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ANTIBACTERIAL ACTIVITY AND MODE OF ACTION OF PLANT FLAVONOIDS AGAINST *PROTEUS VULGARIS* AND *STAPHYLOCOCCUS AUREUS*

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Key Word Index—*Elaeagnus glabra*; Elaegnaceae; (−)-epigallocatechin; flavonoids; antibacterial activity; DNA synthesis; RNA synthesis.

Abstract—(−)-Epigallocatechin, an antibiotic found in *Elaeagnus glabra*, and 28 other related plant flavonoids were tested for their antibacterial activity against *Proteus vulgaris* and *Staphylococcus aureus*. A free 3′,4′,5′-trihydroxy B-ring and a free 3-OH were necessary for antibacterial activity. DNA synthesis was predominantly inhibited by the active flavonoids in *P. vulgaris*, whereas RNA synthesis was inhibited in *S. aureus*.

INTRODUCTION

(−)-Epigallocatechin (compound **27** in Table 1) in the bark of an Okinawan medicinal plant, *Elaeagnus glabra* Thunb., (Elaegnaceae) has been found to be an antibiotic against the human skin bacterium, *Staphylococcus epidermidis* [1]. This flavonoid has also shown to have antibacterial activity against *Proteus vulgaris* (Gram-negative) and *S. aureus* (Gram-positive), and is cytotoxic to HeLa cells [1].

(−)-Epigallocatechin (**27**) belongs to the flavonoid class. Antibacterial and antifungal activities of flavonoids have been reported [2–6], but no systematic study on antibacterial activity of flavonoids has yet been carried out. In this work, **27** and 28 related flavonoids are assayed for antibacterial activity against *P. vulgaris* and *S. aureus*. This paper also reports structure–activity relationship and effect of several active flavonoids on macromolecular and lipid synthesis in the bacteria.

RESULTS

Antibacterial activity

Antibacterial activity of the flavonoids is displayed in Table 1. At 50–200 μg/ml (MIC) (100 μg/ml mostly), six flavonoids were active against *P. vulgaris*, whereas five were active against *S. aureus*. Among the active flavonoids, datiscetin (**8**) and quercetagetin (**14**) were active against *P. vulgaris* only (100 μg/ml) and 7,8-dihydroflavone (**2**) against *S. aureus* only (100 μg/ml). Robinetin (**15**), myricetin (**16**), (+)-dihydrorobinetin (**24**) and (−)-epigallocatechin (**27**) were active against both bacteria (50–200 μg/ml).

Incorporation of radioactive precursors

Proteus vulgaris. The incorporation experiments were performed with **14–16** and **27**. Incorporated radioactivity of the precursors was measured depending upon flavonoid concentration and culture period. Radioactivity at each concentration of **14** showed comparable counts to those of controls at any culture period in all the precursors. This means that neither macromolecular (DNA, RNA and protein) nor lipid synthesis were inhibited by **14**. Radioactivity diminished according to increasing flavonoid concentration in 1 hr culture of **15**, **16** and **27**, the degree (%) of inhibition of macromolecular and lipid synthesis in 1 hr culture is shown in Fig. 1 (A). DNA synthesis was most strongly inhibited, followed by RNA synthesis.

Staphylococcus aureus. 7,8-Dihydroxyflavone (**2**) was used in addition to **15**, **16** and **27**. Similarly, % inhibition of the flavonoids to the syntheses is plotted against flavonoid concentrations on the basis of the data at 1 hr culture, and shown in Fig. 1(B). RNA synthesis was predominantly inhibited by **2**, **16**, and **27** (Fig. 1 A, a, c and d) and DNA synthesis by **15** (Fig. 1 B, b). Compounds **15** and **27** exhibited much weaker inhibitory effect on the syntheses in *S. aureus* than that in *P. vulgaris*.

DISCUSSION

Although antibacterial activity has often been described for flavonoids [2–5], structure–activity relationships are not known as they are in the case of antifungal activity [6].

No specific structural factor was effective against both *P. vulgaris* or *S. aureus* from the present data. However, when the structures of the flavonoids (**15**, **16**, **24** and **27**), which were active against both bacteria, were inspected, several important structural factors are apparent. A free hydroxyl group on the aromatic A- and B-ring was necessary. The flavonoids without such free hydroxyls (**1** and **17**) possessed no activity, while all the active species had them. Furthermore, if the hydroxyls of **27** were masked (**28** and **29**), activity was lost.

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Table 1. Structures and antibacterial activity (MIC) of flavonoids against *Proteus vulgaris* and *Staphylococcus aureus*

	Flavonoid	Substituent on aromatic rings								MIC (μg/ml)§	
		5	6	7	8	2'	3'	4'	5'	*P. vulgaris*	*S. aureus*
Flavones	Flavone (1)	H	H	H	H	H	H	H	H	—	—
	7,8-Dihydroxyflavone (2)	H	H	OH	OH	H	H	H	H	—	100
	6,7-Dihydroxyflavone (3)	H	OH	OH	H	H	H	H	H	—	—
	Chrysin (4)	OH	H	OH	H	H	H	H	H	—	—
	Luteolin (5)	OH	H	OH	H	H	OH	OH	H	—	—
	Apigenin-7,4'-dimethyl ether (6)	OH	H	OCH_3	H	H	H	OCH_3	H	—	—
Flavonols	Galangin (7)	OH	H	OH	H	H	H	H	H	—	—
	Datisetin (8)	OH	H	OH	H	OH	H	H	H	100	—
	Kaempferol (9)	OH	H	OH	H	H	H	OH	H	—	—
	Fisetin (10)	H	H	OH	H	H	OH	OH	H	—	—
	Quercetin (11)	OH	H	OH	H	H	OH	OH	H	—	—
	Rhamnetin (12)	OH	H	OCH_3	H	H	OH	OH	H	—	—
	Morin (13)	OH	H	OH	H	OH	H	OH	H	100	—
	Quercetagetin (14)	OH	OH	OH	H	H	OH	O	H	100	—
	Robinetin (15)	H	H	OH	H	H	OH	OH	OH	100	100
	Myricetin (16)	OH	H	OH	H	H	OH	OH	OH	50	100
Flavanones*	Flavanone (17)	H	H	H	H	H	H	H	H	—	—
	4',5,7-Trihydroxy-flavanone (18)	OH	H	OH	H	H	H	OH	H	—	—
	Eriodictyol (19)	OH	H	OH	H	H	OH	OH	H	—	—
	Homoeridictyol (20)	OH	H	OH	H	H	OCH_3	OH	H	—	—
	Hespertin (21)	OH	H	OH	H	H	OH	OCH_3	H	—	—
Flavanonols†	(−)-Fustin (22)	H	H	OH	H	H	OH	OH	H (2β, 3α)	—	—
	(+)-Taxifolin (23)	OH	H	OH	H	H	OH	OH	H (2α, 3β)	—	—
	(+)-Dihydrorobinetin (24)	H	H	OH	H	H	OH	OH	OH (2α, 3β)	200	200
Catechins†	(+)-Catechin (25)	OH	H	OH	H	H	OH	OH	H (2α, 3β)	—	—
	(−)-Epicatechin (26)	OH	H	OH	H	H	OH	OH	H (2α, 3α)	—	—
	(−)-Epigallocatechin (27)	OH	H	OH	H	H	OH	OH	OH (2α, 3α)	50	100
	Methyl ether of 27 (28)‡	OCH_3	H	OCH_3	H	H	OCH_3	OCH_3	OCH_3 (2α, 3α-OH)	—	—
	Acetate of 27 (29)‡	OAc	H	OAc	H	H	OAc	OAc	OAc (2α, 3α-OAc)	—	—

*All are racemates.

†All are optically active compounds.

‡3-OH is free in **28**, whereas it is acetylated in **29**.

§— means inactive at 200 μg/ml in MIC.

The 3',4',5'-trihydroxy B-ring and the 3-OH were common structural moieties to all the active flavonoids. This type of B-ring coupled with the 3-OH may be, accordingly, the most important structural unit for antibacterial activity against *P. vulgaris* and *S. aureus*. This was confirmed by the data for the following two pairs of flavonoid series: inactive **10** and **26** were changed to active **15** and **27**, respectively, by adding a 5'-hydroxyl in the B-ring.

As with the antifungal activity of flavonoids [6], antibacterial activity of **24** and **27** is given by a non-planar flavonoid structure. This contrasts with the data of ref. [3] which illustrated the importance of the 2,3-double bond (namely, molecular planarity) of flavonoids for the activity. The importance of planarity of structure has also been reported in several pharmacological tests [7–9]. As found previously [1], 2,3-*cis* substituion (**27**) provides greater activity in the non-planar flavonoids series than 2,3-*trans* substitution (**24**).

The 4-carbonyl group was less important, because of the activity of **27**. Elimination of the carbonyl group usually decreases biological activity of flavonoids [9, 10]. However in the case of compound (**27**) which lacks a 4-carbonyl group, the 3-OH might function in controlling the hydrophobicity of the molecule.

It is known that lipid content is greater in the cell wall of Gram-negative bacteria than Gram-positive bacteria. More lipophilic **2** may be trapped into the cell wall of *P. vulgaris*, whereas it passes through the *S. aureus* cell wall; this may explain why **2** is active against *S. aureus* only. (−)-Epigallocatechin (**27**), which is hydrophilic, appears to permeate to *P. vulgaris* and *S. aureus* cell walls

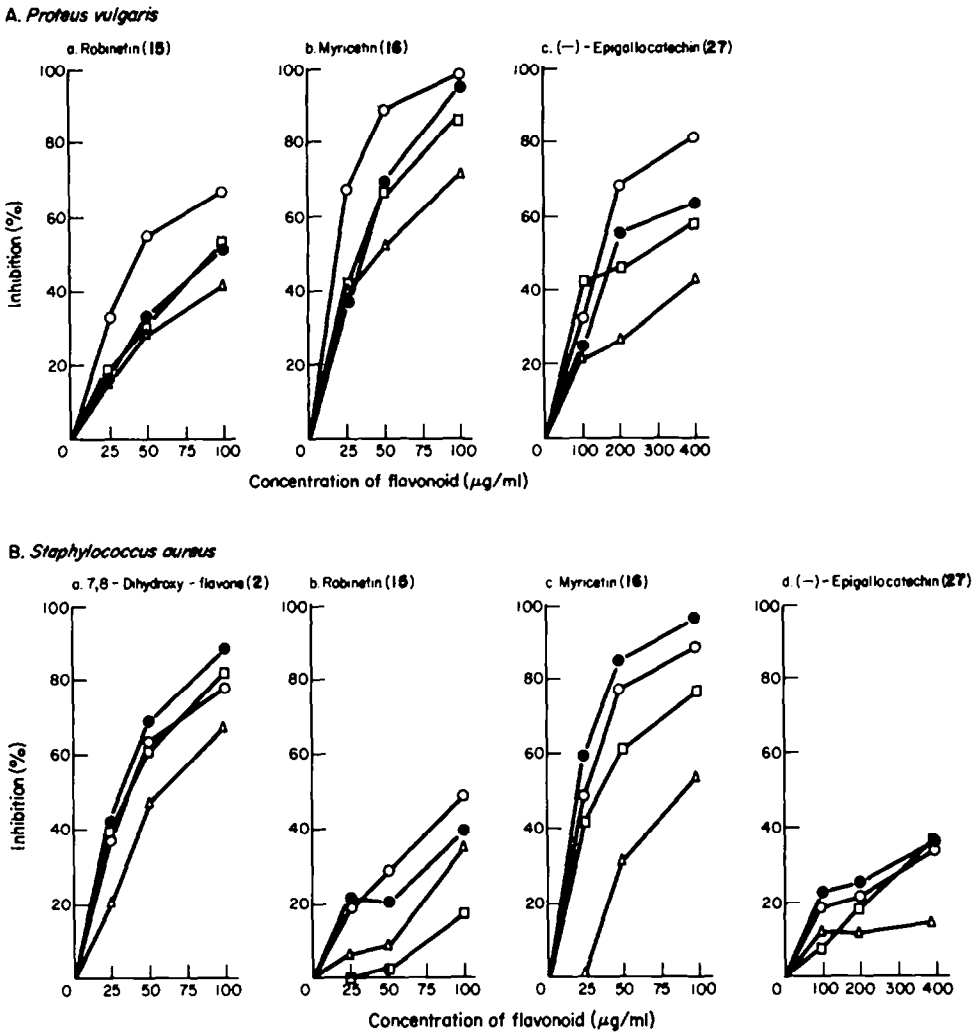

Fig. 1. Inhibitory effect of flavonoids on macromolecular [DNA (O), RNA (●) and protein (Δ)] and lipid (□) synthesis in *Proteus vulgaris* (A) and *Staphylococcus aureus* (B). The bacteria were treated with the indicated concentrations of the flavonoids [25, 50 and 100 μg/ml for **2** (B a), **14**, **15** (A a and B b) and **16** (A b and B c), and 100, 200 and 400 μg/ml for **27** (A c and B d)] together with the radioactive precursors (0.1 mCi/ml) shown in the figure. Inhibition percent was calculated on the basis of the incorporation data at 1 hr culture. Since quercetagetin (**14**) showed no inhibitory action to synthesis in *P. vulgaris*, it is omitted.

at different rates. This is reflected in the discrepancy in inhibitory effect on the syntheses between the bacteria (compare Fig. 1 A, c with Fig. 1 B, d). Similar reasoning would explain the different effects of **15** (Fig. 1 A, a and B, b). Hydrophobicity of **16** is weaker than that of **15**, since there is intramolecular hydrogen bonding between the 4-carbonyl group and 5-OH. As a result, the hydrophobicity of **16** allows it to pass through the cell walls of both *P. vulgaris* and *S. aureus*. With flavonoids, their interaction with the cell surface of microorganisms seems to be important [6].

With regard to the mode of action of the antibacterial flavonoids, it is generally recognized that DNA synthesis is most strongly inhibited by flavonoids in *P. vulgaris*, while RNA synthesis is most effected in *S. aureus*. Flavonoids (e.g. **11**) inhibit the synthesis of DNA, RNA and related macromolecules [9–12, 13 and 14] in other pharmacological experiments. Intercalation and mispairing activity towards the nucleic acid bases [9, 10], have sometimes been proposed because of the similar planar structures of flavonoids and the bases of DNA and RNA. However, in this study, the non-planar flavonoids, **24** and **27**, showed antibacterial activity of the 3′,4′,5′-trihydroxy B-ring coupled with the 3-OH must be important for activity. The B-ring, accordingly, may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases, which is reflected in the inhibitory action on DNA or RNA synthesis.

EXPERIMENTAL

Flavonoids. Catechins, **26** and **27** were isolated from *E. glabra* [1], and **28** and **29** were prepared from **27**. The other flavonoids were obtained commercially. The flavanones (**17**–**21**) were race-

mates, while the flavanonols (**22–24**) and catechins (**25–29**) were optically active [1].

Radioactive precursors. [2-^{14}C]Thymidine (51 mCi/mM), [2-^{14}C]uridine (51 mCi/mM) and L-[U-^{14}C]leucine (348 mCi/mM) were purchased from Radiochemical Centre, Amersham, Buckinghamshire, and [1-^{14}C]acetic acid sodium salt (56 mCi/mM) was obtained from New England Nuclear Corp.

Antibacterial activity. The activity was measured by the standard agar dilution method for MIC determination [15] with a heart infusion agar (E-MC10, Eiken Chemical Co.). Inocula of 1/100 dilution of *Proteus vulgaris* OX-19 or *Staphylococcus aureus* FDA 209 PJC-1, prepared by dilution of a fresh 18–20 hr (37°) broth culture, were applied to agar plates [Petri dish (9 cm in diameter)] each of which had been mixed with a concentration of a flavonoid (200–12.5 μg/1 ml of 5% DMSO). After incubation of the agar plates for 18–20 hr at 37°, the minimal inhibitory concentration (MIC, μg/ml) was determined.

Incorporation of radioactive precursors into bacterial macromolecules and lipid. For the incorporation experiments, flavonoids, **2**, **14–16** and **27** were used: **2** was active against *S. aureus* only, whereas **14** against *P. vulgaris* only. The others were effective against both bacteria.

A bacterium (*P. vulgaris* or *S. aureus*) was grown in a heart infusion broth (E-MC 68, Eiken Chemical Co.) overnight at 37°, after which the culture was diluted to 10 times in volume with the broth and incubated to show 0.2 absorbance at 600 nm. This exponential stage of the bacterium was used for the incorporation experiments (5 ml culture for DNA, RNA and protein synthesis and 7 ml for lipid synthesis).

Each radioactive precursor (0.1 mCi/1 ml culture) and flavonoid (a specified quantity/200 μl DMSO) were added to the culture, and incubated. For measurement of DNA, RNA and protein synthesis, samples (0.5 ml) were harvested periodically (at 1, 1.5 and 2 hr) and the reaction was stopped by adding 5%-TCA (2.5 ml). The TCA-insoluble residue was collected on a glass filter (Whatman GF/C). The filter was washed with TCA and EtOH, dried and counted in a toluene based scintillation liquid (10 ml). For lipid synthesis, a culture sample (1.0 ml) was periodically filtered through the glass filter which was subsequently extracted with $CHCl_3$–MeOH–H_2O (1:2:0.8) (2 ml). After addition of H_2O and $CHCl_3$ (1 ml each), the soln was centrifuged (3000 rpm, 10 min) to separate the $CHCl_3$ layer. Radioactivity of a part (1 ml) of the layer was counted in the scintillation liquid (10 ml).

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