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Research article

Impact of chilling stress on quality and bioactive compounds in young and mature leaves of kratom [*Mitragyna speciosa* (Korth.) Havil.]

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Abstract

Importance of the work: There has been much attention paid to kratom (*Mitragyna speciosa* [Korth.] Havil.) since it was declared a legal plant in Thailand. Recently, the demand for fresh leaves has increased; nevertheless, only a few studies have investigated the postharvest quality management of the leaves.

Objectives: To examine the impact of chilling stress on the quality and alteration of bioactive compounds in young and mature leaves of two cultivars of commercial kratom leaves: 'kan khiao' and 'hang kang'.

Materials and Methods: Fresh kratom leaves of both the kan khiao and hang kang types were sampled from the young and mature leaf stages and stored for 12 d with 85% relative humidity at: 1) 10°C or 2) at room temperature (RT;30°C) . Every 3 d throughout storage, monitoring occurred of the fresh weight loss, superficial leaf color, browning score, electrolyte leakage (EL), malondialdehyde (MDA) content and bioactive compounds (ascorbic acid, antioxidant activity, total phenolic compounds and mitragynine contents).

Results: Chilling injury (CI; based on brown stain on the leaf) was discovered in both types of kratom leaves refrigerated at 10°C. In both types, RT storage resulted in less weight loss and browning. Yong leaves lost less weight loss and CI than mature leaves. CI was positively associated with increased EL and MDA levels in leaves kept at 10°C. In both types, the mitragynine content in the mature leaves was higher than in the young leaves. Both types lost mitragynine, ascorbic acid, antioxidant activity and phenolic compounds due to the cold storage condition.

Main finding: Cold storage induced chilling stress that resulted in leaf browning due to membrane dysfunction, as well as the loss of bioactive compounds and medicinal components, including mitragynine.

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Introduction

Kratom, [*Mitragyna speciosa* (Korth.) Havil.] is a botanical species that is indigenous to Thailand, Malaysia, Indonesia and Papua New Guinea, with its leaves widely used as a traditional medicine, serving as a mild herbal stimulant and reducing common health disorders such as coughing, hypertension, diabetes and pain (Singh et al., 2019). Royal Institute (2003) reported that in Thailand, there are three different types of kratom: 1) 'kan daeng', meaning red, due to color of the veins and petioles; (2) 'kan khiao', meaning pale green, due to the color of the veins and petioles; and 3) 'hang kang', where the apical part of the leaf is unevenly dentate in larger leaves. Ngernsaengsaruay et al. (2022), in their report on the genus *Mitragyna* (Rubiaceae) in Thailand, concluded it remains uncertain whether *M. speciosa* can be classified into three or more varieties, as many differences suggests intraspecific variation rather than distinct varieties. Traditionally, kratom leaves are harvested and masticated while fresh or transformed into a tea or beverage, which is consumed to relieve fatigue and enhance job efficiency (Singh et al., 2016). In 2021, an act of Thai parliament decriminalized kratom by removing it from the list of banned narcotics. This decision was made because kratom had cultural significance in traditional Asian culture, so currently, kratom is allowed for personal possession and use (Charoenratana et al., 2021). Notably, the increased application and use of kratom leaves have expanded all over the world as an alternative source of medicine. In the USA, kratom is currently unregulated and commonly refers to an herbal substance that can produce opioid- and stimulant-like effects to mitigate opioid withdrawal. Some of the main bioactive compounds within kratom leaves are alkaloids, flavonoids and terpenoids (Flores-Bocanegra et al., 2020). Laforest et al. (2023) reported that an alkaloid is unique to *Mitragyna speciosa* (kratom), which accumulates over 50 monoterpene indole alkaloids and oxindole alkaloids in its leaves. While mitragynine is the predominant alkaloid in mature leaves, juvenile leaves accumulate higher amounts of corynantheidine and speciociliatine. In particular, mitragynine has shown promise for development as a treatment for pain, opioid use disorder and opioid withdrawal (Kruegel and Grundmann, 2018). Despite the long period of use of kratom leaves in Southeast Asia, few research studies have been conducted on this plant, with those published mainly concentrating on harvesting index or leaf age and postharvest management during storage, so that there is only limited knowledge available on the physicochemical quality and bioactive compound changes in the kratom leaf. Typically, kratom leaves are stored in a refrigerator or at room temperature (RT) and such storage conditions may have an impact on the leaf quality and the level of the main bioactive compound (mitragynine). Hence, there is a compelling need to study the physicochemical quality changes of kratom leaves during storage in either cold storage (10°C) or at RT. Hence, the purpose of the current study was to investigate the impact of chilling stress on the quality and alteration of bioactive compounds in two famous commercial types (kan khiao and hang kang) of kratom leaves compared to storing the leaves at RT.

Materials and Methods

Plant materials

The fresh kratom leaves used in the experiments were harvested from an orchard located in Pathum Thani province, Central Thailand. The two types of kratom leaves (kan khiao and hang kang) were used at different stages: young (1–2 pairs of leaves from the top) and mature (3–4 pairs of leaves from the top and fully expanding). The leaf samples were delivered within 3 hr by airplane to the laboratory at Kasetsart University, Chalermphrakiat Sakhon Nakhon province campus. Leaves with any physical damage or disease were discarded. The remaining kratom leaves were cleaned using chlorinated water and air-dried before being placed in a cast polypropylene bag with micro-perforated holes and separate samples were stored at refrigerated temperature (10°C) or at RT (30°C) at 85%RH for 12 d. Every 3 d of storage, three biological replications per treatment (10 leaves per replicate) were sampled to evaluate fresh weight (FW) loss, superficial color (L*, a* , b* and chroma *C** values), browning score, electrolyte leakage (EL), malondialdehyde (MDA) content and bioactive compounds such as ascorbic acid, antioxidant activity, total phenolic compounds and mitragynine contents.

Weight loss and superficial color measurement

The FW loss of the kratom leaves was measured before storage and on the different sampling days. The percentage of weight loss during storage was calculated by comparing

the weight at each measurement to the initial pre-storge weight. The superficial color of the kratom leaves was measured using a colorimeter (Minolta CR300; Minolta Co. Ltd; Japan) for the CIELAB L*, a*, and b* values, where the L* value represents lightness to darkness (100–0), the a * value represents redness or greenness (-greenness to +redness), and the b* value represents blueness or yellowness (-blueness to +yellowness). After measuring these values, chroma C*, an indicator of color intensity, was calculated as $(a^*2 + b^*2)^{1/2}$.

Visual browning score evaluation

Chilling injury of the kratom leaf samples during storage was determined based on the brown area on the upper leaf of 30 individual leaves using a score from 1 to 5, where 1 = no occurrence, 2 = mild (1–20% of a leaf affected) 3 = moderate (21–50% of a leaf affected), 4 = severe (51–80% of a leaf affected) and 5 = very severe (81–100% of a leaf affected).

Electrolyte leakage measurement

The EL measurement of each kratom leaf sample was carried out using the method described by Ergun et al. (2007) with a slight modification. Briefly, the kratom leaves were punctured using a 15 mm diameter cork borer and the leaf discs (5g) produced were rinsed with deionized water and then incubated in 20 mL of 0.4 M mannitol at RT for 1 hr. The conductivity of the sample was measured using a conductivity meter (sensION5; Hach Company; USA). Next, the total conductivity of the sample was measured after the sample had been autoclaved at 120°C for 15 min. The percentage of sample electrolyte leakage (EL) was calculated based on: (Final conductivity / Total conductivity) × 100.

Malondialdehyde content assay

The malondialdehyde (MDA) content was used as an index of lipid peroxidation in the kratom leaves based on the method described by Heath and Packer (1968), with a slight modification. Each sample (5 g) was blended in 15 mL of 5% trichloroacetic acid (TCA) and then centrifuged at 10,000×*g* for 10 min. Next, 1 mL of supernatant was reacted with a solution of 2 mL of 15% TCA containing 0.5% thiobarbituric acid for 30 min at 60℃. After that, the sample was immediately placed in an ice bath for 30 min. The absorbance of the supernatant was determined at two wavelengths (532 nm and 600 nm) using an ultraviolet-vis spectrophotometer (GENESYSTM 10-s; Thermo Spectronic; USA). The MDA contents were expressed as nanomoles of MDA·per gram. After absorbance determination, calculations were made according to the formula in Equation 1:

$$MDA = [(OD532 - OD600) \times A \times V] / (a \times E \times W) \quad (1)$$

where A is the total reaction solution + the enzyme extract, OD is the optical density at the specified wavelength in nanometers, V is the total volume of buffer used for enzyme extraction, a is the volume of the enzyme extract used, W is the FW of the sample and E is the constant for MDA (0.155).

Ascorbic acid assay

Each sample (5 g) of the kratom leaves was homogenized with 20 mL of cold 5% metaphosphoric acid and then centrifuged at 12000×*g* for 15 min at 4°C. The supernatant was collected for ascorbic acid determination according to the method of Roe et al. (1948). The extract sample was reacted with 2% di-indophenol, 2% thiourea and 1% dinitrophenol hydrazine. Next, the mixture was incubated at ambient temperature for 3 hr and then 85% of sulfuric acid was added. After incubation for 30 min, the absorbance at 520 nm wavelength was measured. A standard curve was prepared using a series of known ascorbic acid concentrations. Optical density blanching was used; for each sample, the blank value was determined after the addition of 60 µL of ascorbic acid (1 mg/mL) with the aim of measuring the interference due to the sample color. The result was expressed as milligrams of ascorbic acid per gram FW.

Antioxidant capacity measurement

The *2*,*2*-diphenyl-*1*-picrylhydrazyl (DPPH) scavenging capacity was determined according to Brand-Williams et al. (1995). First, a sample (2 g) of frozen kratom leaf powder was homogenized with 3 mL of 85% methanol and then the filtered leaf extract was centrifuged at 6,000×*g* for 10 min at 4°C. Next, 70 µL of supernatant of the kratom leaf extract was added into a test tube and mixed with 2,930 µL of 200 µM DPPH solution in methanol. The reaction mixture was incubated in the dark at RT for 30 min. The absorbance of the solution was read at 517 nm and calculated using the formula: DPPH scavenging capacity (%) = [1 - (A0 / A1)] × 100, where A0 is the absorption of the sample and A1 is the absorption of the blank DPPH solution.

Total phenolic compounds assay

Total phenolic compounds were assayed using the method described by Slinkard and Singleton (1977). Each sample (2 mL) of the extract was reacted with 2 mL of 50 % (volume per volume) Folin-Ciocalteu reagent followed by the addition of 2 mL of concentrated Na_2CO_3 solution. The reaction was incubated for 30 min at RT and then the absorbance was recorded at 750 nm wavelength. Gallic acid was used as the standard and results were expressed as milligrams of gallic acid per gram FW.

Mitragynine content assay

Each sample (5 g) of the kratom leaves was freeze-dried for 5 d to remove the water content, resulting in the lyophilized powder. Then, methanol solvent (200 mL) was added to each sample. Each mixture was consistently shaken in a shaker at 120 revolutions per minute for 2 hr. Next, the mixture was filtered to remove debris and residue, after which, the filtrate was evaporated under reduced pressure to yield the methanolic extract. Each methanolic extract was freeze-dried and kept at -20°C until analysis. The detection of the mitragynine content (in methanol extract, 20 mg/mL*)* was estimated using the gas chromatography-mass spectrometry (GC-MS) method described by Singh et al. (2019). The GC/MS analysis of mitragynine was performed using a gas chromatographic system (GC-2010 Plus; Shimadzu; Japan) coupled with a GCMS-QP2010 ultra mass spectrometry detector (GC-2010 Plus; Shimadzu; Japan) and separated on a column (30.0 m length × 0.25 mm internal diameter × 0.25 µm). The injection volume (1.0 µL) was performed in split flow control mode with the ratio of 1:10. Helium was used as the carrier gas at a flow rate of ± 1 mL/min and the column and oven were maintained at 50°C and 250°C, respectively. The scanned mass range was 40–670 amu. The compounds were identified by comparing their retention times to a mitragynine standard at a major ion of m/z 398.

Statistical analysis

Data were analyzed using factorial analysis comprising three factors: two kratom cultivars, two leaf maturity stages, and two storage conditions. Differences between treatments were determined using Duncan's multiple range test. All tests were considered significant at $p < 0.05$.

Results and Discussion

Visual appearance and chilling injury symptom

Table 1 provides the appearance and CI symptoms of the kratom leaves after storage at RT and 10°C for 12 d. A brown stain was observed on the leaves stored at 10°C. After 12 d, the brown stain on the leaves stored at 10°C was visible along the vein and midrib, whereas all leaves stored at RT had a mild browning of the edge.

Table 1 Visual appearance and chilling injury symptoms of 'kan khiao' and 'hang kang' kratom leaves kept at room temperature (RT) compared to storage at 10°C for 12 days

Kratom cultivar	Storage temperature	Storage time (d)			
		Young leaves		Mature leaves	
		0	12	0	12
Kan khiao	RT				
	10°C				
Hang kang	RT				
	10°C				

Browning of tissues surrounding veins and midribs is generally recognized as a CI characteristic of leaves (Wongsheree et al., 2009), and browning of the edge is induced by the dehydration of lenticels and stomata (Nunes and Emond, 2007). These observations indicated that both types of kratom (kan khiao and hang kang) were sensitive to refrigeration. After 12 d of storage at 10°C, brown stains were evident on the leaves of both types of kratom, covering more than 50% of the leaf blade area. The mature hang kang kratom leaves were more tolerant to CI than the mature kan khiao kratom leaves stored at 10°C. Although Lal Basediya et al. (2013) and Ahmad et al. (2020) suggested that the optimum storage temperature for leafy vegetables was in the range 0–2°C, based on the current results, storage at 10°C caused chilling damage to both types of kratom leaves after storage for 12 d. Kratom is recognized as an indigenous plant in Southeast Asia (Tropical Cancer zone), with all native plants in this region being susceptible to cold temperatures (Wongsheree et al., 2009), so that the primary problem shortening their shelf life in cold storage is a physiological disorder (CI). The current results revealed that kratom leaves were susceptible to cold storage, as their shelf life was shorter than 12 d at 10°C. In addition, this outcome demonstrated that leaf maturity affected the CI resistance of the leaves of both types of kratom. Often the mature leaves were more able to withstand the CI than the young leaves. Additionally, the browning at the leaf edge brought on by the loss of stomatal moisture is a factor linked to the undesirable appearance of kratom leaves during storage at RT, as described by Nunes and Emond (2007).

Weight loss and browning score

The loss of FW in all leaves increased continuously during storage (Table 2). The increased weight loss of leaves stored at 10°C was higher than that of the leaves stored at RT ($p < 0.05$). Generally, refrigeration delays the rates of respiration and transpiration in plants (Ahmad et al., 2020). Under cold stress (such as with an excessively low storage temperature), both respiration and transpiration increase due to the occurrence of CI (Gualanduzzi et al., 2009). Although the respiration rate was not determined in the current study, the increase in FW loss could indicate an increase in respiration rate and tissue damage resulting from chill stress during storage (Vercesi et al., 2006). Tissue damage has been reported to cause an increase in the oxidation reaction of the cell membrane and the depolymerization of the cell wall (Gualanduzzi et al., 2009). In addition, Supapvanich et al. (2015) reported that cold storage induced weight loss and CI in lemon basil leaves during storage at 7°C. Based on the results of the current study, under refrigeration (10°C), the mature leaves of kan khiao and hang kang had significantly higher weight loss than the young ones. The weight loss of the mature kan khiao leaves was presumably greater than that of young leaves while stored at RT, although there was no significant difference. The weight loss of young leaves was predicted to be greater than that of mature leaves for hang kang kratom leaves held at RT; however, on day 12 of storage, the weight losses for both types of mature leaves were significantly greater than that of the young leaves.

Table 2 Interaction effects of kratom cultivar, leaf maturity stage and storage temperature on weight loss and visual browning scores of kratom leaves during 12 d of storage

Kratom cultivar	Leaf stage	Storage temperature	Storage time (d)							
			Weight loss (%)				Browning score			
			Day 3	Day 6	Day 9	Day 12	Day 3	Day 6	Day 9	Day 12
Kan khiao	Mature	10°C	3.12 ± 0.78^{a}	4.38 ± 0.11^{a}	4.96 ± 1.18^{a}	7.20 ± 1.88^{a}	2.20 ± 0.24^{a}	2.80 ± 0.25^{a}	4.10 ± 1.12^{a}	4.50 ± 0.86^{a}
	Young	10°C	1.56 ± 0.33^{b}	3.20 ± 0.15^{b}	3.85 ± 0.83^{b}	5.29 ± 0.83^{c}	2.00 ± 0.02^{a}	2.50 ± 0.33^{a}	3.50 ± 0.54^{b}	3.60 ± 0.75^{b}
	Mature	RT	1.82 ± 0.51^{b}	2.50 ± 0.43^{c}	3.03 ± 1.06^{b}	3.51 ± 1.12^{e}	1.00 ± 0.00^{b}	1.20 ± 0.26^{b}	2.50 ± 0.22^{c}	2.80 ± 0.34^{c}
	Young	RT	1.20 ± 0.47^{b}	1.84 ± 0.20^{e}	2.34 ± 1.20^{c}	2.50 ± 1.58^{f}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.30 ± 0.85^{c}	2.50 ± 0.44^{c}
Hang kang	Mature	10°C	2.04 ± 0.54^{a}	2.65 ± 0.21^{c}	4.46 ± 1.31^{a}	6.61 ± 2.33^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.80 ± 0.38^{c}	3.30 ± 0.51^{b}
	Young	10°C	1.83 ± 1.04^{b}	2.50 ± 0.25^{c}	3.79 ± 1.43^{b}	5.82 ± 1.25^{c}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	2.50 ± 0.41^{c}	2.80 ± 0.21^{c}
	Mature	RT	1.05 ± 0.23^{b}	1.13 ± 0.17^{d}	3.06 ± 0.88^{b}	4.69 ± 2.22^{d}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.20 ± 0.22^{d}	1.80 ± 0.28^{d}
	Young	RT	1.17 ± 0.33^{b}	2.44 ± 0.32^{c}	3.64 ± 0.96^{b}	3.68 ± 1.78^{e}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.20 ± 0.22^{d}	1.60 ± 0.22^{d}
CV (%)			2.12	4.01	2.06	4.01	1.10	1.65	2.53	2.33

RT = room temperature; CV = coefficient of variation; Values (mean ± SD) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different based on Duncan's multiple range test.

The increased weight loss of both types of kratom leaves held at 10°C was clearly related to CI. The increasing weight loss and visual appearance alterations (as shown in Table 1) in both kratom leaves were followed by an increased visual browning score. The visual browning score of the chilled kratom leaves developed quickly and was significantly greater than that of leaves kept at RT. The mature leaves had the highest increased visual browning score, demonstrating that the mature kratom leaves were more susceptible to chilling than the young leaves. There were no significant differences in the enhanced visual browning scores between mature and young leaves for both types of kratom kept at RT. According to Wongsheree et al. (2009), the mature leaves of lemon basil were more sensitive to chilling stress (higher leaf browning) than the young leaves due to the higher membrane lipid peroxidation and the lower activity of antioxidant enzymes. In addition, Pongprasert and Srilaong (2007) reported a higher severity of mature sweet basil leaves than for young leaves during cold storage. Furthermore, Supapvanich et al. (2015) proposed that cold storage-induced chilling stress exacerbated FW loss and the leaf browning score in lemon basil leaves, were due to leaf tissue damage and membrane dysfunction.

Color attributes of kratom leaves

Based on Table 3, the storage temperature affected the superficial color attribute values of kratom leaves. After 3 d of storage, there was a sharp decrease in the L* value of both mature and young kan khiao kratom leaves kept at 10°C, whereas there was a subsequent slight decrease in the L* value of leaves held at RT after 6 d of the storage. After 3 d of storage, both the mature and young kan khiao kratom leaves kept at 10°C had L* values significantly lower than those held at RT. The chilled mature leaves had a lower L* value than the chilled young leaves. All the hang kang kratom leaves had reduced L* values during storage for 3 d and 6 d of storage, after which there was no discernible change in that value. After 6 d, the L* value of the mature and young leaves stored at 10°C had decreased significantly less than for those stored at RT. At the end of storage, the chilled mature leaves had the significantly lowest L* value of all leaves, indicating that refrigeration stimulated increased darkness in both types of kratom leaves that was consistent with the visual appearance (Table 1) and the visual browning score (Table 2). The negative a* value demonstrated the change in the green color of the kratom leaves. There was no significant change in the a* values of the kan khiao and hang kang kratom leaves after they had been stored for 6 d and 3 d, respectively,. After being stored for 6 d, the kan khiao kratom leaves kept at RT had significantly greater a* values in both the mature and young leaves compared to those leaves held at 10°C, indicating that storage at a higher temperature induced a general loss of greenness in the kratom leaves compared to storage at a lower temperature. Regarding this, Ferrante and Maggiore (2007) proposed that storage at a higher temperature induced the loss of greenness and chlorophylls content in leafy vegetables. In the chilled kan khiao kratom leaves, the a * value of the young leaves increased continuously during storage, while there was no discernable change in the mature leaves throughout the storage period. There was a continuous increase in the a* value in the hang kang kratom mature leaves held at RT, which was significantly greater than that of other leaves after 6 d of storage. At the end of storage, the a* value of the chilled young leaves had sharply increased and was significantly higher than that of the young leaves held at RT and of the mature leaves held at 10°C, which both had similar values. An increased a* value indicated the loss of greenness in the leaves. CI induces the degradation of chlorophylls caused by the accumulation of reactive oxygen species (Yamauchi et al., 1995; Ferrante and Maggiore, 2007). The b* value of the leaves indicated yellowness during storage. However, the b* value of both the kan khiao and hang kang kratom leaves stored at RT and 10°C remained constant during storage for 6 d. The b* value of the mature kan khiao leaves stored at RT and 10°C as well as of the chilled young leaves decreased after 9 d of storage, while it maintained a constant for the chilled mature leaves. Similarly, the mature and young hang kang kratom leaves stored at RT for 9 d and 12 d had reduced b* values; however, the b* value of the refrigerated leaves remained consistent during storage. After storage for 9 d, the lowest b* value was in the young leaves held at RT, being significantly lower than for the mature and young leaves held at 10°C. The change in chroma values indicated that storage temperature influenced the leaf color intensity. There were no significant differences in the chroma values between the hang kang and kan khiao kratom leaves before storage, the chroma values of the mature leaves being higher than for the young leaves. However, the storage temperature decreased the chroma values of the kratom leaves during storage. Clearly, storage at 10°C maintained the chroma values of the kratom leaves compared to storage at RT.

Table 3 Interaction effects of kratom cultivar, leaf maturity stage and storage temperature on superficial color values (L^*, a^*, b^* and color intensity of kratom leaves during 12 d of storage

Kratom cultivar	Leaf stage	Storage temperature	Storage time (d)							
			Day 0				Day 3			
			L^*[1]	a^*[2]	b^*[3]	C^*[4]	L^*[1]	a^*[2]	b^*[3]	C^*[4]
Kan khiao	Mature	10°C	35.34±11.8	-10.27±5.1	22.68±8.8	24.90±8.6	32.61±10.0^b	-11.12±9.5^b	22.50±10.0^a	25.10±16.7^a
	Young	10°C	35.10±10.8	-10.81±6.1	22.32±9.0	24.80±10.2	35.75±12.0^a	-10.68±6.6^a	20.75±10.8^b	23.34±13.2^b
	Mature	RT	36.11±10.5	-10.24±5.8	22.10±10.1	24.36±11.0	36.33±12.3^a	-10.69±7.3^a	22.69±12.0^a	25.08±14.3^a
	Young	RT	36.17±11.8	-11.26±6.7	22.62±10.9	25.27±13.3	30.67±12.2^c	-9.84±5.5^a	20.14±11.8^b	22.42±16.1^b
Hang kang	Mature	10°C	33.76±13.3	-8.89±5.1	23.05±11.2	24.70±10.0	30.67±10.5^c	-11.12±6.3^b	22.74±14.0^a	25.31±13.8^a
	Young	10°C	34.72±12.5	-9.07±5.1	23.41±10.8	25.11±11.6	29.75±13.3^c	-10.68±8.3^a	23.47±13.5^a	25.79±13.3^a
	Mature	RT	34.17±12.1	-9.79±4.4	23.12±12.2	25.11±12.0	33.20±12.8^b	-10.69±4.4^a	23.83±13.5^a	26.12±13.0^a
	Young	RT	33.47±10.6	-9.18±4.3	23.37±12.4	25.11±12.1	32.20±12.8^b	-9.84±5.1^a	20.88±13.4^b	23.08±13.6^b
CV (%)			21.01	26.66	28.51	20.07	20.15	20.83	22.12	26.51

Kratom cultivar	Leaf stage	Storage temperature	Storage time (d)											
			Day 6				Day 9				Day 12			
			L^*[1]	a^*[2]	b^*[3]	C^*[4]	L^*[1]	a^*[2]	b^*[3]	C^*[4]	L^*[1]	a^*[2]	b^*[3]	C^*[4]
Kan khiao	Mature	10°C	20.42±6.6^e	-10.68±2.8^c	21.59±6.0^c	24.09±8.0^a	15.01±5.0^e	-8.46±5.3^b	23.09±9.1^a	24.59±6.5^a	15.61±8.3^e	-8.38±5.1^d	22.95±5.8^a	24.43±8.5^a
	Young	10°C	25.21±9.0^d	-8.41±3.8^b	21.00±8.5^c	22.62±8.8^c	20.43±6.3^d	-10.16±2.1^c	19.89±5.5^b	22.33±7.7^b	19.29±8.7^d	-4.95±3.3^b	17.64±4.8^c	18.32±8.0^d
	Mature	RT	36.61±9.7^a	-6.96±3.8^a	22.80±5.9^b	23.84±6.6^b	30.11±8.5^b	-3.03±2.2^a	15.81±5.1^d	16.10±7.1^d	30.20±8.2^a	-2.66±1.1^a	16.24±8.3^c	16.46±8.8^e
	Young	RT	32.56±11.0^b	-9.80±3.3^b	21.10±8.8^c	23.26±6.0^b	29.50±9.3^b	-9.04±3.4^b	13.35±5.8^e	16.12±7.5^d	30.42±6.3^a	-6.89±4.8^c	13.65±8.6^d	15.29±7.3^e
Hang kang	Mature	10°C	29.75±15.1^c	-10.68±3.9^c	20.90±8.8^c	23.47±6.0^b	25.43±9.6^c	-8.46±3.7^b	22.91±6.3^a	24.42±6.2^a	20.22±8.6^d	-8.38±4.2^d	22.85±6.4^a	24.34±6.4^a
	Young	10°C	28.86±13.0^c	-8.41±3.1^b	22.41±9.3^b	23.94±6.3^b	26.55±8.2^c	-10.16±5.2^c	21.96±6.6^b	24.20±6.9^a	25.89±5.9^c	-4.95±2.1^b	21.18±6.1^a	21.75±6.6^b
	Mature	RT	33.53±12.2^b	-6.96±3.3^a	23.53±9.6^a	23.52±8.0^b	30.66±8.8^b	-3.03±2.1^a	20.25±6.8^b	20.48±6.0^c	28.32±7.9^b	-2.66±1.6^a	19.82±6.9^b	20.00±9.2^c
	Young	RT	32.11±12.1^b	-9.80±3.4^b	20.68±8.8^c	22.88±8.4^c	32.46±6.4^a	-9.04±2.8^b	17.57±6.4^c	19.76±6.1^c	30.56±9.4^a	-6.89±5.1^c	17.30±4.5^c	18.62±9.7^d
CV (%)			23.33	25.64	20.10	19.88	19.48	20.61	25.45	23.39	25.08	26.82	21.57	12.02

CV = coefficient of variation; RT = room temperature; Values (mean ± SD) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different based on Duncan's multiple range test.

[1] L^* = lightness of color ranging from black = 0 to white = 100.

[2] larger negative a* value = greener.

[3] larger positive b* value = yellower.

[4] C^* = color intensity $(a^*2 + b^*2)^{1/2}$

Electrolyte leakage and malondialdehyde content of kratom leaves

Elevated oxidation reactions brought on by abiotic and biotic stressors are shown by increases in EL and the MDA content in plant tissues (Wongsheree et al., 2009). Chilling stress is recognized as a kind of oxidative stress-inducing physiological disorder named CI. Table 4 depicts the increased EL and MDA content in the leaves of both kratom cultivars held at RT and 10°C. The EL of the kratom leaves held at 10°C was higher than that of the leaves held at RT. The highest EL was discernible in the chilled young kan khiao kratom leaves, which was significantly greater than in the chilled mature leaves and in all leaves held at RT. There was no significant difference in the EL between the young and mature kan khiao kratom leaves kept at RT. At the end of storage, the EL of the chilled mature kan khiao kratom leaves reached the same level as that of the chilled young leaves. In addition, the EL of the chilled hang kang kratom leaves was significantly higher than that of the leaves held at RT. Furthermore, the chilled mature leaves had the highest EL, which was significantly higher than that of the other leaves during the storage period, followed by the chilled young leaves. Young leaves kept at RT had the lowest increase in EL, which was also significantly lower than that of the mature leaves. The MDA concentration is recognized as a major indicator of membrane lipid peroxidation caused by chilling stress (Kong et al., 2018). Based on the results from the current study, the increased MDA content in the kan khiao kratom leaves was significantly higher than for the hang kang kratom leaves during storage at RT and 10°C. Both the both young and mature kan khiao kratom leaves held at 10°C had significantly higher MDA contents than the leaves stored at RT. The greatest increase in the MDA content was in the chilled mature leaves, followed by the chilled young leaves. After storage for 6 d, the MDA content in the chilled mature leaves was significantly greater than in the other leaves. The MDA content of the mature leaves held at RT increased significantly more than in the young leaves during storage. The MDA concentration of most samples of hang kang kratom leaves increased after the initial day of storage, except for the chilled young leaves, which increased after 3 d. The MDA concentration in the chilled mature leaves increased more than that of other samples during storage, being significantly higher than for the other leaves after day 6 of storage. Notably, the MDA concentration in the mature leaves held at RT remained constant after 3 d. At the end of storage, it was clear that both the mature and young leaves held at 10°C had significantly higher MDA concentrations than those held at RT. This was consistent with the increased visual browning scores, as shown in Table 2. According Wongsheree et al. (2009) and Supapvanich et al. (2015), leaf browning in basils during cold storage was associated with oxidative stress (chilling injury) stimulating membrane degradation and enzymatic browning reaction.

Content of bioactive compounds in kratom leaves

Table 5 shows the changes in mitragynine, ascorbic acid, total phenols and the antioxidant activity of the leaves of both kratom cultivars stored at RT and 10°C. Mitragynine is acknowledged as the primary monoterpenoid indole alkaloid in kratom leaves (Flores-Bocanegra et al., 2020). The mitragynine concentration in mature leaves of both the kan khiao and hang kang cultivars was significantly higher than in the young leaves for both. The mitragynine content in all kratom leaves had declined after storage for 12 d. There was a higher reduction in the mitragynine concentration in all chilled leaves compared to leaves held at RT. On day 12 of storage, the mitragynine concentration of both mature kan khiao and hang kang kratom leaves was significantly greater than for other leaves, followed by the mature leaves of both kratoms stored at 10°C.

Table 4 Interaction effect of kratom cultivar, leaf maturity stage and storage condition on electrolyte leakage and malondialdehyde (MDA) concentration of kratom leaves stored for 12 days at room temperature (RT) or at 10°C

Kratom cultivar	Leaf stage	Storage temperature	Storage time (d)							
			Electrolyte leakage (%)				MDA content (nmol/g FW)			
			Day 3	Day 6	Day 9	Day 12	Day 3	Day 6	Day 9	Day 12
Kan khiao	Mature	10°C	24.04 ± 4.88^{a}	33.50 ± 3.89^{a}	37.65 ± 3.12^{a}	51.71 ± 7.32^{a}	7.13 ± 3.11^{a}	12.16 ± 8.33^{a}	13.32 ± 5.34^{a}	12.34 ± 8.86^{a}
	Young	10°C	19.71 ± 4.11^{b}	27.78 ± 3.85^{c}	35.88 ± 4.88^{b}	53.46 ± 6.31^{a}	8.02 ± 3.85^{a}	10.15 ± 5.89^{b}	10.84 ± 5.35^{b}	10.98 ± 6.26^{b}
	Mature	RT	18.89 ± 3.86^{b}	27.52 ± 4.41^{c}	28.48 ± 5.02^{d}	33.77 ± 5.45^{d}	5.08 ± 3.06^{b}	6.32 ± 4.44^{c}	8.56 ± 5.02^{c}	9.19 ± 5.08^{c}
	Young	RT	12.42 ± 2.36^{d}	29.72 ± 3.29^{b}	30.62 ± 6.02^{d}	35.13 ± 5.36^{c}	4.55 ± 2.56^{b}	5.35 ± 2.26^{c}	5.96 ± 3.32^{d}	8.0 ± 4.34^{c}
Hang kang	Mature	10°C	16.55 ± 2.23^{c}	25.98 ± 4.02^{d}	33.21 ± 8.11^{c}	37.69 ± 6.12^{b}	2.85 ± 1.11^{c}	4.14 ± 2.85^{d}	5.03 ± 2.85^{d}	6.96 ± 3.85^{d}
	Young	10°C	17.46 ± 2.54^{c}	20.84 ± 4.44^{e}	32.53 ± 6.26^{c}	35.41 ± 5.15^{c}	2.03 ± 1.52^{c}	3.11 ± 3.09^{e}	3.85 ± 1.41^{e}	5.88 ± 4.44^{d}
	Mature	RT	13.37 ± 2.76^{d}	14.44 ± 4.36^{f}	20.12 ± 4.67^{e}	30.49 ± 5.54^{e}	2.88 ± 0.92^{c}	3.03 ± 1.17^{e}	3.15 ± 1.09^{e}	3.22 ± 2.72^{e}
	Young	RT	10.37 ± 2.11^{e}	12.30 ± 4.02^{f}	16.23 ± 4.22^{f}	25.43 ± 5.38^{f}	3.03 ± 0.67^{c}	3.16 ± 1.86^{e}	4.08 ± 1.15^{d}	4.11 ± 3.67^{e}
CV (%)			19.58	13.33	10.26	20.23	10.15	10.51	14.39	15.90

FW = fresh weight; CV = coefficient of variation; Values (mean ± SD) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different based on Duncan's multiple range test.

Table 5 Interaction effect of kratom cultivar, leaf maturity stage and storage temperature on mitragynine, ascorbic acid, antioxidant activity and total phenolic compounds of kratom leaves stored for 12 days at room temperature (RT) or at 10°C

Kratom cultivar	Leaf stage	Storage temperature	Storage time (d)							
			Mitragynine (mg/mL)		Ascorbic acid (mg/g FW)		DPPH (%)		Total phenolics (mg/g FW)	
			Day 0	Day 12	Day 0	Day 12	Day 0	Day 12	Day 0	Day 12
Kan khiao	Mature	10°C	6.13 ± 2.11^{b}	4.46 ± 1.16^{b}	0.50 ± 0.22^{a}	0.26 ± 0.16^{a}	3.33 ± 1.17^{a}	1.58 ± 1.02^{c}	21.30 ± 5.45^{b}	16.56 ± 9.26^{b}
	Young	10°C	2.51 ± 0.82^{d}	1.37 ± 0.92^{e}	0.54 ± 0.26^{a}	0.12 ± 0.13^{a}	3.22 ± 1.20^{a}	1.62 ± 1.05^{c}	11.11 ± 3.22^{d}	8.33 ± 5.34^{c}
	Mature	RT	6.02 ± 1.16^{b}	5.98 ± 0.95^{a}	0.51 ± 0.20^{a}	0.43 ± 0.18^{a}	3.52 ± 1.38^{a}	2.53 ± 0.88^{b}	20.09 ± 6.38^{b}	18.32 ± 3.33^{b}
	Young	RT	2.53 ± 1.01^{d}	2.03 ± 0.60^{d}	0.48 ± 0.28^{a}	0.33 ± 0.14^{a}	3.16 ± 1.27^{a}	2.66 ± 0.92^{b}	11.58 ± 4.36^{d}	10.12 ± 4.14^{c}
Hang kang	Mature	10°C	7.01 ± 1.05^{a}	4.35 ± 1.02^{b}	0.49 ± 0.22^{a}	0.35 ± 0.11^{a}	3.11 ± 1.11^{a}	2.58 ± 0.94^{b}	25.38 ± 6.11^{a}	20.39 ± 3.39^{a}
	Young	10°C	3.66 ± 1.32^{c}	2.32 ± 0.83^{d}	0.55 ± 0.24^{a}	0.28 ± 0.12^{a}	3.08 ± 1.87^{a}	2.05 ± 0.81^{b}	15.55 ± 3.34^{c}	10.11 ± 3.38^{c}
	Mature	RT	7.12 ± 1.26^{a}	6.08 ± 1.04^{a}	0.53 ± 0.21^{a}	0.49 ± 0.19^{a}	3.09 ± 1.55^{a}	2.96 ± 0.88^{b}	25.82 ± 8.38^{a}	23.23 ± 5.67^{a}
	Young	RT	3.54 ± 1.17^{c}	3.01 ± 1.02^{c}	0.57 ± 0.25^{a}	0.46 ± 0.12^{a}	3.14 ± 1.83^{a}	3.01 ± 0.86^{a}	16.02 ± 3.33^{c}	15.05 ± 5.89^{b}
CV (%)			45.12	33.06	40.12	42.70	38.98	42.56	41.43	43.45

FW = fresh weight; DPPH = *2,2*-diphenyl-*1*-picrylhydrazyl; CV = coefficient of variation; Values (mean ± SD) within a column superscripted with different lowercase letters are significantly ($p < 0.05$) different based on Duncan's multiple range test.

The young leaves of both cultivars held at RT had higher mitragynine concentrations than those stored at 10°C. Veeramohan et al. (2023) assessed the secondary metabolites in two maturity stages of kratom leaves (young and mature) and reported that mature leaves contained more mitragynine than young leaves. Based on the findings of the current research, the levels of mitragynine in the mature leaves of both the kan khiao and hang kang kratom cultivars were substantially greater than the levels in the young leaves. Chilling stress enhanced the loss of mitragynine in both the mature and young leaves. Matsuura et al. (2014) suggested a link between oxidative stress and monoterpenoid indole alkaloid biosynthesis, in which the H_2O_2 produced as a result of oxidative stress participated in monoterpenoid indole alkaloid synthesis, with the oxidation reaction inducing the degradation of mitragynine (Ramanathan et al., 2015). Based on the results of the current study, cold storage-induced chilling stress (10°C) influenced the changes in the contents of ascorbic acid and total phenols and in the antioxidant activity. However, there were no significant differences in the ascorbic acid content and the antioxidant activity between the mature and young leaves of both kratoms. The total phenol content of the mature leaves was significantly higher than that of the young leaves. In all samples, the ascorbic acid and total phenols contents and the antioxidant activity all decreased after storage for 12 d. The ascorbic acid and total phenols contents and the antioxidant activity of the leaves held at RT were significantly higher than those of the leaves kept at 10°C. The mature leaves of the kan khiao and hang kang kratom cultivars kept at RT had the highest levels of ascorbic acid, total phenols and antioxidant activity after being stored for 12 d. Compared to the kan khiao leaves, the hang kang leaves had lower losses of these compounds during storage at both temperatures. Galani et al. (2017) reported that refrigeration of vegetables at 4°C for 15 d induced losses of total phenols and ascorbic acid and a reduction in antioxidant activity. Additionally, they discovered that the chilling stress accelerated the breakdown of total phenolic compounds in vegetables. Stevens et al. (2008) reported that the ascorbic acid content of tomatoes decreased due to the inhibition of monodehydroascorbate reductase activity from CI.

Cold storage-induced chilling stress impacted the visual quality and content of bioactive compounds in kratom leaves. Weight loss was linked to the browning of the leaves held at RT while CI was linked to the browning of the leaves stored at 10°C. Compared to mature leaves, young leaves of both the kan khiao and hang kang kratom cultivars were more vulnerable to CI. Cold storage (10°C) induced the loss of FW, reductions in the mitragynine, total phenols and ascorbic acid contents and in antioxidant activity, as well as stimulating leaf browning, tissue electrolyte leakage and a reduced MDA content in the leaves of both kratom cultivars comparison to the leaves stored at RT. Based on these outcomes, kratom leaves should be stored at above a temperature that induced cold storage-induced chilling stress (that is, higher than 10°C) to prevent chilling injury and the loss of bioactive compounds during storage.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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