RESEARCH COMMUNICATION

# Suppressive Effects of Okinawan Food Items on Free Radical Generation from Stimulated Leukocytes and Identification of Some Active Constituents: Implications for the Prevention of Inflammation-associated Carcinogenesis

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## Abstract

**Okinawa prefecture in Japan is a distinct area characterized by unique traditional food habits and longevity. Prolonged exposure to activated leukocytes, playing pivotal roles in chronic inflammation-associated carcinogenesis, is known to lead to oxidative and nitrosative damage to macromolecules in the body since they are primary sources of free radicals, such as superoxide anion ($O_2^-$) and nitric oxide (NO). In this study, we estimated anti-oxidative and anti-nitrosative activities of Okinawan food items by employing two cellular experimental systems: (1) phorbol ester-induced $O_2^-$ generation from differentiated HL-60 human promyelocytic leukemia cells; and (2) lipopolysaccharide (LPS)-induced NO generation in RAW264.7 murine macrophages. A total of 138 food items, consisting of 42 samples unique to Okinawa and 96 common in the Japanese main island, were purchased at local markets in Okinawa and extracted with chloroform. When tested at a concentration of 100 μg/ml, 38% (16/42) of the former showed 70% or more inhibition of $O_2^-$ generation while 21% (20/96) of the latter did so. In parallel, 64% (27/42) of the former showed significant NO generation suppression in contrast to 48% (46/96) of the latter . Twenty-one active species were further tested at a concentration of 20 μg/ml, and eleven species, including sugar cane, wild turmeric, and zedoary, were indicated to be most promising items with anti-oxidative and anti-nitrosative properties. In addition, some of the active constituents (chebulagic acid, a resveratrol derivative, and sesquiterpenoids) were identified. Our results suggest that food items typical in the Okinawa area have higher cancer preventive potential than those common in Japan.**

**Key Words**: Free radicals - Okinawa foodstuffs - inflammatory cells - screening

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## Introduction

It is known that plants have potentials to biosynthesize certain chemicals in response to environmental stress stimuli such as ultraviolet (UV) exposure, invading insects, bacteria, and viruses. For instance, they are forced to biosynthesize flavonoids and carotenoids to quench or scavenge free radicals derived from UV light exposure. Based on this principle, it can be hypothesized that the plants exposed to harsher stress must produce more biologically active phytochemicals in terms with quality and quantity. In fact, we have previously shown that extracts from edible plants in subtropical Southeast Asian countries, exposed to intense sun light and thriving microorganisms, show much higher anti-tumor promoting activity as detected by phorbol ester-induced EB virus activation as compared with those common in Japan (Koshimizu, 1988; Murakami, 1995, 1998, 2000a). Also it is important to note that activity-guiding separation of some of the active extracts resulted in isolation and identification of the active compounds with notable cancer preventive potentials and reasonable molecular mechanisms (Nakamura, 1998, 1999, 2000; Murakami, 1996, 2002, 2003, 2004; Tanaka, 1997ab, 2001; Ohata, 1998; Kobayashi, 1998; Miyauchi, 2000; Mofatt, 2000, 2002; Ito, 2004, 2005;

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Ichikawa, 2005; Takada, 2005).

Both endogenous (such as the arachidonate cascade, cytochrome P450, xanthine oxidase, and NADPH oxidase) and exogenous (such as UV light, heavy metals, and food types) oxidative stress are believed to be highly associated with carcinogenesis processes. In immune systems, neutrophils and macrophages are activated in response to exogenous stimuli, including microorganism invasion, to generate free radicals for host protection. It is important, however, to understand that imbalances of redox regulation and prolonged radical generation have been observed in some chronic inflammatory diseases of the digestive tract (Babbs, 1992; Nieto, 2000; Yamada, 1991). In fact, the activated leukocyte-derived oxidative damage may be involved in carcinogenesis by activating procarcinogens (Trush, 1985), participating in malignant transformation (Weitzman, 1985,1990), interacting with human epithelial cells (Kamp, 1989), and modulating DNA bases (Shacter, 1998; Shen, 2000). The NADPH oxidase system is present in inflammatory cells and consists of multiple components to produce superoxide anion ($O_2^-$) (Kobayashi, 2001). Beyond its action as a precursor of various reactive oxygen species, $O_2^-$ itself is involved in several signal transduction pathways (Barrett, 1999; Lee, 1998; Purohit, 1994; Sakai, 1994), while $O_2^-$ generation plays some important roles in colonic inflammation (Tardieu, 2000), cellular transformation (Nakamura, 1998), and carcinogenesis (Schwarz, 1984). On the other hand, there is now ample evidence that excess and/or prolonged generation of nitric oxide (NO) causes carcinogenesis in digestive organs (Goto, 1999; Ambs, 1998). NO is drastically released by the inducible enzyme, inducible NO synthase (iNOS). Moreover, it is of paramount importance to note that peroxynitrite, formed by a non-enzymatic reaction of $O_2^-$ with NO, is markedly mutagenic and responsible for tumor development (Maeda, 1998). Collectively, suppression of leukocytic free radical generation may reduce the risks of carcinogenesis.

The above mentioned background information led us to examine the suppressive effects of Okinawan food items on $O_2^-$ and NO generation from stimulated leukocytes, i.e., respectively differentiated HL-60 and RAW264.7 macrophages. As we had expected, their anti-oxidative and anti-nitrosative activities were remarkably higher than those common in Japan, supporting our hypothesis that food items from subtropical areas are promising and interesting sources of cancer preventive agents based on the differences in environmental conditions. Additionally, the isolation and identification of some active constituents are described.

## Materials and Methods

*Chemicals*

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Research Biochemicals International (Natick, MA). Dulbecco's modified eagle medium (DMEM), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Gibco RBL (Rockville, NY). Lipopolysaccharide (LPS, *E. coli* serotype 0127, B8) was purchased from Difco Labs (Detroit, MI) and interferon (IFN)-γ from Genzyme (Cambridge, MA). Cytochrome C was obtained from Sigma (St. Louis, MO). Chebulagic acid was donated from Dr. Toshio Ichiba, Okinawa Industrial Technology, Center, Okinawa, Japan. All other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless specified otherwise.

*Cells*

HL-60 cells (human promyelocytes) and RAW 264.7 cells (mouse macrophages) were purchased from American Type Culture Collection (Rockville, MA) and cultured respectively in RPMI and DMEM media. Ten percent FBS, 200 U/ml penicillin, and 250 ng/mL streptomycin were added to each, and the cell lines were maintained at 37°C in a 5% $CO_2$ atmosphere.

*Food Samples*

Each sample, purchased at local markets in Okinawa in 2002, was cut into small pieces and soaked in chloroform followed by one-week extraction.

*$O_2^-$ Generation in Differentiated HL-60 Cells*

An inhibitory test of TPA-induced $O_2^-$ generation was performed as previously reported (Murakami, 2002). Briefly, HL-60 cells (5 x $10^5$ cells/ml) were incubated in 1.3% dimethylsulfoxide (DMSO) in RPMI 1640 medium for 6 days. Differentiated HL-60 cells (1 x $10^6$) were incubated with the test compound, then dissolved in DMSO (0.5%, υ/υ) and 1 mL of Hank's buffer for 15 minutes. After washing, the cells were further incubated with 100 nM TPA and 1 mg/mL cytochrome C at 37°C for 60 minutes. Cell viability was measured by trypan blue-dye exclusion test. The level of extracellular $O_2^-$ was then measured by a cytochrome C reduction method. Cells treated with only the vehicle plus cytochrome C and with TPA plus cytochrome C were used as negative and positive controls, respectively.

*$NO_2^-$ Production*

A suppressive test of NO generation was done as previously reported (Murakami, 2002). RAW 264.7 cells (8 x $10^5$ cells/2 mL) in a 60-mm dish were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and specified concentrations of the test compound dissolved in DMSO (0.5%, υ/υ). After 12 hours, the levels of nitrite and cytotoxicity were measured by Griess and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, respectively. Cells treated with only the vehicle and with LPS/IFN-γ were used as negative and positive controls, respectively.

*Inhibitor Rate (IR) and Cell Viability (CV)*

Each experiment was done in duplicate, and the data are expressed by the mean value. The standard errors did not exceed 10% of the means (data not shown). The IR in each assay was calculated using the following equation: IR (%)

= {1- [(test sample data)-(negative control data)][(positive control data)-(negative control data)]$^{-1}$} x 100. Similarly, CV was calculated using the following equation: CV (%) = {1-[(test sample data)-(negative control data)][(positive control data)-(negative control data)]$^{-1}$} x 100. The statistical significance of differences between groups in each assay was assessed by a Student's t-test (two-sided) that assumed unequal variance.

*Isolation and Identification of Active Constituents*
3-methoxy-5-hydroxystilbene

Dried leaves of *Alpinia speciosa* K. schum (1 kg) was extracted with chloroform at room temperature for a week. The extract was filtered and concentrated in vacuo using an evaporator, and then the concentrate (28 g) was subjected to a column chromatography on Wakogel C-200 (increasing the volume of ethyl acetate in *n*-hexane as elute). Eighty-percent ethyl acetate fraction (3.3 g) was then subjected to a reverse phase ($C_{18}$), medium pressure column chromatography on ODS gel (Yamamura Chemical, Kyoto, Japan), increasing the volume of methanol in $H_2O$ as elute. Fifty-percent methanol fraction (106 mg) was purified by preparative thin layer chromatography (25% ethyl acetate in n-hexane) and preparative high performance liquid chromatography (HPLC) (column, J'sphere, Yamamura Chemical; mobile phase, 60% acetonitrile in $H_2O$; flow rate, 7.0 ml/min; detection, 254 nm; retention time, 11.0 min) to afford 3-methoxy-5-hydroxystilbene (purity > 95%). The chemical identity was confirmed by comparing its spectral data (nuclear magnetic resonance, NMR, and mass spectrometry, MS) with those previously reported (Koon-Shin, 1998).

*Sesquiterpenes*

*Curcuma longa* L. (1 kg) was extracted with chloroform at room temperature. The extract was concentrated in vacuo followed by partition between chloroform and water. The chloroform layer thus obtained (3.4 g) was subjected silica gel column chromatography which was eluted with increasing amounts of ethyl acetate in n-hexane. The 2.5% ethyl acetate fraction (800 mg) was further separated on silica gel (0-5% ethyl acetate in *n*-hexane), which had been pretreated with 5% $AgNO_3$ in water and dried. This step allowed us to purify β-atlantone (29 mg). Each fraction was subjected to HPLC (column, YMC ODS AQ-302, Yamamura Chemical; mobile phase, 85% acetonitrile in $H_2O$; flow rate, 1.0 ml/min; detection, 254 nm) to purify sesquiterpenes (retention time, min; and yield, mg): ar-turmerone (4.0, 130 mg), germacrone (4.8, 61 mg), β-turmerone (5.2, 157 mg), and α-termerone (5.3, 100 mg). The retention time of β-atlantone in these HPLC conditions was 5.3 min, and could hardly be separated from those for α- and β-termerones. The chemical identity of isolated sesquiterpenes was confirmed by comparing their spectral data (NMR and MS) with those previously reported (Yang, 2005; Li, 2003; Itokawa, 1984; Golding, 1992). The purity of each compound was > 95%.

## Results and Discussion

*Screening of Okinawan Food Items*

A total of 138 food samples, collected in Okinawa prefecture, consists of 47 families of vegetables, fruits, seaweed, and others. Eight major families are Leguminosae, Solanaceae (10 samples each), Compositae, Rutaceae (9 samples each), Umbelliferae (8 samples), Cucurbitaceae, Gramineae, and Zingiberaceae (7 samples each), and Cruciferae (6 samples) (Table 1). These samples were extracted with chloroform and anti-oxidative and anti-nitrosative activities were evaluated at a concentration of 100 μg/mL for suppression of phorbol ester-induced $O_2^-$ generation in DMSO-differentiated HL-60 cells and that of LPS-induced NO generation in RAW264.7 macrophages, respectively. It is notable that both Gramineae and Zingiberaceae samples showed relatively high frequency of marked suppressive activity (70% > IR) toward both $O_2^-$ and NO generation (5/7, 71% for $O_2^-$ and 6/7, 86% for NO, with respect to each family). These two promising families are followed by Cruciferae (3/6, 50% for $O_2^-$ and 6/6, 100%) and Cucurbitaceae (2/7, 29% for $O_2^-$ and 5/7, 71% for NO). Interestingly, the samples of Umbelliferae has a quite contrastive profile for free radical generation suppression since one out of 8 samples (13%) markedly attenuated $O_2^-$ generation, whereas a high rate (8/8, 100%) was seen for NO generation reduction, suggesting that NO suppressive constituents widely occur in this family, but they have little ability to attenuate $O_2^-$ generation. In this context, it is intriguing to point out that the Umbelliferae plants abundantly biosynthesize the coumarin-related compounds which have been to demonstrated by us to be able to efficiently suppress NO generation in macrophages (Murakami, 1999, 2000b).

Then we classified all samples tested into those commonly seen in Japanese main island and those typical in Okinawan area. Test samples showing 70% or more suppression are designated as 'effective sample'. Figure 1 shows the results of the classification with respect to the suppression of $O_2^-$ (panel A), NO (panel B) and both (panel C), in which the portion of 'effective samples' are hatched in black. It should be noted that Okinawan samples exhibit markedly higher rates of 'effective samples' in each evaluation system. Those results are consistent with our previous work reporting that the extracts from subtropical foods exhibited higher in vitro anti-tumor promoting activity than those from Japanese common ones (Koshimizu, 1988; Murakami, 1995, 1998, 2000a), implying again that subtropical plants produce biologically active chemicals in terms of quantity and quality to protect themselves from invading microorganisms and insects as well as photo-damages by sunlight.

Since the numbers of effective samples in $O_2^-$ and NO assay systems are 31 and 79, respectively, second screening tests, in which sample concentration was decreased to 20 and 4 μg/mL, were done to focus on promising samples which deserve the isolation and identification of active

**Table 1. Suppressive Effects of Okinawan and Common Food Items on TPA-induced $O_2$- generation in Differentiated HL-60 Cells and LPS/IFN-γ-induced NO Generation in RAW265.7 Cells**

| Family/species | Common name | Part tested | $O_2$- IR (%) | CV (%) | NO IR (%) | CV (%) |
|---|---|---|---|---|---|---|
| Actinidiaceae | | | | | | |
| *Actinidia chinensis* Planch. | Kiwi Fruit | Fruit | 16 | 87 | 100 | 100 |
| Anacardiaceae | | | | | | |
| *Mangifera indica* L. | Mango | Fruit | 6.0 | 8.1 | 31 | 100 |
| Auriculariaceae | | | | | | |
| *Auricularia auricula* Underwood. | Cloud ear mushroom# | Whole Part | 92 | 0 | 76 | 97 |
| Araceae | | | | | | |
| *Colocasia esculenta* Schott. | Taro (fresh) | Stalks | 2.2 | 83 | 81 | 79 |
| Taro (powdered) | | Stalks | 0 | 91 | 54 | 94 |
| Bromeliaceae | | | | | | |
| *Ananas comosus* Merrill. | Pineapple* | Fruit | 3.4 | 80 | 30 | 86 |
| Caricaceae | | | | | | |
| *Carica papaya* L. | Papaya* | Fruit | 0 | 69 | 13 | 100 |
| | Papaya* | | 4.2 | 76 | 86 | 99 |
| Cheneopodeaceae | | | | | | |
| *Chenopoofium* L | *Akino-wasuregusa** | Roots | 24 | 81 | 85 | 65 |
| *var.centrorubrum* Mkino. | *Akino-wasuregusa** | Stem | 2.7 | 91 | 43 | 96 |
| *Spinacia oleracea* L. | Spinach | Leaves, Stem | 7.0 | 83 | 100 | 92 |
| Coccoloba | | | | | | |
| *Coccoloba umifera* | *Umi-budo** | Whole Part | 2.8 | 96 | 10 | 100 |
| Combretaceae | | | | | | |
| *Terminalia catappa* | Indian Almond*# | Leaves | 91 | 0 | 100 | 34 |
| Indian Almond* (fermented) | | Leaves | 4.5 | 74 | 100 | 36 |
| Compositae | | | | | | |
| *Artemisia campestris* L. | *Ryukyu-yomogi** | Leaves | 55 | 81 | 100 | 34 |
| *A. princeps* Pamp. | Worm wood | Leaves, Stem | 3.4 | 81 | 87 | 51 |
| *Asparagusu officinalis* L. | Common Asparagus | Stem | 4.6 | 89 | 47 | 100 |
| *Carthamus tinctorius* L. | Safflower | Flower | 27 | 67 | 100 | 34 |
| *Chrysantyemum coronarium* L. | Garland | Leaves, Stem | 0.9 | 51 | 100 | 41 |
| *Cirsium japoricum* | Shima-gobo* | Fruit | 3.9 | 81 | 17 | 55 |
| *Glynura bicolor* DC. | Two-Colored Gynura* | Leaves | 1.2 | 81 | 0 | 84 |
| *Ixeris dentate* Nakai. | Nigana* | Leaves, Stem | 12 | 97 | 81 | 94 |
| *Lacutuca sativa* L. | Lettuce | Leaves | 28 | 94 | 75 | 100 |
| Convolvulaceae | | | | | | |
| *Ipomoea aquatica* Forsk. | Water Convolvulus* | Leaves, Stem | 2.6 | 57 | 77 | 64 |
| *I. batatas* | *Beni-imo* (white)* | Stalks | 36 | 96 | 100 | 100 |
| | *Beni-imo* (brown)* | Stalks | 42 | 76 | 78 | 77 |
| *I batatas* Lam. | Sweet Potato | Root | 6.0 | 86 | 63 | 100 |
| Cruciferae | | | | | | |
| *Brassica chinensis* L. | Qing gin cai | Leaves, Stem | 6.7 | 73 | 100 | 51 |
| *B. compestris* L.var.pervilids. | Komatsuna | Leaves, Stem | 14 | 82 | 86 | 100 |
| *B. jnucea* | Leaf Mustard | Leaves, Stem | 0.4 | 89 | 60 | 45 |
| *B. oleracea var.capitata* L. | Cabbage | Leaves | 2.7 | 85 | 0 | 64 |
| *Nasturtium officinale* RBr. | Creson# | Leaves, Stem | 96 | 11 | 97 | 47 |
| *Raphanus sativus* | Radish | Leaves | 0.8 | 70 | 100 | 69 |
| | Radish# | Sprout | 93 | 93 | 100 | 28 |
| | Radish# | Stem | 92 | 24 | 100 | 31 |
| Cucurbitaceae | | | | | | |
| *Berincasa hispida* Cogn. | White Gourd | Fruit | 1.4 | 89 | 66 | 100 |
| *Cucubita moschata* Poir. *var.meronaeformis* | Pumpkin | Fruit | 18 | 76 | 100 | 100 |
| *Cucumis sabivus* L. | Cucumber | Fruit | 31 | 80 | 80 | 41 |
| *Lagenaria hispida* Hera. | Bottle Gourd# | Fruit | 97 | 0 | 100 | 71 |
| *Luffa cylindrical* Roem. | Sponge Gourd*# | Fruit | 94 | 83 | 79 | 35 |
| *Momordica charantia* L. | Balsam Pear* | Fruit | 3.1 | 100 | 76 | 100 |
| *M. grosvenori* S. | | Stalks | 0 | 74 | 7.4 | 69 |
| Dioscoreaceae | | | | | | |
| *Dioscorea japonica* Thunberg. | Yam | Stalks | 29 | 30 | 89 | 62 |

**Table 1. Continued**

| Family/species | Common name | Part tested | $O_2^-$ IR (%) | CV (%) | NO IR (%) | CV (%) |
|---|---|---|---|---|---|---|
| Elaegnaceae | | | | | | |
| *Elaegunus glabra* Thurb. ex Murray. | *Tsuru-gumi** | Fruit | 31 | 93 | 100 | 86 |
| Gracilariaceae | | | | | | |
| *Gracilaria blodgetti* | *Kubire*#* | Whole Part | 99 | 0 | 100 | 36 |
| Gramineae | | | | | | |
| *Coix lachryma-jobi* L. *var.lachryma-jobi* | Job's Tear*# | Grain | 99 | 48 | 100 | 18 |
| *Oryva sativa* L. | Rice | Grain | 100 | 42 | 39 | 100 |
| | *Mochigome#* | Grain | 91 | 20 | 94 | 95 |
| | *Kurokome#* | Grain | 98 | 66 | 98 | 100 |
| *Cymbopogon citratus* | Lemongrass | Leaves | 0 | 98 | 100 | 47 |
| *Saccharum officinarum* L. | Sugar Cane*# | Stem | 90 | 100 | 100 | 32 |
| *Zizania Latifolia* Stapf | Manchurian Wild Rice* | Grain | 43 | 70 | 100 | 44 |
| Hypneaceae | | | | | | |
| *Hypnea charoides* Lamour. | Carrageenan* | Whole Part | 9.4 | 100 | 100 | 100 |
| Labiatae | | | | | | |
| *Mentha arvensis* | Cool Mint | Leaves | 25 | 74 | 100 | 77 |
| *Orthosiphon stamineus* Benth. | Cats Whiskers* | Leaves | 63 | 68 | 100 | 71 |
| *Perila frubescens Britton var.crispa* Hassk. | Perilla # | Leaves | 89 | 36 | 97 | 42 |
| Lauraceae | | | | | | |
| *Persea americana* Mill. | Avocado | Fruit | 26 | 84 | 100 | 100 |
| Leguminosae | | | | | | |
| *Archis hypogena* L. | Peanut | Nuts | 0.6 | 75 | 13 | 100 |
| *Cassia obtusifolia* L. | Oriental Senna | Leaves | 2.0 | 96 | 100 | 34 |
| *Glycine max* Merr. | Soybean | Beans | 6.0 | 87 | 24 | 51 |
| | | Sprout | 4.9 | 93 | 91 | 100 |
| *Phasedus vulgaris* L. | Common Bean | Pod | 0 | 88 | 29 | 100 |
| *P. vulgaris* L. | *Uzura-mame* | Pod | 13 | 92 | 55 | 100 |
| *Pisum sativum* L. | Pea | Beans | 2.5 | 70 | 25 | 100 |
| *Vaccinium uliginosum* | *Kuromame* bean | Beans | 58 | 89 | 69 | 87 |
| *Vigna angularis* Ohwi et.Chashi. | Adzuki bean | Beans | 8.2 | 89 | 48 | 100 |
| *V. radiata* Rwlczek. | Mung bean | Beans | 7.0 | 99 | 15 | 70 |
| Liliaceae | | | | | | |
| *Allium bakeri* | *Shima-rakkyo* | Bulb | 21 | 97 | 56 | 82 |
| *A sativum* L. | Garlic | Bulb | 20 | 75 | 83 | 100 |
| | | Leaves | 0 | 81 | 100 | 59 |
| *Aloe arborescens* Mill. | Aloe | Leaves | 3.3 | 73 | 18 | 100 |
| Malvaceae | | | | | | |
| *Hibisucus mutabilis* L. | Hibiscus# | Flower | 99 | 0 | 100 | 63 |
| Musaceae | | | | | | |
| *Musa acuminata* Colla L. | Banana | Fruit | 1.9 | 92 | 3.8 | 100 |
| Myrtaceae | | | | | | |
| *Eucalyptus globules* Labill. | Blue Gum# | Leaves | 89 | 0 | 100 | 75 |
| *Psidium guajava* L. | Guava (white)* | Fruit | 1.8 | 80 | 27 | 100 |
| | Guava (pink)* | Fruit | 13 | 100 | 23 | 100 |
| | Guava*# | Leaves | 91 | 0 | 100 | 79 |
| Oxalidaceae | | | | | | |
| *Arerrhoa carambola* L. | Star Fruit | Fruit | 14 | 100 | 27 | 14 |
| Passiflora | | | | | | |
| *Passiflora edulis* Sims. | Passion Fruit | Fruit | 0.6 | 93 | 12 | 100 |
| Pedaliaceae | | | | | | |
| *Sesamum indicum* L. | Sesame (white) | Seeds | 0 | 84 | 22 | 71 |
| | Sesame (black) | Seeds | 0 | 96 | 15 | 84 |
| Pinaceae | | | | | | |
| *Annina atemoya* hort. | Custard apple | Fruit | 98 | 0 | 65 | 52 |
| Piperaceae | | | | | | |
| *Piper retrofracctum* Vahl. | Hihatsu-modoki | Seeds | 3.6 | 82 | 76 | 72 |

(Continued next page)

**Table 1. Continued**

| Family/species | Common name | Part tested | $O_2^-$ IR (%) | CV (%) | NO IR (%) | CV (%) |
|---|---|---|---|---|---|---|
| Plumbaginaceae | | | | | | |
| *Limonium wrightii* O,K, | Ukon-isomatsu*# | Spray | 88 | 100 | 96 | 14 |
| Rhodomelaceae | | | | | | |
| *Digenea simplex* C.Ag. | Digenea*# | Whole Part | 77 | 0 | 100 | 29 |
| Rosaceae | | | | | | |
| *Eriobotrya japorica* Lindl. | Loquat | Fruit | 33 | 60 | 54 | 100 |
| _ *Prunus campanulata* Maxim | *Kanhi-zakura** | Fruit | 10 | 81 | 66 | 100 |
| Rutaceae | | | | | | |
| *Citrus deressa* | Shekwasha* | Fruit | 0 | 91 | 17 | 100 |
| | | Juice | 1.4 | 88 | 18 | 78 |
| *C. limon* Burm. | Lemon# | Fruit | 79 | 0 | 100 | 62 |
| *C. natudaidai* Hayata. | Natsudaidai Orange | Fruit | 0 | 74 | 12 | 68 |
| *Citrus nobilis* | Marcott | Fruit | 2.6 | 93 | 4.1 | 68 |
| *C. tamurana.* hort.ex Tanaka. | Hyuganatsu | Fruit | 1.2 | 87 | 0 | 85 |
| *C. tankan* Hayata. | Tankan | Fruit | 4.4 | 93 | 0 | 90 |
| *Fortunella crassifolia* Swingle. | Kumquats | Fruit | 6.7 | 94 | 36 | 100 |
| *Toddalia asiatica (L.) Lam.* | *Sarukake-mikan** | Bark | 21 | 98 | 100 | 48 |
| Sapotaceae | | | | | | |
| *Pouteria campechiana(HBK)* Baehni. | Canistel | Fruit | 2.7 | 100 | 16 | 85 |
| Saururaceae | | | | | | |
| *Houttuynia cordata* Thunb. | Dokudami | Leaves | 27 | 93 | 100 | 79 |
| Sargassum | | | | | | |
| *Sarugassum fusiforma (Harvey)* Setchell. | Yunabaru-hijiki* | Whole Part | 11 | 80 | 100 | 41 |
| Solanaceae | | | | | | |
| *Capsicum annuum* CV. | Paprica (red) | Fruit | 3.7 | 97 | 0 | 100 |
| | Paprica (yellow) | Fruit | 1.8 | 91 | 71 | 34 |
| *C. annuum var.grossum.*Sendt. | Bell Pepper | Fruit | 3.4 | 92 | 10 | 99 |
| *C. annuum L.var.angulosum* Mill. | Sweet pepper | Fruit | 9.0 | 93 | 39 | 100 |
| *Lycium barbarum* L. | Chinese Wolfberry | Seed | 8.5 | 84 | 75 | 100 |
| *Lycopersicon esculentum* Mill. | Tomato | Fruit | 2.3 | 92 | 92 | 84 |
| | Tomato Cherry | Fruit | 0.6 | 87 | 100 | 22 |
| *Physalis alkekengi L.var.dulce* Pers. | Japanese Lantern Plant | Fruit | 4.0 | 82 | 5.4 | 100 |
| *Solanum melongena* L. | Eggplant | Fruit | 0 | 86 | 53 | 100 |
| *Solanum tuberosum* L. | Potato | Stem | 0 | 71 | 42 | 70 |
| Spermatochnaceae | | | | | | |
| *Nemacystis decipiens* Kuckuck. | *Mozuku#* | Whole Part | 96 | 0 | 100 | 59 |
| Theaceae | | | | | | |
| *Camelia sinensis* O.Kuntze. | Tea | Leaves | 15 | 71 | 65 | 93 |
| Tiliaceae | | | | | | |
| *Corchorus olitorius* | Jew's mallow | Leaves | 48 | 70 | 79 | 53 |
| Tricholomataceae | | | | | | |
| *Lentinus edodes* Singer. | Shiitake mushroom# | Whole Part | 85 | 0 | 100 | 60 |
| *Lyophyllum* decastes. | Shimeji mashroom# | Whole Part | 91 | 100 | 100 | 13 |
| Ulmaceae | | | | | | |
| Celtis sinensis var japonica | Chinese Hackberry | Whole Part | 98 | 0 | 59 | 45 |
| Umbelliferae | | | | | | |
| *Apium Graveolens* L.*var.dulce* Pers. | Celery | Leaves, Stem | 1.8 | 95 | 86 | 42 |
| *Chryptotaenia japonica* Hassk. | Japanese Hornwort | Leaves, Stem | 68 | 49 | 87 | 31 |
| *Daucus carota* L. | Carrot | Root | 7.2 | 91 | 98 | 37 |
| | *Shima-ninjin** | Root | 23 | 85 | 100 | 91 |
| | *Kyo-ninjin* | Root | 7.0 | 85 | 100 | 38 |
| *Foericulum vulgare* Mill. | Fennel | Leaves, Stem | 10 | 67 | 88 | 51 |
| *Peucedanum japoricum* Thurb. | Botan-boufu*# | Root | 100 | 50 | 91 | 49 |
| | Botan-boufu* | Stem | 67 | 89 | 100 | 34 |
| Vitaceae | | | | | | |
| *Vitis vinifera* L. | *Kyoho* | Fuit | 0 | 78 | 8.4 | 77 |
| Zingiberaceae | | | | | | |
| *Alpinia speciosa* K. Schum. | Shell Flower | Leaves | 21 | 81 | 100 | 35 |

(Continued next page)

**Table 1. Continued**

| Family/species | Common name | Part tested | $O_2^-$ IR (%) | CV (%) | NO IR (%) | CV (%) |
|---|---|---|---|---|---|---|
| | (fermented)*# | Leaves | 90 | 45 | 100 | 38 |
| | (powdered)* | Leaves | 44 | 70 | 100 | 33 |
| *Curcuma aromatica* Salisb. | Wild Turmeric*# | Root | 94 | 19 | 93 | 61 |
| *C. longa* L. | Turmeric*# | Root | 94 | 0 | 76 | 52 |
| *C. zedoaria* Rosc. | Zedoary*# | Root | 89 | 0 | 100 | 35 |
| *Zingiber officinale* Rosc. | Ginger | Root | 90 | 14 | 51 | 41 |
| Miscellaneous | | | | | | |
| | *To-fuyo** | | 54 | 93 | 95 | 90 |
| | *To-fuyo* (liquid)* | | 9.0 | 93 | 32 | 100 |
| *Laticauda semifasciata* | Sea snake (dried) * | Whole | 5.0 | 93 | 41 | 93 |

Differentiated HL-60 cells (1 x $10^6$) were incubated with the test compound, then dissolved in DMSO (0.5%, v/v) and 1 mL of Hank's buffer for 15 minutes. After washing, the cells were further incubated with 100 nM of TPA and 1 mg/mL of cytochrome C at 37oC for 60 minutes. Cell viability (CV) was measured using a trypan blue-dye exclusion test. The level of extracellular $O_2^-$ was then measured with a cytochrome C reduction method. Cells treated with only the vehicle plus cytochrome C and those with TPA plus cytochrome C were used as negative and positive controls, respectively. RAW 264.7 cells (8 x $10^5$ cells/2 mL) in a 60-mm dish were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and specified concentrations of the test compound dissolved in DMSO (0.5%, υ/υ). After 12 hours, the levels of nitrite and CV were measured using Griess and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, respectively. Cells treated with only the vehicle and those with LPS/IFN-γ were used as negative and positive controls, respectively. #Samples that showed 70% suppression or more in both assay systems. *Food items that specifically occur in Okinawa prefecture. Data are shown as averages from the results of duplicate experiments.

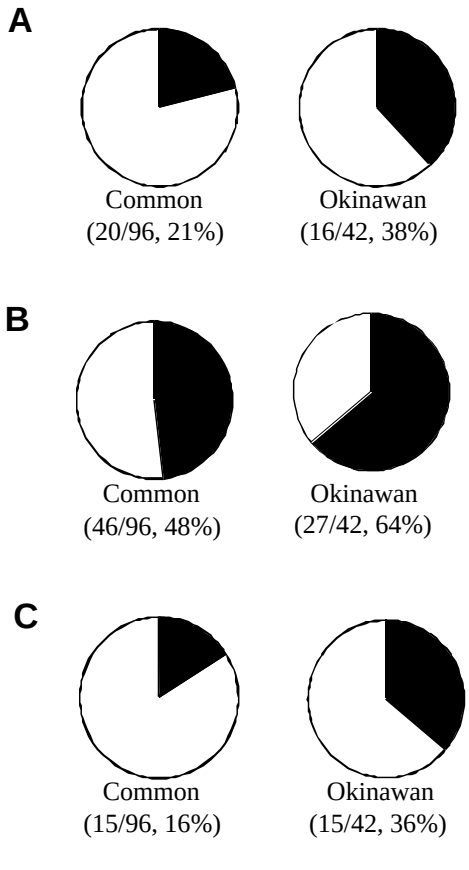


**Figure 1. Comparison of Suppressive Potencies of Okinawan and Common Food Items on Free Radical Generation from Stimulated Leukocytes.** The proportions of food item extracts which showed 70% or more suppression of $O_2^-$ (Panel A), NO (Panel B), and both (Panel C) are shown as the hatched area in each graph.

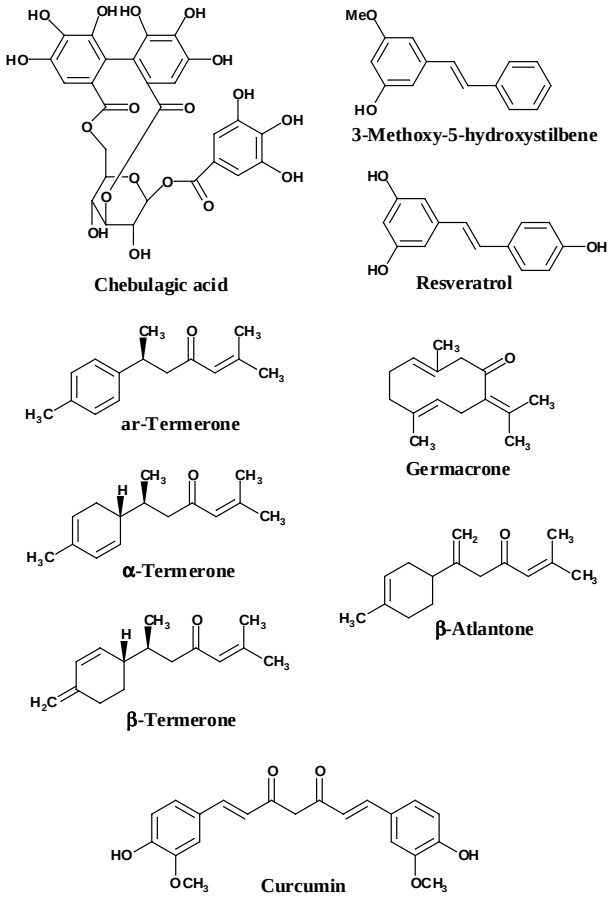


**Figure 2. Chemical Structures of Phytochemicals Examined in an LPS-induced NO Generation Test.** Origins as follows: chebulagic acid (*Terminalia catappa*), 3-methoxy-5-hydroxystilbene (*Alpinia speciosa* K. schum), resveratrol (grape, etc.), ar-turmerone, α- and β-turmerone, β-atlantone, germacrone, and crucumin (Curcuma longa L.).

principles. Twenty-three samples which showed 70% suppression or more in both assays were selected for secondary tests. As the results, 13 samples showed 70% suppression or more toward $O_2^-$ generation at a concentration of 20 μg/ml and only sugar cane and wild turmeric (4 μg/ml) attenuated $O_2^-$ generation by 50% or more (Table 2). On the other hand, most of the samples (21/23, 91%) suppressed NO generation by 70% or more at a concentration of 20 μg/ml, and 39% (9/23) of the total were still suppressive at a concentration of 4 μg/ml. The present results, together with our previous extensive screening for the anti-tumor promoting activities (Koshimizu, 1988; Murakami, 1995, 1998, 2000a) and anti-oxidation activities (Kim, 1998, 2002) of Asian food items, may provide a useful database to search for food materials and its constituents which possess notable cancer preventive potentials derived from their anti-inflammatory properties. To support this notion, it can be indicated that we have so far reported several phytochemicals which are functionally novel and exhibited significant cancer preventive activity in a series of rodent models (Nakamura, 1998, 1999, 2000; Murakami, 1996, 2003, 2004; Tanaka, 1997ab, 2001; Kobayashi, 1998; Miyauchi, 2000). Of great importance, those agents have been proven to show biochemical properties of $O_2^-$ and NO generation suppression in vitro and in vivo (Nakamura, 1998, 1999, 2000; Murakami, 1996, 2002, 2003, 2004; Ohata, 1998). Thus, it is worth making an attempt to isolate and identify the active principles occurring in highly promising food samples listed in Table 2.

*The Active Principles Which Suppress NO Generation*

Based on the present results, literature information, and sample availability, we attempted to identify the active components of three food items, i.e., *getto* (*Alpinia speciosa* K. Schum, Zingiberaceae), turmeric (*Curcuma longa* L., Zingiberaceae), and momotamana (*Terminalia catappa*, Combretaceae). The Spectroscopic data, including those from $^1$H-NMR and MS analyses, they were revealed to be 3-methoxy-5-hydroxystilbene, five known sesquiterpenes, and chebulagic acid (an ellagitannin), respectively, by comparing them with previously published data (Koon-Shin, 1998; Yang, 2005; Li, 2003; Itokawa, 1984; Golding, 1992). 3-Methoxy-5-hydroxystilbene has been isolated another species (*Alpinia katsumadai*, Koon-Shin, 1998) but isolated

**Table 2. Suppressive Activities Toward Free Radical Generation by Okinawan and Common Food Items in the Second Set of Screening Tests**

| Common name | Superoxide | | | | Nitric oxide | | | |
|---|---|---|---|---|---|---|---|---|
| | % suppression | | % cell viability | | % suppression | | % cell viability | |
| | 20 | 4 | 20 | 4 | 20 | 4 | 20 | 4 (μg/mL) |
| Blue gum | 85 | 45 | 28 | 100 | 100 | 100 | 35 | 94 |
| *Botan-boufu1*(stem)* | 33 | 4.6 | 63 | 100 | 100 | 89 | 62 | 100 |
| *Botan-boufu* (root)* | 95 | 29 | 60 | 100 | 100 | 100 | 37 | 16 |
| Bottle gourd | 95 | 0 | 100 | 100 | 93 | 21 | 92 | 100 |
| Cresson | 100 | 40 | 100 | 100 | 100 | 100 | 36 | 87 |
| Digenea* | 100 | 4.5 | 100 | 99 | 100 | 3.6 | 86 | 97 |
| *Getto** | 56 | 4.8 | 100 | 100 | 100 | 74 | 55 | 60 |
| *Getto** (fermented) | 39 | 2.7 | 100 | 100 | 100 | 76 | 27 | 93 |
| Hibiscus* | 82 | 12 | 80 | 100 | 75 | 0 | 100 | 0 |
| Indian almond* | 51 | 14 | 100 | 100 | 100 | 83 | 43 | 77 |
| Indian almond* (fermented) | 44 | 26 | 73 | 100 | 100 | 76 | 44 | 100 |
| Job's tear* | 44 | 0.6 | 100 | 100 | 100 | 45 | 53 | 100 |
| *Kubire** | 89 | 13 | 100 | 100 | 92 | 23 | 85 | 84 |
| Mitsuba | 68 | 0.8 | 92 | 93 | 100 | 100 | 33 | 38 |
| *Moasa** | 95 | 24 | 79 | 100 | 100 | 19 | 67 | 86 |
| Radish (sprout) | 49 | 12 | 100 | 100 | 100 | 56 | 71 | 98 |
| Radish (stem) | 77 | 0 | 63 | 100 | 100 | 69 | 50 | 80 |
| Rice (black) | 52 | 0 | 54 | 100 | 52 | 0 | 90 | 0 |
| Rice (*mochigome*) | 41 | 6.5 | 100 | 100 | 21 | 10 | 96 | 100 |
| *Shiitakem*mashroom | 100 | 1.1 | 0 | 100 | 86 | 19 | 92 | 53 |
| Sugar cane* | 100 | 55 | 100 | 100 | 100 | 89 | 92 | 76 |
| Wild termeric* | 100 | 68 | 8.2 | 100 | 100 | 40 | 39 | 91 |
| Zedoary* | 81 | 18 | 86 | 100 | 100 | 20 | 62 | 92 |

HL-60 cells (5 x $10^5$ cells/ml) were incubated in 1.3% dimethylsulfoxide (DMSO) in RPMI 1640 medium for 6 days. Differentiated HL-60 cells (1 x $10^6$) were incubated with the test compound, then dissolved in DMSO (0.5%, υ/υ) and 1 mL of Hank's buffer for 15 minutes. After washing, the cells were further incubated with 100 nM of TPA and 1 mg/mL of cytochrome C at 37˚C for 60 minutes. Cell viability was measured using a trypan blue-dye exclusion test. The level of extracellular $O_2$- was then measured with a cytochrome C reduction method. Cells treated with only the vehicle plus cytochrome C and those with TPA plus cytochrome C were used as negative and positive controls, respectively. RAW 264.7 cells (8 x $10^5$ cells/2 mL) in a 60-mm dish were treated with LPS (100 ng/mL), tetrahydrobiopterin (10 mg/mL), IFN-γ (100 U/mL), L-arginine (2 mM), and specified concentrations of the test compound dissolved in DMSO (0.5%, υ/υ). After 12 hours, the levels of nitrite and cytotoxicity were measured using Griess and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays, respectively. Cells treated with only the vehicle and those with LPS/IFN-γ were used as negative and positive controls, respectively. *Food items specific to Okinawa prefecture. Data are shown as averages from the results of duplicate experiments.

from *A. speciosa* for the first time. A grape stilbene, resveratrol, and the yellow pigment in turmeric, curcumin, were also subjected to NO generation test because their ability to suppress NO generation has already been reported (Cho, 2002; Brouet, 1995) and because 3-methoxy-5-hydroxystilbene is structurally analogous to resveratrol.

The test agents were added to a RAW264.7 cell culture at the concentrations of 4, 20 and 100 μM. As shown in Figure. 3A-C, chubulagic acid showed a concentration-dependent NO generation suppression without noticeable cytotoxicity. In accordance with the previous report (Cho, 2002), resveratrol markedly suppressed NO generation, and its analog, 3-methoxy-5-hydroxystilbene, was found to be cytotoxic at a higher concentration (Figurs. 3B and 3C). Curcumin abolished NO generation at a concentration of 20 μM whereas it was highly cytotoxic at 100 μM as detected by the value of $NO_2^-$ concentration/cell viability (Figure. 3C). Germacrone, a sesquiterpenoid in turmeric, did not show substantial suppression even at a concentration of 100 μM. NO generation suppressive potencies of α,β-turmerone, ar-turmerone, and β-atlantone were nearly identical and comparable to that of curcumin. Yet, these sesquiterpene are not cytotoxic even at a concentration of 100 μM, in contrast to curcumin, suggesting that they are promising anti-inflammatory and anti-carcinogenic agents in turmeric. In addition, it can be pointed out that their total content in turmeric is comparable to that of curcumin (data not shown).

To our best knowledge, there has been no known reports on the physiological functions of 3-methoxy-5-hydroxystilbene. On the other hand, the NO generation suppressive effects of ar-turmerone, germacrone, and β-turmerone (Hong, 2002; Lee, 2002; Masuda, 1998) have recently and independently been described, but those of other sesquiterpenoids (α-turmerone and β-atlantone) as well as simultaneous evaluation of those five sesquiterpene was done by us for the first time. Yoshikawa et al. demonstrated that the oral activity of germacrone for suppressing D-galactosamine- or tumor necrosis factor-α-induced liver injury in rats (Morikawa, 2002). In addition, an ethanol extract from turmeric, containing curcumin and sesquiterpenoids, significantly ameliorated symptoms of type 2 diabetes genetically diabetic KK-Ay mice (Kuroda, 2005; Nishiyama, 2005). Additionally, chebulagic acid has been shown to inhibit topoisomerase I (Hecht, 1992) and

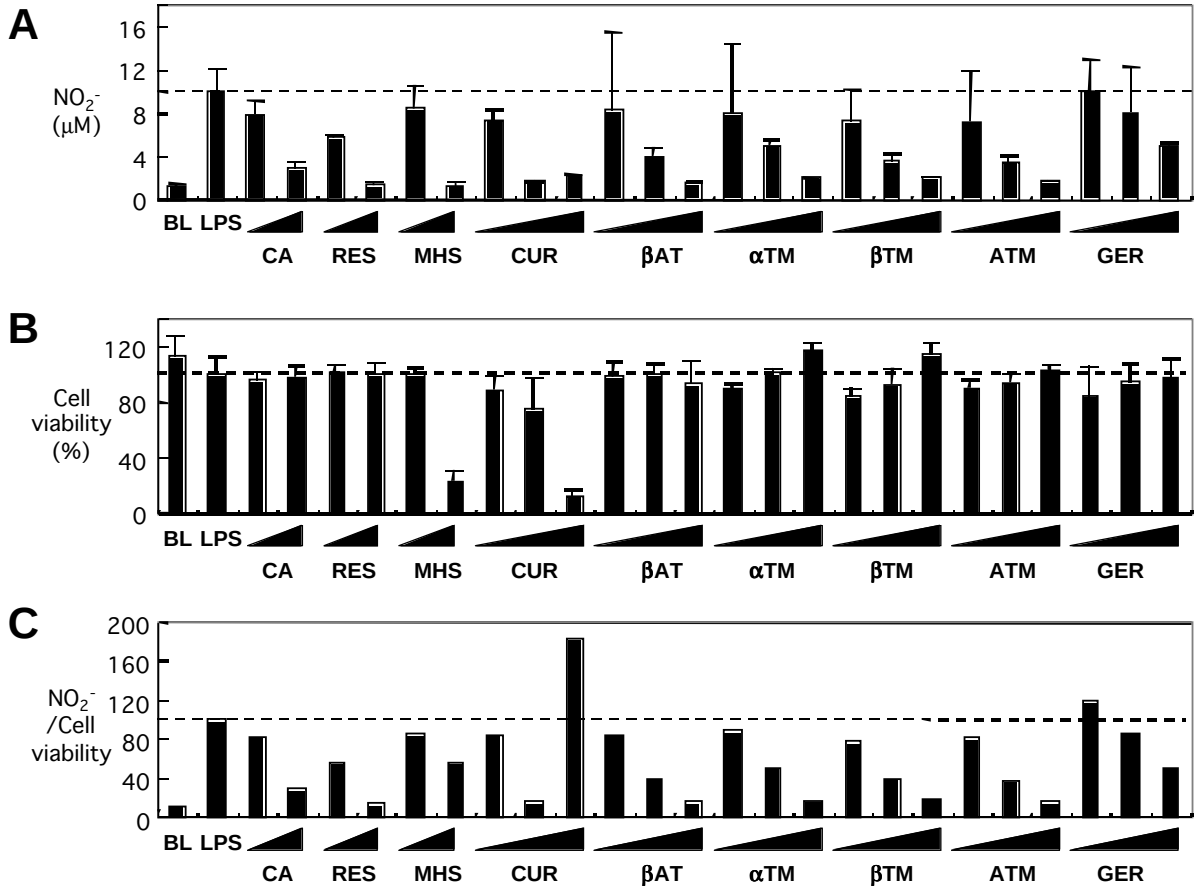


**Figure 3. Effects of Selected Dietary Phytochemicals on LPS-induced NO Generation in RAW264.7 Macrophages.** Panel A, the concentrations of $NO_2^-$ in the media 24 hours after LPS-exposure. Panel B, cell viability as detected by MTT assay. Panel C, the values of $NO_2^-$ concentrations/cell viability which was corrected with the LPS-treated cells as 100. BL, blank; CA, chebulagic acid (20 and 100 μM); RES, resveratrol (20 and 100 μM); MHS, 3-methoxy-5-hydroxystilbene (20 and 100 μM); CUR, curcumin (4, 20 and 100 μM); βAT, β-atlantone (4, 20 and 100 μM); αTM, α-turmerone (4, 20 and 100 μM); βTM, β-turmerone (4, 20 and 100 μM); ATM, ar-turmerone (4, 20 and 100 μM); GER, germacrone (4, 20 and 100 μM).

xanthine oxidase (Fogliani, 2005), to exhibit tumor cell toxicity (Kashiwada, 1992), and to suppress collagen-induced arthritis (Lee, 2005). Together, both the sesquiterpenoids and chebulagic acid are attractive new agents which deserve evaluation of their cancer preventive efficacy.

*Concluding Remarks*

In conclusion, we have shown that Okinawan food items have pronounced potentials to search for effective anti-oxidative new compounds as compared with commonly ingested food items in Japanese main island. In particular, the Gramineae and Zingiberaceae plants appear highly suppressive. Also, chebulagic acid, 3-methoxy-5-hydroxystilbene, and sesquiterpenes have been isolated as NO generation inhibitors from effective plants. Further efforts for identification and chemopreventive studies using those agents are underway in our laboratory.

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