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Anthocyanin and polyphenol contents of *Antidesma thwaitesianum* Müll. Arg. berry juice being stabilized by protein matrices

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Abstract The berry juice of *Antidesma thwaitesianum* Müll. Arg. has been recognized as a rich source of anthocyanin, proanthocyanidin and polyphenol. However, the rapid decrease in polyphenol contents, antioxidant activity and sensory perception during storage hamper its development as health-promoting products. To circumvent this problem, we introduced a method called anthocyanin-protein sorption (APS) to retard polyphenols depletion. The AT berry juice was adsorbed on five different proteins (soy protein isolate (SPI), whey protein isolates (WPI), soybean powder (SBP), black bean powder (BBP), and red bean powders (RBP)), and the matrices obtained after the sorption were evaluated for anthocyanin, proanthocyanidin and total phenolic content along with the antioxidant activity. After four-week storage, all of polyphenol content and the antioxidant activity of AT berry juice decreased dramatically whereas those of anthocyanin-protein matrices were slightly declined (5-21%) over twenty-four-week observation. As protein investigation soy protein isolate (SPI) was most effectively stabilized AT berry juice polyphenols with 5-8% decrease in anthocyanin, proanthocyanidin and total phenoliccontents.

Keywords: *Antidesma thwaitesianum* Müll. Arg., Anthocyanin, Antioxidant, Polyphenol, Soy protein

Introduction

"Coronavirus disease starting in 2019" or COVID-19 that has spread epidemically throughout the world. The people must be carefully protected by strengthening the body's immunity. One of them is to build immunity from food and nutrition. Nutrition has reserved to be an essential role for immunity by interfering with proinflammatory cytokine synthesis, immune cell regulation, and gene expression. Polyphenols are one of categories of natural substances that, exhibit a range of biological activities. Polyphenols promote immunity to

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foreign pathogens via various pathways. It can be used in the immune system effectively (Ding *et al*., 2018).

Antidesma thwaitesianum Müll. Arg. or Ma-Mao is a fruit of the same name as ripe berries in Phyllanthaceae. It found through the region of southeast Asia, especially in some provinces of Thailand. It is a source of high anthocyanin and traditional medicinal plant for a gastric intestinal problem, e.g. diabetes, dysentery, indigestion and constipation (Kassem *et al*., 2013) and is rich in high nutritional, proanthocyanidin, total phenolic compound, flavonoids, amino acid, minerals, vitamins and fiber (Lim, 2012). These are antioxidants that source of bioactivity such as inhibit cancer cell growth and immune stimulation. (Butkhup and Samappito, 2008).

A. thwaitesianum Müll. Arg. was used to treat several diseases. Biological activity of *A. thwaitesianum* Müll. Arg. is expressed cytotoxic activity against lung cancer cell by SRB assay (Tuyoien and Itharat, 2010) and decoction against a panel of six human cancer cell lines (Hansakul *et al.*, 2015) and antibiotic against *Staphyllococus aureus* and *Candida albican* (Tuyoien and Itharat, 2010). In addition, *A. thwaitesianum* Müll. Arg. has an antidiabetic effect, and it improves insulin sensitivity (Mungkhunthod *et al.*, 2016) Thus, the extract *A. thwaitesianum* Müll. Arg. may be use as alternative medicine for type 2 diabetes associated with hypercholesteremic. Puangpronpitag *et al.* (2011) found that *A. thwaitesianum* Müll. Arg. have been recognized to pose a numeral of health-promoting effects including antioxidant, anticarcinogenic, anti-apoptotic and anti-inflammatory activities. Nevertheless, *A. thwaitesianum* Müll. Arg. has been reported to be anti-alpha amylase, anti-alpha glucosidase, anti-apoptotic, anticancer, anti-inflammatory, antimicrobial and anti-viral (Mahomoodally *et al*., 2012; Sireeratawong *et al*., 2012 and Puangpronpitag *et al*., 2011).

Moreover, seeds and marcs or pomace of *A. thwaitesianum* Müll. Arg. which are by products from processed food product exhibited antioxidant activity, alleviate oxidative activity (Puangpronpitag *et al.*, 2008) and antihypertensive effect in hypertensive rats (Kukongviriyapan *et al.*, 2015).

However, polyphenol contents in *A. thwaitesianum* Müll. Arg. processed food products e.g. juice, wine, and jam whice dropped dramatically upon thermal process and storage (Poontawee *et al.*, 2016), leading to reduced antioxidant property and other beneficial effects. In addition, polyphenol oxidase (PPO), sugar contents and organic acids present in *A. thwaitesianum* Müll. Arg. juice also largely involved in precipitation and decomposition of polyphenols.

This approach based on the protein forms tight binding with polyphenols through hydrophobic and hydrogen interactions (Roopchand *et al.*, 2012), while

the affinities of protein and sugars as well as other organic compounds are not observed. In practical, the berry juice was first stirred with protein powder for a short while followed by centrifuge to separate anthocyanin-protein matrices (APMs) from supernatant (Figure 1). Therefore, polyphenols in berry juice were enriched onto protein matrices whereas sugars and other organic compounds were segregated into the supernatant.

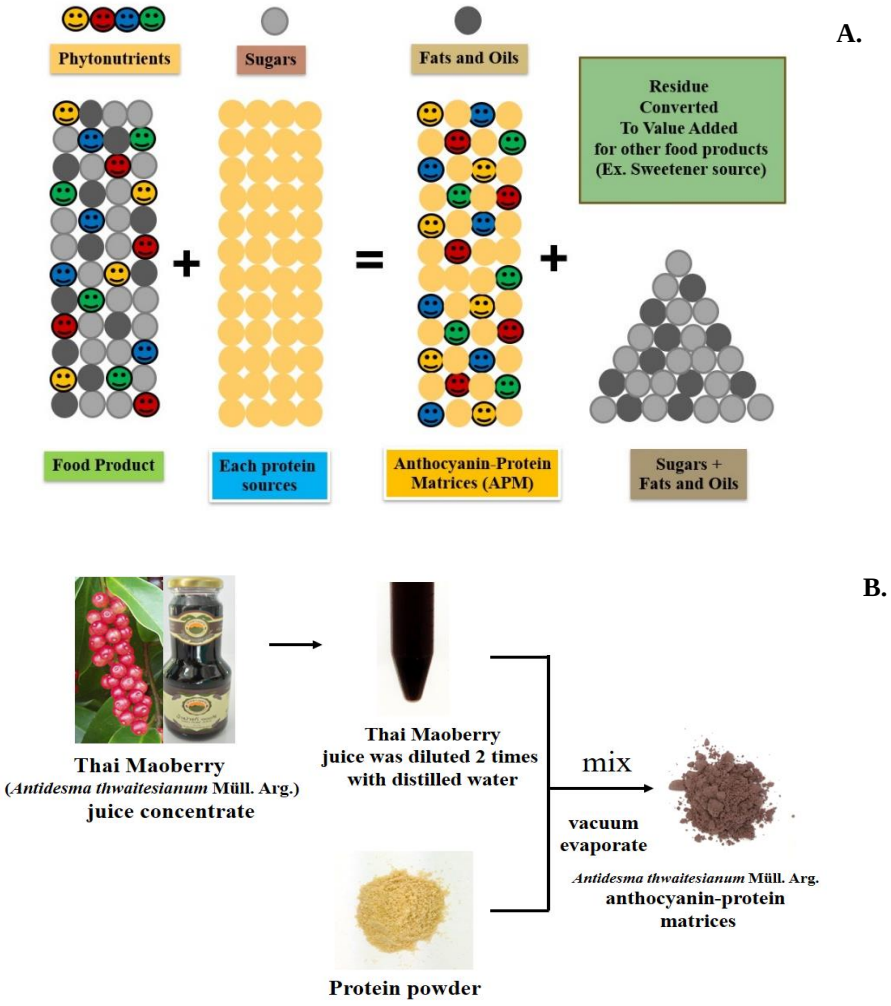

Figure 1. Schematic for production of anthocyanin-protein matrices (A.), Schematic for production of anthocyanin-protein matrices (B.)

The anthocyanin extract in the *A. thwaitesianum* Müll. Arg. was quantified, and calculated amount of each enrich-protein powder

(SPI/WPI/SBP/SGP and WBP) using vacuum evaporation of followed by freeze drying yielded the *A. thwaitesianum* Müll. Arg. anthocyanin-protein matrices.

To circumvent this problem, we introduced the methodology called polyphenol-protein sorption (PPS) to stabilize polyphenol contents in *A. thwaitesianum* Müll. Arg. berry juice without applying thermal conditions. In this study, we examined a variety of protein source for their ability to stabilize each polyphenol contents of AT berry juice through monitoring polyphenol contents and antioxidant property. Furthermore, the stability of polyphenol cotents obtained from anthocyanin-protein matrice (APM) throughout twenty-four-week storage was also evaluated. In addition, the research was studied to prove the possiblility that can be developed to a commercially available product.

Materials and methods

Plant and protein materials

AT berry juice concentrate (20-22 °Brix) was purchased from Sakon Nakorn winery commerce Co., Ltd, Sakon Nakorn Province (Northeastern region), Thailand . Soy and whey protein isolates (SPI and WPI) were imported from ADM, Decatur, IL, USA. Soybean powder (SBP) was taken from Doicham Co., Ltd, Chaing Rai, Thailand, whereas black and red bean powder (BBP and RBP) were purchased from Thanya Farm Co., Ltd, Nonthaburi, Thailand.

Preparation of anthocyanin-protein matrices (APM)

AT berry juice concentrate (24-28 °Brix) was first diluted with distilled water in a ratio of 1:4. The AT berry juice solution (100 mL) was mixed with protein powder (1.2 g) and stirred on a magnetic stirrer for 20 min at room temperature. The resulting mixture was centrifuged 1,500 rpm at 20 °C for 30 min (ROTINA 380/380 R Benchtop Centrifuges classic/cooled) to remove excess juice solution. The remaining anthocyanin-protein matrices were lyophilized and stored dry at 25 °C until use.

Determination of anthocyanin content (ANC)

The pH differential method (Lee *et al*., 2005) was used to measure total monomeric anthocyanin (ANC). The AT-berry anthocyanin-protein matrices

samples were diluted in pH 1 (HCl buffer) and pH 4.5 (NaOH buffer). Absorbance at 520 and 700 nm was measured after 30 min of incubation in the dark at room temperature. The absorbance (A) of the diluted sample was then calculated as (A520 − A700) pH1.0 − (A520 − A700) pH4.5. The absorbance of each sample was measured at 520 nm and 700 nm with a UV/VIS spectrophotometer (UV-7504C; Tsingtao Unicom-Optics Instruments Co., Ltd., China). Concentrations were expressed as mg cyanidin 3-O-glucoside equivalents per L of sample and calculated using the following equation:

ANC (mg/L) =(AxMWxDFx1000)/ (εx1)

where A = (A520 nm-A700 nm) pH 1.0 -(A520 nm-A700 nm) pH 4.5

MW (molecular weight) = 449 g/mol for cyanidin 3-O-glucoside; DF = dilution factor; ε = 26,900 L x mol^{-1} x 9 cm^{-1}, molar extinction coefficient for cyanidin 3-O-glucoside.

Determination of proanthocyanidin (PAC)

Total PAC content was determined colorimetrically using the 4-dimethylaminoinnamaldehyde (DMAC) method (Prior *et al.*, 2010) in a 96 well-plate as previously described. A series of dilutions of standard procyanidin A2 dimer was prepared in 80% ethanol. The anthocyanin-protein matrice (AMP) samples were read at 640 nm against a gallic acid standard curve (Tecan, Infinite F50 Absorbance Microplate Reader, Austria).

Determination of total phenolic compounds (TPC)

Phenolics were determined with Folin–Ciocalteu reagent by the method of Singleton (Singleton and Rossi, 1965). A series of dilutions of standard gallic acid were prepared Folin–Ciocalteu reagent in ranging from 0 to 200 mg (gallic acid)/g. The anthocyanin-protein matrice (APM) each sample were diluted to appropriate concentrations in 96-well plate. The plate reader protocol was set to read the absorbance at 760 nm (Tecan, Infinite F50 Absorbance Microplate Reader, Austria).

Antioxidant activity

Antioxidant activity against ABTS and DPPH was determined using the methods described by Penkumsri (Pengkumsri *et al*., 2015a, 2015b). The antioxidant activity was expressed as milligram of Trolox equivalent per gram of sample.

Stability of Polyphenols at 25 °C

The samples of freeze-dried material (AT-berry juice and anthocyanin-protein matrices) were divided into 5 mL test tube and placed in a 25 °C incubator. At regular intervals over a 24-week period, triplicate sets of samples (1.5 g) were removed (0, 4, 8, 12, 16, 20, and 24 weeks). Total monomeric ANC, PAC, and TPC eluted from the matrices were quantified using the pH differential assay, DMAC assay, and Folin−Ciocalteu assay, respectively. The stability of ANC, PAC and TPC in the anthocyanin-protein matrices (APMs) were evaluated over the course of 24 weeks as described in methods and expressed as a percentage of the original amounts that were eluted on 0 week. Moreover, Antioxidant capacity was determined by performing the ABTS and DPPH assay at 0, 4, 8, 12, 16, 20, and 24 weeks.

Results

Polyphenol contents and antioxidant activity of anthocyanin-protein matrices (APMs)

A variety of APM was prepared from AT berry juice and five different proteins, namely soy protein isolate (SPI), whey protein isolate (WPI), soybean powder (SBP), black bean powder (BBP) and red bean powder (RBP). Anthocyanin-polyphenol contents (Table 1) adsorbed on protein powders were quantified in terms of total phenolic (TPC), anthocyanin (ANC) and proanthocyanidin (PAC). The matrices produced from soy protein isolate (AT-SPI) and whey protein isolate (AT-WPI) contained polyphenol contents, especially ANC values (4.58 ± 0.36 and 4.28 ± 0.26), nearly equivalent to those of AT berry juice (AT-JUI, 4.65 ± 0.42) (Table 1). On the other hand, the matrices AT-SBP, AT-BBP and AT-RBP contained approximately 40-55% lower polyphenol contents than those of AT-JUI. These observations were also consistent with antioxidant activities of AT-SPI and AT-WPI against ABTS and DPPH, which were 12-26% higher than those of AT-SBP, AT-BBP and AT-RBP (Table 2). Polyphenol contents and antioxidant activity retained in APMs were likely to be directly proportional to percentage protein in the matrices.

Stability of polyphenols on storage

How long APMs can stabilize polyphenols in AT berry juice, monitoring polyphenol contents and antioxidant activity every four-week was investigated. The stability of ANC, PAC and TPC in the APMs were evaluated over the

course of 24 weeks as described in methods and expressed as a percentage of the original amounts that were eluted on 0 week (Figure 2.). ANC values of both APMs and AT berry juice slightly reduced (2-6%) within four weeks; however, those of AT berry juice decreased dramatically, and less than 50% of anthocyanin remained in the juice at week 20 (Figure 2A.). The result is the same as PNC and TPC values of APMs and AT berry juice (Figure 2B and 2C). Similarly, antioxidant activity of AT berry juice against DPPH and ABTS was also dropped rapidly after four-week storage (Figure 4).

Table 1. Polyphenol contents adsorbed on AT-protein matrices

Matrices[1]	**% protein**[2]	**ANC**[3]	**PAC**[4]	**TPC**[5]
AB-JUI	-	$4.65^{a} \pm 0.42$	$2.71^{a} \pm 0.57$	$68.15^{a} \pm 0.65$
AT-SPI	90	$4.58^{a} \pm 0.36$	$2.66^{a} \pm 0.73$	$66.95^{a} \pm 0.24$
AT-WPI	85	$4.28^{a} \pm 0.26$	$2.47^{a} \pm 0.15$	$62.72^{a} \pm 0.74$
AT-SBP	31	$2.45^{b} \pm 0.20$	$1.78^{b} \pm 0.04$	$53.71^{b} \pm 0.32$
AT-BBP	24	$2.34^{b} \pm 0.50$	$1.67^{b} \pm 0.19$	$51.56^{b} \pm 0.79$
AT-RBP	22	$2.12^{b} \pm 0.10$	$1.60^{b} \pm 0.11$	$49.44^{b} \pm 0.32$

Note: Mean ± standard deviation (n = 3).; a, b = The differences between the values in the same line are statistically significant ANOVA, ($p < 0.05$).
[1]Flour concentrations were 12 g/L in 2 times diluted AT berry juice
[2]Based on product nutrition label.
[3]Calculated as cyanidin 3-O-glucoside equivalents (mg/g).
[4]Calculated as proanthocyanidin B2 equivalents (mg/g). [5]Calculated as gallic acid equivalents (mg/g).

Table 2. Antioxidant activity of anthocyanin-protein matrices (APM) against ABTS and DPPH

Antioxidant Test	**AT-JUI**	**AT-SPI**	**AT-WPI**	**AT-SBP**	**AT-BBP**	**AT-RBP**
ABTS (% inhibition)	$82.65^{a} \pm 0.32$	$75.34^{a} \pm 0.41$	$72.49^{a} \pm 0.21$	$56.39^{b} \pm 0.35$	$52.41^{b} \pm 0.46$	$51.63^{b} \pm 0.23$
DPPH (% inhibition)	$80.71^{a} \pm 0.28$	$73.61^{a} \pm 0.37$	$70.51^{a} \pm 0.19$	$54.24^{b} \pm 0.43$	$50.67^{b} \pm 0.39$	$49.57^{b} \pm 0.38$

Note: Mean ± standard deviation (n = 3); a, b = The differences between the values in the same line are statistically significant ANOVA, ($p < 0.05$).

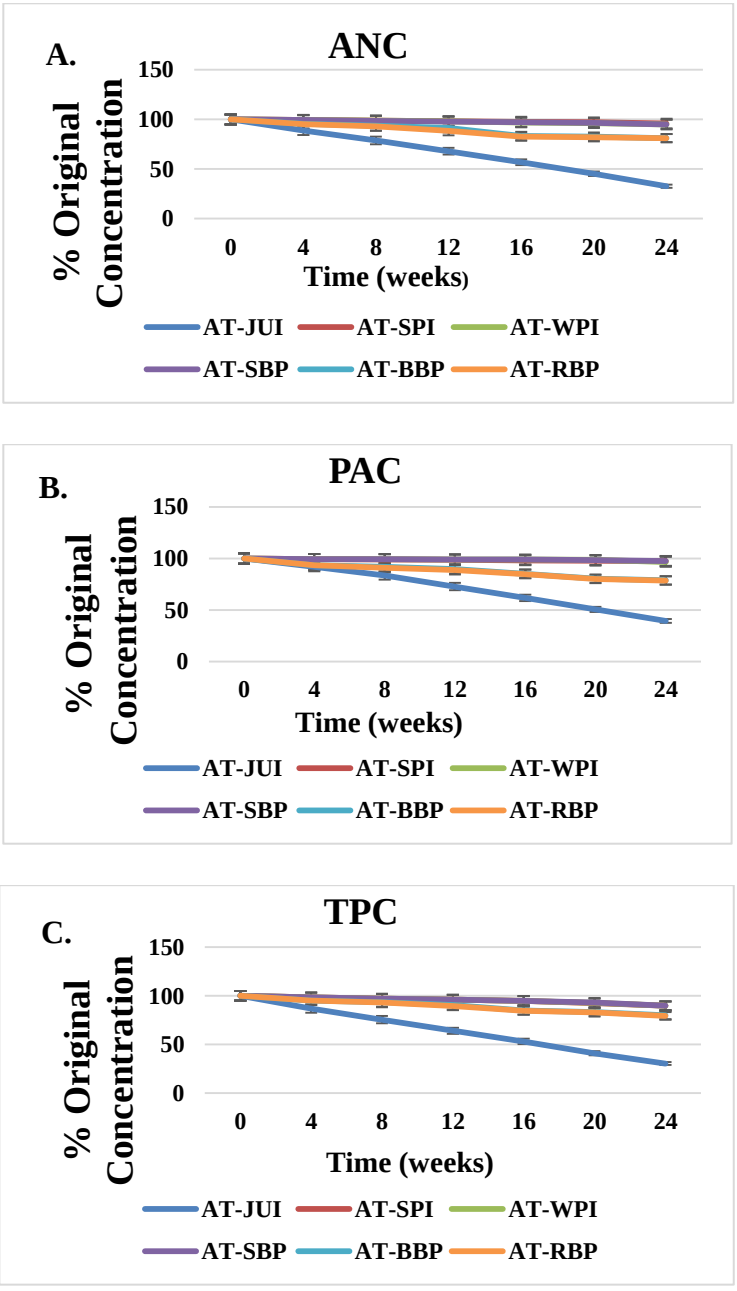

Figure 2. (A) Anthocyanin content (ANC), (B) Proanthocyanindin content (PAC) and (C) total phenolic content (TPC) of AT berry juice and anthocyanin-protein matrices (APMs) stored at 25 ℃ for 24 weeks

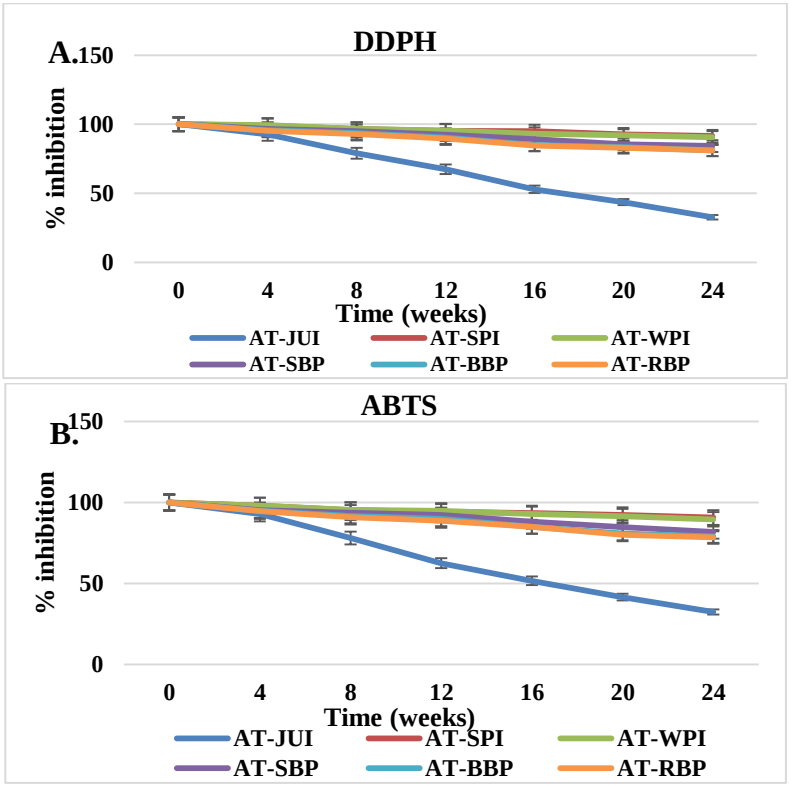

Figure 3. Antioxidant activity of AT berry juice and AMP (A) DPPH and (B) ABTS stored at 25 ℃ for 24 weeks

Product innovation prototype application

The extraction and stabilization of polyphenol compound using SPI, WPI and SBP provides an innovative approach for utilizing AT juice or pomace in the development of novel health food product (anthocyanin-protein matrices (APMs): nutritional enriched food without excess sugars, fat and oil) from this method as a novel innovation technology that can be incorporated in a variety of scientifically validated functional food and dietary supplements. It may be formulated into health foods or dietary supplements innovation product for build body immunity, such as high protein dietary supplement in capsule or tablet, health-high protein yogurt powder, lozenge for health, health-powdered dressing and baked goods. Prototype innovation product from anthocyanin-protein matrices (APMs) for the purpose of health and nutritional were showed in Figure 4. We explored the utility of complexing polyphenols to protein powder to create a novel food ingredient for enriched food or dietary supplements.

Figure 4. Product innovation prototype application of anthocyanin-protein matrices

Discussion

Each anthocyanin–protein matrices (APMs) differed in affinities to AT berry juice that showed a consequence of distinctive physicochemical properties as a result which each protein component in differences in particle size, surface area and degree of solubility were also explained by Roopchand *et al*. (2012). These reasons allow the ability to capture different amounts of polyphenols. Moreover, proteins from soy, pea or whey are considered "fast" digesting, while protein from casein and egg albumin are "slow" digesting (Abou-Samra *et al.*, 2011). Interaction between anthocyanin and protein process efficiently excluded high caloric sugars, fats and oils from the AT berry juice sources (Roopchand *et al.*, 2013; Yousef *et al.*, 2014.)

Anthocyanin contents in both APMs and AT berry juice were slightly reduced (1.6-6.5%) within four-week storage. However, polyphenols (all ANC PAC and TPC) in AT berry juice dropped dramatically after four weeks while those of APMs were slightly reduced within 10%. The reason from anthocyanin-polyphenolic compounds in AT berry juice are particularly unstable and subject to degradation when exposed to extremes in pH or thermal processes as explained by Ferruzzi *et al.*, 2012 and Grace *et al.*, 2013). Levels of ANC, PAC and TPC in the AT-SPI amd AT-WPI were remarkably stable for 24 weeks of incubation at 25 °C with significant differences stated by Grace *et al.*, 2013). Similarly, the antioxidant activity of AT berry juice against DPPH and ABTS also dropped rapidly after four-week storage (Figure 3). The 24 weeks stability study proved that the complexation product stabilizes the anthocyanin-protein matrices (APMs) and results in a new food or drink product with long shelf life.

Anthocyanin in AT berry juice was stabilized by absorption on protein matrices. This method simultaneously prevented degradation of anthocyanin and retained antioxidant activity over 24 weeks on storage. The proteins were examined which soy and whey protein isolates revealed excellent absorption property toward anthocyanin, thus indicating that protein contents play a critical role in stabilizing anthocyanin-polyphenols. This methodology would be served as a potential means to extend shelf life and maintain the beneficial effects of anthocyanin-polyphenols in AT berry juice.

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