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Phenolics and Ascorbic Acid Related to Antioxidant Activity of Mao Fruit Juice and Their Thermal Stability Study (Review Article)

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ABSTRACT

Antioxidant and/or anti-aging activities are always lined with people's minds as major potential benefits concerning human health in the recent commercial features for an economic world of foodstuffs and medical uses. Total phenolics including flavonoids and anthocyanins, and ascorbic acid in the Mao juices are closely related to their antioxidant activity. Numerous research approaches on these functional foods, in particular the colored fruits and vegetables have been investigated. Method validation and determination of the potential compounds have been increasingly developed with highly sensitive and selective procedures and applications including thermal stability of the Mao juice. Their antioxidant activities obtained from different assays related to the contents of both phenolics and ascorbic acid in the anthocyanins-rich Mao juices in Thailand are reported and discussed.

Keywords: Antioxidant activity, Mao juice, Phenolic compounds, Flavonoids, Anthocyanins, Ascorbic acid**,** Thermal stability**.**

INTRODUCTION

Nowadays, there is growing interest in use of polyphenolic antioxidant-rich plants as dietary supplements[1,2]. Health experts recommend to increase the consumption of fruit since there is an evidence linking a diet rich in fruit with reduced incidence of various age relating chronic diseases[3]. The protective effect of fruits has been attributed to their bioactive antioxidant constituents, including polyphenols and vitamins[4]. Polyphenols are secondary metabolites presenting in all plant tissues. They are important antioxidants of human diet[5]. Generally, vitamin C or ascorbic acid is also major reducing agent of the essential phytonutrients for the metabolism of living cells. Both polyphenols

and ascorbic acid are the two major contributors to the total antioxidant activity in vegetable and fruit. It was reported that vitamin C accounted for 65-100% of antioxidant capacity of citrus juices[6]. In addition, some fruits had very high level of total phenolic content and reducing activity[7].

Mao tree (*Antidesma thwaitesianum Muell.* Arg.) is classified in the family *Euphobiaceae.* It is one of many tropical compiled fruits like wild berry which have been known for long time in Thailand, especially in Phu Phan district, Sakon Nakhon province. There are commonly consumed as commercially available products of juice and wine, particularly the fruit juice contains a very rich source of antioxidants. The recent studies have investigated that the health benefits and antioxidant activities of Mao Luang fruits are mainly attributed to almost known phenolic compounds including anthocyanins, proanthocyanidins and tannins, resveratrol and benzoic, caffeic and cinnamic acids[4,8-10]. Total phenolics, antioxidant activity and nutritive values of the Mao fruits have previously been reported. Polyphenolic compounds and proanthocyanidins isolated from the fruit extract exhibited much higher antioxidant activities than that of standard Trolox and had similar antioxidant potential to grape seed proanthocyanidin extract[11]. Fifteen varieties of Mao Luang fruits contained three different flavonoids (catechin, procyanidin B1 and procyanidin B2)[9]. These organic compounds are the major flavonoids in all analyzed fruit samples. The skin contact the Mao Luang red wine had higher amounts of flavonoids, phenolic acids, anthocyanins, organic acids than the non-skin contact one[4]. Since the great importance of antioxidant activity is mainly attributed from polyphenols and ascorbic acid for human health and also because of the growth in commercial fruit juice production and consumption[3]. The part of flavonoids has many beneficial effects on human and animal health. These are the best known natural antioxidants[12-15].

Therefore, this review was aimed to describe total phenolics and ascorbic acid in association with their antioxidant activity and thermal stability of the Mao fruit juices. Their antioxidant activity was comparatively assayed by some antioxidant methods including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2´-azinobis-3-ethylbenzothiazoline-6-sulphonate (ABTS), *N,N*-dimethyl-p-phenylenediamine dihydrochloride (DMPD) and ferric reducing antioxidant power (FRAP). The heating effect on total phenolics of the Mao juice was also investigated. Determination of antioxidant activity, total phenolics, and flavonoids and their correlation studies among total phenolics and flavonoid contents versus antioxidant activity in the fruit juice from various production sources were investigated. Moreover, total anthocyanin was evaluated by using sample dilution method in association with an external calibration curve under optimum conditions by UV-Visible and fluorescence spectrophotometry comparing with pH-differential method[16].

The Mao Fruits

The so-called Mao fruit is classified as a kind of wild berry fruits. There are about eighteen species of *Antidesma* plants. Each fruit is an oval shape with dark green, turns to orange-red and dark-purple at a fully ripe stage and becomes sweet with slightly tart[10]. The ripe fruit is favorable to be consumed and sold in the local market because of its good color and taste. Both Mao juice and its wine have become more popular as healthy nourishment [10]. At present, the health effects of the Mao fruit have been interested. Various publications have been reported as follows.

Organic acids in ripe fruits of fifteen cultivars of Mao Luang from Phuphan valley, Sakon Nakhon were determined. The results showed that there were two groups of organic acids contents in ripe fruit of Mao Luang cultivars. The major group includes tartaric acid, ascorbic acid, citric acid and benzoic acid, and the minor one includes malic acid, lactic acid, oxalic acid and acetic acid[4]. Changes in physico-chemical properties, polyphenolics and antiradical activity in Mao Luang fruit during development and ripening were investigated. At over ripe stage, the fruits had the highest antioxidants. This data attribute a good basis for evaluating the nutritional importance of the Mao fruit[8]. Flavonoids in these ripe fruits also from the Phuphan valley were also determined by RP-HPLC[9].

Polyphenolic contents in both Mao seeds (MS) and Mao marcs (MM) were purified and investigated their radical scavenging activities against DPPH and ABTS radicals and thiobarbituric

acid reactive products (TBARP)[10-14]. The results showed both MS and MM were an abundant source of polyphenols and proanthocyanidins. Their radical scavenging activities of MS and MM against DPPH and ABTS radicals were significantly higher than that of standard Trolox. In addition, the antihypertensive and antioxidative effects of Mao pomace (MP) using hypertensive rats. The MP treatment significantly prevented the increase in blood pressure, hind limb blood flow and vascular resistance of L-NAME treated hypertensive rats[15,16]. The present results provide an evidence for the antihypertensive effect of MP and suggest that MP might be useful as a dietary supplement against hypertension.

Evaluation of the antioxidant activity in Thai fruit wines was done using both off-line DPPH assay and on-line HPLC–MS–DPPH assay for the analysis of phenolic antioxidant compounds[17]. An LC–MS/MS was very useful to quantify the active compounds. This result shows that the on-line HPLC–MS–DPPH assay can be powerful for rapid characterization of antioxidant compounds in plant extracts. The cytotoxic and antioxidant activities of the fruit and fruit waste (residue and marc) extracts of Mao were also studied using chemical and cell-based assays[18]. The results showed that the ethanol extracts of fresh and dried Mao fruits exhibit both cytotoxic and cellular antioxidant activities, and thus possess great potentials for application in the development of effective dietary supplements to prevent oxidative stress-induced diseases.

Chemistry Background of Mao Juice

The chemistry background of the Mao juice including pH, total acidity, volatile acidity, fixed acidity, total solids, specific gravity and buffering capacity was determined following the standard AOAC methods[16]. Total acidity of the fruit juice was determined by titration method. The solution of dilute juice was titrated to pH 8.1 with 0.1 M NaOH standard solution using phenolphthalein. Total acidity was calculated in terms of citric acid using its chemical formula: Acidity (g/100 mL) = (Normality of the juice sample) x (Equivalent weight of citric acid). The pH value of 10% diluted juice was also determined using pH meter.

Volatile acidity was also determined by titration method. The solution of the residual juice sample after heat treatment was titrated with 0.1 M NaOH standard solution until the end point reached at pH 8.1 by pH meter. Fixed acidity is simply the difference between total acidity and its volatile acidity. It refers to the amount of acidity that is not volatile under normal conditions. The fixed acidity was calculated according to the equation: Fixed acidity (as citric acid) = (Total acidity (g/L) as citric acid) – (Volatile acidity, g/100 mL as citric acid x 10 x 1.17).

Buffering capacity is the ability of typical buffer solution to resist changes in pH. The initial pH was measured with pH meter and the buffering capacity was measured by adding 1 M NaOH in the increments of 0.2 mL until the pH value reached at pH 9.0. Buffering capacity is expressed as the molarity of sodium hydroxide required to increase pH value by 1.0 unit[19]. Total solids are a measure of the amount of materials remaining after all the water has been evaporated. It is needed to clarify all dissolved materials including both inorganic (salts) and organic compounds presence in the juice sample. Five mL of the fruit juice was dried in hot oven at 70°C for 24 h. The residual weight was accurately obtained and then calculated as percentage of the sample used.

Free Radicals and Antioxidants Activity

Free radicals are highly reactive chemicals that have the potential to harm cells. They are created when an atom or a molecule (a chemical that has two or more atoms) either gains or loses an electron (a small negatively charged particle found in atoms)[20,21]. Therefore, some may be behaving as oxidants or reductants[22]. The most important oxygen-containing free radicals in many disease states are hydroxyl radical (•OH), oxygen singlet ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), peroxyl (ROO•), hypochlorite (OCl^-), nitric oxide radical (NO), and peroxynitrite radical ($OONO^-$)[23]. The built-up of free radicals over time may contribute to the aging process and the development of health conditions such as cancer, heart disease, atherosclerosis, Alzheimer's disease, inflammation and diabetesand[24].

Antioxidants are molecule that inhibits the oxidation of other molecules. They are interacted with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also

known as "free radical scavengers'[19,20]. They have been traditionally divided into two classes; primary or chain-breaking antioxidants, and secondary or preventative antioxidants.

Primary antioxidants or chain-breaking antioxidants, AH, when present in trace amounts, may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals[25-27].

Chain-breaking mechanisms:

$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$

$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$

$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$

The antioxidant free radical may further interfere with chain propagation reactions by forming peroxy antioxidant compounds:

$A^{\bullet} + LOO^{\bullet} \rightarrow LOOA$

$A^{\bullet} + LO^{\bullet} \rightarrow LOA$

The activation energy of the above reactions increases with increasing A–H and L–H bond dissociation energy. Therefore, the efficiency of the antioxidant increases with decreasing A–H bond strength[27-30]. A classification of antioxidant assays is defined as the type of reaction: hydrogen atom transfer (HAT)-based assays and electron transfer (ET) - based assays

HAT-based assays are used to measure the capability of an antioxidant to quench free radicals by H-atom donation. The HAT mechanisms of antioxidant action in which the hydrogen atom (H) of a phenol (Ar–OH) is transferred to a ROO• radical as summarized by the reaction:

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

where, the aryloxy radical (ArO•) formed from the reaction of antioxidant phenol with peroxyl radical that is stabilized by resonance. Effective phenolic antioxidants need to react faster than biomolecules with free radicals to protect the later from oxidation. The HAT-based assays include oxygen radical absorbance capacity (ORAC) assay, TRAP assay using R-phycoerythrin as the fluorescent probe, crocin bleaching assay using 2,2'-azobis (2-amidinopropane) hydrochloride (AAPH) as the radical generator, and β-carotene bleaching assay[31-32].

ET-based assays (electron transfer) are used to measure the capacity of an antioxidant in the reduction of an oxidant, which changes in color when reduced. The degree of color change is correlated with the sample's antioxidant concentrations. ET-based assays include the total phenols assay by Folin-Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), total antioxidant potential assay using a Cu(II) complex as an oxidant, and DPPH. In addition, other assays are used to measure a sample's scavenging capacity of biologically relevant oxidants such as singlet oxygen, superoxide anion, peroxynitrite, and hydroxyl radical[31-32]. The reaction equations of various ET-based assays can be summarized as follows:

Folin: Mo(VI) (yellow) + e^{-} (from AH)→Mo(V) (blue)

where, the oxidising reagent is a molybdo-phosphotungstic heteropolyacid comprised of $3H_2O–P_2O_5–13WO_3–5MoO_3–10H_2O$ (heteropoly anion: $P_2Mo_5W_{13}O_{62}^{6-}$), in which the hypothesized active center is Mo(VI) with λ_{max} 765 nm.

FRAP: $Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\bullet} + H^{+}$

where, TPTZ: 2,4,6-tripyridyl-s-triazine ligand with λ_{max} 595 nm.

Ferricyanide/Prussian blue

$Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO^{\bullet} + H^{+}$

$Fe(CN)_6^{4-} + Fe^{3+} + K^{+} \rightarrow KFe[Fe(CN)^6]$

where, $KFe[Fe(CN)_6]$: Prussian blue with λ_{max} 700 nm.

ABTS/TEAC: $ABTS + K_2S_2O_8 \rightarrow ABTS^{\bullet+}$

$ABTS^{\bullet+} + ArOH \rightarrow ABTS + ArO^{\bullet} + H^{+}$

where, ABTS is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) with λ_{max} 734 nm and TEAC is Trolox-equivalent antioxidant capacity (also the name of the assay). Although other wavelengths such as 415 and 645 nm have

been used in the ABTS assay, the 734 nm peak wavelength has been predominantly preferred due to less interference from plant pigments.

DPPH: $DPPH\bullet + ArOH \rightarrow DPPH + ArO\bullet + H^+$

where, DPPH• is the [2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl] stable radical with λ_{max} 515 nm.

CUPRAC: $nCu(Nc)_2^{2+} + Ar(OH)_n \rightarrow nCu(Nc)_2^{+} + Ar(=O)_n + nH^+$

where, the polyphenol with suitably situated Ar–OH groups is oxidized to the corresponding quinone, shows absorption maximum at 450 nm [32-35].

Kinetically antioxidants can be classified into six categories as follows

- Antioxidants that break chains by reacting with peroxyl radicals having weak O-H or N-H bonds such as phenol, napthol, hydroquinone, aromatic amines and aminophenols.
- Antioxidants that break chains by reacting with alkyl radicals such as quinones, nitrones, iminoquinones.
- Hydroperoxide decomposing antioxidants such as sulphide, phosphide, thiophosphate.
- Metal deactivating antioxidants: diamines, hydroxyl acids and bifunctional compounds.
- Cyclic chain termination by antioxidants: aromatic amines, nitroxyl radical, variable valence metal compounds.
- Synergism of action of several antioxidants: phenol sulphide in which phenolic group reacts with peroxyl radical and sulphide group with hydroperoxide [36].

Screening Testing for Antioxidant Activity

DPPH free radical scavenging assay

The DPPH assay is based on the reduction of DPPH, a stable free radical with an unpaired electron that is delocalized over the entire molecule. The DPPH radical has been used widely in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants[37]. The free radical DPPH is scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H•. The color changed from purple to yellow after reduction, which can be quantified by its decrease of absorbance at517 nm[38,39].

Measurements are made using a UV-visible spectrophotometer at room temperature, and the scavenging capacity is represented as the percentage of DPPH radical inhibition. The DPPH assay is based on both electron transfer (ET) and hydrogen atom transfer (HAT) reactions[32]. An advantage of the DPPH assay is that it is an easy, rapid and commercial method to evaluate the radical scavenging activity of non-enzymatic antioxidants[16]. Since DPPH is a stable radical, this assay considers not only the concentration of the tested sample, but also the reaction time and the temperature; both of which when controlled carefully enable this assay to be highly reproducible[39]. Antioxidant activity is expressed as BHT equivalent.

DMPD radical scavenging activity assay

N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD) is a compound that is generally used to measure the antioxidant potentials of fruit juice, vegetable and other natural products. This assay focuses on the ability of the antioxidant compounds (AOH) to transfer a hydrogen atom to the colored radical $DMPD^{\cdot+}$ turning it into an uncolored $DMPD^+$ compound[40]. In the presence of ferric iron, it gets converting to $DMPD^{\cdot+}$ radicals, which are scavenged by antioxidant molecules present in the test samples[41]. The maximum wavelength of $DMPD^{\cdot+}$ shows at 505 nm.

DMPD (uncolored) + oxidant (Fe^{3+}) + $H^+ \rightarrow$ $DMPD^{\cdot+}$ (purple)

$DMPD^{\cdot+}$ (purple) + $AOH \rightarrow DMPD^+$ (uncolored) + AO

The reaction is rapid and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Antioxidant ability is expressed as TEAC (Trolox equivalent antioxidant capacity).

ABTS radical cation decolorization assay

The 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonic acid (ABTS) assay is based on the ability of antioxidant molecule to scavenge the stable ABTS radical. In this assay, $ABTS^{\cdot+}$ radical cation

is formed from the reaction between ABTS and potassium persulfate, which is intensely colored. The antioxidant activity is measured as the ability of antioxidant to decrease the color reacting directly with $ABTS^{\cdot+}$ radical cation and can be measured the absorbance at 734 nm. The results are expressed as Trolox equivalent antioxidant capacity (TEAC). Due to $ABTS^{\cdot+}$ radical cation is soluble in both aqueous and organic solvents and is not affected by ionic strength, so it can be used in multiple media to determine both hydrophilic and lipophilic antioxidant activity of sample. Furthermore, it is simpler and cheaper and allowing the evaluation of its rate of consumption with minimal interferences. Therefore, the method has been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixture of beverages and biological fluids[42-46].

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of tripyridyltriazine (TPTZ) forming an intense blue of the Fe^{2+}-TPTZ complex with an absorbance maximum at 593 nm[47]. At low pH, ferric iron (Fe^{3+}) is initially reduced by electron-donating antioxidants present within the sample to its ferrous form (Fe^{2+}). As the ferric to ferrous ion reduction occurs rapidly with all reductants with half reaction reduction potentials above that of Fe^{3+}/Fe^{2+}, the values in the FRAP assay expresses the corresponding concentration of electron donating antioxidants[48]. Increasing absorbance indicates an increase in reductive ability[49,50].

The FRAP assay actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically. Often, FRAP values have a poor relationship to other antioxidant measures. However, in contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive, and robust and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomatic, or manual methods[50-53].

Phenolic and Polyphenolic Compounds

Polyphenols, a large class of chemicals which are found in plants, have attracted much attention in the last decades due to their properties and the hope that they will show beneficial health effects, when taken as a dietary input or as complement[54]. Polyphenols are polyhydroxylated phytochemicals, which have common structures. They are divided into several classes according to the number of phenol rings that they contain and to the structural elements that bind these rings to one another. The main group of polyphenols is flavonoids, phenolic acids, and stilbenoids.

In the present, there is growing interest on plant-derived polyphenols because of their potential antioxidant and antimicrobial properties. Phenolic compounds exhibit considerable free-radical scavenging activity, which is determined by their reactivity as hydrogen- or electron-donating agents, their reactivity with other antioxidants and their metal chelating properties, as well as the stability of the resulting antioxidant-derived radicals. Although determination of polyphenols is hampered by their structural complexity and diversity, several methods have been used to determine polyphenols in sample, then spectrophotometry in the ultraviolet region may be a useful tool to help resolve thisproblem[55].

Colorimetric reactions are widely used in the UV-Visible spectrophotometric method, which is easy to perform, rapid and applicable in routine laboratory use, and low cost[56]. Total phenolic contents are well determined by using Folin-Ciocalteu method with gallic acid as standard. This method is based on the reduction of phosphomolybdic-phosphotungstic acid (Folin) reagent to a blue-colored complex in an alkaline solution occurs in the presence of phenolic compounds. The maximum absorption of the chromophores depends on the alkaline solution and the concentration of phenolic compounds. The absorbance can be measured at 765 nm and the results are expressed as mg gallic acid equivalents.

Phenolic compound + $[P_2W_{18}O_{62}^{7-} + H_2P_2Mo_{18}O_{62}^{6-}]$
$\rightarrow$ Blue complex
Folin-Ciocalteu reagent; Yellow

Flavonoids

Flavonoids are characterized as the group of organic compounds containing two or more aromatic rings, two benzene rings (ring A and B)

joined by a linear three-carbon chain. The central three-carbon chain may form a closed pyran ring (ring C) with one of the benzene rings. It contains one or more phenolic hydroxyl groups. Flavonoids are themselves divided into 6 subclasses, depending on the oxidation state of the central pyran ring: flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols.

Flavonoids have many beneficial effects on human and animal health such as anti-aging, antioxidant, antibacterial and antifungal activities, anticancer, anti-cardiovascular disease and anti-inflammatory[13]. The capacity of the phenolic compounds and flavonoids to act as antioxidants depends upon their position of hydroxyl groups and other features in the chemical structure which is important for their antioxidant and free radical scavenging activities[14, 57,58].

Total flavonoids were determined using aluminium chloride colorimetric method. This method is based on the formation of a complex flavonoid-aluminum. Firstly, aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids. The colored flavonoid-aluminium complex formation obtained can be detected at maximum wavelength of 510 nm. The flavonoid contents were calculated from the linear equation based on the calibration curve and expressed as mg of catechin equivalents[59].

Anthocyanins

Anthocyanins/polyphenolics are the natural colorants responsible for the red, blue, and purple colors of many fruits, vegetables and

Table 1: Antioxidant activity, total phenolics, flavonoids, anthocyanins and ascorbic acid of Mao and other fruit juices

Juice	Antioxidant activity	Total phenolics[92] (mg GAE/100 mL)	Flavonoids (mg CE/L)	Anthocyanins (mg/L)	Ascorbic acid[92] (mg GAE/100 mL)
Mao	26.8*	338	19,899	578	175.3
Orange[98]	9.2	382	-	-	15.86
Pomegranate[99]	20.0**	135	-	-	17.34
Red grape[100]	11.1	64.3	-	-	15.18
Apple[100]	1.20***	42.8	-	-	13.40
White grape	-	33.7	-	-	14.13
Apricot[101]	0.974	18.6	-	-	16.20
Noni[92]	5.85	-	-	-	-
Sour cherry	-	50.1	618.1	368.4	16.44
Peach	-	28.6	-	-	15.63
Sweet cherry[74]	-	-	-	256.6	-
Mango	-	42.9	-	-	12.57
Pineapple	-	35.7	-	-	13.60
Blackcurrant[102]	-	-	975.1	849.8	-
Black grape[102]	-	-	405.2	208.7	-
Elderberry[103]	-	-	1,776	-	-
Strawberry[104]	-	-	-	13.6	-
Raspberry[104]	-	-	-	336.7	-

-: no data; *BHT by DPPH;**[92, 98,99,100]TEAC: Trolox equivalent antioxidant capacity; ***[100,101]AEAC: ascorbic acid equivalent antioxidant capacity;[74]Jakobek et al., 2007;[92]Mahdavi *et al.*, 2010;[98]Gil *et al.*, 2000; [99]Burin *et al.*, 2010; [100]Pernicea *et al.*, 2009; [101]Pisoschi *et al.*, 2009; [102]Mitiæ et al., 2011; [103]Garofuliæ et al., 2012; [104]Lee et al., 2005;

plants. They have several biological activities, including antioxidant, anti-inflammatory, anti-tumor, neuroprotective, anti-diabetic and cancer chemopreventive agents[57-68]. Additionally, they are widely used as colorants in food industry [69]. Anthocyanins are a subclass of flavonoids and represent one of the most widely distributed classes of flavonoids in various plants [70]. They are glycosides of anthocyanidins, which vary with different hydroxyl or methoxyl substitutions at the 3 and 5 positions on the A and C rings in their basic flavylium structure [71,72]. The difference in chemical structure and color of anthocyanins depends on several factors including pH, temperature, light intensity, amount of pigments, metallic ions, ascorbic acid and sugars (glucose, galactose, rhamnose, xylose and arabinose). Moreover, their variations by acylation of the sugar groups with acids occur. In acidic conditions, there are four anthocyanin structures present in equilibrium: flavyliumcation, quinonoidal base, carbinol pseudobase and chalcone[71,73]. Anthocyanins can commonly be found in numerous fruits, especially various kinds of berries, their juices as well as wines [74,75,76].

Evaluation of total anthocyanins in fruits and other plants are usually relied on spectrophotometric methods or HPLC analysis[77-79]. UV-Visible spectrophotometry is one of the most widespread of anthocyanin spectroscopic methods. The method was also applied for the structural change of anthocyanin under the influence of different physicochemical factors and the process of polymerization of anthocyanins [80]. Since their color and stability depend on several factors, much attention has been focused on the development of sensitive and reliable analytical methods for nondestructive quantification of anthocyanins in fruit juices [50].

Fluorescence spectroscopy of anthocyanins has been less studied. In general, anthocyanins are weakly fluorescent in solution, probably due to quenching of the efficient excited state proton transfer to water. Lack of information of anthocyanins luminescence related to their pigments has been limited in the literature[80]. Fluorescence properties of the chalkone isomer of malvidin 3,5-diglucoside in aqueous solution was studied[81]. It was found that long-wavelength fluorescence (centered at 495 nm) observed in the fluorescence spectra of cis-chalcone is ascribed to emitting species formed during the excited state of the chalcone form [81,82]. The fluorescence of cyanidin and malvidin glycosides in aqueous environment was investigated and found that Cya-3-glc exhibits short-wavelength fluorescence at λ_{max}299 nm which was most effectively excited at 220 nm [80]. Similar short-wavelength fluorescence was observed for Cya-3,5-diglc (λ_{max}308 nm) and Mv-3,5-diglc (λ_{max}293 nm) in a binary solvent system. Moreover, the fluorescence approach for measuring anthocyanins and derived pigments in red wine was also reported [77,78].

Determination of Anthocyanins

Effect of pH on color change of the Mao juice

It is widely known that stability of anthocyanins depends on pH of solution. Anthocyanin reversibly changes its color by varying of pH of the solution. In acidic media, anthocyanin exists as the flavylium ion only (AH^+). In base solution, the quinonoidal base may be present as an anion A^-[80]. The sample solution was diluted with buffer solutions of pH 1-11. The electrolyte solutions for pH 1-2 were prepared with 0.2M KCl and 0.2 M HCl. Acetate buffer solutions of pH 3-5 were prepared from 0.2 M CH_3COOH and 0.2 M CH_3COONa. Phosphate buffer solutions of pH 6-8 were consisted of 0.2 M KH_2PO_4 and 0.2 M K_2HPO_4 and carbonate buffer solutions of pH 9-11 were prepared from 0.05 M $NaHCO_3$ and 0.1 M NaOH. The certain pH value was measured with a pH meter. Changes in colored sample solutions were measured spectrophotometrically.

UV-Visible absorption spectrum was recorded at wavelength of maximum absorption for each sample. Fluorescence intensity of both Cya-3-glu and PGD-3-glu was measured at 306 nm with the excitation at 277 nm, while the fluorescence intensity of the sample solution was detected at 309 nm with the excitation at 280 nm.

Determination of anthocyanins by pH-differential method

Total anthocyanins were determined using the pH-differential method [71]. This method is based on Lambert-Beer's law: $A = \varepsilon cl$. The juice samples were diluted in the solution pH 1.0 (0.025 M KCl) and the acetate (0.4 M) buffer solution pH 4.5. The certain pH values were adjusted to pH 1.0 and 4.5

with a drop wise of strong HCl or NaOH solution. The absorbance of each appropriate dilution of the fruit juices was measured at their maximum wavelengths (λ_{max}) in the visible region and at 700 nm for haze background correction. The absorbance (A) of the diluted samples was calculated as followed:

$$A = (A_{\lambda max} - A_{700})_{pH\ 1.0} - (A_{\lambda max} - A_{700})_{pH\ 4.5}$$

where, $A_{\lambda max}$ is the absorbance at the maximum wavelength in the visible region. The total anthocyanin expressed as Cya-3-gluand PGD-3-glu equivalents were calculated with the following formula:

$$Total\ anthocyanins\ (mg/L) = \frac{A \times MW \times DF \times 10^3}{\varepsilon \times l}$$

where, A is the absorbance obtained from pH-differential, including MW 484.84 g/mol for Cya-3-glu and 464.84 g/mol for PGD-3-glu; DF (dilution factor); *l* (path length) in cm; ε = 26,900 molar extinction coefficient for Cya-3-glu and 31,620 for PGD-3-glu in L $mol^{-1}cm^{-1}$; 10^3 (g-to-mg conversion factor). The total anthocyanins are, however, expressed as mg/100 mL of the juice sample.

Determination of anthocyanins by UV-Visible spectrophotometry

Total anthocyanins are calculated as Cya-3-glu equivalent or PGD-3-glu equivalent by means of their calibration curve obtained. Standard solutions of Cya-3-glu (5.0-30 mg/L) in an acidified methanol (1%HCl in methanol) and PGD-3-glu (5.0-25 mg/L) diluted with 1%HCl in deionized water were used for each calibration curve. The absorbance of each sample dilution was measured at 528 nm and 497 nm for Cya-3-glu and PGD-3-glu, respectively. Total anthocyanins were expressed mg/100 mL Cya-3-glu and PGD-3-glu equivalent.

Determination of anthocyanins by spectrofluorophotometry

Cya-3-glu and PGD-3-glu were chosen as the reference compounds. They were diluted in 12% methanol solution pH 2.0 [77-78]. This pH was chosen according to preliminary study on the effect of pH on fluorescence intensity of the anthocyanins. Excitation spectra were recorded from 220 nm to 300 nm for the emission at 306 nm, whereas emission spectra were measured between 310 nm and 450 nm with the excitation at 277 nm. For each sample, excitation spectra were also recorded from 220 nm to 300 nm for the emission at 309 nm, whereas emission spectra were measured between 310 nm and 450 nm with the excitation at 280 nm.

Cya-3-glu (1.0-5.0 mg/L) and PGD-3-glu (0.5-2.0 mg/L) were diluted in buffer solution pH 2.0 for plotting of their calibration curves. All samples were diluted with the same solvent. For both standard and sample solutions, the fluorescence spectra were recorded with the excitation/emission of 277/306 nm for Cya-3-glu and the excitation/emission of 282/311 nm for PGD-3-glu by spectrofluorophotometer.

Method Validation for Anthocyanins

The linearity was tested for the concentration range of 5-30 mg $L^{-1}$of anthocyanins standard for UV-Visible spectrophotometry, 0.5-5 mg L^{-1} for spectrofluorophotometry, while the dilution factor of the Mao juice samples are 200-1600 folds and 1000-64000 folds for UV-Visible spectrophotometry and spectrofluorophotometry, respectively. The calibration curve was constructed and evaluated by its correlation coefficient. The correlation coefficient (R^2) for all the calibration curves was consistently greater than 0.995 [83,84].

Accuracy of the developed method was evaluated by recovery study of total anthocyanins by standard addition method at three spiking levels (low, medium and high of their calibration curve) of each standard of both anthocyanis. The amount of anthocyanins was estimated by applying the obtained values to the regression equation of the calibration curve[84,85].

Ascorbic Acid

Ascorbic acid ($C_6H_8O_6$; L-ascorbic acid)) or vitamin C is an antioxidant, along with vitamin E, β-carotene, and many other plant-based nutrients. It plays an important role in collagen biosynthesis, iron absorption, and immune response activation and is involved in wound healing and osteogenesis. It also acts as a powerful antioxidant which fights against free-radical induced diseases[11]. Ascorbic acid is the enolic form of one β-ketolactone. Ascorbic acid solution is easily oxidized to the diketo form referred to as dehydroascorbic acid, which can easily

be converted into oxalic acid, diketogulonic acid or threonic acid [86,87].

Antioxidants have aromatic ring structures and are able to delocalize the unpaired electron. Vitamin C ($AscH^-$) in the aqueous phase will directly react with or neutralize hydroxyl, alkoxyl and lipid peroxyl (ROO·) radicals and form H_2O, alcohol and lipid hydroperoxides, respectively. Vitamin C turns to a very stable radical (Asc^{-}•), due to its delocalized structure. Moreover, vitamin C can also neutralize the radical form of other antioxidants such as glutathione radical and vitamin E radical, and regenerate these antioxidants. Vitamin C itself is readily regenerated from Asc^{-}• with NADH or NADPH dependent reductases[88-90].

For the determination of ascorbic acid in foods, the method should apply for both, ascorbic acid and dehydroascorbic acid, to give a total value of vitamin C. Many analytical techniques are mentioning in the literature for the determination of vitamin C in different matrices, such as titrimetric, fluorimetric, spectrophotometric, high-performance liquid chromatographic, or enzymatic methods etc.[11].

Spectrophotometric determination of total ascorbic acid is based on coupling with 2,6-dichlorophenol indophenols dye (DCPIP) in different samples of fruits and vegetables. This procedure is one of most simple, accurate and applicable method for determination of total ascorbic acid in fresh foods, such as fruits and vegetables. DCPIP is organic dye with both acid/base and redox properties, which is blue in neutral solution and pink in acidic solution[91,92].

Thermal Stability

Thermal stability of antioxidants is very important in food preservation. The effectiveness of antioxidants varies depending on the food and conditions of processing and storage. The thermal processing of foods involves heating to temperatures ranging from 50°C to 150°C, depending on the pH of the product and the desired shelf life[93]. Some publications have been reported as follows. Fischer et al. (2013)[94] studied on systematic assessment of the factors influencing the anthocyanin stability and color retention of pomegranate juices and less complex model solutions with particular focus on the effects of colorless phenolic co-pigments. The stability of putative health promoting polyphenols of pomegranate juices was not markedly affected by thermal treatment. Unexpectedly, the HMF contents only slightly increased upon forced heating. Therefore, the visual appearance does not adequately reflect the quality and storage stability of pomegranate juices.

Jie et al. (2013)[95] identified thirteen anthocyanins in the purple-fleshed sweet potato cultivar. The enrichment and degradation kinetics of anthocyanins in these matrices were investigated at 80, 90 and 100°C. A higher stability of anthocyanins was obtained in aqueous solutions with pH 3 and 4 and in apple and pear juices. Volden et al. (2008)[96] studied the effects of various thermal processing treatments (blanching, boiling and steaming) of red cabbage. Autoro was assessed for the levels of glucosinolates (GLS), total phenols (TP), total monomeric anthocyanins (TMA), L-ascorbic acid (L-AA) and soluble sugars, as well as for the antioxidant potential by the ferric reducing ability power (FRAP) and oxygen radical absorbance capacity (ORAC) assays. There were significant ($p<0.05$) losses in blanched red cabbage. Boiling gave less extensive reductions: TP, TMA, FRAP, ORAC, L-AA and soluble sugars. Steaming caused no losses for TP, ORAC, FRAP or soluble sugars. Total GLS were severely affected by processing, with reductions of 64%, 38% and 19% in blanched, boiled and steamed red cabbage, respectively.

Sadilova et al. (2007)[97] investigated the structural changes of anthocyanins at pH 3.5 in purified fractions from black carrot, elderberry and strawberry heated over 6 h at 95°C. Degradation products were monitored by HPLC-DAD-MS to elucidate the prevailing degradation pathways. After heating, decline of Trolox equivalent antioxidant capacity (TEAC) was observed in all samples, which was attributed to both anthocyanins and their colorless degradation products following thermal exposure. As deduced from the ratio of TEAC value and anthocyanin content, the loss of anthocyanin bioactivity could not be compensated by the antioxidant capacity of newly formed colorless phenolics upon heating.

CONCLUSION

Total phenolics including flavonoids and anthocyanins, and ascorbic acid in the fruit juice produced from the Mao tree highly related to their antioxidant activity and thermal stability are reviewed, since recent research approaches on the functional foods, in particular the colored fruits and vegetables, have been focused. In analytical aspects, method validation and determination of these mentioned groups of the potential health's benefit compounds have been increasingly developed. Emphasizing on study of the Mao juice products commercially available in Thai markets, compared with other fruit juices as shown Table 1, are reported.

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