Chung Shan Medical Journal 2018; 29:27-40

Original Article

Functionality and characteristic changes of orange juice concentrates due to heat treatment

Chien-Chun Li[1], Shiowshuh Sheen[2], Shih-Han Hsu[3], Hui-Jin Hsu[4] and Shih-Chuan Liu[5*]

[1] *School of Nutrition, Chung Shan Medical University; Department of Nutrition, Chung Shan Medical University Hospital,*

[2] *Eastern Regional Research Center, Agricultural Research Service, Untied States Department of Agriculture*

[3] *School of Health Diet and Industry Management, Chung Shan Medical University;*

[4] *School of Health Diet and Industry Management, Chung Shan Medical University;*

[5] *School of Health Diet and Industry Management, Chung Shan Medical University; Department of Nutrition, Chung Shan Medical University Hospital;*

Fresh orange (*Citrus sinensis* (L.) Osbeck) juice was concentrated at 98±2°C for 0-24 hr, and periodically sampled concentrates of orange juice (COJs) were evaluated to investigate the changes in functionalities and characteristics during the heating process. Phenolic acids and flavonoids at T_{24} (after 24 h heating) were significantly higher than at T_0 (at time 0) except for rutin. The hesperidin content at T_{24} was 1.67 times that at T_0, and the sinensetin content increased 16.36 times over the 24-hour concentration period. EC_{50} values of reducing power at T_{20} and T_{24} were 2.13 and 5.67 times those at T_0 ($p<0.05$), respectively. The 2% and 3% COJs significantly inhibited the growths of *Escherichia coli* O157:H7 and *Listeria monocytogenes*, and the survival potential of *E. coli* in COJs was higher than that of *L. monocytogenes*. Moreover, COJs (at 100 μg/mL level) significantly suppressed TNF-α (1 ng/ml)-induced ICAM-1 protein expression.

Key words: Hesperidin; Antioxidative activity; *Escherichia coli* O157:H7; *Listeria monocytogenes*; ICAM-1 protein expression;

INTRODUCTION

The water content of fresh juice is traditionally removed under vacuum and/or heat to concentrate the juice to around 60-65°Brix and prevent the formation of undesirable substances, such as diacetyl and acetylmethylcarbinol, diketogulonic acid, furfural and 5-hydroxymethylfurfural (HMF), which may affect the flavor, color and other quality factors of concentrated juices[1, 2]. However, some fruit juices are concentrated under high temperature for a relatively long period of time, such as bainiku-ekisu, the concentrate of Japanese apricot juice [3]. In recent years, this has become a popular product in Taiwan and Japan as it possesses many health benefits, such as antioxidant [4], , anti-cancer [6] and antibacterial [7, 8] properties, and improves blood fluidity [5]. Therefore, functionalities of concentrated fruit juices might increase during concentration at high temperature.

In in vitro studies, citrus fruits have demonstrated high antioxidant activity, with that of orange juice largely due to its phenolic content [9]. In Taiwan, 250,000 tons of oranges (*Citrus sinensis* (L.) Osbeck) are produced annually. The total polyphenol and

* Correspondence to: Shih-Chuan Liu
Address: 110, Sec. 1, Jian-Guo North Rd., Taichung, 402, Taiwan
Tel: +886-4-24730022 ext. 11867
Fax: +886-4-23248188

flavonoid contents of these oranges are 37.3±1.53 and 15.7±0.43 mg/g, respectively [10]. Nevertheless, processed orange juice in Taiwan is mostly imported as consumers dislike the color (lemon-yellow) and taste (bitter) of pasteurized orange juice, and imported juice is cheaper than locally produced juice. Therefore, creating a "new" orange juice product with improved functional values that can be consumed year-round is an important issue and challenge for the local food industry.

Orange juice is typically rich in organic acids, sugars, polyphenols and ascorbic acid[9], which makes it a good candidate for the development of a healthy and value-added product. Orange juice concentrated under high temperature is expected to have functional properties similar to those of bainiku-ekisu. To better understand the potential enhancement of functionalities and characteristics of heat-treated concentrates of orange juice (COJs), we investigated the physical and chemical properties, antioxidative activities, antibacterial effects and anti-inflammatory effects of orange juice over various time periods during high-temperature (95-100°C) concentration process.

MATERIALS AND METHODS

2.1 Preparation of orange juice concentrates

Oranges (*Citrus sinensis* (L.) Osbeck) were purchased from Gukeng Township, Yunlin County in central Taiwan. The fruits, each measuring 70±10 mm in diameter, were stored at a refrigerated temperature, cut in half and squeezed to collect juice just before the heating process. The juice was filtered through gauze, placed in a beaker and heated in a 95-100°C water bath for concentration. The juice under heat treatment was sampled at 4-hour intervals, i.e., 0, 4, 8, 12, 16, 20 and 24 hr (samples were coded: T_0, T_4, T_8, T_{12}, T_{16}, T_{20} and T_{24}, with T_0 serving as the control). To determine the physical and chemical properties, phenolic acids, flavonoids and antioxidative activities, all samples were reconstituted to the initial °Brix of fresh juice with de-ionized water.

2.2 Measurements of the physical and chemical properties, phenolic acid and flavonoid contents of fresh and concentrated orange juice

The fresh and concentrated orange juice samples were analyzed for pH value, °Brix, browning index (A_{420}), HMF, total phenolic and flavonoid contents, and phenolic acid and flavonoid compositions.

2.2.1 Determinations of pH value, °Brix and browning index (A_{420})

The pH values of sampled solutions were determined with a pH meter (Model SP-71, Suntex Inc., Taiwan), and °Brix was measured with a hand-held refractometer (S-10E, Atago, Japan). Absorbance at 420 nm (A_{420}) was used as the indicator of the browning of orange juice [11], as determined on spectrophotometer (Spectro UV-Vis Auto, Labomed Inc., CA, USA).

2.2.2 Determination of 5-hydroxymethylfurfural (HMF)

The HPLC method for HMF content measurement [12] was slightly modified in this study. Reconstituted samples were filtered through 0.45μm pore filter. Then 20 μL of each solution were individually analyzed using HPLC instrument (L6200A, Hitachi, Tokyo, Japan) equipped with on-line degasser (Degasys DG-1310, Uniflows, Tokyo, Japan), 250 mm × 4.6 mm x 5 μm (i. d.) C_{18} column (Merck, Darmstadt, Germany) and UV-Vis detector (L4200, Hitachi, Tokyo, Japan). The fractionation program set-up/parameters were as follows: mobile phase, acetonitrile/H_2O/acetic acid, 10/89.5/0.5 (V/V/V); flow rate, 1.0 mL/min; wavelength detection, 280 nm. HMF was used as the standard and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.3 Determination of total phenolic and flavonoid contents

The method used to determine the total phenolic content was described by Julkunen-Titto (1985) [13]. A standard curve was generated with gallic acid, and total phenolic content was expressed as mg gallic acid equivalents per 100 mL sample (GAE mg/100 mL). Total flavonoid contents of the samples were measured using the method of Zhishen et al. (1999) [14], with some modifications. A standard curve was generated with (+)-catechin,

and results were expressed as mg catechin equivalents per 100 mL sample (CE mg/100 mL).

2.2.4 Determination of phenolic acid and flavonoid compositions

The method used to analyze phenolic acid and flavonoid compositions was based on that of Lin et al. (2010) [15]. Waters HPLC system including controlling pump (Model 600, Waters, MA, USA) equipped with on-line degasser (Degasys DG-1310, Uniflows, Tokyo, Japan), C18 column (250 mm × 4.6 mm x 5 μm (i.d.)) (Hypersil GOLD, Thermo Fisher Scientific Inc., Waltham, MA), Waters 2996 photodiode-array detector (PDA) and UV-Vis detector (Waters 486 Tunable Absorbance Detector, Waters, MA, USA) was used. Flow rate was set at 0.8 mL/min. Phenolic acids and flavonoids were determined based on retention time and absorbance, respectively. Results were compared to the standards of phenolic acid (caffeic acid, ρ-coumaric acid, ferulic acid, sinapic acid and chlorogenic acid), flavanone (naringin, hesperidin and neohesperidin), flavonol (quercetin, kaempferol and rutin) and flavone (sinensetin, luteolin and diosmin) (Sigma-Aldrich, St. Louis, MO, USA). Each sample was eluted in a gradient solvent system consisting of methanol (A) and 9% acetic acid (v/v of acid/water) (B). The gradient solvent treatments (resident time; A and B volume % ratio) were as follows: 0 min, 5% A and 95% B; 5 min, 17% A and 87% B; 25 min, 17% A and 87% B; 40 min, 31% A and 69% B; 76 min, 31% A and 69% B; 80 min, 40% A and 60% B; and 120 min, 40% A and 60% B.

2.3 Measurement of antioxidative activities

To determine antioxidative activities, the reconstituted samples from various periods of heat treatment were diluted to 0.1, 0.2 and 0.5x with de-ionized water. Antioxidative activity of catechin was evaluated as a positive control. Antioxidative activities were determined using three indicators: 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, 2,2-azino-bis-[3-ethylbenzothiazoline-6-sulfonic acid] (ABTS) radical scavenging activity and reducing power.

DPPH radical scavenging activity was determined by the modified method of Espín et al. (2000) [16]. Samples of 20 μL each were dispensed onto a 96-well plate, and 200 μL of 0.2 mM DPPH solution (prepared in 100% methanol) were added to each well. The plate was incubated at 37°C in a dark chamber for 30 min and the absorbance at 517 nm (A_{517}) was determined on microplate reader (Multiskan® Spectrum, Thermo, Vantaa, Finland). $ABTS.^+$ radical scavenging effect was determined as described by Scalzo et al. (2005) [17], with some modifications. Peroxidase (13.2 U/mL), H_2O_2 (300 mM) and 2,2-azino-bis- [3-ethylbenzothiazoline-6-sulfonic acid] (ABTS; 150 μM) were mixed to form $ABTS.^+$ solution which was incubated at 30°C in a dark chamber for 1 hour. Then, 300 μL aliquots were dispensed onto a 24-well plate that contained 30 μL of sample solution in each well. Absorbance was determined at 734 nm (A_{734}) on microplate reader after 3 min. Reducing power of samples was determined according to the modified method of Oyaizu (1986) [18]. Sample (250 μL) was added to sodium phosphate buffer (250 μL, 0.2 M, pH 6.6) and potassium ferricyanide (250 μL, 1%). The mixture was incubated at 50°C for 20 min. Then, 250 μL of 10% (g/mL) trichloroacetic acid were added, followed by centrifugation at 3000 rpm for 10 min. A 100 μL sample of the supernatant was mixed with 100 μL of de-ionized water and 25 μL of 0.1% $FeCl_3$. This solution was dispensed into each well of a 96-well plate and incubated at room temperature for 10 min. Then, the plate was placed onto microplate reader for determination of absorbance at 700 nm. The concentration of sample providing 0.5 absorbance values (EC_{50}) was calculated from the curve of absorbance at 700 nm plotted against concentration [19]. EC_{50} was calculated as the effective concentration of sample having 50% of total DPPH and $ABTS.^+$ radical scavenging activity, and the effective concentration of sample having 50% of the maximal DPPH radical scavenging activity.

2.4 Measurement of antibacterial effects

Escherichia coli O157:H7 C9495 and *Listeria monocytogenes* H7779 (Eastern Regional Research Center, Agricultural Research Service, USDA, Wydnmoor, PA) were used to evaluate the

antibacterial effects of high temperature COJs. Various concentrations of T_{24} COJ (1-3%) were mixed well with aseptic growth media (i.e. meat broth), then inoculated with microbes at 10^{8-9} CFU/mL. Cultures were then incubated at 37°C and sampled at specific time intervals to count the number of bacteria using tryptic soy agar (TSA, BD/Difco, Sparks, MD) plate. The detection limit was 1.0 log CFU/mL in the present study. In addition, selective media were used for *E. coli* O157:H7 and *L. monocytogenes* counts with no significant differences between them and TSA (i.e. $p>0.05$).

2.5 Measurement of anti-inflammatory effects

Lipopolysaccharide (LPS)-induced inducible nitric oxide synthase (iNOS) expression and nitric oxide (NO) production in Raw264.7 cells were used to determine whether COJs have anti-inflammatory potential. Intercellular adhesion molecule-1 (ICAM-1), one of the inflammatory biomarkers for predicting cardiovascular disease, is up-regulated by TNF-α in EA.hy 926 endothelial cells [20]. This model was used to confirm the anti-inflammatory effects of COJs.

2.5.1 Cell cultures

Raw264.7 macrophage cells (provided by Dr. K. L. Liu, Chung Shan Medical University, Taichung, Taiwan) were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS in a 5% CO_2,humidified incubator at 37°C. The human endothelial cell line EA.hy926 was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 3.7 g/L $NaHCO_3$, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified, 5% CO_2 incubator at 37 °C.

2.5.2 Nitrite determination

Raw264.7 cells were grown to 60–70 % confluence and treated with various concentrations of COJ (1-100 μg/mL) for 1 h followed by incubation with LPS (100 ng/mL) for an additional 24 h. After each experiment, the conditioned medium was collected and centrifuged at 5,100×g for 5 min. The supernatant containing the nitrite was analyzed on Griess assay, as described by Green et al. (1982) [21]. Then, 100 μL of culture supernatants were mixed with an equal amount (100 μL) of Griess reagent (1% sulphanilamide, 5% phosphoric acid, and 0.1% N-naphthyl ethylenediamine dihydrochloride) and reacted at room temperature for 10 min. Nitrite concentration was determined by measuring the absorbance at 540 nm against a standard curve of sodium nitrite prepared in the culture medium.

2.5.3 Western blotting for iNOS, ICAM-1

Whole cell lysate proteins were prepared from Raw264.7 cells. EA.hy926 cells were used in the procedure described by Yang et al. (2013) [22]. For iNOS and ICAM-1 tests, 10 μg lysate proteins were added to 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel and separated by electrophoresis. The separated proteins were transferred to polyvinylidene difluoride membranes and non-specific binding sites on the membranes were blocked with 5% non-fat dry milk in 15 mM Tris–150 mM NaCl buffer (pH 7.4) at 4°C for 24hr. After blocking, membranes were incubated with anti-iNOS antibody (1:500), anti-ICAM-1 antibody (1:1000), and anti-β-actin (1:4000), respectively, at room temperature for 2 h. These membranes were probed with secondary antibody labelled with horseradish peroxidase. Bands were visualized with enhanced chemiluminescence kit (PerkinElmer Life Science, Boston, MA, USA), then scanned and calculated using luminescent image analyser (LAS-4000, FUJIFILM, Japan).

2.6 Statistical analysis

All experiments were carried out in triplicate. The results are presented as the average of the three replicates. The data were subjected to analysis of variance (ANOVA) and Duncan's multiple range tests with s differences considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

3.1 Changes in the physical and chemical properties of orange juice during concentration

Table 1. ***Changes in characteristics of orange juice samples during concentration at 95-100°C.***

	pH	°Brix	A_{420}	HMF[1] (μg/mL)[4]	Total phenolic[2] (mg/100 mL)	Total flavonoids[3] (mg/100 mL)
T_0[5]	3.94±0.01a[6]	11.60±0.00a	0.14±0.00d	3.54±0.56f	38.75 ± 3.10b	1.36 ± 0.21d
T_4	3.94±0.02a	11.27±0.10c	0.21±0.07d	3.91±0.12f	33.90 ± 2.39c	1.39 ± 0.20cd
T_8	3.92±0.01b	11.40±0.00b	0.29±0.11d	9.64±3.78e	34.69 ± 2.66c	1.71 ± 0.50bcd
T_{12}	3.88±0.01c	11.46±0.08b	0.43±0.01c	8.88±0.81d	38.10 ± 2.68b	1.70 ± 0.21bcd
T_{16}	3.87±0.02c	11.60±0.03a	0.50±0.01c	48.77±17.12c	38.75 ± 2.45b	1.88 ± 0.14bc
T_{20}	3.81±0.01d	11.65±0.07a	0.71±0.05b	118.50±22.85b	39.04 ± 2.70b	2.19 ± 0.16b
T_{24}	3.74±0.01e	10.21±0.10d	1.99±0.24a	212.38±1.61a	60.81 ± 2.26a	4.43 ± 0.25a

[1] *HMF: 5-hydroxymethylfurfural.*
[2] *mg gallic acid equivalent/100 mL sample.*
[3] *mg catechin equivalent/100 mL of sample.*
[4] *Data are presented as the means ± standard deviations (n = 3)*
[5] *T_0, T_4, T_8, T_{12}, T_{16}, T_{20} and T_{24} are the heating times, 0, 4, 8, 12, 16, 20 and 24 hours.*
[6] *Different letters for the individual COJs indicate that the values are significantly different ($p < 0.05$).*

Samples of orange juice were taken at various time intervals (i.e. T_4, T_8, T_{12}, T_{16}, T_{20} and T_{24}, where the subscript indicates process time in hr) during concentration at 95-100°C. Table 1 shows the changes in physical and chemical properties of orange juice during concentration at 95-100°C. The pH values and °Brix of the concentrates gradually decreased as heating time increased, similar to the Maillard and caramelisation reactions observed in a model fructose solution [23, 24]. The products of Maillard and caramelisation reactions, such as formic acid, acetic acid, pyruvaldehyde and glyoxal, etc., may increase the overall acidity [25]. A_{420} and HMF gradually increased prior to T_{20} and accelerated to reach the T_{24} level, indicating that browning reactions mostly occur after T_{20}. However, the formation of HMF content significantly increased after T_{16} with the highest value observed at T_{24} (212.38±1.61 mg/g).

The total phenolic contents at T_4 and T_8 were lower than those at T_0, T_{12}, T_{16}, T_{20} and T_{24}. The highest phenolic content was at T_{24}. The total flavonoid contents gradually increased during the concentration process. There were no significant differences among total flavonoid amounts at T_0, T_4, T_8 or T_{12}, (0-12h), but total flavonoid amounts were higher at T_{16}, T_{20} and T_{24} (12-24 h). In general, ascorbic acid, β-carotene and total phenols are destroyed during high-temperature treatments [26, 27]. In this study, the total phenolic and flavonoid contents increased. Some researchers have reported similar findings. For example, flavanone glycosides of huyou (*Citrus paradisi* Changshanhuyou) peel are destroyed at 120 °C for 90 min or 150 °C for 30 min [28], but several low molecular weight phenolic compounds such as 2,3-diacetyl-1-phenylnaphthalene, ferulic acid, p-hydroxybenzaldoxime, 5-hydroxyvaleric acid, 2,3-diacetyl-1-phenylnaphthalene, and vanillic acid are generated in *Citrus unshiu* peels after heating at 150 °C for 30 min [29].

The differences in phenolic acid and flavonoid contents of fresh orange juice (T_0) and orange juice heated to 95-100°C for 24 hr (T_{24}) are shown in Table 2. Hesperidin was the predominant flavonoid at T_0, and increased after 24 hr heating. Other phenolic acids and flavonoids exhibited similar results, except for rutin. This trend differs from that of hesperidin content of citrus peel, which does not significantly decrease after heating to 90°C for 240 min [30]. Hesperidin and narirutin in freshly squeezed orange (*Citrus sinensis* (L.) Osbeck) juice

Table 2. *Changes in the phenolic acid and flavonoid contents (μg/mL) of fresh (T_0) and heated 24 hr (T_{24}) orange juice at 95-100°C.*

		T_0	T_{24}
Phenolic acid	Chlorogenic acid	4.36±0.00[1]b[2]	21.16±3.23a
	Caffeic acid	2.65±0.24b	6.10±0.08a
	p-Coumaric acid	1.90±0.01b	4.22±0.05a
	Ferulic acid	1.11±0.02b	2.13±0.05a
	Sinapic acid	5.09±0.37b	11.48±0.22a
Flavanone	Naringin	1.60±0.15b	2.00±0.53a
	Hesperidin	26.79±0.30b	44.08±0.03a
	Neohesperidin	1.31±0.12b	6.54±0.39a
Flavone	Diosmin	1.53±0.16b	9.74±1.56a
	Sinensetin	0.36±0.18b	5.89±3.88a
	Luteolin	1.67±0.11b	4.79±2.48a
Flavonol	Rutin	21.58±0.29a	15.57±0.63b
	Quercetin	3.33±0.23b	22.68±1.76a
	Kaempferol	1.54±0.66b	4.55±1.28a

[1] *Data are presented as the means ± standard deviations (n = 3).*
[2] *Different letters for T_0 and T_{24} indicate significant differences ($p < 0.05$).*

remain stable after pasteurization. [31] However, cyanidin-3-glucoside contents of non-thermally treated blood orange juice (Moro cultivar) increase segmentally during heating to 80°C for 6 min and in orange juice heated to 80°C for 1 min. [9]. In the present study, orange juice was at a high temperature (95-100°C) for a long time (24 hr). Therefore, some phenolic components may have been released, and rutin may have been destroyed.

3.2 Changes in antioxidative activities during concentration

The changes in the DPPH radical scavenging activity, ABTS.$^+$ radical scavenging activity and reducing power during the concentration process are shown in Figure 1. Heat treatment reduced antioxidative activities in the early stage (4-16 h) ($p<0.05$). However, these antioxidative activities eventually recovered to T_0 levels, and the reducing power of T_{24} was even higher than that at T_0 ($p<0.05$). EC_{50} values for samples at each time period are shown in Table 3. The DPPH radical scavenging effect was similar to that of the ABTS.$^+$ radical scavenging effect. EC_{50} values of reducing power at T_{20} and T_{24} were significantly lower after 20 hr of heating. The results indicated that heat treatment improves the reducing power of orange juice.

In general, antioxidative activities of fruit juices are reduced during heat treatment. Igual et al. (2010) [27] mentioned that the DPPH radical scavenging capacity of freshly squeezed grapefruit juice is destroyed by thermal treatment (conventional and microwave pasteurization). The DPPH radical scavenging effect decreases segmentally in blood orange juice during heating to 80°C for 6 min and in orange juice heated to 80°C for 1 min [9]. However, improvement in antioxidative activities of fruit juices during heat treatment has also been reported. The bioaccessibility of freshly squeezed orange (*Citrus sinensis* (L.) Osbeck) juice slightly increases by 9–11% after pasteurization. [31] Lee (1992) [32] noted that antioxidative activity of methanol extracts of browning reaction products

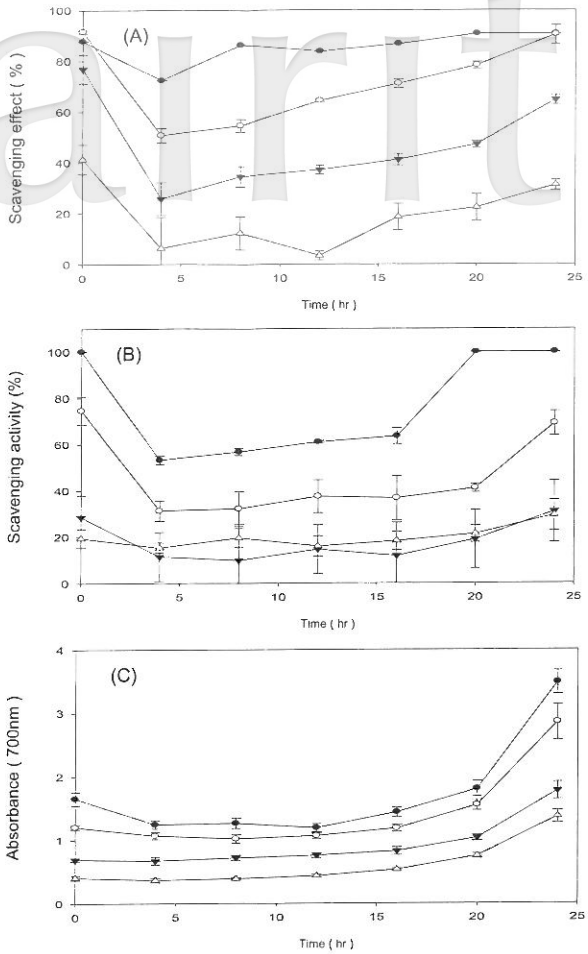

Figure. 1 Changes in DPPH radical scavenging effect (A), ABTS.$^+$ radical scavenging effect (B) and reducing power (C) of orange juice samples (non-diluted juice (●), 0.5-fold diluted juice(□), 0.2-fold diluted juice (▼) and 0.1-fold diluted juice (△)) during high temperature (95-100°C) concentration.

isolated from stored orange juice is higher than that of butylated hydroxyanisole. The antioxidant activity (ABTS.$^+$ radical cation decolorization assay) of cooked grapes decreases due to the loss of the phenolic fraction, but the formation of melanoidins improves the total antioxidant activity of the product [33]. Reconstituted (from concentrate) blood orange juice has higher ABTS.$^+$ radical effect than not-from-concentrate blood orange juice, possibly due to increased amount of carotenoid pigments in reconstituted juice during the thermal concentration process [34]. Ferric reducing antioxidant power (FRAP) of thermally treated (90oC, 4 min) apple juice has been reported to be significantly higher than that of raw juice [35]. This phenomenon might correlate with the formation of browning reaction products and the generation of phenolic acids and flavonoids during thermal treatment.

3.3 The antibacterial effects of COJs

T_{24} had better antioxidative activities and was used as the final product. Therefore, T_{24} was chosen for determinations of antibacterial and anti-inflammatory effects. Figure 2 shows the antibacterial effects of COJ (T_{24}) on *E. coli* O157:H7 (A) and *L. monocytogenes* (B) in meat broth. The 1% COJ could not effectively inhibit the growth of *E. coli* O157:H7. However, higher concentrations (2% and 3% levels) significantly impacted the growth after 8hr incubation. The number of *E. coli* O157:H7 decreased to around 10^2

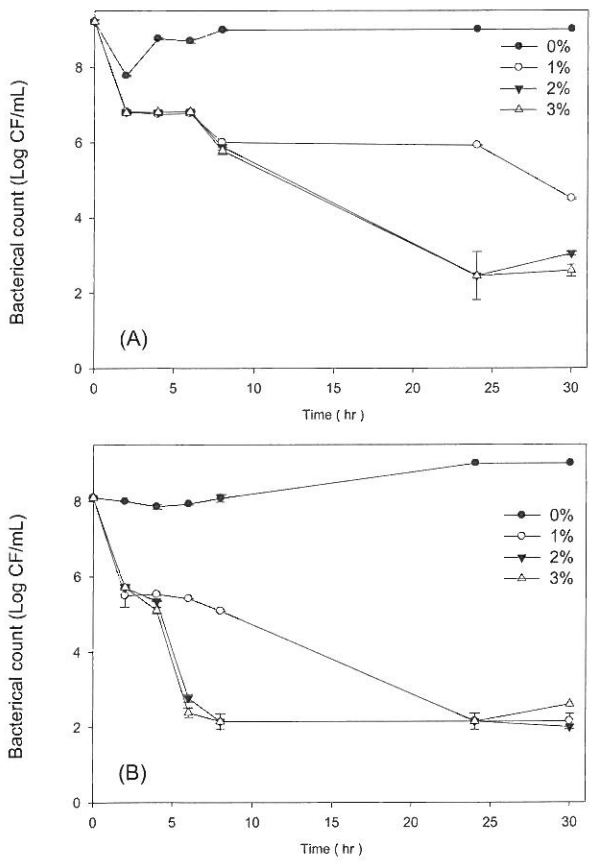

Figure. 2 Growths of Escherichia coli O157:H7 C9495 (A) and Listeria monocytogenes H7779 (B) in thepresence of various concentrates of orange juice (COJs) over 30 hours. ●, Control (0 % COJ); ○, 1% COJ; ▼, 2% COJ; △, 3% COJ.

Table 3. *EC_{50}[1] values of orange juice samples for DPPH radical scavenging, $ABTS.^+$ radical scavenging and reducing power.*

	DPPH (mL sample)[2]	$ABTS^+$ (mL sample)	Reducing power (mL sample)
T_0[3]	0.11±0.02d[4, 5]	0.39±0.03d	0.17±0.01a
T_4	0.48±0.02a	0.93±0.00a	0.19±0.01a
T_8	0.47±0.03a	0.87±0.04a	0.18±0.00a
T_{12}	0.47±0.00a	0.78±0.05b	0.17±0.01a
T_{16}	0.39±0.01b	0.79±0.03b	0.14±0.01a
T_{20}	0.31±0.03c	0.50±0.02c	0.08±0.02b
T_{24}	0.11±0.04d	0.36±0.04d	0.03±0.02b

[1] *EC_{50} defines the effective concentration of sample that has 50% of the DPPH or $ABTS.^+$ scavenging activity, or 0.5 of the total reducing activity.*
[2] *Volume of sample needed.*
[3] *T_0, T_4, T_8, T_{12}, T_{16}, T_{20} and T_{24} are the heating times of 0, 4, 8, 12, 16, 20 and 24 hours.*
[4] *Data are presented as the means ± standard deviations (n = 3).*
[5] *Different letters for the individual COJs indicate that the values are significantly different ($p < 0.05$).*

CFU/mL. *L. monocytogenes* was more sensitive with stronger inhibition than *E. coli* O157:H7. The population of *L. monocytogenes* gradually declined to 10^2 CFU/mL in 1% COJ after 24 hr incubation. Moreover, 2% and 3% COJs significantly inhibited *L. monocytogenes* within 6 h.

Karabiyikli et al. (2014) [36] reported that *L. monocytogenes* and *Salmonella typhimurium* may not survive in un-neutralized sour orange (*Citrus aurantium*) juice (1%) after incubation at 37°C for 7 days. The survival potential of *S. typhimurium* was higher/stronger than that of *L. monocytogenes*. They concluded that the antimicrobial effect of sour orange juice mainly depends on the low pH of the product. Concentrate of Japanese apricot (Bainiku-ekisu) also showed similar antibacterial effects [37]. Addition of bainiku-ekisu inhibited *Enterococcus faecalis*, *Staphylococcus aureus* and *E. coli*, and the minimum inhibitory concentration (MIC) of bainiku-ekisu was 1 mg/mL for all strains. COJs show the potential for use in functional food applications due to their antibacterial and antioxidative effects.

3.4 The anti-inflammatory effects of COJs

As shown in Figures 3A and 3B, LPS (100 ng/mL) significantly induced iNOS protein expression and NO production. The inductions of iNOS and NO levels dose-dependently decreased in the presence of COJ (T_{24}). Maximal inhibition was achieved at 100 μg/mL COJ. Moreover, TNF-α (1 ng/ml)-induced ICAM-1 protein expression was suppressed by COJ at 100 μg/mL. These results indicated that COJ possesses anti-inflammatory properties with the benefits of preventing heart disease. ICAM-1 plays a crucial role in inflammatory vascular diseases as it is capable of inducing monocyte adhesion onto the endothelium in response to oxidized low density lipoproteins or TNF-α [38]. Inhibition of abnormally induced ICAM-1 expression exhibits anti-inflammatory effects on cardiovascular disease [22]. In this regard, we demonstrated that orange juice has beneficial effects for the prevention of cardiovascular disease.

Sterol fortified orange juice and orange juice beverage (1 g sterol /240 ml juice or beverage twice a day) effectively lower inflammatory biomarkers such as interleukin-1b (IL-1b) and IL-6 in healthy humans, but the effects of placebo orange juice and orange juice beverage do not significantly differ [39]. Bergamot juice extract reduces dinitrobenzene sulfonic acid (DNBS)-induced acute colitis in mice

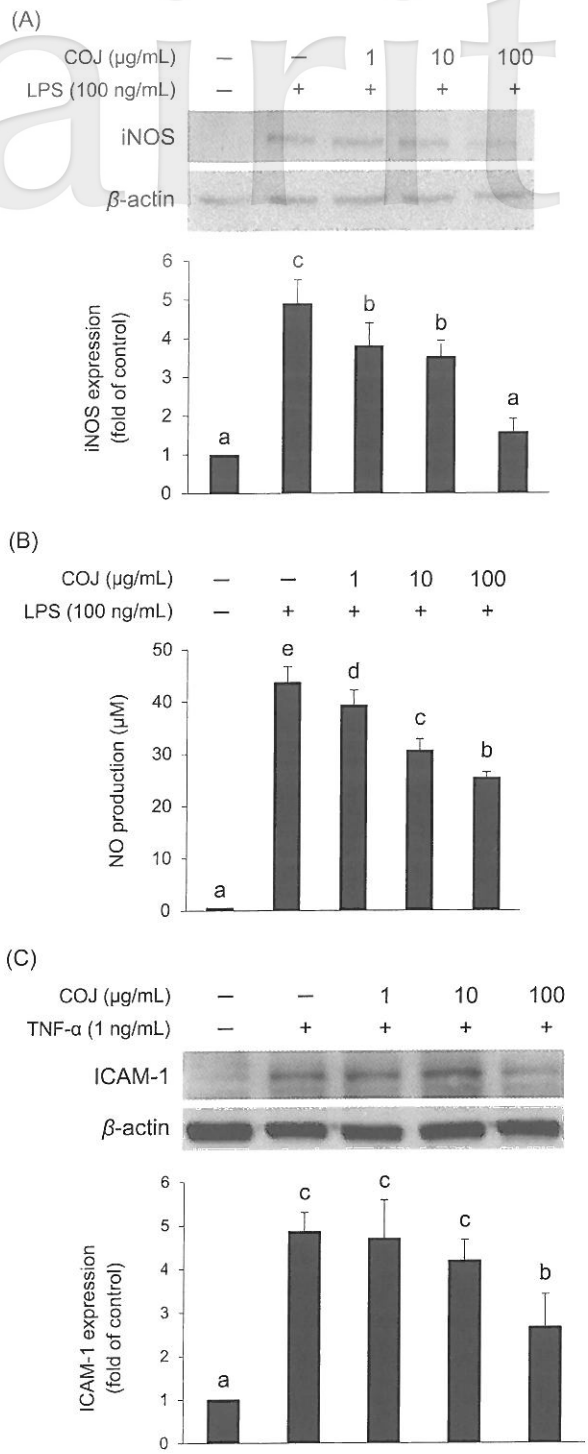

Figure. 3 Effects of concentrates of orange juice (COJs) on LPS-induced iNOS expression and NO production and TNF-α-induced ICAM-1 expression. Raw264.7 cells were pre-treated with various concentrations of COJs for 1 h followed by incubation with 100 ng/mL of LPS for another 24 h. (A) iNOS protein level was determined by Western blotting. (B) The levels of nitrite production were measured on Griess assay. (C) EA.hy 926 cells were pre-treated with various concentrations of COJs for 1 h followed by incubation with 1 ng/mL of TNF-α for another 24 h. ICAM-1 protein levels were determined by Western blotting. One representative experiment out of three independent experiments is shown. Results are presented as mean ± SD of three independent experiments. Results not sharing the same letter are significantly different ($P<0.05$).

by suppressing c-Jun N-terminal protein kinase (JNK) signalling pathway and its downstream NF-kB-mediated ICAM-1 and P-selectin expressions [40]. In this study, COJs inhibited the production of iNOS and NO in RAW264.7 macrophage cells treated with LPS. Moreover, TNF-α-induced ICAM-1 protein expression was suppressed by COJs in EA.hy926 cells, suggesting that down regulation of JNK signalling and NFkB-dependent inflammatory gene transactivation are involved in the anti-inflammatory actions of COJs. A recent study has shown that TNFα-induced ICAM-1 expression is inhibited by andrographolide via suppression of NADPH oxidase activation, induction of glutamate cysteine ligase modifier subunit (GCLM) and hemeoxygenase 1 (HO-1) expression through the PI3K/Akt-mediated AP-1 and PI3K/Akt-mediated Nrf2 pathways in EA.hy926 cells [41]. These results suggest that COJs protect against inflammation via up-regulation of PI3K/Akt-mediated antioxidant enzyme expression. There is no orange juice product such as the COJs reported in the literature. COJs could become a new health food with the potential to prevent heart disease.

3.5 Correlation assays

The correlation coefficients for A_{420}, HMF, total phenolic content, total flavonoid, DPPH scavenging effect, $ABTS.^+$ scavenging effect and reducing power during high-temperature concentration are shown in Table 4.

The correlation coefficients for HMF and total phenolic content and total flavonoid content and antioxidative activities (DPPH scavenging activity, $ABTS.^+$ scavenging activity and reducing power) were similar. In caramelization reaction,

Table 4. ***The correlation coefficients of characteristics of orange juice samples during concentration at 95-100°C.***

	A_{420}	HMF[1]	Total phenolic	Total Flavonoid	DPPH[2]	ABTS.$^+$	Reducing power
A_{420}	--	0.960**[3]	0.965**	0.998**	-0.553	-0.579	-0.929**
HMF		--	0.892**	0.947**	-0.578	-0.645	-0.989**
Total phenolic			--	0.967**	-0.697	-0.684	-0.874*
Total Flavonoid				--	-0.552	-0.569	-0.910**
DPPH					--	0.954**	0.623
ABTS.$^+$						--	0.709
Reducing power							--

[1] *5-hydroxymethylfurfural.*
[2] *EC_{50} of DPPH scavenging activity, ABTS.$^+$ scavenging activity and reducing power.*
[3] **: $p < 0.05$, **: $p < 0.01$.*

the generated HMF may improve DPPH and ABTS.$^+$ scavenging activities [23]. In this study, the correlation coefficient between HMF and reducing power was higher than the correlation coefficients among HMF, DPPH and ABTS.$^+$ scavenging activities.

Similarly, the correlations among total phenolic and flavonoid contents and antioxidative activities (DPPH scavenging activity, ABTS.$^+$ scavenging activity and reducing power) were strong. Rapisarda et al. (1999) [42] noted that a significant proportion of the antioxidant effects of orange juice may be attributed to phenol content. The correlation coefficients among total phenolic and flavonoid contents and reducing power were also high, indicating that some compounds are destroyed, while some are generated, during this process, leading to a net increase in reducing power. As mentioned above, HMF showed stronger correlation with reducing power than with DPPH or ABTS. Therefore, generated HMF may increase reducing power. The specific mechanism has not yet been identified.

CONCLUSIONS

In this study, orange juice was concentrated at higher temperature and for longer time than via traditional means. Reducing power, antibacterial activities and anti-inflammatory effects of COJs increased or were enhanced by heat treatment. Some reactions might occur during heat treatment, such as Maillard reaction, caramelization and phenolic compound breakdown or polymerization. The mechanism underlying the observed changes is complex and not well understood. In addition, the major reactions and compounds for increasing functionalities are also unclear. Nevertheless, this investigation provided some direction for further studies. Concentrated orange juice has the potential to become a new health food with several functionalities or a food ingredient with many potential applications.

Acknowledgements

The laboratory procedures related to foodborne pathogens were performed by Ms. Jennifer Cassidy at ERRC/ARS/USDA, whose work is much appreciated. This study was also supported by Chung Shan Medical University, Taichung, Taiwan (Project No. CSMU-INT-102-14).

References

1. Ting, S. V. and Rouseff, R. L. 1986. Citrus fruits and their products: Analysis and technology. Marcel Dekker, New York, USA.

2. Kim, M. K., Kim, M. Y. and Lee, K. G. 2016. Determination of furan levels in commercial orange juice products and its correlation to the sensory and quality characteristics. Food Chem. 211, 654-660.
3. Nakamura, A. 1995. Organic acids, free amino acids and sugar compositions in ume (Prunus mume) extract, and changes during preparation process of ume extract. Nippon Eiyo Shokuryo Gakkai shi. 48, 232-235.
4. Utsunomiya, H., Takekoshi, S., Gato, N., Utatsu, H., Motley, E. D., Eguchi, K., Fitzgerald, T. G., Mifune, M., Frank, G. D. and Eguchi, S. 2002. Fruit-juice concentrate of Asian plum inhibits growth signals of vascular smooth muscle cells induced by angiotensin II. Life Sci. 72, 659-667.
5. Chuda, Y., Ono, H., Ohnishi-Kameyama, M., Matsumoto, K., Nagata, T., and Kikuchi, Y. 1999. Mumefural, citric acid derivative improving blood fluidity from concentrate of Japanese apricot (*Prunus mume* Sieb. et Zucc). J. Agric.Food Chem. 47: 828-831.
6. Jeong, J. T., Moon, J. H., Park, K. H., and Shin, C. S. 2006. Isolation and characterisation of a new compound from *Prunus mume* fruit that inhibits cancer cells. J. Agric. Food Chem. 54, 2123-2128.
7. Fujita, K., Hasegawa, M., Fujita, M., Kobayashi, I., Ozasa, K., and Watanabe, Y. 2002. Anti-Helicobacter pylori effects of Bainiku-ekisu (concentrate of Japanese apricot juice). Nippon Shokkakibyo Gakkai Zasshi. 99, 379-385.
8. Nakajima, S., Fujita, K., Inout, Y., Nishio, M., and Seto, Y. 2006. Effect of the folk remedy, bainiku-ekisu, a concentrate of Prunus mume juice, on Helicobacter pylori infection in humans. Helicobacter. 11, 589-591.
9. Lo Scalzo, R., Iannoccari, T., Summa, C., Morelli, R. and Rapisarda, P. 2004. Effect of thermal treatments on antioxidant and antiradical activity of blood orange juice. Food Chem. 85, 41-47.
10. Wang, Y. C., Chuang, Y. C. and Hsu, H. W. 2008. The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. Food Chem. 106, 277-284.
11. Chen, S.L., Jin, S.Y. and Chen, C.S. 2005. Relative reactivities of glucose and galactose in browning and pyruvaldehyde formation in sugar/glycine model systems. Food Chem. 92, 597-605.
12. Lee, H. S. and Nagy, S. 1988. Relationship of sugar degradation to detrimental changes in citrus juice quality. Food Tech. 42, 91-94, 97.
13. Julkunen-Tiitto, R. 1985. Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *Journal of Agricultural and Food Chemistry*, 33, 213-217.
14. Zhishen, J., Mengcheng, T. and Jianming, W. 1999. The determination of falconoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. 64, 555-559.
15. Lin, J. T., Liu, S. C., Tsay, G. J. and Yang, D. J. 2010. Composition of flavonoids and phenolic acids in *Glycin tomentella* Hayata cultivated in various soils. Food Chem. 121, 659-665.
16. Espín, J. C., Soler-Rivas, C. and Wichers, H. J. 2000. Characterisation of the total free radical scavenger capacity of vegetable oils and oil fractions using 2,2-diphenyl-2-picrylhydrazyl radical. J. Agric. Food Chem. 48, 648-656.
17. Scalzo, J., Politi, A., Pellegrini, N., Mezzetti, B. and Battino, M. 2005. Plant genotype affects total antioxidant capacity and phenolic contents in fruit. Nutrition. 21, 207-213.
18. Oyaizu, M. 1986. Antioxidative activity of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. *Nippon Shokuhin Kogyo Gakkaishi*. 35, 771-775.
19. Guimarães, R., Barros, L., Barreira, J. C. M., Sousa, M. J., Carvalho, A. M. and Ferreira, I. C. F. R. 2010. Targeting excessive free radicals with peels and juices of citrus fruit: Grapefruit, lemon, lime and orange. Food Chem. Toxicol. 48, 99-106.
20. Chao, C.Y., Lii, C.K., Tsai, I.T., Li, C.C., Liu, K.L., Tsai, C.W. and Chen, H.W. 2011. Andrographolide inhibits ICAM-1 expression and NF-κB activation in TNF-α-treated EA.hy926 cells. J. Agric. Food Chemi. 59,

5263-5271.

21. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S. and Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal. Biochem. 126, 131-138.
22. Yang, Y. C., Lii, C. K., Wei, Y. L., Li, C. C., Lu, C. Y., Liu, K. L., and Chen, H. W. 2013. Docosahexaenoic acid inhibition of inflammation is partially via cross-talk between Nrf2/heme oxygenase 1 and IKK/NF-κB pathways. J. Nutr. Biochem. 24:204-212.
23. Chen, S.L., Yang, D.J., Chen, S.Y. and Liu, S.C. 2009. Effect of hot acidic fructose solution on caramelisation intermediates including colour, hydroxymethoxyfurfural and antioxidative activity changes. Food Chem. 114, 582-588.
24. Liu, S. C., Yang, D. J., Chen, H. Y., Chen, S. L. and Chen, M. L. 2011. Kinetics of fructose on the Maillard brown colour development, pH change, and antioxidative activity development in model fructose/glycine systems. Int. J. Food Sci. Tech. 46, 1768-1774.
25. Martins, S. I. F. S. and Van Boekel, M. A. J. S. 2005. A kinetic model for the glucose/glycine Maillard reaction pathway. Food Chem. 90, 257-269.
26. Dhuique-Mayer, C., Tbatou, M., Carail, M., Caris-Veyrat, C., Dornier, M. and Amiot, M. J. 2007. Thermal degradation of antioxidant micronutrients in citrus juice: Kinetics and newly formed compounds. J. Agric. Food Chem. 55, 4209-4216.
27. Igual, M., García-Martínez, E., Camacho, M. M. and Martínez-Navarrete, N. 2010. Effect of thermal treatment and storage on the stability of organic acids and the functional value of grapefruit juice. Food Chem.118, 291-299.
28. Xu, G., Ye, X., Chen, J. and Liu, D. 2007. Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. J. Agric. Food Chem. 55, 330-335.
29. Jeong, S.-M., Kim, S.-Y., Kim, D. –R., Jo, S.-C., Nam, K. C., Ahn, D. U. and Lee, S.-C. 2004. Effect of heat treatment on the antioxidant activity of extracts from citrus peels. J. Agric. Food Chem. 52, 3389-3393.
30. Garau, M. C., Simal, S., Rossselló, C. and Femenia, A. 2007. Effect of air-drying temperature on physico-chemical properties of dietary fibre and antioxidant capacity of orange (Citrus aurantium v. Canoneta) by-products. Food Chem. 104, 1014-1024.
31. Aschoff, J. K., Kaufmann, S., Kalkan, O., Neidhart, S., Carle, R. and Schweiggert, R. M. 2015. In vitro bioaccessibility of carotenoids, flavonoids, and Vitamin C from differently processed oranges and orange juices [Citrus sinensis (L.) Osbeck]. J. Agric. Food Chem., 63, 578–587.
32. Lee, H. S. 1992. Antioxidative activity of browning reaction products isolated from storage-aged orange juice. J. Agric. Food Chem. 40, 550-552.
33. Piva, A., Di Mattia, C., Neri, L., Dimitri, G., Chiarini, M. and Sacchetti, G. 2008. Heat-induced chemical, physical and functional changes during grape must cooking. Food Chem. 106, 1057-1065.
34. Arena, E., Fallico, B. and Maccarone, E. 2001. Evaluation of antioxidant capacity of blood orange juices as influenced by constituents, concentration process and storage. Food Chem. 74, 423-427.
35. Suarez-Jacobo, A., Rufer, C. E., Gervilla, R., Guamis, B., Roig-Sagues, A. X. and Saldo, J. 2011. Influence of ultra-high pressure homogenisation on antioxidant capacity, polyphenol and vitamin content of clear apple juice. Food Chem. 127, 447-454.
36. Karabiyikli, S., De_Girmenci, H. and Karapinar, M. 2014. Inhibitory effect of sour orange (*Citrus aurantium*) juice on *Salmonella typhimurium* and *Listeria monocytogenes*. LWT - *Food Sci. Tech.* 55, 421-425.
37. Yang, D. J., Chen, H. Y. and Liu, S. C. 2014. Study of antibacterial efficacy of bainiku-ekisu against pathogens. Int. J. Bacteriol. 2014, ID 460395.
38. Kevil C. G., Patel R. P. and Bullard D. C. 2001. Essential role of ICAM-1 in mediating monocyte adhesion to aortic endothelial cells.

Am J Physiol Cell Physiol. 281:C1442- 1447.
39. Devaraj, S., Jialal, I., Rockwood, J. and Zak, D. 2011. Effect of orange juice and beverage with phytosterols on cytokines and PAI-1 activity. Clin. Nutr. 30, 668-671.
40. Impellizzeri, D., Bruschetta, G., Di Paola, R., Ahmad, A., Campolo, M., Cuzzocrea, S., Esposito, E. and Navarra, M. 2014. The anti-inflammatory and antioxidant effects of bergamot juice extract (BJe) in an experimental model of inflammatory bowel disease. Clin. Nutr. 34, 1146-1154.
41. Lu, C. Y., Yang, Y. C., Li, C. C., Liu, K. L., Lii, C. K. and Chen, H. W. 2014. Andrographolide inhibits TNFα-induced ICAM-1 expression via suppression of NADPH oxidase activation and induction of HO-1 and GCLM expression through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways in human endothelial cells. Biochem. Pharmacol. 91:40-50.
42. Rapisarda, P., Tomaino, A., Lo Cascio, R., Bonina, F., De Pasquale, A. and Saija A. 1999. Antioxidant effectiveness as influenced by phenolic content of fresh orange juices. J. Agric. Food Chem. 47, 4718-4723.