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Protective effect of sweet and unshiu mikan oranges and mini tomato on *t*-BHP-induced oxidative stress in HepG2 cells

by

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(오렌지, 토마토, 귤과 **hesperidin, narirutin, rutin**의 **t-BHP** 유도 **HepG2** 세포 보호 효과)

Advisor: Prof. Jae Sue Choi

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List of Abbreviation and symbol

U. mikan orange	: Unshiu mikan orange
S. orange	: Sweet orange
B. tomato	: Boiled tomato
DMSO	: Dimethyl sulfoxide
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
t-BHP	:*tert*-butylhydroperoxide
DCFH-DA	: 2′,7′-dichlorodihydrofluorescein diacetate
HPLC	: High performance liquid chromatography
UV	: Ultraviolet
MEM	: Minimum essential medium
FBS	: Fetal bovine serum
BSA	: Bovine serum albumin
ROS	: Reactive oxygen species
HO-1	: Hemeoxygenase 1
GSH	: Glutathione

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ABSTRACT

The fruits of orange species and mini tomato originated from south East Asia, but are consumed all over the world as healthy foods, and contain bioactive key components. Epidemiological studies indicate that an increase in the consumption of fruits and vegetables associated with a decrease in the incidence of cardiovascular, chronic and degenerative, inflammatory, liver diseases. The aim of this study was to investigate the protective effect of juice powders from sweet orange (*Citrus sinensis*), unshui mikan orange (*Citrus unshiu*), mini tomato (*Solanum lycopersicum*) juice powders and their combinations as well as their major flavonoids such as hesperidin, narirutin and rutin on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative stress on HepG2 cells. The cell viability of juice powders and their combinations as well as their flavonoids were measured by MTT assay. Hepatoprotective effect of juice powders against oxidative stress induced by *t*-BHP on HepG2 cells were investigated through the measurement of cytoprotective effect, intracellular ROS, GSH and the up-regulation of phase-II protein such as heme oxygenase-1 (HO-1) expression through western blot analysis. HPLC was used to analyze the major flavonoids of juice powders.

In the current study, juice powders and their combinations were evaluated for total phenolic and vitamin C content determination. According to HPLC analysis, hesperidin and narirutin are dominantly present in two orange species and rutin in tomato species. The increased ROS and decreased glutathione levels observed in *t*-BHP-treated HepG2 cells were ameliorated by juice powders pretreatment, indicating that the hepatoprotective effects of juice powders, their combinations and the major flavonoids are mediated by the induction of cellular defense action against oxidative stress. The protective effects of juice powders, their combinations and the major flavonoids significantly inhibited the cytotoxic effect induced by 200 μM *t*-BHP on HepG2 cells and also may be associated with positive regulation of GSH levels and decrease in ROS production, thereby preventing cellular damage and the resultant increase in HO-1 expression. These results suggest that the fruit juice powders and their combinations as well as their major flavonoids displayed a significant cytoprotective effect against oxidative stress which may be most likely because of the flavonoids-related bioactive compounds existing therein leading to the normal redox status of cells. Therefore these fruit juice powders and their specific combinations could be advantageous as a bioactive source for the prevention of oxidative injury in hepatoma cells.

I. Introduction

Over the past 20 years there has been growing interest, on the part of the consumer, in minimally processed fruit and vegetables because of their freshness and convenience. Moreover, the benefits of fruit and vegetable consumption are now widely reported in the literature (Kaur & Kapoor, 2001; Scalbert& Williamson, 2000). Especially, orange juice consumption has become a worldwide dietary habit and as a result, the consumption of frozen, concentrated juice has increased steadily over the years. In the world market of concentrated orange and vegetables juice is around 53% and 98% of this production is designated to exportation to European countries. Among the fruit-based beverages, orange juice is the most widely consumed, with a participation of 35% of the fruit beverages market (Neves, 2010). Nowadays, consumers are increasingly aware of diet-related health problems and therefore are demanding natural ingredients, which are expected to be safe and health-promoting. Studies demonstrated the high antioxidant capacity of orange and vegetables, especially due to the expressive presence of vitamin C and phenolic compounds (Lee and Coates, 1999; Jayaprakasha and Patil, 2007). Concentrated orange juice has a greater flavonoid content, including polymethoxylated flavones, hesperidin, rutin, narirutin and naringin, in comparison to fresh juice (Cesar et al., 2010). Sweet orange, the testy, juicy fruits, belonging to the family Rutacae is botanically known as *citrus sinnensis*. *C. sinensis* is one of the most important and widely grown fruits, with total global production reports to be around 120 million tons. Orange trees are widely cultivated in tropical and

subtropical climates for its tasty juice and medicinal value. In the worldwide trades citrus fruits generate about 105 billion dollars per year all over the world. Orange fruits are cultivated in more than 130 countries including, India, UK, France, Germany, Holland, Brazil, China, Korea, USA, and Spain. *C. sinensis* originated from south East Asia, but is consumed all over the world as an excellent source of vitamin C, a powerful natural antioxidant that build the body immune system. Important phytochemicals likes liminoids, hesperidin flavonoids, polyphenols, pectin, and sufficient amount of folacin, calcium, potassium, thiamin and magnesium are also present (Angew, 2007, Etebu and Nwauzoma, 2014). These biologically active compounds prevent arteriosclerosis, oxidative stress, cancer, kidney stones, stomach ulcers, inflammatory, liver, chronic and degenerative diseases, and reduction in cholesterol level and high blood which promote human health (Tripoli et al., 2007; Etebu and Nwauzoma, 2014; Keys, 1995; Di Majo et al., 2005).

In addition, *Citrus unshiu* belonging to the family of Rutaceae. It is one of the most popular citrus fruits and its well-known name is unshiu mikan orange. The unshiu mikan is a small citrus tree with fruits resembling other oranges. Unshiu mikan orange are usually eaten plain or in fruit salads. The ripe fruits are a common food and the dried peels have been used not only in traditional Chinese medicine but also as a skin moisturizing agent in Japanese folk medicine (Higashi-Okai et al., 2002). *C. unshiu* has been reported to contain several biologically active components, such as auraptene, ß-cryptoxanthin, limonine, naringin, narirutin, hesperidin, didymin, tangeretin and nobiletin, (Murakami et al., 2000;

Kita et al., 2003; Sun et al., 2010). Nowadays, pharmacological research has indicated that *C. unshiu* exhibits significant antimutagenic (Arend, 1994), anti-inflammatory (Lin et al., 2003, Da Silva et al., 1994), anti-allergic (Kobayashi and Tanabe, 2006), antioxidant (Majo et al., 2005; Benavente-García et al., 1997), antitumor (Bracke et al., 1991; Bracke et al, 1989), and antiatherosclerosis (Manach et al., 1996; Hertog et al, 1993), functions and reduces phlegm in the lung (Pharmacopoeia Commission China, 2005).

Tomato is one of the most important vegetables all over the world containing a wide range of varieties and types. Originating in the Andes, the tomato (*Solanumlycopersicum* L.) was imported to Europe in the 16th century. At present, it is an important crop plant cultivated all over the world, and its production and consumption continue to increase. Mini tomato (*Solanum lycopersicum*) is a popular type of table tomato with small fruits (1.5-3.5cm in diameter) on long panicles and demand for mini tomato has increased in the market, mainly due to the recognition of their high quality and good taste. The mini tomato is one of the most popular and widely consumed vegetable crops all over the world, and also beneficial to human health because of its high content of antioxidant and phytochemical compounds, including lycopene, β-carotene, flavonoids (rutin, quercetin, kaempferol etc), hydroxycinnamic acid derivatives, Vitamin C, and many essential nutrients (Rosales et al., 2011). Tomato has been recently gaining attention in relation to the prevention of some human diseases. This tomato has achieved tremendous popularity especially in recent years with the discovery of lycopene's anti-oxidative activities and anti-cancer functions (Wu et

al., 2011; Raiola et al., 2014). Thus, tomato production and consumption are constantly increasing. It is noteworthy that tomatoes are not only sold fresh, but also processed as soups, sauces, juices or powder concentrates.

Flavonoids are aromatic secondary plant metabolites, which have been recognized as important due to their physiological and pharmacological role and their health benefits (Buslig and Manthey, 2002; Sharma, 2006). Flavonoids shows a strong antioxidant and radical scavenging activity (Zhang et al., 2006; Pietta, 2000) and appear to be associated with reduced risk for certain chronic diseases (Kris-Etherton et al., 2004), the prevention of some cardiovascular disorders (Gross, 2004) and certain kinds of cancerous processes (Nichenametla et al., 2006; Steinmetz and Potter, 1996). Flavonoids exhibit also antiviral, antimicrobial, anti-inflammatory activities and beneficial effects on capillary fragility and an ability to inhibit human platelet aggregation, antiulcer and antiallergenic properties (Gattuso et al., 2007). Flavonoids are frequently found in citrus fruits, vegetables and cereals (Moufida and Marzouk, 2003; Hollman and Arts, 2000). Flavanones, flavones, and flavonols are three types of flavonoids which occur in citrus fruits (Gattuso et al., 2007). The main flavonoids found in citrus species are hesperidin, narirutin, naringin and eriocitrin (Gattuso et al., 2007). Flavonoids are also found in vegetables, the main flavonoids are rutin, quercetin, kaempferol etc (Slimestad et al., 2008). Hesperidin and narirutin are flavanone *O*-glycosides found so far in juices. These derivatives have a glycosyl substitution exclusively at the C-7 position (on ring A). Furthermore, rutin is a flavone *O*-

glycosides also found in citrus juices and vegetables (Gattuso et al., 2007). Citrus flavanone such as hesperidin has been reported to have antioxidant, anticarcinogenic, antihypotensive, antimicrobial, anti-inflammatory, diuretic, analgesic, and hypolipidemic activities (Garg et al., 2001; Emim et al., 1994; Monforte et al, 1995), while another flavanone naritutin have a wide range of therapeutic properties, including anti-adipogenic (Lim et al., 2015), anti-inflammatory and anti-allergic (Funaguchi et al., 2007), antioxidant, and hepatoprotective activity (Park et al., 2013). Moreover, rutin is a flavone has been reported wide range of biological activities such as, antibacterial (Arima et al., 2002), anti-oxidant and per oxidant (Kessler et al., 203) and antiproliferative activities (Baldisserotto et al., 2015).

Reactive oxygen species (ROS) act as subcellular messengers in such complex cellular processes as mitogenic signal transduction, gene expression, and regulation of cell proliferation (Oakley et al., 2009). However, excessive ROS bring about oxidative stress and attack cellular biomolecules such as lipid, protein and DNA. The induced oxidative stress may disrupt cellular function and membrane integrity, thereby leading to cell death (Shieh et al., 2010). A number of pro-oxidants like *tert*-butyl hydroperoxide (*t*-BHP) have been implicated in the oxidative stress and cell injury that result from intracellular production of ROS (Williams and Jeffrey, 2000). *t*-BHP is widely applied to investigate the mechanism of cell injury initiated by oxidative stress (Rush et al., 1985). It can be metabolized to free radical intermediates by cytochrome P-450 in hepatocytes, which in turn can initiate lipid peroxidation, affect the cell integrity, and mediate DNA damage

(Minotti et al., 1986; Masaki et al., 1989; Martin et al., 2001). These phenomena are similar to the oxidative stress occurring in the cell and/or tissue. Oxidative stress is considered to play a prominent role in the cause of many diseases for example, inflammation, aging, and cancer (Breimer, 1990). Because ROS formation is a naturally occurring process, mammalian cells have developed several protective mechanisms to prevent ROS formation or to detoxify ROS. These mechanisms employ molecules called antioxidants as well as protective enzymes (Chen and Kunsch, 2004). Phase II detoxifying genes not only provide a major mechanism by which cells combat the toxicities of ROS, but their induction is also highly effective and sufficient for protecting cells against oxidative stress as well as the toxic and neoplastic effects of many toxicants and carcinogens (Rushmore and Kong, 2002). Many drugs and naturally occurring chemopreventive phytochemicals are known to increase the transcription of these phase II genes in cell culture and animal models (Surh, 2003). Antioxidant enzymes including hemeoxygenase1 (HO-1), NADPH: quinone oxidoreductase-1 (NQO1) andglutathione-S-transferase (GST) provides protection against the deleterious effects of ROS (Zhao et al., 2010). Among the various antioxidant enzymes, heme oxygenase-1 (HO-1) has recently been highlighted by virtue of its hepatoprotective roles (Kapitulnik, 2004). HO-1 is the rate limiting enzyme in the conversion of heme into biliverdin, releasing free iron and carbon monoxide. Biliverdin is rapidly metabolized to bilirubin, which is a potent antioxidant. There is a large body of evidence suggesting that HO-1 plays a key role in maintaining antioxidant homeostasis during cellular stress (Alam

and Cook, 2003). So, induction of HO-1 is an important cellular mechanism in the strategy against oxidative injury both in vitro and in vivo (Morse and Choi, 2002). HO-1 is also a heat shock protein that has been implicated in a cytoprotective mechanism that protects tissues from oxidative damage (Maines, 1997). Previous studies have demonstrated the potent antioxidant and cytoprotective activities of heme-derived metabolites produced by HO-1 catalysis (Maghzal et al., 2009). In addition, Glutathione (GSH) is the most abundant nonprotein thiol in mammalian cells and has many critical functions, including defense against oxidative stress as a scavenger of ROS and electrophiles. GSH receives electrons to be converted into oxidative GSH which prevents free radicals from releasing electrons. GSH releases electrons after GSH-Px changes H_2O_2 to O_2 and H_2O. Thus, GSH plays a role in maintaining cellular redox homeostasis, scavenging lipid peroxides, and detoxifying reactive intermediates of xenobiotics (Sies, 1999). The synthesis of GSH in cells may be disrupted during aging and under pathologic conditions such as diabetes mellitus, drug resistant tumor growth, and endotoxemia (Wang et al., 2003; Yoshida et al., 1995).

Research on the flavonoids of fruits and vegetables are recognized as an effective scientific approach to the discovery of new source for neutraceuticals on therapeutic agents. The purpose of this study was to investigate the protective effect of fruit juice powders and their specific combinations as well as their major flavonoids for the prevention of oxidative injury in hepatoma cells. Furthermore, we also investigated the roles of HO-1 and GSH in protection against *tert*-butyl hydroperoxide-induced oxidative damage. Apart from these,

the effectiveness of fruit juice powders and their specific combinations in protection against cell injury in a cellular model system caused by oxidative stress has not been previously reported.

II. Materials and methods

2-1. Chemicals

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *t*-BHP, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxyl acid (trolox), bovine serum albumin, quercetin, silymarin, rutin, narirutin and hesperidin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against HO-1 and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Minimum essential medium (MEM), penicillin-streptomycin, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), sodium pyruvate and nonessential amino acids were purchased from Gibco-BRL Life Technologies (Grand Island, NY, USA). The glutathione (GSH) assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). All chemicals and solvents used were of analytical grade.

2-2. Preparation of juice sample

Fresh sweet orange (*Citrus sinensis*L.) unshiu mikan orange (*Citrus unshiu*), mini tomato (*Solanum lycopersicum*) purchased from commercial market in the month March-April, 2014 from Busan, South Korea. First the peel and seed were removed from the fruits and then collect the pulp portion. Pulp section then blend by automated blender and collect the

juice extract. The extract was filtered and removed the debris from juice. Filtered fruits juice was concentrated through the evaporator at temperature 60^{O}C. Freeze-drying lyophilized machine (-52 ^{o}C) is used to remove the water from juice extracts. The dehydrated juice powders were then called fruits juice powders.

2-3. HPLC analysis

Reversed-phase HPLC was performed on the JASCO HPLC system (Tokyo, Japan), consisting of a PU-1580 intelligent HPLC pump, a LG-1580-04 quaternary gradient unit, a UV-1575 intelligent UV/VIS detector, a PG-1580-54 4-line degasser, and a CO-1560 intelligent column thermostat. The BORWIN chromatographic system (Le Fantanil, France) was used for HPLC data analysis. Chromatographic separation was accomplished on a Phenomenex C18 reverse-phase column (Phenomenex, 4.6 x 250 cm, 5 μm) at 30 ^{o}C and monitored at 280 nm. The linear gradient solvent system consisted of 0.5 % acetic acid in water (solvent A) and 100 % CH_3CN (solvent B), and was adjusted from 75 % (solvent A): 25 % (solvent B) to 0 % (solvent A) and 100 % (solvent B) over 60 min at a flow rate of 0.5 mL/min. For preparation of stock solutions, juice powders were dissolved in 100 % MeOH at concentrations of 8 mg/mL, respectively. After filtration through a centrifugal filter device (0.45 μm, Millipore Co., Bedford, MA, USA), 10 μL of each sample was injected into the HPLC system. The retention times of respective compounds were determined using the resulting chromatograms. A calibration curve was drawn using the three standards at concentration ranging from 10 to 500 μg/ml, and the regression equation

was calculated in the form of y =ax-b, where y and x correspond to the peak area and concentration, respectively. The regression equation and correlation coefficient of hesperidine, rutin and narirutin were as follows: y = 2E+07x – 7499.1, r^2 = 0.9991; y = 6E+ 06x + 1803.3, r^2 =0.9993; and y = 8E+ 06x + 311609, r^2 = 0.9957, respectively. The relative of quantities of compounds in each juice powder (mg/g of the juice powder) were calculated from these equations.

2-4. Determination of total phenolic content

The total phenolic content (TPC) of the juice powders was determined with Folin-Ciocalteu reagent using the method of Ainsworth and Gillespie (2007) with slight modifications. Briefly, 0.5 mL of the juice powders (500 μg/mL) was mixed with 2.0 mL Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and neutralized with 4.0 mL sodium carbonate solution (7.5%, w/v). The reaction mixture was then incubated at room temperature for 30 min with intermittent shaking for color development. Absorbance of the resulting blue color was measured at 765 nm using an Ultrospec® 2100pro UV/Visible spectrophotometer with SWIFT II Applications software (Amersham Biosciences, Piscataway, NJ, USA). The TPC was determined from the linear equation of a gallic acid standard curve.

2-5. Determination of total flavonoid content

The TFCs of the juice powders were measured by the colorimetric method (Iqbal and Bhanger, 2006) with minor modifications. Briefly, 1 ml of each sample at various

concentrations (500 μg/mL) was diluted with 4 ml of deionized H_2O in a volumetric flask. Initially, 0.3 ml of 5% $NaNO_2$ was added to each volumetric flask and stored at ambient conditions for 5 min. Next, 0.3 ml of 10% $AlCl_3$ was added and kept for 6 min, followed by the addition of 2 ml of 1 M NaOH. Each reaction flask was then immediately diluted with 2.4 ml of deionized H_2O and mixed. Finally, the absorbance was measured at 510 nm on an Ultrospec®2100pro UV/visible spectrophotometer (Amersham Biosciences, New Jersey, USA). All the experiments were conducted using quercetin as a calibration standard and the results were recorded as mg of quercetin equivalent per g of juice powders (QE, mg/g of each juice powders). The QE values are expressed as means ± SEM of triplicate experiments.

2-6. Vitamin C content determination by DCPIP solution

The test for vitamin C is to utilize the reducing power of vitamin C. In this test, the oxidizing agent is called DCPIP (dichlorophenol indophenol). DCPIP is a dye. It is blue color when in oxidizing form and colorless in reduction form. When DCPIP is added into vitamin C solution, the vitamin C reduces the dye, then, decolorizes the dye. Therefore, the decolorization of DCPIP indicates the presence of vitamin C.

2-7. Cell culture

The HepG2 (human hepatocarcinoma) cell line was purchased from American Type Culture Collection (HB-8065, Manassas, VA, USA). Cells were maintained in MEM containing 2.0 mM_L-glutamine, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate and 10% FBS at 37°C in a humidified atmosphere with 5% CO_2. Medium was changed every 48 h. Samples were dissolved in DMSO before being added to cells; the final concentration of DMSO did not exceed 0.1%.

2-8. Cell viability and cytoprotective assay in HepG2 cells

Cell viability was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] staining assay as previously described (Mosmann, 1983).In brief, HepG2 cells were seeded into a 96-well plate at a density of 2.5×10^4 cells per well and incubated at 37°C for 24 h. The cells were then fed with fresh MEM containing various concentrations of juice powders at concentration of up to 200 μg/mL along with its flavonoid compounds, hesperidin, narirutin and rutinat concentration range of 0-100 μM and incubationcontinued for further 24 h. Controls received vehicle (DMSO) at a concentration equal to that used in sample treated cells. For the cytoprotective assay, after 24 h incubation with tested samples, the medium was replaced with medium containing *t*-BHP (200 μM). Cells were then incubated for 2 h, before the addition of 100 μL of MTT

solution (0.5 mg/ml in phosphate-buffered saline, PBS) following 2 h incubation. To measure the proportion of surviving cells, the medium was replaced with 100 μL of DMSO. Control cells were treated with 0.1% DMSO, this concentration exhibited no cytotoxicity as measured by this assay. Absorbance was measured at 570 nm with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). Triplicate experiments were run for each set and averaged.

2-9. Measurement of the level of intracellular reactive oxygen species

The level of intracellular ROS quantified using the oxidant-sensitive fluorescence probe 2,7-dichlorofluorescein diacetate (DCFH-DA) (Lebel and Bondy, 1990).To determine the extent of intracellular ROS scavenging activity, HepG2 cells were seeded in black 96-well plates at a density of 2.5×10^4 cells/well and incubated with juice powders and flavonoid compounds, hesperidin, narirutin and rutin at concentration range of 0-100 μM for 1 h. Cells were then exposed to *t*-BHP (200 μM) for 30 min to induce ROS production and subsequently incubated with DCFH-DA (20 μM) for 30 min. The resultant fluorescence intensities were measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader (FL×800; Bio-Tek Instruments Inc., Winooski, UT, USA).

2-10. Quantification of glutathione

Measurement of total intracellular glutathione (tGSH) (including the reduced and oxidized forms, GSH and GSSG, respectively; i.e. tGSH= GSH + GSSG) was performed by Tietze's enzymatic recycling method using glutathione reductase according to the instructions in a GSH assay kit (Cayman Chemical, Ann Arbor, MI, USA). In brief, the HepG2 cells were incubated in six-well culture dishes and they were treated with various juice powders at a concentration of 50, 100, and 200 μg/mL in MEM medium for 24 h. Subsequently, the cells were washed twice with PBS (pH 7.4), harvested using a rubber policeman and were homogenized by a freeze-thaw method with PBS in order to extract tGSH. And suctioned supernatant and then add cell lysis buffer with vortexing. And stay for 30min in the ice. And it was performed of centrifuge for 20min at the condition of 15000 rpm. For measurement of the tGSH levels in the sample, and the tGSH concentration was then determined by the end point method according to the procedure mentioned in the assay kit.

2-11. Western blotting analysis and HO activity

After treatment, cells were collected and washed with PBS. The harvested cells were then lysed on ice for 30 min in 100 μL lysis buffer [120 mMNaCl,40 mM Tris (pH 8), 0.1% NP40] and centrifuged at 13,000g for 15 min. Supernatants were collected and protein concentrations were determined using the Bradford method with bovine serum albumin as

the standard (Bradford, 1976).Aliquots of the lysates (40 μg of protein) were boiled for 5min and electrophoresis on a 10% SDS–polyacrylamide gel. Proteins in the gels were transferred onto pvdf membranes, which were then incubated with rabbit polyclonalHO-1 or mouse monoclonal β-actin antibodies. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibody (Beverly, MA). Finally, protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Pierce Biotechnology,Rockford, IL).

2-12. Statistical analysis

Data were expressed as the means ± S.D. from at least three separate experiments unless otherwise indicated. Data were analyzed using one-way ANOVA, followed by each pair of student's *t*-tests for multiple comparisons. Differences were considered significant at $P < 0.05$ and marked by different letters.

III. Results

3-1. HPLC profile of major flavonoids derived from orange species and tomato juice powders

In a phytochemical study, the simultaneous determination of active components is critical to ensure the quality of fruits materials and to find key components manifesting pharmacological effects. Simultaneous HPLC quantitative analysis was conducted to determine the relative presence of flavonoids compounds, hesperidin, narirutin and rutin in orange species and tomato juice powders (Fig. 1). The retention times for standard flavonoid compounds hesperidin, and narirutin were 11.15 and 10.06 min, respectively (Fig. 1). The unshiu mikan orange juice powder exhibited major peaks with retention times in case of hesperdin and narirutin 11.32 and 10.13 min respectively, while sweet orange exhibited major peaks with retention times for hesperdin and narirutin 11.31 and 10.31 min, respectively (Fig. 1). In addition, boiled tomato juice powder exhibited major peaks with retention times for rutin 17.57 min, whereas for standard flavonoid rutin, the retention time was 17.50 min (Fig. 1). The quantitative amount of flavonoid compounds hesperidin, narirutin and rutin in unshiu mikan, sweet orange and boiled tomato juice powder as determined by HPLC was found to be in the order of 6.16, 9.23 mg/g, 6.94, 0.372 mg/g and 5.77mg/g, respectively. So, citrus species contained the highest content of hesperidin and narirutin whereas in case of boiled tomato juice powder, rutin constituted the major portion.

Table 1. Relative amount of compounds in the orange and tomato juice powders

Compounds	Unshiu mikan orange (%)	Sweet orange (%)	Boiled tomato (%)
Hesperidin	6.19	6.94	-
Naririutin	9.23	0.37	-
Rutin	-	-	5.77

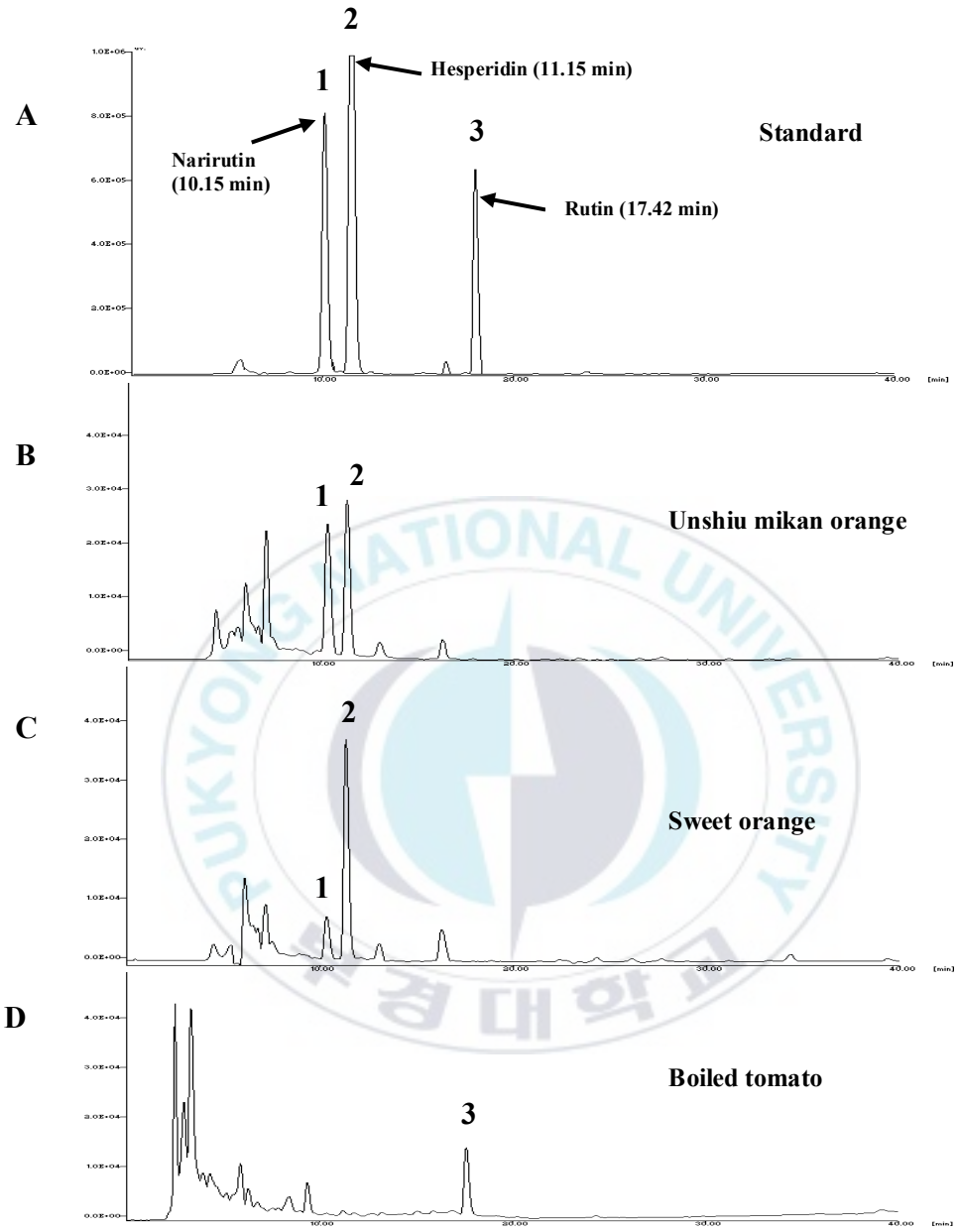


Fig 1. HPLC chromatograms of standard (A), U. mikan orange (B), sweet orange (C), bolied tomato (D). Peaks: 1. Narirutin, 2. Hesperdin, 3. Rutin

3-2. Determination of TPC, TFC and vitamin C contents of the orange species and tomato juice powder

Folin–Ciocalteu reagent and $AlCl_3$ were used to determine the TPCs and TFCs, respectively, in the unshiu mikan orange, sweet orange, raw tomato, boiled tomato, and the specific combination of 2:1:3 (sweet orange: boiled tomato: unshiu mikan orange) juice powder as shown as Table 2. Folin–Ciocalteu reagent (phosphomolybdic–phosphotungstic acid reagent) reacts with all phenolic compounds, including both phenolics and flavonoids, to form a blue color complex that can be detected with a maximum absorbance at 765 nm (Singleton and Rossi, 1965). In the TFC assay, $AlCl_3$ reacts with keto and/or hydroxyl groups on the A or C rings of flavonoids to form an acid stable complex; it also reacts with catechol groups (ortho-dihydroxyl groups) on the A or B rings of flavonoids to form an acid labile complex. The $AlCl_3$–flavonoid complex shows a strong characteristic absorbance at 510 nm (Ardestani and Yazdanparast, 2007). The TPC and TFC results were recorded as mg of gallic acid equivalent per g of dried juice powder (GAE, mg/g extract) and mg of quercetin equivalent per g of dried juice powder (QE, mg/g extract), respectively. As shown in Table 2, the TPC values of the unshiu mikan orange, sweet orange, raw mini tomato, boiled mini tomato, and 2:1:3 were 7.74, 5.48, 6.41, 9.66 and 7.56 GAE mg/g, respectively, whereas TFC values 1.83, 1.00, 0.73, 0.90, 2.16 QE, mg/g, respectively. Boiled tomato exhibited the highest TPCs and combination 2:1:3 showed premier TFC among the tested sample. Ascorbic acid (vitamin C) is a water-soluble vitamin which can

be found in many biological systems and foodstuffs (fresh vegetables and fruits, namely, citrus). Ascorbic acid plays an important role in collagen biosynthesis, iron absorption, and immune response activation and is involved in wound healing and osteogenesis. Many analytical methods can be used for ascorbic acid determination. In the present study we determine vitamin C through dichlorophenol indophenol (DCPIP) titration method. As shown as Fig 2, indicated that citrus orange juice powder showed the most vitamin C and while raw and boiled tomato had very significant vitamin C contents in it.

Table 2. Total phenolic and flavonoid contents of the orange species and tomato juice powders

Sample	Total phenolics (mg/g)[a]	Total flavonoids (mg/g)[b]
Unshiu mikan orange	7.74 ± 0.19	1.83 ± 0.11
Sweet orange	5.48 ± 0.21	1.00 ± 0.14
Raw mini tomato	6.41 ± 0.12	0.73 ± 0.09
Boiled mini tomato	9.66 ± 0.08	0.90 ± 0.21
2:1:3 (sweet orange: boiled tomato: unshiu mikan)	7.56 ± 0.31	2.16 ± 0.17

[a]Gallic acid equivalents (GAE, mg/g of each juice powder) for the total phenolic content.

[b]quercetin equivalents (QE, mg/g of each juice powder) for the total flavonoid content.

The GAE and QE values are expressed as means ± SEM of triplicate experiments.

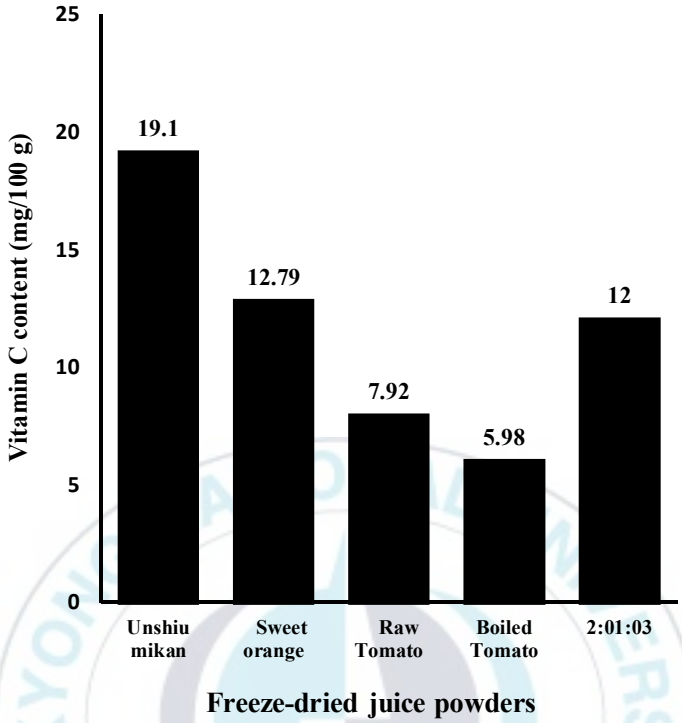


Fig 2. Vitamin C is determined by oxidizing it in acid medium with 2,6-Dichlorophenol indophenol to dehydroascorbic acid.

3-3. Cytotoxicity of unshiu mikan, sweet oranges, raw tomato, boiled tomato and their combination 2:1:3 (sweet orange: boiled tomato: unshiu mikan) juice powders as well as their major flavonoid compounds

Before determining whether the juice powder of unshiu mikan orange, sweet orange, boiled tomato and their combination 2:1:3 (sweet orange: boiled tomato: unshiu mikan orange) along with flavonoid compounds, hesperidin, narirutin and rutin exert any hepatoprotective activity, the cytotoxicity of orange, tomato juice powders and their specific combinations as well as their major flavonoid in HepG2 cells was first measured by the MTT assay. HepG2 cells pretreated with the unshiu mikan orange, sweet orange, boiled tomato juice powders and their combination at concentration up to 200 μg/mL as well as with flavonoid compounds, hesperidin, narirutin and rutin, at concentration range of 0-100 μM, respectively, following incubation for 24 h. Unshiu mikan orange, sweet orange, boiled tomato juice powders and their combination did not possessed any cytotoxicity up to 200 μg/mL as shown in Fig 3. However, the raw tomato exhibited cytotoxicity above 50 μg/mL concentration as shown in Fig 3. The flavonoid compounds, hesperidin at concentration upto 100 μM and rutin at concentration of 20 μM did not showed any cytotoxic effects as shown in Fig 4. Where as, narirutin also did not possessed any cytotoxic at a concentration up to 10 μM as shown in Fig 4. These concentrations were used in subsequent hepatoprotective assays for unshiu mikan orange, sweet orange, boiled tomato juice powders and their combination as well as the bioactive flavonoid compounds.

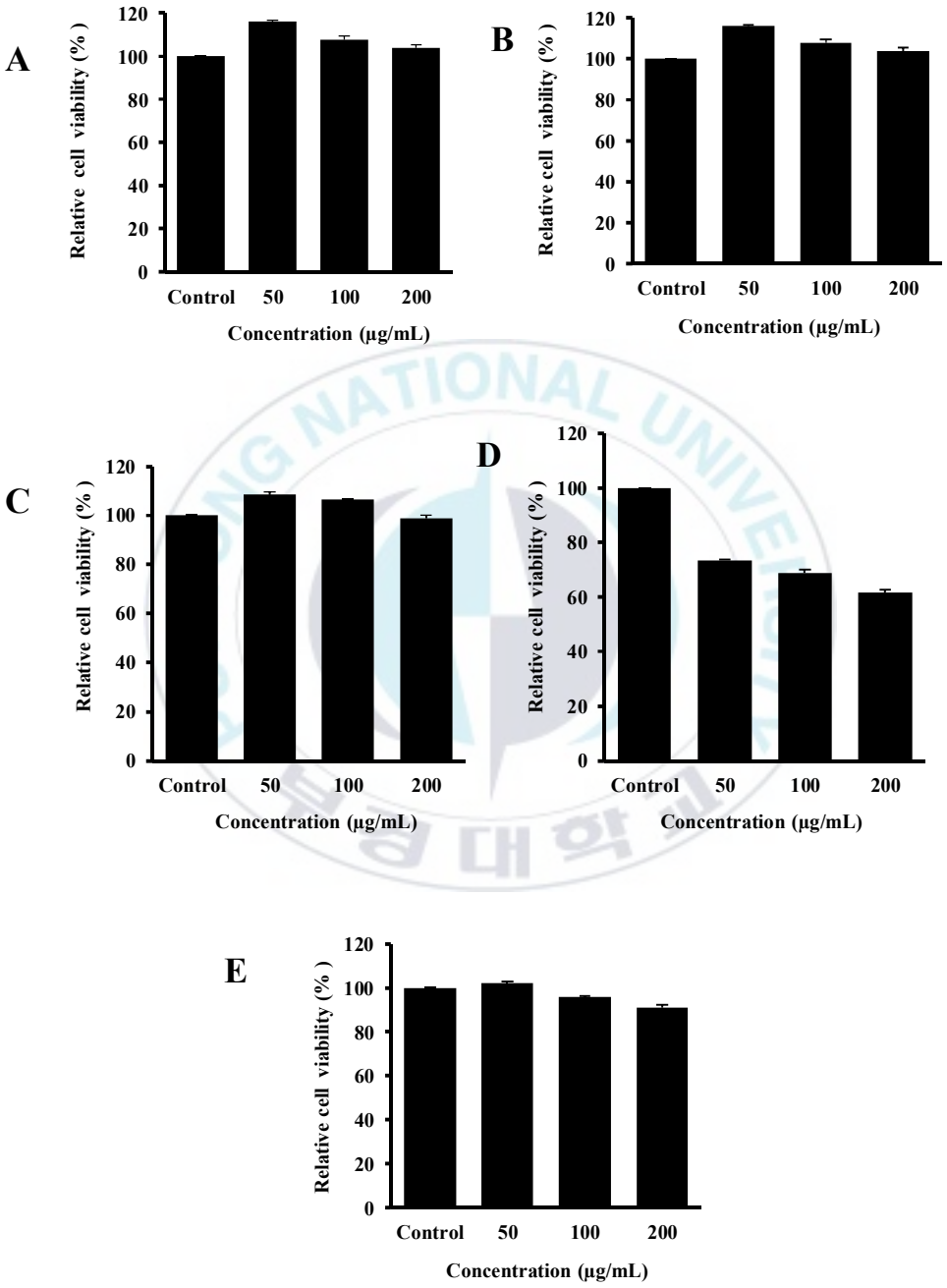
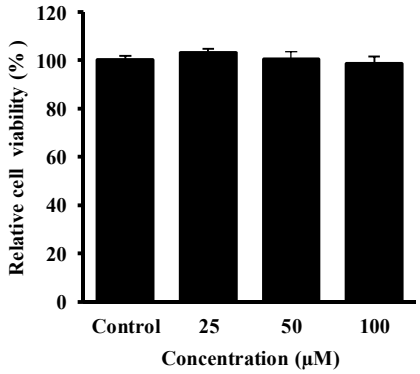
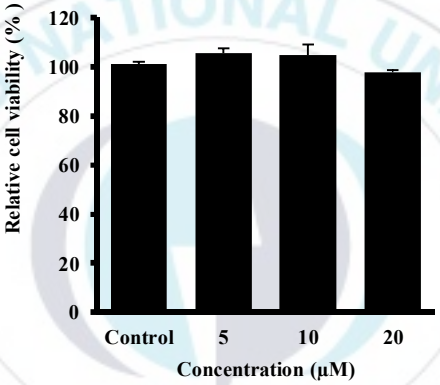

A
B
C
D
E
Relative cell viability (%)
120
100
80
60
40
20
0
Control
50
100
200
Concentration (μg/mL)

Fig 3. Effect of unshiu mikan orange (A), sweet orange (B), boiled tomato (C), raw tomato (D) and 2:1:3 (E) on cell viability in HepG2 cells. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method. Cells were pretreated with the indicated concentrations of 50, 100 200 μg/mL of unshiu mikan orange, sweet orange, boiled tomato, raw tomato and their combination 2:1:3 (sweet orange: boiled tomato: unshiu mikan orange) for 24 hours. Data shown represent means ± standard deviation of triplicate experiments.

A



B



C

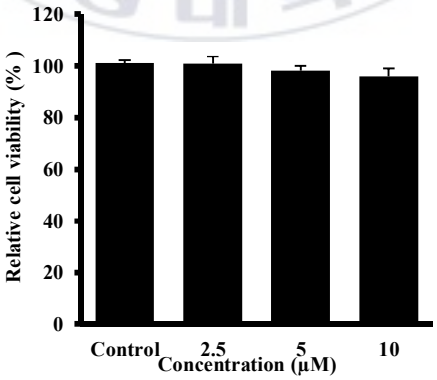


Fig 4. Effect of hesperidin (A), rutin (B) and narirutin (C) on cell viability in HepG2 cells. Cell viability was determined using the MTT method. Cells were pretreated with the indicated concentrations (2.5 to 100 μM) of flavonoids for 24 h. Data shown represent means ± standard deviation of triplicate experiments.

3-4. Cytoprotective effect of unshiu mikan orange, sweet orange, boiled and raw tomato, 2:1:3 and their key flavonoids on *tert*-butyl hydroperoxide induced hepatotoxicity in HepG2

To evaluate whether unshiu mikan orange, sweet orange, boiled tomato and their combination 2:1:3 as well as flavonoid compounds, hesperdin, narirutin and rutin exert any hepatoprotective effect on *t*-BHP treated HepG2 cells, cells were pretreated with unshiu mikan orange, sweet orange, boiled tomato and their combination 2:1:3 at concentrations (50,100, 200 μg/mL) as well as with flavonoid compounds at the concentration range of 2.5-100 μM for 24 h and then treated with *t*-BHP (200 μM). Thereafter, cells were incubated for 2h. Exposure of *t*-BHP (200 μM) significantly decreased the viability of cells to about 51%, while pretreatment of HepG2 cells with unshiu mikan orange, sweet orange, boiled tomato and their combination 2:1:3 at concentrations of 50,100 and 200 μg/mL significantly increased the cell viability and protected cells against *t*-BHP induced cytotoxicity in a dose-dependent manner as shown in Fig 5. However, In case of raw tomato cell viability was not increased to significant levels thus; raw tomato did not exhibit any cytoprotective effects at the indicated concentrations of 50, 100 and 200 μg/mL, respectively. Additionally, when HepG2 cells only treated with *t*-BHP (200 μM), viability levels of cells reduced sharply up to 51%, whereas pretreatment of HepG2 cells with hesperdin, narirutin and rutin at concentrations up to 100 μM significantly increased the cell viability and demonstrated marked cytoprotective effects against *t*-BHP induced

cytotoxicity dose-dependently as shown in Fig 6. The respective cell viabilities achieved upon treatment of unshiu mikan orange, sweet orange, boiled tomato, raw tomato and 2:1:3 at concentrations of 50 100 and 200 μg/mL against *t*-BHP-induced cell damage were in the range of 71 ± 0.1%, 66 ± 0.5%, 62 ± .04%, 69 ± .04% , 65 ± 0.51%, 61 ± 1.0 %, 62 ± 0.2%, 58 ± 0.7%, 55 ± .04%, 57 ± 0.3%, 55 ± 0.2%, 54 ± .01%, 67 ± 0.3%, 64 ± 0.1%, and 61 ± .01%, respectively. Moreover, cell viabilities achieved upon treatment with hesperidin at concentrations of 100, 50 and 25 μM, were found to be 94 ± 1.0%, 88 ± 2.0%, and 84 ± 1.0%, respectively. Additionally, there is no significant difference on cell viabilities achieved upon treatment of hesperidin at concentrations of 25 and 50 μM. In case of rutin the relative cell viabilities achieved with concentrations of 20, 10 and 5μM were in the range of 90 ± 1%, 83 ± 1.1% and 75 ± 2.0%, respectively. Pretreatment of narirutin also increased viability of HpeG2 cells at concentrations of 10, 5 and 2.5 μM were found to be 93 ± 3.1%, 87 ± 2.0%, 78 ± 1.0% respectively. The positive control silymarin enhanced the cell viabilities at concentrations of 12.5 μg/mL up to 85 ± 0.5% respectively. Thus, it is clear that pretreatment of HepG2 cells with unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 and bioactive flavonoid compounds markedly protected cells against *t*-BHP induced cytotoxicity.

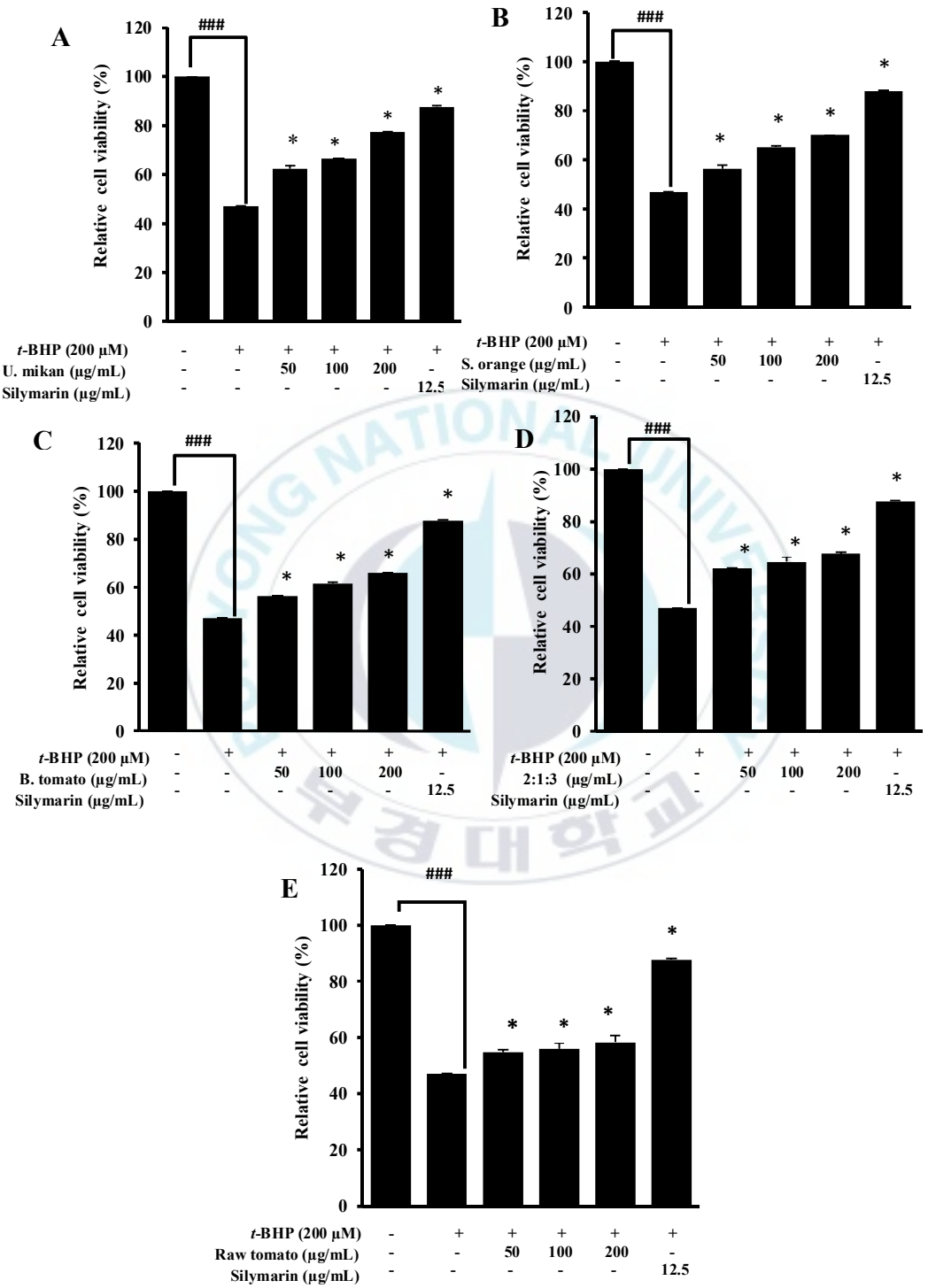

A
B
C
D
E
Relative cell viability (%)
###
*
t-BHP (200 μM)
U. mikan (μg/mL)
S. orange (μg/mL)
B. tomato (μg/mL)
2:1:3 (μg/mL)
Raw tomato (μg/mL)
Silymarin (μg/mL)

Fig 5. Cytoprotective effect of unshiu mikan orange (A), sweet orange (B), boiled tomato (C), 2:1:3 (D) and raw tomato (E) on *t*-BHP-treated HepG2 cells. Cell viability was determined using the MTT method. Cells were pretreated with the indicated concentration (50, 100, and 200 μg/mL) of unshiu mikan orange, sweet orange, boiled tomato, raw tomato and 2:1:3 for 24 h. After that time, cells were treated with *t*-BHP (200μM) and incubated for 2 h. Control values were obtained in the absence of *t*-BHP and unshiu mikan orange, sweet orange, boiled tomato, raw tomato and 2:1:3. Silymarin was used as a positive control. Data are represented as means ± STDEV of triplicate experiments. *$p<0.05$ indicates significant difference from the *t*-BHP- treated group. ###$p<0.001$ indicates significance differences from the control group.

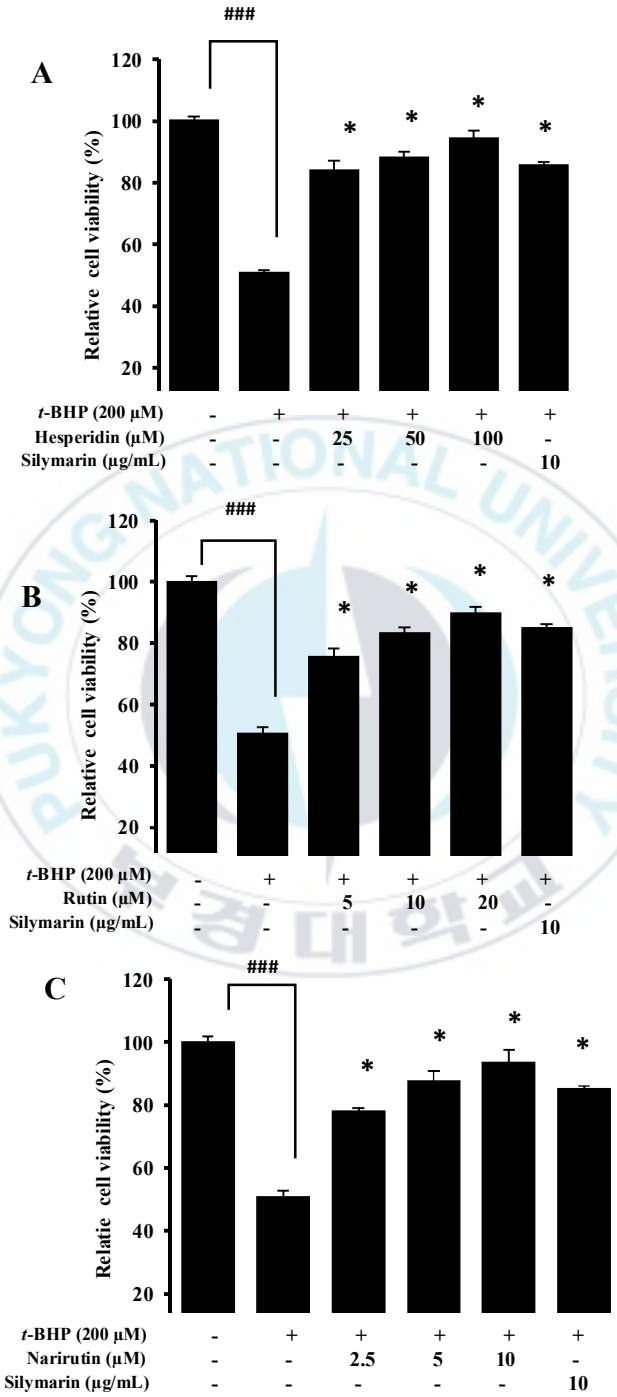

A
###
Relative cell viability (%)
120
100
80
60
40
20
*
t-BHP (200 μM) - + + + + +
Hesperidin (μM) - - 25 50 100 -
Silymarin (μg/mL) - - - - - 10
B
###
Relative cell viability (%)
t-BHP (200 μM) - + + + + +
Rutin (μM) - - 5 10 20 -
Silymarin (μg/mL) - - - - - 10
C
###
Relatie cell viability (%)
t-BHP (200 μM) - + + + + +
Narirutin (μM) - - 2.5 5 10 -
Silymarin (μg/mL) - - - - - 10

Fig 6. Cytoprotective effect of hesperidin (A), rutin (B), narirutin (C) on *t*-BHP-treated HepG2 cells. Cell viability was determined using the MTT method. Cells were pretreated with the indicated concentration (2.5 to 100 μM) of hesperidin, rutin and narirutin for 24 h. After that time, cells were treated with *t*-BHP (200 μM) and incubated for 2 h. Control values were obtained in the absence of *t*-BHP and hesperidin, rutin and narirutin. Silymarin was used as a positive control. Data are represented as means ± STDEV of triplicate experiments. *$p<0.05$ indicates significant difference from the *t*-BHP-treated group. ###$p<0.001$ indicates significance differences from the control group.

3-5. Effect of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 on *t*-BHP-induced ROS generation in HepG2 cells

To recognize whether the observed cytoprotective effect of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 attributed towards the reduction of oxidative stress, we next determined the effect of these juice powders on ROS generation using HepG2 cells exposed to *t*-BHP (200 μM). Exposures of *t*-BHP (200 μM) modify the redox status of cells, which results in generation of ROS. ROS generation was estimated using the ROS-sensitive fluorescence indicator DCFH-DA. ROS generation increased significantly when the cells were treated with *t*-BHP (200 μM) to the levels of about 100%, indicating that *t*-BHP had a strong effect on ROS generation in HepG2 cells. While the pretreatment of HepG2 cells with unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 at concentrations range of 0-200 μg/mL significantly inhibited the ROS generation and protected cells against ROS induced oxidative stress in a concentration-dependent manner compared to *t*-BHP induced HepG2 cells as shown in Fig 7. The relative ROS levels achieved with treatment of unshiu mikan orange at the concentrations of 50, 100 and 200 μg/mL were found to be 84%, 76%, 35% and 61% respectively. Sweet orange at the concentrations of 50, 100 and 200 μg/mL inhibited the ROS levels up to 88%, 78% and 69% respectively. Pretreatment of boiled tomato significantly inhibited the ROS levels at the concentrations of 50, 100 and 200 μg/mL to the levels of 69%, 58% and 46%, respectively. Additionally, combination of 2:1:3 at the concentrations of 50, 100 and 200 μg/mL also showed marked inhibitory

effects toward the ROS generation and the relative inhibited ROS levels achieved upon treatment were found to be 86%, 76% and 64%, accordingly. The positive control trolox at 10 μM concentration inhibited the ROS production about 54%. These results clearly demonstrate that unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powders acts as scavengers of ROS generation induced by *t*-BHP in HepG2 cells.

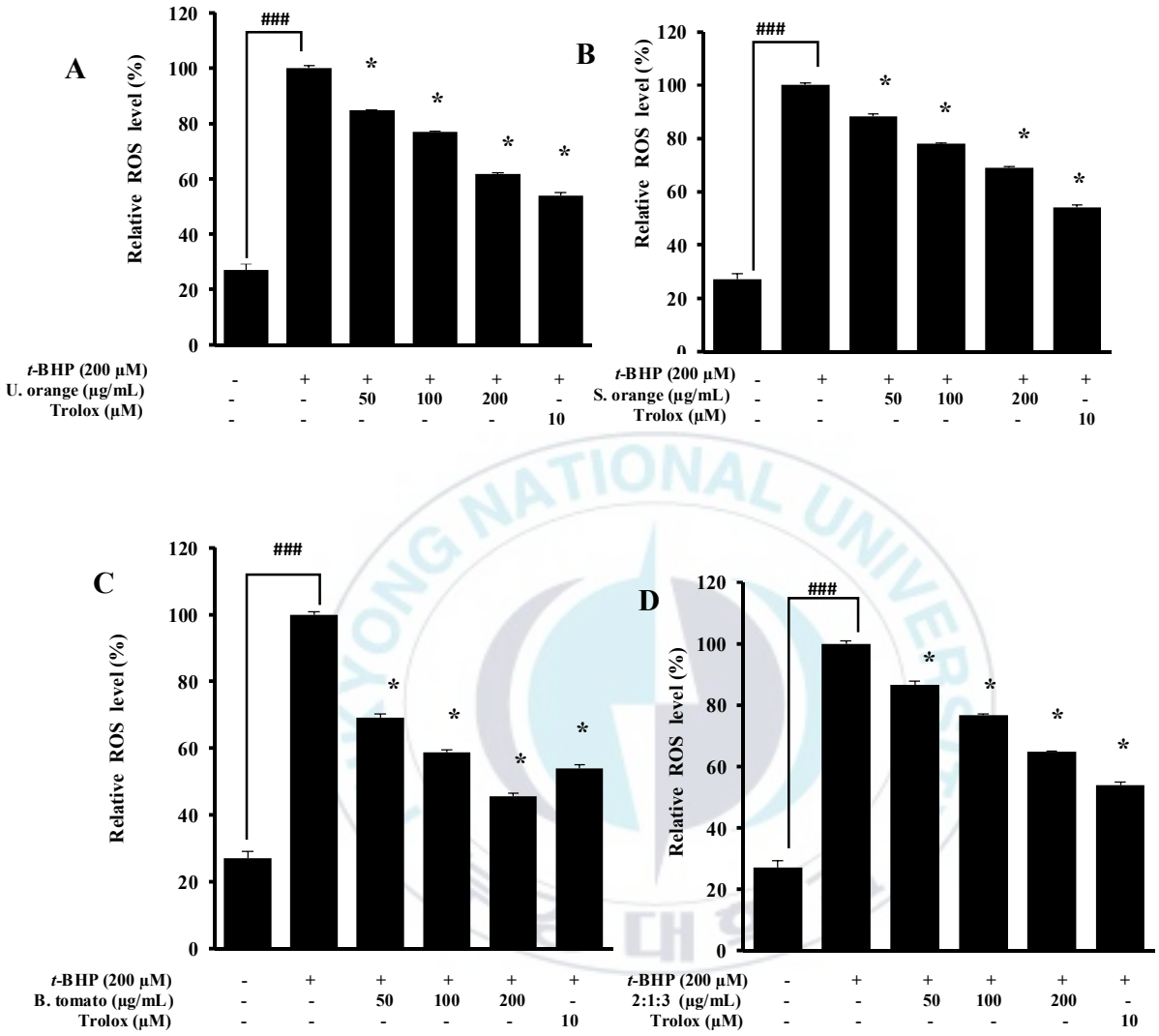
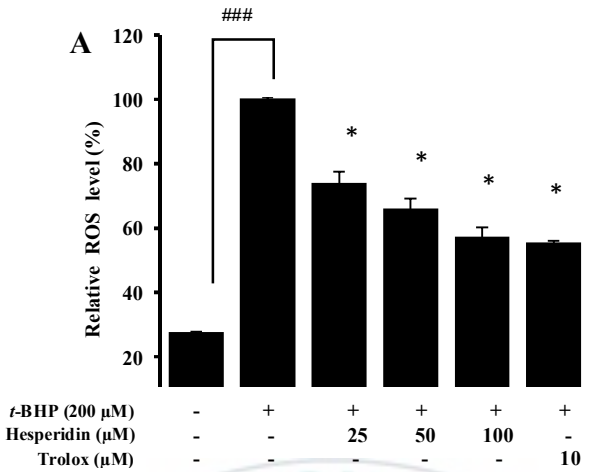
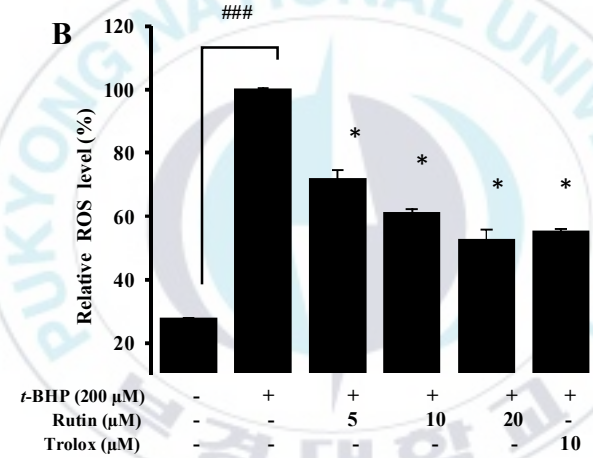

A
B
C
D
Relative ROS level (%)
###
*
t-BHP (200 μM)
U. orange (μg/mL)
S. orange (μg/mL)
B. tomato (μg/mL)
2:1:3 (μg/mL)
Trolox (μM)

Fig. 7. Effect of unshiu mikan orange (A), sweet orange (B), boiled tomato (C) and 2:1:3 (D) on *t*-BHP-induced ROS generation in the HepG2 cells. The cells pretreated with different concentrations (50, 100, and 200 μg/mL) of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 or trolox (10 μM) for 1 h were stimulated with 200 μM *t*-BHP for 30 min. ROS levels were measured by DCFH-DA with fluorescent analysis. The control values were obtained in the absence of *t*-BHP (200 μM) and unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 and after the addition of *t*-BHP (200 μM). Trolox was used as a positive control. Data are expressed as the mean ± SD of three independent experiments. ###$P<0.001$ indicates significant differences from the unstimulated control group. *$P< 0.05$ indicates significant difference from the *t*-BHP-treated group.

3-6. Effect of hesperidin, rutin and narirutin on *t*-BHP-induced ROS generation in the HepG2 cells

To classify with whether the observed cytoprotective effect of flavonoid compounds, hesperidin, rutin and narirutin attributed towards the reduction of oxidative stress, we next determined the effect of these compounds on ROS generation using HepG2 cells exposed to *t*-BHP (200 μM). Exposures of *t*-BHP (200 μM) modify the redox status of cells, which results in generation of ROS. ROS generation was estimated using the ROS-sensitive fluorescence indicator DCFH-DA. ROS generation increased significantly when the cells were treated with *t*-BHP (200 μM) to the levels of about 100%, indicating that *t*-BHP had a strong effect on ROS generation in HepG2 cells. While the pretreatment of HepG2 cells with hesperidin, rutin and narirutin at a concentration range of 0-100 μM significantly inhibited the ROS generation and protected cells against ROS induced oxidative stress in a concentration-dependent manner compared to *t*-BHP induced HepG2 cells as shown in Fig 8. The relative ROS levels achieved with treatment of hesperidin at the concentrations of 25, 50 and 100 μM were found to be 74%, 66% and 57% respectively. Rutin at the concentrations of 5, 10 and 20 μM inhibited the ROS levels up to 72%, 61% and 52%, respectively. Pretreatment of narirutin significantly inhibited the ROS levels at the concentrations of 2.5, 5 and 10 μM to the levels of 67%, 59% and 46%, respectively. The positive control trolox at 10 μM concentration inhibited the ROS production by about 55%. These results clearly demonstrate that bioactive flavonoids hesperidin, rutin and narirutin act as scavengers of ROS generation induced by *t*-BHP in HepG2 cells.


A
###
Relative ROS level (%)
120
100
80
60
40
20
*
*
*
*
t-BHP (200 μM) - + + + + +
Hesperidin (μM) - - 25 50 100 -
Trolox (μM) - - - - - 10


B
###
Relative ROS level (%)
120
100
80
60
40
20
*
*
*
*
t-BHP (200 μM) - + + + + +
Rutin (μM) - - 5 10 20 -
Trolox (μM) - - - - - 10

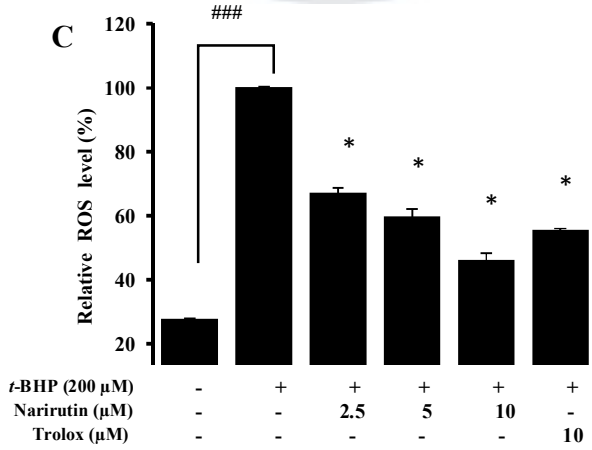

C
###
Relative ROS level (%)
120
100
80
60
40
20
*
*
*
*
t-BHP (200 μM) - + + + + +
Narirutin (μM) - - 2.5 5 10 -
Trolox (μM) - - - - - 10

Fig 8. Effect of hesperidin (A), rutin (B) and narirutin (C) on *t*-BHP-induced ROS generation in the HepG2 cells. The cells pretreated with different concentrations (2.5 to 100 μM) of hesperidin, rutin and narirutin or trolox (10 μM) for 1 h were stimulated with 200 μM *t*-BHP for 30 min. ROS levels were measured by DCFH-DA with fluorescent analysis. The control values were obtained in the absence of *t*-BHP (200 μM) and hesperidin, rutin and narirutin and after the addition of *t*-BHP (200 μM). Trolox was used as a positive control. Data are expressed as the mean ± SD of three independent experiments. ###$P<0.001$ indicates significant differences from the unstimulated control group. *$P< 0.05$ indicates significant difference from the *t*-BHP-treated group.

3-7. Effect of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 on intracellular GSH level in *t*-BHP-treated HepG2 cells

To further illustrate the antioxidant enzymes of HepG2 cells in culture exposed to *t*-BHP (200 μM), the activity of GSH were determined. GSH is ubiquitous intracellular peptide regulating multiple functions ranging from antioxidant defenses, detoxification, conserving thiol status and modulation of cell proliferation (Lu, 2009). Detoxification of exogenous as well as the endogenous toxic substances as well as their metabolites is modulated by GSH. Intracellular GSH regulates important cellular functions through protecting the cellular injury caused by oxidative stress via scavenging free radicals, detoxifying electrophiles, conserving the essential thiol status of proteins by inhibiting oxidation of -SH groups or by decreasing disulfide bonds activated by oxidant stress, possession a reservoir for cysteine and regulating key cell processes such as DNA synthesis, microtubular-associated processes and immune function (DeLeve and Kaplowitz, 1991; Suthanthiran et al., 1990;). In terms of its antioxidant activity, GSH plays a central role to detoxify toxic oxygen radicals such as $O_2\bullet^-$ and H_2O_2, alleviating lipid peroxidation and cellular damage. Exposure of *t*-BHP (200 μM) to HepG2 cells greatly decreased the GSH levels. Nevertheless, the reduced levels of intracellular GSH were significantly rescued and increased by increasing concentrations of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 in a dose-dependent manner as shown in Fig 9. Unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 were markedly increased the depleted GSH levels in

t-BHP (200 μM) treated HepG2 cells. Moreover, unshiu mikan orange and cooked tomato at concentrations of 100 μg/mL and 200 μg/mL restored the GSH levels near to untreated control group. Silymarin was used as positive control; pretreatment with 25 μg/mL silymarin significantly increased the GSH levels. This clearly illustrates that tangerine orange, sweet orange, boiled tomato and 2:1:3 positively regulated the GSH contents in *t*-BHP treated HepG2 cells and provides an antioxidant defense system against oxidative stress.

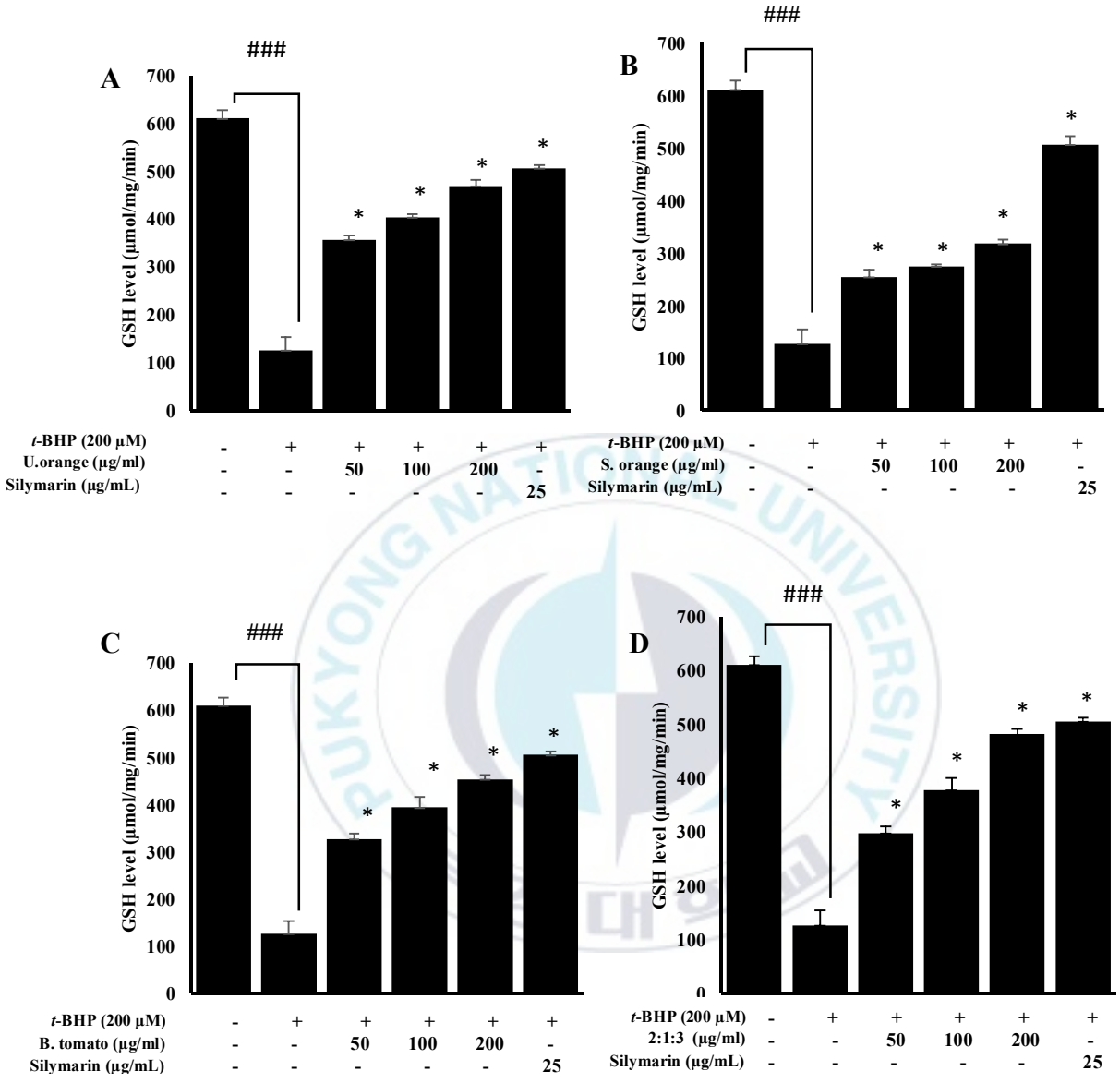
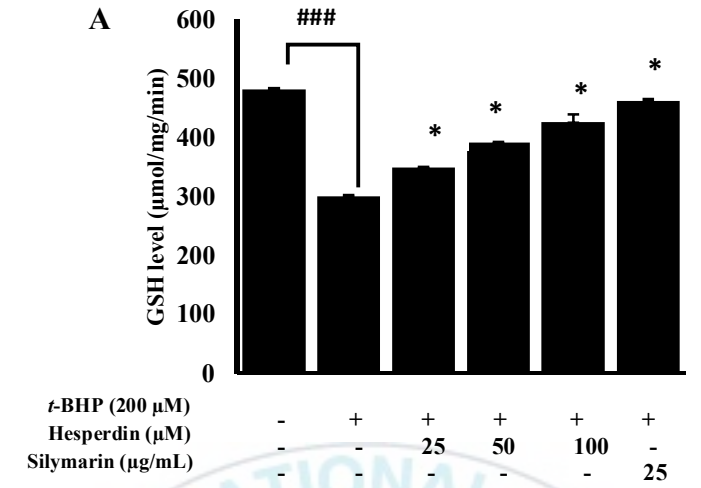

A
###
GSH level (μmol/mg/min)
700
600
500
400
300
200
100
0
*
t-BHP (200 μM) - + + + + +
U.orange (μg/ml) - - 50 100 200 -
Silymarin (μg/mL) - - - - - 25
B
###
GSH level (μmol/mg/min)
700
600
500
400
300
200
100
0
*
t-BHP (200 μM) - + + + + +
S. orange (μg/ml) - - 50 100 200 -
Silymarin (μg/mL) - - - - - 25
C
###
GSH level (μmol/mg/min)
700
600
500
400
300
200
100
0
*
t-BHP (200 μM) - + + + + +
B. tomato (μg/ml) - - 50 100 200 -
Silymarin (μg/mL) - - - - - 25
D
###
GSH level (μmol/mg/min)
700
600
500
400
300
200
100
0
*
t-BHP (200 μM) - + + + + +
2:1:3 (μg/ml) - - 50 100 200 -
Silymarin (μg/mL) - - - - - 25

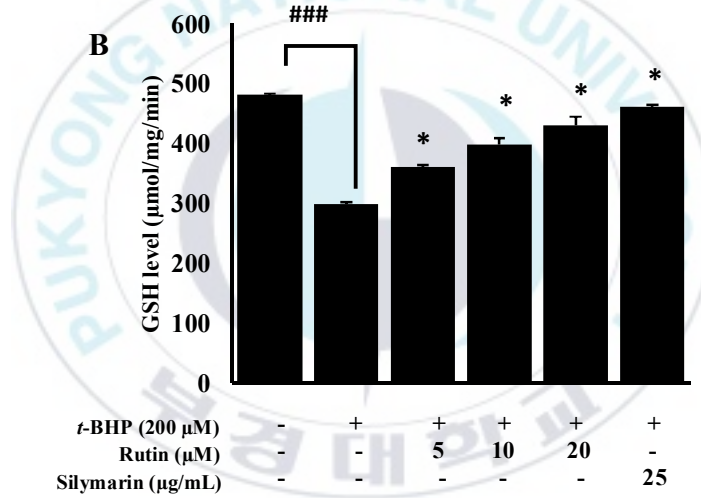
Fig 9. Effect of unshiu mikan orange (A), sweet orange (B), boiled tomato (C) and 2:1:3 (D) on intracellular GSH level in *t*-BHP-treated HepG2 cells. Cells were pretreated with the indicated concentrations (50, 100 and 200 μg/mL) of unshui mikan orange, sweet orange, boiled tomato and 2:1:3 for 24 h. Then *t*-BHP (200 μM) were treated and incubated for 2 h. Whole cell proteins were isolated and used for GSH assay. The data was represented as mean ± STDEV of triplicate experiments.

3-8. Effect of major flavonoids compounds on intracellular GSH levels in *t*-BHP treated HepG2 cells

To further exemplify the antioxidant enzymes of HepG2 cells in culture exposed to *t*-BHP (200 μM), the activity of GSH were determined. GSH is ubiquitous intracellular peptide regulating multiple functions ranging from antioxidant defenses, detoxification, conserving thiol status and modulation of cell proliferation. Detoxification of exogenous as well as the endogenous toxic substances as well as their metabolites is modulated by GSH. Intracellular GSH regulate important cellular functions through protecting the cellular injury caused by oxidative stress via scavenging free radicals, detoxifying electrophiles, conserving the essential thiol status of proteins by inhibiting oxidation of -SH groups or by decreasing disulfide bonds activated by oxidant stress, possession a reservoir for cysteine andregulating key cell processes such as DNA synthesis, microtubular-associated processes and immune function (Hutteret al., 1997). In terms of its antioxidant activity GSH plays a central role to detoxify toxic oxygen radicals such as $O_2\bullet^-$and H_2O_2, alleviating lipid peroxidation and cellular damage (Lu, 1999). Exposure of *t*-BHP (200 μM) to HepG2 cellsgreatly decreased the GSH levels. Nevertheless, the reduced levels of intracellular GSH were significantly rescued and increased by increasing concentrations of hesperdin, rutin and narirutinin a dose-dependent manner as shown in Fig 10. All the flavonoids markedly increased the depleted GHS levels in *t*-BHP (200 μM) treated HepG2 cells. Moreover, narirutin at concentrations of 5 μM and 10 μM restored the GSH levels near to

untreated control group. Silymarin was used as positive control, whereas pretreatment with 25 μg/mL silymarin significantly increased the GSH levels. This clearly illustrates that hesperdin, rutin and narirutin positively regulated the GSH contents in *t*-BHP treated HepG2 cells and provides an antioxidant defense system against oxidative stress.


A
GSH level (μmol/mg/min)
600
500
400
300
200
100
0
###
*
t-BHP (200 μM) - + + + + +
Hesperdin (μM) - - 25 50 100 -
Silymarin (μg/mL) - - - - - 25


B
GSH level (μmol/mg/min)
600
500
400
300
200
100
0
###
*
t-BHP (200 μM) - + + + + +
Rutin (μM) - - 5 10 20 -
Silymarin (μg/mL) - - - - - 25

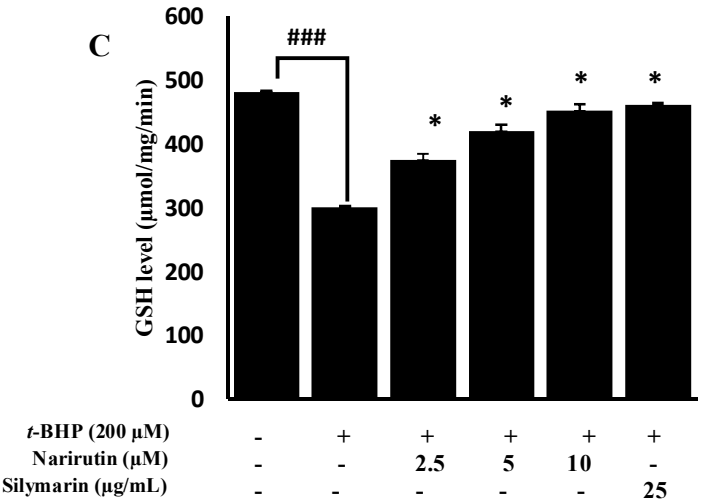

C
GSH level (μmol/mg/min)
600
500
400
300
200
100
0
###
*
t-BHP (200 μM) - + + + + +
Narirutin (μM) - - 2.5 5 10 -
Silymarin (μg/mL) - - - - - 25

Fig 10. Effect of hesperdin (A), rutin (B) and narirutin (C) on intracellular GSH level in *t*-BHP-treated HepG2 cells. Cells were pretreated with the indicated concentrations (2.5 to 100 μM) of hesperdin, rutin and narirutin for 24 h . Then *t*-BHP (200 μM) were treated and incubated for 2 h. Whole cell proteins were isolated and used for GSH assay. The data was represented as mean ± STDEV of triplicate experiments.

3-9. Citrus species and tomato juice powders as well as their key flavonoids induces HO-1 expression in a dose-dependent manner

We first determined the effect of various nontoxic concentrations of juice powder on HO-1 induction. Cells were treated with juice powder for 24 h and showed concentration-dependent increases in HO-1 protein expression (Fig. 11A-D). These increased results were suggesting that juice powder enhances the expression of the HO-1. In addition, we also determined the effect of various nontoxic concentrations of flavonoid compounds on HO-1 induction. Cells were treated with hesperidin, rutin and narirutinfor 24 h and showed concentration-dependent increases in HO-1 protein expression (Fig.12A-C).These increases were suggesting that flavonoid compounds enhance the expression of the HO-1.

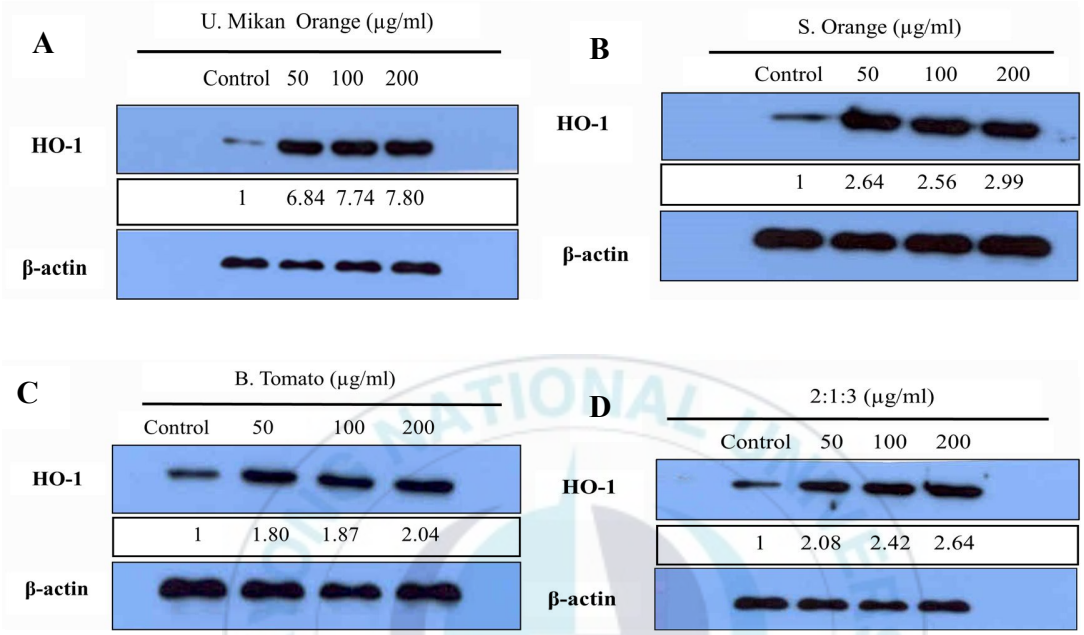


Fig 11. Effect of unshiu mikan orange (A), sweet orange (B), boiled tomato (C) and combination 2:1:3 (D) on HO-1 protein expression. (A-D) Cells were exposed to various concentrations of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 for 24 h and protein expression were analyzed by western blotting. HO-1 protein expression was analyzed by western blotting.

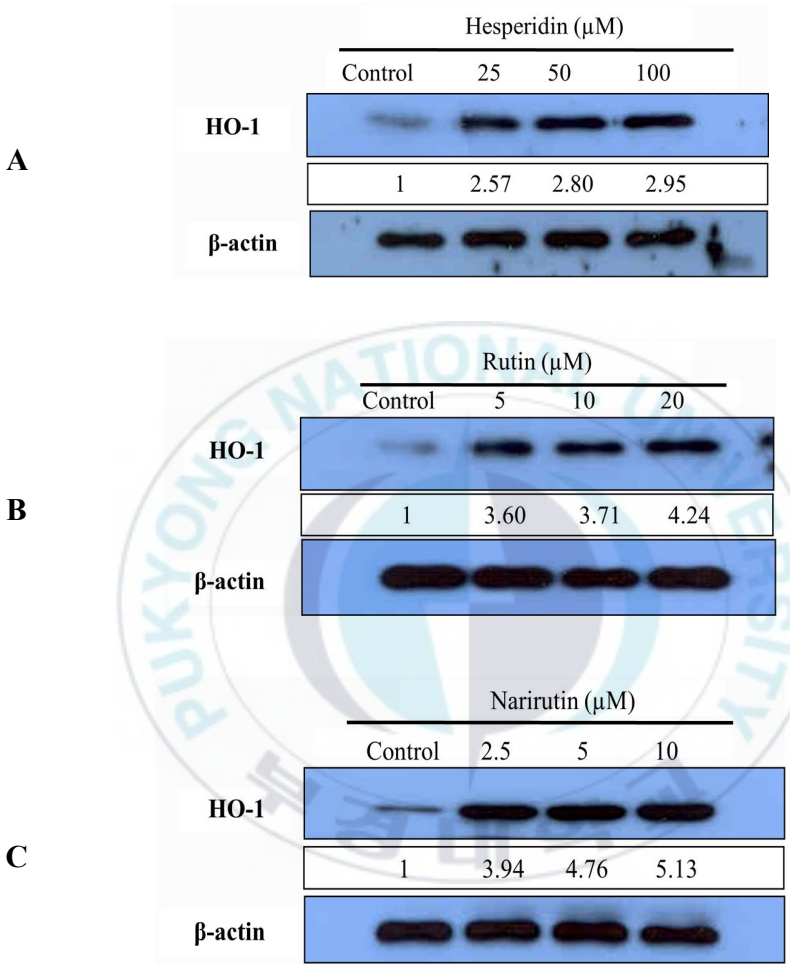


Fig 12. Effect of hesperidin (A), rutin (B) and narirutin (C) on HO-1 protein expression. (A-C) Cells were exposed to various concentrations of hesperidin, rutin and narirutin for 24 h and HO-1 protein expression were analyzed by western blotting.

IV. Discussion

Clinical trials and epidemiological studies have established an inverse correlation between fruit and vegetable dietary intake and the occurrence of diseases such as inflammation, cardiovascular diseases, cancer and aging-related disorders (Doll, 1990; Ames et al., 1993; Willet, 1994). Dietary antioxidants, including vitamin C, polyphenols and carotenoids, are believed to be effective nutrients in the prevention of these oxidative stress-related diseases (Kris-Etherton et al., 2002). Although fruits and vegetables are primary sources for these "nutrient" antioxidants, other dietary components may also be important protective agents. Citrus fruits have commercial importance due to their nutritional value and special flavor. It is estimated that the world production of citrus fruits in 2007–2008 reached 72 million tons; among which, the major commercially important orange fruit accounted for almost 45 million tons (Khan et al., 2010). Citrus fruit juice is rich in vitamin C and other bioactive compounds including flavonoids and phenolic acids, all potentially health-promoting (Widmer and Montanari, 1996). Cirus orange juice is the most popular fruit juice in all over the world. Consumers perceive orange juice as a healthy and natural source of vitamins and other health promoting nutrients, resulting in an increasing worldwide demand and production (Aschoff et al., 2014). Additionally, the convenient packaging and long shelf life of juices are advantageous compared to fresh fruit (Aschoff et al., 2014). Recent intervention studies demonstrated the health benefits of long-term orange juice consumption, such as an increased total antioxidant status, lower total cholesterol levels,

and the prevention of endotoxin increases after meals high in fat and carbohydrate (Aptekmann andCesar, 2013; Foroudi et al., 2014; Ghanim et al., 2010; Sanchez-Moreno et al., 2003). Previously it has been reported that concentrated orange juice has greater flavonoid content, including polymethoxylated flavones, hesperitin, narirutin and naringin, in comparison to fresh juice (Cesar et al., 2010). The most abundant flavonoid in sweet orange and unshiu mikan orange juice is hesperidin and narirutin.

In addition, tomato is another important vegetable and fruit in daily dietary. Several epidemiological studies indicated a beneficial effect of tomato consumption in the prevention of some major chronic diseases, such as some types of cancer and cardiovascular diseases (Giovannucci et al., 1999; La Vecchia et al., 1998). In the present study we investigated the hepatoprotective activity of mini or cherry tomato (*Solanum lycopersicum* L.), because of mini tomato is one of the most important types for fresh consumption in South Korea. Mini tomato is very popular in South Korea due to their superior test than normal size tomato. Mini tomato are commonly characterized by higher dry matter and soluble solids levels than normal-sized tomato, these differences are due to the higher content of sugars and organic acids, which, are major factors in determining the greater sweetness, sourness. Tomato contains not only the nutritional antioxidants such as vitamin A, C and E but also a great quantity of non-nutritional antioxidants, such as betacarotene, carotenoid, flavonoids, flavone, and total phenolics compound, etc. (Chang et al., 2006). Mini tomatoes showed a relatively high level of carotenoids and higher

lipophilic and hydrophilic antioxidative abilities than other typologies of tomatoes commonly used for fresh consumption (Leonardi et al., 2000). On the other hand, in a study on tomato cultivars grown in the United Kingdom, mini tomatoes showed intermediate lycopene and β-carotene levels when compared with other fresh marketed varieties (Hart and Scott, 1995). Crozieret al. (1997) found higher flavonoids concentrations in mini tomatoes compared to normal-sized tomato. Stewart et al (2000) also suggested that the greater skin/volume ratio of mini tomatoes could enhance their flavonoids content, because these compounds occur within the skin of the fruit. Nowadays, most of the tomatoes are consumed raw, in salads, or after cooking at home, although tomatoes produced worldwide are used for tomato paste, an ingredient in different processed tomato products such as ketchup, sauces, and soups. Preliminary we used raw mini tomato for our experiments but raw mini tomato showed significant cytotoxic effects on HepG2 cells. Next we had chosen boiled freeze-drying mini tomato for our experiments. Previously, Stahl and Sies (1992) suggested that a greater content of lycopene was found in heat-processed tomato juice than that in unprocessed one. Chen et al (2000) has been reported that a higher antioxidative activity was obtained through boiled or heat-processed tomato fruits juice than that in unprocessed one. Meanwhile, Wang et al. (1996) observed that heat processed tomato juice had a much higher antioxidant activity than the fresh ones. From the above-mentioned reports, it was observed that nutritional value of tomato could be increased through the heating processed. Previously, Chang et al (2006) has been also reported that freeze-drying

or hot-air-drying tomatoes increase the antioxidative properties due to the enhanced of ascorbic acid, total phenolics, total flavonoids, and lycopene contents compared to raw tomato. In this study we noticed that boiled mini tomato increase total phenolics, total flavonoids contents, compared to raw mini tomato in Table 1. Above all the particular reasons we chose the boiled mini tomato for our experiments.

Hepatic injury induced by *t*-BHP has been well characterized and is commonly used as a model for screening the hepatoprotective activities of drugs (Yen et al., 2004). *t*-BHP triggers the generation of harmful free radical intermediates, e.g., peroxyl and alkoxyl radicals, which readily cross cellular membranes and lead to the production of the highly reactive hydroxyl radicals. The hydroxyl radicals in turn react with macromolecules such as DNA, proteins, and lipids and thus damage cells (Grunberger et al., 1988).The antioxidant and free radical scavenging activities of many substances have been assessed, and many substances that possess anti-hepatotoxic activity also show strong antioxidant activity (Luper, 1998). In this study, an intracellular system was employed to test the cytotoxic effect of unshiu mikan orange, sweet orange, raw tomato, boiled tomato and their specific combination of 2:1:3 (sweet orange: boiled tomato: unshiu mikan orange) juice powder as well as their key flavonoids. However, raw tomato shows significant cytotoxic effects on HepG2 cells. For this reason, all other juice powders except raw tomato were chosen for subsequent experiments that aim to evaluate the cytoprotective effects against oxidative damage. In this experiment we also test a specific combination of three types juice powder,

because of nowadays combine juice is very popular and good for health. Combination of different types of fruit juice is more significant because of each individual fruits juice contain different types ingredients or phytochemicals. The combination of 2:1:3 (sweet orange, boiled tomato, unshiu mikan orange) juice powder showed significant hepatoprotective activity because of three different types of fruits content various types ingredients or phytochemicals, which may be acts a synergistically. In the case of cell viability, oxidatively damaged by *t*-BHP, the unshiu mikan, sweet orange, boiled tomato and 2:1:3 showed significant protective activity. However, In case of raw tomato, cell viability was not increased to significant levels thus; raw tomato did not exhibit any cytoprotective effects at the indicated concentrations. However, unshiu mikan, sweet orange, boiled tomato and 2:1:3 prevented *t*-BHP-induced cell death, evidenced by MTT test indicating that they have cytoprotective activities. Moreover, hesperidin, narirutin and rutin which are major flavonoid compounds present in unshiu mikan, sweet orange, boiled tomato juice powders, and displayed cell protection against *t*-BHP-induced oxidative stress. These protective effects of hesperidin, rutin and narirutin may in part be responsible for the cytoprotection of juice powder. Numerous studies noted that *t*-BHP, an organic hydroperoxide, induces an array of cellular dysfunctions, including generation of peroxyl radicals, peroxidation of membrane lipids, glutathione and protein thiol deletion, and DNA damage, and eventually leading to cell death (Garcia-Alonso, Ros, &Periago, 2006).

Oxidative stress can be defined as an imbalance between the oxidant and antioxidant system. Under normal circumstances the levels of ROS are low enough to be removed by the natural defense systems of the cell. However, when ROS is induced by oxidants to such an extent that cellular defenses are overwhelmed, the cells are exposed to oxidative stress, consequently leading to cell injury (Alia et al., 2005). Thus, phytochemical or antioxidant therapy is therefore regarded as a promising strategy to prevent cells from oxidative damage (Kaliora et al., 2006). In addition, apoptosis of the cells can be induced by ROS, leading to pathological cell death. According to the results, 200 μM *t*-BHP treatment induced ROS generation. As expected, treatment with unshiu mikan, sweet orange, boiled tomato and 2:1:3 juice powder, hesperidin, rutin and narirutin decreased ROS generation. These results suggest that the protective effect of unshiu mikan, sweet orange, boiled tomato and 2:1:3 juice powder, hesperidin, rutin and narirutin on the cytotoxicity of HepG2 cells may in part be attributed to the scavenging ROS, consequently preventing *t*-BHP-induced oxidative damage in HepG2 cells. Chen et al (2012) has been previously reported that sweet orange peel extract and bioactive flavonoid hesperdin decreased ROS generation, which is similar to our results.

Glutathione (GSH), widely distributed in animal tissues, plants and microorganisms, is well known to function both as a reductant and as a nucleophile due to its side-chain sulfhydryl (SH) residue in cysteine of GSH (Anderson, 1985). Numerous studies showed that GSH levels may be induced to increase by some extracts and naturally occurring

phenolic and flavonoid compounds (Yu et al., 2007; Chen et al., 2012). In an attempt to further explain the observed cytoprotective effect of unshiu mikan, sweet orange, boiled tomato and 2:1:3 juice powder, we determined the GSH levels in *t*-BHP-induced HepG2 cells co-incubated with unshiu mikan, sweet orange, boiled tomato and 2:1:3 juice powder and their key flavonoid compounds hesperidin, rutin and narirutin. According to the data obtained, t-BHP was significantly cytotoxic to HepG2 cells, accompanied by ROS generation and a marked depletion in GSH levels. Indeed, severe GSH depletion leaves cells more vulnerable to oxidative damage and is normally associated with calcium homeostasis disruption, which ultimately causes cell death (Lima et al., 2007). However, treatment with unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powder as well as flavonoid hesperidin, rutin and narirutin prevented decrease in GSH levels induced by t-BHP. Among the juice powder unshiu mikan orange, a specific combination 2:1:3 and boiled tomato markedly increased the depleted GHS levels in *t*-BHP treated HepG2 cells, due to the high phenolic and flavonoid contents herein in Table 1. Moreover, the flavonoid narirutin manifestly increased the depleted GSH levels in *t*-BHP treated HepG2 cells, among the tested flavonoid. Chen et al (2012) has been previously reported that hesperidin markedly increased the depleted GSH levels in *t*-BHP treated HepG2 cells, which is similar to our results. Evidently, the protection against GSH depletion was probably the highly relevant effect of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 providing an effective cellular reducing agent, and thereby leading to the detoxification of xenobiotics.

The induction of the phase II enzyme system is an important event in the cellular stress response during which a diverse array of electrophilic and oxidative toxicants can be eliminated or inactivated before they damage critical cellular macromolecules (Rushmore and Kong, 2002). Antioxidant agents can either scavenge ROS or stimulate the detoxification mechanism within cells, resulting in removal of ROS. HO-1 is a key enzyme of the antioxidant defense system. Increased HO-1 activity leads to enhanced protection against free radicals produced by intrinsic or extrinsic stimuli. HO-1 is the inducible form of HO that catalyzes the conversion of heme into biliverdin, carbon monoxide, and free iron as rate-limiting enzyme (Choi and Alam, 1996). Many studies have demonstrated the potent antioxidant and cytoprotective activities of heme derived metabolites produced by HO-1(Ryter et al., 2002). Therefore, enhanced activities of HO-1 may protect HepG2 cells against possible oxidative damage. The cytoprotective properties of antioxidants have been partially attributed to their ability to induce cytoprotective enzymes. Among the various cytoprotective enzymes, HO-1 expression has been considered an adaptive and beneficial response to oxidative stress in a wide variety of cells (Song and park, 2014; Qaisiya et al., 2014).The diversity of stimuli that can induce HO-1 suggests that the molecular mechanisms that regulate HO-1 are complex. Several studies have described the regulatory sites and transcription factors required for activation of the HO-1 promoter (Hill et al., 2000). Our results showed that unshiu mikan orange, sweet orange, boiled tomato and 2:1:3

juice powder as well as flavonoid hesperidin, rutin and narirutin increased HO-1 protein expressions in HepG2 cells (Figs. 11 and 12). The increase of HO-1 expression by unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powder as well as flavonoid hesperidin, rutin and narirutin conferred cytoprotection against *t*-BHP-induced oxidative stress. These results suggest that unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powder as well as flavonoids hesperidin, rutin and narirutin-induced anti-oxidant gene expression might serve as an important mechanism for the cytoprotective effects of unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powder as well as flavonoids hesperidin, rutin and narirutin. There are many reports that HO-1 is induced by various phytochemicals (Garg et al., 2008; Kim et al., 2010), the present result is the first demonstration that unshiu mikan orange, sweet orange, boiled tomato and 2:1:3 juice powders as well as flavonoids hesperidin, rutin and narirutin is a potent inducer of HO-1 expression in HepG2 cells.

V. Conclusion

The beneficial effect of flavonoids on human health is universally accepted nowadays. *Citrus* fruits and vegetable juices remain one of the most readily available dietary sources for their intake. Consumer demand for healthy food products provides an opportunity to develop foods rich in antioxidants as new functional foods. In the present study, our results indicate that the juice powders of orange and boiled tomato, which does not show significant toxicity up to 200 μg/mL, could serve as a candidate with strong hepatoprotective effects in *t*-BHP-induced hepatoma cell damage, by scavenging ROS production. To the best of our knowledge, this study is the first report on the evaluation of cytoprotective effect of unshiu mikan orange, sweet orange, boiled tomato and their combination 2:1:3 (sweet orange: boiled tomato: unshiu mikan orange) in *t*-BHP-induced HepG2 cells. The possible mechanism is that juice powders displayed significant scavenging ROS in *t*-BHP-induced HepG2 cells. The decrease in ROS generation appeared in parallel with up-regulation of GSH levels and antioxidant enzyme activity. Moreover, the direct scavenge of ROS by juice powders may increase the expression level of HO-1. The protective effects of juice powders, their combinations and the major flavonoids in 200 μM *t*-BHP-induced HepG2 cells significantly inhibited the cytotoxic effect and also may be associated with positive regulation of GSH levels and decrease in ROS production, thereby preventing cellular damage and the resultant increase in HO-1 activity. These results suggest that the fruit juice powders and their combinations as well as their major flavonoids

displayed a significant cytoprotective effect against oxidative stress which may be most likely because of the flavonoids-related bioactive compounds existing therein leading to maintenance of the normal redox status of cells. Therefore these fruit juice powders and their specific combinations could be advantageous as a bioactive source for the prevention of oxidative injury in hepatoma cells. This study showed that freeze-dried orange species and tomato juice powders are good sources of antioxidant which may be attributed to the presence of flavonoids, phenolic compounds and vitamin C existing therein.

VI. References

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