The Journal of Microbiology, October 2007, p. 473-477
Vol. 45, No. 5

In vitro Activity of Kaempferol Isolated from the *Impatiens balsamina* alone and in Combination with Erythromycin or Clindamycin against *Propionibacterium acnes*

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(Received April 25, 2007 / Accepted October 23, 2007)

The *in vitro* antibacterial activity against antibiotic-resistant *Propionibacterium acnes* of kaempferol isolated from the *Impatiens balsamina* alone and in combination with erythromycin or clindamycin antibiotics was investigated. The antibiotic combination effect against antibiotic-resistant *P. acnes* was studied by checkerboard test. Kaempferol and quercetin demonstrated antibacterial activities against *P. acnes*. Minimum inhibitory concentrations (MICs) for both compounds were ≤32 μg/ml and ≤64 μg/ml for clindamycin-sensitive and -resistant *P. acnes*, respectively. The four combination formulations (kaempferol and either erythromycin or clindamycin; quercetin and either erythromycin or clindamycin) exhibited a synergic inhibition of *P. acnes* growth. The combination of kaempferol with quercetin showed an indifferent effect. The combination of clindamycin with kaempferol or quercetin showed a greater synergic effect than that of erythromycin with kaempferol or quercetin. Thus, these combinations demonstrated the potential to treat acne.

Keywords: *Propionibacterium acnes*, kaempferol, erythromycin, clindamycin, checkerboard test

Propionibacterium acnes is a non-spore forming, gram positive anaerobic, pleomorphic rod, belonging to the human cutaneous normal flora (Eady and Ingham, 1994). *P. acnes*, a major etiologic agent of acne vulgaris, stimulates the production of the proinflammatory cytokines and enhances the production of an inflammatory mediator like prostaglandins (PGs), consequently inducing acne (Vowels *et al.*, 1995). The conversion of arachidonic acid to PGs is catalyzed by the enzyme cyclooxygenase (COX). Magnolol decreases PG production, which had been increased by *P. acnes* by inducing the activation of COX-2 (Lee *et al.*, 2005). *P. acnes* could enhance the activated macrophage in chronic inflammatory lesions (Bialecka *et al.*, 2005). It was recently suggested that *P. acnes* is an excellent candidate as the causative agent of prostate cancer (Shannon *et al.*, 2006).

Many topical and systemic treatment methods have been proposed for the treatment of acne vulgaris. Retinoids are most commonly used to treat acne, but their main side effect is skin irritation (Krautheim and Gollnick, 2004; Chivot, 2005). Clindamycin and erythromycin are the most frequently used topical anti-*P. acnes* agents. Topical antibiotics reduce the population of *P. acnes* and exert anti-inflammatory activity, but feature the major disadvantage of dramatically increasing bacterial resistance. Extensive use of antibiotics to treat acne has built up widespread resistance in cutaneous propionibacterium (Coates *et al.*, 2002; Eady *et al.*, 2003; Ross *et al.*, 2003; Bojar and Holland, 2004; Oprica and Nord, 2005). A new approach for preventing antibiotic resistance is the use of combination antibiotic therapy (Mayer and Nagy, 1999; Wu *et al.*, 1999; Sobieszczyk *et al.*, 2004; Jung *et al.*, 2005). Although trials have shown that antibiotic combination prevented the emergence of resistance and enhanced the synergic effects, some limitations remain in combination treatment (Gould and Milne, 1997). Thus, further studies investigating optimal combinations are needed.

Medicinal plants have been used worldwide as various kinds of therapeutic agents since ancient times. Various antimicrobial activities of natural flavonols have been reported (Arima *et al.*, 2002; Lin *et al.*, 2005; Sung *et al.*, 2006). The flowers of *Impatiens balsamina* contain flavonoids such as kaempferol and quercetin which are known to possess antifungal, anticancer, and antioxidant activities (Yang *et al.*, 2001; Wang *et al.*, 2006). In Korea, *Impatiens balsamina* has been used in traditional oriental medicine to treat scrofulosis, carbuncles, and dysentery (Kang and Moon, 1992). Kaempferol was a markedly active inhibitor of transcriptional activation of COX-2 (Liang *et al.*, 1999). Kaempferol and quercetin have inhibitory activities against melanin synthesis (Lim *et al.*, 2006). In the present study, we have also discovered the antibacterial activity of kaempferol against an acne-inducing agent, *P. acnes*.

The aim of the present study was to evaluate the *in vitro* antimicrobial activity of kaempferol isolated from *Impatiens balsamina* and to investigate the synergic effects of kaempferol combined with erythromycin or clindamycin against antibiotic-resistant *P. acnes*. Although kaempferol is well known, this is the first report of combination effect against antibiotic-resistant *P. acnes*.

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Materials and Methods

Bacterial strains

P. acnes KCTC 3314 (ATCC 6919) was purchased from the Korean Collection for Type Culture (Daejon, Korea). *P. acnes* CCARM 9010 (clindamycin-resistant strain) was from the Culture Collection of Antimicrobial Resistant Microbes (Seoul, Korea).

Antibiotics

Clindamycin, penicillin G, chloramphenicol, erythromycin, and tetracycline were purchased from Sigma (USA). Ampicillin was purchased from USB (USA).

Plant materials and chemicals

The flowers of *Impatiens balsamina* were collected in Koyang-si, Kyunggi-do, Korea, during July and August, 2003. A voucher specimen was deposited at the Department of Clinical Laboratory Science, College of Health Sciences, Korea University, Korea. Kaempferol was obtained from our previous study, which was isolated from the methanol extract of the flowers of *Impatiens balsamina* (Lim *et al.*, 2006). It was purified by solvent fractionations, TLC, and HPLC procedures. The purified compound was identified as kaempferol by ^{1}H NMR, ^{13}C NMR, direct electrospray ionization (ESI), and electron impact-mass spectrometry (EI-MS). Quercetin was purchased from Sigma (USA).

Determination of growth inhibition of P. acnes

P. acnes was adjusted to the McFarland 0.5 standard and used to inoculate GAM agar (Nissui, Japan) plate. Each disk containing either 30 μg, 60 μg of kaempferol, or dimethylsulfoxide (DMSO) only was placed on the plate. The plates were incubated in an anaerobic gas generating pouch (GasPak EZ; Becton, Dickinson and Company, USA) at 37°C for 48 h. The incubation atmosphere contained 5% CO_2. The inhibition zone was determined by measuring diameter.

Determination of MICs

MICs of kaempferol, quercetin, and six antimicrobial agents were determined by the CLSI (formerly NCCLS) microbroth dilution methods (National Committee for Clinical Laboratory Standards, 1997). Antimicrobial agents were dissolved in DMSO. A dilute suspension of bacteria was inoculated into each well of a 96-well microplate, each containing a different concentration of the antimicrobial agent being tested. We performed doubling dilutions of the antimicrobial agents. The range of antibiotic dilutions was 1024 μg/ml to 0.0625 μg/ml in the GAM broth and a final concentration of 1×10^5 cfu/ml of test bacteria was added to each dilution. The plates were incubated in an anaerobic gas generating pouch at 37°C for 48 h. The incubation atmosphere contained 5% CO_2. MIC was defined as the lowest concentration of antimicrobial agent that inhibited bacterial growth, as indicated by the absence of turbidity. Antimicrobial agent-free broths containing 5% DMSO were incubated as growth controls. Minimum bactericidal concentration (MBC) was determined by inoculating onto GAM agar plates a 10 μl of medium from each of the wells from the MIC test which showed no turbidity. MBCs were defined as the lowest concentration of antimicrobial agent where was no bacterial growth on the plates.

Checkerboard titrations

For the checkerboard titration the concentrations tested for kaempferol, quercetin, erythromycin, and clindamycin ranged from six two-fold dilutions below the MIC to twice of the MIC for the test strains. GAM broth was used for the checkerboard tests and a final concentration of 1×10^5 cfu/well was inoculated. MICs of the combinations were determined after incubation at 37°C for 48 h. Fractional inhibitory concentration (FIC) indices, determined by averaging all of the FIC values of wells along the growth-no-growth interface, were calculated at 48 h. An index of less than 0.5 was considered as synergism and of greater than 2.0 as antagonism (Pillai *et al.*, 2005).

All experiments were independently repeated three times, and the data in tables are expressed as averages.

Results

Antimicrobial susceptibility

Kaempferol was isolated as a yellow powder from the methanol extract of the flowers of *Impatiens balsamina* (Fig. 1). Kaempferol is relatively insoluble in water and was therefore dissolved in DMSO. To investigate the antimicrobial activity of kaempferol, we examined its growth inhibition

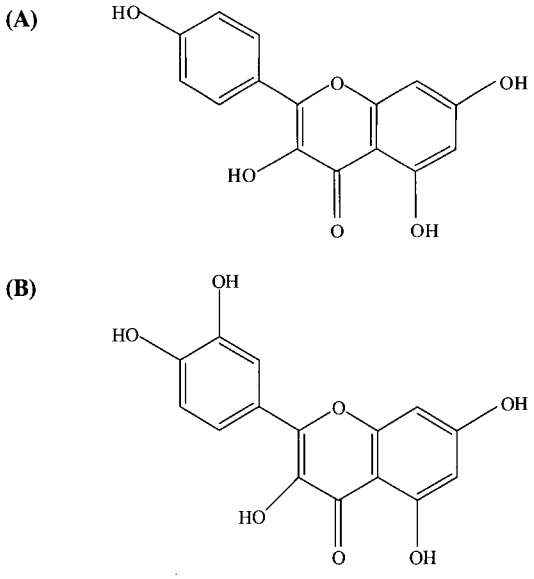

Fig. 1. Structures of kaempferol (A) and quercetin (B).

Table 1. Growth inhibition of kaempferol against *P. acnes* KTCT 3314

Amount used (μg)[a]	Inhibition zone (mm)
0[b]	0
30	20.33±0.44
60	25.83±0.22

[a]Dissolved in DMSO
[b]DMSO only

effect against *P. acnes*, and found that it acted in a concentration-dependent manner (Table 1). The antimicrobial activity of kaempferol was evaluated and compared with that of quercetin which has a very similar structure with kaempferol (Fig. 1).

The MICs and MBCs of kaempferol and quercetin against *P. acnes*, along with ampicillin, penicillin G, chloramphenicol, erythromycin, tetracycline, and clindamycin, are shown in Table 2. Under the conditions used, the clindamycin-resistant strain required twice the concentration of kaempferol or quercetin that the clindamycin-sensitive strain required to inhibit their growth. However, there was no difference between clindamycin-sensitive and -resistant strains in their degree of sensitivity to kaempferol and quercetin, which were 32 μg/ml and 64 μg/ml for clindamycin-sensitive and -resistant strains, respectively. In the case of MBC, the clindamycin-resistant strain required kaempferol or quercetin at a concentration of twice that required by the clindamycin-sensitive strain. The MICs of clindamycin and erythromycin were both ≤0.0625 μg/ml against clindamycin-sensitive *P. acnes* and were ≤32 μg/ml and ≤512 μg/ml, respectively, against antibiotic-resistant *P. acnes*. The drug-free positive growth control demonstrated good growth. To check any contaminating organisms, aerobic purity plates, nutrient agar and sheep blood agar plates were incubated at 37°C in either ambient air or 5% CO_2 for 48 h. There was no growth in any of the conditions. The cell-free negative growth control was also free of any growth.

Assessment of synergy

To investigate the efficacy of a combination therapy containing an antibiotic and a non-antibiotic antimicrobial agent on the treatment of antibiotic-resistant *P. acnes*, checkerboard tests were performed with antibiotics and flavonols. Combination tests against clindamycin-sensitive *P. acnes* were not performed, because the growth of sensitive strains is easily inhibited with a very low concentration of clindamycin or erythromycin alone. The results of the checkerboard tests are summarized in Table 3. All the combinations except that of kaempferol with quercetin were synergic according to the FIC index. The combination of kaempferol and quercetin showed an additive effect. The two compounds have a similar structure, suggesting that they probably inhibit the growth of *P. acnes* by the same mechanism of action. From the most synergic concentrations of antibiotics in the checkerboard results, the amount (4 μg/ml) of clindamycin combined with kaempferol or quercetin required to inhibit antibiotic-resistant *P. acnes* was less than an eighth of the amount (32 μg/ml) required using clindamycin alone. In this case, the amounts of kaempferol and quercetin required to inhibit antibiotic-resistant *P. acnes* were also reduced from 64 μg/ml to 4 μg/ml and from 64 μg/ml to 8 μg/ml, respectively. In the case of combining erythromycin with kaempferol or quercetin, the amount of erythromycin was reduced to a fourth of the amount required when used alone and the amounts of kaempferol and quercetin required were both 1 μg/ml.

Discussion

Clindamycin and erythromycin are commonly used topical antibiotics for treatment of acne (Krautheim and Gollnick, 2004). One of the major problems in the use of topical antibiotics is the development of resistance. Clindamycin inhibits protein synthesis with the same mechanism of action

Table 2. Antimicrobial activity of kaempferol, quercetin, and other antibiotics against clindamycin-sensitive and -resistant *P. acnes*

Antimicrobial agent	Clindamycin-sensitive strain		Clindamycin-resistant strain	
	MIC (μg/ml)	MBC (μg/ml)	MIC (μg/ml)	MBC (μg/ml)
Kaempferol	≤32	≤128	≤64	≤256
Quercetin	≤32	≤64	≤64	≤128
Chloramphenicol	≤0.25	≤0.5	≤0.5	≤0.5
Erythromycin	≤0.0625	≤0.0625	≤512	≤1024
Tetracycline	≤0.25	≤4	≤0.25	≤4
Penicillin G	≤0.0625	≤0.0625	≤0.0625	≤0.0625
Ampicillin	≤0.0625	≤0.0625	≤0.0625	≤0.0625
Clindamycin	≤0.0625	≤0.0625	≤32	≤64

Table 3. Checkerboard results for clindamycin-resistant *Propionibacterium acnes* strain

Drug combination[a]	FIC index	Most synergic concentration (μg/ml)	Maximum concentration tested (μg/ml)
KAE/QUE	0.516-0.750	No synergy	128/128
KAE/ERY	0.266-1.125	1/128	128/512
KAE/CLI	0.187-0.562	4/4	128/64
QUE/ERY	0.266-1.125	1/128	128/512
QUE/CLI	0.250-0.562	8/4	128/64

[a]KAE, kaempferol; QUE, quercetin; ERY, erythromycin; CLI, clindamycin

as erythromycin, which provides a latent potential for the creation of cross resistance. Clindamycin-resistant *P. acnes* used in this study also expressed erythromycin resistance. Clindamycin- and erythromycin-resistant strains of *P. acnes* have been isolated worldwide (Coates *et al.*, 2002). Therefore, research trials on the use of combination treatments of topical antibiotics have been conducted to reduce the incidence of antibiotic-resistant *P. acnes*. Our combination treatments studies demonstrated that the four combination formulations (kaempferol and either erythromycin or clindamycin; quercetin and either erythromycin or clindamycin) exhibited a synergic inhibition of *P. acnes* growth. Although the combination of erythromycin with kaempferol or quercetin showed synergic effects, those combinations are not clinical practical because a high concentration of erythromycin is required to produce a synergic effect, which results in hepatotoxicity (Viluksela *et al.*, 1996). However, in the combination, the most synergic concentrations of kaempferol and quercetin were lower than those required when they were used with clindamycin.

Clindamycin and erythromycin are bacteriostatic antibiotics which interact with 23S rRNA in 50S ribosomal subunits and inhibit protein synthesis. Flavonols, morin, and quercetin are known to inhibit the synthesis of DNA (Meltz and MacGregor, 1981; Arima *et al.*, 2002). On the other hand, the antibacterial inhibitory mechanism of kaempferol has not been elucidated. However, we assume that kaempferol has the same mechanism of inhibition as quercetin has, because the structure of kaempferol is very similar to that of quercetin and the MICs of both are the same. The synergic effects in combinations of flavonols with erythromycin or clindamycin shown in Table 3 were based on the cooperation action of the inhibition of the DNA synthesis of flavonols and the inhibition of the protein synthesis of erythromycin or clindamycin. In addition, although the MICs of kaempferol and quercetin against antibiotic-resistant *P. acnes* were twice those against antibiotic-sensitive *P. acnes*, it could not be confirmed whether *P. acnes* causes resistance against them (Table 2). Further studies are needed to elucidate the clear inhibitory mechanism of kaempferol.

Although clindamycin-resistant *P. acnes* is susceptible to ampicillin and penicillin, the antibiotics for the treatment of acne are generally limited to lipid-soluble compounds. In this respect, kaempferol and quercetin have an advantage of being applicable to treat acne because of their lipid-soluble feature. Retinoids are used to treat acne alone or in combination with antibiotics. However, it has been reported that they cause skin irritation and are associated with teratogenicity (Krautheim and Gollnick, 2004). The extract of *Impatiens balsamina* has been regulated as being an approved food additive in Korea and Japan, which ensures the safety of kaempferol. Flavonols from natural sources have been widely used for antimicrobial agents. The combinations of kaempferol or quercetin with rutin, a flavonol which does not show antimicrobial activity in itself, enhanced the antibacterial activities against *Salmonella enteritidis* (Arima *et al.*, 2002). Myricetin, a natural flavonoid, inhibited extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* at a high concentration (MIC_{50} value, 128 μg/ml) and it showed a significant synergic effect in combination with antibiotics against ESBL-producing *K. pneumoniae* (Lin *et al.*, 2005). Kaempferol has a moderate antibacterial activity, in addition to its strong anti-melanin synthesis activity and inhibition activity on COX (Liang *et al.*, 1999; Lim *et al.*, 2006). Therefore, in combination with its approved safety, kaempferol is very attractive in the treatment of acne by inhibiting the growth of *P. acnes* and also inhibiting PG synthesis via the inhibition of COX activity.

In conclusion, kaempferol and quercetin showed synergic effects in the presence of clindamycin or erythromycin, although we could not clearly elucidate their inhibitory mechanisms against *P. acnes*. The results obtained here cannot be applied directly in clinical trials, but we consider that the combination treatment of flavonols and antibiotics will prove to be helpful to treat antibiotic-resistant *P. acnes*.

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