# ARTICLES

## Antiproliferative Effects of the Readily Extractable Fractions Prepared from Various *Citrus* Juices on Several Cancer Cell Lines

Satoru Kawaii, Yasuhiko Tomono, Eriko Katase, Kazunori Ogawa, and Masamichi Yano*

National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka 424-0204, Japan

To eliminate the masking effect by flavonoid glycosides, which comprise ~70% of conventionally prepared sample, the readily extractable fraction from *Citrus* juice, which was prepared by adsorbing on HP-20 resin and eluting with ethanol and acetone from the resin, was subjected to antiproliferative tests against several cancer cell lines. Screening of 34 *Citrus* juices indicated that King (*Citrus nobilis*) strongly inhibited proliferation of all cancer cell lines examined. Sweet lime and Kabuchi inhibited three of the four cancer cell lines. In contrast, these samples were substantially less cytotoxic toward normal human cell lines.

**Keywords:** *Citrus; readily extractable fraction; A549; B16 melanoma 4A5; CCRF-HSB-2; TGBC11TKB; human foreskin keratinocytes; human umbilical vein endothelial cells*

### INTRODUCTION

It is commonly accepted that cancer formation can be prevented by the consumption of certain foods (Stavric, 1994), and flavonoids in *Citrus* fruits and juices are one of the most prominent cancer-preventing agents (Attaway, 1994). From a viewpoint of health promotion by dietary habits, cancer preventative activity in the *Citrus* juices is more important than that found in other inedible parts. However, few studies have focused on the biological activities in *Citrus* juices, possibly because the measurable activities in juices tend to be hindered by the more abundant substances that have no activity in vitro. Sample preparation by the conventional solvent extraction of a freeze-dried fruit using a methanol/dimethyl sulfoxide solvent mixture [MeOH/DMSO (1:1)] is a relatively powerful and accurate way to determine various substances in *Citrus* fruit and is therefore suitable for chemotaxonomic study. Biological activity of such extracts, however, is hindered by the presence of high proportions of flavonoid glycosides, namely naringin, neoeriocitrin, and hesperidin (50−80% of total flavonoids) (Kanes et al., 1992), because these compounds had weak antiproliferative activity toward cancer cell lines (Kawaii et al., 1999b).

To eliminate the masking effect by the flavonoid glycosides, we have prepared the readily extractable fractions of *Citrus* juices by adsorbing on HP-20, a porous polymer resin, and succesive elution from the resin with ethanol and acetone (Kawaii et al., 1999a). The main constituents of the readily extractable fraction of *Citrus* juices were flavonoids as indicated by HPLC analysis, and this procedure could efficiently reduce the total proportion of naringin, neoeriocitrin, and hesperidin from ~90% of the total flavonoid in the MeOH/DMSO extract to 57% of the readily extractable fraction of King juice. In this study, the readily extractable fractions from 34 representative and/or economically important *Citrus* species, according to Tanaka's classification (Hodgson, 1967; Tanaka, 1969), were tested for their antiproliferative activity against several cancer cell lines. Among 34 *Citrus* juices examined, King, Sweet lime, and Kabuchi demonstrated potent antiproliferative activity against cancer cell lines with less cytotoxicity toward normal human cell lines.

### MATERIALS AND METHODS

**Fruit Samples.** All fruits that were harvested from trees at the National Institute of Fruit Tree Science, Okitsu, Shimizu, Shizuoka, Japan, on December 9−10, 1996, were juiced by hand-squeezing. Two hundred milliliters of juice was adsorbed on 250 g of HP-20 (Mitsubishi Chemicals, Tokyo, Japan), which was then eluted with ethanol (750 mL) and acetone (750 mL). The combined eluates were concentrated and dissolved in DMSO at a concentration of 100 mg/mL as a stock solution.

**Cell.** Cancer cell lines used in this study were obtained from the Riken Gene Bank (Tsukuba, Japan) and are as follows: A549, human lung carcinoma; B16 melanoma 4A5, melanin pigment producing mouse melanoma; CCRF-HSB-2, T-cell leukemia; TGBC11TKB, human gastric cancer cell and lymph-node metastasized. A549 and B16 melanoma 4A5, TGBC11TKB, and CCRF-HSB-2 were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), DMEM supplemented with 5% FBS, and RPMI1640 medium supplemented with 10% FBS, respectively. Culture kits of normal human umbilical vein endothelial cells (HUVE) and normal human foreskin keratinocytes (HFK) were purchased from the Morinaga Institute of Biological Science (Yokohama, Japan). The maximum concentration of DMSO in the culture medium (0.4%) did not influence cellular growth.

**Cell Proliferation Assay.** The level of cellular proliferation for each cell line grown in 96-well microtiter plates was

* Corresponding author (fax +81-543-69-2115; e-mail ym6082@okt.affrc.go.jp).

10.1021/jf9812228 CCC: $18.00 
Published on Web 06/26/1999

**Table 1. Antiproliferative Activity of the Readily Extractable Fractions from *Citrus* Juices on Several Cancer Cell Lines**

| name[a] | | | inhibition of cellular proliferation (%) at 100 μg/mL | | | |
|---|---|---|---|---|---|---|
| conventional[b] | scientific | yield[c] (mg) | A549 | B16 | CCRF-HSB-2 | TGBC 11TKB |
| II. Tahiti lime | *C. latifolia* | 1203 | 5.1 ± 15.8 | −5.3 ± 2.8 | 75.5 ± 22.1* | 32.3 ± 7.3* |
| III. Sweet lime | *C. limettioides* | 908.9 | 7.8 ± 3.9 | 28.3 ± 3.9* | 93.1 ± 2.2* | 40.3 ± 3.8* |
| II. Bergamot | *C. bergamia* | 1024 | −4.2 ± 2.2 | 10.8 ± 4.9 | 10.4 ± 5.8 | 28.3 ± 6.9* |
| III. Eureka lemon | *C. limon* | 1178.2 | −0.2 ± 0.3 | −11.1 ± 5.1 | −6.5 ± 5.7 | −15.1 ± 4.1 |
| IV. Hirado buntan | *C. grandis* | 525.1 | 4.9 ± 5.2 | −20.4 ± 3.7 | 48.1 ± 3.8* | 0.2 ± 4.3 |
| IV. Marsh grapefruit | *C. paradisi* | 977.2 | −4.4 ± 5.3 | −5.6 ± 3.8 | 26.7 ± 4.7* | 7.6 ± 2.8 |
| IV. Red blush | *C. paradisi* | 1829.1 | 0.1 ± 2.2 | 7.1 ± 2.4 | 27.1 ± 7.8* | 4.8 ± 3.2 |
| IV. Kinukawa | *C. glaberrima* | 240.9 | 1.3 ± 0.6 | 0.8 ± 5.3 | 45.9 ± 10.1* | −10.8 ± 4.9 |
| IV. Hassaku | *C. hassaku* | 627.5 | 15.2 ± 4.2 | 15.3 ± 4.1* | −0.2 ± 6.5 | 9.4 ± 2.7 |
| V. Natsudaidai | *C. natsudaidai* | 1198.6 | 0.4 ± 3.1 | 13.3 ± 5.8 | −3.2 ± 2.9 | −3.6 ± 8.4 |
| V. Sanbokan | *C. sulcata* | 620.2 | 12.4 ± 0.9 | −5.1 ± 0.1 | 31.8 ± 5.4* | 12.8 ± 13.3 |
| V. Sour orange | *C. aurantium* | 898.3 | −2.8 ± 2.6 | −4.9 ± 2.1 | −2.8 ± 7.2 | 5.3 ± 8.7 |
| V. Valencia | *C. sinensis* | 610.9 | 6.5 ± 0.6 | 7.8 ± 0.6 | 23.7 ± 2.6* | 8.1 ± 2.0 |
| V. Morita navel | *C. sinensis* | 565.1 | −6.8 ± 12.8 | −4.3 ± 3.3 | 0.1 ± 10.7 | 5.8 ± 0.6 |
| V. Oto mikan | *C. sinograndis* | 549.6 | −0.1 ± 0.1 | 9.9 ± 8.5 | 12.7 ± 5.4 | 8.9 ± 7.3 |
| V. Iyo | *C. iyo* | 606.7 | −3.2 ± 2.6 | −3.4 ± 2.2 | −10.8 ± 1.2 | 1.9 ± 4.9 |
| V. Hyuganatsu | *C. tamurana* | 1073.8 | −0.2 ± 12.9 | 14.3 ± 10.1* | −22.8 ± 5.1 | 6.8 ± 2.8 |
| VI. Yuzu | *C. junos* | 1023.3 | −0.5 ± 10.3 | −0.8 ± 2.1 | −12.8 ± 5.4 | 0.1 ± 1.3 |
| VII. Kunenbo | *C. nobilis* | 415 | 33.2 ± 5.5* | −5.6 ± 2.2 | 16.7 ± 1.2 | 17.5 ± 5.3* |
| VII. King | *C. nobilis* | 1172 | 48.1 ± 3.2* | 50.6 ± 3.1* | 87.8 ± 2.8* | 65.3 ± 1.8* |
| VII. Sugiyama unshiu | *C. unshiu* | 520.3 | 4.9 ± 0.3 | 5.4 ± 4.1 | −15.4 ± 4.8 | 3.0 ± 2.6 |
| VII. Okitsu wase | *C. unshiu* | 171.8 | 0.1 ± 2.2 | −13.4 ± 6.4 | 56.7 ± 5.8* | −2.3 ± 4.3 |
| VII. Yatsusiro | *C. yatsusiro* | 541.2 | 9.9 ± 3.2 | 22.8 ± 5.2* | 7.4 ± 5.2 | 7.3 ± 1.2 |
| VII. Kabuchi | *C. keraji* | 375.3 | 20.3 ± 5.0* | 20.3 ± 4.3* | 19.8 ± 4.8 | 35.9 ± 3.0* |
| VII. Ota ponkan | *C. reticulata* | 510.1 | −22.3 ± 15.3 | −10.3 ± 0.8 | 15.7 ± 7.3 | −6.8 ± 8.3 |
| VII. Dancy tangerine | *C. tangerina* | 180.3 | −22.3 ± 5.1 | 0.1 ± 2.1 | 25.2 ± 1.4* | 18.8 ± 0.8 |
| VII. Clementine | *C. clementina* | 586.6 | 8.3 ± 2.1 | 23.8 ± 6.6* | 20.3 ± 5.3* | 32. 7 ± 3.2* |
| VII. Jimikan | *C. succosa* | 1473 | 19.3 ± 2.6* | 8.8 ± 14.2 | 2.6 ± 2.2 | −14.7 ± 8.9 |
| VII. Shikaikan | *C. suhuiensis* | 581.7 | 10.9 ± 5.3 | 11.3 ± 4.6 | 12.3 ± 7.2 | 8.8 ± 0.9 |
| VII. Kobeni mikan | *C.erythrosa* | 231.4 | −3.2 ± 6.8 | −3.0 ± 2.8 | 14.4 ± 5.1 | 13.8 ± 1.2 |
| VII. Hirakishu | *C. kinokuni* | 637.4 | −4.3 ± 9.6 | 2.5 ± 2.5 | 5.4 ± 7.2 | 6.8 ± 2.2 |
| VII. Shiikuwasha | *C. depressa* | 458.6 | 13.6 ± 3.3 | −1.1 ± 0.8 | −11.7 ± 8.5 | 39.5 ± 4.1* |
| VII. Koji | *C. leiocarpa* | 369.4 | −8.1 ± 6.8 | −13.6 ± 6.8 | 42.1 ± 3.8* | 35.8 ± 2.8* |
| VIII. Shikikitsu | *C. madurensis* | 1012.2 | 3.2 ± 2.1 | −9.3 ± 4.4 | −14.2 ± 5.4 | −21.9 ± 10.8 |

[a] The classification and the nomenclature of *Citrus* plants were based on Tanaka's classification (Hodgson, 1967; Tanaka, 1969). [b] Roman numerals before each common name indicate Tanaka's categorical numbers. [c] Yield from 100 mL of juice. Asterisks (*) indicate significant differences of growth inhibition relative to the untreated controls at $p < 0.01$ by Student's $t$ test.

measured by using alamar Blue (Biosource International, Lewisville, TX), an oxidation−reduction indicator. Staining with alamar Blue absorbance linearly correlated with cell numbers of each cell lines; the coefficients of correlation between the alamar Blue staining and the cell numbers of A549, B16 melanoma 4A5, CCRF-HSB-2, TGBC11TKB, HFK, and HUVE are 0.993, 0.995, 0.998, 0.995, 0.999, and 0.998, respectively (Kawaii et al., 1999c). To each well were added 2 $\times 10^3$ cells/100 μL of A549, B16 melanoma 4A5, TGBC11TKB, HUVE, or HFK or $10^4$ cells/100 μL of CCRF-HSB-2 cell suspension; these were grown for 24 h, and then mixed with 100 μL of medium containing serial dilution of samples to be assayed. After 3 days of incubation, 20 μL of alamar Blue was asceptically added to each well and incubation was continued for a further 6 h (for monolayer-cultured cell lines, i.e., A549, B16 melanoma 4A5, TGBC11TKB, HFK, and HUVE) or 24 h (for suspension-cultured cell line, i.e., CCRF-HSB-2). Inhibition of cellular proliferation (percent of untreated control) was calculated with the equation

$$\text{inhibition (\%)} = 100 - \frac{[(A_{570} - A_{595}) \text{ of test agent dilution}] - [(A_{570} - A_{595}) \text{ of blank}]}{[(A_{570} - A_{595}) \text{ of positive growth control}] - [(A_{570} - A_{595}) \text{ of blank}]} \times 100 \quad (1)$$

where $A_{570}$ and $A_{595}$ are the absorbances at 570 and 595 nm, respectively.

## RESULTS AND DISCUSSION

Antiproliferative effects of the readily extractable fractions prepared from 34 *Citrus* juices on A549, B16 melanoma 4A5, CCRF-HSB-2, and TGBC11TKB are summarized in Table 1. King (scientific name *C. nobilis*) demonstrated the most potent antiproliferative activity, showing highest growth inhibition. This was followed by Sweet lime (*C. limettioides*), Shiikuwasha (*C. depressa*), Kabuchi (*C. keraji*), Koji (*C. leiocarpa*), Clementine (*C. clementina*), Tahiti lime (*C. latifolia*), and Bergamot (*C. bergamia*). As to CCRF-HSB-2, Sweet lime had the most potent activity, followed by King, Tahiti lime, Okitsu wase (*C. unshiu*), Hirado buntan (*C. grandis*), Kinukawa (*C. glaberrima*), and Koji.

A549 and B16 melanoma 4A5 showed higher degrees of resistance to the antiproliferative activity of the readily extractable fractions. King inhibited cellular proliferation of the four cancer cell lines, followed by Sweet lime and Clementine, which inhibited B16 melanoma 4A5, CCRF-HSB-2, and TGBC11TKB, and by Kabuchi, which inhibited A549, B16 melanoma 4A5, and TGBC11TKB. Tahiti lime and Koji inhibited two of four cell lines. According to Tanaka's classification (Hodgson, 1967; Tanaka, 1969), *Citrus* species can be classified into eight groups (systematic position: I, Papeda; II, Limonellus; III, Citrophorum; IV, Cephalocitrus; V, Aurantium; VI, Osmocitrus; VII, Acrumen; VIII, Pseudofortunella). Roughly speaking, potent antiproliferative activity on the cancer cell lines was mainly observed in group VII, followed by groups IV, II, and III.

Antiproliferative effects of the readily extractable fractions of King, Sweet lime, and Kabuchi on normal human cell lines were also examined by using human umbilical vein endothelial cells (HUVE) and human

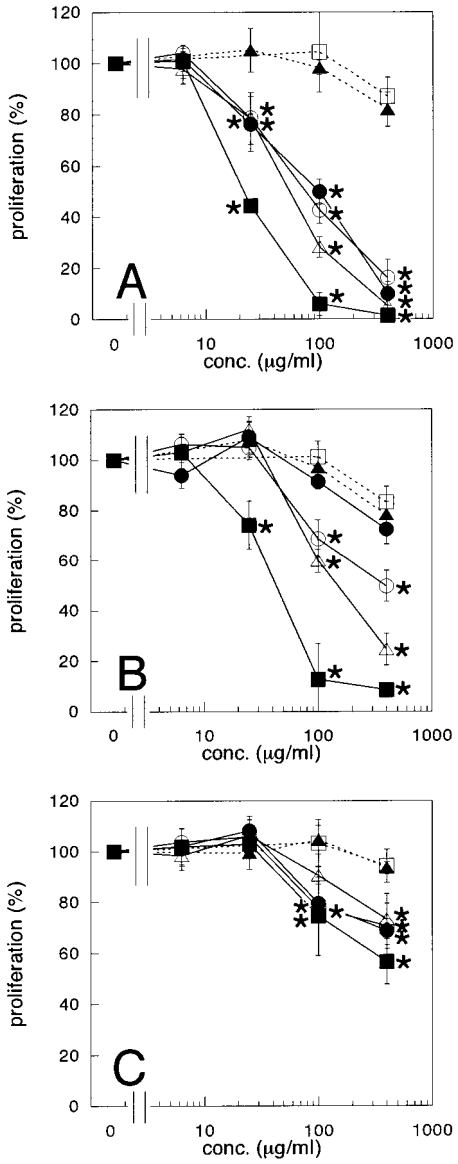


**Figure 1.** Dose−response of the readily extractable fractions of King (A), Sweet lime (B), and Kabuchi (C) on clonal proliferation of several cell lines: growth (percent of control) of A549 (●), B16 melanoma 4A5 (○), CCRF-HSB-2 (■), TGBC11TKB (△), HUVE (□), and HFK (▲); the solid and dotted lines represent cancer cell lines and normal human cell lines, respectively. Each point represents the mean of triplicates of experiments. Vertical bars indicate standard deviations. Asterisks (*) indicate significant differences of cancer cell growth relative to HFK, a normal human cell line at $p < 0.01$ by the Tukey−Kramer honestly significant difference test.

foreskin keratinocytes (HFK). Concentration−response effects of the readily extractable fractions on the proliferation of HFK and HUVE as well as A549, B16 melanoma 4A5, CCRF-HSB-2, and TGBC11TKB cell lines are shown in Figure 1. These fractions, by contrast, are substantially less cytotoxic toward the normal human cell lines.

HPLC analysis has indicated that King juice was rich in polymethoxylated flavones (Kawaii et al., 1999a), which had potent anticancer activity (Kandaswami et al., 1991). The test medium of the readily extractable fraction of King juice contained totally 1.8 μM polymethoxylated flavones (NBL, 0.41 μM; HPT, 0.76 μM; NTD, 0.33 μM; TNG, 0.33 μM). Because the total concentration of the polymethoxylated flavones was very close to their minimum effective concentration (2.5 μM) (Kawaii et al., 1999a), we considered that these polymethoxylated flavones mainly contributed the antiproliferative activity of the readily extractable fraction of King juice. Our results suggested that the readily extractable polymethoxylated flavones are important candidates for cancer-protective action. Epidemiological studies have indicated that flavonoid consumption is associated with a reduced risk of cancer (Wattenberg, 1985, 1990; Verma et al., 1988; Wei et al., 1990), and anticancer activity of flavonoids has been reported (Deschner, 1992; Sugiyama et al., 1993; Tanaka et al., 1997; Benavente-García et al., 1997).

The present study was intended to survey the manifestation of antiproliferative activity of the readily extractable fraction prepared from 34 *Citrus* juices. This procedure could efficiently reduce the total proportion of hesperidin, naringin, and neoeriocitrin from 90% of the total flavonoid in the MeOH/DMSO extract from the freeze-dried endocarp to 57% in the readily extractable fraction prepared from King fruits (Kawaii et al., 1999a), and thus we could detect the antiproliferative activity in *Citrus* juices. In summary, these results demonstrate the feasibility of physiological screening on the readily extractable fraction to identify *Citrus* species and cultivars rich in anticancer substances and will aid in horticultural breeding program selection of varieties rich in anticancer substances.

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Received for review November 10, 1998. Revised manuscript received May 3, 1999. Accepted May 10, 1999. This work was supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences from the Bio-oriented Technology Research Advancement Institution.

JF9812228