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Assessment of validation and antioxidant activities of novel 12 Korean strawberry cultivars

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Abstract

In this study, antioxidant activities of extracts of 12 Korean strawberry cultivars were evaluated and validation of an analytical method for ellagic acid, a marker compound, was carried out. Cultivar 'Josaenghongshim' had the highest total polyphenol and flavonoid contents. In addition, cultivar 'Josaenghongshim' had the highest value and cultivar 'Seolhyang' showing the highest activity. Ellagic acid contents of 'Suhong', 'Okme', and 'Dahong' cultivars were the highest ellagic acid content. Correlation coefficient of the calibration curve was 0.9999, showing very high linearity. The HPLC-DAD method for the quantitation of ellagic acid content showed high linearity at various concentration ranges, with a limit of detection of 2.35 μg/mL. The limit of quantification was 7.13 μg/mL. Relative standard deviations from intra- and inter-day precision were less than 5.13%. Recovery rates of ellagic acid at 10, 50, and 100 μg/mL, respectively, were 99.0-100.1% with RSD values less than 5.30%. These results provide viable information for the validation of antioxidant capacities of 12 cultivars of Korean strawberries.

Keywords: antioxidant; validation; marker compounds; Korean strawberries; ellagic acid.

Practical Application: Development of a new variety of strawberry with excellent antioxidant effect.

1 Introduction

Reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydroxy radical ($\cdot OH^-$), and hydrogen peroxidase (H_2O_2) produced by various metabolic processes. There are known to cause human diseases and aging (Hwang et al., 2014). Excessively produced ROS can lead to severe physiological disabilities by damaging tissues and cells, and inhibiting protein breakdown and DNA synthesis (Halliwell et al., 1992). Living organisms have antioxidant systems such as superoxide dismutase (SOD), catalase, glutathione reductase, glutathione, and thioredoxin to protect cells against damages caused by ROS (Sies, 1993). However, needs of safe and effective antioxidants are increased for stressed-out modern people (Lee & Lee, 2016). Recently, due to improvement of living standard and change in consumer perception, functional food materials developed from natural product and related research are increased (Lee et al., 2015).

Strawberry (*Fragaria ananassa* Duch.), a perennial plant belonging to the rose family, has high contents of sugars, organic acids, and bioactive compounds such as ascorbic acid, polyphenols, quercetin, ferulic acid, ellagic acid, and flavonols (Kim et al., 2015). Consuming strawberries can reduce the risk of chronic diseases such as cancers (Seeram et al., 2006), cardiovascular diseases (Azzini et al., 2010), and memory loss (Giampieri et al., 2012). It can also decrease mutation and cholesterol level (Meyers et al., 2003). Among all polyphenols in strawberries, ellagic acid has a strong antioxidant efficacy by scavenging free radicals (Türk et al., 2010). It also possesses antiviral and antibacterial properties. It can also protect cells against apoptosis (Xu et al., 2003). Ellagic acid may produce positive effects in the control of estrogen receptor, collagen expression, and inhibition of photoresistance damage in fibroblasts (Kim et al., 2016; Türk et al., 2010; Papoutsi et al., 2005). In addition, ellagic acid can prevent tissue damage by repress peroxide lipid formation and control carcinogenesis by promoting the activity of detoxification enzyme (Maas et al., 1991). It also has protective effect against skin cancer (Mukhtar et al., 1986), and liver cancer (Shimogaki et al., 2000).

Although many studies have been conducted on ellagic acid using HPLC, only a few studies have been conducted on strawberry varieties (Yong et al., 2019). The objective of this study was to investigate antioxidant effects of 12 cultivars of strawberries grown and harvested by the National Institute of Horticultural and Herbal Science and chungcheongnamdo agricultural research & extension. In addition, we performed a validation of content analysis method for ellagic acid to obtain basic data that would aid the development of natural antioxidants and marker components of ellagic acid in various strawberry varieties.

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2 Materials and methods

2.1 Chemical and reagents

Folin-Ciocalteu reagent, tannic acid, quercetin, ellagic acid, 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and (+/-)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). For ellagic acid analysis, methanol and acetonitrile were obtained from J. T. Baker (Phillipsburg, NJ, USA). All other chemicals used were special grade reagents.

2.2 Plant materials and extraction

Twelve cultivars of Korean strawberries grown and harvested by the National Institute of Horticultural and Herbal Science and chungcheongnamdo agricultural research & extension in 2017 were used as samples in this study (Table 1). These 12 cultivars of strawberry samples used in this study were harvested from red-colored hard-boiled fruits with similar growth period. Strawberry samples were extracted with 10 vol (v/w) of water at 80 °C for 3 h, and extraction was repeated 3 times. Extracts were concentrated with vacuum evaporator (COSMOS660-50L, Kyungseo Machines Co, Incheon, Korea), and frozen in a deep freezer at -70 °C for 24 h, freeze-dried and stored at 4°C for use in the experiment. Each dried extraction, it was then dissolved in a pretreatment solvent (ethanol: distilled water: HCI = 3:1:1) and hydrolyzed for 3 h at 90°C. The extracted solution was then cooled to at room temperature and filtered with a syringe filter (0.45 μm, Hyundaimicro Co., Ltd, Korea) to obtain as a test solution.

2.3 Determination of total polyphenol content

Total polyphenol contents were measured applying the modified method of Folin-Ciocalteu method (Singleton et al., 1999). Each sample solution (500 μL) was mixed Folin-Ciocalteu reagent (500 μL) for 3 min, added with 10% Na_2CO_3 (500 μL), incubated at room temperature for 1 h, and then subjected to measurement of absorbance at 760 nm using a UV/VIS spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). Total polyphenol content (mg TAE/100 g fresh weight (FW)) was obtained using a standard curve prepared with tannic acid as the standard substance.

Table 1. Parentage and Origin of 12 strawberry cultivars.

NO	Cultivar	Parentage	Origin
1	Berrystar	Ssanta×07-S-28	Korea (2014)
2	Chodong	Halunoka×Palcheondae	Korea (1986)
3	Dahong	Sachinoka×Maehyang	Korea (2007)
4	Dummy	Unknown	Korea
5	Johong	Yeobong×Akihime	Korea (2002)
6	Josaenghongsim	Honghag×Useusino	Korea (1982)
7	Manhyang	Yeobong×Akkanekko	Korea (2003)
8	Mihong	Toyonoka×Yeohong	Korea (1996)
9	Okme	Toyonoka×Maehyang	Korea (2010)
10	Seolhyang	Akihime×Red Pearl	Korea (2005)
11	Sinseolmae	Seolhyang×Geumhyang	Korea (2012)
12	Suhong	Hogyojosaeng×Chunhyang	Korea (1985)

2.4 Determination of total flavonoid contents

Total flavonoid contents were determined using the Moreno method (Moreno et al., 2000). Briefly, each sample (10 μL) was diluted with 80% ethanol (990 μL). Then 100 μL of this sample solution was mixed with 80% ethanol solution (4.3 mL) containing of 10% aluminum nitrate and 1 M potassium acetate for 40 min. Absorbance was then measured at 415 nm using a spectrophotometer (Mecasys Co., Daejeon, Korea). Total flavonoid content (mg QE/100 g FW) was obtained using a standard curve prepared with quercetin as the standard substance.

2.5 DPPH radical scavenging activity

DPPH radical scavenging activity is one indicator of electron-donating ability. It was measured applying the modified method of Brand-Williams et al. (1995). The sample solution (10 μL/well) and 0.2 mM DPPH (190 μL/well) were added into a 96-well plate. After incubation at 25°C for 30 min, absorbance was measured at 515 nm using an ELISA reader (Thermo Fisher SCIENTIFIC, Multiskan Sky, KOREA).

2.6 ABTS radical scavenging activity

ABTS radical scavenging activity is one indicator of electron-donating ability. It was measured with modified method of Thaipong et al. (2006). Briefly, 7 mM ABTS and 2.45 mM potassium persulfate were mixed at a ratio of 1: 1 (v /v) to react in a dark room at 25 °C for 24 h to generate radicals. Radical stock solution was diluted with distilled water so that the absorbance value at 734 nm was 0.70 ± 0.02. After incubation in a dark room at 25 °C for 5 min, absorbance was measured at 734 nm using a UV/VIS spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea).

2.7 Prepare of standard solution

Ellagic acid analytical standard with a purity of 95% was dissolved in 1×10^{-2} N NaOH and store at 4°C. A standard solution was made by dilution so that the concentration of this solution was in the range of 10-100 μg/mL.

2.8 Method validation

Validation of analysis methods for marker components was performed by determining linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision according to guidelines of analysis methods used for medicines (Korean Food and Drug Administration, 2015).

- ***Linearity, limit of detection, and limit of quantitation*** Ellagic acid standard solution (five concentrations) was used to determine coefficient (R^2) using retention time and regression equation (y = ax + b, y: peak area, x: concentration (μg/mL)) shown on HPLC. Limit of detection and limit of quantitation for each component were determined based

on the standard deviation and the slope of the calibration curve using chromatogram of the standard solution;

- ***Accuracy and precision*** Accuracy and precision were evaluated to determine inter-day and intra-day variations of the index component at the same concentration. Accuracy refers to the proximity of average test results obtained by the analysis method. Relative standard deviation was calculated by measuring each sample three times for each concentration (10 μg/mL, 50 μg/mL, and 100 μg/mL). Precision indicates the proximity between measured values when a sample is taken several times and measured under specified conditions. It was measured with relative standard deviation which was obtained by measuring the standard solution at each concentration (0, 50, and 100 μg/mL) three times;
- ***Stability*** To check chemical stability of ellagic acid according to the storage period, each sample was kept at room temperature for 24 h, and then ellagic acid content was measured.

2.9 Ellagic acid content analysis

Ellagic acid contents in strawberry extracts were analyzed using high performance liquid chromatography (HPLC, Agilent Technologies, Santa Clara, CA, USA) with an autosampler and a DAD detector. Analysis conditions are shown in Table 2. Analysis was done using YMC ODS column (4.6×250 mm, Kyoto, Japan) and tertiary distilled water containing 0.1% formic acid (solvent A) and 100% acetonitrile (solvent B) as mobile phase. Measurements were made at 254 nm wavelength with a flow rate of 1 mL/min and 10 μL of sample for injection.

2.10 Statistical analysis

Experimental data of this study are expressed as mean ± standard deviation after three repeated experiments. Difference between groups was determined by one-way variance analysis and Duncan's multiple range test using SPSS (statistical package for the social sciences, ver. 25). Statistical significance was considered at $p < 0.05$.

Table 2. Analytical conditions of HPLC for ellagic acid analysis.

Parameters		Conditions	
Column		YMC ODS 5 μm 250 × 4.6 mm	
Flow rate		1 mL/min	
Injection volume		10 μL	
UV detection		254 nm	
Run time		40 min	
	Time (min)	**A (%)**	**B (%)**
Gradient condition	0	90	10
	5	90	10
	25	40	60
	30	20	80
	35	90	10
	40	90	10

3 Results and discussion

3.1 Determination of phenolic compounds

Phenolic compounds contained in fruits provide general quality (such as color and taste) and health functions (such as antioxidant) of fruits. They are important ingredients that can protect plants from infections by bacteria and viruses. They can also extend the storage period (Tosun et al., 2009). In this study, total polyphenol contents present in strawberry extracts were measured using tannic acid as a reference substance for quercetin (Table 3). Total polyphenol contents ranged from 21.79 to 118.85 mg TAE/100 g FW, with 'Josaenghongsim' cultivar showing the highest total polyphenol contents among all cultivars tested, followed by 'Okme' and 'Chodong', whereas 'Manhyang', 'Sinseolmae', and 'Johong' cultivars had the lowest total polyphenol contents. Significant (p<0.05) differences in total polyphenol content were found among strawberry cultivars. Scalzo et al. (2005) have reported that total polyphenol contents in six Italian strawberry cultivars ('Don', 'Idea', 'Camarosa', 'Onda', 'Patty', and 'Svev') are in the range of 1,093~2,128 mg GAE/L. Pineli et al. (2011) have also reported that total polyphenol contents in 'Osogrand' and 'Camino Real' are 2,234.62 mg GA/kg FW and 1,743.47 mg GA/kg FW, respectively. These results were higher than ours. However, Lim et al. (2016) have reported that total polyphenol contents in 'Sulhyang' and 'Janghee' are 229.18 and 39.11 mg GAE/100 g FW, respectively. Chaves et al. (2017) have shown that total polyphenol contents in seven strawberry cultivars are in the range of 1.6~2.54 mg TAE/g FW. These results were similar to ours.

In the present study, 'Josaenghongsim' had the highest total flavonoid contents at 599.56±9.82 mg QE/100 g FW, followed by 'Mihong' at 288.28±75.24 mg QE/100 g FW and 'Seolhyang' at 202.00±2.34 mg QE/100 g FW (Table 3). Significant ($p < 0.05$) differences in total flavonoid contents were found among strawberry cultivars. Lim et al. (2016) have reported that total flavonoid contents in 'Sulhyang' and 'Janghee' strawberrries

Table 3. Content of total polyphenols and flavonoids in 12 Korean strawberry cultivars.

Sample	Total polyphenol contents (mg TAE[1)]/100g FW[2)])	Flavonoid contents (mg QE[3)]/100 g FW)
Berrystar	36.83 ± 4.96^{ef}[4)5)]	17.57 ± 0.01^{e}
Chodong	61.01 ± 6.01^{c}	16.80 ± 0.10^{e}
Dahong	61.01 ± 17.1^{cd}	17.83 ± 0.11^{e}
Dummy	34.20 ± 5.25^{ef}	19.41 ± 0.10^{e}
Johong	23.72 ± 1.08^{f}	125.78 ± 5.39^{d}
Josaenghongsim	118.85 ± 1.97^{a}	599.56 ± 9.82^{a}
Manhyang	21.7 ± 0.64^{f}	17.38 ± 0.01^{e}
Mihong	56.35 ± 15.11^{cde}	288.28 ± 75.24^{b}
Okme	74.46 ± 2.33^{b}	18.29 ± 0.11^{e}
Seolhyang	39.03 ± 0.47^{ef}	202.00 ± 2.34^{c}
Sinseolmae	23.56 ± 0.64^{f}	123.38 ± 0.67^{d}
Suhong	42.75 ± 3.81^{def}	220.53 ± 18.97^{c}

[1)] TAE: tannic acid equivalent; [2)] FW: fresh weight; [3)] QE: quercetin equivalent; [4)] Values are mean ± standard deviation (n = 3); [5)] Values with different letters within the same row are significantly different by Duncan's multiple range test at p < 0.05.

are 52.32 and 39.11 mg CE/100 g, lower than ours. Bae et al. (2019) have reported that total flavonoid content in 'Sulhyang' is 546.12 mg CHE/100 g sample, same as 'Josaenghongsim'. Kim et al. (2018) have determined antioxidant activities of various berries and reported that total flavonoid contents in raspberry and wild raspberry are 731.08 and 362.41 mg QE/100 g FW, respectively, higher than our results. Lee et al. (2014) have shown that total flavonoid contents in wild raspberry were in the range of 10.91~14.90 mg QE/100 g FW, similar to our results. These findings indicate that total polyphenol and flavonoid contents are different according to the variety.

3.2 Antioxidant activity

Antioxidant activity was measured based on DPPH and ABTS free radical scavenging activities widely used to find antioxidants from various natural materials (Lee et al., 2014). DPPH free radical scavenging activity consists in the reduction of DPPH radical by substances present in the extraxt, leading to the formation of reduced DPPH-H, which changes in color from deep purple to loss of color (Chaves et al., 2017). DPPH free radial scavenging activities were measured using DPPH and expressed as trolox equivalent (μmol TEAC/100 g FW). DPPH free radial scavenging activities of 12 cultivars of strawberries used in this experiment were in the range of 1,381.7±12.7 to 1,095.3±37.1 μmol TEAC/100 g FW, with 'Josaenghongsim', 'Johong', 'Sinseolmae', 'Seolhyang', and 'Berrystar' having the highest values (Table 4). Significant ($p < 0.05$) differences in DPPH free radial scavenging activity were found among strawberry cultivars. Nowicka et al. (2019) have determined DPPH free radical scavenging activities of 90 strawberries grown in Poland and found that the average activity is 751.57 μmol TEAC/100g. Pineli et al. (2011) have reported that DPPH free radical scavenging activity of 'Osogrand' and 'Camino Real' are 12.83 μmol TEAC/g and 10.10 μmol TEAC/g, respectively, somewhat lower than our results.

Another effective method to measure radical scavenging activity is the ABTS radical cation decolorization assay. This method can be used to obtain results in a short time. It can be applied to both hydrophobic and hydrophilic sample (Kim et al., 2019). ABTS free radial scavenging activities of 12 strawberry extracts were compared. Results are shown in Table 4. ABTS free radical scavenging activities of these extracts were in the range of 9,179.0±1.0 to 4,262±15.3 μmol TEAC/100 g FW, with 'Seolhyang' showing the highest activity, followed by Suhong, Manhyang, and Johong. There were significant ($p < 0.05$) differences in ABTS free radical scavenging activity among strawberry cultivars. Kim et al. (2016) have reported that ABTS free radical scavenging activities of raspberry and blackberry are 72 μmol TE/g and 20 μmol TE/g, respectively, lower than ours. Lee et al. (2015) have reported that ABTS free radical scavenging activities of black raspberry, blueberry, and raspberry are 2.99 mmol TEAC/100 g FW, 1.47 mmol TEAC/100 g FW, and 0.77 mmol TEAC/100 g FW, respectively. These results suggest that antioxidants present in these strawberry extracts could eliminate highly reactive free radicals to prevent oxidation of important biomolecules such as unsaturated fatty acids, genes, and proteins (Fan et al., 2011).

Table 4. Antioxidant activities of 12 Korean strawberry cultivars.

Sample	DPPH (μmol TEAC1)/100 g FW2))	ABTS (μmol TEAC/100 g FW)
Berrystar	1316.0 ± 30.3[a3)4)]	4715.7 ± 5.8[g]
Chodong	1207.5 ± 18.7[bc]	4732.3 ± 20.8[g]
Dahong	1178.2 ± 56.6[cd]	4472.7 ± 5.5[i]
Dummy	1145.1 ± 23.2[d]	4262.3 ± 15.3[h]
Johong	1364.0 ± 20.6[a]	5209.0 ± 0.1[c]
Josaenghongsim	1381.7 ± 12.7[a]	5052.7 ± 11.8[d]
Manhyang	1243.7 ± 4.8[b]	5239.0 ± 10.0[b]
Mihong	1095.3 ± 37.1[d]	4942.3 ± 37.9[f]
Okme	1126.8 ± 13.4[d]	4719.1 ± 0.1[g]
Seolhyang	1328.2 ± 19.4[a]	9179.0 ± 1.0[a]
Sinseolmae	1346.6 ± 3.7[a]	5019.0 ± 10.0[e]
Suhong	1114.6 ± 45.2[d]	5252.4 ± 5.7[b]

[1)] TEAC: Trolox equivalent antioxidant capacity. [2)] FW: fresh weight. [3)] Values are mean±standard deviation (n = 3). [4)] Values with different letters within the same row are significantly different by Duncan's multiple range test at $p < 0.05$.

3.4 Method validation

- ***Analysis conditions*** HPLC analyses of ellagic acid contents in strawberries were performed using an Agilent HPLC system (Agilent Technology, Santa Clara, CA, USA). The mobile phase consisted of solvent A (tertiary distilled water containing 0.1% formic acid) and solvent B (acetonitrile). The flow rate for the mobile phase was set at 1 mL/min. UV detection was performed at wavelength of 254 nm;
- ***Linearity, limit of detection (LOD), and limit of quantitation (LOQ)*** Standard calibration curves for ellagic acid standard by HPLC were obtained in the range of 10-100 μg/mL using five different concentrations (n = 3). Ellagic acid standard was used to calculate a linear regression equation with a correlation coefficient (R^2) of 0.9999. LOD was found to be 2.35 μg/mL for ellagic acid standard and LOQ of the standard were found to be 7.13 μg/mL. Thus, detection and quantification of ellagic acid are possible even if its amount is small (Table 5). Results of this study can be used to verify quantitative and detection limits of marker component analysis for the standardization of strawberry extract;
- ***Accuracy, precision, and recovery*** The precision of the optimized method using standard compounds was verified at three concentrations (10, 50 and 100 μg/mL). Relative standard deviations (RSDs) were 0.20-5.13% and 0.82-5.31% by HPLC for inter-day and intra-day variations (n=3), respectively (Table 6). Regarding accuracy, the average recovery rate of ellagic acid was 99.0% to 100.1%. RSD value was in the range of 0.8% to 5.3% (Table 7). Thus, the analytic method was confirmed to have excellent precision and accuracy.
- ***Stability and Contents*** Chemical stability of ellagic acid, a marker compound, was determined based on the change in content of ellagic acid at a concentration of 100 ug/mL after it was left at room temperature for 24 hours (Table 8). Results revealed that the average recovery rate of ellagic

acid was 100.4%. Its content change rate was 1.50% after 24 hours of storage at room temperature. Thus, ellagic acid was confirmed to have chemical stability for 24 hours at room temperature. Also, the ellagic acid content in all varieties of strawberries was found to be in the range of 12.8±0.2 -16.6±0.3, respectivlely (Table 9).

Table 5. Calibration curve, linearity, limit of detection (LOD), limit of quantitation (LOQ) of ellagic acid.

Concentration (μg/mL)	Regression equation	R2	LOD (μg/mL)	LOQ (μg/mL)
10-100	y=133.22x-320.63	0.9999	2.35217	7.12778

Table 6. Precision of ellagic acid

Concentration (μg/mL)	Inter-day		Intra-day	
	Mean ± SD[1)] (μg/mL)	RSD[2)] (%)	Mean ± SD (μg/mL)	RSD (%)
10	9.83 ± 0.50	5.13	9.82 ± 0.51	5.31
50	47.91 ± 0.31	0.66	47.55 ± 0.42	0.83
100	96.56 ± 0.19	0.20	95.04 ± 0.79	0.82

[1)] Value are mean ± standard deviation (n = 3); [2)] Relative standard deviation.

Table 7. Accuracy of ellagic acid.

Spiked amount (μg/mL)	measured amount (μg/mL)	RSD (%)	Recovery (%)	Recovery average
10	9.53 ± 0.50	5.3	93.8	100.0
			103.0	
			103.0	
50	47.20 ± 0.41	0.9	98.0	99.0
			99.2	
			99.7	
100	95.50 ± 0.79	0.8	99.7	100.1
			101.1	
			96.6	

Recovery (%) = (amount found-original amount)/amount spiked × 100%.

Table 8. Analytical result of ellagic acid stability test

Compound	0 h	24 h	Recovery (%)
Ellagic acid	95.04	96.41	100.4

Table 9. Contents of ellagic acid in 12 Korean strawberry cultivars

Sample	Ellagic acid Content (μg/mL)
Berrystar	15.1 ± 0.1[c1)2)]
Chodong	15.1 ± 0.2[c]
Dahong	16.5 ± 0.2[a]
Dummy	14.2 ± 0.2[e]
Johong	16.1 ± 0.0[b]
Josaenghongsim	12.8 ± 0.2[f]
Manhyang	14.2 ± 0.1[e]
Mihong	14.8 ± 0.1[d]
Okme	16.6 ± 0.3[a]
Seolhyang	14.5 ± 0.1[e]
Sinseolmae	14.5 ± 0.1[e]
Suhong	16.6 ± 0.1[a]

[1)] Values are mean ± standard deviation (n = 3); [2)] Values with different letters within the same row are significantly different by Duncan's multiple range test at $p < 0.05$.

5 Conclusions

In this study, strawberry fruit extracts from 12 Korean cultivars showed high antioxidant activities. Such high activities are thought to be due to their high contents of total polyphenol and flavonoid that can inhibit oxidation of free radicals. Results of this study could be used as basic data for establishing a standard for determining the development potential and performing content analysis of strawberry extracts based on quantitative analysis using ellagic acid as a marker compound for the validation and standardization of natural antioxidants.

Conflict of interest

All of the authors agree with submission to FSB and we have no conflict of interest to declare.

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