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Research Article

Evaluation of Butterfly Pea (*Clitoria ternatea*) Extract with Different Ethanol-Water Solutions for Potential Natural Colorant Indicator

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Abstract: Dried Butterfly Pea (DBP) flowers were subjected to extraction using different concentrations of ethanol in water (0, 30, 50, 70, and 100%) through agitation in a water bath. The Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Monomeric Anthocyanin Content (TMAC), antioxidant and antimicrobial properties, color, and pH sensitivity of the extracts were then evaluated. All extracts possessed antioxidant properties and showed potential antimicrobial activity. The highest TPC and TMAC were observed for the DBP extract in 30% ethanol, at 58.55 mg GAE/g d.w. and 3.53 mg/g d.w. of extract, respectively ($p < 0.05$). The 30% ethanol extract had the highest sensitivity to pH changes compared to the 100% ethanol extract. The findings of this study indicate that BP extracted with 30% ethanol has the potential to be used as a natural food dye.

***Keywords*:** solvent extraction, butterfly pea, bioactive substances, anthocyanin, natural pigment, pH indicator

1. Introduction

The Butterfly Pea (BP) (*Clitoria ternatea*) flower, a member of the Fabaceae family, is commonly known as *telang* (Malaysia), *kordofan* pea (Sudan), *cunha* (Brazil), or *pokindang* (Philippines). The structure of the plant consists of solitary, axillary papilionaceous bright blue petals with white to light yellow at the center, pinnate compound leaves, flat and pointed pods, and nodular roots. The plant has a high growth rate, is easy to maintain, and can withstand drought. Its flowers are capable of blooming nearly year-round [1].

Due to health concerns and the rarity of blues, these flowers are widely used as a natural colorant in food and beverages, courtesy of their vibrant blue color. The BP flower also exhibits higher pH sensitivity and a broader range of color variations, and its extracts have a longer shelf life compared to other plant-based colorants [2]. In addition, the blue-colored extracts from these flowers have been reported to possess potential antioxidant, antimicrobial, hypolipidemic, and anti-inflammatory properties [3]. These properties of BP are likely attributed to the pigmented phenolic compounds, particularly anthocyanins, which are present in the plant.

Several factors influence the extraction of anthocyanins from plants, including the sample-to-solvent ratio, time, temperature, extraction methods, as well as the types of solvent used [4-5]. Ethanol is widely used in extraction applications in the food or pharmaceutical industry, as it is Generally Recognized As Safe (GRAS) by the Food and

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Drug Administration (FDA). Previous studies have also reported the effectiveness of ethanol as a solvent for extracting anthocyanins from various plants such as red cabbage, purple sweet potato, red rice bran, blackberry, and grape skins [6].

When the pH is below 3, anthocyanins predominantly exist in the stable red flavylium cation form. As the pH increases between 4 and 5, the colorless carbinol pseudobase forms, resulting from the rapid hydration of the flavylium cation. At a pH of 6 to 7, the flavylium cation loses a proton, leading to the creation of a neutral quinonoidal base that exhibits a purple to violet color. Finally, at a pH range of 7 to 8, an anionic quinonoidal base forms, displaying a blue color [7]. Due to the sensitivity towards various pH levels, the anthocyanins are widely used as a part of the smart packaging system as pH indicators for monitoring food quality. These indicators respond to the metabolites released from microbial and chemical changes during the deterioration of food through visual color changes of the smart film. The incorporation of anthocyanins as a smart indicator film has been reported to monitor the freshness of fish, shrimp, and milk [8].

Most research has focused on the comparison of different organic solvents, acidified solvents, or the use of assisted technology for extracting anthocyanins from BP flowers [5, 9, 10]. A previous study demonstrated that, in terms of antioxidants, 50% ethanol was the best for extracting fresh BP flowers, which is similar to that of water extraction combined with heat treatment for 1 h [10]. However, that study did not reveal the antimicrobial potential and pH sensitivity of these BP flowers for further applications. Therefore, our research emphasises the use of varying concentrations of ethanol in water for the extraction of dried double-petal BP flowers through the agitation method, with a focus on their phytochemical, antioxidant, antimicrobial properties, and pH sensitivity. This work features the best parameters for the application of the BP flower extract as a potential natural colouring indicator, aligning with the United Nations Sustainable Development Goals (UNSDGs) 12: Responsible Consumption and Production, which promotes the use of eco-friendly and renewable resources over environmentally harmful alternatives.

2. Materials and methods

2.1 *Materials*

Dried double-petal Butterfly Pea (DBP) flowers were purchased from a local market (Negeri Sembilan, Malaysia). The acetic acid, ethanol (99.8%), sodium hydroxide, hydrochloric acid, and potassium hydrogen phthalate were purchased from Chemiz (Selangor, Malaysia). Aluminium chloride, ferric chloride, ferrous sulphate, gallic acid, sodium carbonate, sodium acetate, sodium nitrate, potassium peroxodisulfate, quercetin, and 2,4,6-Tri(2-pyridyl)-S-triazine (TPTZ) were obtained from R & M Chemicals (Selangor, Malaysia). Folin-ciocalteau reagent, 2,2-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were bought from Merck (Darmstadt, Germany).

2.2 *Sample extraction*

The DBP flowers with an average moisture content of 11.36% (Moisture content analyzer, AND MX50, Japan) were ground using a blender (Blendforce Maxi BL233, Tefal, France) and sieved through a 40-mesh sieve. The DBP powder was then extracted according to Mehmood et al. [11] with slight modifications. The DBP with a solid-solvent ratio of 1:15 was extracted with different concentrations of ethanol in water (30, 50, 70, and 100%) and water (0% ethanol) at 50 °C for 150 min in the water bath shaker. After that, the extracts were filtered using Whatman filter paper no. 1. The removal of ethanol from the filtrate was carried out by using a rotary evaporator under vacuum at 45 °C, and the extracts were kept at -80 °C before freeze-drying. The freeze-dried Butterfly Pea flower Extracts (BPE) were kept away from light in a silica gel-filled desiccator at room temperature (25 °C). The BPE was dissolved with distilled water at a concentration of 5 mg/mL beforehand and was used for each analysis. The maximum wavelength (λmax) for the 100% ethanol extract was observed around 400 nm, while the hydroalcoholic and water extracts exhibited values between approximately 570 and 580 nm.

2.3 *Total Phenolic Content (TPC)*

The TPC of BPE was evaluated according to Mehmood et al. [11]. About 5.9 mL of distilled water, 0.5 mL of

Folin-ciocalteau reagent, and 1.5 mL of 20% (w/v) Na_2CO_3 were added to 0.1 mL of BPE. The mixture was added with an additional 2 mL of distilled water before being heated at 70 °C for 10 min in the water bath. After heating, the samples were cooled to room temperature before measuring absorbance at 765 nm using a cuvette (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA). All analyses were performed in at least triplicate. A calibration curve was established using gallic acid at concentrations ranging from 0.05 to 1 mg/mL, yielding a linear equation of $y = 0.001x + 0.0766$ ($R^2 = 0.99$). The results were expressed as mg gallic acid equivalent/g of extract dry weight (mg GAE/g d.w).

2.4 *Total Flavonoid Content (TFC)*

The TFC of BPE was measured at least in triplicate by adding 1 mL of 2% $AlCl_3$ to 1 mL of BPE [12]. Distilled water was used as a blank. The mixture was incubated for 10 min at room temperature before being measured in a cuvette at 450 nm by using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA). The reference standard used was quercetin at different concentrations ranging from 0.00 to 1 mg/mL with the linear equation of $y = 0.0118x + 0.0697$ ($R^2 = 0.98$). The results were expressed as mg quercetin equivalent/g of extract dry weight (mg QE/g d.w.).

2.5 *Total Monomeric Anthocyanin Content (TMAC)*

The anthocyanin content of BPE was evaluated using the pH differential method reported [5]. The extracts were diluted with pH 1.0 potassium chloride buffers and pH 4.5 potassium hydrogen phthalate buffers with a ratio of 1:10 (extract: pH solution). Then, the absorbance for each buffer was measured in a cuvette at 520 nm and 700 nm by using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA), respectively. Distilled water was used as a blank, and the analysis was performed at least in triplicate. The TMAC was expressed as mg cyanidin-3-glucoside equivalent per gram of dry extract (mg/g) and was calculated as:

$$\text{TMAC (mg/g)} = \frac{A \times Mw \times DF \times 1{,}000}{\varepsilon \times L}$$

Where, A is absorbance = $(A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$;
Mw = 449.2 g/mol for cyanidin-3-glucoside;
DF = dilution factor;
ε = 26,900 L/mol/cm is the molar extinction coefficient;
L = path length (1 cm).

2.6 *Antioxidant activities of BPE*

2.6.1 *DPPH scavenging activity*

The scavenging activity of BPE was measured at least in triplicate by % DPPH scavenging activity [13]. In 0.5 mL of BPE extract, about 1.95 mL of 0.1 mM ethanolic DPPH solution was added. The mixture was incubated at room temperature in a dark condition for 30 min before being measured in a cuvette at 517 nm by using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA). The % DPPH scavenging activity was calculated as:

$$\%\ \text{DPPH} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100$$

Where A is absorbance, A_{blank} is the absorbance of the DPPH solution, and A_{sample} is the absorbance of BPE with the DPPH solution.

2.6.2 *ABTS radical scavenging activity*

The ABTS stock solution was prepared by adding an equal volume of 7 mM ABTS with 2.45 mM $K_2S_2O_8$ and was kept for 12 to 16 h. Then, the ABTS stock solution was diluted with ethanol until the absorbance was adjusted to 0.70 ± 0.02 at 734 nm and was left at room temperature to stabilize. The % of ABTS scavenging activity was determined according to Zhou et al. [14], where the ratio of BPE reacted with the ABTS solution is 1:4 (v/v). Then, the mixture was incubated in a dark condition for 10 min before being measured in a cuvette at 734 nm by using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA). The analysis was performed at least in triplicate, and the % of ABTS scavenging activity was calculated as:

$$\% \text{ ABTS} = \left(\frac{A_{blank} - A_{sample}}{A_{blank}} \right) \times 100$$

Where A represents absorbance, A_{blank} is the absorbance of ABTS solution, and A_{sample} is the absorbance of BPE reacted with ABTS solution.

2.6.3 *Ferric-Reducing Antioxidant Power (FRAP)*

The FRAP solution was prepared fresh by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3 \cdot 6H_2O$ at a ratio of 10:1:1 of the solutions, respectively. Then, the solution was heated in a 37 °C water bath for 30 minutes. The FRAP assay was conducted according to Sik et al. [15]. About 0.1 mL of BPE was reacted with 3 mL of FRAP solution before the mixtures were heated in a 37 °C water bath for 4 min. The analysis was conducted at least in triplicate. The absorbance was measured in a cuvette at 593 nm by using a spectrophotometer (Genesys 10S UV-Vis Spectrophotometer, Thermo Scientific, USA) and was expressed as Fe^{2+} mmol/g of extract d.w.

2.7 *Optical measurement*

The color of the BPE was expressed as the mean (n = 3) of the luminosity (L*)-, red-green index (a*)-, and yellow-blue index (b*)-values and was measured by using the Lovibond LC100 handheld spectrocolorimeter (Lovibond, China). A standard white tile (L*-: 92.4, a*-: 0, b*-: 1.53) was used for calibration before being measured according to the International Commission on Illumination (CIE) color scale. The chroma (C*) and hue angle (H°) were also measured according to the equation below [16]. Then, the images of the BPE extracts with different solutions were photographed using a phone camera (Samsung Galaxy S10+, Korea).

$$\text{Chroma } \left(C^* \right) = \sqrt{a^2 + b^2}$$

$$\text{Hue angle } \left(H^\circ \right) = \text{Tan}^{-1} \left(\frac{b^*}{a^*} \right)$$

2.8 *pH sensitivity*

The pH sensitivity of BPE was conducted for 100% ethanol and 30% ethanol extracts, according to their lowest and highest value of TMAC. About 1 mL of BPE was reacted with 2 mL of 13 different types of pH-adjusted solutions prepared from 1 M HCl and 1 M NaOH, ranging from pH 1 to pH 13. The images of the extracts reacted with different pH buffers were photographed using a phone camera (Samsung Galaxy S10+, Korea). The color measurements of the extracts at different pH buffers of pH 1 to 13 were expressed as the mean (n = 3) of CIE color space L*-, a*-, and b*-values by using the Lovibond LC100 handheld spectrocolorimeter (Lovibond, China).

2.9 *Antimicrobial activity*

The antimicrobial activity of the BPE was evaluated using an agar well diffusion method described by Tessema et al. [17] with slight modifications. The Mueller-Hilton Agar (MHA) plates were streaked with four different testing microorganisms (*Listeria monocytogenes* (ATCC 19112), *Staphylococcus aureus* (ATCC 29737), *Escherichia coli* (ATCC 25922), and *Salmonella enterica* serovar Typhi (ATCC 14028)) that had been adjusted to a 0.5 McFarland standard (OD about 0.08-0.10 at 635 nm) by using a sterile cotton swab. Then, 6 mm diameter wells were made on the agar by using a sterile stainless steel core borer. About 20 µl of the various extracts (50 mg/mL) were filled into the well. The same volume of sterile distilled water and streptomycin (10 µg/mL, Oxoid, UK) was filled into the well as the negative and positive controls, respectively. After that, the plates were left at room temperature for at least an hour to allow diffusion of the extracts into the medium before incubating at 37 °C for 24 h. The antimicrobial activity of the extracts was expressed as the average values of the diameter of the inhibition zones (mm).

2.10 *Statistical analysis*

Statistical analyses were performed using one-way Analysis of Variance (ANOVA) in Minitab (Version 19, Minitab, Pennsylvania, USA). Each experiment was carried out in triplicate (n = 3) and expressed as mean ± standard deviation. The differences between means of different types of solvent extractions were compared using Tukey's test method of comparison at a 95% significance level.

3. Results and discussion

3.1 *TPC*

The effect of different ethanol concentrations on the TPC of BPE is shown in Table 1. The TPC values ranged between 46.15 and 58.55 mg GAE/g d.w. extract. The result showed a significant difference ($p < 0.05$) among the solvents tested, with 30% ethanol yielding the highest TPC value. Interestingly, water (0% ethanol) also proved to be an effective solvent for extracting polyphenolic compounds under these extraction conditions, exhibiting higher polyphenol content compared to 100% ethanol. Water is particularly effective in extracting multiple hydroxyl groups due to its high polarity. Water also allows efficient release of polyphenols while preserving their stability under mild heating and controlled extraction time. In contrast, although ethanol can disrupt the cell membranes [18] and promote the release of bioactive compounds, it may also contribute to the breakdown of phenolics under higher temperatures and prolonged exposure [19]. This suggests that extraction efficiency is not solely contributed by solvent selectivity towards polyphenol, but likely the combined factors such as extraction temperature and duration. The lower ethanol content in the 30% hydroalcoholic solution probably created a balanced environment to preserve the extracted polyphenols from degradation at the applied temperature and extraction time, which led to higher TPC values. Moreover, the hydroalcoholic solvents could enhance the extraction efficiency of BPE by offering a range of polarities. High water content favors the recovery of polar compounds like phenolic acids and glycosylated flavonoids, while higher ethanol ratios improve solubilization of semi-polar phenolic diterpenes and flavones [20]. The results obtained were slightly different from those reported by Jaafar et al. [21], who found that the optimum ethanol concentration for TPC extraction of BPE was 37% (v/v) by using a water bath shaker at 45 °C for 90 min. Meanwhile, Jeyaraj et al. [10] observed that 50% ethanol was the most effective solvent for BPE extraction compared to methanol and acetone. Apart from solvent concentration, various factors such as extraction time, temperature, pH, as well as extraction method also influence the yield of TPC [5, 22].

Table 1. TPC, TFC, and the TMAC of the butterfly pea extracts with different solvent extraction

	Water (0% ethanol)	100% ethanol	70% ethanol	50% ethanol	30% ethanol
TPC (mg GAE/g d.w.)	52.71 ± 0.68^{b}	46.15 ± 0.46^{d}	50.71 ± 0.85^{c}	49.92 ± 0.61^{c}	58.55 ± 0.08^{a}
TFC (mg QE/g d.w.)	14.57 ± 0.04^{e}	29.14 ± 0.48^{a}	21.44 ± 0.17^{b}	19.96 ± 0.84^{c}	17.58 ± 0.64^{d}
TMAC (mg/g d.w.)	2.96 ± 0.11^{bc}	0.56 ± 0.06^{d}	3.17 ± 0.06^{b}	2.87 ± 0.11^{c}	3.53 ± 0.10^{a}

Values are given as mean ± standard deviation (n = 3). Means in the same row with different letters are significantly different ($p < 0.05$).

3.2 *TFC*

Flavonoids are secondary metabolites in plants that play key roles in responses to both biological and non-biological environmental factors. In this study, the TFC of BPE ranged between 14 and 29 mg QE/g d.w. The TFC for BPE extracted with 100% ethanol was double in amount ($p < 0.05$) compared to the water extract. Notably, the TFC values lowered ($p < 0.05$) as ethanol percentage decreased, highlighting the influence of solvent selectivity on flavonoid extraction. The flavonoids, mainly aglycones, are largely influenced by the solvent polarity. While polar solvents like water can form hydrogen bonds with the hydroxyl groups of flavonoids, the predominantly hydrophobic regions of these compounds are poorly compatible with water, limiting their extraction. In contrast, ethanol had a moderate polarity and amphiphilic nature, which interacted effectively with both hydrophilic and hydrophobic regions of the flavonoids. Thus, ethanol or hydroalcoholic solvents can simultaneously dissolve non-polar aromatic structures and form hydrogen bonds with hydroxyl groups, resulting in higher TFC compared to water alone. Previous studies have identified flavonoids present in BP flowers, including myricetin, epicatechin, rutin, kaempferol, quercetin, and procyanidin A2 [23-25]. Similar trends were reported by Yusuf et al. [26], who observed an increase in TFC values when increasing ethanol concentration up to 80% at 25 °C using ultrasound-assisted extraction for 20 min.

3.3 *TMAC*

Anthocyanins are a colorful subgroup of flavonoids responsible for the red, pink, purple, and blue pigments found in flowers, fruits, and vegetables. Previous studies have identified the delphinidin-based anthocyanins, particularly ternatins, as the compounds responsible for the characteristic blue color of the butterfly pea flowers [27, 28]. In this study, the TMAC of the BPE ranged between 0.56 and 3.53 mg/g, with the highest TMAC ($p < 0.05$) observed for 30% ethanol extraction. Azman et al. [29] reported that a high solvent-to-water ratio is effective for extracting anthocyanins due to their polar nature and solubility. This may be attributed to the high solubility of glycosylated anthocyanins that form stable anthocyanins in aqueous environments. This effect was evident in our study, as water and hydroalcoholic solvents yielded darker extracts with lower a* (red index) and b* values (blue index), indicating a higher presence of red-blue pigments. In contrast, the brightness of the sample extracted with 100% ethanol was significantly ($p < 0.05$) higher than water due to the low a* value (red index) and a high b* value (yellow index), suggesting the predominance of yellow-green compounds. The hydrophobic regions of the polyphenolic anthocyanin structure also enable it to solubilize in organic solvents such as ethanol. Interestingly, Liao et al. [30] reported a significant decrease in TMAC when the ethanol concentration was increased up to 80% for anthocyanin from purple eggplant, highlighting that excessive ethanol can reduce anthocyanin yield.

3.4 *Antioxidant activity*

The antioxidant activities of the BPE were evaluated by DPPH, ABTS, and FRAP assays. The antioxidant activities of BPE are shown in Figure 1(a) and (b). Results have shown that the % DPPH for BPE was the highest ($p < 0.05$) for 100% ethanol, followed by 70% ethanol, 50% ethanol, 30% ethanol, and water. Also, the DPPH scavenging activity for ethanol with a higher water mixture (50% and 30% ethanol) was the same as water extraction. A similar trend was also

observed for % ABTS of all BPE extraction solvents with a remarkable radical scavenging activity above 90%. Both DPPH and ABTS assays had the same electron transfer mechanism and involved the decolorization of the free radicals. However, stronger scavenging activity by the ABTS assay could contribute to its diverse ability to determine antioxidant capacity for both hydrophilic and hydrophobic compounds simultaneously compared to the DPPH assay, which occurs preferably in a hydrophobic system [31]. Interestingly, both DPPH (r = 0.973) and ABTS (r = 0.885) strongly resonated with the result of TFC, indicating a possibility that the flavonoids in the extract play a role in the radical scavenging activity. Meanwhile, the FRAP assay worked differently from both DPPH and ABTS, which involved a non-radical color formation reaction from the reduction of the ferrous complex by the active compounds. The highest value of FRAP observed was 30% ethanol with 0.32 mmol Fe^{2+}/g extract. Siti Azima et al. [32] reported that the reducing power ability was associated with total anthocyanins.

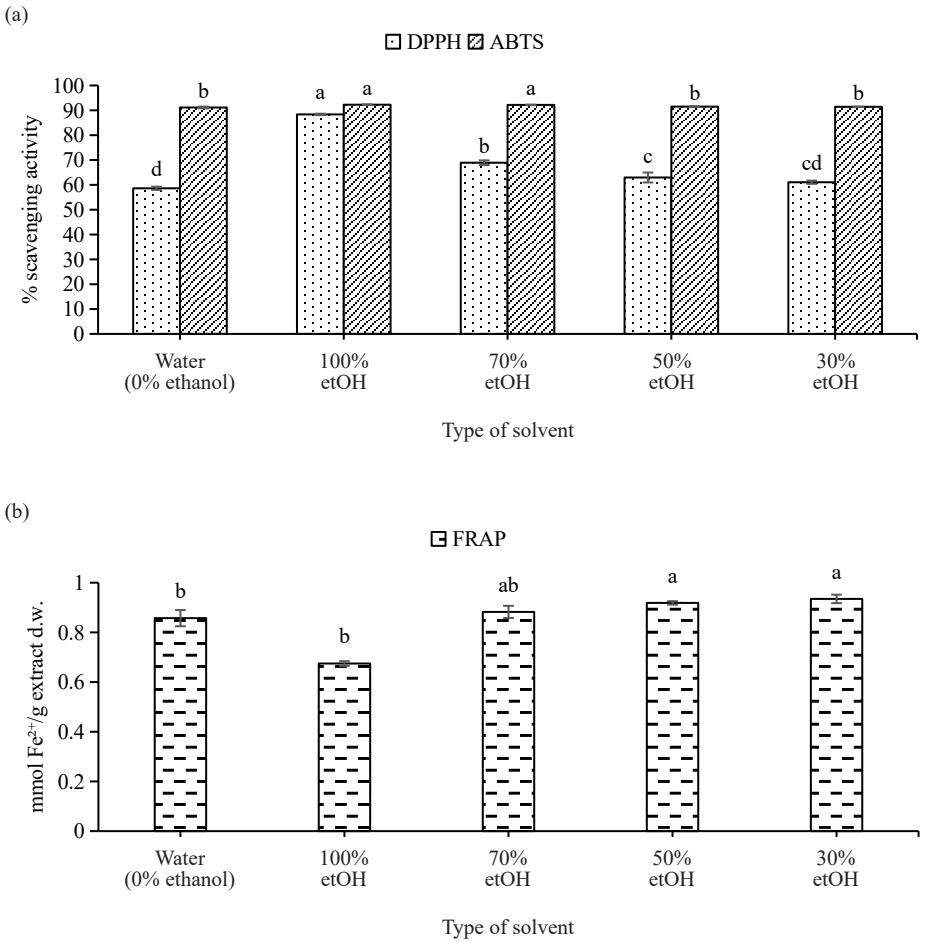

Figure 1. The antioxidant activities of BPE in different concentrations of ethanol in water. (a) The DPPH and ABTS % scavenging activity and (b) FRAP analysis

3.5 *Optical properties*

The optical properties of BPE, such as the color (CIE lab color space), chroma (color intensity or vividness), and hue angle (degrees of actual color) of BPE, were measured and presented in Table 2. BPE extracted with 100% ethanol was significantly ($p < 0.05$) different from other solvent extractions. The 100% ethanol extract had higher lightness values, and a slight red hue compared to the BPE extracted using water and ethanol at 30%, 50% and 70% concentrations. This indicated that 100% ethanol has lower anthocyanin presence, aligning with the reduction in

pigment extraction under highly non-polar conditions as observed in the lowest TMAC values. The low b* values for all extracts except for 100% ethanol highlight a strong blue hue of the anthocyanins. Besides that, 100% ethanol exhibited the highest chroma (more intense in color) and a shift in hue towards yellow (84.64°), consistent with the yellowish tone observed in Figure 2(a). However, the hydroalcoholic and water extracts showed chroma values ranging from 21 to 24, indicating vivid coloration alongside hue angles near 315 to 323°, which correspond to bluish-purple tones of the anthocyanins. The major anthocyanin responsible for the blue color in the plant is known as delphinidin [33]. The polyacylated derivatives of delphinidin 3,3',5'-triglucosides, also known as ternatins, were particularly responsible for the vibrant blue color of the BP flowers [34].

Table 2. Optical properties of BPE at different extraction solvents

	Water (0% ethanol)	100% ethanol	70% ethanol	50% ethanol	30% ethanol
L*-value	5.10 ± 0.10^{d}	46.20 ± 0.00^{a}	8.10 ± 0.10^{b}	6.67 ± 0.15^{c}	4.90 ± 0.00^{d}
a*-value	17.10 ± 0.10^{bc}	3.60 ± 0.10^{d}	17.37 ± 0.32^{b}	19.13 ± 0.12^{a}	16.73 ± 0.06^{c}
b*-value	-12.43 ± 0.15^{b}	38.40 ± 0.30^{a}	-17.37 ± 0.32^{d}	-14.67 ± 0.06^{c}	-14.73 ± 0.06^{c}
Chroma (C*)	21.14 ± 0.16^{d}	38.57 ± 0.31^{a}	24.56 ± 0.46^{b}	24.11 ± 0.13^{b}	22.30 ± 0.04^{c}
Hue angle (H°)	323.98 ± 0.26^{a}	84.64 ± 0.10^{e}	315.00 ± 0.00^{d}	322.53 ± 0.06^{b}	318.64 ± 0.18^{c}

Values are given as mean ± standard deviation (n = 3). Means in the same row with different letters are significantly different ($p < 0.05$).

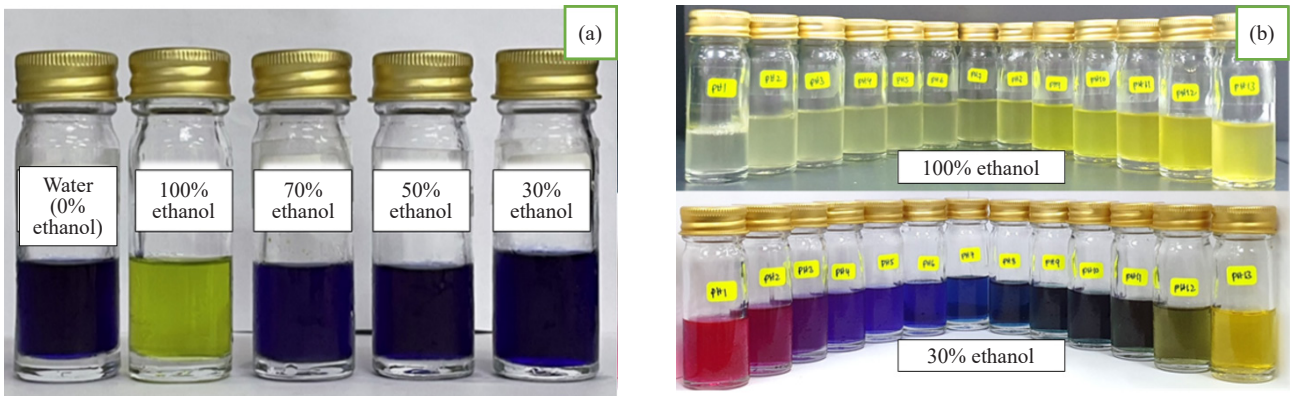

Figure 2. (a) The image of butterfly pea flower extracts in different ethanol concentrations of ethanol in water, and (b) Butterfly pea extracts of 100% ethanol and 30% ethanol at different pHs ranging from pH 1 to pH 13

3.6 *pH sensitivity*

The pH sensitivity of BPE extracted with 100% ethanol and 30% ethanol was evaluated based on their TMAC values, the lowest and the highest anthocyanin contents, respectively, since anthocyanins are the major pigments in these extracts. The initial pH values for 100% ethanol and 30% ethanol were 4.02 and 5.26, respectively (data not listed). Based on the observation in Figure 2(b), the BPE extracts at 30% exhibited notable variations across a wide pH range (1 to 13), transitioning from red, magenta, purple, blue, green, and yellow as the environment shifted from highly acidic to highly alkaline. These observations are consistent with previous reports on BP extracts [13] and reflect the structural transformations of anthocyanins in response to pH changes. In contrast, 100% ethanol extract displayed minimal color changes and consistently appeared lighter across the pH range, contributed by its high yellow index (b* > 0). Meanwhile, the extract obtained with 30% ethanol was darker in color due to its intense blue hue (b* < 0).

This difference in color suggests that 30% ethanol facilitated a more efficient anthocyanin extraction due to its high polarity, resulting in deeper blue hues. Table 3 shows that the 30% ethanol extracts appear more red (high a*-value) under strongly acidic conditions, which is characteristic of the red-purple flavylium cation form of anthocyanins. As the pH approached neutrality, the a*-values rapidly shifted negatively, reflecting a transition from red to bluish green as the flavylium cation undergoes structural transformation, converting into quinoidal base and an anionic quinonoid base. Conversely, 100% ethanol maintained a very low a*-values (near zero) across lower pH values towards neutrality, indicating less anthocyanin content within the low-polarity solvent. Additionally, green compounds are readily extracted from both solvents in decreasing amounts under alkaline conditions (pH 7 to 12). Notably, 100% ethanol displayed an increased shifting of positive b*-values, indicating stronger yellow tones across all pH levels, as shown in Figure 2 (b), peaking at pH 12. This may be due to pigment instability or possible oxidation. In contrast, the 30% ethanol extract exhibited a dramatic shift towards negative b*-values peaking at pH 5, which aligned with the formation of quinonoidal and anionic base at mildly acidic to neutral conditions. Increasing alkalinity shifts towards a higher b*-value (yellow index), peaking at pH 13, suggesting the formation of chalcones that impart a yellowish discoloration of BPE. These colorimetric trends align with the known pH-dependent anthocyanin transformations. Generally, anthocyanins exist as red flavylium ions at low pH (pH ≤ 3). Increasing pH makes the flavylium ion undergo deprotonation and hydration. The initial deprotonation of the flavylium ion, which occurred in mildly acidic to neutral conditions, yields a purple quinonoid base, followed by further deprotonation to form an anionic quinonoid base that imparts a greenish color. Hydration of the flavylium ion leads to the formation of a colorless hemiketal, which will further transform into yellow *cis*-chalcones and *trans*-chalcones (pH 12-13) [13, 35].

Table 3. The L*-, a*-, and b*-values of pH sensitivity for 100% ethanol and 30% ethanol BPE extracts

	L*-values		a*-values		b*-values	
	100% ethanol	30% ethanol	100% ethanol	30% ethanol	100% ethanol	30% ethanol
pH 1	60.43 ± 0.32^{a}	28.17 ± 0.06^{b}	1.40 ± 0.17^{b}	54.30 ± 0.10^{a}	14.03 ± 0.29^{b}	6.13 ± 0.06^{b}
pH 2	58.83 ± 0.12^{a}	23.10 ± 0.10^{b}	1.63 ± 0.06^{b}	45.67 ± 0.15^{a}	20.20 ± 0.00^{a}	-8.23 ± 0.06^{b}
pH 3	59.30 ± 0.00^{a}	18.97 ± 0.06^{b}	1.13 ± 0.06^{b}	34.47 ± 0.06^{a}	20.50 ± 0.00^{a}	-26.73 ± 0.06^{b}
pH 4	60.10 ± 0.01^{a}	18.07 ± 0.06^{b}	1.10 ± 0.00^{b}	29.73 ± 0.12^{a}	20.53 ± 0.21^{a}	-36.30 ± 0.27^{b}
pH 5	59.87 ± 0.06^{a}	19.50 ± 0.10^{b}	1.00 ± 0.01^{b}	27.33 ± 0.06^{a}	20.87 ± 0.06^{a}	-39.70 ± 0.10^{b}
pH 6	60.63 ± 0.12^{a}	17.03 ± 0.15^{b}	0.43 ± 0.06^{b}	10.73 ± 0.35^{a}	22.50 ± 0.00^{a}	-34.33 ± 0.40^{b}
pH 7	60.87 ± 0.06^{a}	20.07 ± 0.06^{b}	-1.97 ± 0.15^{a}	-14.77 ± 0.06^{b}	31.00 ± 0.10^{a}	-19.60 ± 0.10^{b}
pH 8	60.53 ± 0.15^{a}	16.17 ± 0.06^{b}	-3.83 ± 0.06^{a}	-19.20 ± 0.10^{b}	40.60 ± 0.00^{a}	-9.70 ± 0.00^{b}
pH 9	59.77 ± 0.10^{a}	30.83 ± 0.23^{b}	-3.97 ± 0.06^{a}	-12.87 ± 0.15^{b}	52.40 ± 0.00^{a}	-4.57 ± 0.06^{b}
pH 10	59.60 ± 0.00^{a}	8.00 ± 0.10^{b}	-4.63 ± 0.15^{a}	-6.30 ± 0.10^{b}	51.00 ± 0.10^{a}	-3.90 ± 0.00^{b}
pH 11	57.67 ± 0.15^{a}	7.67 ± 0.17^{b}	-1.70 ± 0.10^{a}	-3.90 ± 0.10^{b}	55.50 ± 0.10^{a}	0.90 ± 0.10^{b}
pH 12	57.10 ± 0.00^{a}	18.23 ± 0.21^{b}	-0.57 ± 0.06^{a}	-3.27 ± 0.21^{a}	58.04 ± 0.06^{a}	9.67 ± 0.15^{b}
pH 13	55.87 ± 0.06^{b}	57.80 ± 0.00^{a}	2.87 ± 0.13^{b}	5.93 ± 0.15^{a}	51.13 ± 0.15^{b}	59.80 ± 0.10^{a}

Values are given as mean ± standard deviation (n = 3). Means in the same row of pH value for each L*-, a*-, and b*-values between 100% ethanol and 30% ethanol with different letters are significantly different ($p \leq 0.05$).

3.7 *Antimicrobial activity*

In this study, the antimicrobial activities of BPE at 50 mg/mL were evaluated using the agar well diffusion method against two Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and two Gram-negative bacteria (*E. coli* and *S.* Typhi). The diameter of the inhibition zones shows (Table 4) that most of the BPE samples were able to inhibit both Gram-positive and Gram-negative bacteria. The highest inhibition zones for *L. monocytogenes*, *S. aureus*, and *E. coli* were exhibited by BPE with 70% ethanol, whereas the water extract showed the greatest inhibition against *S.* Typhi. However, it was also noticeable that 100% ethanol extracts had no inhibitory effect on *S. aureus* and *S.* Typhi. This lack of activity could be attributed to the inefficient extraction of polar anthocyanins and other hydrophilic compounds that contribute to antibacterial action. The antimicrobial effect may be due to their polyphenolic compounds, which can interact with the bacterial membrane proteins by sequestering essential ions for protein stability, as well as through electron transfer processes at the membrane interface involving both hydrophobic interaction and hydrogen bonding. These mechanisms ultimately disrupt bacterial membranes, leading to antimicrobial effects [36].

Table 4. The antimicrobial activity of BPE

	The diameter of inhibition zones (mm)						
	Streptomycin (+ve control)	Sterile distilled water (-ve control)	Water (0% ethanol)	100% ethanol	70% ethanol	50% ethanol	30% ethanol
L. monocytogenes	20.17 ± 1.65^{a}	0.00	7.38 ± 0.88^{b}	7.00 ± 1.41^{b}	9.58 ± 1.53^{b}	9.50 ± 2.12^{b}	9.50 ± 2.12^{b}
S. aureus	20.00 ± 1.89^{a}	0.00	7.37 ± 0.05^{b}	0.00	9.40 ± 0.09^{b}	8.33 ± 0.00^{b}	8.67 ± 0.00^{b}
E. coli	21.33 ± 0.47^{a}	0.00	7.67 ± 0.47^{b}	6.50 ± 0.71^{b}	9.50 ± 1.18^{b}	8.03 ± 0.05^{b}	8.50 ± 0.24^{b}
S. Typhi	20.00 ± 0.94^{a}	0.00	10.33 ± 0.94^{b}	0.00	10.00 ± 0.00^{b}	6.98 ± 0.73^{b}	9.77 ± 1.74^{b}

Values are given as mean ± standard deviation (n = 3). Means in the same row with different letters are significantly different ($p < 0.05$).

4. Conclusion

In this study, the butterfly pea was extracted using ethanol-water solutions of varying concentrations. Results showed that solvent polarity affected the phytochemical properties of the butterfly pea extracts. Low polarity solvents had a significant effect on the TFC, whereby higher solvent polarity influenced the total phenolic and anthocyanin contents. All BPEs exhibited antioxidant properties with strong radical scavenging activity, alongside good antimicrobial effects. Furthermore, higher water content in the ethanol-water solutions displayed an intense blue pigment and showed high sensitivity to pH changes. These findings indicate that BPEs with a proper ratio of water-solvent solutions are promising natural coloring agents with color-changing abilities in response to pH, as well as antioxidant and antimicrobial properties.

CrediT authorship contribution statement

Nor Adilah Abdullah: Conceptualization, Methodology, Investigation, Writing—Original Draft preparation, Writing—Reviewing and Editing. Chong Gun Hean: Supervision, Validation. Ezzat Mohamad Azman: Methodology, Validation, Writing—Reviewing and Editing. Nur Hanani Zainal Abedin: Methodology, Validation, Writing—Reviewing and Editing, Supervision.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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