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Enzyme-assisted extraction of cashew nut (*Anacardium occidentale* L.) testa

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Abstract

Enzyme-assisted extraction (EAE) has been successfully applied to extract polyphenols from various food materials and by-products. Hence, the present research aimed to valorize the cashew nut (*Anacardium occidentale* L.) testa by EAE method using an enzyme cocktail of cellulase and pectinase. The effects of different processing parameters, including the pH (3.0 - 6.0), temperature (30 - 70°C), extraction time (60 - 150 min), solid -to-solvent ratio (1/5 - 1/85), and enzyme concentration (0.1 - 0.4% v/v) on the total polyphenol content (TPC) and antioxidant capacity of the testa extracts were investigated. The results showed that the TPC of the testa extracts positively correlated with the antioxidant capacity. The EAE performed at pH 4.0, a temperature of 50°C, an extraction time of 60 mins, a solid-to-solvent ratio of 1/55, and an enzyme concentration of 0.2% (v/v) resulted in the highest TPC (168.84 g GAE/kg DW) with DPPH and ABTS antioxidant activity of 885.56 and 1513.60 (mmol TE/kg DW), respectively. The EAE technique holds significant promise as a sustainable approach to mitigate the underutilization and undervaluation of cashew nut testa waste, which could otherwise contribute to environmental pollution.

1. Introduction

Fruit by-products are one of the major sources of organic waste materials that cause environmental problems. The cashew nut (*Anacardium occidentale*) testa, accounting for 1-3% of its total weight (Sharma *et al.*, 2020), is typically removed as a by-product during processing because it contributes to a bitter and astringent taste, affecting the sensorial quality of nut products. A substantial amount of cashew nut testa waste remains underutilized or undervalued. The discarded testa accumulates in storage areas, contributing to soil pollution, contamination of underground water, and air quality degradation through combustion. Previous studies reported that cashew nut testa is a valuable source of polyphenols including catechin, epigallocatechin, and epicatechin that can be used for nutraceutical applications (Viswanath *et al.*, 2016; Salehi *et al.*, 2019). It was also proven that the polyphenolic compounds appear to contribute to the observed antioxidant activity of cashew testa (Sruthi and Naidu, 2023).

A range of conventional solvent-based extraction methods is predominantly being used to extract the bioactive compounds from cashew testa. However, the conventional extraction method has several disadvantages including low extraction efficiency, being time-consuming, high solvent consumption, and negative environmental impact (Thi and Tai, 2023). The adoption of non-conventional techniques for extracting polyphenolic compounds from cashew nut testa has been limited.

Recently, enzyme-assisted extraction (EAE) has been successfully applied to extract polyphenols from various food materials and by-products, demonstrating its superiority over other conventional extraction. The composition of plant cell walls as in the case of cashew nut predominantly comprised of cellulose, hemicellulose, lignin, and pectin, constitutes the principal impediment to bioactive compound extraction (Kaur *et al.*, 2023; Anoopkumar *et al.*, 2024). Therefore, enzymes such as cellulase and pectinase play an important role in degrading these cell wall components, facilitating the release of polyphenolic compounds (Gardossi *et al.*, 2010). EAE can significantly increase the overall extraction yield and enhance the antioxidant capacity of the extract. It was reported that EAE had been successfully applied to extract phenolic compounds from various plant sources, including pistachio green hull

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(Ghandahari Yazdi *et al.*, 2019), Japanese peppermint (Shimotori *et al.*, 2020), sunflower wastes (Ricarte *et al.*, 2020), watermelon rind (dos Santos *et al.*, 2022), blackcurrant press cake (Granato *et al.*, 2022), and chili pepper seeds (Cortes-Ferre *et al.*, 2022).

However, to the best of our knowledge, a limited number of available articles in the literature currently exist on the EAE of polyphenolic compounds from cashew nut testa by using an enzyme cocktail of commercial cellulase and pectinase. Therefore, the main objective of the present study was to screen the EAE of cashew nut testa at various key operational parameters, including pH, temperature, extraction time, solid-to-solvent ratio, and concentration of an enzyme mixture of pectinase and cellulase.

2. Materials and methods

2.1 Chemicals

2,2-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy−2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and Folin–Ciocalteu reagent were supplied from Merck (Darmstadt, Germany). Standard gallic acid was obtained from Sigma-Aldrich (Missouri, USA). Analytical-grade water was purified using a Milli-Q purification system (Millipore, Massachusetts, USA). Pectinex® Ultra SP-L (pectinase, ≥3,800 PGU/ml) and Celluclast® 1.5L (cellulases, 700 EGU/ml) were obtained from Novozymes (Denmark). All remaining chemicals employed in the experiments adhered to analytical-grade standards.

2.2 Cashew nut testa

Cashew nut testa was supplied by the Kimmy Farm Company (Ho Chi Minh City, Viet Nam). The values of total polyphenol content (TPC) of the raw material testa were obtained at 198.1±2.1 g GAE/kg DW which was reported in our recent study (Tai *et al*., 2024). The testa underwent pulverization, followed by sieving through a 0.5 mm sieve to obtain a fine powder. The resulting powder was stored in sealed polyethylene bags and kept in the dark at -10°C until required for the extraction process.

2.3 Enzymatic extraction procedure

An amount of 1.00±0.01 g of cashew nut testa powder was combined with distilled water used as the solvent for the extraction in a 50 mL beaker. Sodium acetate buffer was used to adjust the pH. Celluclast® 1.5L and Pectinex® Ultra SP-L were used at a ratio of 1:1 (v/v). Before conducting the main experiments, a series of preliminary trials were undertaken to assess the influence of each extraction parameter on the efficiency of polyphenol extraction. These trials helped establish the range of parameters to investigate. Consequently, a typical enzymatic extraction was carried out at pH 4.5, 50°C for 60 mins, the solid-to-solvent ratio of 1/15 (w/v), and enzyme mixture concentration of 0.2% (v/v) in a thermostatic water bath (Memmert, Germany). After the extraction, the enzymes were deactivated at 90°C for 5 mins. The samples were centrifuged at 25°C for 10 mins at 5000 rpm by Universal 320R centrifuge (Hettich, Germany), and the supernatant was collected for further analysis. The efficiency of polyphenol extraction based on the total polyphenol content and antioxidant capacity of the testa extracts was assessed.

2.4 Total phenolic content

The assessment of total phenolic content (TPC) was conducted using the Folin-Ciocalteu assay, as described by Tai *et al.* (2022) with slight modification. Briefly, a volume of 0.1 mL of cashew testa extract was combined with 1.8 mL of Folin-Ciocalteu reagent (10%). After a brief shaking, the mixture was allowed to stand at room temperature for 5 mins before the addition of 6.9 mL of water and 1.2 mL of Na_2CO_3 (15%). Incubation occurred in the dark at room temperature for 90 mins. The color formation was quantified by measuring absorbance at 765 nm using a UV-Vis spectrophotometer (Jenway 7305, UK). A control with water was employed, and a standard curve was developed using gallic acid at different concentrations with an R^2 value of 0.9997. The results were calculated as grams of gallic acid equivalent (GAE) per kilogram dry weight (DW).

2.5 Antioxidant capacity

2.5.1 DPPH assay

The DPPH assay was performed following the method of Tai *et al.* (2022). Briefly, a volume of 0.1 mL of cashew testa extract was blended with 0.9 mL of ethanol and 4.0 mL of 0.1 mM DPPH solution. After a brief shaking, the mixture underwent incubation at room temperature for 30 mins in the dark. The color formation was determined by measuring absorbance at 517 nm using a UV-Vis spectrophotometer (Jenway 7305, UK). A water control was utilized, and a standard curve was constructed at various concentrations of Trolox resulting in an R^2 value of 0.9995. The results were expressed as millimoles of Trolox equivalent (TE) per kilogram DW.

2.5.2 ABTS assay

Similarly, the ABTS assay was conducted following the method of Tai *et al.* (2022) with slight modifications. In brief, 7 mM ABTS solution was stirred with 2.45 mM potassium persulfate solution for 16 hours in the dark.

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The resulting stock solution was diluted with water to achieve an absorbance of 0.7±0.02 at 734 nm. For the assay, a volume of 0.1 mL of cashew testa extract was combined with 1.9 mL of ethanol and 3.0 mL of the stock solution. Following a brief shaking, the mixture was incubated at room temperature for 15 mins in the dark, and color formation was quantified by measuring absorbance at 734 nm using a UV-Vis spectrophotometer (Jenway 7305, UK). Water control was used, and a standard curve was established at various concentrations of Trolox with an R^2 value of 0.9974. The results were calculated as millimoles of TE per kilogram DW.

2.6 Experimental design

The experiments employed a single-factor and completely randomized design, with EAE parameters (pH, temperature, extraction time, solid-to-solvent ratio, and enzyme concentration) as independent variables. Total polyphenol content (TPC) and antioxidant activities by DPPH and ABTS assays were used as response variables. The condition yielding the highest TPC and antioxidant capacity was chosen as the control variable for subsequent experiments.

2.7 Statistical analysis

All experiments in this study were conducted in triplicate. The results are presented as mean values ± standard deviation bars. Analysis of variance (ANOVA) was used to determine the significant difference in experimental data among samples with a 95% confidence level (or $p \leq 0.05$) using STATGRAPHICS Centurion XV Version 15.1.02 software.

3. Results and discussion

3.1 Effect of pH

In the enzymatic extraction process, pH plays an important role as it affects the solubility of dissolved compounds and the stability of enzymes. The impact of pH on the efficiency of cashew nut testa extraction is depicted in Figure 1. It was observed that the polyphenol content and antioxidative capacity of the cashew nut testa showed a direct correlation. It is agreed with Thi Lan Khanh *et al*. (2018) who reported that total phenolic compounds are a major contributor to the antioxidant capacity of plant materials.

The results indicate that varying the extraction pH from pH 3.0 to pH 4.0 significantly increases the TPC and antioxidant capacity (both scavenging against DPPH and ABTS). The polyphenol content significantly rises from 136.59 g GAE/kg dry matter (DW) to 163.06 g GAE/kg DW. At the same time, the DPPH radical scavenging activity increases from 436.0 mmol TE/kg DW to 756.44 mmol TE/kg DW, and ABTS radical scavenging activity rises from 760.86 mmol TE/kg DW to 1049.38 mmol TE/kg DW. However, as the extraction pH is further increased to pH 6.0, the extract's TPC and antioxidant activity decrease. The polyphenol content decreases to 145.64 g GAE/kg DW, and antioxidant activity decreases to 514.89 mmol TE/kg DW in the DPPH assay. In the same trend, antioxidant activity drops to 720.28 mmol TE/kg DW in the ABTS assay. Therefore, the highest TPC and antioxidant capacity are achieved at pH 4.0. This can be explained by the optimal pH range of the studied enzymes, Pectinex® Ultra SP-L and Celluclast® 1.5 L, which are closely aligned (Lim *et al*., 2024). In general, these two enzymes have closely matched optimal pH values, making them suitable for blending to enhance the extraction efficiency. Each enzyme functions optimally within its suitable pH range, and deviations from this pH range can result in enzyme denaturation and reduced interaction with organic compounds, hindering polyphenol release and diminishing extraction efficiency (Munde *et al.*, 2017). The optimal pH found in this work nearly agreed with the study by Gómez-García *et al*. (2012) which investigated the extraction of grape (*Vitis vinifera* L.) residues using a combination of Pectinex® Ultra and Celluclast® 1.5 L at pH 3.5. The slight disparity may be attributed to the distinct nature of the raw material. Consequently, with the maximum TPC and antioxidant activity obtained at pH 4.0, this pH was considered proper for further experiments to investigate the extraction of the polyphenols from cashew nut testa.

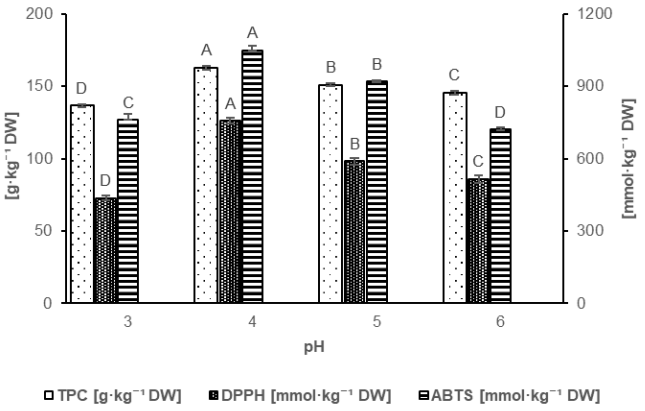

Figure 1. Effect of pH on the total polyphenol content (TPC) and antioxidant capacity (DPPH and ABTS assays) at 50°C for 60 mins, solid-to-solvent ratio of 1/15 (w/v), and 0.2% (v/v) enzymes. Bars with different notations are statistically significantly different by the Tukey-Kramer HSD test ($P<0.05$).

3.2 Effect of temperature

Temperature plays a crucial role in affecting enzyme activity, consequently affecting the overall efficiency of the extraction process. As shown in Figure 2, concerning

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the polyphenol content of the cashew testa extracts, an increase in temperature from 30°C to 50°C results in a significant increase in TPC from 110.67 g GAE/kg DW to 162.88 g GAE/kg DW. However, with a further increase in temperature to 70°C, the TPC decreases to 131.72 g GAE/kg DW. Regarding antioxidant activity, there is a proportional increase in polyphenol content as the temperature rises from 30°C to 50°C, with antioxidant activity in the DPPH assay increasing from 650.67 mmol TE/kg DW to 758.11 mmol TE/kg DW and antioxidant activity in the ABTS assay increasing from 942.78 mmol TE/kg DW to 1114.93 mmol TE/kg DW. Nevertheless, when the temperature continues to rise to 70°C, the DPPH antioxidant activity decreases to 455.44 mmol TE/kg DW, and the ABTS antioxidant activity decreases to 779.75 mmol TE/kg DW. These results can be explained by the presence of pectin and hemicellulose compounds that commonly exist in the cell walls of cashew testa. During extraction processes, these compounds contribute to the extract's viscous state, impeding the escape of polyphenol compounds. To enhance the extraction efficiency, increasing the temperature and reducing viscosity becomes necessary. Additionally, Pectinex® Ultra SP-L is optimally effective within a temperature range of 30°C to 45°C, while Celluclast® 1.5 L is most effective within a range of 30°C to 55°C. Consequently, these two products possess closely aligned optimal temperature ranges, making them suitable for combination to enhance extraction efficiency.

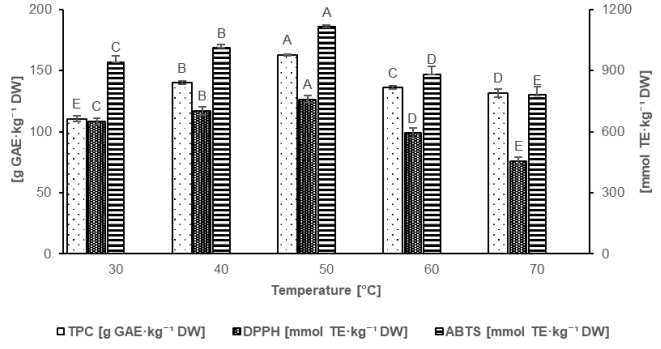

Figure 2. Effect of temperature on the total polyphenol content (TPC) and antioxidant capacity (DPPH and ABTS assays) at pH 4.0 for 60 mins, solid-to-solvent ratio of 1/15 (w/v), and 0.2% (v/v) enzymes. Bars with different notations are statistically significantly different by the Tukey-Kramer HSD test (P<0.05).

As elucidated by Dai and Mumper (2010), an increase in temperature reduces viscosity, fostering enhanced interaction between enzymes and solvents, facilitating continuous convective diffusion, and enhancing extraction capability. However, continued elevation of the temperature may induce enzyme denaturation and the degradation of phenolic compounds. This dual effect can elevate the solubility of unfavorable solutes, resulting in diminished TPC of the extract. Munde *et al*. (2017) also stated that each type of enzyme operates optimally within a specific temperature range. Deviations from this range can lead to enzyme denaturation, reducing their interaction with organic compounds and subsequently diminishing extraction efficiency. Conversely, an increase in temperature can accelerate polyphenol oxidation reactions. The same phenomena have been reported by Laroze *et al*. (2010), which employed a temperature of 50°C for the extraction of phenolic compounds from raspberry wastes, resulting in a 35% increase in extracted content and 50% and 15% enhancement in DPPH and ABTS antioxidant activities, respectively. Additionally, dos Santos *et al*. (2022) used a temperature of 50°C for enzyme-assisted extraction of albedo extract obtained from watermelon rind, which showed the highest polyphenolic potential and antioxidant capacity. Similarly, Zheng *et al*. (2009) employed a temperature of 50°C to extract polyphenols from unripe apples. Therefore, to achieve the best polyphenols extraction efficiency from cashew testa, a temperature of 50°C will be selected for the next optimization experiments.

3.3 Effect of extraction time

The extraction time can positively and negatively affect the extraction process. A short extraction time results in incomplete extraction. Conversely, a prolonged extraction time coupled with high temperature can lead to the degradation of certain heat-sensitive compounds (Thi and Tai, 2023). Therefore, the extraction time must be carefully selected to ensure optimal TPC and antioxidant activity. As observed in Figure 3, in the initial phase of extraction, both the TPC and antioxidant activity gradually increase with time, reaching maximum values at 60 mins, with TPC at 162.10 g GAE/kg DW, and antioxidant activity according to DPPH and ABTS assays at 735.22 mmol TE/kg DW and 1162.81 mmol TE/kg DW, respectively.

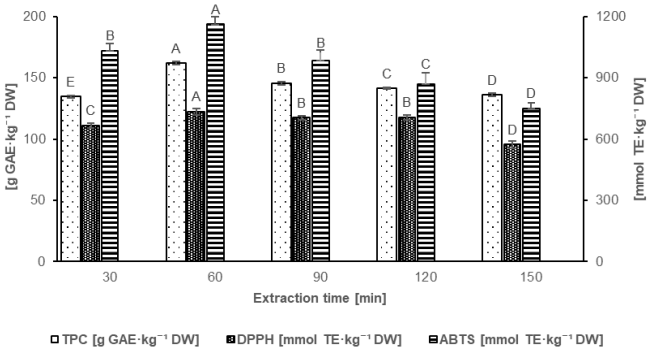

Figure 3. Effect of extraction time on the total polyphenol content (TPC) and antioxidant capacity (DPPH and ABTS assays) at pH 4.0, 50°C, solid-to-solvent ratio of 1/15 (w/v), and 0.2% (v/v) enzymes. Bars with different notations are statistically significantly different by the Tukey-Kramer HSD test (P<0.05).

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This result aligns with the principle of enzyme hydrolysis reactions, where an appropriate duration is needed for enzymes to act upon the entire organic material, hydrolyzing pectin and cellulose within the plant tissue structure. This facilitates the release of intracellular substances and enhances their diffusion, making the process more efficient. However, when the extraction time is extended to 150 mins, the TPC and antioxidant activity decreases. Specifically, the TPC decreases to 136.22 g GAE/kg DW, and the DPPH and ABTS scavenging activities decrease to 573.11 mmol TE/kg DW and 750.11 mmol TE/kg DW, respectively. It was reported that as the extraction time lengthens, both the TPC and antioxidant activity decrease because polyphenols come into contact with oxygen that is susceptible to oxidation (Munde *et al.*, 2017). On the other hand, when compared to the conventional ethanol extraction of cashew nut testa with rotary shaking at 37° C, Kamath and Rajini (2007) achieved a total phenolic content of 243 g GAE/kg testa powder after 180 mins. The variance in phenolic content observed may also be attributed to differences in analysis methods, the polarity of extraction solvents, and notably, the genetic diversity of cashew nut testa varieties.

The study outcomes also agreed with the findings of Hai *et al*. (2016), who also used a 60-min extraction time to achieve the highest extraction efficiency. Therefore, at an extraction time of 60 mins, the TPC and antioxidant activity reach their maximum values and will be used in subsequent experiments.

3.4 Effect of solid-to-solvent ratio

In the course of the extraction process, the capillary structure inherent in the material facilitates the infiltration of solvent molecules into its core. The migration of polyphenol molecules from the innermost regions to the surface is propelled by the existing gradient in polyphenol concentration. As shown in Figure 4, increasing the solvent ratio from 1/5 to 1/55 results in a significant increase in TPC, from 107.09 g GAE/kg DW to 164.50 g GAE/kg DW. Similarly, the antioxidant activity shown in the DPPH assay increases from 423.25 mmol TE/kg DW to 875.44 mmol TE/kg DW, and antioxidant activity detected by ABTS exhibits a proportional increase from 836.75 mmol TE/kg DW to 1535.61 mmol TE/kg DW.

However, further increasing the solvent ratio from 1/55 to 1/85 does not lead to a further increase in TPC. Instead, TPC decreases to 152.36 g GAE/kg DW, and both DPPH and ABTS antioxidant activities decrease to 775.00 mmol TE/kg DW and 1200.43 mmol TE/kg DW, respectively. Comparatively, by using pectinase, cellulase, and tannase. Ghandahari Yazdi *et al*. (2019) reported that the amount of extracted phenolic compounds from pistachio green hull increased with the increase of the solid-to-solvent ratio from 1:20 to 1:80, while further increasing this ratio from 1:80 to 1:150 decreased the extraction yield. It can be observed that inadequate solvent relative to the quantity of raw material is insufficient to fully dissolve the extracted compounds. The enhancement of polyphenol extraction efficiency correlates with an increase in solvent volume. However, upon reaching the optimal threshold of solvent volume, polyphenols are completely solubilized. Beyond this point, further escalation of solvent volume leads to polyphenol dilution within the solvent, consequently diminishing antioxidant activity. Thi and Tai (2023) also found that heightened solvent volume elevates dissolved oxygen content, causing reductions in both TPC and antioxidant activity. Moreover, increasing the solvent volume may inadvertently extract undesired compounds. Based on these observations, the solid-to-solvent ratio 1/55 has been chosen as the fixed parameter for subsequent experiments to extract polyphenols from cashews testa.

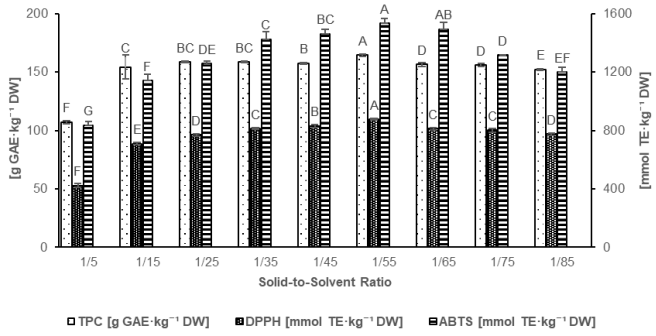

Figure 4. Effect of solid-to-solvent ratio on the total polyphenol content (TPC) and antioxidant capacity (DPPH and ABTS assays) at pH 4.0, 50°C for 60 mins, and 0.2% (v/v) enzymes. Bars with different notations are statistically significantly different by the Tukey-Kramer HSD test ($P<0.05$).

3.5 Effect of enzyme concentration

The enzyme concentration is one of the important factors affecting the extraction process. The efficiency of polyphenol extraction will increase as the enzyme concentration used increases because of the ability to disrupt more cell walls of the cashew testa, creating favorable conditions for releasing polyphenols within the cell matrix. The results in Figure 5 demonstrate that initially, as the enzyme concentration increased from 0.1% to 0.2%, the TPC of the testa cashew extract increased significantly from 136.49 g GAE/kg DW to a maximum of 168.84 g GAE/kg DW. Correspondingly, DPPH antioxidant activity increased from 635.56 mmol TE/kg DW to 885.56 mmol TE/kg DW, and ABTS antioxidant activity increased from 1300.76 to 1513.60 mmol TE/kg DW.

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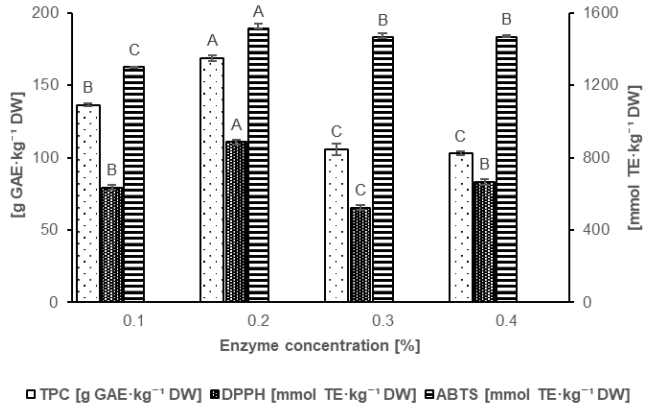

Figure 5. Effect of enzyme concentration on the total polyphenol content (TPC) and antioxidant capacity (DPPH and ABTS assays) at pH 4.0, 50°C for 60 mins, solid-to-solvent ratio of 1/55 (w/v). Bars with different notations are statistically significantly different by the Tukey-Kramer HSD test ($P<0.05$).

This observation is also consistent with Drevelegka and Goula (2020), that increasing the amount of enzyme accelerated the rate and efficiency of the extraction of phenolic compounds. However, as the enzyme concentration continued to rise to 0.3% and 0.4%, both the TPC and the DPPH and ABTS antioxidant activities exhibited a decreasing trend. Upon reaching a certain enzyme concentration, saturation occurs within the extraction environment, causing the production of compounds to remain unchanged or potentially decrease due to the influence of oxidative agents (Munde *et al.*, 2017). Moreover, from an economic perspective, a high enzyme concentration significantly increases the cost of the extraction process. Because both TPC and antioxidant activity obtained maximal values at the enzyme concentration of 0.2%, this was selected for the optimized process of cashew testa extraction.

4. Conclusion

The preliminary study of using an enzyme cocktail comprised of Celluclast® 1.5 L (cellulase) and Pectinex® Ultra SP-L (pectinase) demonstrated advancing sustainable technique for extracting valuable polyphenols with antioxidant properties from the by-product, cashew testa. The results indicate that optimizing pH within a specific range positively impacts TPC and antioxidant capacity. Additionally, elevated temperatures and prolonged extraction times enhance antioxidant activities; however, beyond certain thresholds, further increases in temperature and extraction time do not significantly impact TPC and antioxidant capacity. The solid-to-solvent ratio and enzyme concentration also emerged as influential factors in the extraction process. Employing extraction conditions with a pH of 4.0, a temperature of 50°C, an extraction time of 60 min, a solid-to-solvent ratio of 1/55, and an enzyme concentration of 0.2% (v/v) resulted in a testa extract characterized by high TPC and strong antioxidant activities.

Conflict of interest

The authors declare no conflict of interest.

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