International Food Research Journal 29(3): 631 - 645 (June 2022)
Journal homepage: *http://www.ifrj.upm.edu.my*

Synergistic effects of *Garcinia mangostana* and *Clitoria ternatea* extract mixture on antioxidant activities, colour, and anthocyanin stabilities

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Article history

Received:
22 April 2021
Received in revised form:
28 September 2021
Accepted:
25 November 2021

Keywords

anthocyanin,
co-pigmentation,
half-life,
rate constant,
synergistic antioxidant

Abstract

The present work determined the antioxidant activities, and colour and anthocyanin stabilities of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures. The plants were extracted at three different ratios of GMP:CTF at three different extractant pH levels (3.0, 3.5, and 4.0). The highest synergistic effects of ORAC, FRAP, ABTS, and DPPH radical scavenging capacity were observed in the mixture of 25% GMP and 75% CTF (25GMP:75CTF) extracted at pH 3.5. Based on the kinetic degradation of anthocyanin, and the L^*, C^*, and $h°$ values, 25GMP:75CTF extracted at pH 3.5 exhibited a significantly lower rate constant ($-k$ = 2.27) and higher half-life ($t_{1/2}$ = 5.09 h) over 100°C, thus indicating the most stable mixture ratio and extractant pH condition of those tested. Therefore, the mixture of GMP and CTF at a ratio of 25:75 and an extractant pH of 3.5 produced the most stable extract with a good synergistic effect.

Introduction

The global anthocyanin market was valued at around US$ 500 million in 2018, and was projected to reach about US$ 735.9 million by 2026 due to the emerging of new product innovation and development from anthocyanin (Transparency Market Research, 2019). Post COVID-19 is expected to positively impact the global anthocyanin market due to the increasing awareness and demand for natural health as well as chemical-free products (Allied Market Research, 2021). Anthocyanin has a promising potential in diverse applications such as additives, ingredients, and colourants in food, pharmaceutical, and cosmeceutical industries, as well as an indicator in active and smart packaging (Yong and Liu, 2020).

However, the instability of anthocyanin towards pH, temperature, oxygen, sulphur dioxide, bleaching agent, and enzymes limits the application of anthocyanin in food products and processing (Shipp and Abdel-Aal, 2010). The degree of acylation and methoxylation, co-pigmentation complexes, and metal-anthocyanin complexes have been reported to enhance anthocyanin stability by the formation of inter- and intramolecular forces (Buchweitz *et al.*, 2012a; 2012b). Co-pigmentation is a phenomenon in which the pigment and other colourless organic compounds or metallic ions form molecular or complex associations, thus generating a change or an increment in the colour intensity (Rein, 2005). The co-pigments are a rich system in π-electrons which are able to associate with flavylium ions, and are rather poor in electrons (Castañeda-Ovando *et al.*, 2009). This association gives protection for the water nucleophilic attack in the second position of the flavylium ion and sulphur dioxide in the four positions (Darias-Martín *et al.*, 2001; Castañeda-Ovando *et al.*, 2009). When the co-pigment is mixed with an anthocyanin solution, an interaction is carried out by producing a hyperchromic effect (increasing the absorption intensity) and bathochromic shift (increasing the wavelength) in the absorption spectra of the UV-Vis region (Sharara, 2017).

The incorporation of polyphenolics (*i.e.*, hydrocinnamic acid, chlorogenic acid, ferulic acid,

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DOI:
https://doi.org/10.47836/ifrj.29.3.15

and C-glycosyl flavone) and their extracts into the anthocyanin as co-pigment has been reported to increase the anthocyanin stability during thermal processing and storage of model as well as fruit juice systems (Bimpilas *et al.*, 2016; Gras *et al.*, 2016; 2017; Chatham *et al.*, 2020; Pangestu *et al.*, 2020). Apart from polyphenolics, interactions between anthocyanin-anthocyanin were also reported to enhance anthocyanin stability (He *et al.*, 2012; Fernandes *et al.*, 2015). However, the use of pure polyphenolic standards as co-pigments is expensive and not applicable in the food industry. Therefore, the use of crude natural polyphenolic and a combination of natural anthocyanin extracts as cofactors for anthocyanin stability is more cost-effective and applicable in the food industry. There are several studies on the effects of crude polyphenolic addition to the anthocyanin-containing products, but the reports on the combination of anthocyanin-anthocyanin plant samples are scarce (Sari *et al.*, 2012; Bobinaitė *et al.*, 2016).

Kinetic modelling is consistently employed to predict the influence of processing on the quality parameters including colours, nutrients, and vitamin degradations, whereby the information on the degradation kinetics, including reaction order, rate constant, and half-life are vital in predicting food quality loss during storage, as well as thermal process treatments (Patras *et al.*, 2011). The change in physical properties associated with the loss of quality and nutrients is an important factor to consider in food processing. For example, the loss of original colour in a strawberry jam (Patras *et al.*, 2011) and blueberry juice (Buckow *et al.*, 2010; Kechinski *et al.*, 2010) processing was due to the anthocyanin degradation affected by high temperatures. Therefore, kinetic studies are essential to minimise undesired changes, and optimise the quality of natural colourants during processing.

Therefore, the present work aimed at producing defined mixtures from *G. mangostana* (mangosteen) peel and *C. ternatea* (bluebellvine) flower extracts, and to determine whether the crude mixture might improve the antioxidant, colour, and stability properties of anthocyanin at a milder acidic pH, than that commonly used for extraction (pH 2.0 or lower). Next, we hypothesised that mixing plant extracts might produce different tonalities and hues which could not be achieved with any single extract at a given pH. To achieve these objectives, we determined the colour properties, spectral properties, anthocyanin contents, and antioxidant activities of the mixed plant extracts that were extracted at different pH levels (3.0, 3.5, and 4.0). Kinetics studies of the anthocyanin and colour degradation of mixed plant extracts in aqueous medium at 100°C for 120 min, were also investigated.

Materials and methods

Chemicals

Aluminium chloride hexahydrate ($AlCl_3$•$6H_2O$), sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_3$), Trolox, and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium chloride (KCl), hydrochloric acid (HCl), iron chloride hexahydrate ($FeCl_3$•$6H_2O$), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Merck & Co. (Darmstadt, Germany). Additionally, food-grade citric acid and sodium citrate were purchased from Meilun Food Chemical Sdn. Bhd., Selangor, Malaysia.

Raw materials

The *G. mangostana* fruits were obtained from Sg. Siput, Perak, Malaysia, and selected for uniformity of colour at maturity index 6, where the *a**(red)/*b**(green) ratio was between 13 and 16 (Palapol *et al.*, 2009). The exocarps were separated from the mesocarps by using a stainless-steel spoon, and the exocarps were selected for use in the present work (*G. mangostana* peel; GMP). Additionally, the *C. ternatea* flowers (CTF) were obtained from Santan, Perlis, Malaysia. Obtained samples were packed in low-density polyethylene (LDPE) zipper-lock plastic packaging, and stored in the icebox at a controlled temperature (4°C) for approximately 7 h during transportation to Universiti Teknologi MARA, Shah Alam Selangor, Malaysia. The samples were stored for less than 2 w at -20°C in a freezer, prior to extraction.

Sample preparation

The extracts of individual and mixtures of fresh GMP and CTF extracts were prepared in three different ratios (75:25, 50:50, and 25:75; w/w), and labelled 75GMP:25CTF, 50GMP:50CTF, and 25GMP:75CTF, respectively. The extracts of individual GMP and CTF were labelled 100GMP and 100CTF, respectively. The samples were stirred for

10 min at 100°C in 100 mM citrate buffer, at different pH levels (3.0, 3.5, and 4.0). The ratio of plant parts to buffer was 1:4 (w/v). The residue was subsequently extracted twice with the extractant, and the extracts were combined. The extracts were then filtered by using a Whatman filter paper No. 1 and Buchner funnel equipped with a vacuum pump (Gast DAA-V715A-EB, Vatech, Malaysia). The filtrates were collected and evaporated by using a rotary evaporator at 60°C and 114 mbar. The concentrated filtrates were lyophilised and stored in amber bottles at -20°C prior to further analysis.

The blanching technique (heating at 100°C) employed in the present work may enhance the stability of anthocyanin and phenolic compounds by the inactivation of PPO that stabilise the bioactive compounds during processing and storage; furthermore, blanching causes structural changes in plant tissue that lead to the increase in the cell wall porosity thus enhancing the mass transfer and extraction yield of phenolic compounds (Rossi *et al.*, 2003; Brownmiller *et al.*, 2008; De Aguiar Cipriano *et al.*, 2015; Deylami *et al.*, 2016).

UV-Vis spectra characterisation

Accurately, 100 g/L of the samples were prepared by dissolving each of the anthocyanin extract mixtures in distilled water, and diluted to 10 g/L with the respected extractant, which was buffer. Visible absorption spectra of the samples were measured by a spectrophotometer (Perkin-Elmer Lambda 35, USA) from 400 to 700 nm. The samples were allowed to equilibrate for 15 min before the measurements. All the experimental procedures were conducted in minimal light, and the samples were also protected from light.

Colour properties

Approximately 100 g/L of the samples were prepared by dissolving each of the anthocyanin extract mixtures in distilled water. The visual colours of the mixture samples were measured by Minolta CR-400 (Konica Minolta Inc., Mahwah, NJ) colorimeter at 8 mm contact area. The CIE colour was expressed in lightness (L^*), chroma (C^*), and hue angle ($h°$).

Anthocyanin content

The total anthocyanin content (TAC) was determined following the methods described by Cinquanta *et al*. (2002) and Lee *et al*. (2005). The observed TAC value of each mixture was compared with the expected value, which was a mathematical sum of the TAC derived from the individual extract, analysed at three ratios of GMP and CTF. The means of the observed and expected values were compared by using a *t*-test. The difference between the two means was considered significant at $p < 0.05$, and statistically analysed by SAS (2002).

Antioxidant activities

The antioxidant activities of the extract mixtures were determined using ABTS radical scavenging assay (Kim *et al.*, 2010), DPPH radical scavenging assay (Brand-Williams *et al.*, 1995), FRAP assay (Benzie and Strain, 1996), and ORAC assay (Zen-Bio, 2011).

To compare the antioxidant activities of individual and mixture of extracts, the synergistic effect (SE) was calculated as SE = (Experimental value) / (Theoretical value). The theoretical values were calculated as the mathematical sum of the antioxidant activity derived from the individual extract analysed at three ratios of GMP and CTF. SE > 1, SE = 1, and SE < 1 represent synergism, additivity, and antagonism, respectively (Fuhrman *et al.*, 2000). The DPPH radical scavenging activity of the extract mixtures was expressed in EC_{50} with SE > 1, SE = 1, and SE < 1 representing antagonism, additivity, and synergism, respectively.

Anthocyanin and colour stabilities

Approximately 100 g/L of the samples were prepared by dissolving the extract mixture in distilled water. The influence of heating temperature on the anthocyanin and CIE colour stabilities was determined in the cap boiling tubes wrapped with aluminium foil, and stored in 100°C oven for 0, 15, 30, 45, 60, 75, 90, 105, and 120 min. Then, 5 mL of the samples were taken from the boiling tube, cooled, and equilibrated for 15 min at room temperature, prior to obtaining the measurements. The percentages of the remaining anthocyanins were calculated using Eq. 1. The percentages of the remaining anthocyanins were plotted as a function of time (min).

$$\text{Percentage of residual anthocyanin} = \frac{[A]}{[A_0]} \times 100 \quad \text{(Eq. 1)}$$

where, $[A_0]$ = total anthocyanin content before the treatment, and [A] = total anthocyanin after the treatment.

The percentages of the remaining anthocyanins were plotted as a function of time (min). Based on the plotted graph, the r^2 showed that the remaining anthocyanin *versus* time fit the first-order reaction kinetic. Since the degradation of the sample fit to the first-order reaction, so the rate constant and half-life were calculated using Eqs. 2 and 3, respectively.

$$\ln\left(\frac{A_t}{A_0}\right) = -k \times t \quad \text{(Eq. 2)}$$

$$t_{1/2} = -\frac{\ln(2)}{k} \quad \text{(Eq. 3)}$$

where, A_t = percentage of the remaining anthocyanin, A_0 = percentage of the initial anthocyanin, k = rate constant (obtained from the slope of the graph), t = time, and $t_{1/2}$ = half-life.

The CIE colour stabilities were expressed in L^*, C^*, and $h°$, and the parameters were plotted as a function of time (min). Based on the plotted graph, the r^2 showed that the remaining anthocyanin and C^* value *versus* time fit the first-order reaction kinetics, while L^* and $h°$ values *versus* time fit to the zero-order reaction kinetics. Hence, the changes in L^* and $h°$ values were modelled based on zero-order kinetics, and the rate constant and half-life of L^* and $h°$ were calculated using Eqs. 4 and 5 (Reyes and Cisneros-Zevallos, 2007). Meanwhile, the rate constant and half-life of C^* value were calculated using Eqs. 6 and 7, respectively.

$$L^* \text{ or } h^o = k \times t \quad \text{(Eq. 4)}$$

$$t_{1/2} = \frac{L^*_{initial} \text{ or } h^o_{initial}}{2k} \quad \text{(Eq. 5)}$$

$$\ln\left(\frac{C^*_t}{C^*_0}\right) = -k \times t \quad \text{(Eq. 6)}$$

$$t_{1/2} = -\frac{\ln(2)}{k} \quad \text{(Eq. 7)}$$

where, C^*_t = final chroma value, and C^*_0 = initial chroma value.

Statistical analysis

Data were analysed from three triplicate samples. Statistical analyses were conducted using Statistical Analysis System 9.1.3 software package (SAS, 2002). Analysis of variance (ANOVA) in a completely randomised design and Duncan's multiple range tests were used to compare any significant differences between samples. The least significant difference (LSD) at 5% level was calculated to compare the differences between the means following a significant ANOVA effect. Values were expressed as the means ± standard deviations (SD).

Results and discussion

UV-Vis spectra

The visible spectrum of GMP exhibited maximum absorption wavelength at 515.85, 516.08, and 514.97 nm at pH levels of 3.0, 3.5, and 4.0, respectively. The maximum absorption wavelength of GMP at 514.97, 515.85, and 516.08 nm might have been due to the presence of cyanidin 3-glucoside and cyanidin 3-sophoroside, as previously reported by Siti Azima *et al.* (2017). This is also consistent with the findings of Vergara *et al.* (2009) who reported that cyanidin 3-glucoside and cyanidin 3-sophoroside exhibited maximum absorption at 516 and 518 nm, respectively. Meanwhile, delphinidin and polymeric anthocyanin (ternatin) were the main anthocyanins found in CTF (Siti Azima *et al.*, 2017; Escher *et al.*, 2020).

The CTF extract exhibited maximum wavelength absorption at 573.95 and 619.26 nm, 573.38 and 619.12 nm, and 574.59 and 624.80 nm, at pH levels of 3.0, 3.5, and 4.0, respectively. The absorption band at 619 nm in CTF extract might have been due to the formation of metal quinonoidal complexes as described in Lee *et al.* (2011). The addition of CTF to GMP at a certain ratio and pH resulted in the bathochromic shift and the hyperchromic or hypochromic effect of the original sample, which might suggest the self-association or co-pigmentation effect of anthocyanin and phenolic compounds in GMP and CTF, thus forming anthocyanin-anthocyanin or anthocyanin-phenolic compound complexes. Previous studies reported that phenolic compounds found in CTF were protocatechuic acid, gallic acid, chlorogenic acid, myricetin, cyanidin, delphinidin, epicatechin, rutin, kaempferol, and quercetin (Kaisoon *et al.*, 2011; Siti Azima *et al.*, 2017; Escher *et al.*, 2020). Meanwhile, GMP was found to contain chlorogenic acid, vanillic acid, protocatechuic acid, *p*-coumaric acid, ferulic acid, gallic acid, caffeic acid, epicatechin, catechin, cyanidin 3-sophoroside, cyanidin 3-glucoside, myricetin, rutin, kaempferol, and quercetin (Zarena and Sankar, 2012; Siti Azima *et al.*, 2017). Therefore, the mixture of these two plant extracts, which possess

structurally diverse phytochemicals, might have enhanced the anthocyanin stability in the individual sample (GMP or CTF) by self-association or co-pigmentation. Furthermore, Gras *et al.* (2017) reported that the mixture of purple sweet potato anthocyanin extract with phenolic from apple and rosemary extracts also resulted in a hyper- or hypo-chromic effect.

The most evident hyperchromic shift (ΔA = 0.45 AU) and bathochromic shift (Δλmax = 56.95 nm) was observed in 25GMP:75CTF with the extractant pH of 3.5, as shown in Table 1. The mixture of purple sweet potato extract with phenolic acid, apple extract, and rosemary extract imparted a strong co-pigmentation effect (hyperchromic shift), being particularly powerful at pH 3.6 and 4.6 (Gras *et al.*, 2017). Gonnet (1999) also reported that the most intense effects of co-pigmentation were observed at pH 3.5. A major bathochromic shift from 619 to 624 nm, and hyperchromic effect of CTF as the extractant pH increased from 3.0 to 4.0, is consistent with the results obtained by Lee *et al.* (2011) which might have been due to the formation of the ionic bond between citric acid and anthocyanin chromophores (Houghton and Hendry, 2012).

Table 1. Bathochromic and hyperchromic effects of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium.

pH	Bathochromic/hyperchromic effect					
	λ_{max}			ΔA_{max}		
	75GMP:25CTF	**50GMP:50CTF**	**25GMP:75CTF**	**75GMP:25CTF**	**50GMP:50CTF**	**25GMP:75CTF**
pH 3.0	11.35 ± 1.11^{Cc}	56.84 ± 1.22^{Ba}	57.74 ± 1.09^{Aa}	0.15 ± 0.02^{Aa}	0.15 ± 0.03^{Bb}	0.41 ± 0.02^{Ab}
pH 3.5	54.08 ± 1.21^{Bb}	56.29 ± 1.53^{Ba}	56.95 ± 1.51^{Aa}	0.06 ± 0.01^{Cc}	0.25 ± 0.03^{Ba}	0.45 ± 0.02^{Aa}
pH 4.0	57.96 ± 1.12^{Ab}	59.03 ± 1.30^{Aa}	59.35 ± 1.29^{Aa}	0.11 ± 0.01^{Bb}	0.19 ± 0.02^{Ab}	0.19 ± 0.02^{Ab}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios.

The most common indication of anthocyanin colours is based on the maximum absorption of the visible wavelength of a spectrophotometer. Since anthocyanin occurs as a flavylium cation at a strong pH, the maximum wavelength is reasonably representative of colour at these pH levels. However, as the pH increases, each anthocyanin occurs as a mixture of various equilibrium forms. Therefore, at these pH levels, the mixture of maximum wavelength and shoulder may be the representative colour of the solution (Torskangerpoll and Andersen, 2005). As a result, as compared to only using the wavelength of maximum absorbance, a CIE colour system that measures the reflection or transmission spectrum of an object at all wavelengths is preferable and more accurate in representing the colour of the solution.

Colour properties and anthocyanin content

Table 2 shows the L^* value, C^* and $h°$, and anthocyanin contents of the extract mixtures at different extractant pH levels. The L^* value of the extract mixtures significantly decreased ($p < 0.05$) as the concentration of CTF increased, which indicated that the darkness of the sample increased as the concentration of CTF increased. Previous study showed that the L^* value of anthocyanin extract obtained from *Syzygium cumini* fruit decreased with the addition of polyphenolic from rosemary extract, which acted as a co-pigment (Sari *et al.*, 2012). The addition of co-pigments (phenolics from apple and rosemary extracts) resulted in a significant decrease in the L^* value, which was pronounced at elevated co-pigment dosages (Gras *et al.*, 2017).

A significant decrease in the C^* value was observed at pH 3.0 as the concentration of GMP decreased from 100 to 50%, but an increasing trend was observed as the concentration of CTF increased from 50 to 100%. At pH 3.5, a decreasing trend in the C^* value was observed as the ratio of GMP decreased from 100 to 75%, but the chromaticity increased as the concentration of CTF increased from 25 to 100%. At pH 4.0, an increasing trend of the C^* value was observed as the concentration of CTF increased from 25 to 100%. This indicated that increasing the concentration of CTF at a specific ratio and pH significantly increased the colour saturation or intensity of the extract mixtures. The addition of red beet, where the major pigment is betalain, to the anthocyanin-based samples, namely black carrot, strawberry, and elderberry, resulted in the increase in the C^* value as the concentration of red beet

Table 2. Colour properties and anthocyanin content of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium.

Colour properties and anthocyanin content	pH	Samples				
		100GMP	**75GMP:25CTF**	**50GMP:50CTF**	**25GMP:75CTF**	**100CTF**
Lightness (L^*)	pH 3.0	39.66 ± 0.03^{Ca}	32.15 ± 0.16^{Cb}	27.95 ± 0.01^{Cc}	26.48 ± 0.03^{Bd}	24.76 ± 0.01^{Ce}
	pH 3.5	40.95 ± 0.38^{Ba}	32.81 ± 0.05^{Bb}	28.65 ± 0.02^{Bc}	26.37 ± 0.03^{Cd}	25.42 ± 0.01^{Ae}
	pH 4.0	44.30 ± 0.23^{Aa}	35.39 ± 0.03^{Ab}	29.93 ± 0.01^{Ac}	26.81 ± 0.01^{Ad}	25.31 ± 0.01^{Be}
Chroma (C^*)	pH 3.0	31.92 ± 0.14^{Aa}	24.92 ± 0.18^{Ad}	23.99 ± 0.05^{Ae}	26.59 ± 0.11^{Bc}	30.08 ± 0.06^{Bb}
	pH 3.5	21.81 ± 0.58^{Bd}	20.47 ± 0.04^{Be}	22.60 ± 0.06^{Bc}	26.83 ± 0.09^{Bb}	29.61 ± 0.08^{Ca}
	pH 4.0	14.75 ± 0.39^{Ce}	15.81 ± 0.05^{Cd}	23.89 ± 0.05^{Ac}	29.03 ± 0.05^{Ab}	32.61 ± 0.06^{Aa}
Hue angle ($h°$)	pH 3.0	28.35 ± 0.09^{Ad}	2.48 ± 0.09^{Ce}	339.55 ± 0.05^{Aa}	325.39 ± 0.03^{Ab}	316.31 ± 0.06^{Ac}
	pH 3.5	21.35 ± 0.10^{Be}	343.75 ± 0.06^{Aa}	323.85 ± 0.06^{Bb}	315.92 ± 0.08^{Bc}	311.95 ± 0.07^{Bd}
	pH 4.0	17.45 ± 0.10^{Cd}	324.93 ± 0.08^{Ba}	310.28 ± 0.05^{Cb}	308.38 ± 0.05^{Cc}	308.38 ± 0.07^{Cc}
Total anthocyanin content (mg/g cyanidin 3-glucoside)	pH 3.0	23.83 ± 0.17^{Aa}	$22.66 \pm 0.62^{Ab}*$	$21.39 \pm 0.56^{Ac}*$	$19.42 \pm 0.54^{Ad}*$	16.08 ± 0.01^{Ae}
	pH 3.5	23.56 ± 0.49^{Aa}	$22.41 \pm 0.57^{Ab}*$	$21.70 \pm 0.41^{Ac}*$	$20.11 \pm 0.71^{Ad}*$	16.32 ± 0.51^{Ae}
	pH 4.0	22.97 ± 0.89^{Ba}	21.25 ± 0.74^{Bb}	$19.86 \pm 0.61^{Bc}*$	$18.42 \pm 0.54^{Bd}*$	14.98 ± 1.09^{Be}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios. Asterisk (*) indicates a significant difference between observed and expected values ($p < 0.05$). The expected value for the total anthocyanin of the mixtures were: 75GMP:25CTF at the extractant pH 3.0, 3.5, and 4.0 were 21.89 ± 0.13, 21.73 ± 0.37, and 20.98 ± 0.70, respectively; 50GMP:50CTF at the extractant pH 3.0, 3.5, and 4.0 were 19.95 ± 0.09, 19.91 ± 0.32, and 18.98 ± 0.67, respectively; and 25GMP:75CTF at the extractant pH 3.0, 3.5, and 4.0 were 18.01 ± 0.04, 18.08 ± 0.37, and 16.98 ± 0.83, respectively.

increased (Stintzing *et al.,* 2006). The co-pigmentation of anthocyanins in *S. cumini* fruit with non-coloured phenolics (sinapic, ferulic, and caffeic acids) decreased the *L** and increased the *C** values, thus demonstrating that the colour of co-pigmented anthocyanin in the beverage model was more intense and saturated than the colour of natural anthocyanin (Sari *et al.,* 2012).

The hue angle of the samples shifted from red (28.35°) to magenta (308.38°) as the concentration of CTF increased from 25 to 100%. The shift in *h*° as a result of an intermixture of anthocyanin from plant extract with other plant extract is consistent with Stintzing *et al.* (2006) and Sari *et al.* (2012). The mixture of purple sweet potato anthocyanins with apple and rosmarinic extracts produced a wide range of colour shades between magenta and yellow as a result of co-pigmentation effects and the genuine colour of extracts (Gras *et al.,* 2017). The shift in *h*° is also consistent with the bathochromic shift observed in the UV-Vis spectra. The *h*° of the samples of 50GMP:50CTF at pH 3.0 and 75GMP:25CTF at pH 3.5 shifted from red to crimson. Therefore, this shift might be attributed to the bathochromic shift and the formation of three peaks in the visible spectra of 50GMP:50CTF at pH 3.0 (528.20, 572.69, and 618.74 nm, respectively) as well as 75GMP:25CTF at pH 3.5 (530.81, 570.16, and 618.71 nm, respectively). Additionally, the magenta colour of extract mixtures of 25GMP:75CTF at pH 3.0; 50GMP:50CTF and 25GMP:75CTF at pH 3.5; 75GMP:25CTF, 50GMP:50CTF, and 25GMP:75CTF at pH 4.0; and 100CTF might have been due to the equilibrium mixtures of red flavylium cations that appeared as a small shoulder at 529 - 532 nm, and the two tautomers of neutral blue quinonoidal bases with two absorption bands at 573 - 574 and 619 - 624 nm, as reported by Lee *et al.* (2011).

The observed values of all extract mixtures and extractant pH levels were significantly higher ($p < 0.05$) in TAC as compared to the expected values, except for the observed value of 75GMP:25CTF at pH 4.0, which exhibited no significant difference ($p > 0.05$) in TAC as compared to that of the expected value (Table 2). Additionally, the highest percentage of deviation between the observed and the expected values was noted in 25GMP:75CTF at pH 3.5 (11.23%). The protective effect on the anthocyanin recovery of the extract mixture was likely related to the phenomenon of co-pigmentation, which was more evident in 25GMP:75CTF at pH 3.5.

Antioxidant activities

Mechanism of actions of antioxidant assays can be categorised either into hydrogen atom transfer (HAT), or single electron transfer (SET) (Gupta, 2015). According to Prior *et al.* (2005), antioxidants can deactivate radicals both by hydrogen atom transfer and single electron transfer, and the mechanism depends on the antioxidant structure and properties, solubility and partition coefficients, and solvent system. After all, the major factors that determine the mechanism of antioxidants are bond dissociation energy (BDE) and ionisation potential (IP) (Wright *et al.,* 2001). Based on the mechanism of antioxidant, ORAC assay is considered as HAT-based assay while FRAP assay is considered as SET-based assay (Prior *et al.,* 2005; Craft *et al.,* 2012). Although the DPPH and ABTS radical scavenging activities are classified as SET-based assays, these radicals may be neutralised either by direct reduction via electron transfer or by radical quenching via H atom transfer, thus considered as mixed HAT/SET assay (Prior *et al.,* 2005; Apak *et al.,* 2013). Structurally diverse phytochemicals in plant-based foods may possess similar, overlapping, or different but complementary effects of their antioxidant activities (Wang *et al.,* 2011). Therefore, the combination of different plant-based foods or extracts may exhibit additive, synergistic, or antagonist effects.

The antioxidant activities and the synergistic effects of the extract mixtures at different extractant pH levels are shown in Tables 3 and 4, respectively. The addition of 75% CTF at pH 3.0, and 25% CTF at pH 4.0 showed a significant decrease in the ABTS radical scavenging activities. Additionally, at pH 3.5, no significant decrease was noted in the extract mixtures when the percentage of CTF was increased from 25 to 75%. The extract mixtures showed synergistic and additive effects on the ABTS radical scavenging activity. Moreover, the highest synergistic effect of the extract mixtures on the ABTS radical scavenging activity was noted in 25GMP:75CTF at pH 3.5.

According to Mena *et al.* (2013), the mixture of 75% Mollar de Elche pomegranate juice with 25% Wonderful pomegranate juice significantly increased the ABTS radical scavenging activities, but the mixture of 75% Mollar de Elche pomegranate with 25% lemon juices significantly decreased the ABTS radical scavenging activities. Koa and Sopade (2012)

Table 3. Antioxidant activity of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium.

Antioxidant activity assay	pH	Samples				
		100GMP	75GMP:25CTF	50GMP:50CTF	25GMP:75CTF	100CTF
ABTS radical scavenging activity (μM TEAC/g FEW)	pH 3.0	5.20 ± 0.01^{Ca}	4.93 ± 0.17^{Ca}	4.45 ± 0.27^{Ca}	3.94 ± 0.06^{Cb}	3.02 ± 0.13^{Cc}
	pH 3.5	6.53 ± 0.09^{Ba}	6.33 ± 0.14^{Ba}	6.10 ± 0.10^{Ba}	5.81 ± 0.22^{Ba}	3.65 ± 0.13^{Bb}
	pH 4.0	7.63 ± 0.11^{Aa}	7.12 ± 0.08^{Ab}	6.89 ± 0.20^{Ab}	6.65 ± 0.48^{Ab}	4.2 ± 0.05^{Ac}
DPPH radical scavenging activity (mg/ml)	pH 3.0	0.11 ± 0.01^{Be}	0.19 ± 0.01^{Bd}	0.22 ± 0.01^{Bc}	0.28 ± 0.02^{Bb}	0.49 ± 0.01^{Aa}
	pH 3.5	0.11 ± 0.01^{Be}	0.18 ± 0.01^{Cd}	0.23 ± 0.02^{Bc}	0.28 ± 0.02^{Bb}	0.48 ± 0.01^{Ba}
	pH 4.0	0.12 ± 0.01^{Ae}	0.20 ± 0.01^{Ad}	0.27 ± 0.01^{Ac}	0.32 ± 0.01^{Ab}	0.48 ± 0.01^{Ba}
FRAP value (mM TEAC/g FEW)	pH 3.0	79.37 ± 0.77^{Aa}	63.49 ± 0.41^{Bb}	46.74 ± 0.32^{Bc}	31.26 ± 1.33^{Cd}	13.33 ± 0.28^{Ce}
	pH 3.5	79.90 ± 0.35^{Aa}	66.59 ± 1.16^{Ab}	49.07 ± 1.16^{Ac}	37.18 ± 1.65^{Ad}	14.09 ± 0.21^{Be}
	pH 4.0	78.08 ± 0.15^{Ba}	62.94 ± 0.63^{Bb}	47.04 ± 0.21^{Bc}	35.47 ± 0.96^{Bd}	14.59 ± 0.37^{Ae}
ORAC value (μmol TEAC/g FEW)	pH 3.0	18.79 ± 0.45^{Ba}	17.47 ± 0.82^{Bb}	16.57 ± 1.04^{Abc}	15.54 ± 0.74^{Bc}	11.83 ± 1.59^{Ad}
	pH 3.5	19.46 ± 0.40^{Aba}	19.09 ± 0.55^{Aa}	17.01 ± 0.87^{Ab}	16.34 ± 0.93^{Ab}	12.33 ± 1.48^{Ac}
	pH 4.0	19.76 ± 0.64^{Aa}	19.11 ± 0.47^{Aab}	17.75 ± 0.75^{Abc}	16.72 ± 0.47^{Ac}	12.92 ± 1.48^{Ad}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios.

Table 4. Synergistic antioxidant effect of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium.

Antioxidant activity assays	pH	Extract mixture		
		75GMP:25CTF	50GMP:50CTF	25GMP:75CTF
ABTS radical scavenging activity	pH 3.0	1.06 ± 0.04^{Aba}	1.08 ± 0.08^{Ba}	1.11 ± 0.18^{Ba}
	pH 3.5	1.09 ± 0.03^{Ac}	1.20 ± 0.04^{Ab}	1.33 ± 0.09^{Aa}
	pH 4.0	1.05 ± 0.01^{Bc}	1.16 ± 0.02^{Ab}	1.30 ± 0.09^{Aa}
DPPH Radical scavenging activity	pH 3.0	0.89 ± 0.06^{Ba}	0.73 ± 0.04^{Cb}	0.69 ± 0.06^{Bb}
	pH 3.5	0.88 ± 0.05^{Ba}	0.78 ± 0.05^{Bb}	0.71 ± 0.05^{Bc}
	pH 4.0	0.96 ± 0.03^{Aa}	0.90 ± 0.04^{Ab}	0.84 ± 0.05^{Ac}
ORAC Assay	pH 3.0	1.02 ± 0.02^{Ba}	1.08 ± 0.07^{Aa}	$1.16 \pm 0.15_{Aa}$
	pH 3.5	1.08 ± 0.03^{Aa}	1.07 ± 0.04^{Aa}	1.17 ± 0.13^{Aa}
	pH 4.0	1.06 ± 0.05^{Aba}	1.09 ± 0.07^{Aa}	1.15 ± 0.11^{Aa}
FRAP Assay	pH 3.0	1.01 ± 0.01^{Bb}	1.01 ± 0.01^{Bb}	1.05 ± 0.05^{Ba}
	pH 3.5	1.05 ± 0.02^{Ab}	1.04 ± 0.02^{Ab}	1.22 ± 0.05^{Aa}
	pH 4.0	1.01 ± 0.01^{Bb}	1.02 ± 0.01^{Bb}	1.16 ± 0.03^{Aa}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios.

reported that the increased concentration of sorghum in a sorghum-barley mixture decreased the ABTS radical scavenging activities. However, in the same study, a synergistic effect of the sorghum-barley mixtures was noted in 20 sorghum:80 barley and 40 sorghum:60 barley. Oszmianski and Wojdylo (2009) investigated the effect of blackcurrant and two varieties of apple mash, and found that apple juices mixed with 20% blackcurrant had much higher ABTS radical scavenging activity than that of mashes made from only apples, and a synergistic effect on the ABTS radical scavenging activity was also noted in the mixture of apple-blackcurrant.

The DPPH radical scavenging activity showed that CTF mixed with GMP had a significantly lower ($p < 0.05$) EC_{50} than that of 100% CTF, with a noticeable decrease in EC_{50} in 25GMP:75CTF at pH 3.0 (46.94%) and pH 3.5 (43.06%). Lower EC_{50} indicated higher DPPH radical scavenging activity. Therefore, the extract mixtures resulted in higher DPPH radical scavenging activities than those of the individual CTF extract. In addition, the extract mixture also resulted in the synergistic effects on the

DPPH radical scavenging activity. The DPPH radical scavenging activity of the extract mixture was expressed in EC_{50}. The 25GMP:75CTF (at pH 3.0 and 3.5) and 50GMP:50CTF (at pH 3.0) extracts had significantly lower SE values than those in the other extract mixtures (SE = 0.69, 0.71, and 0.73, respectively).

Wang *et al.* (2011) studied the synergistic, additive, and antagonist effects of combining several foods on the antioxidant capacities, and reported that a mixture of apple with purple cauliflower and a mixture of raspberry with adzuki bean demonstrated a synergistic effect on DPPH radical scavenging activity. In the same study, an additive and antagonistic effect was also noted in the combinations of other foods. Oszmianski and Wojdylo (2009) studied the mixture of blackcurrant with two varieties of apples (Shampion and Idared), and demonstrated higher DPPH radical scavenging activity than pure apples. In the same study, a synergistic effect on the DPPH radical scavenging activity was observed in the combination of 80% Shampion apple and 20% blackcurrant, while an antagonistic effect was observed in the combination of 80% Idared apple and 20% blackcurrant. However, Nedamani *et al.* (2015) asserted that the tertiary combination of green tea, oak, and rosemary did not show any synergistic effect on the DPPH radical scavenging activity; thus, only additive and antagonistic effects were noted.

Based on the ORAC assay, the extract mixtures had significantly higher ORAC values than those of the 100CTF. The significant increase in the ORAC value was noted in 25GMP:75CTF at pH 3.0, 3.5, and 4.0 with an increase of 31.36, 32.52, and 29.41%, respectively. In addition, the mixture of GMP and CTF also resulted in synergistic and additive effects on the ORAC values. All combinations of GMP and CTF exhibited a synergistic effect on ORAC, with an SE value > 1, except for 75GMP:25CTF at pH 3.0, which showed an additive effect, with SE value = 1.

Wang *et al.* (2011) reported significant synergistic effects on the oxygen radical antioxidant capacity when combining several foods: soybean-adzuki bean, apple-tomato, raspberry-soybean, raspberry-adzuki bean, raspberry-black bean, apple-adzuki bean, apple with black bean, broccoli-adzuki bean, mushroom-black bean, and purple cauliflower-black bean. Parker *et al.* (2009) evaluated the synergistic antioxidant potential of complex mixtures of eight phenolic compounds found in honey and papaya, and reported that most of the combinations provided additive effects with some combinations providing synergistic effects on the oxygen radical antioxidant capacity.

The extract mixtures had significantly higher FRAP values at all extractant pH levels than those of 100CTF, thus indicating that the addition of GMP to CTF improved the FRAP value of the extract. In addition, the mixture of GMP and CTF demonstrated additive and synergistic effects, with the highest synergistic effect noted in 25GMP:75CTF at pH 3.5 (SE = 1.22 ± 0.05). Wang *et al.* (2011) investigated the effect of combining several foods on the ferric-reducing antioxidant power, and found that combining apple with black bean and raspberry with adzuki bean resulted in a significant synergistic effect (SE = 1.08 and 1.19, respectively). Nedamani *et al.* (2015) reported that all the tertiary combinations of green tea, oak, and rosemary showed a synergistic effect on the ferric-reducing power. Additionally, the combinations of 20% blackcurrant with 80% Idared apple demonstrated an additive effect on the ferric-reducing power, but the combination of 20% blackcurrant with 80% Shampion apple showed an antagonistic effect (Oszmianski and Wojdylo, 2009).

In general, only 25GMP:75CTF showed the highest synergism in the four antioxidant assays employed in the present work. Meanwhile, none of the extract mixture exhibited an antagonist effect in the four assays employed. To the best of our knowledge, there are limited studies conducted on the anthocyanin co-pigmentation and synergistic antioxidant effect. Since the crude anthocyanin extract was used in the present work, which might also contain another type of phenolics, previous studies conducted on the combination of non-anthocyanin phenolic and non-anthocyanin crude might also be useful to support the present work. Nedamani *et al.* (2015) reported that all mixture ratios of green tea, rosemary, and oak fruit exhibited a synergistic effect on the ferric-reducing power and total antioxidant capacity. However, they also found that all the mixtures exhibited an antagonist effect on the DPPH radical scavenging activity. The binary combination of gallic acid, rosmarinic acid, caffeic acid, chlorogenic, rutin, and quercetin showed synergistic effects while no antagonist effect was observed (Hajimehdipoor *et al.,* 2014). There are several factors that contribute to the synergistic, additive, and antagonist effects on the antioxidant activity of the mixtures. Peyrat-Maillard *et al.* (2003) investigated the synergistic and antagonist effects of

phenolic compounds in 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)-induced oxidation, and suggested three theories: (1) combinations of a weak antioxidant with a strong antioxidant, where the weak antioxidant may regenerate the strong antioxidant, thus improving overall radical quenching ability of the combination; (2) antagonism may be explained by the strong antioxidant regenerating the weak antioxidant, which in turn quenches the radical, and decreases the overall antioxidant strength of the combination; and (3) in a combination of a strong antioxidant with another strong antioxidant, the two compounds may regenerate each other, thus improving the overall antioxidant strength. In another study, Freeman *et al.* (2010) suggested that synergistic and antagonist interaction between phenolic compounds found in Navel oranges was mainly due to the: (1) functional groups, (2) reduction potentials, and (3) relative concentration.

Anthocyanin and colour stability

Anthocyanin stability of the extract mixtures in aqueous medium at 100°C for 120 min is shown in Table 5. The residual anthocyanin decreased as a function of time, and followed first-order reaction kinetics. Anthocyanin degradation of chokeberry, blackcurrant, and crowberry juices and their mixtures also followed a first-order kinetic reaction (Hellström *et al.*, 2013). Reyes and Cisneros-Zevallos (2007) reported that the thermal degradation of anthocyanin and chroma in purple flesh potato, red flesh potato, grape, and purple carrot followed first-order kinetic reactions. Anthocyanin degradations under isothermal heating were reported to follow first-order kinetics for juice and concentrate of sour cherry (Cemeroglu *et al.*, 1994), strawberries (Garzón and Wrolstad, 2002), and blackberries (Wang and Xu, 2007).

The extract mixtures at pH levels 3.0 and 3.5 showed a decreased rate constant but increased half-lives. Perhaps, at this pH, the co-pigmentation effectively suppressed the degradation rate of anthocyanin, therefore increasing the stability of anthocyanin and chromaticity. A notably higher $t_{1/2}$ value was observed in extract mixture at pH 3.5 as compared to at pH 3.0 and 4.0. Additionally, 25GMP:75CTF at pH 3.5 had the lowest *k* value and highest $t_{1/2}$ value as compared to the other individual and extract mixtures, thus indicating that 25GMP:75CTF at pH 3.5 was the most stable as compared to the other mixtures. The stability of 25GMP:75CTF could be interrelated with co-pigmentation and protective effects.

The lightness, chroma, and hue angle stability of extract mixtures in aqueous medium at 100°C for 120 min is shown in Table 6. The lightness and hue angle increased throughout the time of heating, following the zero-order reaction. This finding is consistent with Mohamad *et al.* (2011) and Reyes and Cisneros-Zevallos (2007) who reported that the degradation of lightness and hue angle values followed a zero-order kinetic degradation. According to Reyes and Cisneros-Zevallos (2007), the increase in the *L** values could be related to the formation of translucent extract due to the colour fading, while the increase in hue might be due to the formation of yellow chalcone species. The chroma stability of the extract mixture at different pH levels followed a first-order kinetic reaction, and decreased with the function of time. These findings are consistent with those of Sari *et al.* (2012) who also reported that the degradation of the *C** value of natural and co-pigmented (with rosemary extract) anthocyanin in *S. cumini* fruit also followed a first-order kinetic reaction.

Table 5. Anthocyanin stability of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium 100°C for 120 min.

Samples	Rate constant ($-k$) × 10^{-3}			$t_{1/2}$ (h)		
	pH 3.0	pH 3.5	pH 4.0	pH 3.0	pH 3.5	pH 4.0
100GMP	3.13^{Ab}	3.07^{Ac}	7.30^{Aa}	3.69^{Db}	3.76^{Ea}	1.58^{Ec}
75GMP:25CTF	2.80^{Cb}	2.70^{Bc}	6.90^{Ba}	4.13^{Bb}	4.28^{Da}	1.67^{Dc}
50GMP:50CTF	2.83^{Bb}	2.60^{Cc}	6.67^{Ca}	4.08^{Cb}	4.44^{Ca}	1.73^{Cc}
25GMP:75CTF	2.60^{Db}	2.27^{Ec}	6.30^{Da}	4.44^{Ab}	5.09^{Aa}	1.83^{Bc}
100CTF	2.60^{Db}	2.43^{Dc}	6.20^{Ea}	4.44^{Ab}	4.75^{Ba}	1.86^{Ac}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios.

Table 6. Colour stability of *Garcinia mangostana* peel (GMP) and *Clitoria ternatea* flower (CTF) extract mixtures at different extractant pH levels and ratios in aqueous medium at 100°C for 120 min.

Colour properties	Samples	RFate constant (k) × 10^{-3}		
		pH 3.0	**pH 3.5**	**pH 4.0**
Lightness (L^*) stability	100 GMP	45.70^{Ab}	40.90^{Ac}	59.50^{Aa}
	75GMP:25CTF	39.00^{Bb}	34.70^{Bc}	50.90^{Ba}
	50GMP:50CTF	36.00^{Cb}	33.40^{Cc}	49.80^{Ca}
	25GMP:75CTF	31.70^{Eb}	30.90^{Ec}	48.80^{Ea}
	100CTF	32.00^{Db}	31.10^{Dc}	48.90^{Da}
Chroma (C^*) stability	100GMP	5.90^{Ab}	5.10^{Ac}	9.50^{Aa}
	75GMP:25CTF	5.30^{Bb}	5.00^{Bc}	7.10^{Ba}
	50GMP:50CTF	4.20^{Cb}	3.80^{Dc}	5.70^{Ca}
	25GMP:75CTF	3.90^{Eb}	3.20^{Cc}	4.30^{Ea}
	100CTF	4.00^{Bd}	3.20^{Cc}	4.40^{Da}
Hue angle ($h°$) stability	100GMP	40.90^{Ab}	39.90^{Ac}	43.10^{Aa}
	75GMP:25CTF	34.20^{Bb}	32.00^{Bc}	42.80^{Ba}
	50GMP:50CTF	33.00^{Cb}	30.80^{Cc}	34.60^{Ca}
	25GMP:75CTF	28.30^{Eb}	27.50^{Ec}	29.20^{Ea}
	100CTF	29.80^{Db}	29.00^{Dc}	31.40^{Da}

Means followed by uppercase superscripts in a column are significantly different ($p < 0.05$) between pH levels. Means followed by lowercase superscripts in a row are significantly different ($p < 0.05$) between GMP:CTF ratios.

Conclusion

The most evident co-pigmentation effect was observed in the 25GMP:75CTF sample extracted at pH 3.5, with a significantly higher protective effect on the anthocyanin recovery than the other samples. This mixture of plant extracts produced the most stable extract at 100°C for 120 min with a good synergistic effect. Therefore, the results presented herein may have direct implications to the colourant industry to solve the instability of anthocyanin under high temperatures. Moreover, these findings could also be used to extend the application of natural colourants in high-temperature foods such as bakery products.

Acknowledgement

The authors would like to thank the Faculty of Applied Sciences, Universiti Teknologi MARA, Malaysia for funding and laboratory facilities.

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