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Nitrite Scavenging Effect by Flavonoids and Its Structure-Effect Relationship

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Abstract □ Nineteen flavonoids, five phenolics, two coumarins, maltol and L-ascorbic acid were tested as scavenger of nitrite which is believed to participate in the formation of N-nitroso compounds. Many were found to be potent scavenger and the five most potent ones were (+)-catechin, (−)-epicatechin, phloroglucinol, caffeic acid and L-ascorbic acid. The nitrite scavenging effect was higher at pH 1.2 than pH 3.0 and increased when the incubation time was longer. The possible relationship of structures to scavenging effect of the flavonoids tested was discussed.

Keywords □ Nitrite scavenging effect, flavonoids, phenolics, maltol, L-ascorbic acid, structure-effect relationship

Nitrite are used in many countries as deliberate food additives. These serve to stabilize the color of cured meats, contribute flavor and protect against the danger of botulism.[1,2] In addition, nitrates which are widely distributed in human environment are also used often as deliberate food additives.[3] The potential toxicity of nitrate to animals as nitrate poisoning has been recognized by nitrite which is formed from nitrate by bacterial and salivary reduction either prior to ingestion or within the gastrointestinal tract.[4]

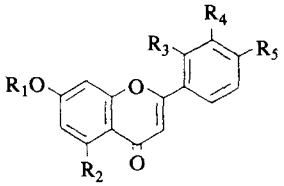
Unlike nitrate, which is relatively inert chemically, nitrite is very reactive, especially at low pH in its protonated form, nitrous acid (pKa = 3.4). Nitrous acid can react both as a nitrosating agent and as an oxidizing agent.

Nitrite is intrinsically toxic. The exact lethal dose of nitrite in humans is not known, but it is estimated to be about 1 gram sodium nitrite in adults.[5] The acute toxic effect of nitrite administration is the induction of infant methemoglobinemia. The nitrite reacts with oxyhemoglobin to convert it from its ferrous form to the ferric form(methemoglobin) that is unable to bind oxygen. The presence of a certain fraction of methemoglobin also distorts the oxygen dissociation curve of residual hemoglobin so that it transports oxygen less effectively.[6]

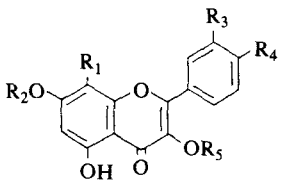
Carcinogenic N-nitroso compounds are also produced by the acid-catalyzed reaction of nitrite with certain nitrogen compounds.[7] Humans may be exposed to N-nitroso compounds in tobacco products[8] and in contaminated air[9], water[9,10], and food[9,11,12] as well as from the nitrosation of exogenous and endogenous amines in the stomach and possibly other tissues,[13-15] but direct evidence linking N-nitroso compounds with human cancer causation is still scant. Since the presence of nitrite is a requisite on the formation of N-nitroso compounds, any compound that could compete successfully with the secondary amine for the available nitrite would reduce the possibility of N-nitroso compound formation.

It was reported that certain compounds such as ascorbate[16-20], erythobate[18], and its esters [21,22], α-tocopherol[23,24], sorbic acid[25], and other reducing agents (sodium bisulfite, tannic acid, thiols such as cysteine and 2-mercaptoethanol and NADH)[20,26] which are endogenous to foodsfuffs or may be added to foods for preservative purposes, have been shown to inhibit the formation of N-nitrosamines. Ascorbate and sorbic acid have been shown to react with nitrite to reduce the available nitrite in the nitrosation.

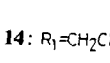
It was also appeared that a number of naturally occurring phenolic compounds may react as catalyst or inhibitor on the formation of N-nitroso compouds.[27-31] The extent of catalysis and inhibition are dependent on the pH and on the relative con-



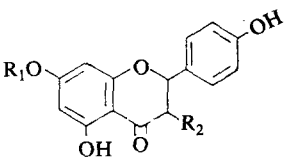
1: $R_1=R_2=R_3=H$, $R_4=R_5=OH$
2: $R_1=R_3=R_4=H$, $R_2=R_5=OH$
3: $R_1=R_3=H$, $R_2=R_4=R_5=OH$
4: $R_1=R_4=H$, $R_2=R_3=R_5=OH$
5: $R_1=R_3=R_4=H$, $R_2=OH$, $R_5=OCH_3$
6: R_1=glucose, $R_2=R_5=OH$, $R_3=R_4=H$
7: R_1=glucose, $R_2=R_4=R_5=OH$, $R_3=H$
8: R_1=glucose, $R_2=OH$, $R_3=R_4=H$, $R_5=OCH_3$



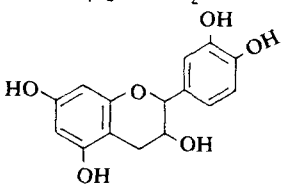
9: $R_1=R_2=R_3=R_5=H$, $R_4=OH$
10: $R_1=R_2=R_5=H$, $R_3=R_4=OH$
11: $R_1=R_2=R_3=H$, $R_4=OH$, R_5=glucose
12: $R_1=R_2=H$, $R_3=R_4=OH$, R_5=galactose
13: $R_1=R_2=H$, $R_3=R_4=OH$, R_5=rutinose



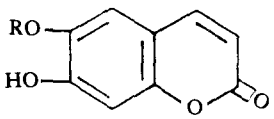
14: R_1= , R_2=glucose, $R_3=H$, $R_4=OCH_3$, R_5=rhamnose



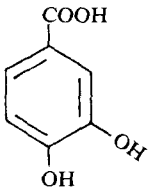
15: $R_1=R_2=H$
16: $R_1=H$, $R_2=OH$
17: R_1=glucose, $R_2=H$



18(2α,3β)
19(2α,3α)



20: R = H
21: R = glucose



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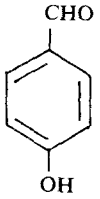

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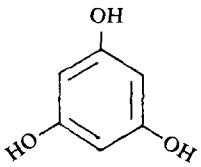

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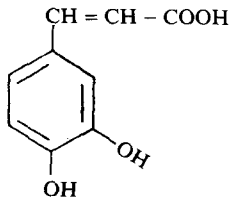

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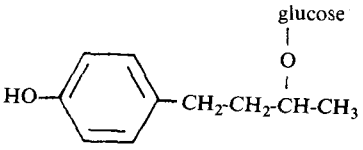

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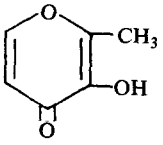

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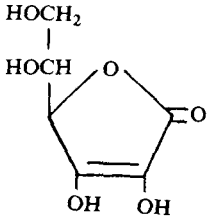

28

centrations of nitrite and of the phenolic compounds.

It is very interesting that phenolic compounds such as chlorogenic acid, caffeic acid and ferulic acid are contained in our customary diets.

Flavonoids, which are among the most ubiquitously distributed compounds in the plant kingdom (as well as some mosses, liverworts, fungi and ferns) have been shown to possess a variety of biochemical and pharmacological activities.[32-34]

Since flavonoids occurr in all higher plants they are, and always have been, a common constitutent of diet. It has been estimated that the "average" daily diet contains about 1 gram of flavonoids.[35] Because many flavonoids contain benzoyl and cinnamoyl system, we investigated as to whether they affect the nitrosation of proline by nitrite *in vitro*.[36]

As already mentioned, N-nitroso compounds are formed by the interaction of nitrogenous compounds with nitosating agents, the most important of which is acid nitrite. In view of this, it may be worthwhile to begin on the nitrite scavenging effect reducing the formation of N-nitroso compounds.

The present paper describes nitrite scavenging effect by nineteen flavonoids (3′,4′,7-trihydroxy flavone (**1**), apigenin (**2**), luteolin (**3**), norarthocarpetin (**4**), acacetin (**5**), apigenin-7-glucoside (**6**), luteolin-7-glucoside (**7**), linarin (**8**), kaempferol (**9**), quercetin (**10**), astragalin (**11**), hyperin (**12**), rutin (**13**), icariin (**14**), naringenin (**15**), dihydrokaempferol (**16**), prunin (**17**), (+)-catechin (**18**), (−)-epicatechin (**19**)), two coumarins (esculetin (**20**), esculin (**21**)), five phenolics (protocatechuic acid (**22**), hydroxybenzaldehyde (**23**), phloroglucinol (**24**), caffeic acid (**25**), rhododendrin (**26**)), maltol (**27**), and L-ascorbic acid (**28**). Some structure-effect relationships are also discussed.

RESULTS AND DISCUSSION

Although nitrite scavenging effect has often been described for phenolic compounds, there is no report in the case of flavonoids. The nitrite scavenging effect of flavonoids, phenolic compounds, coumarins, maltol and L-ascorbic acid at several concentrations(mM) were measured. The nitrite scavenging percentages of individual compounds are presented in Tables I-V.

As shown in Tables I-IV, the flavonoids except (+)-catechin and (−)-epicatechin showed weak effect than phenolics. But from this, we can deduce some structure-effect relationships.

A free hydroxyl group on the aromatic B ring was necessary for effect. The flavonoids without such free hydroxyls (**5**,**8** and **14**) possessed weak or no effect, while all the active species except **17** had them. Furthermore, if the two hydroxyls were at-

Table I. Nitrite scavenging effect of flavones and flavone glycosides at pH 1.2

Flavonoids	Concentration (mM)	% of nitrite scavenging effect*
1	0.1	31.6
	0.2	55.9
	0.3	80.0
	0.4	—
2	0.1	10.6
	0.2	17.1
	0.3	23.7
	0.4	—
3	0.1	9.0
	0.2	12.9
	0.3	16.8
	0.4	20.7
	0.5	24.7
4	0.1	26.8
	0.2	45.9
	0.3	65.0
	0.4	84.1
	0.5	96.3
5		NE
6	0.1	5.4
	0.2	10.2
	0.3	15.0
	0.4	19.7
	0.5	24.5
7	0.1	11.9
	0.2	18.0
	0.3	27.4
	0.4	35.1
	0.5	42.8
8	0.1	3.5
	0.2	7.0
	0.3	10.6
	0.4	—

*Values are means of three experiments
NE; No effect, —; Interfered the absorbance at 540 nm

Table II. Nitrite scavenging effect of flavonol and flavonol glycosides at pH 1.2

Flavonoids	Concentration (mM)	% of nitrite scavenging effect*
9	0.1	22.0
	0.2	—
10	0.1	62.2
	0.2	—
11	0.1	5.0
	0.2	9.4
	0.3	13.9
	0.4	18.3
	0.5	22.8
12	0.1	20.9
	0.2	32.5
	0.3	44.2
	0.4	55.9
	0.5	67.5
13	0.1	20.3
	0.2	30.4
	0.3	46.4
	0.4	—
14		NE

* Values are means of three experiments.
NE; No effect, —; Interfered the absorbance at 540 nm

Table III. Nitrite scavenging effect of flavanone, flavanonol and flavanone glycoside at pH 1.2

Flavonoids	Concentration (mM)	% of nitrite scavenging effect*
15	0.1	8.0
	0.2	13.7
	0.3	19.4
	0.4	25.1
	0.5	30.8
16	0.1	5.5
	0.2	7.9
	0.3	10.4
	0.4	12.8
	0.5	15.3
17		NE

* Values are means of three experiments.
NE; No effect

Table IV. Nitrite scavenging effect of catechins at pH 1.2

Flavonoids	Concentration (mM)	% of nitrite scavenging effect*
18	0.1	>100
19	0.1	>100

* Values are means of three experiments.

Table V. Nitrite scavenging effect of coumarins, maltol, L-ascorbic acid and phenolic compounds at pH 1.2

Compounds	Concentration (mM)	% of nitrite scavenging effect*
20	0.1	22.3
	0.2	29.4
	0.3	36.4
	0.4	43.4
	0.5	50.4
21	0.1	4.0
	0.2	6.3
	0.3	8.5
	0.4	10.7
	0.5	13.0
22	0.1	14.3
	0.2	18.3
	0.3	22.4
	0.4	26.4
	0.5	30.4
23		NE
24	0.1	37.6
	0.2	60.4
	0.3	83.1
	0.4	98.2
	0.5	>100
25	0.1	>100
26		NE
27	0.1	8.6
	0.2	16.1
	0.3	24.3
	0.4	32.6
	0.5	40.8
28	0.1	50.6
	0.2	86.8
	0.3	>100

* Values are means of three experiments.
NE; No effect

tached, effect was enhanced. However, flavone (**3**) with a catechol (*ortho*) hydroxyls in ring B was much less potent than flavone(**4**) where the hydroxyl groups have a resorcinol (*meta*) orientation.

In comparison with flavone and flavonols with or without a glycosyl group, flavonol compounds were relatively strong effect. If the hydroxyl at C-3 position of flavonols (**9** and **10**) was masked(**11-13**), effect was reduced. Thus, the number and the orientation of hydroxyls at B ring and 3-OH were common structural moieties to all active flavonoids. This type of B ring coupled with the 3-OH may be, accordingly, the most important structural unit for nitrite scavenging effect. But, in case of flavanone and flavanonol compounds(**15** and **16**), this contrasts with the above suggestions, which illustrated the important of the 2,3-double bond of flavonoids for the effect.

Elimination of free hydroxyl at C-5 of **3**, effect was also increased due to lack of hydrogen bond between free hydroxyl at C-5 and adjacent carbonyl group. As found **18** and **19** which has no carbonyl group at C-4 provided greater effect, regardless of the 2,3-double bond absence, the role of hydrogen bond between free hydroxyl at C-5 and adjacent carbonyl goup at C-4 seemed to be more important factor. And, it does not show any different effect on the 2,3-trans substitution (**18**) and 2,3-cis substitution (**19**).

Based on these results, some partial structure-

Table VI. Nitrite scavenging effect of some compounds at pH 3.0

Compounds	Concentration (mM)	% of nitrite scavenging effect*
2		NE
3		NE
7	0.1	11.5
	0.2	18.0
	0.3	24.4
	0.4	30.9
	0.5	37.3
12	0.1	14.7
	0.2	22.0
	0.3	29.4
	0.4	36.7
	0.5	44.0
13	0.1	13.5
	0.2	21.9
	0.3	30.2
	0.4	38.6
	0.5	47.0
15		NE
16		NE
18	0.1	41.2
	0.2	63.8
	0.3	86.5
	0.4	>100.0
19	0.1	34.8
	0.2	57.5
	0.3	80.1
	0.4	>100.0
20	0.1	11.9
	0.2	19.5
	0.3	27.2
	0.4	34.8
	0.5	42.5
21		NE
22	0.1	9.0
	0.2	12.7
	0.3	16.4
	0.4	20.1
	0.5	23.8
24	0.1	12.4
	0.2	28.1
	0.3	43.9
	0.4	59.6
	0.5	75.3
25	0.1	28.5
	0.2	49.3
	0.3	70.1
	0.4	90.9
	0.5	>100
27	0.1	7.0
	0.2	11.7
	0.3	16.3
	0.4	21.0
	0.5	25.7

* Values are means of three experiments.
NE: No effect

effect relationships can be brought out.

1. The more hydroxyls at B ring, the more effect was indicated. Among them, *meta* dihydroxy orientation possess more scavenging property as compared to *ortho* dihydroxy orientation.
2. The aglycone flavonols are more potent than its corresponding 3-O-glycosylated compounds and possess more effect as compared to flavones.
3. Absence of hydrogen bond between free hydroxyl at C-5 and adjacent carbonyl group at C-4 is more important factor for effect enhancement.
4. Flavanone, flavanonol and its corresponding glycosides has weak or no effect indicating the importance of 2,3-double bond.

It is known that phenolic acids exhibited high nitrite scavenging effect. Similar results are also obtained in our experimental conditions and adopt the above structure-effect relationships deduced from flavonoids.

As shown in Table V, caffeic acid(**25**) showed greater effect than any other tested compounds. Phloroglucinol(**24**) has high effect but less than caffeic acid. This means α,β-unsaturated aromatic rings are more important than the number of hydroxyls at aromatic ring. When caffeic acid which has another conjugated double bond compared to protocatechuic acid (**22**), the effect was more potent as shown in flavonoids, while mono hydroxylation at aromatic ring such as **23** and **26** lead to more reduction of the effect. In the case of maltol (**27**) which has similar structure of L-ascorbic acid (**28**) but lack of *ortho* dihydroxyls, the effect was reduced significantly.

Of the two coumarins, aesculetin (**20**) are more potent effect (but less than phenolics) than its corresponding glycoside, aesculin(**21**). This also means the importance of the number of free hydroxyls. The nitrite scavenging effect of some compounds on the time intervals and different pH range are also measured. As shown in Table VI and Table VII, the effect was decreased with increasing pH and increased with longer incubation time. These data are well consistent with the previous results of some phenolics or other compounds.[19,37] Detailed mechanistic scavenging effect and effect on the direct nitrosamine formation by these compounds are in progress.

Table VII. Nitrite scavenging effect of some compounds at several incubation time (pH 1.2)

Compounds	Concentration (mM)	Nitrite scavenging effect at several incubation time (%)* 0.5 hr.	1 hr.	2 hr.	4 hr.
1	0.07	16.5	28.5	31.2	78.1
6	0.46	20.2	22.4	36.1	88.1
7	0.22	16.5	23.8	37.4	82.0
10	0.07	43.0	58.9	84.1	94.1
12	0.11	9.0	24.9	49.0	97.8
13	0.10	25.5	32.8	45.2	62.7
15	0.33	19.8	31.3	50.7	75.6
16	0.35	14.4	25.2	42.0	63.3
18	0.014	47.9	59.3	71.6	83.8
19	0.014	43.3	52.2	65.2	77.8
20	0.22	28.1	35.6	54.9	95.3
22	0.33	9.7	24.8	35.2	97.0
25	0.028	62.4	65.7	70.5	100<

* Values are means of three experiments.

EXPERIMENTAL METHODS

Materials

Flavonoids (**1, 3-5, 7,8-10, 12, 15-17**) and **26** were isolated from various plants.[38-42] Other chemicals were also purchased from the commerical sources indicated. Naphthylethylene-diamine·HCl, sulfanilamide, sodium nitrite, L-ascorbic acid, *p*-hydroxybenzaldehyde, rutin, phloroglucinol, (+)-catechin, (−)-epicatechin and caffeic acid (Sigma); esculetin, esculin and maltol (Aldrich). Compounds **2, 6, 11, 14,** and **22** were obtained through the courtesy of Professors Won Sick Woo, Sam Sik Kang and Hye Suk Yoon (Choi) of Natural Products Research Institute, Seoul National University.

Sulfanilamide reagent was prepared by adding 5 gram in a mixture of 50 m*l* conc. HCl and about 300 m*l* distilled water and diluted to 500 m*l* with distilled water. N-(1-naphthyl) -ethylenediamine dihydrochloride (0.5 gram) was dissolved in 500 m*l* of distilled water. All other chemicals were reagent grade.

Apparatus

Ultraviolet absorption spectrum was measured with a Shimadzu Double Beam Spectrophotometer.

Assay of nitrite scavenging effect

Determination of nitrite were conducted according to the procedure of Standard Methods[43], except for the addition of DMSO for sample preparation as shown in Fig. 1. Because of poor water solubility of many of compounds, some com-

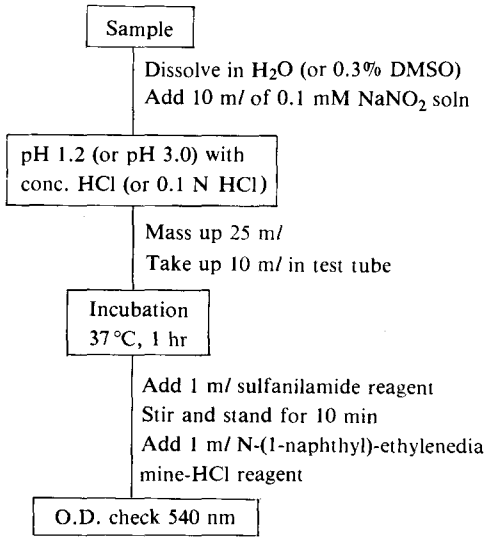

Fig. 1. Assay of nitrite scavenging effect

pounds (**2-5, 8-10, 12, 15, 16**) were dissolved in 0.3% DMSO solution. DMSO at 0.5% showed weak nitrite scavenging effect but did not affect the scavenging effect of compounds (<2%). Sample solutions and control solution, all containing 0.1 mM $NaNO_2$, were adjusted to an indicated pH with concentrated hydrochloric acid. Each mixture (10 ml) placed in a screw cap tube was incubated at 37 °C. After incubation, the solutions were reacted with sulfanilamide reagent (1 ml) and naphthylethylene diamine reagent (1 ml) and the nitrite concentration was determined by the absorbance at 540 nm.

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