

Ethanol-modified supercritical CO₂ extraction of cashew (*Anacardium occidentale*) nut testa

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Summary

Cashew nut (*Anacardium occidentale* L.) testa, a by-product derived from cashew nut processing, is a valuable source of polyphenols with antioxidant properties. This study aimed to screen the extraction of cashew nut testa with supercritical CO₂ and ethanol as a co-solvent. The impacts of various key operating parameters, including co-solvent concentration (5–20 %, v/v), temperature (40–60 °C), pressure (15–30 MPa) and extraction time (30–120 min) on the total phenolics content (TPC) and the antioxidant activity of the extract were investigated. The resulting extract from cashew nut testa exhibited high TPC and antioxidant activity values when employing a 15 % (v/v) ethanol co-solvent concentration, a temperature of 40 °C, a pressure of 25 MPa and an extraction time of 90 min. Under these conditions, the extract of cashew nut testa achieved TPC of 63.97 g·kg⁻¹ on dry weight (DW) basis expressed as gallic acid equivalent and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity of 1522.72 mmol·kg⁻¹ DW expressed as Trolox equivalent. Ethanol-modified supercritical CO₂ extraction demonstrated promising potential as a sustainable approach for recovering the phenolic compounds from the cashew nut testa.

Keywords

supercritical extraction; co-solvent; cashew nut testa; polyphenol; antioxidant activity

The cashew nut (*Anacardium occidentale*), belonging to *Anacardiaceae* family, is a tropical evergreen cultivated tree. The testa, a thin layer covering the cashew nut, constitutes approximately 1–3 % of its total weight and possesses a bitter and astringent taste that can negatively impact the sensory quality of food products [1]. Consequently, the testa is typically removed as a by-product during cashew nut processing.

Recent research has highlighted the potential of cashew nut testa as a valuable source of polyphenols that find significant applications in the food and nutraceutical industries [2–4]. The major valuable flavonoids identified in cashew nut testa include catechin, epicatechin and epigallocatechin [5].

In most studies, conventional techniques, particularly maceration, are commonly employed for extracting bioactive compounds from cashew nut testa. Maceration involves soaking the sample in an appropriate solvent in a closed system,

followed by agitation at room temperature and subsequent separation of solid components from the solvent through filtration or centrifugation. Solvents including water [6], methanol [7, 8] and ethanol [5, 9] have been employed for bioactive compound extraction from cashew nut testa. However, despite its advantage of being simple, maceration has the disadvantage of being time-consuming and requiring large volumes of solvents [10].

In recent years, innovative and sustainable techniques such as microwave-assisted extraction, ultrasound-assisted extraction, enzymatic extraction, pressurized liquid extraction or supercritical fluid extraction (SFE), have been developed for extracting bioactive compounds from natural by-products [11]. These advanced technologies aim to overcome the limitations associated with conventional techniques. Among these techniques, SFE has emerged as a promising alternative for extracting bioactive compounds from various plant

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sources. This technique offers several advantages over conventional ones, including reduced organic solvent consumption, improved product quality and lower operational costs [12]. Carbon dioxide is commonly employed as a supercritical fluid due to its non-toxic and inert properties, as well as its low critical temperature and pressure. The addition of a polar substance, such as alcohol as a co-solvent, is employed to enhance the extraction efficiency of polar compounds from plant matrices due to the non-polar nature of CO₂ [13]. It was reported that modified-supercritical CO₂ extraction was successfully used to extract bioactive compounds from fruit and vegetable by-products [14–16].

However, to the best of our knowledge, no literature currently exists on the extraction of polyphenols from cashew nut testa by supercritical CO₂ extraction. Therefore, the main objective of this study was to screen the extraction of cashew nut testa by using ethanol-modified supercritical CO₂. The effects of key operating parameters, including ethanol concentration, temperature, pressure and extraction time, on the efficiency of polyphenols extraction based on the total polyphenols content and antioxidant activity, were investigated.

MATERIALS AND METHODS

Chemicals

2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and Folin–Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Standard gallic acid was supplied by Sigma-Aldrich (St. Louis, Missouri, USA). Analytical water was obtained by a Milli-Q purification system (Millipore, Bedford, Massachusetts, USA). All other chemicals were of analytical grade.

Preparation of cashew nut testa

Cashew nut testa was obtained from Kimmy (Ho Chi Minh City, Viet Nam). The testa was pulverized, then passed through a 0.5 mm sieve to obtain a fine powder and kept in sealed polyethylene bags. The testa powder was stored in the dark at –20 °C for a maximum of 3 weeks until used for extraction.

Ethanol-modified supercritical CO₂ extraction procedure

The extraction of cashew nut testa with supercritical CO₂ and ethanol as a co-solvent was performed on a Spe-ed SFE-2 supercritical fluid ex-

traction system (Applied Separation, Allentown, Pennsylvania, USA) equipped with a modifier-liquid pump (Series 1500) following the method described by TAI et al. [13] with a slight modification. Briefly, the prepared sample (5.00 ± 0.03 g testa powder) was put into a stainless-steel extraction tube and then installed into a CO₂ supercritical extractor. To prevent particles from flowing into the system tubing, glass cotton was used at both ends of the extraction tube before the caps were screwed onto. The flow rate of CO₂ was kept at 4.0 l·min⁻¹. The supercritical extraction experimental layout followed a completely randomized design with a single factor including co-solvent concentration (5–20 %, v/v), temperature (40–60 °C), pressure (15–30 MPa) and extraction time (30–120 min). After the extraction, the extract was collected and diluted with distilled water before determining the total polyphenols content and antioxidant activity. After each experiment, the processing parameter level resulting in the highest content of polyphenols and the highest ABTS radical-scavenging activity was chosen as the control variable for the next experiment. Each experiment was repeated three times.

Composition analysis

The following methods were used for testa composition analysis: AOAC 930.15 for moisture content, AOAC 942.05 for total ash, AOAC 2003.05 for crude fat (lipid) and AOAC 2001.11 for crude protein [17].

Polyphenolic compounds determination

To determine the polyphenols available in the raw material testa, extraction of soluble phenolic compounds was performed following the method of CHANDRASEKARA and SHAHIDI [5] with minor modifications. An amount of 6 g of defatted testa was mixed with 100 ml ethanol (80 %, v/v) and heated for 40 min under reflux conditions in a thermostatic water bath at 60 °C. The supernatant was collected after centrifugation at 4000 ×g for 5 min (Universal 320R; Hettich, Tuttlingen, Germany) and extraction was repeated four times. Then, the combined supernatants of the cashew nut testa extract were used to determine the total phenolic content by Folin–Ciocalteu assay.

Total phenolics content

Total phenolics content (TPC) was determined using Folin–Ciocalteu assay as described by PHAN et al. [18] with modifications. Briefly, a volume of 0.1 ml of cashew testa extract (after dilution) was subjected to the reaction with 1.8 ml of Folin–Ciocalteu reagent (10 %, v/v). The solu-

tion was shaken and incubated for 5 min. Then, 1.2 ml of 150 g·l⁻¹ sodium carbonate and 6.9 ml of distilled water were added. After 1 h incubation at room temperature (25 ± 0.1 °C) in dark conditions, absorbance was measured at 765 nm using a PhotoLab 6600 UV–Vis spectrophotometer (WTW, Weilheim, Germany). Various concentrations of gallic acid were used to construct the standard curve ($R^2 = 0.9983$) and distilled water was used as a control. The experiment was carried out in triplicate and results were expressed as gram of gallic acid equivalent (GAE) per kilogram dry weight (DW).

Antioxidant activity

Quantitative determination of the antioxidant capacity of cashew testa extracts by the ABTS method was performed according to the method described by THI and TAI [11] with modifications. In brief, 7 mmol·l⁻¹ ABTS solution was mixed with 2.45 mmol·l⁻¹ potassium persulfate solution (ratio of 1:1, v/v). The mixture was kept in the dark at room temperature (25 ± 0.1 °C) for 16 h and diluted with distilled water to reach an absorbance of 0.7 ± 0.02 at 734 nm to obtain the ABTS stock solution. A volume of 0.1 ml of cashew testa extract was mixed with 3.0 ml of ABTS stock solution and 0.9 ml of ethanol (95 %, v/v). The mixture was shaken briefly and incubated at room temperature (25 ± 0.1 °C) for 15 min in dark conditions. The colour formation was determined by measuring the absorbance at 734 nm using a PhotoLab 6600 UV–Vis spectrophotometer. Various concentrations of Trolox were used to construct the standard curve ($R^2 = 0.9929$) and distilled water was used as a control. The experiment was carried out in triplicate and results were expressed as ABTS radical-scavenging activity in millimoles of Trolox equivalent (TE) per kilogram DW.

Tab. 1. Composition of cashew nut testa used in this work.

Component	Content
Moisture [%]	7.5 ± 0.5
Ash [%]	3.6 ± 0.3
Lipid [%]	20.5 ± 0.4
Protein [%]	10.1 ± 0.2
Soluble polyphenols [g·kg ⁻¹]	198.1 ± 2.1

Average values of triplicate analyses \pm standard deviation. Soluble polyphenols are expressed as grams of gallic acid equivalents per kilogram dry weight.

RESULTS AND DISCUSSION

Characteristics of cashew nut testa

The composition of cashew nut testa varies with the maturation stage and size of the cashew nut. Tab. 1 presents the average composition of cashew nut testa used in this study as the input material for supercritical CO₂ extraction. The obtained results indicated that the moisture, ash, lipid and protein contents of the testa were similar to values reported previously [19, 20]. It could be observed that cashew nut testa is a promising source for polyphenols recovery. The soluble polyphenols content of 198.1 g·kg⁻¹ DW (expressed as GAE) found in this study fell within the range reported by KAMATH and RAJINI [9] with 243.0 g·kg⁻¹ DW. Disparities in these values may be attributed to cashew varieties, cultivation conditions and the process of separating the cashew nuts. Notably, the high lipid content of 20.5 % found in the cashew nut testa may adversely impact the extraction of polyphenols using supercritical CO₂.

Effects of ethanol on the extraction

In modified supercritical CO₂ extraction, the concentration of the co-solvent has a significant impact on the extraction process. Fig. 1 describes extraction efficiency at various concentrations of ethanol as a co-solvent for extraction of polyphenols from the cashew nut testa by supercritical CO₂. As the ethanol concentration increased from 5 % (v/v) to 15 % (v/v), the total polyphenols content increased from 10.97 g·kg⁻¹ DW to 46.71 g·kg⁻¹ DW. With the same trend, the ABTS-scavenging activity increased from 658.85 mmol·kg⁻¹ DW to 1110.42 mmol·kg⁻¹ DW (expressed as TE). The positive effects of co-solvent on the recovery of TPC and antioxidant activity could be explained by improving the extractability and facilitating the analytes solubilization [21]. The co-solvent induces changes via intra-crystalline and osmotic swelling of the cellular matrix whose more open structure can bring about more rapid and efficient extraction [22].

Some previous research also reported similar improvements in the recovery of bioactive compounds extracted with supercritical CO₂ by applying a certain amount of co-solvent. MURGA et al. [23] obtained the highest value of phenolic compounds from grape seed at an ethanol concentration of 15 % (v/v). QUINTANA et al. [24] observed an increase in TPC of licorice extract by increasing ethanol concentration from 0 % (v/v) to 20 % (v/v). The study by DÍAZ-REINOSO et al. [16] indicated that an increase in ethanol concen-

tration in the supercritical CO₂ extraction led to a significant increase in the global extraction yield and antioxidant activity.

However, the efficiency of the co-solvent to a certain extent could be lost. As could be observed in this study, a further increase in ethanol concentration by up to 20 % (v/v) resulted in no significant change in *TPC* and antioxidant activity. The result of this work is similar to the study by DE CAMPOS et al. [25] which evaluated the effect of various ethanol concentrations on free radical-scavenging of grape pomace extracts from Cabernet sauvignon (*Vitis vinifera*), the highest antioxidant potential was obtained with 15 % (v/v) ethanol in supercritical CO₂ and then decreased at 20 % (v/v) ethanol. The negative effect of co-solvent at high concentrations could be explained by possible hypotheses that when ethanol concentration in the supercritical mixture is high, there is not enough energy to separate the hydrogen-bonded ethanol molecules to form hydrogen bonds between the solute and the solvent. Therefore, fewer solute molecules are solubilized, which reduces extraction efficiency [26]. According to TAI et al. [13], increasing the co-solvent concentration would increase the overall flow rate through the sample, thus reducing the interaction between solute and solvent. Due to a good recovery of bioactive compounds, 15 % (v/v) ethanol concentration was chosen as the fixed parameter for the next experiment in this study.

Effects of temperature on the extraction

The effect of temperature on the recovery of total phenolics and antioxidant activity of cashew testa extract obtained by ethanol-modified supercritical CO₂ is shown in Fig. 2. The highest total phenolics content (46.71 g·kg⁻¹ DW) and antioxidant activity (1090.79 mmol·kg⁻¹ DW) were obtained at 40 °C. Overall, it could be observed that the increase in temperature caused a decrease in both *TPC* and antioxidant activity. Up to 60 °C, compared to the highest values at 40 °C, both *TPC* and antioxidant activity decreased significantly by nearly 27 % and 33 %, respectively. The results of this study are in agreement with CADENA-CARRERA et al. [27] regarding the biological activities of extracts obtained by using supercritical CO₂. In that study, an increase in temperature from 45 °C to 60 °C at 15 MPa caused a decrease in both the *TPC* and antioxidant activity of guayusa leaf extract. In another work, SATO et al. [28] obtained the highest antioxidant activity from strawberry leaves at 40 °C and 20 MPa by using supercritical CO₂ and a further increase in temperature caused a decrease in antioxidant activity.

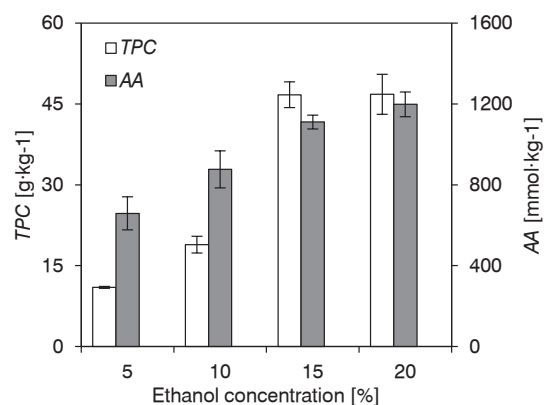


Fig. 1. Effects of ethanol concentration on the extraction of polyphenols and antioxidant activity.

TPC – total phenolics content (expressed as gram of gallic acid equivalent per kilogram dry weight), *AA* – 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity (expressed as millimoles of Trolox equivalent per kilogram dry weight).

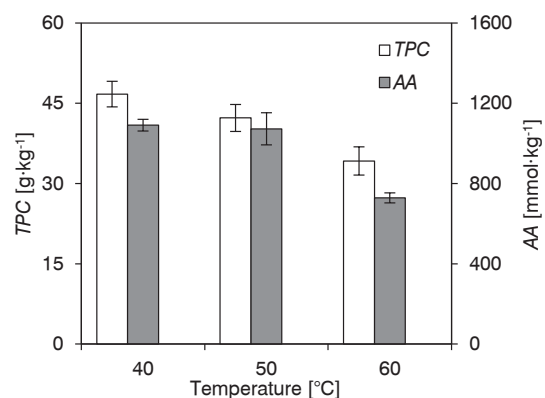


Fig. 2. Effects of temperature on the extraction of polyphenols and antioxidant activity.

TPC – total phenolics content (expressed as gram of gallic acid equivalent per kilogram dry weight), *AA* – 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity (expressed as millimoles of Trolox equivalent per kilogram dry weight).

The negative effect of temperature on *TPC* and antioxidant activity on extract obtained by supercritical CO₂, in this case could be explained by the change in supercritical fluid density and volatility of the solid solute. In general, the increase in supercritical fluid temperature at isobaric conditions could cause two opposing phenomena: (i) a decrease in density leading to decrease in molecular interactions between the solvent and the solute, and as a result, a decrease in solubility of bioactive compounds; (ii) an increase in vapour pressure of the solute and hence an increase in solubility by the volatility effect [29]. Therefore, if the extraction pressure is not sufficiently high,

a decrease in density and solvent power with increasing temperature would prevail, resulting in reduced solubility of the solute and lower extraction efficiency. This could explain the fact that the observed *TPC* and antioxidant activity of cashew testa extract in this work decreased with the further increase in extraction temperature. Besides, the increasing temperature might cause some undesirable chemical reactions and degradation of some heat-sensitive bioactive compounds [30]. Additionally, increasing the extraction temperature would increase the energy cost of the process. Therefore, a temperature of 40 °C was chosen as the operating temperature for subsequent experiments.

Effects of pressure on the extraction

Pressure is among the most important processing parameters in supercritical extraction. To investigate the effect of pressure on *TPC* and antioxidant activity, the experiments of ethanol-modified supercritical CO₂ extraction of cashew testa were conducted at a pressure ranging from 15 MPa to 30 MPa (Fig. 3).

The results showed that the lowest *TPC* and antioxidant activity were obtained at the pressure of 15 MPa. As the pressure increased from 15 MPa to 20 MPa, *TPC* did not change significantly, while the antioxidant activity shown in the ABTS assay slightly increased to 1178.38 mmol·kg⁻¹ DW. Upon further increasing the pressure to 25 MPa, *TPC* and antioxidant activity increased by nearly 20 % and 25 % to reach the maximum values of 55.8 g·kg⁻¹ DW and 1338.47 mmol·kg⁻¹ DW, re-

spectively. However, beyond 30 MPa, the changes in *TPC* and antioxidant activity were statically insignificant. It can also be observed in Fig. 3 that the trends of *TPC* and antioxidant activity concerning pressure change can be described in three stages: (1) an initial pressure increase from 15 MPa to 20 MPa that did not result in a significant improvement in *TPC* and antioxidant activity; (2) a subsequent increase to 25 MPa, during which both *TPC* and antioxidant activity were maximized; and (3) a further increase in pressure (up to 30 MPa), which did not significantly affect the values of *TPC* and antioxidant activity. Generally, the increase in pressure will lead to an increase in fluid density and power of solvation that could enlarge the solubility of soluble compounds [31]. However, in the first stage, the increase from 15 MPa to 20 MPa might not have provided a sufficient density effect to significantly elevate the values of *TPC* and antioxidant activity. However, as the pressure further increased up to 25 MPa, the density, in particular the attractive forces between the solvent and solute molecules, became strong enough to significantly enhance both *TPC* and antioxidant activity values. In the final stage, the failure to improve extraction efficiency by increasing pressure up to a threshold point of 30 MPa could be attributed to an increase in viscosity and a decrease in diffusion coefficients resulting in reduced contact of the supercritical fluid with pores in the raw material, thereby potentially reducing solute dissolution [29]. Previous studies showed that at a higher pressure, the system is becoming over-compressed and repulsive solute-solvent interactions become dominant over attractive interactions [32]. Moreover, an increase in pressure could cause the extraction of undesirable compounds other than phenolic substances [33].

The observed trends in *TPC* and antioxidant activity concerning pressure changes at a constant temperature and amount of co-solvent are in line with previous research findings. In the context of ethanol-modified supercritical CO₂ extraction of custard apple peel, the study of TAI et al. [13] showed that both *TPC* and antioxidant activity increased within the pressure range of 15 MPa to 25 MPa, but decreased when pressure was raised to 30 MPa. In the study of BENELLI et al. [33] focusing on the supercritical extraction of orange pomace, at a constant temperature of 50 °C, antioxidant activity reached a maximum value at 20 MPa and then decreased with a further increase in pressure. Based on these observations, a pressure of 25 MPa was chosen for subsequent experiments.

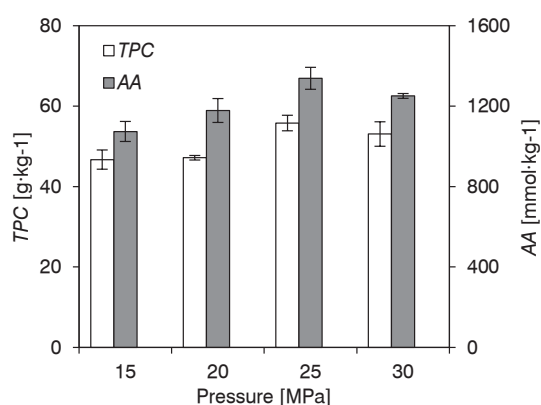


Fig. 3. Effects of pressure on the extraction of polyphenols and antioxidant activity.

TPC – total phenolics content (expressed as gram of gallic acid equivalent per kilogram dry weight), *AA* – 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity (expressed as millimoles of Trolox equivalent per kilogram dry weight).

Effects of time on the extraction

Understanding the effects of extraction time on the extraction efficiency of cashew testa is crucial for optimizing the supercritical CO₂ extraction process. The effect of extraction time on the recovery of total phenolics and antioxidant activity was investigated at 30-minute intervals from 30 min to 120 min. The results are depicted in Fig. 4.

The overall extraction curves of cashew nut testa exhibited three distinct stages, similar to those described by DA SILVA et al. [34]. In the first stage (up to 60 min), both *TPC* and antioxidant activity sharply increased by 58 % and 92 %, respectively. During this period, the extraction rate remained constant as the external surface of the particles is covered with solute, allowing for easy accessibility. The mass transfer was primarily driven by convection. In the second stage (from 60 min to 90 min), the rate of increase in *TPC* and antioxidant activity was significantly lower compared to the first stage. *TPC* and antioxidant activity continued to increase by approximately 15 % and 7 %, respectively, compared to their values at 60 min of extraction time. During this phase, failures in the external surface solute layer appeared and diffusion mechanisms started to operate concurrently with convection, leading to a falling extraction rate. In the final stage (from 90 min to 120 min), there were no significant changes in both *TPC* and antioxidant activity. This stage represented a low extraction rate, where the external layer of solute disappeared and the mass transfer dominantly occurred through diffusion inside the solid particles [34].

Overall, the extraction efficiency of cashew testa could be enhanced by prolonging the extraction time, as observed from 30 min to 90 min during stages 1 and 2. However, further increase in extraction time did not lead to significant changes in extraction yield beyond a certain threshold, as evidenced by 90 min to 120 min during stage 3. BRUNNER [29] suggested two possible explanations for this decrease: (i) depletion of solute near the outer membrane of the solid material and (ii) insufficient length of the cell containing the solid material, limiting the maximal solvent loading capacity. Comparable overall extraction curves were observed in previous studies with supercritical extraction of chestnut burs [16] and yacon leaves [35].

Optimal extraction conditions

Based on the experimental results, a 15 % (v/v) ethanol co-solvent concentration, a temperature of 40 °C, a pressure of 15 MPa and an extrac-

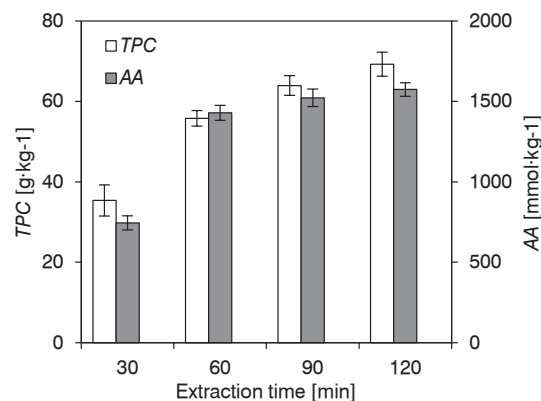


Fig. 4. Effects of extraction time on the extraction of polyphenols and antioxidant activity.

TPC – total phenolics content (expressed as gram of gallic acid equivalent per kilogram dry weight), *AA* – 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical-scavenging activity (expressed as millimoles of Trolox equivalent per kilogram dry weight).

tion time of 90 min were finally selected as a set of conditions for ethanol-modified supercritical CO₂ extraction of cashew testa. Under these conditions, the values of *TPC* and antioxidant activity were obtained at 63.97 g·kg⁻¹ DW and 1522.72 mmol·kg⁻¹ DW, respectively. The *TPC* value obtained in this study was similar to the work of FIGUEROA-VALENCIA et al. [7] who extracted polyphenols from the testa of red and yellow cashew by methanol extraction. However, *TPC* and antioxidant activity obtained through supercritical CO₂ extraction in this research were relatively lower than those reported by CHANDRASEKARA and SHAHIDI [5] when using the conventional method with 80 % (v/v) ethanol to extract polyphenols from defatted cashew testa. The observed differences in *TPC* obtained by supercritical extraction can be attributed to various factors, including the origin of the raw materials and the defatting pre-treatment method employed, as well as polarity of the solvent used for extraction [21].

CONCLUSIONS

The present preliminary study demonstrated the potential of supercritical CO₂ extraction using ethanol as a co-solvent for a sustainable recovery of valuable polyphenols as antioxidants from cashew nut testa. The results indicated that increasing the ethanol concentration within a certain range positively influenced *TPC* and antioxidant activity. Moreover, higher pressures exerted

a positive effect on antioxidant activities of the extracts. However, beyond specific thresholds, further increase in pressure and ethanol concentration did not significantly affect TPC and antioxidant activity. Additionally, a lower extraction temperature was found to be favourable for cashew nut testa extraction. Relatively good antioxidant activities of the extract were obtained at the supercritical CO₂ extraction condition involving 15 % (v/v) ethanol co-solvent concentration, a temperature of 40 °C, a pressure of 25 MPa and an extraction time of 90 min. Nevertheless, to enhance the efficiency of polyphenols extraction, it is recommended to incorporate an additional pre-treatment step such as defatting in future studies.

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