J. Microbiol. Biotechnol. (2007), **17**(3), 530–533

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Antimicrobial Effects of Flavone Analogues and Their Structure-Activity Relationships

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Received: October 3, 2006
Accepted: November 26, 2006

Abstract It has been well known that the use of *Saccharomyces cerevisiae* can cause fungemia in critically ill patients and flavone shows an antimicrobial effect on *S. cerevisiae*. Therefore, we have investigated the activities of thirteen flavone analogues on *S. cerevisiae* in our studies. Because flavonoids including flavones have antioxidative effects, we try to carry out the activity studies of flavone analogues *in vitro* and *in vivo*. In addition, the relationships between the structures of flavone analogues and their biological activities, such as antimicrobial and antioxidative effects, were elucidated using Comparative Molecular Field Analysis calculations. Of the flavone analogues tested here, 3,2'-dihydroxyflavone showed both good antimicrobial and antioxidative activities.

Keywords: Antimicrobial, flavonoid, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, antioxidative effect

From ancient times, *Saccharomyces cerevisiae* has been the most important yeast because of its use in baking and brewing, and one of the most studied eukaryotic organisms [4, 5, 9, 14, 17]. Furthermore, it was the first eukaryotic genome that was completely sequenced. Its genome is composed of about 13,000,000 base pairs and 6,275 genes, although only about 5,800 of these are believed to be true functional genes [3]. It is estimated that yeast shares about 23% of its genome with that of humans and reproduces by a division process known as budding. In addition, it is comparatively similar in structure to human cells, both being eukaryotic, in contrast to the prokaryotes. Apparantly, many important proteins in human biology were first discovered by studying their homologues in yeast [4, 10, 11, 16].

S. cerevisiae can cause disease in humans. In particular, it is marketed as a probiotic supplement (Ultralevura) for the treatment of *Clostridium difficile* colitis [2]. Unfortunately, its use can cause fungemia in critically ill patients [8]. Because its usefulness is much greater than its harmfulness, however, it is still used in baking and brewing.

It has been reported that flavone, one of natural products originated from plants, showed antimicrobial effect on *S. cerevisiae* [13]. Flavone belongs to the flavonoids, which are found from various plant sources and composed of a C6-C3-C9 skeleton. According to the oxidation condition of the pyran ring placed at the center of flavonoids, flavonoids can be further subdivided into five major subclasses as follows: flavonols, flavanols, flavones, isoflavones, and anthocyanidins [7]. Flavonoids show many biological activities such as antioxidative effects and proliferative effects. In this study, fourteen flavone analogues were tested for their antimicrobial and antioxidative effects, and those analogues are as follows: flavone (**1**), 2',3'-dihydroxyflavone (**2**), 2',4'-dihydroxyflavone (**3**), 2',4'-dimethoxyflavone (**4**), 2'-dihydroxyflavone (**5**), 3',4'-dihydroxyflavone (**6**), 3',4'-dimethoxyflavone (**7**), 3,2'-dihydroxyflavone (**8**), 3,2'-dimethoxyflavone (**9**), 3,4'-dimethoxyflavone (**10**), 3-hydroxy-2'-methoxyflavone (**11**), 3-hydroxy-3'-methoxyflavone (**12**), 5-hydroxyflavone (**13**), and 6-hydroxyflavone (**14**) (Fig. 1).

In order to test antimicrobial effects, *Saccharomyces cerevisiae* was cultured in YM broth (3 g yeast extract, 3 g malt extract, 10 g peptone, 10 g dextrose in 1 l distilled water) at 30°C, 180 rpm for 24 h. One ml of cultured broth was spread on the square plate (245×245×20 mm) with Sabouraud dextrose agar. On the top of the cultured broth, paper discs (Ø 6 mm) were placed and flavone analogue samples were dropped. After 24 h at 30°C, inhibitory zones were measured. Because flavone analogues were dissolved in dimethylsulfoxide (DMSO), it was used as a control, and kanamycin and hygromycin were tested to compare with known antimicrobial agents. The concentrations of samples and two antimicrobial agents were 100 mM and 50 mg/mL, respectively. Aliquots of 1 μl and 10 μl of each

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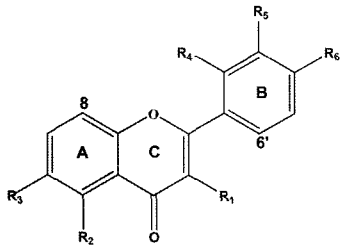

Flavone analogues	Substituents	Name
analogue 1	R_1=R_2=R_3=R_4=R_5=R_6=H	Flavone
analogue 2	R_1=R_2=R_3=R_6=H, R_4=R_5=OH	2',3'-Dihydroxyflavone
analogue 3	R_1=R_2=R_3=R_5=H, R_4=R_6=OH	2',4'-Dihydroxyflavone
analogue 4	R_1=R_2=R_3=R_5=H, R_4=R_6=OCH_3	2',4'-Dimethoxyflavone
analogue 5	R_1=R_2=R_3=R_5=R_6=H, R_4=OH	2'-Hydroxyflavone
analogue 6	R_1=R_2=R_3=R_4=H, R_5=R_6=OH	3',4'-Dihydroxyflavone
analogue 7	R_1=R_2=R_3=R_4= H, R_5=R_6=OCH_3	3',4'-Dimethoxyflavone
analogue 8	R_2=R_3=R_5=R_6=H, R_1=R_4=OH	3,2'-Dihydroxyflavone
analogue 9	R_2=R_3=R_5=R_6=H, R_1=R_4=OCH_3	3,2'-Dimethoxyflavone
analogue 10	R_2=R_3=R_4=R_5= H, R_1=R_6=OCH_3	3,4'-Dimethoxyflavone
analogue 11	R_2=R_3=R_5=R_6=H, R_1=OH, R_4=OCH_3	3-Hydroxy-2'-methoxyflavone
analogue 12	R_2=R_3=R_4=R_6= H, R_1=OH, R_5=OCH_3	3-Hydroxy-3'-methoxyflavone
analogue 13	R_1=R_3=R_4=R_5=R_6=H, R_2=OH	5-Hydroxyflavone
analogue 14	R_1=R_2=R_4=R_5=R_6=H, R_3=OH	6-Hydroxyflavone

Fig. 1. Structures and nomenclatures of flavone and its thirteen analogues.

sample were dropped on paper discs. All experiments were performed twice and their results were exactly consistent with each other. The results showing the inhibitory effects are listed in Table 1 and the results of their paper discs are shown in Fig. 2.

Table 1. The antimicrobial effects of flavone analogues.

Derivative	Name	Antimicrobial effects (diameter/mm)	
		1 μl	10 μl
1	Flavone	ND	2 mm
2	2',3'-Dihydroxyflavone	ND	ND
3	2',4'-Dihydroxyflavone	ND	ND
4	2',4'-Dimethoxyflavone	ND	ND
5	2'-Hydroxyflavone	ND	ND
6	3',4'-Dihydroxyflavone	ND	ND
7	3',4'-Dimethoxyflavone	ND	ND
8	3,2'-Dihydroxyflavone	1 mm	3 mm
9	3,2'-Dimethoxyflavone	ND	1 mm
10	3,4'-Dimethoxyflavone	ND	1 mm
11	3-Hydroxy-2'-methoxyflavone	ND	1 mm
12	3-Hydroxy-3'-methoxyflavone	ND	1mm
13	5-Hydroxyflavone	ND	ND
14	6-Hydroxyflavone	ND	ND
	Kanamycin	ND	ND
	Hygromycin	2 mm	5 mm
	DMSO	ND	ND

ND: not detected.

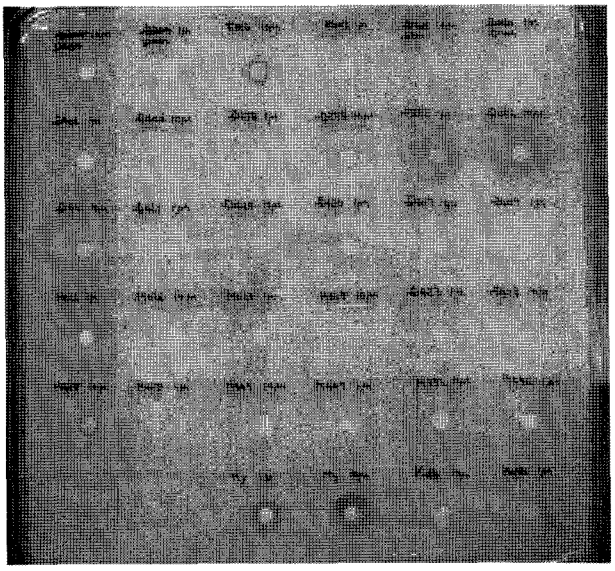

Fig. 2. The antimicrobial effects of flavone analogues. The concentration of the samples was 100 mM. Aliquots of 1 μl and 10 μl of each sample were dropped on paper discs.

The analogue with the strongest effect was 3,2'-dihydroxyflavone (**8**), which showed the effect at 10 μl as well as 1 μl. In order to elucidate the relationships between the antimicrobial effects and the structures, structure-activity relationships (SAR) were carried out on a Linux Pentium 3.2 GHz PC using Comparative Molecular Field Analysis (CoMFA) calculations with a set of 14 analogues. The software for the calculation was Sybyl 7.2 (Tripos, St. Louis, MO, U.S.A.). The CoMFA contour map represents the steric field values that correlate with the biological activity and it is illustrated in Fig. 3, where the contours are shown in green (more bulk favored) and yellow (less

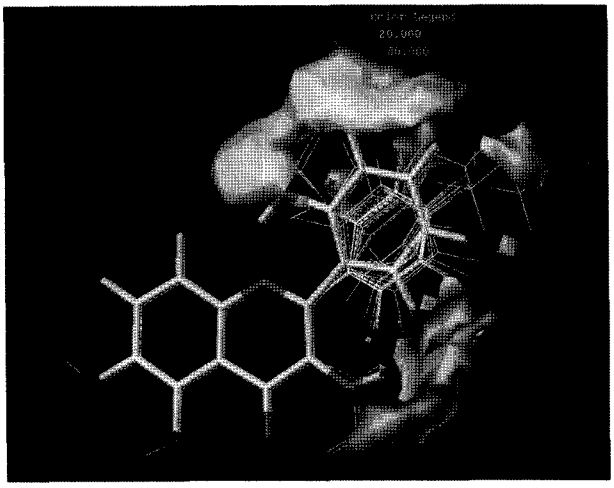

Fig. 3. The CoMFA contour map representing the steric field values by showing the contours in green (more bulk favored) and yellow (less bulk favored).

bulk favored). The analysis revealed two green colored contours and one yellow colored contour. The favorable green contour is seen near the C-3 position of the C-ring, so that the analogues with bulky C-3 substituents show good antimicrobial activity. Furthermore, those compounds that satisfy this requirement are as follows: 3,2'-dihydroxyflavone (**8**), 3,2'-dimethoxyflavone (**9**), 3,4'-dimethoxyflavone (**10**), 3-hydroxy-2'-methoxyflavone (**11**), and 3-hydroxy-3'-methoxyflavone (**12**). Another green contour is placed at the surface area of C-2', C-3', and C-4' of the B-ring. Likewise, analogues **8**, **9**, **10**, **11**, and **12** contain substituents at C-2', C-3', and C-4'. On the other hand, the yellow contour is seen near the center of B-ring, so that plane B-ring is favorable.

In order to investigate antioxidative effects of flavone analogues, 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical-scavenging effects were tested according to the methods previously reported by Ahn *et al.* [1]. The data are reported as the mean of three independent experiments, and were evaluated by Student's *t* test. Values of $P<0.05$ were considered to be statistically significant. The scavenging effect of vitamin C used as a reference was 88.0%, and the effects of 14 flavone analogues are listed in Table 2, where 2',3'-dihydroxyflavone (**2**), 3',4'-dihydroxyflavone (**6**), and 3,2'-dihydroxyflavone (**8**) showed statistically significantly ($P<0.05$) good antioxidative effects. In order to elucidate structure-activity relationships, SAR was performed as the method mentioned above. The analysis of the electrostatic contours revealed three red contours (electronegative substituents favored), which were near the 3-hydroxyl group in the C-ring and C-2' and C-4' in the B-ring (Fig. 4). It means that the molecules including **2**, **6**, and **8** could exhibit good activity. Even though dihydroxylated flavone analogues showed good activities, the position of dihydroxyl groups was important. 2',4'-Dihydroxyflavone contains two hydroxyl groups, but it showed worse activity than **2**, **6**, and **8**. Therefore, it seems that *ortho* dihydroxyl groups exhibit good activity, whereas *meta* dihydroxyl groups do not. Among 14 flavone analogues tested in this study, analogue **2**, 2',3'-dihydroxyflavone satisfying the conditions mentioned above, showed the best scavenging effect for DPPH radicals (85.8%).

Table 2. The effects of flavone analogues in DPPH radical-scavenging.

Derivative	Name	Antioxidative effects (%)
1	Flavone	4.2
2	2',3'-Dihydroxyflavone	85.8
3	2',4'-Dihydroxyflavone	40.4
4	2',4'-Dimethoxyflavone	2.2
5	2'-Hydroxyflavone	4.5
6	3',4'-Dihydroxyflavone	80.6
7	3',4'-Dimethoxyflavone	1.4
8	3,2'-Dihydroxyflavone	83.7
9	3,2'-Dimethoxyflavone	2.2
10	3,4'-Dimethoxyflavone	0.9
11	3-Hydroxy-2'-methoxyflavone	15.4
12	3-Hydroxy-3'-methoxyflavone	45.5
13	5-Hydroxyflavone	2.1
14	6-Hydroxyflavone	13.5
	Vitamin C	88.0

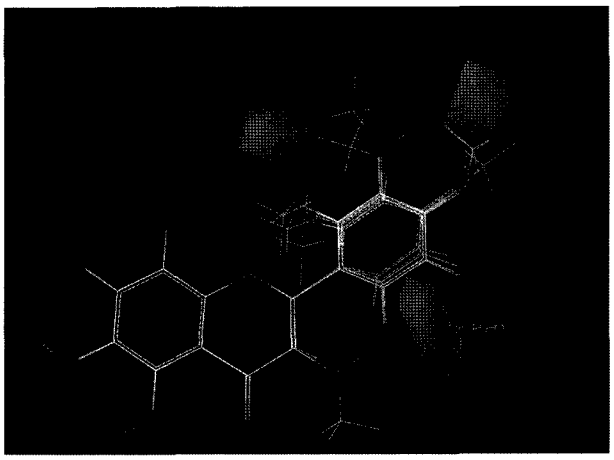

Fig. 4. CoMFA contour specification showing electrostatic sites (Red contours: negative potential is favored).

To evaluate *in vivo* antioxidative activities of flavone analogues, *Caenorhabditis elegans* was used. The experimental procedure followed the methods reported previously [12, 15]. Because flavone was dissolved in DMSO, worms were also grown on plates containing 0.5% DMSO. The activities of several flavone analogues tested in *C. elegans* were detected by examining survival rates of worms after treatment with paraquat (1,1'-dimethyl-4-4'-bipyridinium dichloride hydrate; Sigma-Aldrich, St. Louis, MO, U.S.A.) to generate oxidative stress. The survival rate of worms grown in normal plate treated in 0.5% DMSO was 26.1%. According to the analysis of the oxidative stress study, the survival rate was influenced by the nature and positions of substituents around the B-ring in flavone analogues and the results are as follows: analogue **2** with *ortho* dihydroxyl groups, 2.3-fold; analogue **3** with *meta* dihydroxyl groups, 1.5-fold; analogue **12** with one hydroxyl group and one methoxyl group, 1.4-fold; and analogue **4** with dimethoxyl groups, almost 0-fold increase in comparison with the survival rate in normal plates. As a result, the *in vivo* antioxidative activities are consistent with *in vitro* activities.

Consequently, the antimicrobial activity of 3,2'-dihydroxyflavone on *S. cerevisiae* can compete with that of hygromycin, and the DPPH radical-scavenging effect of 2',3'-dihydroxyflavone also can compete with that of vitamin C. Therefore, 3,2'-dihydroxyflavone has both good antimicrobial and antioxidative activities.

Acknowledgments

This work was supported by grant KRF-2006-005-J03402 (KRF), grant R01-2004-000-10688-0 (KOSEF), and a grant from the second BK21 (MOE). Young, Park, and Lee contributed equally to this work.

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