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Comparative Study on Pigment Components (Flavonols and Anthocyanins) and Antioxidant Capacity in Different Colored Double-petal Lotus

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Abstract:

Lotus, an edible flower with high ornamental values, takes forms in many sizes, shapes, and colors. Among which, double-petal lotus not only has higher ornamental values but also gives a yield advantage in exploitation due to the large number of petals. Flower color is an important ornamental indicator, and flavonoids are the key basis for color presentation as well as important active components in lotus. This research used petals of four different colored double-petal lotus cultivars to identify their petal color, pigment localization, and pigment components (flavonols and anthocyanins), as well as the antioxidant activity of the petal extraction solution using the ABTS method. It was discovered that the white YMQ was the brightest but had the lowest Chroma and the third-highest Hue angle, while the dark red WFMH was the darkest but had both the highest Chroma and Hue angle. Most of the colored pigments were located in all the thin-walled cells immediately adjacent to both the upper and lower epidermis. The yellow MLQS had the highest amount of Quercetin derivatives in flavonols but the lowest antioxidant activity, the dark red WFMH had the highest amount of anthocyanin, and the pink MDZ had the highest antioxidant activity. This research offers insights into the potential medical usage of certain double-petal lotus cultivars like MLQS and WFMH, hopefully providing a reference for the identification of raw materials for extracting pigments to improve sleep patterns and prevent Alzheimer's Disease.

Keywords: Nelumbo, flower color, antioxidant activity, flavonoids

1. Introduction

Lotus, one of the ten most famous Chinese traditional flowers, has a history of nearly 3000 years of cultivation in China (Wang et al., 2023). Based on morphological characters, the lotus consists of two species, *Nelumbo nucifera* Gaertn. (Asian lotus) which is distributed in Asia and the north of Australia, and *N. lutea* Willd. (American lotus) which is distributed in the northeast of North America and the north of South America (Deng et al., 2013). Not only does lotus possess high ornamental, cultural, and religious values, but it also exhibits a well-known homology for food and medicine. The petals are edible as a fried dish, the seeds as fruits, and both the leaves and embryos can be made into tea (Liu et al., 2023). In addition, various parts play a significant role in Chinese medication. For example, the Nelumbinis Semen can help with palpitation and insomnia, while the Nelumbinis Folium can stop bleeding (Chinese Pharmacopoeia Commission., 2020.).

Lotus flower mostly appears in white, yellow, pink, and red. Flower color is observed as a petal (or other flower organ) is exposed to light and the pigment layer is penetrated. The light is partially absorbed, while the remaining is reflected by the sponge tissue and passes back to the pigment layer, sensed as colors by our eyes (Zhao & Tao, 2015). There are many factors that can influence the formation of flower colors, among which colored pigments are the most significant factors. Some of the most common pigments in lotus include flavonols and anthocyanins, which are two major subclasses of flavonoids and are beneficial to both plants and human bodies (Deng et al., 2013). Apart from helping plants to survive, reproduce, and adapt to the environment, flavonoids can help to improve eyesight and memory, prevent cancer and atherosclerosis, lower blood sugar, and exhibit physiological activities including anti-hyperlipidemia, anti-oxidation, and anti-aging effects (Yin et al., 2015). Flavonoids may be

detected and analyzed by High-Performance Liquid Chromatography (HPLC) and Waters ACQUITY Ultra Performance Liquid Chromatograph (UPLC I-CLASS, Waters) system, while their total antioxidant capacity (T-AOC) can be measured using ABTS assay, DPPH assay, ferric reducing antioxidant power (FRAP) assay, and Oxygen Radical Absorbance Capacity (ORAC) assay (Chen et al., 2013; Liu et al., 2023; Wang et al., 2023)

Previous studies have investigated anthocyanin biosynthetic pathways and key genes, as well as the physical and chemical factors that can regulate flavonoid pigments to mediate flower color development (Zhao & Tao, 2015). The ornamental value of lotus has already been fully explored, and its pro-healthy activity from the edible parts like the seeds has also been confirmed by reviewed studies using in vitro models and human trials (SkrajdaBrdak et al., 2020). However, for double-petal lotus especially, as some cultivars of it produces little seeds but a high number of petals, there is still a lot of room left to explore its value in petals, either in medical, healthcare, or cosmetic products, for example. Four different colored double-petal lotuses were used to fill in the gap. We measured perceivable colors on the petals using a CIE L*a*b* device, conducted a microscopic observation of the sliced colored petals, detected and analyzed flavonoids using HPLC, and measured the antioxidant activity of the extracted solutions. This research is conducted to explore the numerical differences of perceivable colors in lotus petals, the localization of different colored pigments as well as how their compositions lead to the formation of different colors, variations among the level of antioxidant activities, and whether they are correlated to the amount of flavonoids detected in the lotus. Hopefully the findings can provide valuable references for the comprehensive utilization of lotus in regard to the color differences in petals, and promoting the application of natural pigments in cosmetics, healthcare, and medications.

2. Materials and Methods

2.1 Plant materials

Four different colored double-petalled lotus cultivars from Chenshan Botanical Garden were used in the present study, including Nelumbo 'Yimengqu' (YMQ) which is white, Nelumbo 'Weifangmohong' (WFMH) which is dark red, Nelumbo 'Modouzi' (MDZ) which is pink, and Nelumbo 'Molingqiuse' (MLQS) which is yellow.

2.2 Detection of Perceivable Colors on Petals

Three petals from the inner ring of each lotus sample were taken for the detection of perceivable colors. The petal colors were characterized by the *CIE L*a*b** color measurement system and the three parameters were determined using the Konica Minolta Portable Spectrophotometer CM-2300d (Konica Minolta, Japan). The L^* value indicates the lightness ranging from 0-100, the a^* value indicates the color spectrum from red to green, and the b^* value indicates the color spectrum from blue to yellow. Chroma (C^*) is calculated from $C^* = (a^{*2} + b^{*2})^{1/2}$ and the Hue angle (h) is calculated from $h = \arctan(b^* / a^*)$.

2.3 Microscopic Observation of the Sliced Colored Petals

A freehand section was conducted on the petals to elucidate petal structure as well as the distribution of pigments among the petals. The petal section was observed under the Olympus BX43 Upright Microscope (Olympus, Japan). Three levels of magnification were taken, specifically 4x, 10x, and 20x.

2.4 Extraction of Flavonoids

The extraction solution was prepared with the volume ratio of formic acid, methanol, and water at 2:80:18. Fresh petals were ground into powders under liquid nitrogen conditions. 300 mg sample powder was added into a 2 mL tube, and then 1 mL extraction solution was also added in. The mixture was mixed thoroughly with a vortex oscillator. The tube was placed in an ultrasonic cleanser for 30 minutes for better extraction. After sonication, the tube was centrifuged at 12,000 rpm for 10 minutes. The supernatants were transferred into the 5 ml centrifuged tubes, and the extraction procedure was repeated twice. A final of 3 ml of supernatant for each sample was collected and stored in a -40 ℃ refrigerator for further measurements.

2.5 Flavonoid Detection and Identification

High-Performance Liquid Chromatography with the Agilent 1260 HPLC System was used to detect the flavonoids with the column TSK gel ODS-80Ts QA C18 (250 mm × 4.6 mm) (Tosoh, Japan). The wavelength for detecting flavonol was 350 nm, and the wavelength for detecting anthocyanin was 520 nm, while the spectra range of detection was between 200 and 600 nm. 2% formic acid solution (A) and 100% acetonitrile (B) were used for the mobile phase. A gradient elution program followed: 0 min, 8% B; 6 min, 16% B; 14 min, 20% B; 24 min, 24% B; 30 min, 36% B; 32 min, 92% B; 34–38 min, 8% B. The column temperature was held constant at 30 ℃, with a detection speed of 0.8 mL/min and sample injection volume of 10 μL. An Agilent 6520 HPLC system combined with an accurate-mass Q TOF LC/MS was used for flavonoid identification. Both negative and positive modes were determined.

A standard curve equation of quercetin 3-*O*-glucuronide

(y = 27526x, R^2 = 0.9797) was plotted to determine the flavonol content (mg/g FW), while a standard curve equation of cyanidin 3-*O*-glucoside (y = 29463x - 28.374, R^2 = 0.9936) was plotted to determine the anthocyanin content (mg/g FW).

2.6 Antioxidant Activity Assessment

The total antioxidant capacity was measured using the ABTS assay test box. ABTS is oxidized to green ABTS+ with appropriate oxidants, but the production of ABTS+ is inhibited under the presence of antioxidants. The total antioxidant capacity is determined by measuring the absorbance of ABTS+ at 405 nm. A series gradient concentration of Trolox solution was tested to obtain a standard curve equation (y = -0.8879x + 1.0027, R^2 = 0.9957). The total antioxidant capacity of the extract was expressed as mM Trolox/g FW.

2.7 Statistical Analysis

Each experiment contained three repeats and all data were reported as mean values ± SDs. Microsoft Office Excel 16.88 was used to calculate the average values and standard errors. Microsoft Office Excel 16.88, SigmaPlot 12.0 (SigmaPlot, Sysat software company, Kratt, Germany), and OriginPro 9.0 were used for plotting. SPSS 25 was used for Pearson correlation coefficient analysis.

3. Results

3.1 Petal color measurement by CIE L*a*b* color coordinate

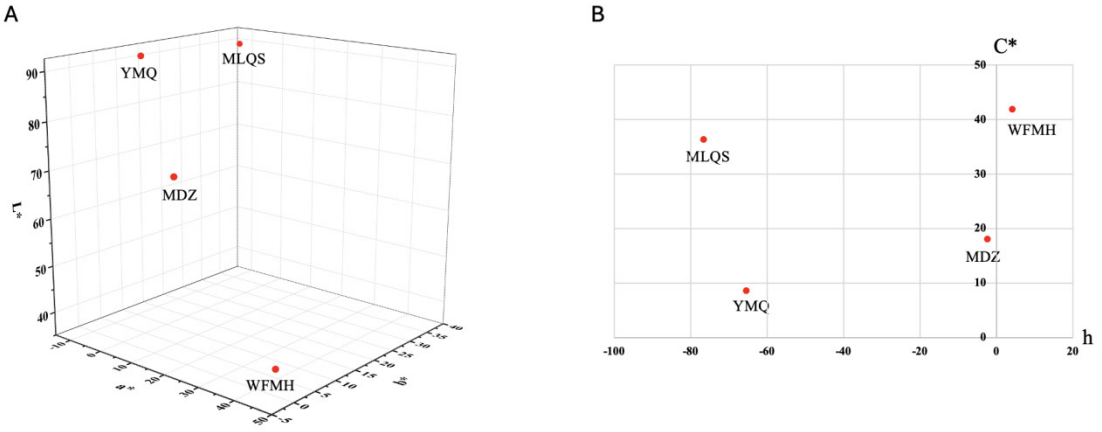

Fig 1. *CIE L*a*b** **color measurement system values**

A. Three-dimensional visualization of the *L**, *a**, and *b** values; B. Two-dimensional visualization of the calculated C* and h values.

The *L** value indicates the lightness ranging from 0-100, where the numerical value and brightness are positively correlated. The white YMQ is the brightest with the highest value of 92. The yellow MLQS is also very bright and has a value of 90, very close to YMQ. The pink MDZ has a value of 73 (Fig. 1A), while the dark red WFMH has a value of 38, much lower than MDZ.

The *a** value indicates the color spectrum from red to green, where the more negative the value, the deeper the color of green; the more positive the value, the deeper the color of red. The dark red WFMH has the deepest red with a value of 42, which is followed by the pink MDZ with a value of 18. Both the yellow MLQS and the white YMQ have no red color, as their values are both negative, -8 and -3, respectively (Fig. 1A).

The *b** value indicates the color spectrum from blue to yellow, where the more negative the value, the deeper the color of blue; the more positive the value, the deeper the color of yellow. The yellow MLQS has the deepest yellow with a value of 35, which is then followed by the white YMQ and the dark red WFMH, with values of 8 and 3, respectively. The pink MDZ has the deepest blue with a value of -1 (Fig. 1A).

Among all, the dark red WFMH has both the highest Chroma and Hue angle. The yellow MLQS has the second-highest Chroma but the lowest Hue angle. The pink MDZ has the third-highest Chroma and the second-highest Hue angle. The white YMQ has the lowest Chroma and the third-highest Hue angle (Fig. 1B).

3.2 Microscopic Observation of Pigment Location in Petal

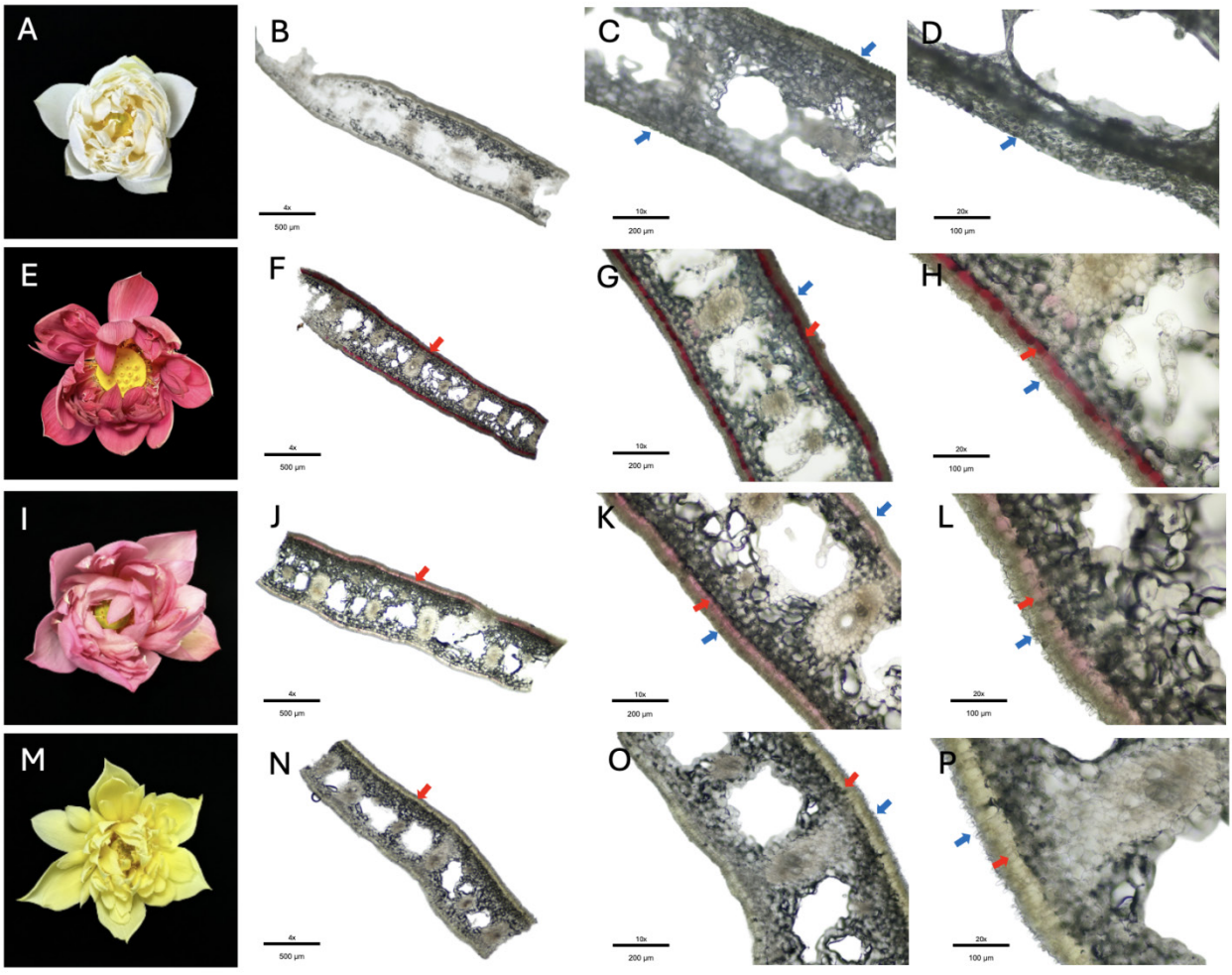

Fig 2. Microscopic observation of Sliced Colored Lotus Petals

A. E. I. M. Lotus flowers in white, dark red, pink, and yellow, (YMQ, WFMH, MDZ, and MLQS) respectively; B. C. D. Transection of the white petal; F. G. H. Transection of the dark red petal; J. K. L. Transection of the pink petal; N. O. P. Transection of the yellow petal. The red arrows showed where pigments were presented and the blue ones showed the conical epidermal cells.

The microscopic observation showed that the lotus petal structures did not have fenestrated tissue but mainly upper and lower epidermal cells as well as spongy tissue. There was a layer of regularly arranged thin-walled cells within each upper and lower epidermal cells. The white petals were free of colored pigments (Fig. 2B, C, D). The dark red petals were rich with red pigments in all the thin-walled cells immediately adjacent to both the upper and lower epidermis (Fig. 2F, G, H). Both the pink and yellow petals had pigments presented on both sides of the epidermal cells (Fig. 2J, K, L, N, O, P), but both had more and deeper colored pigments on the petal adaxial surface than on the adaxial surface.

3.3 Pigment extraction

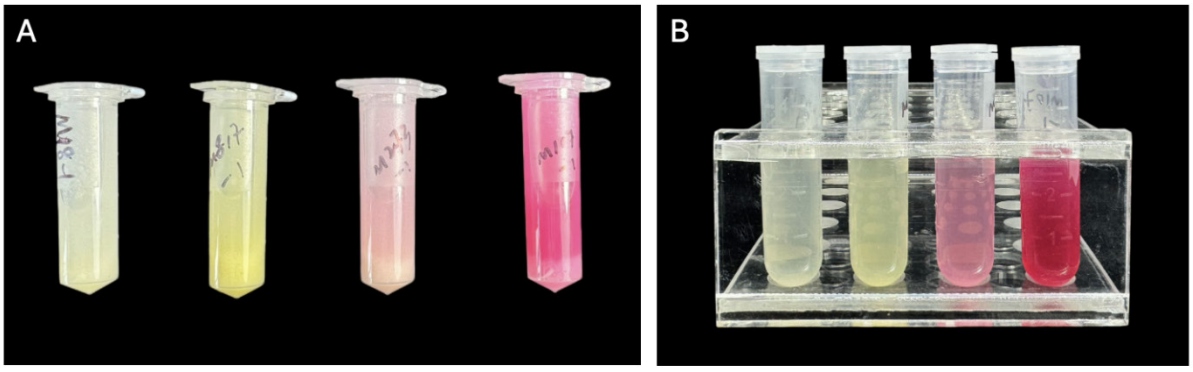

Fig 3. Pigment extractions

A. Centrifuged pigment extractions; B. Extracted supernatants of the pigment extractions.

All pigment extractions had corresponding colors with each of the colored lotus. The white YMQ extracted milky white pigments; the yellow MLQS extracted lemon yellow pigments; the pink MDZ extracted peach pink pigments; and the dark red WFMH extracted rose red pigments (Fig. 3A). The extracted supernatants had similar but lighter and clearer colors (Fig. 3B). All colored pigments are shown to be extracted when the powders turn white. However, yellow powders remained, indicating there are still some colored pigments left, possibly carotenoids.

3.4 Flavonoid detection

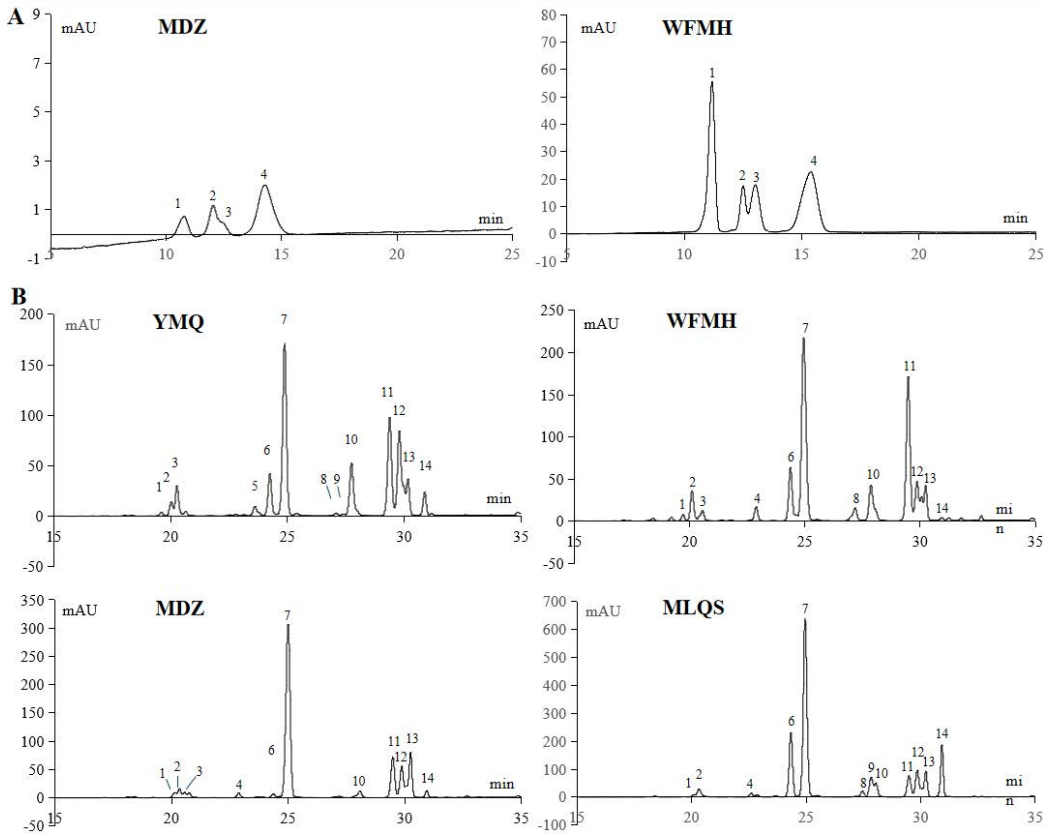

Fig 4. HPLC chromatograms at 520 nm of anthocyanins (A) and at 350 nm of flavonol (B)

A. Four anthocyanins (peaks 1-4) were separated in MDZ and WFMH; B. Totally 14 flavonols (peaks 1-14) were separated from the four cultivars.

An abundant amount of anthocyanins was only present in

WFMH and consisted of 4 sub-kinds, delphindin 3-*O*-glucoside (peak1), cyanidin 3-*O*-glucoside (peak2), petunidin 3-*O*-glucoside (peak3), and malvidin 3-*O*-glucoside (peak4) (Fig. 4A). MDZ also had an absorption peak at 520nm, but the amount of anthocyanin was too low to be integrated.

A total of 14 flavonols were separated from the four cultivars:p1 Myricetin 3-*O*-galactoside; p2 Myricetin 3-*O*-glucoside; p3 Quercetin 3-*O*-arabinopyranosyl-(1→6)-galactopyranoside; p4 Quercetin 3-*O*-rutinoside; p5 Quercetin 3-*O*-galactoside; p6 Quercetin 3-*O*-glucoside; p7 Quercetin 3-*O*-glucuronide; p8 Kaempferol-3-*O*-robinobioside; p9 Kaempferol 3-*O*-galactoside; p10 Isorhamnetin 3-*O*-rutinoside; p11 Kaempferol 3-*O*-glucoside; p12 Kaempferol 3-*O*-glucuronide; p13 Isorhamnetin 3-*O*-glucoside; p14 Isorhamnetin 3-*O*-glucuronide (Fig. 4B). The four lotus cultivars varied in their pigment compositions: YMQ lacked Quercetin 3-*O*-rutinoside; WFMH lacked Quercetin 3-*O*-galactoside and Kaempferol 3-*O*-galactoside; MDZ lacked Quercetin 3-*O*-galactoside, Kaempferol-3-*O*-robinobioside, and Kaempferol 3-*O*-galactoside; while MLQS lacked Quercetin 3-*O*-arabinopyranosyl-(1→6)-galactopyranoside and Quercetin 3-*O*-galactoside. Quercetin 3-*O*-glucuronide remained the highest content among all four cultivars, while Kaempferol 3-*O*-glucoside content was also quite high for YMQ and WFMH.

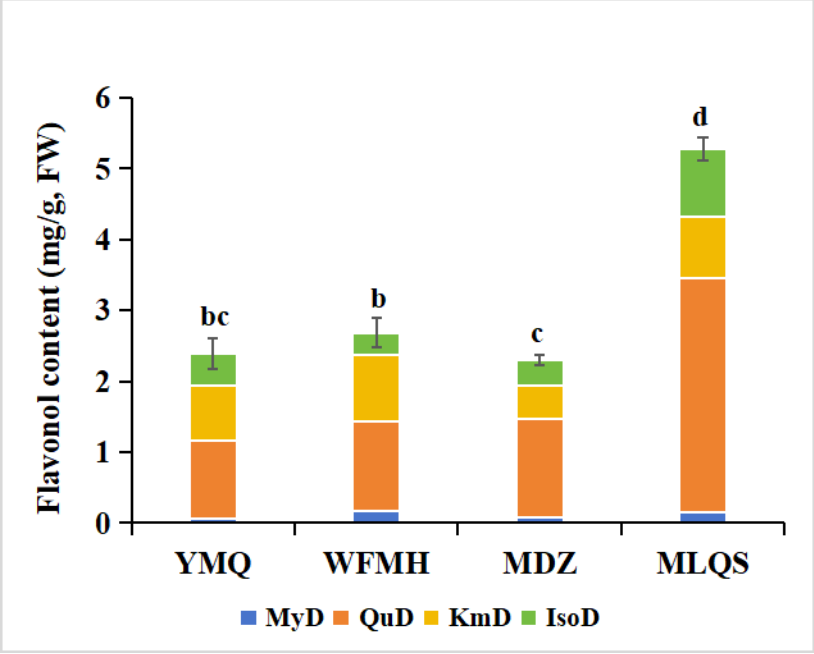

Fig.5 Flavonol quantity detection

MyD, Myricetin derivatives; QuD, Quercetin derivatives; KmD, Kaempferol derivatives; IsoD, Isorhamnetin derivatives

All the flavonols belonged to 4 subclasses: Myricetin derivatives, Quercetin derivatives, Kaempferol derivatives, and Isorhamnetin derivatives. Through horizontal comparison, Quercetin derivatives remained at the highest level across 4 lotus cultivars, with the highest level of quantity in MLQS. Myricetin derivatives remained at the lowest level for all 4 Lotus cultivars, with the highest level of quantity in WFMH. For YMQ, WFMH, and MDZ, there were higher levels of Kaempferol derivatives than Isorhamnetin derivatives, but the opposite remained in MLQS. At p=0.05, the total flavonol content in MLQS was significantly different from the rest, and WFMH and MDZ were significantly different from each other, While YMQ and WFMH had no significant difference. (Fig. 5).

3.5 Antioxidant activity identification by 2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid ammonium salt (ABTS) test

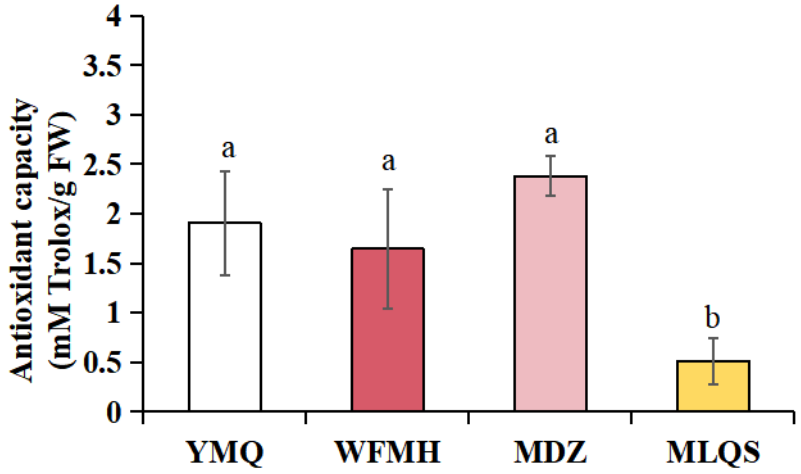

Fig 6. Total antioxidant capacity from ABTS test.

The yellow MLQS had the lowest antioxidant activity of 1.28 mM Trolox/g FW. The dark red WFMH had the third-highest concentration of 4.11 mM Trolox/g FW. The white YMQ had the second-highest concentration of 4.76 mM Trolox/g FW. The pink MDZ had the highest antioxidant activity of 5.96 mM Trolox/g FW. At p=0.05, MLQS was significantly different from the rest of the samples (Fig. 6). The activities and detected total flavonoids were negatively correlated with a statistically significant value of -0.804 at p=0.01 two-ended. This indicated there should be some other chemicals that are more related to its antioxidant activity.

4. Discussion

The petal tissue structure is similar to those of the leaves, containing the upper epidermis, fenestrated tissue, spongy tissue, and lower epidermis, among which the floral pigments are usually found in the epidermal cells (Zhao et al., 2015). Research has also shown that the structure of the epidermal cells in the petals affects the colors by altering optical properties. The lotus petal epidermal cells are cone-shaped, which increases the amount of incident light hitting the epidermal cells, enhancing light absorption by the pigments, which in turn deepens petal colors and increases flower saturation (Yoshida et al., 2009).

Previous studies have shown that flavonols were important second metabolites in lotus petals. They played the role of either co-pigment in red or pink flowers containing anthocyanins or imparted their own color to the white or yellow flowers containing no anthocyanins (Liu et al., 2023). Anthocyanins can be detected in pink, red, purple, and blue flowers, where the higher build-up of the pigment can lead to darker flower colors (Deng et al., 2013). In this study, the dark red WFMH has much more anthocyanin than the pink MDZ. Throughout the flower's development, the variation tendency of the total flavonol content was coincident with the variation of yellow colored-petals (Liu et al., 2023). In this study, the yellow MLQS has much more flavonol than the white YMQ, among which the Quercetin derivatives play a significant role in the differences, which is possibly why the lotus petals appear yellow.

The four phenolic compounds identified in the lotus cultivars, Myricetin derivatives, Quercetin derivatives, Kaempferol derivatives, and Isorhamnetin derivatives, have all been reported to possess anti-inflammatory activity (Cho et al., 2016). NO and PGE2 have been considered as an effector of inflammation, while myricetin can effectively inhibit NO production and downregulate inflammatory mediators expressions, thereby contributing to the inhibition of inflammatory activity (Cho et al., 2016). Quercetin derivatives are shown to enhance sleeping quantity and quality through their potential interaction with the GABAA receptor (Kim et al., 2021). Quercetin may indirectly promote GABA neurotransmission by enhancing chloride ion permeability, which leads to hyperpolarization and suppression of synaptic transmission ((Kim et al., 2021). Kaempferol derivatives and Isorhamnetin derivatives, as well as cyanidin from anthocyanin, may have the potential to prevent Alzheimer's Disease by inhibiting key enzymes in the occurrence of Alzheimer's Disease, including butyrylcholinesterase (BChE), and beta-secretase (BACE-1) (Temviriyanukul et al., 2020). The yellow MLQS has the highest amount of Quercetin derivatives, which could become a raw material for the extraction of Quercetin derivatives for natural herbal hypnotics, becoming a healthier

alternative for sleeping pills. The amounts of Kaempferol derivatives are also quite high for the yellow MLQS and the dark red WFMH, and with the dark red WFMH containing cyanidin and the relatively high amount of Isorhamnetin derivatives in the yellow MLQS, both lotus cultivars can be raw materials for extracting those pigments for the prevention of Alzheimer's Disease.

However, it is also noted that according to the correlation analysis, ABTS showed a significant negative correlation with TF. This is possibly due to the existence of other chemicals like procyanidine, flavone, or other polyphenol substances, or the use of FW which had higher water content.

5. Conclusion

This research explored the composition of different colored pigments as well as the potential usage of double-petal lotus beside their ornamental values through HPLC to analyze flavonols and anthocyanins and ABTS to measure the total antioxidant capacity, hopefully providing insights into how extractants from certain double-petal lotus cultivar like MLQS and WFMH can help to improve sleep patterns and prevent Alzheimer's Disease. However, limitations remain as there were limited samples and only ABTS was used to measure the total antioxidant capacity, which led to the finding of a negative correlation. Nonetheless, such findings provided a reasonable conjecture that other colored pigments that had stronger antioxidant activity remained in the lotus. For future studies, more samples should be used for each double-petal lotus cultivar; multiple methods for detecting total antioxidant capacity should be implemented for confirmation, including DPPH, FRAP, and ORAC, to detect and determine how procyanidine, flavone, or other polyphenol substances play a role in antioxidant activity.

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