone	Ampicillin	Cefotaxime	Chloramphenicol
Gram-positive				
E. faecium ATCC 19434	8	4	2	4
S. aureus ATCC 25923	4	4	4	4
S. mutans ATCC 3065	32	8	8	16
Gram-negative				
E. coli O-157 ATCC 25922	8	4	2	4
E. coli ATCC 43895	16	8	8	4
P. aeruginosa ATCC 27853	8	4	2	2

Table 2. Combinational activities of amentoflavone and antibiotics against bacterial strains.

Bacterial strains	AME-AMP		AME-CEF		AME-CHL	
	Individual MIC for combination (μg/ml)	FICI [a]	Individual MIC for combination (μg/ml)	FICI	Individual MIC for combination (μg/ml)	FICI
Gram-positive						
E. faecium ATCC 19434	2/0.5	**0.375(S)** [b]	4/0.5	0.75(NI)	2/0.5	**0.375(S)**
S. aureus ATCC 25923	1/0.5	**0.375(S)**	2/1	0.75(NI)	0.5/1	**0.375(S)**
S. mutans ATCC 3065	8/4	0.75(NI)	8/4	0.75(NI)	8/8	0.75(NI)
Gram-negative						
E. coli O-157 ATCC 25922	4/1	0.75(NI)	2/0.25	**0.375(S)**	2/0.5	**0.375(S)**
E. coli ATCC 43895	4/2	**0.5(S)**	4/1	**0.375(S)**	4/1	**0.5(S)**
P. aeruginosa ATCC 27853	2/1	**0.5(S)**	2/0.5	**0.5(S)**	2/0.25	**0.375(S)**

[a]Fractional inhibitory concentration index (FICI) was calculated by the following formula: FICI = ($MIC_{Drug\ A}$ in combination/$MIC_{Drug\ A}$ alone) + ($MIC_{Drug\ B}$ in combination/$MIC_{Drug\ B}$ alone).

[b]*S*, synergistic; *NI*, no interaction; *AN*, antagonism.

AME, Amentoflavone; AMP, Ampicillin; CEF, Cefotaxime; CHL, Chloramphenicol.

acid cycle. This depleted the intracellular NADH, which is related to a commensurate rise in the production of reactive oxygen species such as peroxides and superoxides. These reactive oxygen species interacted with Fe^{2+} *via* Fenton chemistry to generate highly toxic oxygen radicals, which react with DNA and proteins, resulting in the death of bacteria. Based on these studies, we investigated whether bacterial strains treated with amentoflavone generated hydroxyl radicals and examined how it influenced the synergistic effects. As shown in Fig. 1, amentoflavone induced the formation of hydroxyl radicals. Additionally, each of two different classes of bactericidal antibiotics, ampicillin and cefotaxime, induced hydroxyl radical formation by showing different degrees against bacterial strains. The cells treated with AME-AMP exhibited a higher degree of hydroxyl radical formation than that of all other combinations against all the bacterial strains, except for *E. coli* O-157. In contrast, the bacteriostatic antibiotic, chloramphenicol, did not sufficiently stimulate hydroxyl radical formation. However, cells treated with a combination of AME-CHL exhibited increased hydroxyl radical formation. Therefore, we suggest that the hydroxyl radical played a key role in the synergistic effect between amentoflavone and each antibiotic. Furthermore, all combinations against *S. mutans*, showing no inhibition, exhibited the lowest hydroxyl radical products (Fig. 1).

Kohanski *et al.* [12] demonstrated that Gram-positive bacteria (*S. aureus*) as well as Gram-negative bacteria (*E. coli*) produce hydroxyl radicals in response to bactericidal antibiotics such as ampicillin. The authors showed that ampicillin stimulated hydroxyl radical formation in bacteria by catabolic NADH depletion. Therefore, using the NAD^+ cycling assay, we observed the change in the NAD^+ and NADH in the bacterial strains. *S. aureus* (Fig. 2A) and *E. coli* (Fig. 2B) were selected as the model organisms for this experiment because these strains produced more hydroxyl radicals than other strains. The result showed a large relative drop in NADH accompanied by a modest increase in NAD^+. To investigate how amentoflavone induced hydroxyl radicals, we calculated the NAD^+/NADH ratios in cells treated with and without antibacterial agents. Bactericidal agents including amentoflavone or ampicillin increased the NAD^+/NADH ratio of the bacterial organisms by more than 2-fold, 30 min after the agents were added. This ratio returned to untreated levels 1 h after the treatment. This spike was not observed in the untreated culture, in which the NAD^+/NADH ratio remained tightly bound (Fig. 2). The transient depletion of NADH causes the hyperactivation of the electron transport chain, and hydroxyl radical formation is serially produced by the Fenton reaction. Therefore, we suggest that the formation of hydroxyl radicals by NADH depletion due to the treatment with amentoflavone and antibiotics contributed to bacterial cell death (Fig. 2).

In conclusion, this study showed the antibacterial effects of amentoflavone and its synergistic capacity with antibiotics

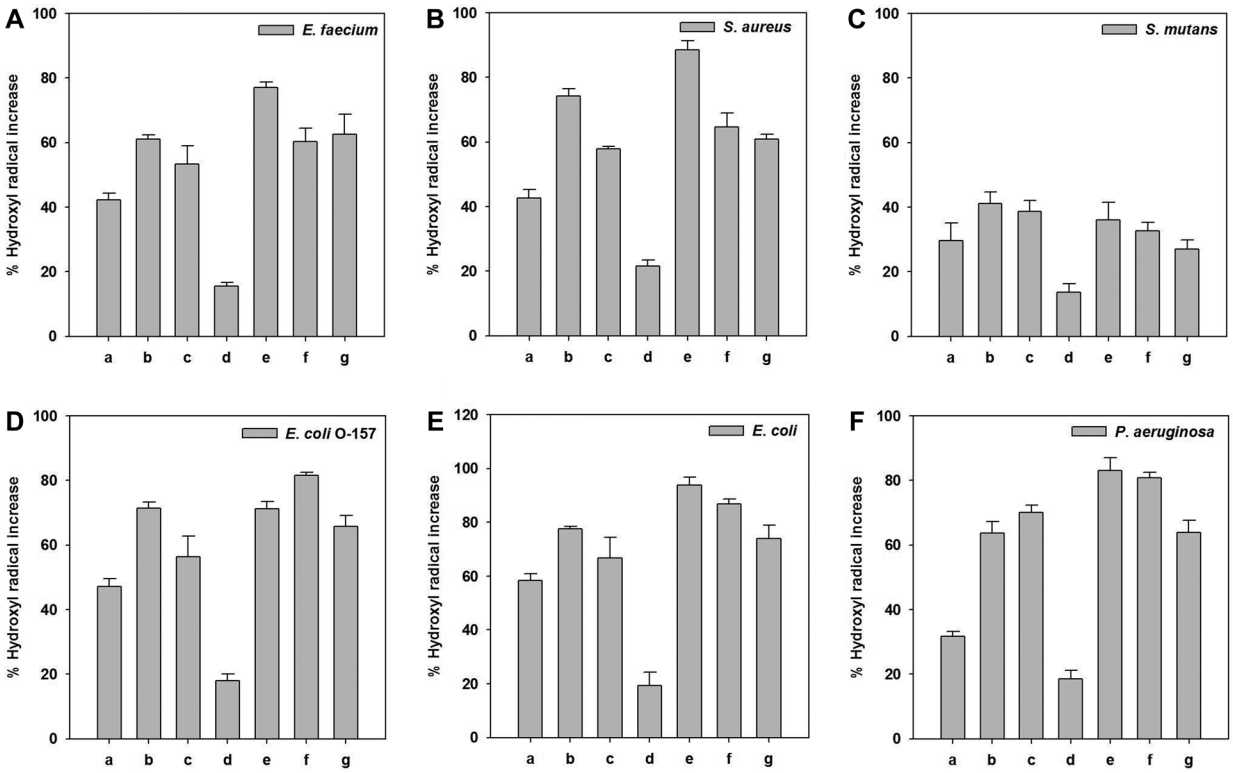

Fig. 1. The percentage of hydroxyl radical increase, checked by the HPF staining method.
Bacterial cells were incubated with agents for 2 h. The data represent the mean and standard deviation values for three independent experiments. (a) MIC of amentoflavone, (b) MIC of ampicillin, (c) MIC of cefotaxime, (d) MIC of chloramphenicol, (e) MIC in combination of amentoflavone and ampicillin, (f) MIC in combination of amentoflavone and cefotaxime, and (g) MIC in combination of amentoflavone and chloramphenicol.

against various representative pathogenic bacteria. Through the hydroxyl radical formation assay and NAD^+ cycling assay, the results suggest that the amentoflavone-induced generation of hydroxyl radicals by NADH depletion and the dysfunction of the protective action are important factors in the antibacterial effect of amentoflavone and its synergism with antibiotics. In summary, the findings of this study support that amentoflavone has considerable effective antibacterial activity, deserving further investigation for clinical applications against infectious diseases.

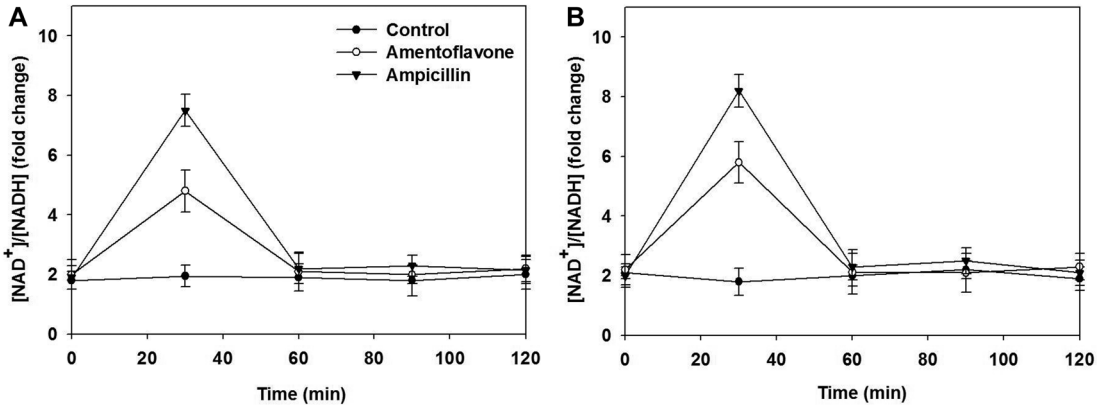

Fig. 2. Fold change in NAD^+/NADH (nM/nM) following treatment with the MIC of the agents at 0, 30, 60, 90, and 120 min.
The data represent values from three independent experiments. (**A**) *S. aureus*, (**B**) *E. coli*.

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