



RESEARCH ARTICLE

Morphological and physiological properties of kratom (*Mitragyna speciosa*) leaves: Macronutrients, phytochemicals, antioxidants, and mitragynine content

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Abstract

Morpho-physiological characteristics of leaves are significantly associated with photosynthetic capacity and leaf growth. This study was designed to evaluate the relationship between leaf functional traits, nutrients, and their active compounds throughout the developmental stages of kratom leaves. Five growth stages were identified: S1 (7-15 days), S2 (15-30 days), S3 (30-45 days), S4 (45-60 days), and S5 (60-75 days). A comparison of leaf-group stages was conducted based on morpho-physiological traits, macronutrient content, phytochemical analysis, and antioxidant activity. The results revealed that leaf weight and leaf area increased from S1 to S5, with a slight decrease observed in S5. Stomatal density remained similar across all stages. In contrast, chlorophyll and carotenoid contents showed a steady increase up to the S5 stage. The maximum assimilation rate (A_{max}) and light-saturated photosynthetic rate (P_{max}) were achieved at the S2 and S3 stages. Macronutrient levels (N, P, and K) were highest in the younger leaf-group stages (S1 to S2) and significantly different from the older leaf-group stages (S4 to S5). The highest amount of phenolics, flavonoids, and antioxidant activity were found in the middle leaf-group stage (S3). However, anthocyanin content tended to decrease with leaf-group stages. Moreover, the mitragynine content continuously decreased with leaf age, with the highest content found in the young (S1 to S2) and middle (S3) group stages of leaves. Therefore, the productive phytochemical contents in the fully expanded leaves should be considered, especially mitragynine content, which is mainly used in medicinal products.

Keywords

leaf development; leaf nutrients; leaf traits; medicinal plant; photosynthetic capacity

Introduction

Kratom (*Mitragyna speciosa* (Korth.) Havil.), an indigenous tropical plant of Southeast Asia countries, is commonly used as traditional medicine (1, 2). Some people believe that kratom leaves can enhance their workability and productivity (1). It is suggested that the leaves have high potential as a valuable source of analgesic medications due to their pain-relieving properties (3). Because of these medicinal properties, the demand for kratom leaves in herbal medicine is rapidly increasing (4).

There are around 57 active compounds found in kratom leaves, of which 40 are alkaloids. The dominant alkaloids are mitragynine, which makes up 66% of the total, and 7-hydroxymitragynine, accounting for 2%

(5). Mitragynine is the primary active alkaloid, contributing to the significant medicinal benefits of the kratom leaf, such as stimulating workability and analgesic and relaxing effects (6). Various studies have reported that the alkaloid profile of kratom leaves varies significantly by different geographical locations (5, 7). Additionally, alkaloid levels in the kratom leaves are influenced by the agroclimatic environment and the leaf growth stages (8).

Through the alkaloid profiles of the kratom leaves, the explicit composition of phytochemicals is determined, including flavonoids, polyphenols, and antioxidant (9, 10). The leaves of medicinal plants are essential for their growth, function, and survival. The light-absorbing capacity of leaves influences morphological, physiological, and phytochemical traits (11, 12). Studies in *Robusta* coffee (13), grapevine (14), and some woody plants (15) have shown that the development of leaves depends on their age or growth stage. These factors play a crucial role in the morphological construction and physiological functions of kratom plants and serve as fundamental indicators for precise cultivation, ensuring the quality and production of kratom leaves.

This study aimed to identify the developmental stages of kratom leaves and their relationships with leaf morpho-physiological characteristics and phytochemicals. The goal is to obtain fresh raw materials of kratom leaves containing various important substances for further processing and medical use, particularly mitragynine, which is commonly used in medicinal products.

Materials and Methods

Experimental site and material

The experiment was conducted from January to December 2022 at a farm in Sichon district, Nakhon Si Thammarat province, Thailand (latitude 9°2'7.23"N, longitude 99°50'59.86" E, and altitude 10 m above sea level). Ten kratom trees, aged ten years old and exhibiting various leaf development stages, were randomly selected for leaf sample collection. The sample leaves were categorized into five growth stages based on their ages in days: S1 (7-15 days), S2 (15-30 days), S3 (30-45 days), S4 (45-60 days), and S5 (60-75 days) (Fig. 1).

Leaf area, leaf weight, and leaf stomatal density measurements

The individual leaf area (LA) was measured using a leaf area meter (LI-3000C, LI-COR, Lincoln, NE, USA). After harvesting, the sample leaves were weighed to determine their fresh weight (FW), followed by oven drying at 65 °C for 72 hours to measure the leaf dry weight (DW). The fresh and dry weights of leaves at each stage were recorded to establish their relationships. Stomatal quantification was conducted using the nail polish method (16), where nail polish was applied to the adaxial surface of each sample leaf and subsequently peeled off with clear tape. The replicas were then detached, placed on microscope slides, and examined under a compound microscope (Zeiss CP-Achromat 40x Objective) (Carl Zeiss Microscopy, USA). This method provides information about the density and distribution of stomata on the leaf surface.

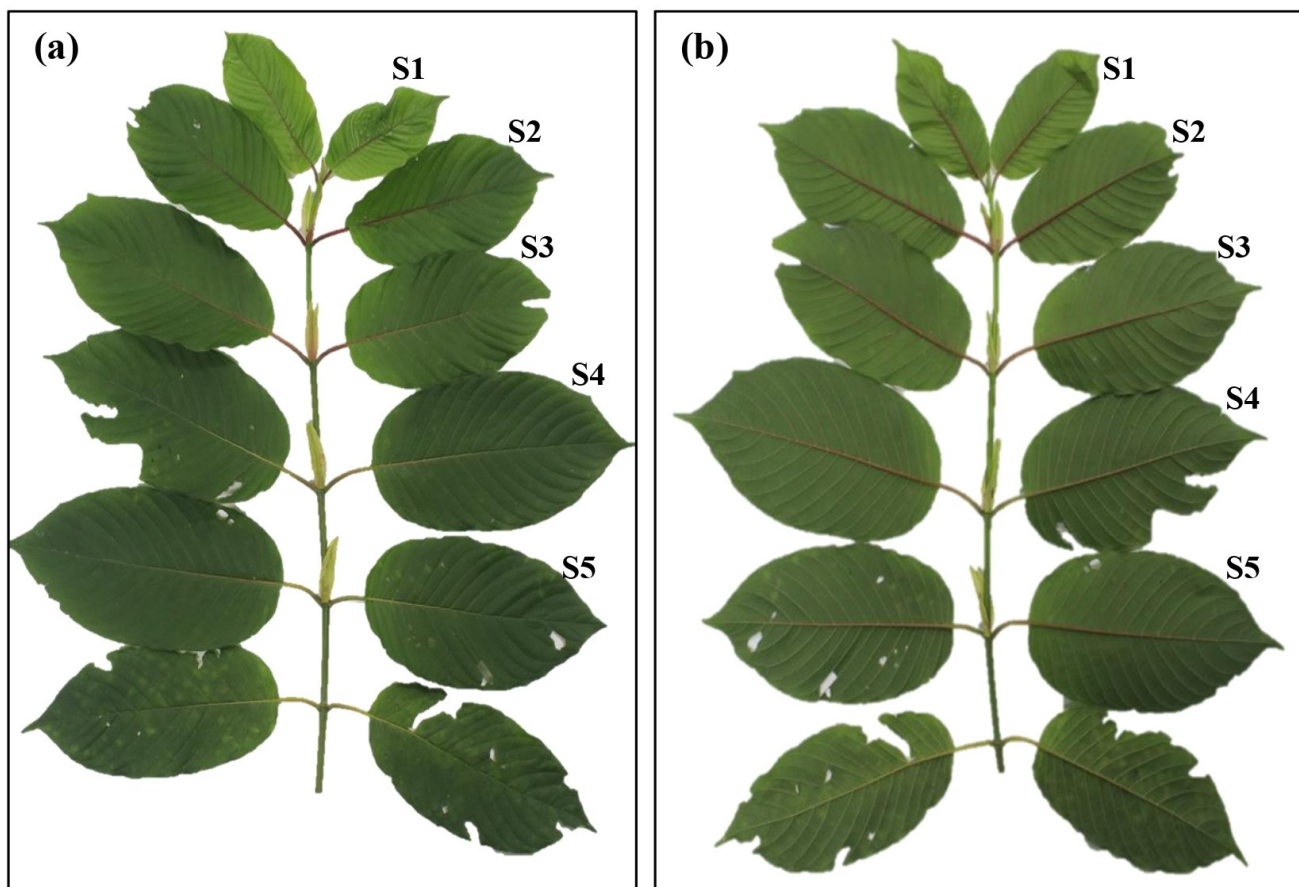


Fig. 1. The sample of growth stages of kratom leaves: S1 (7-15 days), S2 (15-30 days), S3 (30-45 days), S4 (45-60 days), and S5 (60-75 days) of adaxial (a) and abaxial (b) leaf surfaces

Leaf chlorophyll and carotenoids measurements

The photosynthetic pigments, namely, chlorophyll a (Chl_a), chlorophyll b (Chl_b), and total chlorophyll (Chl_{total}), as well as carotenoids (Car), were extracted using *N,N*-dimethylformamide (DMF) following a modified method of Netto et al. (17). The linear correlations between the contents of pigments (chlorophyll and carotenoid) and their respective chlorophyll meter readings (Dualox, DX19007, Force A, France) were measured and calculated using the following equations: $Chl_a = 0.5113x - 3.7332$ ($r^2 = 0.894$), $Chl_b = 0.194x - 1.0394$ ($r^2 = 0.869$), $Chl_{total} = 0.7053x - 4.7726$ ($r^2 = 0.890$), and $Car = 0.094x - 0.3657$ ($r^2 = 0.941$).

Leaf photosynthetic measurements

Leaf photosynthesis at various stages of leaf development was analyzed in field trial conditions using a portable photosynthesis system (LI-6800, Li-Cor Inc., Lincoln NE, USA). The quantitative responses associated with leaf photosynthesis were obtained for the light-saturated photosynthetic rate (P_{max}), dark respiration (R_d), light compensation point (I_c), and light saturation point (I_s). Photosynthetic curves were determined under an altered ambient LED light source at intervals of 0 to 2,000 $\mu\text{mol}/\text{m}^2/\text{s}$. The ambient CO_2 concentration and leaf chamber temperature were constantly controlled at 400 $\mu\text{mol}/\text{mol}$ and 28°C, respectively. Additionally, CO_2 concentration was regulated from 0 to 2,000 $\mu\text{mol}/\text{mol}$ initially. Moreover, CO_2 compensation (τ) and mesophyll conductance (g_m) were also determined using a curve fitting method (14).

Leaf macronutrients measurements

The leaf samples were collected from each treatment and dried at 65 °C for 72 hours. The samples were then ground and sieved through 20- and 40-mesh sieves. Finely ground tissues were digested with H_2SO_4 to determine the total leaf nitrogen content (N) using the Kjeldahl method. Additionally, they were mixed with HNO_3 and HClO_4 ($\text{HNO}_3:\text{HClO}_4 = 3:1$ v/v) for P, K, Ca, and Mg analyses using spectrometry (18).

Analysis of phytochemical compositions and antioxidant activity in kratom leaves

Leaf samples preparation

The leaf samples from each group of stages (S1 to S2, S3, and S4 to S5) were first dried and powdered. The sample powders were then soaked in 95% ethanol solvent at a sample-to-solvent ratio of 1:10. Afterward, the solution was sieved, and the extracts and solvent were separated using a centrifuge (Cryste, Bucheon-si, Korea) at 4,000 rpm for approximately 15 minutes. The sediments were evaporated using a rotary evaporator (Buchi, Flawil, Switzerland) in a water bath (Alpha A12, Lauda, Germany) to remove the remaining ethanol. Then, the extracted samples were placed into bottles respectively. A 0.025 g sample was taken from each bottle and mixed with ethanol in preparation for analysis.

Antioxidant activity

To determine the antioxidant capacity of the sample, the Ferric Ion Reducing Antioxidant Power (FRAP) assay was

used for measurement. It was prepared by mixing 300 mM sodium acetate, 10 mM TPTZ solution, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution at a 10:1:1 ratio (19). The sample and FRAP reagent were mixed in a ratio of 0.1:4.5 ml, and the mixture was shaken for 10 min in the dark at room temperature. After 30 minutes of incubation at 37 °C, the solution's absorbance was measured at 593 nm (13).

Total phenolic content (TPC) and total tannin content (TTC)

These parameters were analyzed using the modified Folin-Ciocalteu method (13, 20). For TPC determination, the phenolic standard was mixed with 2 mL of Folin-Ciocalteu reagent and 1.5 mL of 7.5% NaCO_3 . The solution was then shaken and kept in the dark for 2 hours. The same process was carried out for TTC, but with different solvent volumes. The tannin standard was mixed with 1.6 mL of Folin-Ciocalteu reagent and 2 mL of 7.0% NaCO_3 , then agitated and placed in the dark for 1.5 hours. A standard curve was constructed using 25 mg of gallic acid. The absorbance of the solution was measured at 751 nm and 760 nm for TPC and TTC, respectively, using a spectrophotometer (13).

Total flavonoid content (TFC)

To analyze TFC, an aluminum chloride colorimetric assay was employed (21). The sample solution was prepared by combining 0.4 mL of the sample with 4 mL of distilled water, 0.3 mL of 5% NaNO_2 , and 0.3 mL of 10% AlCl_3 . After incubating for 5 minutes, the solution was left to stand for an additional 6 minutes. Subsequently, 2 mL of 1 mol/L NaOH was added, and distilled water was added to adjust the volume to 10 mL. After allowing the solution to stand for 15 minutes, absorbance was measured using a spectrophotometer at a wavelength of 510 nm (13).

Total anthocyanin content

Anthocyanin content was determined using the spectrophotometric method (22). All samples were macerated with a mixture of 95% ethanol and 1.5 mol HCl/L in an 85:15 ratio and left to rest for 12 hours at 4 °C. The solution was then adjusted to a volume of 100 ml (23). Total anthocyanin content (mg/g) in the leaves was calculated using the following formula: Total anthocyanin = $(A_{530} \times \text{dilution factor})/98.2$, where, 98.2 is the molar absorption value of cyanidin-3-glucoside for the acid-ethanol solvent. Absorbance readings at 530 nm were taken using a UV-VIS spectrophotometer (UV-1900i, Shimadzu, Japan), then converted to mg/g using the formula (24).

Mitragynine and alkaloid contents

Each leaf-group stage (S1 to S2, S3, and S4 to S5) of leaf powder was extracted with 80% methanol (CH_3OH) at a 1:10 (w/v). The mixture was vigorously shaken and allowed to stand overnight. Subsequently, the solution was centrifuged at 3,000 rpm for 15 minutes to separate the precipitate from the extract. The methanol was evaporated using a rotary evaporator under vacuum until nearly completely evaporated, resulting in a dry crude alkaloid extract. A standard solution of mitragynine in methanol was prepared according to the kratom extraction method (25, 26). High-

Performance liquid chromatography (HPLC) at the scientific Equipment Center, Prince of Songkla University, was used to analyze mitragynine concentrations. This procedure included the use of a pump based automated injection system (Waters 2695, Waters Associates, Milford, MA, USA), with photodiode array detection (Waters 996) at 225nm to monitor the column effluent.

Statistical analysis

Data were presented as mean values along with their standard deviations. ANOVA analysis was conducted to assess the variance in leaf morpho-physiological, leaf nutrients and phytochemical parameters across stages of kratom leaf development. Duncan's Multiple Range Test (DMRT) was employed for comparing the data, with significance considered at a p -value ≤ 0.05 .

Results and Discussion

Leaf growth performance at different stages of kratom leaves

The leaf growth performance was assessed based on fresh weight, dry leaf weight, and leaf area. Significant relationships were observed between leaf dry weight and fresh weight of kratom leaves across various stages (Fig. 2a). The results indicated a significant influence of leaf fresh weight on its dry weight ($y = 0.2784x + 0.0437$, $r^2=0.939$). Additionally, the leaf area, measured using a leaf area meter based on width and length, exhibited a linear relationship ($y = 0.7103x + 3.3955$, $r^2=0.987$) (Fig. 2b). Notably, during the initial growth stages, there was a sharply increase in leaf area (from S1 to S2), followed by steady growth in fully expanded leaves (S3) (Fig. 2c). This suggests that the fresh mass proportionately increased with the increase in leaf dry weight until the leaf area reached its maximum size before senescence due to ongoing expansion and transition acceleration (27, 28). Given the critical role of leaf area in productivity and its peak size before senescence, harvesting leaves during stages S2 to S4 is recommended.

Chlorophyll and carotenoid contents, and stomatal density in different stages of kratom leaves

Differences in chlorophyll (Chl_a , Chl_b , and Chl_{total}) and carotenoid (Car) contents across various stages (S1 to S5) of kratom leaves are depicted in Fig. 3a. Notably, all chlorophyll contents exhibited a consistent pattern, with the lowest levels observed in the youngest leaves (S1), and the highest levels in the oldest at stage S5. This trend mirrors finding from a study on Robusta coffee plants (13), which reported a gradual increase in chlorophyll content in older leaves. Similarly, changes in carotenoid contents across different stages (S1 to S5) followed a pattern akin to chlorophyll content. Previous research has highlighted a correlation between carotenoid and chlorophyll levels (29). Although there were no significant difference in stomatal density among the stages, the oldest leaves (S5) exhibited higher stomatal density compared to the preceding stage (Fig. 3b). This increase in stomatal density during leaf senescence is associated with the regulatory mechanism of leaves. Stomatal density adjustment is recognized as an

important trait for plant tolerance under environmental stress (16).

Physiological process of kratom leaves at different stages

Differences in leaf growth stages significantly influenced CO_2 concentration for assimilation rates. As depicted in Fig. 4a, the assimilation rate gradually increased with rising CO_2 concentration. The peak CO_2 concentration (800-1,200 $\mu molCO_2/mol$) corresponded to a high assimilation rate (A_{max}) ranging between of 20-45 $\mu molCO_2/m^2/s$ across all leaf growth stages (S1 to S5). The highest CO_2 concentration was associated with the A_{max} (14,30). Notably, all growth stages exhibited a similar pattern of assimilation rate, with fully expanded leaves demonstrating the highest value.

In contrast, the highest photosynthesis rate (P_{max}) varied across leaf ages, with the peak light intensity observed around 1,200 $\mu mol/m^2/s$ (Fig. 4b). Interestingly, the study revealed differences in light compensation points between young and mature kratom leaves. This finding aligns with observations in grapevine leaves, where similar light responses were observed across all stages of leaf development throughout the growing season, indicating an increase in carbon assimilation with increasing Photosynthetic Photon Flux Density (PPFD) (31, 32).

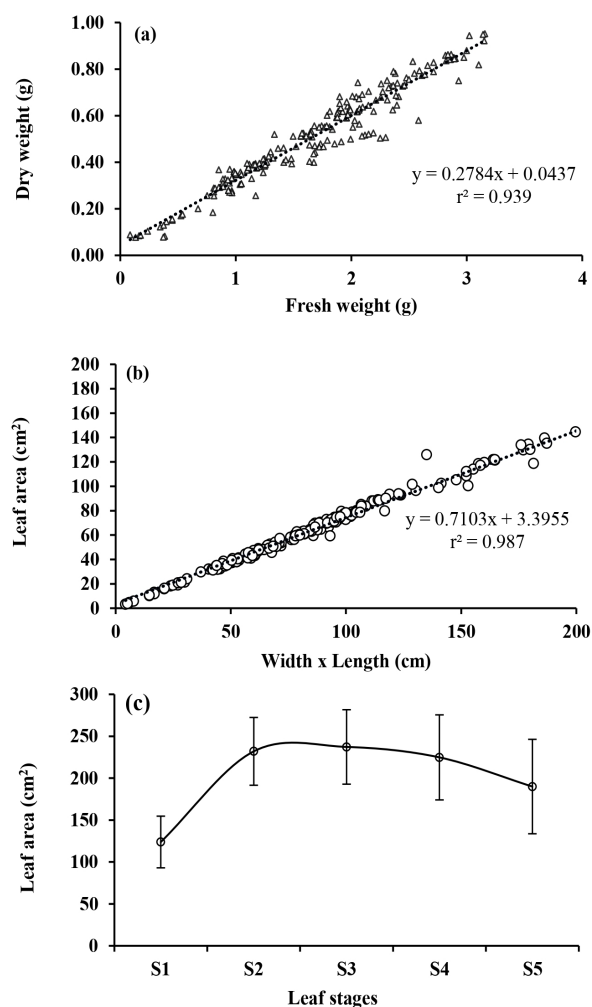


Fig. 2. The relationship between fresh weight and dry weight (a), linear regression relationships between measured leaf area (width x length, cm^2) and leaf area meter (cm^2) (b), and changes in leaf area (cm^2) in difference stages (S1-S5) of kratom leaves (c)

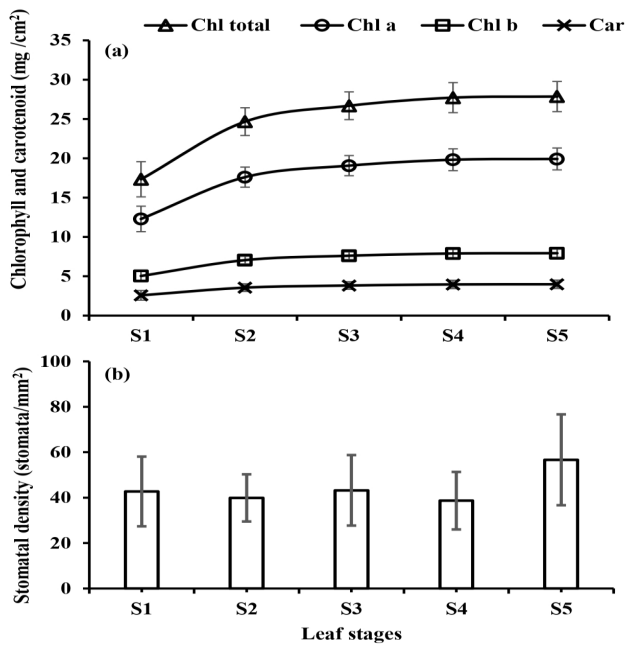


Fig. 3. Changes in the contents (a) of chlorophyll (Chl_{total}), chlorophyll a (Chl_a), chlorophyll b (Chl_b) and carotenoid (Car), and stomatal density (b) in difference stages (S1-S5) of kratom leaves

The pattern of light-saturated photosynthetic rate (P_{max}), dark respiration (R_d), light compensation point (I_c), light saturation point (I_s), CO_2 compensation point (τ), and mesophyll conductance (g_m) response across different stages of kratom leaves (Fig. 5). P_{max} exhibited a similar trend to R_d , I_c , I_s , and g_m throughout leaf development stage, with peak value observed at stages S2 to S3, followed by a gradual decline towards the end of leaf growth (S5). The distinct pattern was observed in τ during the early stage, characterized by a sharp decline from the onset of leaf development at stage S1, reaching its lowest values at stages S2 to S3. Subsequently, a gradual increase was observed until stage S5. These findings suggest that younger kratom leaves exhibit higher metabolic activity compared to older leaves, likely attributable to their greater photosynthetic capacity. This phenomenon is consistent with previous research indicating that photosynthetic capacity increases during leaf development and declines thereafter, potentially influenced by changes in mesophyll thickness and chloroplast distribution across the leaf surface (14, 33).

Macronutrient, phytochemical and mitragynine content of kratom leaves at different stages

The macronutrient content of kratom leaves at different stages is presented in Fig. 6a, encompassing N, P, K, Ca, and Mg, observed across three leaf growth stages (S1 to S2, S3, and S4 to S5) as representative groups. Significant differences were observed in all macronutrient content. Higher macronutrient values were evident in the early leaf stages (S1), while lower content was observed during leaf senescence (S5), with the exception of Ca, which exhibited a contrasting trend. The source-sink transition progresses basipetally along the leaf, coinciding with changes in physiological, biochemical, and anatomical properties (34). Variations in macronutrient content (such as N, P, K, and Mg) throughout kratom leaf development may stem

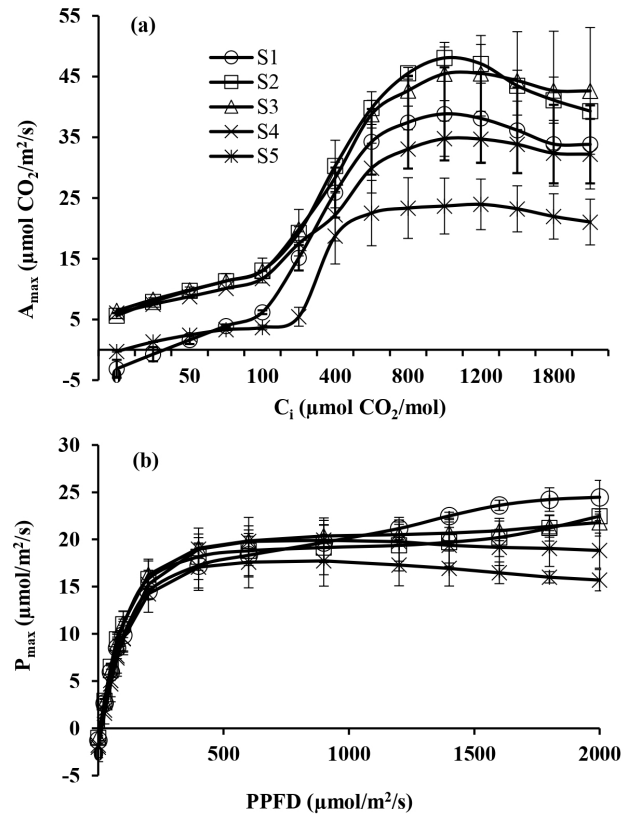


Fig. 4. Carbon response curves (A_{max}) in difference CO_2 concentration (a), and light-saturated photosynthetic response curves (P_{max}) in difference light intensity (b) in different stages (S1-S5) of kratom leaves

from the mobilization of these nutrients from older to younger leaves. Conversely, Ca, known for its relative immobility in the phloem of most plant species (35, 36, 37), showed a different distribution pattern. Furthermore, nutrient content and photosynthetic capacity in kratom leaves exhibited a parallel trend with leaf age, peaking upon full leaf expansion and declining during leaf senescence.

The content of phenolics, flavonoids, and antioxidants exhibited a consistent trend, characterized by an initial increase during the early leaf growth stages (S1 to S2, and S3), followed by a decline in the senescence stages (S4 to S5) (Fig. 6b). This pattern mirrors physiological traits such as photosynthesis, which plays a crucial role in the biosynthesis of secondary metabolites. Additionally, a significant positive correlation exists between the production of total phenolics, flavonoids, and antioxidant activity and photosynthetic functions (38, 39). The tannin content exhibited a distinct pattern compared to other phytochemicals, showing a tendency to increase up to the leaf senescence group stage (S4 to S5). This finding contrasts with observations in Robusta coffee leaves (13) and mangrove (40) leaves, where tannin content was higher in young leaves than in old leaves. In contrast, the anthocyanin content showed only a slight decline as leaves matured from stage S1 to S5. This suggests that anthocyanin production may be sustained even during senescence, unlike other compounds that are typically more susceptible to photoinhibition in mature leaves (41). Such findings underscore the dynamic nature of kratom's secondary metabolite profile throughout its leaf development.

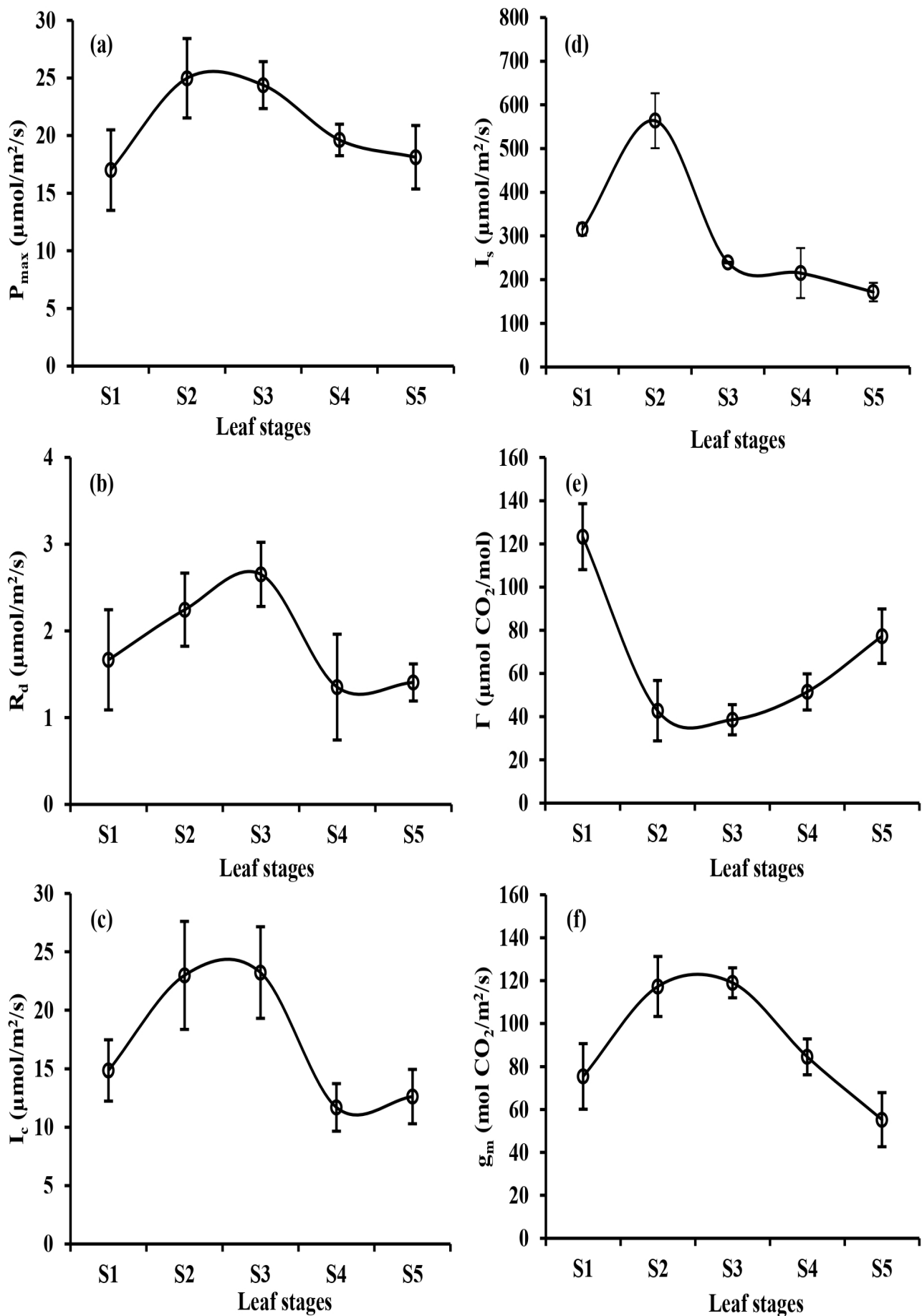


Fig. 5. Changes in light-saturated photosynthetic rate (P_{max}) (a) dark respiration (R_d) (b), light compensation point (I_c) (c), light saturation point (I_s) (d), CO_2 compensation point (Γ) (e), and mesophyll conductance (g_m) (f) in different stages (S1-S5) of kratom leaves

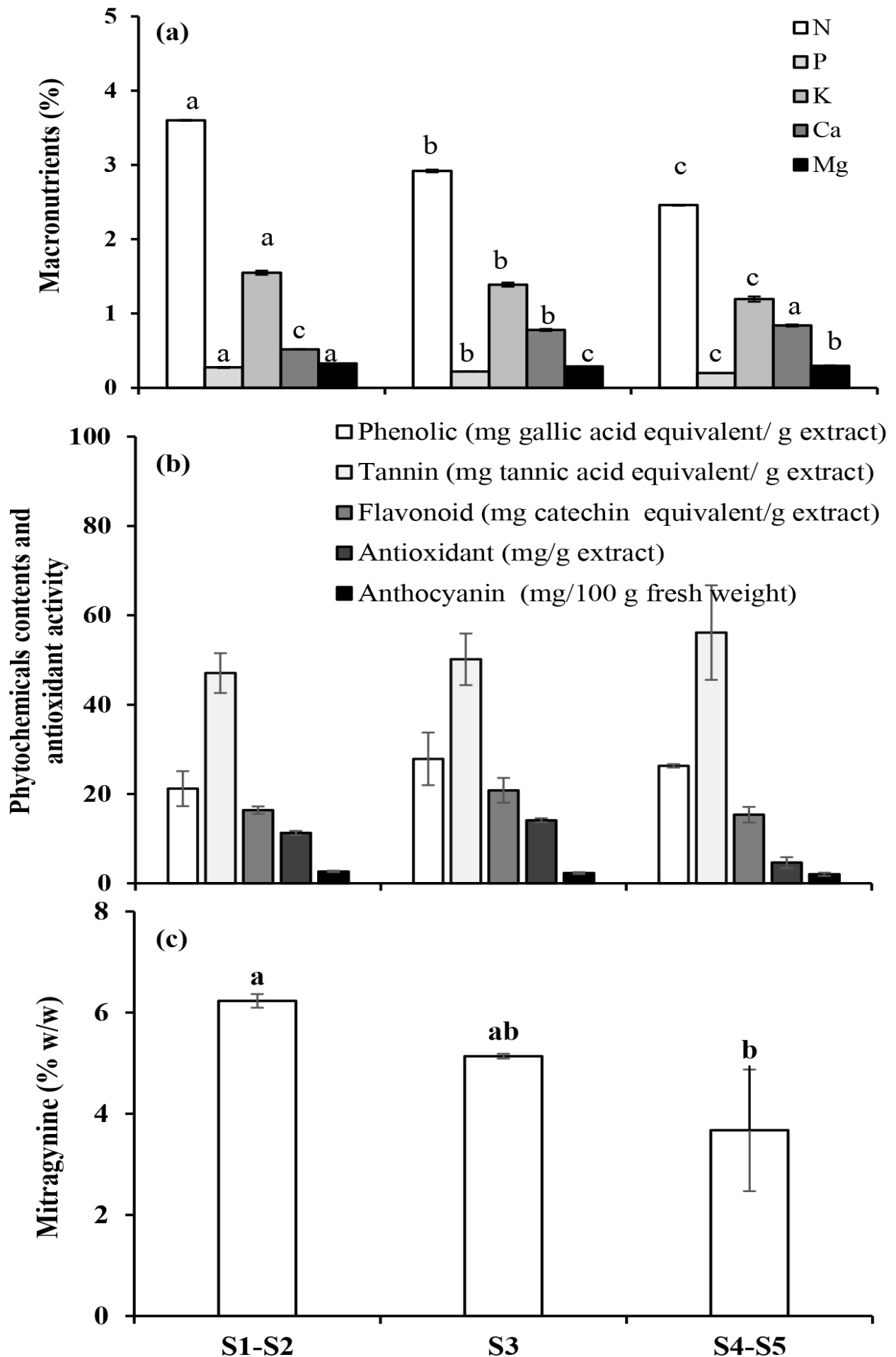


Fig. 6. The contents of macronutrients (a) phytochemicals and antioxidant activity (b), and mitragynine (c) in different stages (S1 to S2, S3, and S4 to S5) of kratom leaves

Furthermore, the mitragynine content of kratom leaves exhibited a significant difference across various leaf stages (Fig. 6c). The content of mitragynine decreased continuously with leaf age, with the highest levels observed in young leaves and the lowest in older ones. This pattern shared similarities with the development trend of nitrogen content in kratom leaf growth stages. Previous studies have highlighted the influence of nitrogen on mitragynine levels (42). However, it's worth noting that the mitragynine content in kratom leaves is influenced by various factors, including geographical and climatic conditions, as well as leaf growth dynamics (43, 44, 45). Therefore, the variation in mitragynine content across leaf development stages underscores the importance of timing of leaf harvesting to optimize production and potency.

Hence, the distinct growth stages of kratom leaves notably influence their morpho-physiological functions, often correlating with variations in phytochemical contents. Determining the optimal harvest timing for kratom leaves should be guided by the specific active compounds desired.

Conclusion

This study examined how leaf development influences the morphological and physiological properties of kratom leaves. The findings revealed a distinct pattern: most traits increased from the youngest stage (S1) to the fully expanded leaf stage (S3), then declined during leaf senescence (S5), with peak values occurring between stages S2 and S3. Macronutrient content (N, P, and K) also varied significantly across leaf growth stages, with young leaves consistently displaying higher levels of these nutrients compared to older leaves. Notably, the content of phenolics, flavonoids, and antioxidant activity followed a different pattern, tending to increase with leaf age before decreasing during senescence. However, mitragynine content peaked in fully expanded leaves before declining in older leaves. Considering these findings, optimal harvesting of kratom leaves should be timed to maximize phytochemical and mitragynine content, with leaves harvested during the fully expanded stage (S3) likely to be most beneficial.

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Authors' contributions

WP & ID drafted the manuscript. WP, CS, AC & RC carried out the field survey, morpho-physiological studies, and performed the statistical analysis. The conceptualization, supervision of experiments, and edited manuscript were participated by RC. Also, SS assisted in analysis of the

mitragynine content. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors have declared no conflicts of interest.

Ethical issues: None.

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