Tropical Journal of Pharmaceutical Research May 2025; 24 (5): 575-582
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
https://dx.doi.org/10.4314/tjpr.v24i5.1

Original Research Article

Antioxidant activity and correlation analysis of phenolic compounds in novel varieties of *Lactuca sativa* L.

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Sent for review: 22 February 2025 *Revised accepted: 20 May 2025*

Abstract

Purpose: *To determine the antioxidant efficacy of three lettuce (Lactuca sativa L. Asteraceae) varieties: Cheongchima JLS201206-12 (a traditional variety), and two newly bred varieties, Cheongharang Ⅰ and Cheongharang Ⅱ.*

Method: *Extraction was done with 100 mL of water and ethanol at various concentrations (20, 40, 60, 80, and 100 %) using a reflux extractor at 100 °C for 60 min. Total polyphenol content (TPC) and total flavonoid contents (TFC) were determined. Antioxidant activity of the lettuce extracts was evaluated in vitro using DPPH, ABTS, PMA, and FRAP assays with ascorbic acid and trolox as positive controls at 5 and 800 µg/mL, respectively.*

Results: *At 60 % ethanol, Cheongchima I also showed significantly higher phenolic (14.33 ± 0.00 mg GAE/g) and flavonoid (16.81 ± 0.00 mg CE/g) contents compared to Cheongchima, Cheongharang Ⅱ, and the standard controls ($p < 0.05$). Furthermore, Cheongharang Ⅰ exhibited significantly higher antioxidant activity compared to the traditional variety Cheongchima in DPPH, ABTS, PMA, and FRAP assays ($p < 0.05$).*

Conclusion: *Aqueous and ethanol extracts of Cheongharang Ⅰ demonstrate significantly higher antioxidant effects than other varieties, suggesting its strong potential as a natural source of antioxidants used in functional foods.*

Keywords: *Lactuca sativa L. Antioxidants, FRAP assay, DPPH assay, Correlation analysis*

Tropical Journal of Pharmaceutical Research is indexed by Scopus, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

INTRODUCTION

Oxidative stress, primarily driven by reactive oxygen species (ROS), is a major contributor to the development and progression of numerous diseases in modern times. Reactive oxygen species (ROS), which include free radicals and other reactive intermediates, are generated as by-products of cellular metabolism and play crucial roles in various physiological processes, such as gene transcription, signal transduction, and immune responses [1]. However, an imbalance between ROS production and the body's antioxidant defense leads to excessive ROS levels [2]. This imbalance causes significant oxidative damage to critical biomolecules, including lipids, proteins, and DNA, ultimately leading to cellular dysfunction and disease [3]. Free radicals, characterized by their unpaired electrons, are particularly reactive, inducing oxidative stress, which may result in cellular damage and death [4]. Their role in reducing oxidative stress is vital for maintaining overall human health and preventing a wide range of

diseases associated with oxidative damage [5]. Increasing awareness of the protective effects of antioxidants has led to their widespread use in food and medicine. Furthermore, a growing interest in natural sources of antioxidants due to their perceived safety and efficacy compared to synthetic alternatives [6]. Natural antioxidants, derived from plants and other dietary sources, have gained prominence due to concerns about toxicity and side effects of synthetic antioxidants. Research into natural antioxidants has expanded to include various medicinal and agricultural substances known for their minimal side effects and potent antioxidant properties [7]. Among such substances, lettuce (*Lactuca sativa* L.), a member of the Asteraceae family, stands out due to its diverse varieties and potential health benefits. *Lactuca sativa L.* has elongated, oval-shaped leaves, available in both green and red varieties. It is primarily cultivated in mild climates and grown throughout the year, except during winter. Lettuce contains several bioactive compounds, including sesquiterpene lactones (such as lactucin, deoxylactucin, and lactucopicrin), which are known for their beneficial activities [8]. Additionally, lettuce is rich in secondary metabolites such as polyphenols, flavonoids, vitamin C, carotenoids, and tocopherols, all of which contribute to its antioxidant properties [9]. Also, caffeic acid and chlorogenic acid, which are present in high concentrations in lettuce, offer a range of pharmacological benefits including antibacterial, antioxidant, and anti-inflammatory effects [10]. This study investigated the antioxidant efficacy of two newly developed lettuce varieties, Cheongharang Ⅰ and Cheongharang Ⅱ, which have been bred through pure line selection from the traditional Cheongchima lettuce (JLS201206-12) by the Jeonnam Agricultural Research and Extension Services. By assessing the antioxidant properties of these new varieties, the study seeks to validate their potential as natural sources of antioxidants and contribute to the development of functional foods and products.

EXPERIMENTAL

Materials

The three types of lettuce used in this study (Cheongchima, Cheongharang Ⅰ, Cheongharang Ⅱ) were provided by Jeonnam Agricultural Research and Extension Services. All analytical solvents used, including distilled water and ethanol, were of HPLC grade. Additionally, a reflux extractor (MS-DM, MISUNG Co., Seoul, Korea) was used for the extraction process.

Extraction

For the preparation of extracts, the lettuce varieties were ground into a powder using a grinder (NFM-3561SN, NUC Co., Daegu, Korea). Each 10 g lettuce sample was extracted with 100 mL distilled water (DW), 20, 40, 60, 80, and 100 % ethanol using a reflux extractor (MS-DM, MISUNG Co., Seoul, Korea) at 100 °C for 60 min. The extracts were filtered using a vacuum filter with Whatman 1 filter papers (Cytiva, 100 circles, diameter: 90 mm), and the filtrate was further filtered through a syringe filter (0.45 μm) and stored for further use.

Antioxidant contents

Total polyphenol contents (TPC)

Total polyphenol content was measured using the Folin-Ciocalteu colorimetric assay [11]. Each 500 μL of aqueous and ethanol extracts from the lettuce varieties was mixed with 500 μL of Folin reagent and reacted at room temperature for 3 min. Thereafter, 500 μL of 10 % Na_2CO_3 (sodium carbonate) solution was added and mixed, followed by a 1 h reaction in a dark room. The absorbance was measured at 760 nm using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). The amount of GAE (gallic acid equivalent) per gram of extract was calculated based on the standard calibration curve of gallic acid ($y = 0.0548x + 0.1432$, $R^2 = 0.9992$).

Total flavonoid contents (TFC)

Total flavonoid content was measured following the method of Saleh and Hameed [12]. Each 200 μL of aqueous and ethanol extracts was mixed with 800 μL of 80 % ethanol. Thereafter, 60 μL of 5 % $NaNO_2$ (sodium nitric oxide) was added and left to stand at room temperature for 5 min, and 60 μL of 10 % $AlCl_3$ (Aluminium chloride) was added, mixed and left to react for 5 min at room temperature. Finally, 400 μL of 1M NaOH (sodium hydroxide) solution was added, and the absorbance was measured at 510 nm using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). Catechin was used as the standard solution at 25 and 800 μg/mL, and the amount of CE (Catechin equivalent) per gram of extract was calculated based on the standard calibration curve of catechin ($y = 0.0068x – 0.0799$, $R^2 = 0.9991$).

Ferric-reducing antioxidant power (FRAP)

Ferric reducing antioxidant power was evaluated following the method of Benzie and Strain [13]. A working solution was prepared by mixing 300

mM pH 3.6 acetate buffer, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine), and 20 mM $FeCl_3 \cdot 6H_2O$ in a ratio of 10:1:1 and incubating at 37 °C for 10 min. Each 35 µL lettuce extract was mixed with 1050 µL of the working solution and reacted at 37 °C for 30 min. Absorbance was measured at 595 nm using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). Ascorbic acid was used as the standard solution at 25 and 800 µg/mL, and the amount of AAE (ascorbic acid equivalent) per gram of extract was determined based on the standard calibration curve of Trolox ($y = 0.0004x + 0.0568$, $R^2 = 0.999$).

Phosphomolybdenum antioxidant activity (PMA)

The phosphomolybdenum antioxidant assay was done following the method of Prieto and Pineda [14]. Each 100 µL of both aqueous and ethanol extract was placed in an Eppendorf tube (EP tube), and 1 mL working solution was prepared by mixing 4 mM ammonium molybdate, 28 mM sodium phosphate, and 0.6 M sulfuric acid in a ratio of 1:1:1. The mixture was then reacted in a water bath at 90 °C for 90 min. After cooling to room temperature, the absorbance was measured using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). The amount of AAE (ascorbic acid equivalent) per gram of extract was determined based on the standard calibration curve prepared with L-ascorbic acid ($y = -0.0011x – 1.3646$, $R^2 = 0.9997$).

1,1-diphenyl-2-picrylhydrazyl assay (DPPH assay)

The DPPH radical scavenging activity was evaluated following the method of Yen and Chen [15]. A DPPH working solution was prepared by mixing 0.2 mM DPPH reagent with 50 mL of methanol (MeOH). Each 50 µL of lettuce ethanol and aqueous extracts was mixed with 950 µL of the DPPH working solution and reacted in a 25 °C incubator for 30 min. Thereafter, absorbance was measured at 515 nm using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). The DPPH radical scavenging activity was expressed as TE (Trolox equivalent) per gram of extract, calculated based on the standard calibration curve of Trolox ($Y = -0.0045X + 1.3646$, $R^2 = 0.9995$).

2,2'-azino-bis-3-ethylbenzthiazoling-6-sulphonic acid assay (ABTS assay)

The ABTS radical scavenging activity was evaluated following the method of Pellegrini *et al* [16]. A radical solution was formed by mixing 7.4 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid; ABTS) with 2.4 mM potassium persulfate and allowing it to stand in the dark at room temperature for 24 h. The ABTS solution was then diluted with distilled water to achieve an absorbance of 0.7 (± 0.1) at 734 nm, forming the ABTS working solution. Each 150 µL of aqueous and ethanol extract was mixed with 1,350 µL of ABTS working solution and left to stand in the dark for 5 min, and absorbance was measured at 734 nm using a spectrophotometer (Neogen, Optizen 2120 UV, Sejong, Korea). The ABTS radical scavenging activity was expressed as TE (Trolox equivalent) per gram of extract, calculated based on the standard calibration curve of Trolox ($y = -0.0116x + 0.5841$, $R^2 = 1.0000$).

Statistical analysis

Data was analyzed using Statistical Package for the Social Sciences (SPSS version 22.0, IBM, Armonk, NY, USA). Determinations were done in triplicate and data were expressed as the mean ± standard deviation (SD). Comparison was done using one-way analysis of variance (ANOVA). If significance was found, Tukey's test was used for post-hoc analysis, and $p < 0.05$ was considered statistically significant. Correlations were indicated using Pearson's correlation coefficient.

RESULTS

Total polyphenol contents (TPC)

Total phenolic content of different lettuce cultivars was measured and expressed as the equivalent amount of gallic acid. The 60 % ethanol (EtOH) extract of Cheongharang Ⅱ showed the highest total phenolic content at 14.85 ± 0.03 mg GAE/g (Table 1; $p < 0.05$).

Total flavonoid contents (TFC)

Total flavonoid content was highest in the 60 % EtOH extract of Cheongharang Ⅰ (16.81 ± 0.00 mg CE/g; $p < 0.05$). Furthermore, 60 % EtOH extract of Cheongharang I also exhibited significantly higher flavonoid content (12.08 ± 0.00 mg CE/g; $p < 0.05$). Therefore, Cheongharang Ⅰ extracts demonstrated the highest flavonoid content (Table 1).

Ferric-reducing antioxidant power (FRAP)

The FRAP assay revealed that the 60 % EtOH extract of Cheongharang Ⅰ exhibited significantly higher FRAP antioxidant activity ($p < 0.05$). Reducing the power of Cheongharang Ⅰ was the highest on average (Table 2).

Table 1: Total polyphenol contents (TPC) and total flavonoid contents (TFC) of *Lactuca sativa L.* extracts

Extract	TPC (mg GAE/g)			TFC (mg CE/g)		
	Chengchima	*Chengharang Ⅰ*	*Chengharang Ⅱ*	*Chengchima*	*Chengharang Ⅰ*	*Chengharang Ⅱ*
Aqueous	6.04±0.00^{d,3}	9.96±0.01^{b}	3.45±0.01^{b}	10.92±0.00^{b}	15.61±0.00^{b}	8.67±0.00^{a}
20% EtOH	5.87±0.00^{d}	11.95±0.01^{c}	7.06±0.01^{d}	12.71±0.00^{d}	16.51±0.00^{e}	8.87±0.00^{b}
40% EtOH	5.45±0.01^{c}	13.36±0.00^{c,d}	4.79±0.00^{c}	13.23±0.00^{e}	16.12±0.00^{d}	9.55±0.00^{d}
60% EtOH	6.46±0.01^{e}	14.33±0.00^{d}	14.85±0.03^{e}	12.62±0.00^{d}	16.81±0.00^{f}	12.08±0.00^{f}
80% EtOH	4.42±0.00^{b}	13.06±0.02^{c,d}	4.25±0.00^{c}	11.17±0.00^{c}	15.92±0.00^{c}	10.41±0.00^{e}
100% EtOH	3.14±0.01^{a}	3.97±0.07^{a}	2.70±0.00^{a}	7.57±0.00^{a}	9.11±0.00^{a}	9.28±0.00^{c}

GAE: Gallic acid equivalent. CE: Catechin equivalent. Values were expressed as mean ± SD. Means with different letters (a - f) in the same column are significantly different at $p < 0.05$

Table 2: Ferric reducing antioxidant power (FRAP) and phosphomolybdenum complex assay (PMA) of *Lactuca sativa L.* extracts

Extract	FRAP (mg TE/g)			PMA (mg AAE/g)		
	Chengchima	*Chengharang Ⅰ*	*Chengharang Ⅱ*	*Chengchima*	*Chengharang Ⅰ*	*Chengharang Ⅱ*
Aqueous	87.93±0.00^{e,3}	64.00±0.00^{b}	11.26±0.00^{b}	1.81±0.00^{b}	4.35±0.00^{e}	1.80±0.00^{b}
20% EtOH	67.45±0.00^{b}	123.76±0.00^{d}	44.71±0.00^{d}	1.50±0.00^{a}	3.74±0.00^{c}	2.19±0.00^{c}
40% EtOH	81.02±0.00^{c}	126.38±0.00^{e}	61.50±0.00^{f}	2.53±0.00^{e}	3.46±0.00^{a}	1.76±0.00^{a}
60% EtOH	82.33±0.00^{d}	129.12±0.00^{f}	5.79±0.00^{a}	2.51±0.00^{d}	4.06±0.00^{d}	4.41±0.00^{f}
80% EtOH	80.43±0.00^{c}	117.21±0.00^{c}	49.60±0.00^{e}	3.08±0.00^{f}	4.48±0.00^{f}	3.75±0.00^{d}
100% EtOH	16.86±0.00^{a}	32.69±0.00^{a}	20.79±0.00^{c}	2.18±0.00^{c}	3.55±0.00^{b}	4.19±0.00^{e}

TE: Trolox equivalent, AAE: Ascorbic acid equivalent. Values are expressed as mean ± SD. Means with different letters (a - f) in the same column are significantly different at $p < 0.05$

Phosphomolybdenum antioxidant activity (PMA)

The results revealed that the 80 % EtOH extract of Cheongharang Ⅰ exhibited significantly higher PMA activity ($p < 0.05$; Table 2).

DPPH and ABTS assay

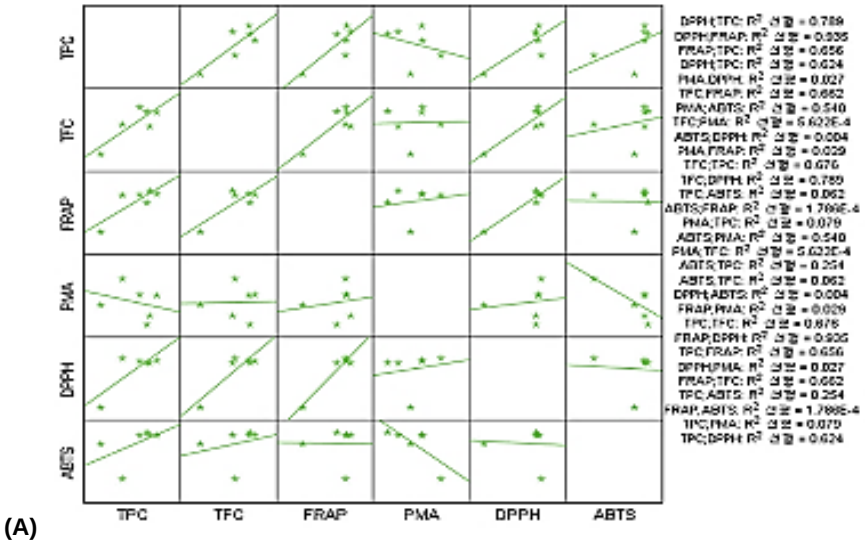
The assay results revealed that Cheongharang Ⅰ at 80 % EtOH extract exhibited significantly higher DPPH scavenging activity ($p < 0.05$). These results suggest that Cheongharang Ⅰ extract has a slightly higher capacity to neutralize DPPH radicals compared to other extracts. The highest ABTS scavenging activity was observed in the Cheongharang Ⅰ at 40 % EtOH extract, the aqueous extract of Cheongharang Ⅱ, and Cheongchima at 20 % EtOH extract, all of which showed the same scavenging activity of 0.50 ± 0.00 mg TE/g (Table 3).

Correlation analysis

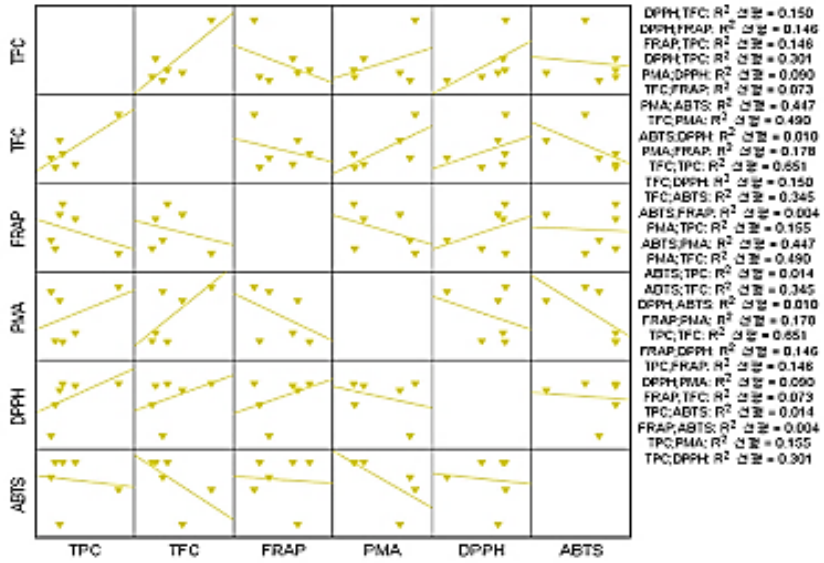
The correlation analysis aimed to determine the relationships between TPC, TFC, and antioxidant activities (FRAP, PMA, DPPH, and ABTS; Figure 1; Tables 4 - 6). For Cheongchima, a strong positive correlation was observed between TPC and TFC (R = 0.822, $p < 0.05$), TFC and FRAP (R = 0.814, $p < 0.05$), TFC and DPPH (R = 0.888, $p < 0.05$), but a minimal correlation with ABTS scavenging activity, suggesting that flavonoids in Cheongchima were more effective at scavenging DPPH radicals compared to ABTS radicals.

Table 3: DPPH radical scavenging activity and ABTS radical scavenging activity of *Lactuca sativa L.* extracts

Extract	DPPH (mg TE/g)			ABTS (mg TE/g)		
	Chengchima	*Chengharang Ⅰ*	*Chengharang Ⅱ*	*Chengchima*	*Chengharang Ⅰ*	*Chengharang Ⅱ*
Aqueous	4.60±0.01^{e,2}	4.59±0.00^{d}	3.00±0.03^{d}	0.49±0.01^{b}	0.49±0.00^{c}	0.50±0.00^{a}
20% EtOH	4.62±0.01^{d}	4.92±0.01^{c}	4.84±0.00^{b}	0.50±0.01^{a}	0.47±0.00^{d}	0.50±0.00^{b}
40% EtOH	4.79±0.00^{c}	4.98±0.01^{b,c}	5.01±0.01^{a,b}	0.49±0.00^{c}	0.50±0.00^{a}	0.50±0.00^{c}
60% EtOH	4.87±0.00^{b}	5.01±0.00^{b}	5.06±0.00^{a}	0.49±0.00^{d}	0.50±0.00^{b}	0.28±0.00^{e}
80% EtOH	5.09±0.00^{a}	5.29±0.00^{a}	4.41±0.00^{c}	0.34±0.00^{e}	0.30±0.00^{f}	0.00±0.00^{f}
100% EtOH	0.00±0.00^{f}	1.34±0.00^{e}	0.00±0.00^{e}	0.46±0.00^{f}	0.37±0.00^{e}	0.38±0.00^{d}



(A)



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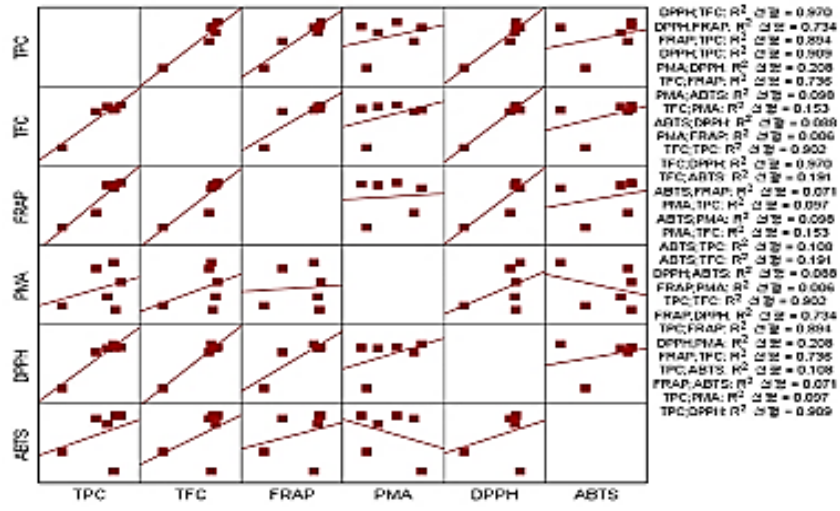

(C)

Figure 1: Correlation between antioxidant contents (TPC, TFC) and transitional metal ion reducing power (PMA, FRAP) and radical scavenging activity (DPPH, ABTS) of *Lactuca sativa L.* extracts. (A) Chengchima, (B) Changharang Ⅰ, (C) Chenghara

For Cheongharang Ⅰ, a strong positive correlation was found between TPC and TFC (R = 0.950, $p < 0.01$), TPC and FRAP (R = 0.945, $p < 0.01$), and TPC and DPPH (R = 0.953, $p < 0.01$). For Cheongharang Ⅱ, TPC had a positive correlation with DPPH (R = 0.548), but a negative correlation with ABTS (R = -0.117). This indicates that, in Cheongharang Ⅱ, phenolic compounds were more effective at scavenging DPPH radicals than ABTS radicals, though lower.

DISCUSSION

Lettuce is a self-pollinated annual plant with a deep taproot and large horizontal lateral roots for water and nutrient absorption. It is diverse in color, shape, surface, margin, texture of leaves, and rich in flavonoids [17]. Previous studies revealed that total flavonoid content in lettuce was significantly higher compared to spinach and cabbage [18]. Phytoconstituents in lettuce, such as polyphenols and carotenoids, are also susceptible to different growth conditions and show variations among cultivars [19]. Therefore, this study evaluated TPC, TFC, and antioxidant activity of three *Lactuca sativa L*. cultivars using multiple *in vitro* assays (DPPH, ABTS, FRAP, and PMA). The results revealed that 60 % ethanol extract of Chengharang Ⅰ exhibited significantly higher TPC and TFC compared to Chengchima and Chengharang Ⅱ ($p < 0.05$).

Various studies have used different concentrations of ethanol for extraction, resulting in various degrees of antioxidant activity. For example, 70 % ethanol extract of red lettuce showed higher TPC [20], optimized TPC level was obtained at 70 % ethanol [21].

Table 4: Correlation between antioxidant contents and antioxidant activity of *Lactuca sativa L.* (Chengchima) extracts

Factor	TPC	TFC	FRAP	PMA	DPPH	ABTS
TPC	1	0.822*	0.810	-0.281	0.790	0.504
TFC		1	0.814*	0.024	0.888*	0.248
FRAP			1	0.170	0.967**	-0.013
PMA				1	0.163	-0.735
DPPH					1	-0.064
ABTS						1

TPC: Total polyphenol content, TFC: Total flavonoid content, FRAP: Ferric-reducing antioxidant power, PMA: Phosphomolybdenum complex assay, DPPH: DPPH radical scavenging activity, ABTS: ABTS radical scavenging activity. Correlation was significantly different (*$p < 0.05$ and **$p < 0.01$)

Table 5: Correlation between antioxidant contents and antioxidant activity of *Lactuca sativa L.* (Chengharang Ⅰ) extracts

Factor	TPC	TFC	FRAP	PMA	DPPH	ABTS
TPC	1	0.950**	0.945**	0.311	0.953**	0.328
TFC		1	0.858*	0.391	0.985**	0.437
FRAP			1	0.080	0.857*	0.267
PMA				1	0.456	-0.312
DPPH					1	0.297
ABTS						1

TPC: Total polyphenol content, TFC: Total flavonoid content, FRAP: Ferric-reducing antioxidant power, PMA: Phosphomolybdenum complex assay, DPPH: DPPH radical scavenging activity, ABTS: ABTS radical scavenging activity. Correlation was significantly different at *$p < 0.05$ and **$p < 0.01$.

Table 6: Correlation between antioxidant contents and antioxidant activity of *Lactuca sativa L.* (Chengharang Ⅱ) extracts

Factor	TPC	TFC	FRAP	PMA	DPPH	ABTS
TPC	1	0.807	-0.382	0.394	0.548	-0.117
TFC		1	-0.270	0.700	0.388	-0.587
FRAP			1	-0.422	0.382	-0.065
PMA				1	-0.301	-0.668
DPPH					1	-0.099
ABTS						1

This showed that ethanol concentration significantly affects the total phenolic and flavonoid content of *Lactuca sativa* (lettuce) extracts. The TPC determined for the studied samples was different from TPC values reported in previous studies [22,23]. This may be due to environmental effects such as irrigation, soil composition, and other phenolic characteristics on the growth of lettuces [19]. The study further revealed that Cheongharang Ⅰ consistently demonstrated the highest antioxidant capacity, at 60 % ethanol extract, indicating a strong correlation between phenolic composition and antioxidant efficacy. Each assay provides insights into different antioxidant mechanisms.

The DPPH and ABTS assays assess free radical scavenging, where Cheongharang Ⅰ showed superior activity, reflecting its rich flavonoid content. The FRAP assay, which measures reducing power, was highly correlated with TPC and TFC, especially in Cheongharang Ⅰ, suggesting strong electron-donating ability. The PMA assay confirmed broad-spectrum antioxidant capacity, particularly in 80 % ethanol extracts.

Therefore, Cheongharang Ⅰ holds promise as a functional food material and potential natural antioxidant source. Its consistent performance across assays supports its application in health products targeting oxidative stress.

CONCLUSION

This study has demonstrated that aqueous and ethanol extracts of various lettuce cultivars show different antioxidant activities, closely linked to their phenolic and flavonoid content. Cheongharang Ⅰ exhibited the highest total polyphenol content, total flavonoid content, which strongly correlated with its antioxidant activity, highlighting its potential as a natural antioxidant source. Cheongharang Ⅰ emerges as a strong candidate for pharmaceutical applications, particularly for products targeting oxidative stress-related conditions.

DECLARATIONS

Acknowledgement/Funding

The authors thank the technicians who participated in this study.

Ethical approval

None required.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Conflict of interest

No conflict of interest associated with this work

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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