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Effect of Sulfite-treated Daylily (*Hemerocallis fulva* L.) Flower on the Production of Nitric Oxide and DNA Damage in Macrophages

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ABSTRACT

The inhibitory effect of fresh or dried daylilies extract on the generation of nitric oxide, and the effects of sulfur dioxide in dried daylily on nitric oxide production and DNA damage in Raw 264.7 macrophages were examined. The extracts from sulfite-treated and untreated dried daylily flowers exhibited stronger scavenging effects on nitric oxide production by sodium nitroprusside (SNP) than that of fresh flower. Fresh and untreated dried daylily flowers exhibited strong inhibitory effect on nitric oxide induced by lipopolysaccharide (LPS) in macrophages, but the sulfite-treated dried daylily did not. The scavenging effect of sulfite on nitric oxide production by SNP was dose-dependent, and sulfite also exhibited low inhibitory effect on nitric oxide induced by LPS in macrophages. Sodium hydrogen sulfite also slightly induced DNA damage in macrophages. Although sulfur dioxide in dried flowers is volatilized during heating, its residual adverse effects should still be concerned.

Key words: daylily flower, *Hemerocallis fulva* L., nitric oxide, sulfur dioxide, DNA damage

INTRODUCTION

Daylily (*Hemerocallis fulva* L.), also know as "golden needle" in Chinese, has been used as food and in traditional medicine for thousands of years in Asia. As the roots and leaves of daylily were used in the treatment of inflammation and jaundice, its flowers are widely consumed as vegetable in eastern Asian. Several constituents such as naphthalene glycoside, anthraquinone, and steroidal saponin have been identified from roots, leaves or flowers of daylily[1-4]. Those compounds exhibited strong antioxidant activity[4-5] and inhibitory effects on the proliferation of human tumor cell[6]. The daylily flowers are used as vegetables by two different ways. The edible part of fresh daylily is immature green flower; however, the dried daylily is processed from mature yellow flower by soaking in sulfite solution and drying. In our previous study[7], the extracts from daylily flowers exhibited strong inhibition on nitric oxide (NO) generation. The extracts from dried flower exerted well scavenging effects on NO derived from sodium nitroprusside (SNP). However, the extracts from fresh flower expressed stronger inhibition on NO production in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. Therefore, the sulfite remained in dried flower may influence the generation of NO *in vitro* or *in vivo*.

Sulfites (widely defined as the sulfiting agents, sulfur dioxide gas, metabisulfite, bisulfite and sulfite) are often used in foods to control the enzymatic or nonenzymatic browning, to inhibit the growth of microorganism, or as an antioxidant and bleaching agent[8-10]. Sulfite can cause allergic reactions in humans, of which the bronchoconstriction in asthmatics is the most common[11]. Harvey and Nelsestuen[12] reported that the reaction of NO with sulfites may be the major source of biological toxicity of sulfites. In addation, sulfite and peroxynitrite ($ONOO^-$) are synergistically toxic to neurons or other tissues[13]. NO has been reported to act as a messenger molecule mediating various physiological functions[14]. However, the overproduction of NO was found associated with various pathophysiological processes including inflammation, atherosclerosis and carcinogenesis[15-17]. Thus, the objectives of this study were to investigate the inhibitory effects of water extracts from various fresh or dried daylily flowers on the generation of NO, and the effect of sulfite on the generation of NO and DNA damage in cells.

MATERIALS AND METHODS

I. *Materials*

SNP, LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), N-(1-naphthyl)-ethylene-

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diamine dihydrochloride, sulfanilamide, sodium nitrite, agarose, N-lauroyl sarcosinate, ethidium bromidine (EtBr), triton X-100, bromphenol blue and gallic acid were purchased from Sigma Co. Ltd. (St Louis, MO, USA). NaN_3 and disodium ethylenediaminetetraacetic acid (Na_2-EDTA) were purchased from Osaka Co. Ltd. (Japan). Dimethyl sulfoxide (DMSO), sodium hydrogen carbonate and *ortho*-phosphoric acid were purchased from Merck Co. Ltd. (Darmstadt, Germany). Sodium hydrogen sulfite ($NaHSO_3$) was purchased from Wako Co. Ltd. (Japan). PSN antibiotic mixture (penicillin-streptomycin-neomycin), Dulbecco's Modified Essential Medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA (T/E), low melting point agarose (LMA) and normal melting point agarose (NMA) were purchased from Gibco Ltd. (Grand Island, NY, USA). RAW 264.7 cells, the murine macrophage cell line, were obtained from the Bioresource Collection and Research Center, Food Industry Research and Development Institute (Hsinchu, Taiwan).

II. *Plant Material and Extraction*

Six daylily samples used in this study were purchased from a local market in Taichung, Taiwan. They included sulfite-treated dried flowers (No. 1, No. 2 and No. 3), which were mature flowers soaked in sulfite solution and dried, sulfite-untreated dried flower (No. 4), which was mature flower dried directly without soaking in sulfite solution, and fresh flowers (No. 5 and No. 6), which were immature flowers. Plant materials were weighed and finely minced in 4 volumes of deionized water, followed by grinding with homogenizer for 10 min. The mixture was then centrifuged at 9000 ×g for 30 min at 4°C. The supernatants of extracts were freeze-dried and then stored at -20°C.

III. *Scavenging Effects on NO*

The scavenging effects of water extracts from daylily flowers or sodium sulfite on NO were measured by the method of Marcocci *et al*.[18] Four milliliters of extracts solution were added to 1 mL of SNP solution, (25 mM), and incubated for 150 min at 37°C. An aliquot (0.5 mL) of the incubation mixture was added to 0.3 mL of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% naphthylethylenediamine dihydrochloride). The absorbence of the chromophore, which was formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbence of standard solutions of sodium nitrite treated in the same way with Griess reagent. Scavenging effect (%) was calculated as [1-(nitrite conc. in sample with 5 mM SNP/nitrite conc. in control)] × 100.

IV. *Cell Culture*

The murine macrophage cell line RAW 264.7 were cultured in 25 or 75 cm^2 plastic flasks with DMEM-supplemented with 10% heat-inactivated FBS and antibiotics. These cells were activated with 1 μg/mL LPS (*Escherichia coli*, Serotype 0.55:B5) and cultured for 20 hr at 37°C in 5% CO_2.

V. *Inhibitory Effects on NO Production Activated by LPS*

The inhibitory effects of water extracts from daylily flowers or sodium sulfite on NO production were estimated by the method of Dirsch *et al*.[19]. Cells were seeded in a 96 well-plate (8×10^4/200 μL), cultured for two days and then treated with or without LPS (1 μg/mL) in the absence or presence of daylily flower extracts (200 μg/mL) or sodium sulfite at 37°C for 20 hr. As a parameter of NO synthesis, nitrite concentration was assessed in the supernatant of macrophages RAW 264.7 by Griess reaction. Briefly, 100 μL of cell culture supernatant was removed and combined with 60 μL of 1% sulfanilamide in 5% H_3PO_4 and 60 μL of 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H_2O in a 96 well plate, followed by absorbance measurement at 570 nm using a SPECTRA microplate reader (SLT-Labinstruments). The nitrite concentration was determined by comparison with a sodium nitrite standard curve.

To understand whether the observed NO inhibition was not false positive due to cytotoxic effects, cell respiration, an indicator of cell viability, was evaluated through the mitochondrial-dependent reduction of MTT to formazan. After the supernatants from the plate was removed for nitrite determination, the cells were incubated with MTT (0.5 mg/mL) for 45 min at 37°C. The medium was aspirated and the cells were resuspended in DMSO (250 μL) for at least 2 hr in the dark. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm.

VI. *Analysis of DNA Damage (Comet Assay)*

(I) *DNA Damage of Sodium Sulfite toward RAW 264.7 Cells*

The RAW 264.7 cells were incubated with (0.1-1.0 μg/mL) or without sodium sulfite for 1 hr at 37°C in dark. The cells were recovered by centrifugation at 800 rpm and resuspended in LMA for comet analysis.

(II) *Inhibitory Effects on DNA Damage Induced by SNP*

Cells were incubated with or without SNP in the absence or presence of 200 μg/mL daylily flower extracts for 1 hr at 37°C in dark. The cells were pelleted by centrifugation and resuspended in LMA as described above.

(III) *Comet Assay*

The DNA damage was estimated by Comet assay using single cell gel electrophoresis[20]. Briefly, fully frosted slides were covered with 0.5% NMA as the first

layer, with a mixture of cell suspension and 0.5% of LMA as the second layer, and finally with 0.5% of LMA (without cell) as the third layer. After solidification at 4°C, all slides were immersed in the lysing buffer (2.5 M NaCl and 100 mM EDTA, pH 10, with freshly added 1% Triton X-100 and 10% DMSO) at 4°C for 1 hr. The slides were then placed in a horizontal electrophoresis tank filled with freshly prepared electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 10) at 4°C. The slides were left in the solution for 20 min to allow the DNA unwinding and expression of alkali labile damage before electrophoresis. Electrophoresis was then run at 4°C for 20 min using 25 V and 300 mA. After electrophoresis, the slides were neutralized in the neutralization buffer, stained with ETBr, kept in a humidified airtight container and examined using a fluorescence microscope. Images of 100 randomly selected cells from each slide were analyzed. The degree of DNA damage is expressed in the tail moment value. The tail moment was calculated as: tail length × percentage of tail DNA/100. Tail length is the maximal distance the damaged DNA migrates from the center of the cell nucleus; the percentage of tail DNA is the percent DNA that migrates from the nucleus into the comet tail.

VII. *Determination of Sulfur Dioxide*

The sulfur dioxide was distilled from the acidified food and collected in neutral hydrogen peroxide solution, where sulfur dioxide was oxidized to sulfuric acid. The latter was then titrated with standard NaOH. SO_2 (ppm) = (a × F × 3200) / S
3200: 1 mL of 0.1 N NaOH = 3200 μg SO_2.
a: the titrated value of 0.1 N standard NaOH.
F: the force value of 0.1 N standard NaOH.
S: weight of sample.

VIII. *Determination of Total Phenolic Compounds*

The total polyphenolic compounds of water extracts from daylily flowers was determined according to the method of Taga *et al.*[21] using gallic acid as standard. The water extracts from daylily flowers (0.1 g) was dissolved in 5 mL of 0.3% HCl in methanol/water (60:40, v/v). One hundred microliter was aliquoted and added to 2 mL of 2% Na_2CO_3. After 2 min, 100 μL of 50% Folin-Ciocalteu reagent was added and the mixture was incubated for 30 min. Absorbance was measured at 750 nm and results were expressed as milligrams per gram of gallic acid equivalents (GAE).

IX. *Determination of Flavonoids*

The content of flavonoids of water extracts from daylily flowers was determined according to the method of Jia *et al.*[22]. One milliliter of water extracts (200 μg/mL) was added to 6 mL of 5% $NaNO_2$ in deionized H_2O. Three milliliter of $AlCl_3$ (1:10) was added 5 min later. After 6 min, 2 mL of mixture solution was added to 2 mL of NaOH (1 N). Absorbance was measured at 510 nm. Quercetin was used as the standard for calibration.

X. *Statistical Analysis*

Each experiment was performed at least triplicate from individual experiments. Statistical analyses were carried according to the SAS Institute User's Guide. Variance was analyzed using the ANOVA procedure. Significant differences ($P < 0.05$) between the means were determined using Duncan's multiple range test.

RESULTS AND DISCUSSION

I. *Effect of Daylily Flower Extracts on the Production of NO in SNP Solution and LPS-activated Macrophages*

The daylily flower extracts were checked for their scavenging effects on NO derived from SNP. SNP will decompose and produce NO in aqueous solution at physiological pH. Under aerobic conditions, NO reacts with oxygen to produce the stable products nitrate or nitrite, which can be determined using Griess reagent[18]. The scavenging effects of extracts from sulfite-treated dried flower and untreated dried flower on NO were 33-69%, while the extracts from fresh flower showed less 10% scavenging effects at a concentration of 200 μg/mL as shown in Figure 1. One reason for the discrepancy between fresh and dried flowers is the edible part of fresh daylily is immature flower whereas the dried daylily is

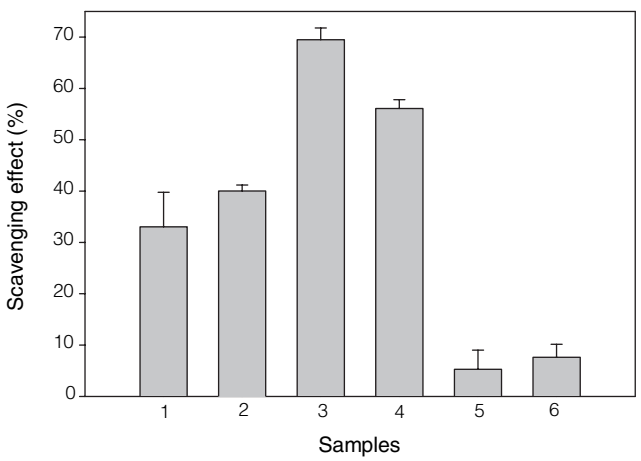

Figure 1. Scavenging effect of water extracts from various fresh daylily and dried daylily flowers on nitric oxide derived from SNP (5 mM). Concentration of sample used for treatment was 200 μg/mL. Samples 1-3 are water extracts from various sulfite-treated dried daylilies. Sample 4 is water extracts from untreated dried daylily. Samples 5-6 are water extracts from various fresh daylilies. Results are shown as mean ± SD (n = 3).

processed from mature flower. To understand the effects of sulfite treatment on the production of NO, we examined the inhibitory effects of both sulfite-treated and -untreated dried daylily flower on NO generation. The scavenging effect of untreated dried flower on NO derived from SNP was between those of sulfite-treated dried flower samples. Therefore, the components responsible for inhibitory effect on NO generation may be altered in the flower maturation process or may be related to the sulfite treatment and drying.

Macrophages will produce NO upon activation by LPS. As shown in Figure 2, the water extracts from fresh flower displayed over 70% inhibition on NO generation stimulated by LPS, and the untreated dried flower also showed about 60% inhibitory effects. However, the sulfite-treated dried flower exhibited almost no inhibitory effects on NO generation (-9.2~5.6%). Therefore, sulfite treatment obviously diminished the inhibitory effects of dried flower against NO generation stimulated by LPS.

As shown in Figures 1 and 2, the results of inhibitory effects on NO generation in LPS stimulated RAW 264.7 macrophages were different from that of scavenging effects on NO derived from SNP. Sulfite-treated dried flower exhibited strong scavenging effects on NO, but no inhibitory effects on NO generation stimulated by LPS in macrophages. In contrast, fresh flower exhibited more inhibitory effects on NO generation in LPS-activated macrophages than in SNP solution system. One reason is that the activated macrophages generate NO from L-arginine via the catalysis of iNOS. Therefore, the mechanism of inhibition on NO generation in LPS-activated macrophages may be attributed not only to the scavenging effects on NO but also to the action of iNOS. In our previous study[7], the western blotting of iNOS enzyme indicated that the strong inhibition on NO production, exhibited by the extracts from fresh flower, was contributed by the ability to reduce the iNOS induction. The fresh flower extracts (200 μg/mL) completely abolished the iNOS induction by LPS (1 μg/mL). The other vegetable extracts from pea sprout and eggplant suppressed the NO production by scavenging NO and inactivating iNOS enzyme.

II. *Effect of Sulfite on the Production of NO in SNP Solution and LPS-activated Macrophages*

The amount of sulfur dioxide in daylily flower extracts is shown in Table 1. For all extracts from sulfite-treated dried flowers, the sulfur dioxide content was between 476-878 μg/g. On the other hand, SO_2 was not detectable in extracts from fresh and untreated dried flowers. For sulfite-treated dried flower extracts, the positive correlation (R = 0.713) was observed between the sulfur dioxide content and the scavenging effects on NO.

In our preliminary study, 460 mg sulfur dioxide could be distilled from 1 g $NaHSO_3$ by the same method. Therefore, the inhibitory effects of $NaHSO_3$, that could

Table 1. The sulfur dioxide content of water extracts from various fresh daylily and dried daylily flowers

Samples	SO_2 content (μg/g)*
Sulfite-treated dried daylily (No.1)	476 ± 40
Sulfite-treated dried daylily (No.2)	839 ± 39
Sulfite-treated dried daylily (No.3)	878 ± 33
Sulfite-untreated dried daylily (No.4)	0 ± 0
Fresh daylily (No.5)	0 ± 0
Fresh daylily (No.6)	0 ± 0

*Results are shown as mean ± SD (n ≧ 3).

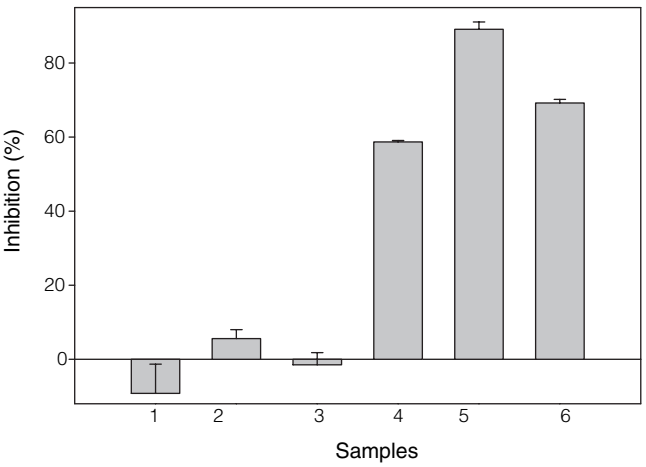

Figure 2. Effects of water extracts from various fresh daylily and dried daylily flowers on the production of nitric oxide induced by LPS (1 μg/mL) in RAW 264.7 macrophages. Concentration of sample used for treatment was 200 μg/mL. Samples 1-3 are water extracts from various sulfite-treated dried daylilies. Sample 4 is water extracts from untreated dried daylily. Samples 5-6 are water extracts from various fresh daylilies. Results are shown as mean ± SD (n = 3).

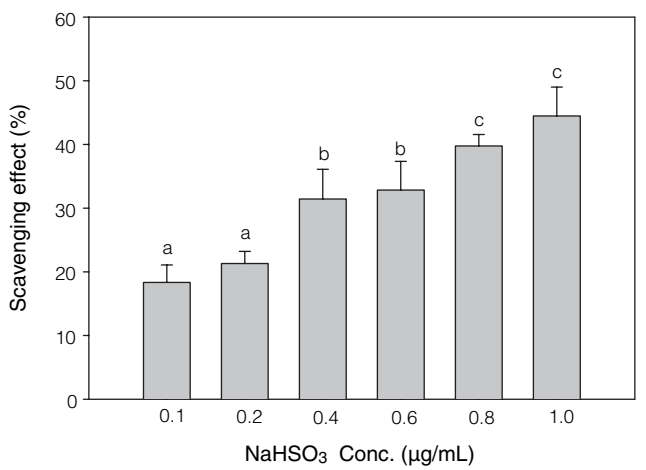

Figure 3. Scavenging effect of sodium hydrogen sulfite on nitric oxide derived from SNP (5 mM). Results are shown as mean ± SD (n = 3). [a-c]Values with differing alphabetic superscripts are significantly different from each other ($p < 0.05$).

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release same amount of sulfur dioxide in sulfite-treated dried flower extracts, on the generation of NO was investigated. The results indicated that the scavenging effect of $NaHSO_3$ on the production of NO by SNP was dose-dependent (Figure 3). As the same sulfur dioxide equivalent, three sulfite-treated dried flower extracts at the concentration of 200 μg/mL were equal to 0.21-0.38 μg/mL $NaHSO_3$. As the results in Figures 1 and 3 were compared, the scavenging effects of extracts from three sulfite-treated dried flowers on the generation of NO were 33-69%; however, the scavenging effects of 0.2-0.4 μg/mL $NaHSO_3$ were 20-30%. In addition to sulfur dioxide, some other components in daylily flower may be responsible for their scavenging effects on NO.

$NaHSO_3$ also exhibited 13-25% inhibitory effect on NO induced by LPS in macrophages (Figure 4). There was no significantly difference ($P > 0.05$) in the inhibitory effect of $NaHSO_3$ at various concentrations. The results in Figures 2 and 4 indicate that the loss of inhibitory effect of sulfite-treated dried flower extracts on NO induced by LPS was not due to the existence of sulfite, but related to the change of components in the bleaching processes. However, the change of components or chemical reactions in the bleaching processes may result in the promotion on NO generation and thus counteract the action of sulfite. It remains as an open matter to be studied further.

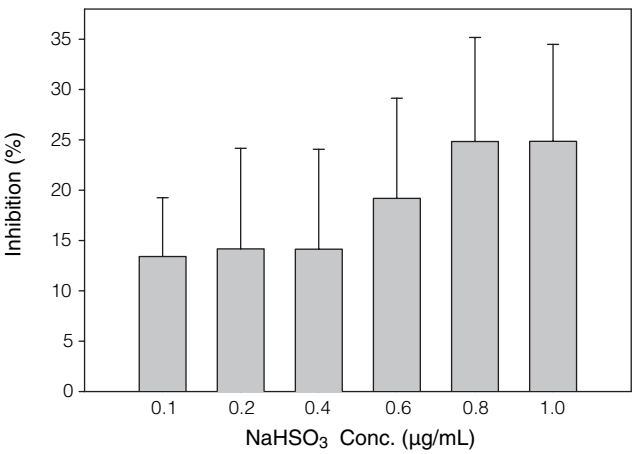

Figure 4. Effects of sodium hydrogen sulfite on the production of nitric oxide induced by LPS (1 μg/mL) in RAW 264.7 macrophages. Results are shown as mean ± SD (n = 3).

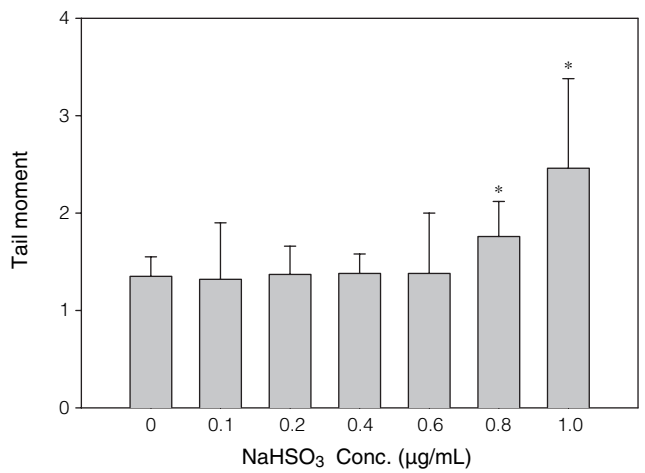

Figure 5. Effect of sodium hydrogen sulfite on DNA damage in RAW 264.7 macrophages. The tail moment was calculated as: tail length × percentage of tail DNA/100. Results are mean ± SD for n = 3. *Significantly different from control at $P < 0.05$.

In the present study, $NaHSO_3$ inhibited NO generation in SNP solution and LPS-activated macrophages, which may be related to reaction of NO and sulfite. Littlejohn *et al.*[23] had showed that the reactions of hydrogen sulfite or sulfite ions with NO in aqueous solution led to the formation of N-nitrosohydroxylamine-N-sulfonate [$^{-}ON(NO)SO_3^{-}$]. However, the inhibitory effects of daylily flower extracts may also be contributed by other components.

III. *Effect of Sulfite on SNP-mediated Macrophages DNA Damage*

DNA damage induced by various concentrations of sodium hydrogen sulfite measured by the comet assay, a simple and sensitive technique used to detect DNA damage in individual cells, is shown in Figure 5. A positive increase in the tail moment value was observed following 0.8 and 1.0 μg/mL $NaHSO_3$ treatment of macrophages in comparison with the untreated control ($P < 0.05$). The DNA damages induced by $NaHSO_3$ at test doses were still low as compared with that by other DNA damage reagents.

Sulfites enter the body by foods, beverages or drugs because sulfating agents are widely used as preservatives. In addition, sulfur dioxide is an air pollutant released into the atmosphere from the combustion of fossil fuel. Exposure to sulfite will result in the change of NO synthesis and regulatory function. There are increasing lines of evidence indicating that NO and its donors could induce DNA strand breaks *in vitro* and *in vivo*[24-25]. Harvey and Nelsestuen[12] reported that the reaction of sulfites with NO or proposed carriers of NO may be a major source of sulfite toxicity. The ability of NO and these carriers of NO to inhibit platelet aggregation could be reversed by low concentrations of sulfites. Koksala *et al.*[26] suggested that high concentrations of NO and cytokines may play a role in the pathogenesis of sulfur dioxide induced asthma-like syndrome, which was caused by sulfur dioxide exposure during apricot sulfurization processes. The toxicity of sulfite can markedly exacerbate the damage by peroxynitrite ($ONOO^{-}$) on a neuronal cell line[27]. Therefore, the effects of $NaHSO_3$ on SNP-mediated DNA damage were also measured in this study. However, as shown in Figure 6, $NaHSO_3$ had no significant effect on SNP-induced DNA damage in RAW 264.7 macrophages.

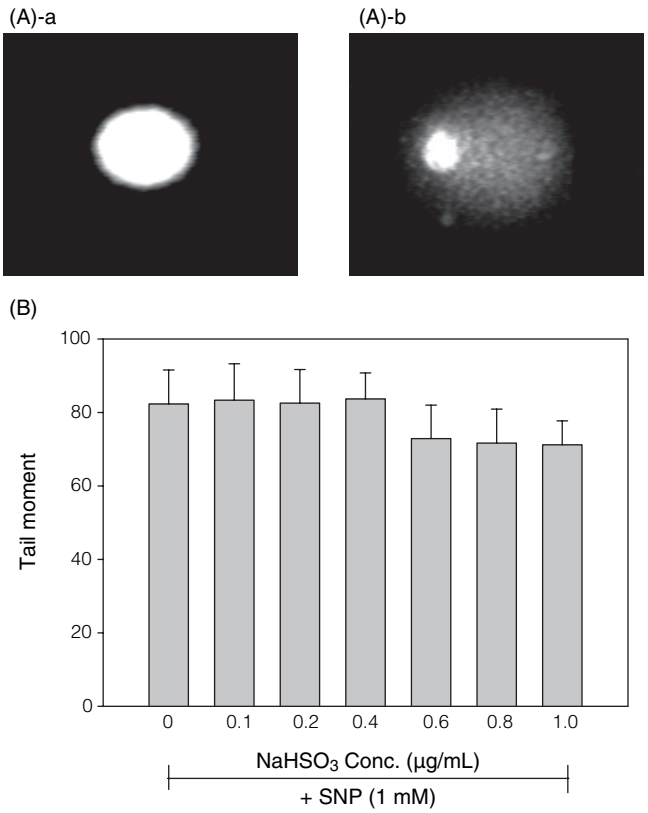

Figure 6. Effect of sodium hydrogen sulfite on sodium nitroprusside (SNP) induced DNA damage in RAW 264.7 macrophages. The tail moment was calculated as: tail length × percentage of tail DNA/100. Results are mean ± SD for n = 3.

IV. *Determination of Phenolic Compounds*

The phenolic compounds such as flavonoids and phenolic acids are important plant constituents because of their antioxidant activity[28-29]. Several researches also indicated that natural antioxidants such as flavonoids[30] and tea catechins[31] scavenged NO and proved their protective effects toward cell injury in pathophysiological processes. The analysis of phenolic compounds in daylily flowers is shown in Table 2. The contents of total phenolic compounds and flavonoids in the flower extracts were illustrated in the order of untreated dried daylily ≥ fresh daylily > sulfite-treated dried daylily. Therefore, the contents of phenolic compounds may be influenced by the mature period and bleaching treatment. Both carotenoids and flavonoids contribute to the yellow color in dried daylily flowers. Tai and Chen[32] indicated that the amounts of most carotenoids were higher in the $NaHSO_3$-soaked daylily flowers than in unsoaked ones. Moreover, air-drying resulted in a higher loss of carotenoids. The flavonoids and phenolic compounds reduced by the processes of bleaching. Cichewicz and Nair[4] isolated kaempferol, quercetin, isorthamnetin glycosides and naphthalene glycosides from edible daylily flower. All of these compounds possessed antioxidant properties. Flavonoids were also very potent NO scavengers *in vitro*[33]. Kim *et al.*[34]. evaluated the effects of various naturally occurring flavonoids on NO production in LPS-activated RAW 264.7 cells. Flavonoids such as apigenin, wogonin, luteolin, tectorigenin, and quercetin inhibited NO production in LPS-activated RAW 264.7 cells, and their inhibitory activity might be due to the reduction of inducible nitric oxide synthase (iNOS) expression. In our previous study, the extract from fresh daylily flower exerted the strongest inhibition on NO production, which was attributed to the ability to reduce the iNOS induction[7]. However, The differences of components between mature and immature daylily flowers are still unclear and need to be studied further.

Table 2. The contents of total polyphenol compounds and flavonoids of water extracts from various fresh daylily and dried daylily flowers

Samples	Total phenolic compounds (mg/g)	Flavonoids (mg/g)
Sulfite-treated dried daylily (No.1)	14.78^{b}*	17.38^{c}**
Sulfite-treated dried daylily (No.2)	9.47^{c}	15.94^{d}
Sulfite-treated dried daylily (No.3)	6.88^{d}	14.88^{d}
Sulfite-untreated dried daylily (No.4)	23.33^{a}	27.60^{a}
Fresh daylily (No.5)	22.68^{a}	22.44^{b}
Fresh daylily (No.6)	21.78^{a}	23.44^{b}

* Values in a column with the different superscripts are significantly different ($P < 0.05$).

In the present study, the inhibitory effects of daylily flower extracts on NO were different in various model system, and may be influenced by the mature period and bleaching process. Sulfite is a colorless, water soluble chemical used for bleaching and preserving foods, fruits and vegetables. Although sulfur dioxide could be volatilized in heating, the effects of residual sulfur dioxide on people, especially the patients with asthma should be considered. There should be a restricted quantity for the dried daylily flower intake. The fresh daylily flower is a potent inhibitor on NO generation, and its inhibitory effects may be due to reduction of iNOS enzyme expression by flavonoids.

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