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Antioxidant Potential, Phenolic Content, and Nitrate/Nitrite Content in Various Lettuce Varieties

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ABSTRACT

Ten lettuce varieties are analyzed for antioxidant activity, total phenolic, nitrate, and nitrite contents, and the effects of harvest maturity and cold storage in selected varieties are determined. Antioxidant activity and total phenolic content (TPC) are the highest in Red Oak, Red Coral, Red Rapids, and Grand Rapids; intermediate in Green Oak, Green Cos, and Frillice Iceberg; and the lowest in Butterhead, Mini Green Cos, and Head lettuce. Nitrate content is the highest in Red Coral, while the other 9 varieties have comparably lower contents. Nitrite content is inadequate and does not differ with variety. Harvest maturity of 45 days after transplanting (DAT) produced the highest antioxidant activity and TPC, much higher in Red Bowl and Red Butterhead varieties than in Mini Green Cos, Butterhead, Frillice Iceberg, and Green Big Bowl varieties. Storage at 8°C for 21 days has no remarkable effects on antioxidant activity, TPC, nitrate, and nitrite contents. Stored Red Bowl lettuce has higher antioxidant activity and TPC than Butter-head and Green Big Bowl varieties. Nitrate content decreases at the end of storage, while nitrite content is below 1 mg/kg FW during the entire storage period, regardless of variety.

INTRODUCTION

Lettuce (*Lactuca sativa* L.) varieties are diverse in size, shape, color, texture, and flavor, and those cultivated worldwide are categorized into Crisphead, Butterhead, Cos, and Loose-leaf (Assefa et al., 2021). Thailand grows more types of lettuce, including Green Oak, Red Oak, Frillice Iceberg, Iceberg, Butterhead, Cos, Red Batavia, Rocket Salad, Wild Rocket, Green Coral, Red Coral, Red Rapids, and Grand Rapids. Lettuce is grown in soil or soilless culture, and better agricultural practices for crop management are available to produce high-quality crop yields to serve domestic and export markets (Talubnak et al., 2017). As with other vegetables, lettuce is rich in vitamins, minerals, fiber, and phytochemicals responsible for organoleptic and biological properties (Deng et al., 2013). Lettuce contains many bioactive compounds that confer antioxidant activity associated with lowered risk of human chronic diseases, such as cancer, heart attack, diabetes, obesity, and micronutrient deficiency disorders.

Antioxidants are compounds inhibiting oxidative chemical reactions that could otherwise generate free radicals and chain reactions damaging the cells of living organisms (Sen & Chakraborty, 2011). Even at low concentrations, antioxidants can considerably impede toxic oxidative processes that lead to aging and human diseases. Antioxidant substances act as free radical scavenging molecules (Gan & Azrina, 2016). In particular, phenolic compounds are one of the most important natural antioxidants with significant

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bioactivities that protect cells against the oxidative damage caused by free radicals (Yosefi et al., 2010; Veljković et al., 2019; Adegbaju et al., 2020). Phenolic compounds have anticarcinogenic, antimicrobial, and anti-inflammatory properties (Flávia et al., 2015). Many reports revealed that lettuce and other vegetables have high concentrations of phenolic compounds (Deng et al., 2013).

Nitrates and nitrites induce free radical formation (Hmelak Gorenjak & Cencič, 2013). Nitrate and nitrite are inorganic compounds in soil, water, and food, especially plant foods and vegetables. Nitrate levels in vegetables vary greatly and can range from 10-10,000 mg/kg, while the levels of nitrites are low (Reinik et al., 2008). Nitrate does not directly affect human health but has a potentially hazardous risk when reduced to the nitrite form, resulting in carcinogenic N-nitrosamine production. Daily vegetable intake can have up to 90% nitrates, 5-20% of which are reduced to nitrite with the action of anaerobic bacteria in the oral cavity and gastrointestinal tract (Reinik et al., 2008; Hmelak Gorenjak & Cencič, 2013; Veljković et al., 2019). However, vegetables contain protective substances against the damaging effects of nitrite, such as ascorbate, vitamin E, and phenolic compounds (Lidder & Webb, 2013). Nitrate content can accumulate differently in vegetables depending on the cultivar, plant production process, duration of the growth period, harvesting time, and nitrogen sources.

Many factors contribute to the nutrient content of vegetables, including genetics, growth conditions, production practices, maturity at harvest, and postharvest handling conditions (Barrett et al., 2010). Different vegetables have different phytochemical contents and antioxidant capacities (Deng et al., 2013). Yosefi et al. (2010) reported that the genotype, leaf type, and plant part are sources of variability in antioxidant capacity and nitrate content. Adegbaju et al. (2020) find quantitative differences in the concentration of phytochemical compounds and antioxidant capacity of Celosia plants at different growing seasons and phases of growth. Phytochemical contents may change during the postharvest period (Serrano et al., 2011). Nie et al. (2019) show variations in phytochemical contents and antioxidant activity in response to different harvest times, while Galani et al. (2017) show these variations in response to refrigerated storage. In addition, prolonged cold storage of vegetables may lead to nitrates' conversion to nitrites, though nitrate reduction was delayed relative to non-refrigerated storage. This study aims to compare the variations in antioxidant activity, total phenolics, nitrate, and nitrite contents of different varieties of lettuce available in Thai markets and determine the effects of harvest maturity and cold storage on the parameters in selected lettuce varieties.

MATERIALS AND METHODS

Experiments and Plant Materials

Three experiments were conducted to determine the variations in lettuce varieties' antioxidant activity, total phenolics, nitrate, and nitrite contents. Experiment 1 in completely randomized design (CRD) with 8 replicates (one whole plant/replicate) used 10 varieties: Green Oak, Red Oak, Frillice Iceberg, Butterhead, Green Cos, Mini Green Cos, Head, Red Coral, Red Rapids, and Grand Rapids (Fig. 1); 80 samples of these varieties were collected between January-February 2021 from supermarkets in Bangkok, Thailand, and were subjected to the analyses.

Fig. 1. Lettuce varieties used in Experiment 1

Experiment 2 determined the effects of harvest maturity based on the number of days after transplanting (DAT). It was laid out in Randomized Complete Block Design (RCBD) with three replications. The treatments included six lettuce varieties: Red Bowl, Red Butterhead, Mini Green Cos, Butterhead, Frillice Iceberg, and Green Big Bowl (Fig. 2). Certified seeds from Maejo 68 Seed Co., Ltd., Chiang Mai, Thailand, were used. They were germinated in trays of 104 cells with peat moss as substrate. After 14 days of sowing, the seedlings were transplanted into the greenhouse at the Faculty of Agricultural Technology, Valaya Alongkorn Rajabhat University, under the Royal Patronage, Pathum Thani Province, Thailand. The soil was a mixture of loam soil, rice husk, chopped coconut husk, coconut flakes, dried cow dung, and dried chicken dung at the same ratio. Fertilizer application used complete fertilizer 15-15-15 (N–P_2O_5–K_2O) at 2.5 g per plant applied weekly, starting after transplanting until a week before harvesting. The different harvesting periods were 40, 45, and 50 DAT. The harvested plants were then subjected to the analyses.

Experiment 3 in CRD with three replicates used Red Bowl, Butterhead, and Green Big Bowl varieties harvested at 45 DAT and then stored at 8ºC, 85% RH under 12 h light/12 h darkness for 21 days. Measurement of parameters was performed every three days.

Sample Extraction

Lettuce samples were extracted with 70% ethanol at a ratio of 20% (w/v) and homogenized at 14,000 rpm for 2 minutes using a homogenizer (IKA, Germany). The homogenized sample was incubated for 3 days at room temperature, shaking twice a day using a vortex mixer. The filtrate was obtained via Whatman™ filter paper No.1. The ethanolic extract was used to analyze antioxidant activity and total phenolics content.

For nitrate and nitrite analysis, sample extraction followed the official method of the Association of Official Analytical Chemists (AOAC, 1995). A 10 g finely chopped lettuce sample was extracted with 200 ml of distilled water and homogenized at 14,000 rpm for 2 minutes using a homogenizer. This extract was moved to a hot water bath at 80°C for 120 minutes and then cooled to room temperature. The volume was adjusted to 200 ml with distilled water, and the mixture was allowed to stand for 5 minutes at room temperature before filtering using Whatman™ 42 filter paper. This filtrate was used for the analyses.

Fig. 2. Lettuce varieties are grown for Experiment 2

Analysis of Antioxidant Activity

The antioxidant capacity of the ethanolic extract was determined using the 1,1–diphenyl–2–picrylhydrazyl (DPPH) free radical scavenging method (Yingngam et al., 2014). Briefly, 100 µl of sample and standard solution were pipetted into a test tube. Then, 100 µl of 0.2 mM DPPH solutions in absolute ethanol was added. The solution was shaken and placed in the dark at room temperature for 30 minutes. Afterward, the solution's absorbance was measured at 517 nm using a 96-well microplate reader (Model: Multiskan GO, Thermo Scientific, Finland); as blank, 70% ethanol was used. The 50% inhibition concentration (IC_{50}) value is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower IC_{50} value indicates the greater overall effectiveness of the antioxidant, which was calculated by the probit method. The equation 1 calculated the percent inhibition:

% Inhibition = [(Acontrol-Asample)/(Acontrol)]×100 .....1)

where Asample and Acontrol are the absorbances of the sample ethanolic extracts solution and control reaction, respectively.

Analysis of Total Phenolic Content

The Folin determined total phenolic content (TPC)–Ciocalteu colorimetric method using Gallic acid as the standard measured (Yingngam et al., 2014), which is the most widely used to determine TPC in plant extracts (Blainski et al., 2013; Veljković et al., 2019). A 200 µl of the ethanolic extract from lettuce was mixed with 200 µl of 10% Folin-Ciocalteu reagent, 2.5 ml of distilled water (DW), and 2.0 ml of 7.5% sodium carbonate (Na_2CO_3) and 70% ethanol as a control. The mixture was allowed to incubate at room temperature for 90 minutes. The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (Model: Genesys 10S UV-Vis, Thermo Fischer Scientific, USA). A standard curve was prepared using a concentration range of 20–120 µg/ml of Gallic acid in ethanol. Standard solutions of gallic acid were designed using the same procedure as the sample extraction method. The standard curve equation was y = 0.00277x + 0.0014, R^2 = 0.9999. The TPC was expressed as milligrams per kilogram fresh weight (mg/kg FW) of Gallic acid equivalent as formula 2:

TP = 100a/c ....2)

where TP = concentration of total phenolics content in µg/g FW or mg/kg FW, a = concentration obtained from the standard curve in µg/mL, c = percentage of sample extract.

Analysis of Nitrate and Nitrite Contents

Nitrate and nitrite analysis followed the AOAC (1995) method. For nitrate analysis, 1 ml of aqueous extract solution sample was transferred to a test tube, and 1 ml of 5% salicylic acid in sulfuric acid (H_2SO_4) was added and mixed using a vortex mixer. The mixture was left to stand for 15 minutes at room temperature; then, 10 ml of 4.0 M sodium hydroxide (NaOH) was added to the mixing. After 15 minutes, the absorbance at 410 nm was determined. The 5% salicylic acid in sulfuric acid was executed by dissolving 5 g of salicylic acid in 95 ml of H_2SO_4. Simultaneously, 4.0 M NaOH was prepared by dissolving 160 g of NaOH in 1 l of DW.

A 2 ml of the aqueous extract solution was transferred to a test tube for nitrite analysis, and 2 ml of the sulfanilamide reagent was added and mixed with a vortex mixer. After allowing the mixture to remain at room temperature for 15 minutes, 2 ml of NED reagent was added. The absorbance at 520 nm was measured after 15 minutes. A 0.5 g of sulfanilamide was dissolved in 100 ml of 2.4 N HCl in water to create the reagent. Furthermore, 0.3 g of N–(1–naphthyl)–ethylene diammonium dichloride was dissolved in 100 ml of 0.12 N HCl in water to create the NED reagent. The absorbance was taken using a UV-Vis spectrophotometer (Model: Genesys 10S UV-Vis, Thermo Fischer Scientific, USA). A standard curve was prepared using a concentration range of 25–125 µg/ml of nitrate and 0.1–1.0 µg/ml of nitrite in distilled water. Standard solutions of nitrate and nitrite were prepared with the same procedure for sample extraction for standard curve plotting, using the equation y = 1.2562x + 0.0259, R^2 = 0.9990, for nitrate and y = 0.2389x + 0.0007, R^2 = 0.9999, for nitrite. Nitrate and nitrite contents were expressed as milligrams per kilogram fresh weight (mg/kg FW) as formula 3:

N = xa/m ....3)

where N = concentration of nitrate or nitrite in µg/g FW or mg/kg FW, x = total volume of distilled water using extracted in ml, a = concentration obtained from the standard curve in µg/ml, m = mass of plant extract used in the assay in gram.

Statistical Analysis

The results of the experiments were statistically analyzed by performing an analysis of variance (ANOVA) and treatment mean comparison by Duncan's Multiple Range Test (DMRT). The antioxidant capacity in IC_{50} was calculated by the probit method.

RESULTS AND DISCUSSION

Responses of 10 Lettuce Varieties (Experiment 1)

In this study, the antioxidant activities of ten lettuce varieties are evaluated, revealing a wide range of activity levels as measured by IC_{50} values, which varied from approximately 7% to 38% (Fig. 3).

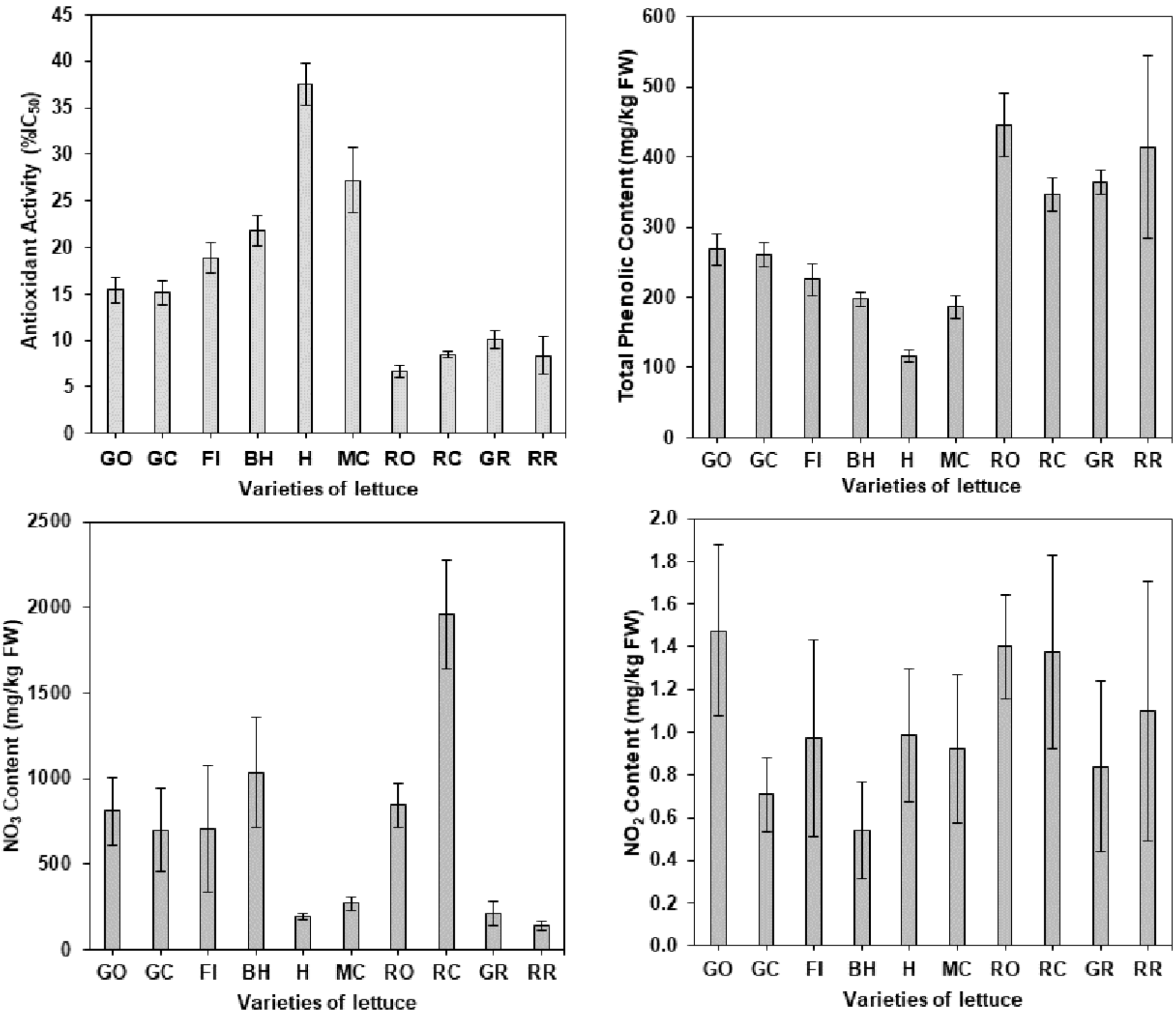

Fig. 3. Antioxidant activity, total phenolics, nitrate, and nitrite contents of 10 lettuces Varieties-Green Oak (GO), Green Cos (GC), Frillice Iceberg (FI), Butterhead (BH), Head (H), Mini Green Cos (MC), Red Oak (RO), Red Coral (RC), Grand Rapids (GR) and Red Rapids (RR), collected between January-February 2021 from supermarkets in Bangkok, Thailand. Mean separation by DMRT (*P*<0.05)

Red Oak, Red Coral, Red Rapids, and Grand Rapids stand out, exhibiting the most potent antioxidant activities, as evidenced by the lowest IC_{50} values, all at or below 10%. In contrast, Green Oak, Green Cos, and Frillice Iceberg show moderate antioxidant activities, with IC_{50} values of 15% to 19%. The varieties Butterhead, Mini Green Cos, and Head lettuce have the highest IC_{50} values, indicating the lowest antioxidant activities, ranging between 22% and 38%. Total phenolic content (TPC) closely mirrors the antioxidant activity trends. The varieties mentioned above with the highest antioxidant activities also had the highest TPC, with values ranging between 347 and 445 mg/kg fresh weight (FW).

Conversely, the varieties with the lowest antioxidant activities have the lowest TPC, ranging from 116 to 268 mg/kg FW. There is notable variability in nitrate levels across the various lettuce varieties. The Red Coral variety demonstrates the highest nitrate content at 1958.8 mg/kg FW, surpassing the nine varieties with lower nitrate concentrations ranging from 141 to 1033 mg/kg FW. Conversely, nitrite content does not significantly vary among the different lettuce varieties, ranging from 0.54 to 1.48 mg/kg FW.

Following prior research findings, red leaves lettuce cultivars such as Red Oak, Red Coral, and Red Rapids demonstrate heightened antioxidant capacity and total phenolic content (TPC) in comparison to their green leaf counterparts (Heimler et al., 2007; Zapata-Vahos et al., 2020). This enhanced performance can be attributed to the diverse array of phenolic compounds in these varieties, encompassing lignin, tannins, phenolic acids, flavonoids, and other derivatives. These compounds are pivotal in determining various plant characteristics, including bitterness, astringency, color, and odor (Horax et al., 2005; Wu et al., 2013). A noteworthy observation in the current experiment is the comparable antioxidant capacity and TPC exhibited by Grand Rapids, a green-leaf variety, aligning its performance with the red-leaf varieties.

Harvest Maturity Effects on 6 Lettuce Varieties (Experiment 2)

This research primarily investigates the influence of harvest maturity on the antioxidant capacity of six lettuce varieties. Red Bowl and Red Butterhead consistently demonstrate heightened antioxidant capacities, with IC_{50} values ranging from 6-10% when harvested within 40 to 50 days after transplanting (DAT). In contrast, Mini Green Cos, Butterhead, Frillice Iceberg, and Green Big Bowl exhibit relatively lower antioxidant capacities, with IC_{50} values ranging from 20-23%, as presented in Table 1. The analysis of antioxidant activity across different harvest maturities reveals that the highest antioxidant activity is generally obtained at 50 DAT for all varieties studied. Specifically, the IC_{50} values were 8.2%, 5.7%, and 6.9% for Red Bowl and 10.1%, 6.0%, and 6.7% for Red Butterhead at 40, 45, and 50 DAT, respectively.

The other varieties do not display a consistent trend in antioxidant activity with the progression of harvest maturity from 40 to 50 DAT (Table 2). A significant interaction effect is noted, where antioxidant activity initially decreases from 40 DAT to 45 DAT, then increases at 50 DAT, especially noticeable in Mini Green Cos, Frillice Iceberg, and Green Big Bowl (Table 2). TPC, Red Bowl, and Red Butterhead had the highest contents (458.7 and 426.7 mg/kg FW, respectively), over twice as high as that found in Mini Green Cos, Butterhead, Frillice Iceberg, and Green Big Bowl. Interestingly, TPC is the highest at 45 DAT compared to 40 or 50 DAT (Table 1), suggesting an optimal harvest time for maximizing phenolic content. The interaction effect between variety and harvest maturity is insignificant in this context.

Regarding the relationship between antioxidant activity and TPC, this study's findings align with those of previous research (Li et al., 2009; Yosefi et al., 2010; Deng et al., 2013; Rumainum et al., 2018; Veljković et al., 2019), highlighting TPC as a major factor contributing to the antioxidant properties of vegetables. Specifically, lettuce varieties harvested at 45 DAT exhibited higher TPC and antioxidant activity than those harvested at 40 and 50 DAT. However, no significant changes are observed during cold storage, except for a slight decline in TPC and antioxidant activity in the Red Bowl variety, which may be attributable to the enzymatic breakdown of polyphenolic compounds (Al-Weshahy et al., 2013).

Effects of Cold Storage (Experiment 3)

The effects of cold storage on the antioxidant properties of lettuce varieties were examined. Red Bowl lettuce maintains high antioxidant activity (low IC_{50}) for the initial 15 days of storage, after which a slight decrease is observed, indicated by an increase in IC_{50} (Fig. 4). In contrast, Butterhead and Green Big Bowl varieties exhibited much lower antioxidant activities, which remained stable throughout the 21-day storage period. This stability is also reflected in their TPC, which is significantly lower than that of the Red Bowl variety. In particular, it is noteworthy that the TPC of Red Bowl lettuce declined, dropping below its initial levels after 18-21 days of storage. Red Bowl exhibits higher nitrate and nitrite concentrations than the other two varieties. These contents did not undergo significant changes throughout the cold storage period, except for a reduction in nitrate content towards the conclusion of the storage duration. Nitrite content consistently remains below 1 mg/kg FW for all varieties during storage. The study unveils variations in nitrate and nitrite contents among lettuce varieties, with red-leaf varieties generally displaying higher levels than their green-leaf counterparts. Notably, these contents demonstrate relative stability during cold storage, aligning with prior findings in spinach, where storage at 10°C has negligible effects on nitrate content (Yang et al., 2017).

Conversely, J.-C. Chung et al. (2004) and W. Q. Chung et al. (2013)'s research suggested that cold storage at 5°C does not significantly impact nitrate and nitrite levels in certain vegetables over 7 days. However, storage at ambient temperatures (22°C) resulted in a decline in nitrate levels and an increase in nitrite levels. Lower temperatures may contribute to a reduction in nitrate reductase activity and alterations in the internal electron transport of nitrate (Yaneva et al., 1996). Previous studies have highlighted the impact of genetics, environmental variables, and agricultural practices on the uptake and accumulation of nitrate in vegetable tissues (Santamaria et al., 2001).

Table 1. Antioxidant activity, total phenolics, nitrate and nitrite contents of six lettuce varieties harvested at 40-50 days after transplanting (DAT)

Treatments	Antioxidant activity (%IC_{50})	Total Phenolics (mg/kg FW)	Nitrate (mg/kg FW)	Nitrite (mg/kg FW)
Variety				
Red Bowl	6.9c	458.7a	2220.0b	1.05a
Red Butterhead	7.6c	426.7b	2335.2ab	0.72b
Mini Green Cos	23.0a	151.3e	1367.8d	0.36c
Butterhead	20.1b	186.0cd	1816.5c	0.50bc
Frillice Iceberg	20.6b	161.7de	2517.1a	0.40c
Green Big Bowl	20.3b	201.0c	1385.4d	0.25c
Harvest maturity (DAT)				
40	17.1a	248.6b	1986.4b	0.50b
45	14.9b	280.7a	2205.0a	0.36b
50	17.2a	263.3ab	1629.6c	0.79a

Remarks: Mean separation within columns per variable by DMRT (P<0.05)

Table 2. Interaction effect of lettuce variety and harvest maturity on antioxidant activity and nitrate content

Parameter/Variety	Harvest maturity (Days after transplanting)		
	40	45	50
Antioxidant activity (%IC_{50})			
Red Bowl	8.2fg	5.7g	6.9g
Red Butterhead	10.1f	6.0g	6.7g
Mini Green Cos	23.4ab	21.3b-d	24.2a
Butterhead	20.3c-e	19.2de	20.8cd
Frillice Iceberg	21.1b-d	18.1e	22.7a-c
Green Big Bowl	19.4de	19.4de	22.2a-c
Nitrate content (mg/kg FW)			
Red Bowl	2156.4cd	2525.5bc	1978.2de
Red Butterhead	2499.1bc	2732.4ab	1774.0e-h
Mini Green Cos	1420.6h-k	1534.0g-j	1148.7k
Butterhead	1798.9d-g	1862.7d-g	1787.9d-h
Frillice Iceberg	2653.1ab	2981.2a	1917.0d-f
Green Big Bowl	1390.2i-k	1594.0f-i	1171.9jk

Remarks: Mean separation within parameter by DMRT (P<0.05)

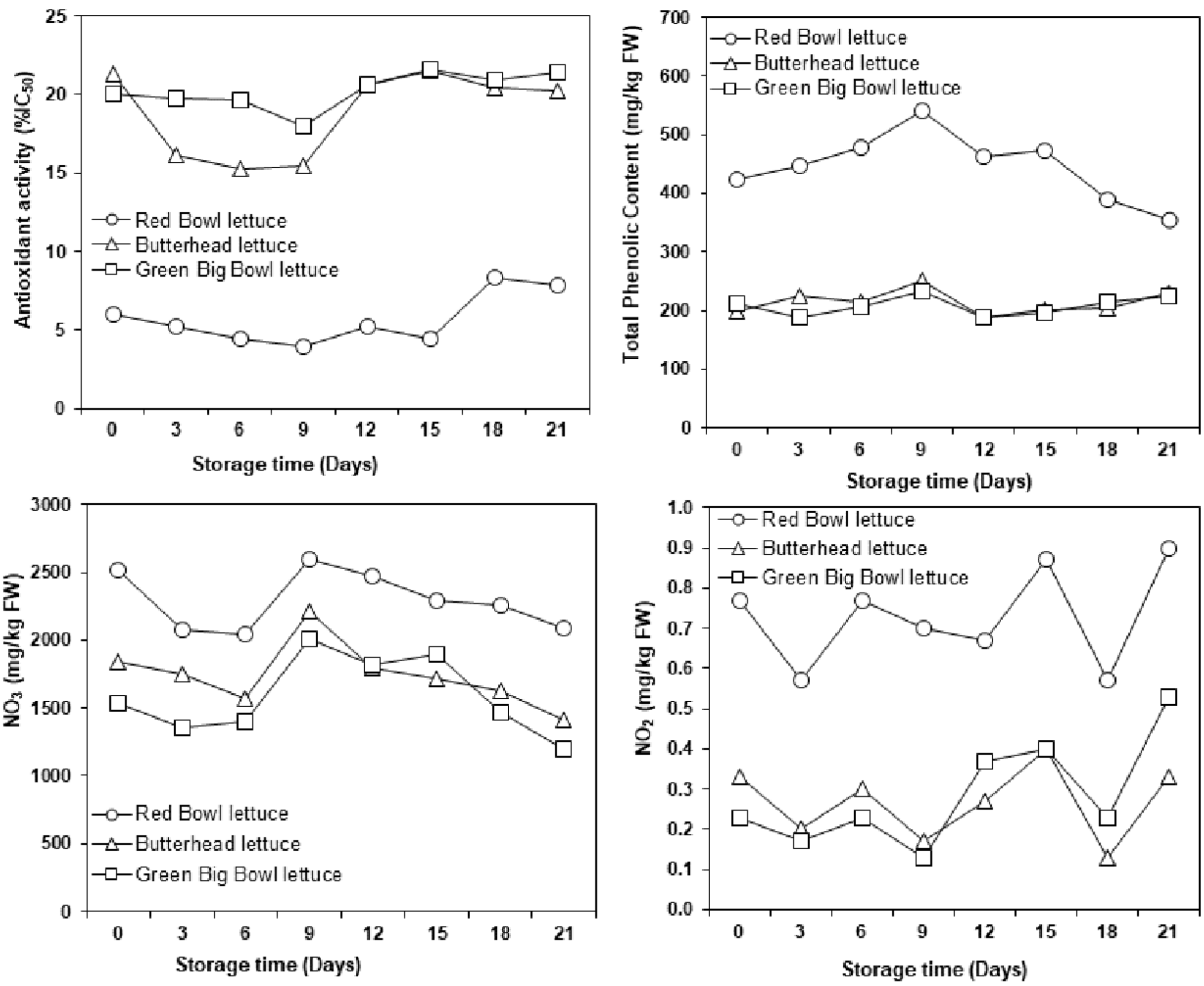

Fig. 4. Antioxidant activity, total phenolics, nitrate, and nitrite contents of three lettuce varieties during storage at 8°C for 21 days

CONCLUSION

The variety of lettuce mainly influenced antioxidant activity and TPC, which are higher in red leaf than green leaf varieties. Still, the former contained higher nitrate and nitrite levels than the latter. Harvest maturity also affects antioxidant activity and TPC, which seems better when plants are harvested at 45 DAT. Cold storage has a relatively minor effect on antioxidant capacity, TPC, nitrate, and nitrite contents. Consuming more colorful lettuces harvested at proper maturity and stored under refrigeration can positively impact consumers' health.

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