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Influence of Some Flavonoids on N-Nitrosoproline Formation *In vitro* and *In vivo*

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Abstract

Some compounds including flavonoids were tested as scavenger of nitrite which is believed to participate in the formation of N-nitroso compound. Many were found to be potent scavengers and the most potent ones were ascorbic acid, potassium thiocyanate, chlorogenic acid, catechin, morin, luteolin, luteolin-7-glucoside, and naringenin. To evaluate the influence of the above compounds on the endogenous formation of N-nitroso compound, the amount of nitrosoproline (NPRO) was examined by co-incubation of nitrite, proline, and test compounds at various concentrations. The analysis of NPRO by High Performance Liquid Chromatography (HPLC) was newly developed. Most compounds except ascorbic acid and chlorogenic acid were found to be no effects or activatory effects on NPRO formation. From the results obtained, it was suggested that most flavonoids which are contained in our customary diets were not associated with the inhibition of NPRO formation.

Key words : flavonoids, nitrite scavenger, nitrosoproline formation, HPLC

INTRODUCTION

Nitrites 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 the human environment, are also often used as delibrate food additives[3].

Carcinogenic N-nitroso compounds are produced by the acid-catalyzed reaction of nitrite with certain nitrogen compounds[4]. Humans may be exposed to N-nitroso compounds in tobacco products[5] and in contaminated air[6], water[6,7], and food[6,8,9] as well as from the nitrosation of exogenous and endogenous amines in the stomach and possibly other tissues[10~12], but direct evidence linking N-nitroso compounds with human cancer causation is still scant.

Since the presence of nitrite is a requisite for 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[13~17], erythrobate[15], and its esters[18,19], α-tocopherol[20,21], sorbic acid[22], and other reducing agents (sodium bisulfite, tannic acid, thiols such as cysteine and 2-mercaptoethanol and NADH)[17,23] which are endogenous to foodstuffs or may be added to food 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.

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[24~26].

Since flavonoids occur in all higher plants, they are, and always have been, a common constituent of diet. It has been estimated that the "average" daily diet contains about 1 gram of flavonoids[27].

In the present study, we investigated whether they affect the nitrosation of proline by nitrite *in vitro* and *in vivo*. As mentioned above, N-nitroso compounds are formed by the interaction of nitrogenous compounds with nitrosating agents, the most important of which is

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acid nitrite. Preliminary report on the nitrite scavenging effect by flavonoids and its structure-effect relationship has already been reported by the authors[28].

MATERIALS AND METHODS

Materials

The used flavonoids (Scheme 1) were isolated from *Prunus davidiana*[29] and *Ixeris sonchifolia*[30]. Other chemicals were purchased from the commercial sources indicated. Naphthylethylene-diamine-HCl, sulfanilamide, sodium nitrite, proline and L-ascorbic acid (Sigma), chlorogenic acid and KSCN (Wako).

Apparatus

Ultraviolet absorption spectrum was measured with a Shimadzu Double Beam Spectrophotometer. High Performance Liquid Chromatography(HPLC) was used with Waters Associates HPLC systems and instrumental condition of analysis are shown in Table 1.

In vitro assay of nitrite scavenging effect and the NPRO formation

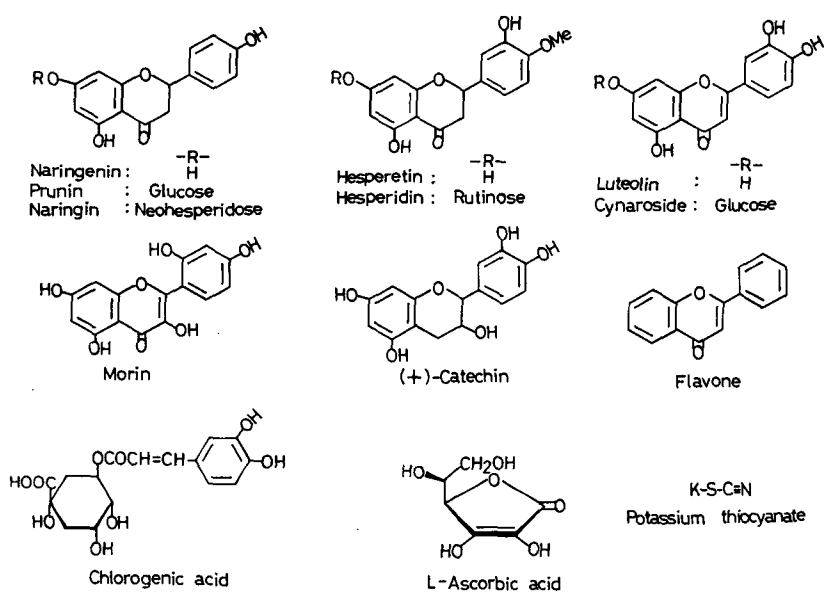
Determination of nitrite and NPRO were conducted according to the procedure of Standard Methods Committee[31] and Mirvish et al.[13], except for the addition of DMSO for sample preparation as shown in Fig. 1. Because of poor water solubility of many compounds, some compounds were dissolved in 10% DMSO solution.

In vivo assay of NPRO[32,33]

Male Sprague-Dawley rats (250~300g) were supplied by Hae Eun trade company and were housed individually in wire-bottomed cages. The animals were maintained at 22~24°C on a 12hr light-dark cycle with chow diet and water available *ad libitum*. Rats were dosed orally with 0.75M proline and $NaNO_2$ to generate NPRO. The animals were administered the test materials (3mM~9mM) by oral gavage in rapid succession, once a day on two consecutive days. The 48hr urine sample was collected. To prevent artificial formation of NPRO, 1ml of 10% ammonium sulfamate (AS) solution was immediately added to the urine sample and was extracted 3 times with an equal volume of a ethylacetate (EtOA-

Table 1. Instrumental condition for HPLC analysis of N-nitrosoproline(NPRO)

Instrument : Waters Associates HPLC system
Column : μBondapak™ C_{18} (3.9 × 300mm, i.d.)
Detector : UV 238 or 343nm
Mobile Phase : 0.02 % H_3PO_4 : CH_3CN =47 : 10
Flow rate : 0.7ml/min.



Scheme 1. Structures of test compounds.

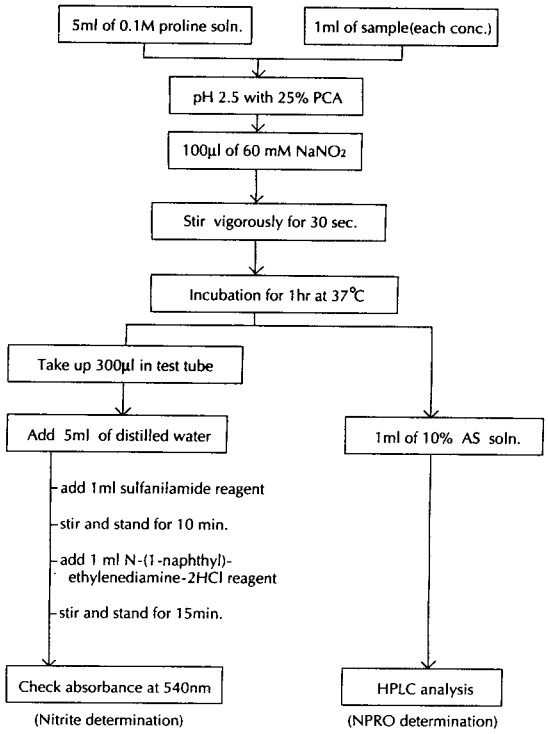


Fig. 1. Schematic procedure for determination of nitrite and N-nitrosoproline(NPRO).

c). The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated to dryness by a rotary evaporator. The residue was used to determine NPRO in a HPLC. An authentic sample of NPRO kindly supplied by Dr. Bartsch was used as the reference compound for quantitation.

RESULTS AND DISCUSSION

Nitrite scavenging effect

The nitrite scavenging effect of flavonoids, KSCN, ascorbic acid and chlorogenic acid at several concentrations(mM) was measured. The percentages of nitrite remaining in individual compounds are presented in Figs. 2~4. As shown in Fig. 2, ascorbic acid and KSCN appeared to inhibit the percentages of nitrite remaining. In the case of flavonoids(Figs. 3 and 4), flavanone compounds except naringenin showed weak or no effect. In comparison with flavanone compounds, flavonol compounds such as luteolin and morin possess more scavenging effect as

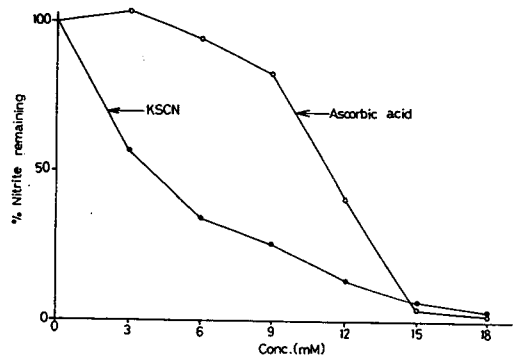


Fig. 2. Nitrite scavenging effect of ascorbic acid and KSCN at various concentrations.
Each point represents means of two or three experiments.

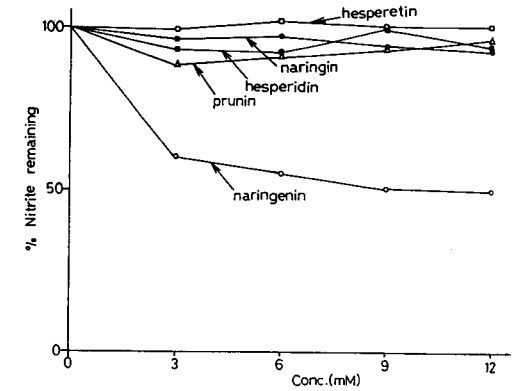


Fig. 3. Nitrite scavenging effect of flavanone and flavanone glycosides at various concentrations.
Each point represents means of two or three experiments.

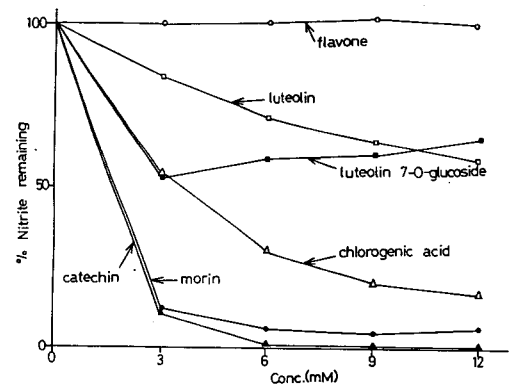


Fig. 4. Nitrite scavenging effect of flavone, flavone glycoside and other compounds at various concentrations.
Each point represents means of two or three experiments.

compared to ascorbic acid. These data agree with the previous report[28].

Effect of ascorbic acid, KSCN and flavonoids on the NPRO formation

In recent studies, Ohshima and Bartsch[32] administered high doses of proline and nitrite to human subjects and rats, and these investigators found an increased formation of NPRO in both species, a noncarcinogenic nitrosamine that is excreted virtually unchanged in the urine when given to rats. Measurement of increased urinary excretion of NPRO after the administration of proline and nitrate has been proposed as an index of nitrosation in humans. Ohshima and Bartsch used the gas chromatogram for identification of NPRO. The increased urinary excretion of NPRO in humans that occurs after the administration of proline and nitrate can be blocked or accelerated by ascorbic acid and KSCN[34]. Thiocyanate ions reported a powerful catalyst of solution nitrosation of amines through the generation of nitrosyl thiocyanate intermediate. The present study was carried out to investigate whether the flavonoid compounds would be useful for the inhibition of the endogeneous formation of NPRO, and also to search for a more simple and rapid method for the identification of NPRO by

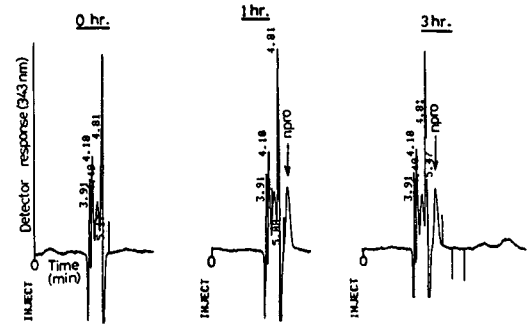


Fig. 5. N-nitrosoproline formation by incubation time.

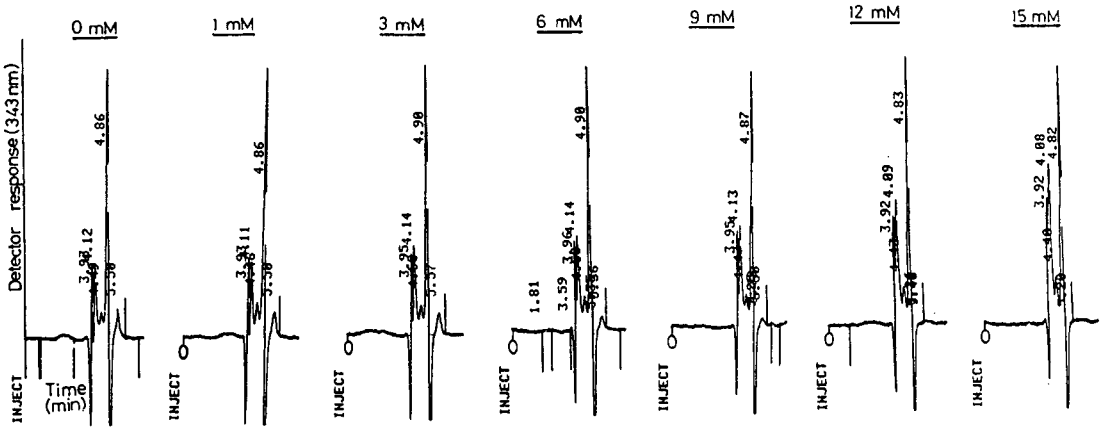


Fig. 6. Effect of ascorbic acid on NPRO formation at various concentrations.

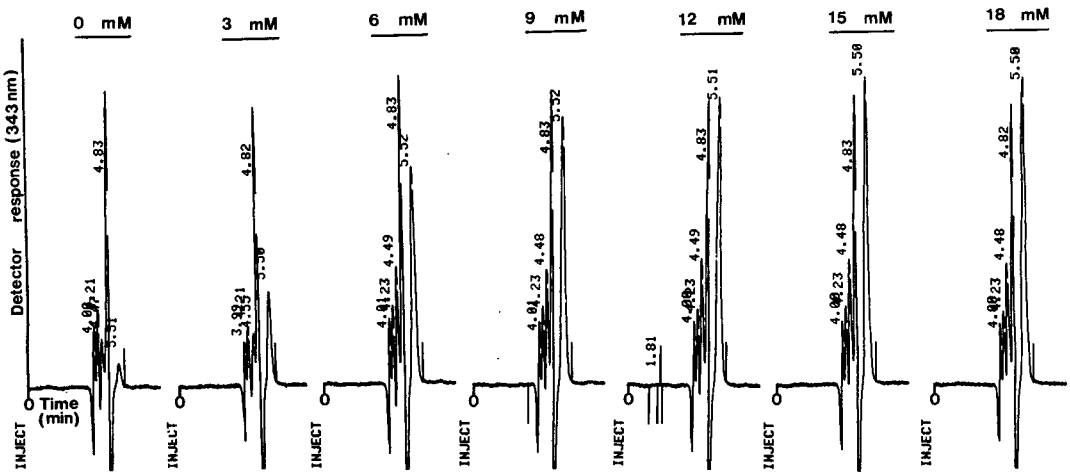


Fig. 7. Effect of KSCN on NPRO formation at various concentrations.

use of HPLC systems. In order to investigate the HPLC conditions for analyzing the NPRO formation, we compared the ascorbic acid (inhibitor) and KSCN (catalyst) on the NPRO formation. First, we investigated the amount of NPRO formed from the reaction mixtures(proline and $NaNO_2$) under the normal conditions at 37° C on three different time intervals such as 0hr, 1hr, and 3hr. From 1hr of incubation, it showed maximum level of produced NPRO and maintained until 3 hrs of incubation (Fig. 5). At 0 time, it didn't show any formation of NPRO. Fig. 6 and Fig. 7 showed the yield of NPRO in the presence of various concentrations of ascorbic acid and KSCN with the 1hr incubation time determined by Fig. 5. The inhibition of NPRO formation by ascorbic acid was most marked at the concentration of 12mM ; whereas KSCN exerted a marked catalytic effect on the formation of NPRO over the concentration tested. Thus we thought that the conditions for HPLC and incubation were good for our experiment. The effect of flavonoids on the NPRO formation was shown in Tables 2~4. Generally, the flavonoid used in this experiment did not affect the formation of NPRO *in vitro* condition. In the case of morin, naringenin, catechin and luteolin 7-glucoside exhibited an increasing effect (Table 2 and 3), while chlorogenic acid showed a decreasing effect (Table 4). Most of the compounds such as flavone, naringin, hesperidin, hesperetin, luteolin, and prunin did not affect the formation of NPRO. Among the flavonoid compounds, naringenin and catechin showed the catalytic effect on the NPRO formation similar to *in vitro*. The present work also demonstrates that *in vivo* NPRO formation is catalyzed in a manner similar to that observed in *in vitro* experiments, although to a lesser extent (Table 5). It can be expected, however, that *in vivo* phenomena are more complex and involve other parameters, particularly since nitrite is absorbed on the stomach mucosa. Nevertheless, this work shows that *in vitro* experiments with these important catalytic inhibitory substances can approximately represent *in vivo* phenomena. Flavonoids used in this study showed no effect in the inhibition of NPRO formation, although these results changed

Table 2. Nitrite scavenging activity and NPRO formation by flavone and flavone glycosides

Flavonoids	Conc. (mM)	Nitrite remaining (%)	NPRO formation (%)	(ng)
Control	0	100.0	100.0	(299.9)
Flavone	3	100.3	83.9	(238.9)
	6	100.5	87.7	(253.6)
	9	102.0	96.8	(287.7)
	12	100.4	100.6	(302.3)
Morin	3	12.3	3057.5	(8970.0)
	6	5.9	3137.5	(9296.9)
	9	4.4	3137.5	(9296.9)
	12	6.1	3900.0	(11661.0)
Luteolin	3	83.8	148.4	(374.2)
	6	71.7	158.0	(403.5)
	9	64.1	162.0	(415.7)
Luteolin-7-glucoside	3	53.4	380.4	(1081.2)
	6	58.4	384.0	(1092.2)
	9	59.9	379.2	(1077.6)
	12	64.6	436.0	(1250.7)

Values are means of two or three experiments
Figures in parenthesis are quantity of NPRO

Table 3. Nitrite scavenging activity and NPRO formation by flavone and flavone glycosides

Flavonoids	Conc. (mM)	Nitrite remaining (%)	NPRO formation (%)	(ng)
Control	0	100.0	100.0	(269.4)
Naringin	3	96.8	100.0	(269.0)
	6	97.5	127.2	(341.6)
	9	94.2	90.9	(242.1)
	12	93.5	89.1	(239.4)
Naringenin	3	60.8	409.8	(1270.1)
	6	55.4	448.4	(1416.4)
	9	50.1	479.8	(1538.3)
	12	49.9	503.2	(1623.6)
Hesperidin	3	94.1	108.8	(299.9)
	6	96.3	126.3	(360.8)
	9	92.5	131.6	(399.1)
	12	93.5	122.8	(348.6)
Hesperetin	3	99.3	79.4	(212.8)
	6	100.4	85.0	(228.6)
	9	100.9	93.8	(250.5)
	12	108.4	115.6	(309.6)
Catechin	3	11.1	890.0	(2394.1)
	6	1.7	857.1	(2305.3)
	9	1.0	948.6	(2550.1)
	12	0.0	2172.8	(5842.7)
Prunin	3	96.6	108.4	(290.5)
	6	93.7	147.8	(395.4)
	9	91.7	144.0	(387.3)
	12	88.5	133.0	(357.8)

Values are means of two or three experiments
Figures in parenthesis are quantity of NPRO

Table 4. Nitrite scavenging activity and NPRO formation of chlorogenic acid

Conc. (mM)	Nitrite remaining (%)	NPRO formation (%)	(ng)
0	100.0	100.0	(321.8)
3	54.2	61.0	(165.8)
6	30.8	58.2	(154.8)
9	20.8	64.0	(178.0)
12	17.2	61.0	(165.8)

Values are means of two or three experiments
Figures in parenthesis are quantity of NPRO

Table 5. Effect of some compounds on NPRO formed *in vitro* and *in vivo*

Experiment	Control (%)	Ascorbic acid[a] co-administered (%)	KSCN[b] (%)	Naringenin[b] (%)	Catechin[b] (%)
In vivo	40	0	150	200	250
In vitro	100	0	400	400	900

[a] 9mM [b] 3mM

according to the experimental conditions.

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시험관 및 생체조건하에서 니트로소프로린 생성에 미치는 수종 플라보노이드 화합물의 영향

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요 약

우리들의 식이중에 함유되어 있는 플라보노이드 화합물들이 니트로소화합물의 생성에 미치는 영향을 알아보기 위하여 생체내에서 일어나는 니트로소화 과정을 아질산염, 프로린과 시료를 동시에 투여하여 생성된 니트로소프로린 함량을 시험관 및 생체조건하에서 HPLC로서 측정하였다. 그 결과, 잘 알려진 ascorbic acid, KSCN과 더불어 catechin, chlorogenic acid, morin, luteolin, luteolin-7-O-glucoside, naringenin 등은 아질산염 소거에는 상당한 효과가 있는 것으로 나타났으나, 니트로소프로린 생성에 대해서는 ascorbic acid와 chlorogenic acid를 제외한 나머지 화합물들은 거의 영향을 미치지 않거나 오히려 그 생성을 촉진시키는 것으로 나타났다.