Antibacterial Difference of Plant Flavonoids to Gram-Negative and -Positive Bacteria

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Article

Antibacterial Difference of Plant Flavonoids to Gram-Negative and -Positive Bacteria

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Abstract: The antibacterial activities of plant flavonoids have increasingly paid a more attention to. In our previous works, the antimicrobial quantitative structure-activity relationship was established for plant flavonoids against gram-positive bacteria, and its major action site was confirmed as the cell membrane. To understand the antimicrobial difference of plant flavonoids to gram-negative and -positive bacteria, here the minimum inhibitory concentrations (MICs) of thirty-seven plant flavonoids to *Escherichia coli* were determined using microdilution broth method, and then the correlation between their lipophilic parameter ACD/LogP or $LogD_{7.40}$ values and their MICs were analyzed. Combined with those correlations analyses from forty-six plant flavonoids reported, the results showed that there is also correlation between their LogP values and MICs to Gram-negative bacteria, while it is difficult to predict the MIC of plant flavonoids against gram-negative from their lipophilic parameters like against Gram-positive bacteria. Simultaneously, the antibacterial activities of plant flavonoids to Gram-negative bacteria are generally weak. After compared the character of regression curves established from plant flavonoids against gram-negative and -positive bacteria, it was found that the antibacterial activities of most plant flavonoids to Gram-negative bacteria are greater than to Gram-positive bacteria when their LogP values are less than about 3.0, but less than which when their LogP values are more than about 3.6. Moreover, different from mainly acting on the cell membrane of Gram-positive bacteria, there are probably multiple action modes of plant flavonoids against Gram-negative bacteria, and among them the cell membrane is also an important action.

Keywords: flavonoids; antimicrobial; lipophilicity; LogP; MIC; correlation; bacterium; *Escherichia coli*; gram-negative bacteria

1. Introduction

Antimicrobial resistance (AMR) has been seriously threatened human public health and global economic development, and the COVID-19 pandemic has further accelerated this problem [1]. Thereout, new antimicrobial agents desperately need to be developed [2,3]. After antimicrobial agents have been used for the treatment of bacterial infection, most of them would bring some side effects to the body, and eventually the resistant mutants to them would be also emerged [4]. However, some plant secondary metabolites not only have antimicrobial activities, and also show good safety for human body since they exist in all sorts of plant derived foods and beverages [5,6]. Among them, plant flavonoids have gradually paid a close attention to [7–11].

Flavonoids are an important class of secondary metabolites widely distributed in various plants, and many of them have different degrees of inhibitory activity to many pathogenic bacteria. More importantly, some of them can enhance the antimicrobial activities of some antimicrobial agents, and/or even reverse the AMR [12,13]. Various antibacterial mechanisms involving the synthesis

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inhibitions to DNA, proteins and cell envelope, and the damage of cell membrane were reported for plant flavonoids. Simultaneously, many structure-activity relationships were summarized, while some of them were contradictory [13-15]. Recently, Yuan, *et al*. established that the antimicrobial quantitative relationship of plant flavonoids to Gram-positive bacteria [16], using the statistical analysis and validation of large samples. Based on this relationship, the minimum inhibitory concentrations (MICs) of plant flavonoids against Gram-positive bacteria can be calculated. Moreover, it was pointed out that the cell membrane is the major site of plant flavonoids acting on Gram-positive bacteria, and which includes the damage of phospholipid bilayers and likely involves the inhibition of the respiratory chain, or some others [16,17]. Next, the interfered experiments with menaquinone-4 or menaquinones extracted from *Staphylococcus aureus* further proposed that the quinone pool is a key target [18].

From above, it was spontaneously wondered whether the antimicrobial activities of plant flavonoids to Gram-negative bacteria are related to their lipophilicities, and whether the antimicrobial quantitative relationship can be also established for predicting their MICs. Is there antibacterial difference of plant flavonoids to gram-negative and -positive bacteria? such as their antibacterial effects and mechanisms. Here, the antibacterial structure-activity relationship and possible mechanisms of plant flavonoids to Gram-negative bacteria were further explored according to similar procedure reported [16,17].

2. Results

2.1. Structure, antibacterial activity, and physicochemical parameter

Thirty-seven plant flavonoids (Table 1 and Figure S1), with seven structural subtypes including dihydroflavones, flavones, flavonols, chalcones, isoflavones, isoflavanes and xanthones, were selected for the antimicrobial susceptibility assay and the correlation analyses for the antimicrobial activity MIC and the physicochemical parameter LogP or $LogD_{7.40}$. Their MIC values (μM) against Escherichia coli and S. aureus were respectively listed in Table 1, together with their LogP and $LogD_{7.40}$ values. The results showed that the antimicrobial activities of all these flavonoids to E. coli are very weak with the MICs ranged from 1206.15 to more than 6820.53 μM. This indicated these plant flavonoids with the LogP and $LogD_{7.40}$ values ranged from 1.26 to 6.70 have weak inhibitory activity to Gram-negative bacteria. However, they presented various degrees of antibacterial activities to S. aureus, with the MICs ranged from 9.42 to 13552.14 or more than 7578.45 μM. According to the antimicrobial quantitative relationship reported [16], the MICs (Table 1) of these plant flavonoids to Gram-positive bacterial S. aureus were simultaneously calculated by the equation $y = -0.1285\ x^6 + 0.7944\ x^5 + 51.785\ x^4 - 947.64\ x^3 + 6638.7\ x^2 - 21{,}273\ x + 26{,}087$ [16], and the results showed that the calculated MIC values of 81.1% plant flavonoids can be accepted comparing with those tested. This once again verified that the antimicrobial activities of plant flavonoids to Gram-positive bacteria can be effectively calculated or predicted.

Simultaneosly, the MIC values of fifty-two plant flavonoids (Figure S2) against Gram-negative bacterial E. coli were collected from sixteen papers [19-34], and showed in Table 2, for acquiring wider analysis data. These flavonoids includes eight structural subtypes of plant flavonoids, such as dihydroflavones, flavones, biflavones, flavonols, chalcones, isoflavones and xanthones. Intuitively observed from Table 2, these flavonoids, with the LogP values ranged from 1.53 to 8.03, have various degrees of antibacterial activities to E. coli, with the MICs ranged from 1.49 to 3308.19 or more than 1722.53 μM.

Table 1. Tested plant flavonoids together with their structure types, physicochemical parameters and antibacterial activities.

Compounds (No.)[a]	Structure types	LogP[b]	$LogD_{7.40}$ [b]	*E. coli* ATCC 25922		*S. aureus* ATCC 25923	
				MIC (μM)[c]	Log_{10}(MIC)[c]	MIC (μM)[c]	Predicted MIC (μM)[d]

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Sophoraflavanone G (**1**)	Dihydroflavones	6.52	6.33	1206.15	3.0814	9.42	16.37
Neohesperidin (**2**)	Dihydroflavones	2.44	1.99	1677.12	3.2246	>1677.12	1816.01
Naringin (**3**)	Dihydroflavones	2.73	2.30	1763.88	3.2465	>1763.88	1151.90
Hesperidin (**4**)	Dihydroflavones	1.78	1.33	1677.12	3.2246	>1677.12	4440.63
Methyl hesperidin (**5**)	Dihydroflavones	2.54	2.10	3278.95	3.5157	>3278.95	8410.66
Eriodictyol (**6**)	Dihydroflavones	2.59	2.34	7104.70	3.8515	>7104.70	1442.76
Eriocitrin (**7**)	Dihydroflavones	1.47	1.03	> 3433.13	> 3.5357	>3433.13	6397.02
Hesperitin (**8**)	Dihydroflavones	2.90	2.65	6775.18	3.8309	>6775.18	863.94
Naringenin (**9**)	Dihydroflavones	3.19	2.96	7527.47	3.8766	3763.74	509.64
didymin (**10**)	Dihydroflavones	2.72	2.29	3444.51	3.5371	>3444.51	1170.98
Narirutin (**11**)	Dihydroflavones	2.07	1.65	3527.75	3.5475	>3527.75	3063.61
Baicalein (**12**)	Flavones	3.31	2.60	> 3789.22	> 3.5785	>3789.22	404.47
Licoflavone C (**13**)	Flavones	4.20	3.77	3026.36	3.4809	3026.36	85.1
Tangeritin (**14**)	Flavones	2.73	2.73	2749.95	3.4393	>2749.95	1151.9
Nobiletin (**15**)	Flavones	2.8	2.80	2544.73	3.4056	>2544.74	1025.21
Vitexin (**16**)	Flavones	1.28	0.45	2368.29	3.3744	2368.29	7888.23
Isovitexin (**17**)	Flavones	1.28	0.15	> 2368.29	> 3.3744	2368.29	7888.23
Diosmin (**18**)	Flavones	2.05	1.23	1682.69	3.2260	>1682.69	3146.25
Rhoifolin (**19**)	Flavones	1.72	0.91	3540.07	3.5490	>3540.07	4777.21
Apigenin (**20**)	Flavones	2.10	1.57	7578.45	3.8796	>7578.45	2942.82
Diosmetin (**21**)	Flavones	3.10	2.55	> 6820.53	> 3.8338	>6820.53	603.30
5-Demethylnobiletin (**22**)	Flavones	2.60	2.28	5273.32	3.7221	>5273.32	1420.23
4',5,7-Trimethoxyflavone (**23**)	Flavones	3.35	3.35	6557.38	3.8167	>6557.38	373.96
Sinensetin (**24**)	Flavones	3.40	3.40	5499.91	3.7404	>5499.91	338.78
Orientin (**25**)	Flavones	1.58	0.72	> 4567.55	> 3.6597	>4567.55	5639.27
Isoorientin (**26**)	Flavones	1.58	0.41	4567.55	3.6597	>4567.55	5639.27
Quercetin (**27**)	Flavonols	2.07	1.40	> 3388.04	> 3.5299	13552.14	3063.61
Galangin (**28**)	Flavonols	2.83	2.16	3789.22	3.5785	>3789.22	974.47
Icaritin (**29**)	Flavonols	5.09	4.54	2779.66	3.4430	2779.66	75.22
Rutin (**30**)	Flavonols	1.95	1.22	> 1677.26	> 3.2246	1677.26	3585.77
Quercitrin (**31**)	Flavonols	2.36	1.63	4567.55	3.6597	>4567.55	2043.95
Isoliquiritigenin (**32**)	Chalcones	3.40	3.26	3995.94	3.6016	3995.94	338.78
Licochalcone A (**33**)	Chalcones	4.95	4.85	3026.00	3.4809	11.82	74.44
Formononetin (**34**)	Isoflavones	3.15	2.91	3817.05	3.5817	>3817.04	549.61
Puerarin (**35**)	Isoflavones	2.14	1.59	4918.58	3.6918	614.82	2787.56
Glabridin (**36**)	Isoflavanes	4.18	4.18	3156.79	3.4992	49.32	86.81
α-Mangostin (**37**)	Xanthones	6.70	6.10	2494.70	3.3970	4.87	8.17

[a]: The structures of these compounds were shown on Figure S1 in supplementary materials. [b]: The LogP and $LogD_{7.40}$ values were calculated using software ACD/Labs 6.0. [c]: MIC, minimum inhibitory concentration; log_{10}(MIC) means Log_{10} of MIC. [d]: The MIC values of plant flavonoids were predicted from the equation the equation $y = -0.1285\ x^6 + 0.7944\ x^5 + 51.785\ x^4 - 947.64\ x^3 + 6638.7\ x^2 - 21{,}273\ x + 26{,}087$ [16].

Table 2. Reported plant flavonoids together with their structure types, physicochemical parameters, and inhibitory antibacterial activities to E. coli ATCC 25922.

Compounds (No.)[a]	**Structure types**	**LogP**[b]	**$LogD_{7.40}$** [b]	**MIC (μM)**[c]	**Log_{10}(MIC)**[c]	**References**
Candidone (**38**)	Dihydroflavones	5.64	5.64	11.35	1.0540	19
Atalantoflavone (**39**)	Flavones	4.31	3.58	761.13	2.8815	19
2'-hydroxyatalantoflavone (**40**)	Flavones	3.87	3.10	726.57	2.8613	19
Neocyclomorusin (**41**)	Flavones	4.30	2.87	18.33	1.2632	19
Neobavaisoflavone (**42**)	Isoflavones	4.68	4.69	23.64	1.3736	19
Daidzein (**43**)	Isoflavones	3.14	2.61	236.83	2.3744	19
Isowighteone (**44**)	Isoflavones	4.78	4.23	380.57	2.5804	19
Isoneorautenol (**45**)	Dihydroisoflavones	4.16	4.16	794.14	2.8999	19
Abyssione-V 4′-*O*-methyl ether (**46**)	Dihydroflavones	8.03	7.79	9.23	0.9652	20
6,8-diprenylgenistein (**47**)	Isoflavones	7.33	7.16	19.19	1.2831	20
Alpinumisoflavone (**48**)	Isoflavones	5.80	4.93	11.60	1.0645	20
Eriodictyol (**49**)	Dihydroflavones	2.59	2.34	867.27	2.9382	21

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Hesperetin (**50**)	Dihydroflavones	2.90	2.65	3308.19	3.5196	21
Neohesperidin (**51**)	Dihydroflavones	2.44	1.99	>1637.81	>3.2143	21
Neoeriocitrin (**52**)	Dihydroflavones	2.13	1.68	1341.07	3.1275	21
Naringin (**53**)	Dihydroflavones	2.73	2.30	>1722.53	>3.2362	21
Naringenin (**54**)	Dihydroflavones	3.19	2.96	2938.37	3.4681	21
5-hydroxy-7,4'-dimethoxyflavone (**55**)	Flavones	3.40	2.78	167.62	2.2243	22
Genkwanin (**56**)	Flavones	2.36	1.75	351.78	2.5463	22
Quercetin-5,3'-dimethylether (**57**)	Flavonols	2.30	1.74	151.38	2.1801	22
Rhamnazin (**58**)	Flavonols	2.51	1.73	302.76	2.4811	22
Rhamnocitrin (**59**)	Flavonols	2.56	1.82	166.52	2.2215	22
7,4', 7'', 4'''-tetramethoxy amentoflavone (TMA) (**60**)	Bioflavones	5.80	4.10	420.47	2.6237	23
Isoginkgetin (IGG) (**61**)	Bioflavones	5.68	4.32	242.29	2.3843	23
Podocarpusflavone A (PFA) (**62**)	Bioflavones	4.15	2.53	452.5	2.6556	23
Nobiletin (**63**)	Flavones	2.80	2.80	439.86	2.6433	24
Kaempferol (**64**)	Flavonols	2.05	1.40	87.34	1.9412	24
Licoflavone C (**65**)	Flavones	4.20	3.77	23.08	1.3632	25
Derrone (**66**)	Isoflavones	5.55	4.82	23.22	1.3659	25
Epimedokoreanin B (**67**)	Flavones	6.59	6.14	>151.49	>2.1804	26
Auriculasin (**68**)	Isoflavones	7.70	6.89	9.51	0.9782	26
Pomiferin (**69**)	Isoflavones	7.44	7.06	19.03	1.2794	26
Gancaonin L (**70**)	Isoflavones	5.03	4.58	22.58	1.3537	26
Mopanin (**71**)	Flavonols	1.94	1.04	>214.59	>2.3316	26
Luteolin (**72**)	Flavones	2.40	1.85	349.36	2.5433	27
Quercetin (**73**)	Flavonols	2.07	1.40	1323.45	3.1217	28
Quercetin 3-*O*-*β*-D-glucosyl (1→4)-*α*-L-rhamnoside (**74**)	Flavonols	2.92	2.20	40.02	1.6023	29
Quercetin-3-*O*-*α*-L-rhamnoside or quercitrin (**75**)	Flavonols	2.51	1.78	108.13	2.0339	29
Entadanin (**76**)	Flavonols	2.00	0.086	36.31	1.5600	29
Kaempferide (**77**)	Flavonols	3.00	2.33	333.03	2.5225	30
Kaempferide-3-*O*-*β*-D-glucoside (**78**)	Flavonols	2.31	1.60	432.52	2.6360	30
Galangin (**79**)	Flavonols	2.83	2.16	370.04	2.5682	30
Tiliroside (**80**)	Flavonols	4.02	3.32	3.36	0.5263	31
Quercetin-3,7-*O*-*α*-L-dirhamnoside (**81**)	Flavonols	1.53	0.53	3.36	0.5263	31
Kaempferol-3,7-*O*-*α*-L-dirhamnoside (**82**)	Flavonols	1.73	0.76	3.46	0.5391	31
Scandenone (**83**)	Isoflavones	7.90	7.12	4.94	0.6937	31
Angusticornin B (**84**)	Chalcones	4.31	4.07	2.87	0.4579	32
Bartericin A (**85**)	Chalcones	5.46	5.77	1.49	0.1732	32
2',4',2-(OH)$_3$-chalcone (**86**)	Chalcones	3.95	3.66	476.08	2.6777	33
2',4',3-(OH)$_3$-chalcone (**87**)	Chalcones	3.35	3.05	179.51	2.2541	33
Isobavachalcone (**88**)	Chalcones	5.49	5.44	>394.6	>2.5962	34
α-Mangostin (**89**)	Xanthones	6.70	6.10	>311.84	>2.4939	34

[a]: The structures of these compounds were shown on Figure S2 in supplementary materials. [b]: The LogP and LogD$_{7.40}$ values were calculated using software ACD/Labs 6.0. [c]: MIC, minimum inhibitory concentration; log$_{10}$(MIC) means Log$_{10}$ of MIC.

2.2. Correlation and regression analyses for the MICs and the physicochemical parameters

To discover some correlations between the physicochemical parameters and the MICs of plant flavonoids against Gram-negative, those flavonoids in Tables 1 and 2 were respectively selected for the statistical analyses. As we pointed out [17], the antimicrobial activities of a compound against different pathogenic bacteria were varied, and even against the same one in different determination conditions. Thereby, the regression analyses were performed for these flavonoids tested and reported, respectively. The regression equations together with their correlation coefficients (r) and coefficients of determination (R^2) were listed in Table 3, and the regression curves from the LogP and MIC or log$_{10}$(MIC) were shown on Figure 1. From Table 3, it indicated that all the r values for euqations (1) to (4) were more than the corresponding critical values of $r_{0.975}$ (35) or $r_{0.975}$ (44), and the parameter LogP was better than the LogD$_{7.40}$ to be selected for the correlation analyses. These indicated that there are correlations between the lipophilic parameter LogP (x) and the MIC or log$_{10}$(MIC) (y) of plant flavonoids to gram-negative bacteria especially E. coli, and that the antibacterial activities of plant flavonoids to gram-negative bacteria are related to their lipophilicities, whether resulted from plant flavonoids tested here or reported. However, all the R^2 values for euqations (1) to (6) were less than 0.45, far less than 1.0. Generally, the closer the R^2 is to 1, the higher the goodness of fit, and the closer the calculated value is, on the whole, to the actual one [16]. Thereby, the smaller R^2 values indicated that the goodness of fit of these regression equations were too low to

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be effectively used for predicting the MIC values of a certain plant flavonoid to Gram-negative bacteria.

Table 3. Regression equations for the correlation between the physicochemical parameter (x) and the antimicrobial activity (y) to gram-negative bacteria especially *E. coli*, of plant flavonoids.

Equation number	Sample numbers (n)	Parameters[a] (x)	Regression equation (r[b])	Coefficient of determination (R^2)
(1)	37	LogP	$y = 154.74x^4 - 2328.1x^3 + 11774x^2 - 23630x + 20574$ (0.3736) [c]	0.1396
(2)			$y = -0.0022x^6 + 0.0574x^5 - 0.5624x^4 + 2.6776x^3 - 6.4916x^2 + 7.5985x + 0.2396$ (0.4714) [d]	0.2222
(3)	37	$LogD_{7.40}$	$y = 51.533x^4 - 648.46x^3 + 2399.6x^2 - 2834x + 5407.4$ (0.3412) [c]	0.1164
(4)			$y = 3.4251x^6 - 99.079x^5 + 1128.5x^4 - 6389x^3 + 18606x^2 - 25841x + 13552$ (0.4108) [c]	0.1688
(5)	46	LogP	$y = -0.0358x^4 + 0.7264x^3 - 5.2356x^2 + 15.438x - 13.244$ (0.6670) [d]	0.4449
(6)			$y = 0.0621x^3 - 0.9417x^2 + 4.0824x - 2.9354$ (0.5875) [d]	0.3452
(7)	46	$LogD_{7.40}$	/ [e]	

[a]: The physicochemical parameter (x) was calculated using software ACD/Labs 6.0. [b]: r, correlation coefficient; the significant level α was set as 0.05, and the critical values of $r_{0.975}(35)$ and $r_{0.975}(44)$ were equal to 0.33 and 0.29, respectively. [c]: The antimicrobial activity (y) was the MIC of a certain flavonoid to Gram-negative bacteria especially *E. coli*. [d]: The antimicrobial activity (y) was the $\log_{10}$(MIC) of a certain flavonoid to Gram-negative bacteria especially *E. coli*. [e]: No correlation could be established.

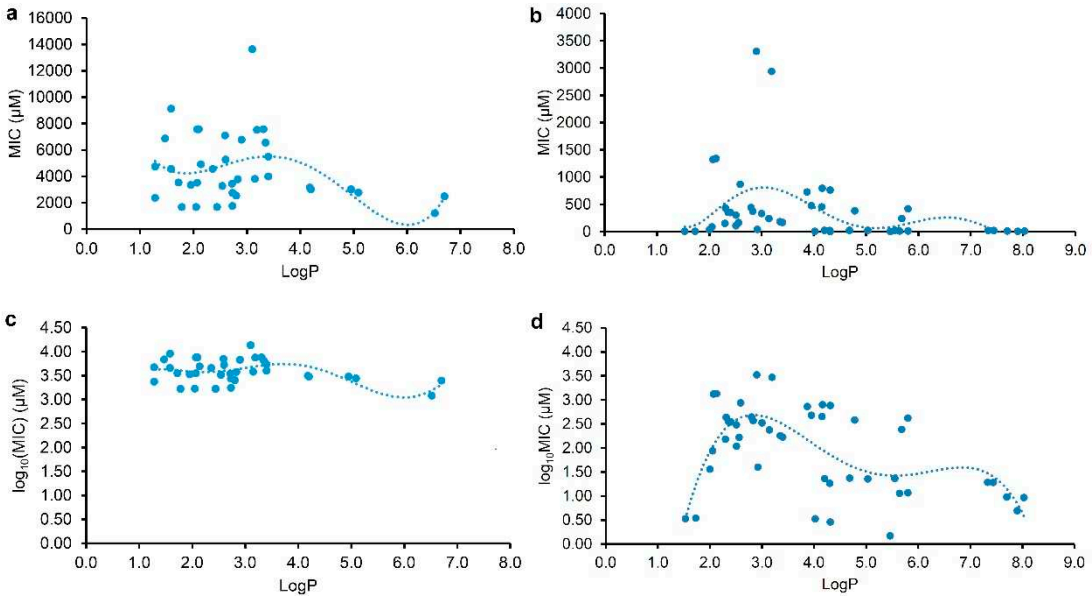

Figure 1. Polynomial regression analyses for the physicochemical parameter LogP (x) and the MIC (up) or $\log_{10}$(MIC) (down) (y) to Gram-negative bacteria mainly E. coli, of plant flavonoids tested (left column, n = 37) and reported (right column, n = 46).

From Figure 1, both curves (Figures 1a and 1c) regressed from the MIC or $\log_{10}$(MIC) and LogP value of tested plant flavonoids presented biconcave characters, and have two minimum values. However, another two curves (Figures 1b and 1d) regressed from those of reported plant flavonoids have three minimum values. Excluding the difference in the range of LogP value, it was found that all of them showed similar characters when the LogP values of plant flavonoids were less than 7.0. Namely, the left two curves have minimum MIC or $\log_{10}$(MIC) values when the logP values of tested plant flavonoids were about 2.0 and 6.0, and while the right two curves have minimum MIC or

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log_{10}(MIC) values when the logP values of reported plant flavonoids were about 1.5 and 5.5. Differently, the right two curves have the third minimum MIC or log_{10}(MIC) values. This was mainly attributed to that the LogP values of some reported plant flavonoids were more than 7.0. Although there were a little difference between the curve shapes of Figure 1a and 1c, another effective fitting equation (6) with a smaller r and R^2 than equation (5) could be also established from the LogP and log_{10}(MIC) of reported plant flavonoids, and its regression curve (Figure S3) is similar to that of Figure 1c. Thereby, these above indicated that that keeping appropriate lipophilicity, probably with the LogP value of about 2.0 (or 1.5), 6.0 (or 5.5), or 7.8, is necessary for plant flavonoids to obtain greater antibacterial activities to gram-negative bacteria, while not like plant flavonoids to gram-positive bacteria that the more the LogP value (from 2.0 to 8.9), the greater the antimicrobial activity presented overall.

2.3. Different actions of plant flavonoids to Gram-negative and Gram-positive bacteria

As Yuan, et al. mentioned [16,17], many factors involving the methods and details of MIC test would have an influence on the experimental results, it is usual fact that the MICs of a compound against the same pathogen are varied large in different labs. Thereby, the MIC data tested in our lab have stronger consistency than those collected from the literature, and so the regression curve of Figure 1c was further selected to compare with the regression curves from the LogP and log_{10}(MIC) of plant flavonoids against Gram-positive bacteria in our previous work [16], for discovering their difference. From Figure 2, the obvious difference of two curves was that most flavonoids likely have greater antimicrobial activities to Gram-negative than Gram-positive bacteria when the LogP value was less than about 3.0. However, most flavonoids likely have greater antimicrobial activities to Gram-positive than Gram-negative bacteria when the LogP value was more than about 3.6. Another, the biconcave (Figure 2a) or even triple concave (Figure 1d) character indicated that there are probably multiple action modes of plant flavonoids against Gram-negative bacteria, while a main cell membrane action of plant flavonoids against Gram-positive bacteria only presents one concave character. As two curves present simialr decreasing character along with the increase of LogP values ranged from about 3.6 to 6.0 (Figure 2), the cell membrane action was probably an important mechanism for some plant flavonoids against Gram-negative bacteria, especially for those with the LogP values ranged from about 3.6 to 6.0 (Figure 1c), or from about 2.8 to 5.5 (Figure 1d).

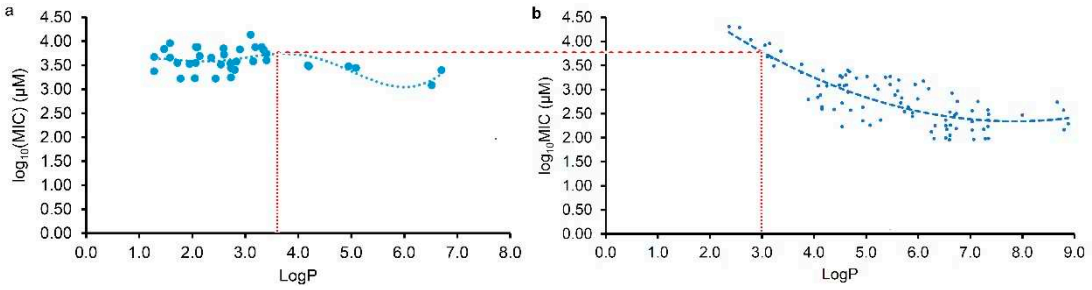

Figure 2. Comparing the regression curves from the LogP and log_{10}(MIC) of plant flavonoids against Gram-negative (**a**) and -positive bacteria (**b**). **a**, same to Figure 1c; **b**, same to the left figure on Figure 3 of our previous work [16].

3. Discussion

To explore whether the antimicrobial activities of plant flavonoids to Gram-negative bacteria can be also predicted from the lipophilic parameter ACD/LogP values like to Gram-positive of plant flavonoids, the correlation and regression analyses were performed respectively using thirty-seven tested and forty-six reported data pairs of the LogP values and MICs of plant flavonoids against Gram-positive bacteria. The results indicated the antibacterial activities of plant flavonoids to Gram-negative bacteria are related to their lipophilicities. However, they cannot be predicted from their LogP values.

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Although both regression curves (Figures 1a and 1b) overall presented similar character along with the change of LogP values, it was noted that the MICs of tested plant flavonoids were obviously higher than those of reported plant flavonoids. This was probably attributed to different determination condition, methods, details, and selected pathogenic strains. There were nine same compounds among tested and reported plant flavonoids, and so their antibacterial activities to *E. coli* ATCC 25922 were reorganized and listed in Table S1 for comparing the difference of tested and reported data [21,24,25,28,30,34]. The results indicated that only the MICs of compound **13** (Licoflavone C) presented more than 10 times difference between tested (3026.36 μM) and reported (23.08 μM) values [25]. Thereby, using broth microdilution method in 96-well plates, the antibacterial activity of this compound to *E. coli* ATCC 25922 was determined by six repeats, and all the MICs of compound **13** against *E. coli* ATCC 25922 were 3026.36 μM.

As mentioned in section 2.3, the antibacterial activities of plant flavonoids, with the logP value less than about 3.6, to Gram-negative bacteria were statistically more than those to Gram-positive ones, and they would also increase overall along with the increase of LogP values when which more than about 3.6. However, the antibacterial activities of most plant flavonoids to Gram-negative bacteria were weak overall. Probably, it is difficult to discover plant flavonoids with very strong antibacterial activities to Gram-negative bacteria.

4. Materials and Methods

4.1. Materials, Chemicals and Reagents

Thirty-seven plant flavonoids: icaritin (>98%), isoliquiritigenin (98%), formononetin (98%), isoliquiritigenin (98%), galangin (98%), baicalein (98%), diosmin (95%), hesperetin (97%), puerarin (98%), apigenin (≥95%), diosmetin (98%) and naringenin (97%) were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China); α-mangostin (>98.0%), licochalcone A (>98.0%), nobiletin (≥98.5%), tangeritin (≥98.5%), quercitrin (98%), sinensetin (98%), narirutin (98%), orientin (99%) and isoorientin (98%) were purchased from Chengdu Push Bio-technology Co., Ltd. (Chengdu, China); naringin (95%), neohesperidin (≥98%) and hesperidin (95%) were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China); eriodictyol (≥98%), eriocitrin (≥98%), rhoifolin (≥98%) and licoflavone C (≥98%) were purchased from Wuhan ChemFaces Biochemical Co., Ltd. (Wuhan, China); glabridin (99.8%) and sophoraflavanone G (>98%) were purchased from Shanghai TopScience Co., Ltd. (Shanghai, China); quercetin (97%) was purchased from Meryer (Shanghai) Biochemical Technology Co., Ltd. (Shanghai, China); methyl-hesperidin (95%) was purchased from Shanghai Acmec Biochemical Co.,Ltd. (Shanghai, China); didymin (≥98%), 5-demethylnobiletin (≥98%), 4',5,7-trimethoxyflavone (≥98%), vitexin (≥98%) and isovitexin (≥98%) were purchased from Sichuan Weikeqi Biological Technology Co.,Ltd. (Sichuan, China). All the compounds were stored at −20°C. The stock solutions of above plant flavonoids were prepared by dissolving in a certain volume of dimethyl sulfoxide (DMSO), and diluted with Mueller Hinton broth (MHB) to obtain a concentration of 4096, 8192 or 16384 μg/mL. The stock solution was mixed well, and then diluted to the desired concentrations with MHB immediately before use. In another, the DMSO concentrations in all the test systems were kept to less than 5.0%, and all those in the blank controls were 5.0%.

Casein hydrolysate (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China), starch soluble, beef extract and agar powder (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China) were used for preparing the media. Mueller Hinton agar (MHA) consisted of casein hydrolysate 17.5 g/L, starch soluble 1.5 g/L, beef extract 3.0 g/L, and agar powder 17.0 g/L dissolving in purified water, and the pH value of 7.40 ± 0.20. MHB was prepared without agar powder according to the same composition and procedure to MHA. DMSO and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China), and 96-well plates were purchased from Shanghai Excell Biological Technology Co., Ltd. (Shanghai, China). All reagents were analytical or biochemical ones. All TopPette Pipettors (2~20 μL and 20~200 μL) were purchased from DLAB Scientific Co., Ltd., Beijing, China.

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4.2. Bacterial Strains and Growth Condition

E. coli ATCC 25922 and *S. aureus* ATCC 25923 were purchased from American Type Culture Collection, Manassas, VA, USA, and this organism was stored in Microbank™ microbial storages (PRO-LAB diagnostics, Toronto, Canada) at −20°C. Prior to use, *E. coli* and *S. aureus* were cultured onto MHA plate at 37°C, and then pure colonies from the plate were inoculated into MHA at 37°C for 24 h on a rotary shaker (160 rpm). A 1:100 dilution of the overnight culture was made into fresh MHB, and then incubated at 37°C until the exponential phase for the following experiments. MHB was used for the antimicrobial susceptibility tests.

4.3. Antimicrobial Susceptibility Assay

According to the standard procedure described by the Clinical and Laboratory Standards Institute (CLSI) [35], the exponential phase culture was diluted with MHB to achieve a bacterial concentration approximately 1.0×10^6 CFU/mL, and then the susceptibility of plant flavonoids against *E. coli* ATCC 25922 or *S. aureus* ATCC 25923 was determined using the broth microdilution method on the 96-well plates in triplicate [4]. Depending on the preliminary MIC values of plant flavonoids, the initial concentration 1024, 2048 or 4096 μg/mL of each compound was respectively set. After the 96-well plate were incubated at 35°C for 24 h, a 20 μL of MTT (4.0 mg/mL) was added into each well, shaking well, and stayed for 30 min at ambient temperature. The MIC, defined as the lowest concentration of compounds that completely inhibited bacterial growth in the micro-wells, was judged from no color change when the bacterial growth in blank wells was sufficient [18].

4.4. Structures and MICs of plant flavonoids reported

As the antimicrobial activities of a certain compound against different pathogenic strains of Gram-negative bacteria were varied, the data collection focused on plant flavonoids having inhibitory activities to E. coli, a representative Gram-negative bacterium. The structures, the MICs to E. coli, and other related information of plant flavonoids were unsystematically searched from Google academic search engine, and several databases SciFinder, Medline, Elsevier, ACS, ScienceDirect, Wiley Online Library, and Springer-Link, using keywords flavonoid and E. coli, or and antimicrobial, or and antibacterial, and or and Gram-negative bacteria. Furthermore, the relevant references in the obtained literature were also tracked. Next, the structures, the MIC values to E. coli, and other related information of plant flavonoids were collected from the obtained literature. Finally, the structures of collected compounds were drawn using software ChemBioDraw Ultra 14.0.

4.5. Correlation and regression analyses

The physicochemical parameters ACD/LogP and $LogD_{7.40}$ of plant flavonoids measured in section 4.3 or previously reported were calculated using software ACD/Labs 6.0. Subsequently, the antimicrobial activities MICs (μM), and the LogP and $LogD_{7.40}$ values of flavonoids measured or reported were respectively listed in an excel table. After this, the correlation analyses between the calculated LogP or $LogD_{7.40}$ values (x) and the MICs (y) of plant flavonoids in the table were performed using Microsoft Excel software. Simultaneously, the corresponding regression equations were established for further verifications, and the value of correlation coefficient (r) was also calculated. It is noting that those compounds (Table 2) without accurate MIC values (such as more than the measuring concentration) were not considered for the regression analyses, while their related information can be used for the following discussion.

The correlation between the antimicrobial activities MICs of plant flavonoids to Gram-negative bacteria and their physicochemical parameters LogP and $LogD_{7.40}$ was validated by r-test, and the coefficient of determination (R^2) was also calculated for judging the fitting precisions according to equation (5) in previous work [16].

4.7. Comparision for the characters of regression curves

Referred previous work [16], the MIC was further transformed to the $\log_{10}$(MIC), and subsequently the regression analysis between the $\log_{10}$(MIC) (y) and the LogP or $LogD_{7.40}$ (x) was further performed. Subsequently, the characters of their regression curves were compared with those of the cell envelope of Gram-negative and -positive bacteria for exploring possible reasons of the antibacterial difference of plant flavonoids to gram-negative and -positive bacteria.

5. Conclusions

In summary, the antibacterial activities of plant flavonoids to Gram-negative bacteria are related to their lipophilicities, while they cannot be statistically predicted from their ACD/LogP values like to Gram-positive bacteria. Simultaneously, the antibacterial activities of plant flavonoids to Gram-negative bacteria are generally weak, and most are greater than those to Gram-positive ones when the lipophilic parameter LogP values are less than about 3.0, but less than which when their LogP values are greater than about 3.6. Different from mainly acting on the cell membrane of Gram-positive bacteria, there are probably multiple action modes of plant flavonoids against Gram-negative bacteria, and among them the cell membrane is also an important action.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Chemical structure of thirty-seven tested plant flavonoids; Figure S2: Chemical structure of fifty-two plant flavonoids reported; Figure S3: Another effective regression curve for equation (6) established from the LogP of reported plant flavonoids and their $\log_{10}$(MIC) values.

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