RJC

RASĀYAN *J. Chem.*
Vol. 15 | No. 3 |1693-1701| July - September | 2022
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

INFLUENCE OF MATURITY STAGE ON TRYPTOPHAN, PHENOLIC, FLAVONOID, AND ANTHOCYANIN CONTENT, AND ANTIOXIDANT ACTIVITY OF *Morus alba* L. FRUIT

C. Yongram[1], P. Panyatip[2], P. Siriparu[3], J. Ratha[3], B. Sungthong[4], S. Tadtong[2] and P. Puthongking[3,5,✉]

[1]Division of Cannabis Health Science, College of Allied Health Sciences, Suansunandha Rajabhat University, Samut Songkhram, Thailand, 75000
[2]Department of Pharmacognosy, Faculty of Pharmacy, Srinakharinwirot University, Nakhon Nayok, Thailand, 26120
[3]Melatonin Research Group, Khon Kaen University, Khon Kaen, Thailand, 40002
[4]Integrative Pharmaceuticals and Innovation of Pharmaceutical Technology Research Unit Faculty of Pharmacy, Mahasarakham University, Maha Sarakham, Thailand, 44150
[5]Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand, 40002
✉Corresponding Author: pploenthip@kku.ac.th

ABSTRACT

Mulberry (*Morus alba* L.) fruits have been suggested to be a good nutritional source of alkaloids, phenolic compounds, flavonoids, and anthocyanins, which can provide health benefits. However, limited studies have addressed how changes in health promotion compounds and mulberry fruit quality are affected by harvest season. Therefore, phytochemical profiles of mulberry fruit at different maturity stages were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mulberry fruit extracts were also analyzed for their antioxidant potential and total phenolic, flavonoid, and anthocyanin contents. The results established that purple fruit had the highest total phenolic, flavonoid, and anthocyanin contents (608.87±8.07 mg GAE/g DW, 11.39±0.17 mg GAE/g DW, and 22.04±0.54 mg/ 100 g DW, respectively). Purple fruit also exhibited the strongest antioxidant activity in DPPH, ABTS, and FRAP assays. Quantitative analysis by LC-MS/MS revealed that purple fruit had the highest cyanidin-3-O-glucoside, rutin, gentisic acid, protocatechuic acid, and gallic acid contents, whereas green fruit had the highest caffeic acid, chlorogenic acid, and tryptophan contents. Our result suggests that later developmental stages of mulberry fruit have increased phenolic, flavonoid, and anthocyanin contents, and antioxidant capacity while tryptophan content was high in both green and purple mulberry fruits.

Keywords: Maturity stage, Tryptophan, Phenolics, Anthocyanins, PCA, LC-MS/MS.

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INTRODUCTION

Mulberry (*Morus alba* L.) is the dominant species of the Moraceae family. All parts of mulberry are useful: leaves are used for silkworm breeding, roots are used in Traditional Chinese Medicine (TCM), and mulberry fruits are widely consumed both fresh and as an ingredient in food products.[1] The consumption of mulberry products, especially mulberry fruit is increasing rapidly because of their rich nutritional value. Moreover, many pharmacological properties of mulberry fruits have been reported such as antioxidant, antiatherosclerosis, immunomodulatory, antitumor, antihyperglycemic, hypolipidemic, and neuroprotective activities.[2] For folk medicines, mulberry fruits have been used for anti-fever, diuretic, liver protection, and cardiovascular disease prevention.[3] Mulberry fruits contain abundant nutrients including proteins, lipids, carbohydrates, fiber, minerals, and vitamins.[1] Nine essential amino acids are found in mulberry fruits. The ratio between essential amino and total amino acids is 42%, which is the value for an ideal protein source according to the Food and Agriculture Organization (FAO) and World Health Organization (WHO) recommendations.[4] Moreover, mulberry fruits are also rich sources of bioactive compounds such as phenolics, anthocyanins, flavonoids, carotenoids, and polysaccharides.[5] Most of the

http://doi.org/10.31788/RJC.2022.1536958

phytochemical compounds found in mulberry fruit are produced from the shikimate biosynthetic pathway. In higher plants, aromatic amino acids including phenylalanine, tyrosine, and tryptophan, which are important precursors for primary and secondary metabolite biosynthesis, are also produced from the shikimate pathway.[6] Phenolics and anthocyanins synthesized from phenylalanine protect plants against environmental conditions that affect plant growth. These compounds have pharmacological properties including antioxidant and anti-inflammatory activities, which can provide health benefits.[7] A previous report showed that phenolic levels in mulberry fruit are higher than those in blackberry, blueberry, raspberry, or strawberry.[8] The phenolic compounds isolated from mulberry fruits include gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, chlorogenic acid, syringic acid, and others.[9] Ripened mulberry fruit contains high amounts of anthocyanins such as cyanidin and delphinidin derivatives and rutin is the most abundant flavonoid compound found in mulberry fruit.[10] In addition, the antioxidant capacity of mulberry fruit correlates with the phenolic and flavonoid contents.[11] Tryptophan is a precursor of several plant growth regulators including auxin, alkaloids, and indoles.[12] It is converted to melatonin by three pathways via serotonin, *N*-acetyltryptamine, 5-hydroxytryptophan, and 5-methoxytryptamine intermediates.[13] Tryptophan is the precursor compound for a number of important hormones in humans including the pineal hormone, melatonin.[14] Melatonin, predominantly participates in the regulation of circadian rhythms and sleep-wake cycles but also exhibits pharmacological activities including potent antioxidant[15,16] and anti-inflammatory activity[17] and neuroprotective effects against Alzheimer's disease.[18] Disruption of the sleep-wake cycle due to the aging process is related to a decrease in melatonin levels that negatively affects sleep efficiency and health.[19] Several reports have suggested that edible plants are good sources of phytochemicals and can play a role in the promotion of good health.[20,21] The consumption of a tryptophan-enriched diet has been reported to increase serum melatonin levels, which improved sleep quality and elevated antioxidant capacity.[22] Therefore, this study aimed to determine the content of tryptophan and its metabolites 5-methoxytryptamine and melatonin, in each maturity stage of the mulberry fruit. Furthermore, phenolic, flavonoid, and anthocyanin contents and antioxidant activity were investigated using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Correlation analysis of the phytochemical profile and antioxidant capacity of each mulberry fruit maturity stage was carried out by principal component analysis (PCA).

EXPERIMENTAL

Plant Extraction

The mulberry fruit (*Morus alba* L.) was collected in Khon Kaen province, Thailand. The fresh fruit was divided into 3 maturity stages including green, red, and purple fruit (Fig.-1). Each maturity stage of mulberry fruit was macerated with methanol to obtain extracts of green, red, and purple fruits with yields of 4.61%, 3.80%, and 4.94%, respectively.

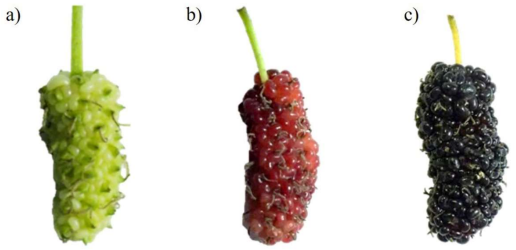

Fig.-1: Three Stages of Mulberry Fruits as (a) Green, (b) Red and (c) Purple Mulberry Fruit

Analysis of the Phytochemical Contents by LC-MS/MS

The tryptophan, 5-methoxy tryptamine, and melatonin contents in mulberry fruit extracts were determined by liquid chromatography-tandem mass spectrometry (LC-MS 8030, Shimadzu Corp, Kyoto, Japan) with column C-18 (150×2.1 mm, 3 μm) (Inertsil ODS-3 C18; GL Sciences Inc., Japan). The method was modified from a previous report.[23] Briefly, the mobile phase consisted of 0.45% formic acid and acetonitrile in a ratio of 1:1 with a flow rate of 0.2 ml/min, and an injection volume of 2 μL. The MS detection was performed on a triple quadrupole mass spectrometer with positive mode and operated in electrospray ionization (ESI) mode. Concentrations of metabolites were determined by multiple reaction monitoring

(MRM) with monitored mass transitions of tryptophan (m/z 205 to188), 5-methoxytryptamine (m/z 191 to 174), and melatonin (m/z 233 to 174). The method for anthocyanin, phenolic acid, and flavonoid content was modified from a previous report.[24,25] The isocratic system for mobile phase A (1% acetic acid in water) and mobile phase B (acetonitrile) with a flow rate of 0.2 ml/min with column C-18 (150×2.1 mm, 3 µm) at 30°C for anthocyanin and 40°C for phenolic acids and flavonoids and injection volume as 2 µL. The mass spectrometer was operated in the positive ESI mode (ion source temperature of 400 °C) with MRM and monitored mass transitions of gallic acid (GA; m/z 169 to 125), caffeic acid (CFA; m/z 179 to 135), gentistic acid (GTA; m/z 153 to 108), protocatechuic (PCCA; m/z 153 to 109), chlorogenic acid (ChA; m/z 353 to 191), rutin (RU; m/z 609 to 300), and myricetin (MyT; m/z 317 to 151).

Determination of Chemical Content

The total phenolic content (TPC) of mulberry fruit extract was evaluated following the previous method using the Folin-Ciocalteu colorimetric method.[26] The absorbance of the reactant was read at 700 nm by a microplate reader. The TPC was calculated as milligram (mg) of gallic acid equivalent per gram (g) of dry weight. The flavonoid content (TFC) of mulberry fruit extract was modified from the previous method.[27] In brief, the mixture of 2% $AlCl_3$ and sample (1:1) were added to a 96-well plate. The mixture solution was stood at room temperature for 20 min. The absorbance was read at 415 nm using a microplate reader. The total flavonoid contents were calculated as quercetin equivalents unit (mg QE/g DW). Total anthocyanin content (TAC) was determined by dissolving the mulberry fruit extracts (10 mg) in two buffers (0.025 M potassium chloride pH 1.0 and 0.4 M sodium acetate pH 4.5) at a volume of 10 ml and left for 15 min at room temperature. The absorbance of the reactant was read with a microplate reader at 520 and 700 nm. Deionized water was used for a blank. Total anthocyanin content was calculated as mg cyanidin-3-O-glucoside equivalents per dry weight as eqn.-1.

$$TAC\ (mg/100g) = (Abs \times MW \times D \times V \times 100)/(eL \times G) \quad (1)$$

Where: Abs is absorbance ((A510-A700) pH 1.0 - (A510-A700) pH 4.5); MW is molecular weight of anthocyanin (449.2 Da); D is dilution factor; V is final volume (mL); e is cyanidin-3-O-glucoside molar absorbance (26900); L is cell path length (0.6 cm); G is sample dry weight (mg).[28]

Antioxidant Capacities

The DPPH (1,1-diphenyl-2-picrylhydrazyl) was dissolved in ethanol at a concentration of 200 µM. Then 200 µM DPPH was mixed with different concentrations of mulberry fruit extract (100 µL each) in 96-well plates and incubated in dark conditions at room temperature for 30 min. The absorbance of the mixtures was measured by a microplate reader at 490 nm. Trolox was used as the positive control. The IC_{50} was calculated by plotting the percentage of inhibition and concentration of samples.[29] The ABTS [2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid)] radical cation was prepared by mixing 28 mM ABTS with 2.45 nM potassium persulfate in purified water. The ABTS reagent was kept at room temperature in the dark condition for 18 h before use. The mulberry fruit extracts (50 µL) at different concentrations were mixed with ABTS (150 µL) in 96-well plates and incubated at room temperature for 10 min. The absorbance of mixtures was measured by a microplate reader at 415 nm. Trolox was used as the positive control. The IC_{50} was calculated by plotting the percentage of inhibition and concentration of samples.[30] FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 20 mM $FeCl_3$ solution, and 10 mM TPTZ in 40 mM HCl in a ratio of 10:1:1. The mulberry fruit (10 µL) extracts were mixed with ultrapure water (30 µL) and FRAP reagent (300 µL) in 96-well plates. The mixtures were incubated at 37°C for 4 min. The absorbance of the mixtures was measured by a microplate reader at 595 nm. Trolox was used as the positive control. The FRAP values are shown in mmole/100 g DW unit.[31]

Statistical Analysis

The data are presented as mean ± standard deviation (SD). The data were analyzed by one-way analysis variance (ANOVA) with SPSS 26.0 software for Windows. Tukey's HSD (Tukey's Honest Significant Difference) test was used to determine significant differences between samples ($p<0.05$). The Pearson's correlation coefficient (r) and multivariate exploratory techniques of Principle Component Analysis (PCA) were analyzed by the XLSTAT (Student 2021.3.1.1178) software.

RESULTS AND DISCUSSION

Phytochemical Constituents in the Mulberry Fruit Maturity Stage

In plants, the essential amino acid tryptophan plays a crucial role in the biosynthetic pathways of growth-promoting metabolites such as auxins, glucosinolates, alkaloids, and indoles.[12] Melatonin, which is an indole derivative, is produced from the precursor tryptophan and protects the plant from extreme temperature change, UV radiation, pollution, and oxidative stress.[32] The biosynthesis of phytomelatonin differs from plants to animals, although both begin with tryptophan. In plants, tryptophan goes through many intermediates including tryptamine, 5-hydroxytryptophan, serotonin, *N*-acetyl-serotonin, and 5-methoxytryptamine.[13] Therefore, the contents of tryptophan and its metabolites at each stage of mulberry fruit's maturity were evaluated in this study. The LC-MS/MS analysis of tryptamine, 5-methoxytryptamine, and melatonin contents are shown in Table-1. Green and purple mulberry fruit presented the highest tryptophan content (0.06 and 0.05 mg/g extract, respectively) followed by red fruit (0.03 g/g extract). For 5-methoxytryptamine content, it was found that green fruit showed the highest content, but it had no significant difference between purple and red fruit. Melatonin was not detected in the mulberry fruit extracts. A previous study of the effect of the fruit-development stage on melatonin levels examined fruits in stage I (day 1-24), stage II (day 25-46), and stage III (day 47-56). They found that melatonin level was high during the seed developed stage (stage-I) and proposed that this was because the germ tissue is susceptible to oxidative damage in stage I and melatonin might be produced in high amounts to protect the germ tissue against free radicals and oxidative stress. The level of melatonin decreased in the other stages.[33] For our study, we divided the fruit maturity stages by color. The green fruits in our study correspond to stage II of the previous study. Thereby, melatonin content was undetectable in our experiment.

Table-1: Chemical Composition of Mulberry Fruit Extracts

Compounds	Chemical Composition (mg/g crude extract)		
	Green	Red	Purple
Tryptophan	0.06 ± 0.00^{a}	0.03 ± 0.00^{b}	0.05 ± 0.00^{a}
5-Methoxytryptamine	$1.6\times10^{-4}\pm0.00^{a}$	$1.3\times10^{-4}\pm0.00^{a}$	$1.3\times10^{-4}\pm0.00^{a}$
Cyanidin-3-O-glucoside	0.02 ± 0.00^{c}	0.58 ± 0.03^{b}	16.77 ± 0.14^{a}
Gallic acid	1.63 ± 0.16^{c}	2.54 ± 0.28^{b}	3.93 ± 0.35^{a}
Caffeic acid	8.38 ± 0.44^{a}	7.88 ± 0.06^{a}	3.91 ± 0.22^{b}
Gentisic acid	15.81 ± 0.56^{b}	15.29 ± 0.65^{b}	28.66 ± 0.56^{a}
Chlorogenic acid	5450.40 ± 59.89^{a}	2297.65 ± 27.97^{c}	4372.56 ± 21.05^{b}
Protocatechuic acid	4.81 ± 0.27^{c}	9.41 ± 0.37^{b}	16.18 ± 1.27^{a}
Rutin	254.07 ± 3.19^{c}	301.94 ± 3.72^{b}	372.21 ± 4.38^{a}
Myricetin	ND	ND	ND

Note: ND is not detected. Letters indicates the significant difference in data between a column in the same rows at $p<0.05$ using one-way ANOVA with Tukey HSD.

The TPC, TFC, and TAC of mulberry fruit extracts are shown in Table-2. Purple fruit demonstrated the highest total phenolic content (608.87±8.07 mg GA/g DW), which was higher than green fruit and red fruit. TPC in fully mature mulberry fruits has previously been reported to range from 2.2-164 mg/g DW and 181-1,650 mg/ 100 g FW.[5,34-38] Another study that investigated the effect of mulberry fruit maturity stage on phenolic content divided the fruits into 4 ripening stages and found that stage II fruit presented the lowest TPC. They suggested that the number of phenolic compounds decreased during the development of the fruits.[11] This was similar to a study of the trend line of 14 stages of sweet cherry that showed TPC declined in the early stages and increased in the later stages of fruit development.[39] The TFC of mulberry fruit increased from green, red, and purple fruits as 3.51±0.05, 7.08±0.06, and 11.69±0.17 mg QE/g DW, respectively. Our findings show a high TFC content in all maturity stages and a higher level than in a previous report that showed low TFC (0.64-2.11 mg QE/g DW) in the fruit of 8 cultivars of mulberry.[40] In addition, mulberry fruit presented increased TFC from semi-ripened to fully-ripened with TFC values of 3.92 and 6.25 mg CE/g DW, respectively.[41]

The ripened purple fruits presented the highest content of TAC (22.04±0.54 mg/ 100 g DW). The TAC of the green and red fruits was small, and there was no significant difference. Remarkably, the production and accumulation of anthocyanins occur in only a short time in the growing period. Ripened fruit extracts have

been reported to contain the highest level of anthocyanins.[42] This result indicates that the amount of TAC depends on the development of the mulberry fruit. The level of 5 phenolic acids (gallic acid, caffeic acid, gentisic acid, chlorogenic acid, protocatechuic acid), 2 flavonoids (rutin and myricetin), and 1 anthocyanin (cyanidin-3-O-glucoside) were analyzed using LC-MS/MS (Table 1). The highest chlorogenic acid content was in the range 2297.65±27.97 - 5450.40±59.89 mg/g crude extract, which was found in green fruit followed by purple and red fruit, respectively. Caffeic acid also had the highest content in green fruit. In contrast, gallic acid, protocatechuic acid, gentisic acid, rutin, and cyanidin-3-O-glucoside showed increasing content in green, red, and purple fruits, respectively. However, myricetin content was not found in these mulberry fruits.

Table-2: Chemical Contents and Antioxidant Capacities of Mulberry Fruit Extracts

	Green	Red	Purple	Trolox
Chemical contents				
TPC (mg GA/g DW)	464.75 ± 10.32^{b}	406.13 ± 8.40^{c}	608.87 ± 8.07^{a}	-
TAC (mg/ 100 g DW)	0.31 ± 0.05^{b}	0.14 ± 0.00^{b}	22.04 ± 0.54^{a}	-
TFC (mg QE/g DW)	3.51 ± 0.05^{c}	7.08 ± 0.06^{b}	11.69 ± 0.17^{a}	-
Antioxidant capacities				
DPPH IC_{50} (µg/mL)	258.86 ± 2.17^{d}	251.20 ± 0.88^{c}	234.27 ± 0.62^{b}	6.37 ± 0.02^{a}
ABTS IC_{50} (µg/mL)	125.39 ± 0.71^{c}	127.33 ± 1.99^{c}	42.94 ± 0.24^{b}	3.75 ± 0.04^{a}
FRAP (mmole/100 g DW)	$8.84\pm0.68^{b,c}$	7.55 ± 0.22^{c}	18.16 ± 0.35^{b}	234.07 ± 7.16^{a}

Note: Letters indicate the significant difference in data between a column in the same rows at $p<0.05$ using one-way ANOVA with Tukey HSD.

Antioxidant Capacities

The antioxidant activities of mulberry fruit are shown in Table-2. The DPPH free radical can accept hydrogen from antioxidant molecules to become a stable diamagnetic molecule.[43] A color change in the test system reflects the quantity of DPPH radicals in the test environment, which indicates the radical scavenging property of the test compound.[44] The DPPH radical scavenging activities of the three stages of mulberry fruit were low compared with Trolox. The extracts showed a dose-dependent effect at a concentration of 100 mg/mL. The IC_{50} value of the mulberry fruit extracts is shown in Table-2. Purple mulberry fruit exhibited higher activity than other fruits. The ABTS radical cation is generated by potassium persulfate. It can accept an electron to produce stable ABTS, which is observed from a color change.[45,46] The ABTS radical scavenging activities of the fruit extracts are presented in Table 2. The IC_{50} values of the green, red, and purple fruit extracts were 125.39, 127.33, and 42.94 µg/mL, respectively. The purple mulberry fruit exhibited more activity than green and red fruit, but it was lower activity than Trolox.

Fe (III) is reduced to Fe (II) by antioxidant compounds.[47] The mulberry fruit extracts showed low ferric reducing antioxidant power activity compared with Trolox. At a concentration of 500 µg/mL, the reducing power activities of green, red, and purple fruit extracts were 8.84, 7.55, and 18.16 mmole/100 g DW, respectively.

Principal Component Analysis (PCA)

Correlation among the phytochemical components and antioxidant activities of mulberry fruits at different maturities were analyzed by principal component analysis (PCA) and the results are presented in Table-3. The antioxidant capacities (DPPH, ABTS, FRAP) of the mulberry fruit extracts correlated with TAC, TFC, and TPC. Strong and significant ($p<0.05$) correlations were found between ABTS and TAC, cyanidin-3-O-glucoside, and gentisic acid contents. In contrast, the tryptophan, 5-methoxytryptamine, caffeic acid, and chlorogenic acid contents showed negligible correlation with the DPPH, ABTS, and FRAP activities. These findings accord with a previous report that the phenolic components in mulberry juice were responsible for its high antioxidant activity.[48] The Pearson's correlation coefficients suggest that the antioxidant capacity of the mulberry fruit extracts depended on phenolics, flavonoid compounds, and anthocyanins to scavenge the radicals. Principal component analysis discovers interesting relations in data patterns. Figure-2 shows the PCA biplot for the mulberry fruit analysis including the correlation of the variables and the scatter plot of the observations. Green mulberry fruit was found to have the highest tryptophan, 5-methoxytryptamine, caffeic acid, and chlorogenic acid contents. Purple mulberry fruit was found to be rich in total anthocyanin, phenolic, and flavonoid contents, as well as having high antioxidant capacities, and the highest contents of

some phenolics and flavonoid compounds. The results of the chemical components analysis indicated that cyanidin-3-O-glucoside, gallic acid, gentisic acid, protocatechuic acid, and rutin were related to the mulberry fruit ripening stage. The variables in F2, consisting of tryptophan, 5-methoxytryptamine, and chlorogenic acid, were highest in green mulberry fruit and are the precursor compounds for other secondary metabolites in the biosynthesis pathway. The relationships of phytochemical compounds and their antioxidant capacities in mulberry fruit at different stages were also evaluated using agglomerative hierarchical clustering (AHC) based on their highest variations. The results are divided into 2 clusters. Cluster I (purple mulberry fruit) had the highest TAC, TPC, TFC, cyanidin-3-O-glucoside, gallic acid, gentisic acid, protocatechuic acid, and rutin contents and the highest antioxidant capacities. Cluster II (green and red mulberry fruits) contained the lowest TAC, TPC, and TFC and the lowest antioxidant capacity, confirming the relationship between phytochemical compounds and their antioxidant capacities in mulberry at different maturity stages.

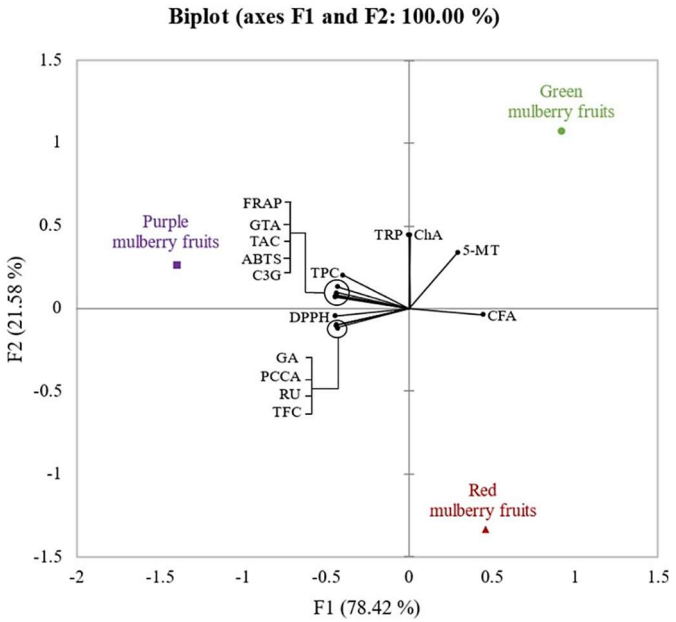

Fig.-2: Biplot of Principal Component Analysis at The Differential Stages of Mulberry Fruit

Table-3: Pearson's Correlation Coefficient Between Antioxidant Activities and Chemical Compositions

Chemical compounds	Antioxidant capacities		
	DPPH	ABTS	FRAP
Total phenolic content	0.841	0.954	0.985
Total flavonoid content	0.987	0.909	0.847
Total anthocyanin content	0.957	1.000*	0.995
Tryptophan	-0.096	0.170	0.297
5-Methoxytryptamine	-0.724	-0.516	-0.400
Cyanidin-3-O-glucoside	0.967	1.000*	0.990
Gallic acid	0.993	0.927	0.870
Caffeic acid	-0.983	-0.997	-0.977
Gentisic acid	0.949	0.999*	0.997*
Chlorogenic acid	-0.105	0.161	0.288
Protocatechuic acid	0.992	0.923	0.865
Rutin	0.992	0.923	0.865

Note: Values in * are different from 0 with a significance level alpha = 0.05.

CONCLUSION

The maturity stage of mulberry fruit significantly affected the number of bioactive compounds. The green fruit had the highest content of the essential amino acid, tryptophan, and its related metabolites. The ripened purple fruit had similar tryptophan content to the green fruit, but with higher phenolic and anthocyanin

contents. The number of bioactive compounds in the fruit extracts, especially TPC, TFC, and TAC, correlated with antioxidant activities. For instance, purple fruits had the strongest ABTS radical scavenging activity which correlated with their high TPC and TAC. It can be concluded that mulberry fruits are a valuable source of essential amino acids and bioactive compounds especially, tryptophan, phenolic compounds, and anthocyanins, and can be used as a food supplement to promote good health.

ACKNOWLEDGEMENT

This research has received funding support from the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation [grant number B05F630053], Khon Kaen University, Thailand. The authors thank Dr. Glenn Borlace, Faculty of Pharmaceutical Sciences, Khon Kaen University for English language assistance.

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[RJC-6958/2022]