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# Antioxidant ability of *Chenopodium formosanum* extracted using an ethanol–ammonium sulfate two-phase system

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## Abstract

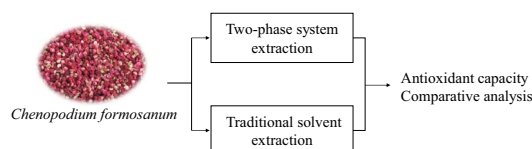
**Background:** *Chenopodium formosanum* (CF) provides the human body with numerous nutritional components. This study used the two-phase system to identify an efficient method to obtain CF extracts. CF extraction was performed using an ethanol–ammonium sulfate two-phase system. The efficacy of different CF extracts with five types of antioxidant ability was tested and compared with traditional aqueous and alcohol extractions.

**Results:** The results showed that a separated top of the two-phase system extract had higher total phenol content ( $120.35 \pm 5.80$  mg gallic acid equivalent/g dry extract), total flavonoid content ( $447.06 \pm 16.57$  mg quercetin equivalent/g dry extract) and reducing ability ( $284.48 \pm 4.60$  mg vitamin C equivalent/g dry extract) than those of other extracts. Furthermore, the separated top of the two-phase system extract and the top of the two-phase system extract had higher 1,1-diphenyl-2-picrylhydrazyl free radical scavenging ability and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging ability than those of the water extract, alcohol extract, bottom of two-phase system extract, and separated bottom of two-phase system extract.

**Conclusions:** The results indicate that CF has great potential for use in natural plant health supplements and skin care products and that the two-phase extraction system can yield an effective CF extract.

**Keywords:** Antioxidant, Extract, *Chenopodium formosanum*, Two-phase

## Graphical Abstract



## Background

*Chenopodium formosanum* (CF), a grain native to Taiwan, has long been regarded as an important plant by indigenous people; however, in the past it was often

confused with quinoa [1]. CF is mainly distributed throughout southern and eastern Taiwan, and the plant can grow up to 2 m tall. Farmers usually plant CF in spring or autumn and can harvest it in 100 days.

CF has a bright color that comes from betalains [2]. Betalains include purple-red anthocyanins (betacyanins) and orange-yellow betaxanthins, which have a strong coloring ability and are believed to have positive physiological effects on humans [3]. Betanin has

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also proven to be an effective antioxidant component in CF, with the strongest antioxidant capacity and stability with a pH of 5 [1]. CF also contains phenolic compounds and flavonoids, and a high-performance liquid chromatography chart showed that 8 phenolic compounds and 14 flavonoids were found in dehulled CF seeds [4]. CF has also proven tentatively effective in disease prevention. For example, feeding CF to rats with colon cancer lesions demonstrated some efficacy in preventing colon cancer progression [5]. CF extracts can also protect the skin from damage caused by ultraviolet rays [6]. Additionally, CF water extract can reduce hypertension [7], and the brightly colored grain is used as an insect repellent and as a decoration by indigenous people in Taiwan [1].

The aqueous two-phase system (ATPS) is a liquid–liquid extraction technology that has recently attracted considerable attention. Studies have reported the successful use of this system for plant extraction, sewage treatment, separation of precious metals, and other applications [8, 9]. In addition, because of its characteristics of stratification, environmental protection, extraction selectivity, and easy scale-up, the ATPS has been gradually applied to the separation and purification of small molecules [10].

ATPS has been successfully applied to the extraction of components from plants such as aloe leaf [11], blueberry [12], eucalyptus [13], garlic powder [14], grape seed [15], ginseng [16], honeysuckle [17], *Lilium davidii* var. *unicolor* Salisb [18], *Lentinus edodes* [19], ramie [20], *Radix Sophorae Tonkinensis* [21], *Schisandra chinensis* Baill [22] and wheat valley [23]. Compared with other liquid–liquid extractions, ATPS is simpler and lower in cost due to the high recovery of phase-forming components [22]. The lignan purity of *Schisandra chinensis* Baill extracted using an optimized ATPS increased from 0.98% to 4.49% [22]. The purity of allicin extracted from garlic by using an ATPS (68.4%) was much higher than that obtained using ethanol extract (31.8%), and the bioactive tests on extracts by an ATPS displayed an effective antioxidant activity [24]. Comparing the extraction results from *Tagetes erecta* L. by using an ATPS and by using Soxhlet extraction, ATPS extraction manifested a superior total polyphenol content and antioxidant activity [25].

ATPS is a novel technology for plant extraction; however, no report has yet described ATPS extraction for CF. Compared with traditional extraction methods, ATPS has the advantages of low cost and easy scale-up. Consequently, this study used the ethanol–ammonium sulfate two-phase system for CF extraction and compared the results with those from traditional extraction.

## Materials and methods

### Reagents

Aluminum (III) chloride, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and quercetin were obtained from Alfa Aesar (Tewksbury, MA, USA). Iron (III) chloride hexahydrate was provided by J. T. Baker (Phillipsburg, NJ, USA). Trichloroacetic acid, sodium carbonate, and sodium phosphate dibasic dehydrate were purchased from Riedel-de Haën (Seelze, Germany). Folin–Ciocalteu reagent was supplied by Fisher Scientific (Loughborough, Leicestershire, UK). Potassium ferricyanide was purchased from First Chemical Co. (Pasca-goula, MS, USA). Gallic acid was obtained from Fluka (Neu-Ulm, Germany). Ammonium sulfate and potassium persulfate were procured from Showa Chemical Co. (Tokyo, Japan). Vitamin C was obtained from Acros (Geel, Belgium). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and sodium nitrite were provided by Sigma-Aldrich (St. Louis, MO, USA). Ethanol and sodium hydroxide were obtained from Echo Chemical Co. (Miaoli, Taiwan) and Choneye Chemical Co. (Taipei, Taiwan), respectively. Sodium phosphate monobasic was purchased from Shimadzu's Pure Chemical Co. (Osaka, Japan).

### Preparation of extract

#### Material

CF was sourced from the Hearty Farm, Yunlin, Taiwan. The CF seeds were dried whole in a hot air circulating oven at 30 °C for 72 hours for water removal and mold prevention. The processed CF seeds were stored in a freezer at −30 °C prior to experiments.

#### Water extraction

Deionized (DI) water was the solvent used for extraction. 1 g CF seeds and 100 g DI water were combined, stirred at 25 °C for 1 hour, and then filtered to obtain the water extract (WE) of CF.

#### Ethanol extraction

CF extraction was performed using 32.5 wt % ethanol as the solvent. The proportion of ethanol extract used was based on the ethanol ratio used in the two-phase system. The weights of CF seeds and ethanol aqueous solution were 1 g and 100 g, respectively. The solution was stirred at 25 °C for 1 hour and subsequently filtered to obtain a 32.5 wt% ethanol extract (AE) of CF.

#### Two-phase system extraction

The two-phase solution was prepared from 32.5 g of ethanol, 52.5 g of DI water, and 15 g of ammonium sulfate. 1 g CF seeds and 100 g two-phase system were mixed. After the two-phase CF extract was stirred at 25 °C for 1 hour and then filtering, the filtered two-phase CF extract was

poured into a separating funnel to separate the top phase from the bottom phase. After standing for 5 min, the top of the two-phase system extract (TTE) and the bottom of the two-phase system extract (BTE) were obtained.

#### Separated single-phase extraction of two-phase system

The above-mentioned two-phase system solution was prepared. After the top and bottom phases were separated, CF was extracted from each phase. The weights of CF seeds and the top phase or the bottom phase was set 1 g and 100 g, respectively, and the solution was stirred for 1 h at 25 °C then filtered. The separated top of the two-phase system extract (STTE) and the separated bottom of the two-phase system extract (SBTE) were obtained for comparison with the two-phase system extraction, TTE, and BTE. Figure 1

shows the six CF extracts by different experimental procedures in this study.

#### Total phenolic content

According to the test method of Huang [26] and Huang [27], 0.3 mL of CF extract was added to 1.5 mL of 1 N Folin–Ciocalteu reagent and 1.2 mL of 15% Na<sub>2</sub>CO<sub>3</sub> for reaction in darkness for 30 minutes, and a spectrophotometer at a wavelength of 765 nm was used to detect its absorbance value. The experimental standard chemical used was gallic acid, and the total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per gram of CF dry extract.

#### Total flavonoid content

This experiment referred to the research method of Chan [28] and Lin [29]. In brief, 1 mL of CF extract was added to 0.15 mL of 5% NaNO<sub>2</sub> and mixed until homogeneous. After 5 minutes, 0.3 mL of 10% AlCl<sub>3</sub> was added, and the

mixture was continuously stirred for 5 minutes. Subsequently, 1.5 mL of 1 M NaOH was added to the mixture to react for 60 minutes. The spectrophotometer was used to detect its absorbance at a wavelength of 510 nm. This method used quercetin as the standard, and the total flavonoid content was expressed as milligrams of quercetin equivalent (QE) per gram of CF dry extract.

#### DPPH radical scavenging ability

This experiment referred to the test method of Tsai [30] and Chang [31] in which 2 mL of 0.2 mM DPPH prepared with 95% ethanol solution was added to 2 mL of CF extract and mixed evenly. After 30 minutes of reacting in darkness, the absorbance of the solution was measured at 517 nm, and DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging ability (\%)} = \left( 1 - \frac{\text{Abs.sample}}{\text{Abs.blank}} \right) \times 100\%.$$

The IC<sub>50</sub> of the DPPH radical scavenging activity was calculated from the curve of DPPH radical scavenging ability versus concentration. IC<sub>50</sub> indicates how many samples are needed for the DPPH radical scavenging ability to reach 50%. The lower the IC<sub>50</sub>, the more effective the antioxidant capacity of the sample is.

#### ABTS radical scavenging activity

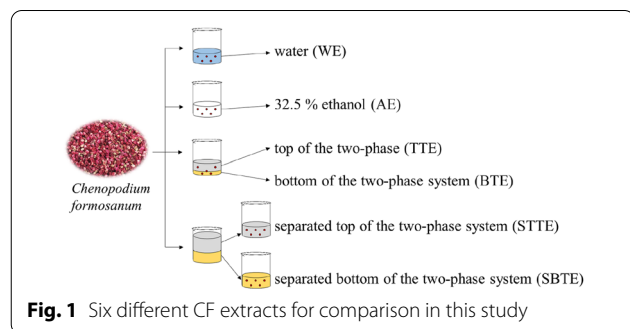
With reference to a previously described method [27, 32], an equal volume of 7 mM ABTS aqueous solution and 2.45 mM potassium persulfate solution was mixed uniformly and placed in an environment at 4 °C in darkness for 16 hours. The background absorbance was diluted to 0.7 ± 0.02 by using 95% ethanol. Subsequently, 0.4 mL of CF extract and 3.6 mL of ABTS solution were mixed evenly in darkness for 10 minutes. The absorbance was measured at 734 nm, and the ABTS radical scavenging activity was calculated using the following equation:

$$\begin{aligned} \text{ABTS radical scavenging ability (\%)} \\ = \left( 1 - \frac{\text{Abs.sample}}{\text{Abs.blank}} \right) \times 100\%. \end{aligned}$$

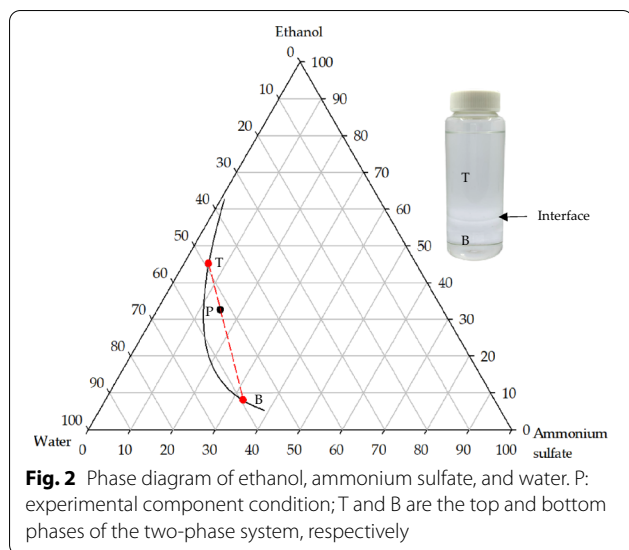
The method for attaining the IC<sub>50</sub> of the ABTS radical scavenging activity was the same as that of the DPPH radical scavenging activity.

#### Ferric reducing power

With reference to a previously reported method [29, 33], 1 mL of CF extract, 0.5 mL of 0.2 M phosphate buffer solution (pH 6.6) and 0.5 mL of 1% K<sub>3</sub>Fe(CN)<sub>6</sub> solution



**Fig. 1** Six different CF extracts for comparison in this study



were mixed at 50 °C for 20 minutes in a water bath then rapidly cooled. Subsequently, 0.5 mL of 0.1%  $\text{CCl}_3\text{COOH}$  solution, 2.5 mL of DI water, and 0.5 mL of 0.1%  $\text{FeCl}_3$  solution were added to the mixture. After 10 minutes of standing in darkness, the mixture was analyzed using the spectrophotometer at a wavelength of 700 nm. Vitamin C was used as the standard for determining the ferric reducing power. The ferric reducing power was expressed as the milligrams of vitamin C equivalent (VCE) per gram of CF dry extract.

## Results and discussion

### Phase diagram

Figure 2 shows the phase diagram of ethanol, ammonium sulfate, and water at 25 °C. The black curve in the figure is the theoretical equilibrium phase diagram of the three components as described in the literature [34, 35]. The black dot (P) in the phase diagram represents the experimental conditions of this study. The two red dots represent the top phase (T) and the bottom phase (B) of the two-phase system. The interface in the vial between the

top phase (pH = 5.3) and the bottom phase (pH = 5.9) is clearly demarcated. The volume ratio of the top and bottom phases in the vial corresponds to the segment ratio of T to P and P to B in the phase diagram called the tie-line lever rule. The acidic pH values of the top phase and the bottom phase are beneficial for the extraction of CF [1].

### Extraction yield

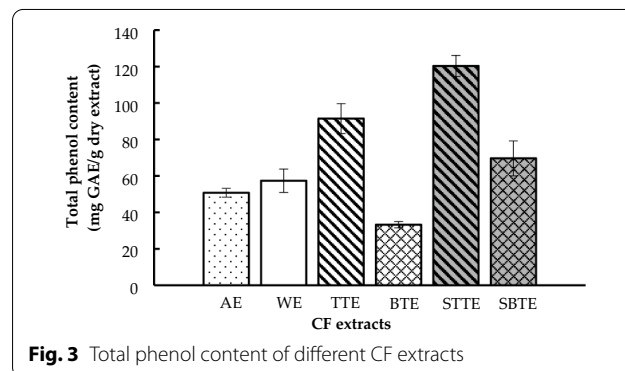
The extraction yield of CF depended on the extraction solution. Table 1 presents the extraction yields by dry weight of different extracts. The extraction yield of WE ( $12.38 \pm 1.11\%$ ) was the highest of the different extraction solutions. The combined yields (9.28%) of TTE (3.55%) and BTE (5.73%) comprise the overall yield in the two-phase system, and this value was between that of AE ( $7.96 \pm 1.6\%$ ) and WE ( $12.38 \pm 1.11\%$ ). The yield of TTE was lower than that of BTE, and that of STTE was lower than SBTE. A possible reason of this result may be that CF has more hydrophilic than hydrophobic components. This finding is consistent with the result of polysaccharides in extract from *Gentiana scabra* Bunge [36].

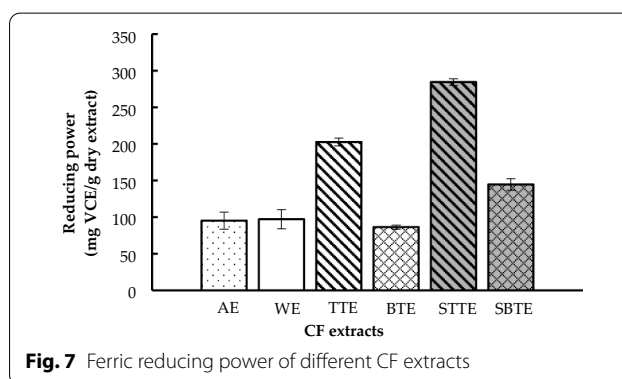
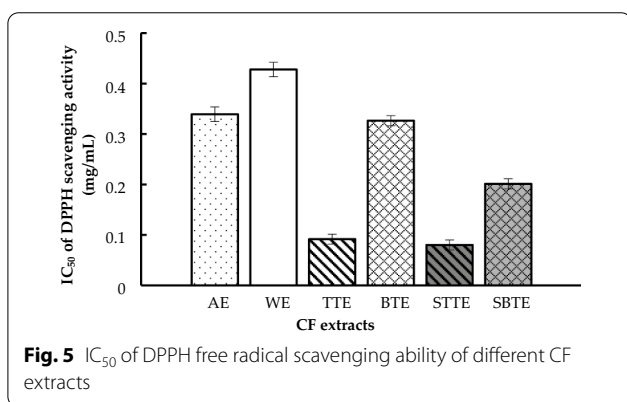
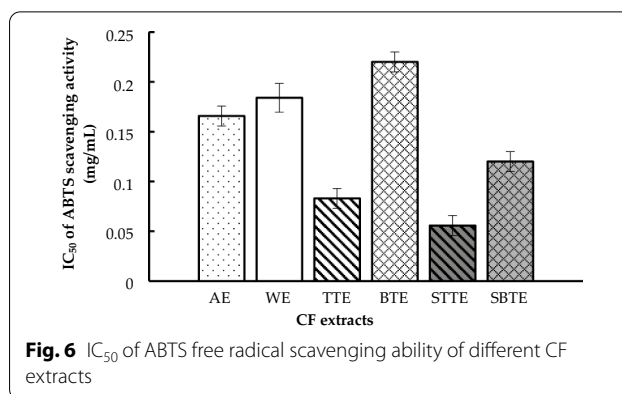
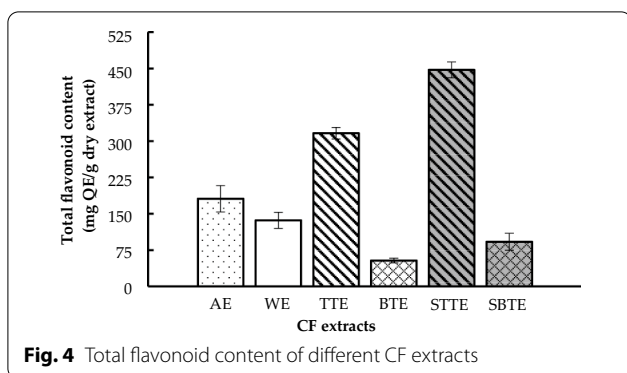
### Total phenol content

Phenolic compounds are often used as indicators of antioxidant capacity because they have a strong ability to scavenge free radicals. Figure 3 shows the amount of GAE per gram of dry weight of CF extract. STTE had the highest total phenol content ( $120.35 \pm 5.80$  mg of GAE/g of dry extract), followed by TTE ( $91.47 \pm 8.23$  mg GAE/g dry extract), and BTE with the lowest total phenol content ( $33.22 \pm 1.71$  mg GAE/g dry extract). The total phenol contents of AE and WE were  $50.74 \pm 2.43$  and  $57.37 \pm 6.41$  mg of GAE/g of dry extract. TTE and STTE had higher total phenol content than BTE and SBTE, respectively. A possible reason is that phenol migrated to the top phase in acidic conditions [37]. This result indicates

**Table 1** Extraction yield (%) of CF in different extraction methods

| Extract | Yield (%)        |
|---------|------------------|
| AE      | $7.96 \pm 1.6$   |
| WE      | $12.38 \pm 1.11$ |
| TTE     | $3.55 \pm 0.96$  |
| BTE     | $5.73 \pm 0.53$  |
| STTE    | $3.70 \pm 0.68$  |
| SBTE    | $8.59 \pm 0.47$  |





the consistency of CF extraction by using a two-phase system (TTE and BTE) and a separated single-phase system (STTE and SBTE).

#### Total flavonoid content

Flavonoids are often used as indicators of antioxidant capacity. If a sample has flavonoids, it will react with the developer to form an orange-red aluminum chelate. Fig. 4 shows the result of QE per gram of dry CF extract by using different extraction methods. STTE had the highest total flavonoid content ( $447.06 \pm 16.57$  mg QE/g dry extract), followed by TTE ( $316.22 \pm 11.89$  mg QE/g dry extract) and BTE with the lowest content ( $53.39 \pm 4.89$  mg QE/g dry extract). The total flavonoid contents of AE, WE, BTE, and SBTE were much lower than that of STTE. Moreover, the two-phase extract and the separated single-phase system registered a similar result; the top phase had a higher flavonoid content than the bottom phase. This result was the same as *Crotalaria sessiliflora* L extracted using an ATPS [38].

#### DPPH radical scavenging ability

The DPPH radical is a stable free radical at a wavelength of 517 nm. When the DPPH radical accepts electrons,

the color changes from dark purple to light yellow. Fig. 5 illustrates that the lowest IC<sub>50</sub> of the different extraction methods were achieved for STTE ( $0.08 \pm 0.01$  mg/mL) and TTE ( $0.09 \pm 0.01$  mg/mL), followed by SBTE ( $0.20 \pm 0.01$  mg/mL). The IC<sub>50</sub> values of AE, WE, BTE were far higher than those of STTE and TTE, between  $0.33 \pm 0.01$  and  $0.43 \pm 0.01$  mg/mL. The result evinced a similar trend; the top phase had higher DPPH radical scavenging activity than the bottom phase did for the two-phase system (TTE and BTE) and the separated single-phase system (STTE and SBTE). The experimental data were compared with the literature [39], and the IC<sub>50</sub> of AE and WE are nearly equal to the IC<sub>50</sub> of water extract and organic solvent extract of CF, corroborating the results in this experiment.

#### ABTS radical scavenging activity

When an ABTS cation radical, which is stable at a wavelength of 734 nm, is scavenged by a sample, the ABTS solution color changes from blue-green to colorless. The lighter the color of the ABTS solution, the higher the ABTS free radical scavenging ability of the sample is. The IC<sub>50</sub> results in Fig. 6 indicate that STTE ( $0.06 \pm 0.01$  mg/mL) and TTE ( $0.08 \pm 0.01$  mg/mL) displayed a superior antioxidant capacity because of their low IC<sub>50</sub> values. The



IC<sub>50</sub> values of AE, WE, and BTE were between 0.17 and 0.22 mg/mL, considerably higher than those of STTE and TTE. This result demonstrates that the top phases (TTE and STTE) have a higher ABTS free radical scavenging ability than do the bottom phases (BTE and SBTE), and this outcome is consistent with a previous report [23].

#### Ferric reducing power

If a sample manifests ferric reducing power, the oxidizing substance can stimulate electrons to terminate the oxidation reaction. Considering the amount of Prussian blue produced as an indicator, the more Prussian blue that is produced, the higher the antioxidant capacity is. Fig. 7 shows that STTE had the strongest reducing ability (284.48 ± 4.60 mg VCE/g dry extract), followed by TTE (202.61 ± 5.34 mg of VCE/g of dry extract). Furthermore, the two-phase extracts (TTE and BTE) and the separated single-phase system (STTE and SBTE) registered a similar result; the top phase had higher ferric reducing power than did the bottom phase.

Overall, TTE and STTE had the best antioxidant outcomes. However, TTE and STTE also showed the lower CF extraction yield. This consequence may result from pH of CF extracts. Ammonium sulfate has weak acidity in aqueous solutions, and this is extremely appropriate to separate bioproducts stable [34]. The pH values of the top phase and bottom phase are 5.3 and 5.9, respectively. Betanin, an effective antioxidant component in CF, has the strongest antioxidant capacity and stability with a pH of 5 [1]. Besides, phenolic compounds migrated to the top phase in acidic conditions [37]. The total phenol content and flavonoid content related to antioxidant capacity of TTE and STTE were higher than BTE and SBTE.

There are numerous papers on antioxidant activity of CF that have come out in recent years [40–47]. However, conventional methods to extract CF may be not efficient. ATPS has advantages of effective and economically viable separation for plant extraction [8, 9]. Purifying of target compounds [22, 24], bioactive ingredients and antioxidant activity [24, 25] can be enhanced in ATPS compared with conventional methods. Therefore, APTS can provide a potential approach for large-scale production of bioactive compounds from CF in the future.

#### Conclusions

CF is endemic to Taiwan and considered a plant high in functional components. This study used a two-phase extraction system for CF and compared it with traditional solvent extraction. The results indicated that a two-phase extraction system has greater antioxidant capacity than traditional solvent extraction. CF extracted

with the top phase of a two-phase system had superior antioxidant capacity in terms of total phenolic content, total flavonoid content, DPPH radical scavenging ability, ABTS radical scavenging ability, and ferric reducing power. The result confirms that the ethanol–ammonium sulfate–water two-phase system for extraction from CF can not only provide more appropriate extraction conditions, but can also deliver excellent performance in the antioxidant capacity of an extraction.

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#### Authors' contributions

Conceptualization, W-HW and Y-SL; methodology, Y-SL; formal analysis, W-HW, C-HS, M-YC, S-LH and Y-SL; data curation, W-LL and C-YC; writing—original draft preparation, Y-SL; writing—review and editing, Y-SL and W-HW. All authors read and approved the final manuscript.

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#### Availability of data and materials

Not applicable.

#### Declarations

#### Ethics approval and consent to participate

Not applicable.

#### Consent for publication

All authors have read and agreed to the version of the manuscript.

#### Competing interests

The authors declare that they have no competing interests.

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