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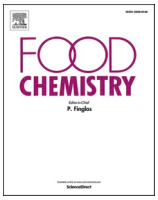

Effects of polyphenols from *Tasmannia lanceolata* on structural, emulsifying, and antioxidant properties of pea protein

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ABSTRACT

The effects of polyphenols from Tasmanian pepper (*Tasmannia Lanceolata*) leaf and berry on the functional properties of pea protein were investigated in flaxseed oil-in-water emulsions. Phenolic acids and flavonols in Tasmanian pepper leaf with smaller molecular weights led to stronger non-covalent interactions with pea protein, while anthocyanins from Tasmanian pepper berry induced protein aggregation under acidic condition and co-existed with proteins in neutral and alkaline conditions. The total phenolic content was significantly increased with incorporation of polyphenols from Tasmanian pepper leaf (334.94–445.92 μg/mL) and berry (72.89–153.03 μg/mL) to pea protein (4.19–15.59 μg/mL). The oxidative stability of emulsions at pH 3 and 7 was enhanced, reducing TBARS value from 1.54 to 2.68 mg MDA/kg in pea protein to 0.56–0.85 mg MDA/kg after 2 weeks storage. These findings illustrated the distinct interactions between pea protein and different polyphenols from Tasmanian pepper leaf and berry to enhance the antioxidant capacity of pea protein.

1. Introduction

Polyphenols are strong antioxidants with multiple hydroxyl groups attached to aromatic rings as key functional groups (Bhuyan & Handique, 2022). These compounds help prevent a number of diseases such as cancer, heart disease, diabetes, and neurodegenerative disorders due to antioxidant and anti-inflammatory properties, while when applied to food matrices, macromolecules such as proteins and polysaccharides can interact with polyphenol, altering their structural and functional properties (Luna-Guevara et al., 2018). Native Australian plants and fruits are unique and contain significant amounts of secondary metabolites that function as antioxidants due to the distinct climate conditions in Australia (Mani et al., 2021). In 2010, native Australian herbs and spices including anise myrtle, lemon myrtle, bush tomato, wattle seed, Tasmanian pepper leaf and berry were found to represent rich sources of phenolic compounds including benzoic/cinnamic acids and flavonoids (Konczak et al., 2010). More studies on the composition and antioxidant activity of native Australian plants and their therapeutic effects have been reported over a decade (Mani et al., 2021). However, no study to date has examined the potential applications of native Australian plants as functional ingredients in food.

Tasmannia lanceolata, commonly known as Tasmanian pepper, is primarily grown in Tasmania and parts of other states in Australia. Tasmanian pepper leaf and berry, traditionally used in food as tea, essential oils, flavoring agents, and spice blends, are becoming more widely cultivated due to growing demand in food, cosmetic, and health-related sectors (Sultanbawa, 2016). Especially, Tasmanian pepper leaf is abundant in phenolic acids and flavonols including chlorogenic acid, quercetin, and *p*-coumaric acid, whereas its berry is rich in anthocyanins such as cyanidin 3-rutinoside and cyanidin 3-glucoside (Konczak et al., 2010). The anthocyanin content of Tasmanian pepper berry is 50–100 % higher compared to blueberries and other fruits (Netzel et al., 2007) and Tasmanian pepper leaf also demonstrate superior antioxidant capacity with higher levels of polyphenols than other Australian native herbs (Konczak et al., 2010), suggesting significant potential as natural and healthful food ingredients. Existing literature on Tasmanian pepper predominantly focused on antioxidant capacity and medicinal effects, but not on their potentials to prevent lipid oxidation in food formulations.

While numerous studies on protein-polyphenol complexes are available, interactions between plant proteins and polyphenols have gained special attention for their potentials in improving the

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functionality of plant proteins and applications as antioxidant emulsifiers, edible film additives, nanoparticles, and hydrogels. Polyphenol incorporation promotes the structural rearrangement of plant proteins, thereby improving their techno-functional properties (Kim et al., 2024). The pH levels and the type of polyphenols are key factors that influence the interaction dynamics between plant proteins and polyphenols, impacting the antioxidant and emulsifying properties of plant proteins (Lima et al., 2023). For example, rice protein complexed with anthocyanins displayed the highest emulsifying properties at pH 3 with a rod-like structure forming stable interfacial layer compared to pH 7 and 9 (Li, Zhao, et al., 2020). Moreover, pea protein and quercetin were found to mainly interact by hydrogen bonding due to phenolic hydroxyl groups in quercetin, while pea protein formed complexes with curcumin and resveratrol via hydrophobic interaction with greater hydrophobicity of curcumin and resveratrol (Zhang et al., 2022). Molecular structure of polyphenol including molecular weight, hydrophobicity, and the number and location of hydroxyl groups plays a key role in determining the binding capacity with proteins (Kim et al., 2024).

Emulsion is the most common application of plant protein-polyphenol complexes because polyphenol addition generally promotes protein unfolding, making them readily adsorb to oil/water interface and prevent lipid from oxidation with enhanced antioxidant capacity (Quan et al., 2019). Moreover, the addition of excess polyphenols leads to protein aggregation, resulting in unstable emulsions with low physical and oxidative stability (Kim et al., 2024). However, most studies only focused on a single type of polyphenol while varying the polyphenol concentration. This limit underscores the need to examine how the interactions of plant proteins with different polyphenols influence the physical and oxidative stability of the emulsions.

Legume proteins have been extensively studied as natural emulsifiers (Kim, Wang, & Selomulya, 2020). Nonetheless, commercial legume protein isolates composed of 80–90 % protein, 10–15 carbohydrates, and 1–5 % fat are vulnerable to lipid-derived deterioration, raising an uncertainty on their ability to protect lipid from oxidation (Soendjaja & Girard, 2024). Polyphenol incorporation into food emulsions is well-known to be effective in protecting lipids by neutralizing free radicals and reactive oxygen species (Wu & Zhou, 2021). The effects of polyphenols according to their concentrations on structural modification and emulsifying ability of legume proteins have been well-documented. However, the impact of different types of polyphenols on the structure, antioxidant capacity, and emulsifying properties of legume protein across a range of pH remain underexplored (Dai et al., 2020; Hao et al., 2022). The pH should be taken into consideration as a critical factor because the structure and functionality of plant proteins are generally more sensitive to pH compared to animal proteins, which influence the binding capacity of polyphenols. Among legume proteins, pea protein, with lower allergenicity, higher digestibility, and more sustainable as a crop than soy protein, has emerged as a popular plant-based protein source, albeit challenges in their sensory properties including beany, bitter, and astringent flavor. By incorporating polyphenols to mask their flavor, the sensorial properties of pea protein may be improved. Here we investigated the interactions between pea protein and polyphenols extracted from Tasmanian pepper leaf and berry on their structure and antioxidant capacity, and their impact on the physical and oxidative stability of flaxseed oil-in-water emulsions. Specifically, two types of polyphenols compared here are distinctive because polyphenols in the Tasmanian pepper leaf, rich in phenolic acids and flavonols, contain compounds with relatively smaller molecular weights than the anthocyanin-rich polyphenols from Tasmanian pepper berry.

2. Materials and methods

2.1. Materials

Commercial pea protein isolate was kindly donated from Rouquette (S85F, 85 % protein, Rouquette, Geneva, IL, USA). Tasmanian pepper (*Tasmannia lanceolata*) leaf and berry were purchased from Wild Pepper Isle (Hobart, Australia). Flaxseed oil was purchased from local supermarket (Sydney, Australia). All samples were prepared using deionized Milli-Q water (Millipore Corporation, MA, USA). All chemicals used here were of analytical grade.

2.2. Polyphenol extraction from Tasmanian pepper leaf and pepper berry

Polyphenols were extracted from powdered formulations of Tasmanian pepper leaf and berry using an ethanol-Milli-Q water solution 70:30, filtrated with a Büchner-funnel and ethanol evaporation at ~63 °C and vacuum, using a rotary evaporator. The polyphenol concentration of Tasmanian pepper leaf and berry were 15.12 mg/mL and 6.20 mg/mL as quantified by the Folin-Ciocalteu method. The extracts were stored at −18 °C before use.

2.3. Conjugation between pea protein and polyphenols from Tasmanian pepper leaf and berry

A stock solution of pea protein isolate (2 %, *w/v*) was prepared in Milli-Q water with 0.02 % sodium azide, and the ultrasound treatment was applied to dissolve pea protein precipitates at 96 W intensity with on/off time of 5 s/5 s for 10 min. The preparation of protein-polyphenol complex was followed by a modified method of Dai et al. (2022). Complexation between pea protein and polyphenols was accomplished by adding polyphenols to pea protein dispersion to achieve protein: polyphenol ratio of 10:1 before adjusting the pH to 3, 5, 7, and 9. The solutions was stirred (500 rpm) in an oxygen-free environment for 2 h to allow polyphenols to bind with pea protein at 20 °C. The samples were dialyzed for 24 h with 8 changes of Milli-Q water at ambient temperature. The control group (pea protein only) was named PP3, PP5, PP7, PP9, and pea protein-polyphenol complex was named either PL3, PL5, PL7, PL9 (using Tasmanian pepper leaf) or PB3, PB5, PB7, PB9 (using Tasmanian pepper berry) depending on polyphenol source and pH.

2.4. Emulsion formation

Pre-emulsions consisting of 20 % (*v/v*) flaxseed oil and 80 % colloidal solutions prepared in section 2.3 were fabricated using high-shear mixer (IKA Ultra-Turrax T25, Staufen, Germany) at 7000 rpm for 5 min. After mixing, the emulsions were homogenized using high-pressure homogenizer Panda PLUS 2000 (GAE Niro Soavi, Parma, Italy) at 20 MPa for three passes.

2.5. Interaction dynamics of polyphenols with pea protein

2.5.1. Measurement of polyphenol binding equivalents

The polyphenol binding equivalents were estimated by measuring total phenolic content using the Folin–Ciocalteu reducing capacity method with slight modifications (Liu et al., 2021). In brief, 1 mL of the pea protein-polyphenol complex (10 times diluted) was mixed with 0.5 mL of 0.2 N Folin–Ciocalteu reagent for 5 min, and then 1 mL of 15 % sodium carbonate was added. Next, the samples were kept in a dark room for 60 min before measuring the absorbance at 760 nm by using an ultraviolet–visible spectrophotometer. The total phenolic content in each sample was calculated using a calibration curve of gallic acid, and the results are expressed as microgram polyphenol per gram of sample.

2.5.2. Total anthocyanin content

The total anthocyanin content was determined as described by Papillo et al. (2018). Pea protein-polyphenol complex samples were opportunely diluted 10 times with potassium chloride buffer (0.025 M), pH 1.0, where the absorbance of the sample at 520 nm was within the linear range of the spectrophotometer. The solution was kept for 5 min and the absorbance was measured at 520 nm and 700 nm. The total anthocyanin content was expressed as cyanidin-3-glucoside (CY-3-Glu)

equivalents and the concentration calculated through the *Eq. (1)*:

$$\text{Total anthocyanin content } (\mu g/mL) = \frac{(A_{520nm} - A_{700nm}) \times 449.2 \times DF \times 1000}{\varepsilon \times 1} \quad (1)$$

where: 449.2 = molecular weight of CY-3-Glu; 1000 = conversion factor from mg to μg; ε (molar extinction coefficient of CY-3-Glu) = 26,900 L mol^{-1} cm^{-1}.

2.5.3. Free amino group content

The free amino group content was determined through the *ortho*-phthaldialdehyde (OPA) method (Kim, Wang, Ma, et al., 2023). The OPA reagent was prepared by dissolving 80 mg of OPA in 2 mL of methanol and then adding 5 mL of 20 % (*w*/w) SDS, 50 mL of 0.1 mol/L borax, and 200 μL of β-mercaptoethanol. Distilled water was then added until the volume of the mixture reached 100 mL. One milliliter of the OPA reagent and 50 μL of the samples were mixed and the absorbance value A_{340} was measured at 340 nm. L-leucine was used as a standard to calculate the free amino group content (nmol/g).

2.5.4. Molecular docking

The interactions between pea protein and polyphenols were estimated using Autodock vina tools (version 1.5.7) (Huey et al., 2012) and PyRx (version 0.9) (Dallakyan & Olson, 2015). The simulations were carried out using protein fractions including vicilin (7U1I), convicilins (7U1J), and prolegumin (3KSC) as receptors, representing the major components in pea proteins. The ligands included the structures (.pdb format) of chlorogenic acid and quercetin representing polyphenols from Tasmanian pepper leaf and cyanidin-3-rutinoside and cyanidin-3-glucoside representing the polyphenols from Tasmanian pepper berry, which were downloaded from PubChem-NCBI repository. These structures were reconstructed for its missing atoms, solvated, pKa adjusted and assigned with atomic charges using APBS server and PARSE force field (Jurrus et al., 2018). Vina exhaustiveness was set to 64 for simulation and analyzed using BIOVIA Discovery Studio Visualizer (version 24.1.0.23298, Dassault Systèmes Biovia Corp., San Diego). The most stable conformations and interactions observed in these simulations at pH 7 have been illustrated.

2.6. Antioxidant capacity

The ferric reducing antioxidant power (FRAP) assay was using the protocol described previously (Naik et al., 2024). The free radical scavenging capacity of the samples was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) read at 517 nm using a UV/Visible spectrophotometer as described previously (Liu et al., 2021). For both method, Trolox was used as a standard.

2.7. Structural properties

2.7.1. Microfluidic modulation spectroscopy

Microfluidic modulation spectroscopy (MMS) was used to quantify protein secondary structure (Kim, Wang, Ma, et al., 2023). Protein samples were centrifuged at 10,000 rpm (equivalent to 12,298 ×*g*) for 10 min to remove large particles. The samples were analyzed using an AQS3pro (RedShiftBio, Boxborough, MA). Triplicates were taken for each sample at ambient temperature of 23 °C, at a modulation rate of 1 Hz, with a backing pressure of 5 psi; and all advanced parameters set as default. The Differential Absorbance Unit (Diff AU) was recorded at each wavenumber across the amide I band of the Infrared (IR) spectrum. The RedShiftBio delta control software was used for sample analysis and spectral processing. The higher order structural (HOS) elements were calculated using Gaussian curve fitting.

2.7.2. Surface hydrophobicity

The surface hydrophobicity was estimated following a modified method of Kim, Wang, Ye, et al. (2023). In brief, the samples were serially diluted from 0.05 to 0.2 mg/mL and twenty microliters of 8 mM 8-anilino-1-naphthalenesulfonic acid (ANS) solution were added to 1 mL of the protein solution. The excitation and emission wavelengths were set to 370 nm and 490 nm, respectively. A plot of fluorescence intensity versus protein concentration was drawn and the surface hydrophobicity index (dimensionless) was calculated as the slope of initial fluorescence intensity vs. protein concentration curve.

2.7.3. Particle size distribution and zeta potential measurements

The particle size and zeta potential of the protein complex dispersions were evaluated using a Malvern Zetasizer (Nano ZS, Malvern Instrument, UK) with a refractive index of 1.52. Samples were diluted 100 times with Milli-Q water.

2.7.4. Transmission electron microscopy

The microstructure of the samples was observed using transmission electron microscopy (TEM Tecnai G2, FEI). A 10 μL drop of a 25-fold diluted dispersion was applied to a carbon-coated electron microscope grid (300 mesh). After 5 min, any excess liquid was blotted away with a filter paper. The sample was then stained with 2 % uranyl acetate for 30 s. Excess stain was removed, and the grids were allowed to air dry for one hour before being examined under the TEM.

2.8. Emulsion properties and stability

2.8.1. Physical stability

Samples were stored in glass test tubes (16 × 150 mm), and the creaming index was measured during 7 days of storage. Then, the creaming index was calculated by the height of cream (top) layer divided by the height of the total emulsion. The droplet size distribution of the emulsions was analyzed by Mastersizer 3000 (Malvern Instruments, Malvern, UK) with 10–18 % obscuration range.

Confocal laser scanning microscopic (CLSM) images were obtained using a Zeiss LSM 800 confocal microscope (Jena, Germany). The protein in water phase and oil phase were colored by Nile blue A and Nile Red solutions, respectively. A drop of colored emulsions was located in a microscope slide glass and covered by a micro cover glass. Images were obtained using 10 × objective lens by exciting at 488 nm for Nile Red and 633 nm for Nile Blue A.

2.8.2. Oxidative stability

An oxidation initiator system was prepared to accelerate lipid oxidation by separately dissolving F_2SO_4 and EDTA (8×10^{-2} mol/L) in water. Equal volumes of both solutions were mixed, and iron-EDTA complexes could form in the dark under moderate stirring for 1 h. Emulsion aliquots (10 g) were put in 15-mL centrifuge tubes and the initiator solution (60 μL) was added.

The primary oxidation of flaxseed oil was quantified by lipid hydroperoxide. The emulsion samples (0.2 mL) and mixed solvent (1.0 mL) of isooctane: isopropanol (2:1, *v*/v) were centrifuged at 3500 rpm for 10 min. The supernatant (200 μL), methanol: n-butanol (30 μL, 3:1, v/v), and ammonium thiocyanate: ferrous solution (30 μL, 1:1, v/v) were added to 2 mL centrifuge tube in turn. The mixture solution was then

incubated in the dark for 20 min, after which the absorbance was measured at 510 nm. The peroxide value (POV) of the samples was calculated according to the *Eq. (2)*:

$$POV\left(\frac{meq}{kg}\right) = \frac{A \times K \times n \times 0.5 \times 1000}{55.86 \times m \times 2} \quad (2)$$

where A was the absorbance of the sample solution; K was the slope of the standard curve of Fe^{3+}; n was the volume fraction of the supernatant; m was the weight of the oil in the sample.

Thiobarbituric acid-reactive substances (TBARS) values were estimated by a modified method (Kim, Ryu, & Kim, 2020). An emulsion sample (0.1 mL) was added to 1 mL of a mixture of 15 % trichloroacetic acid/0.02 M 2-thiobarbituric acid and homogenized for 5 min at 5000 rpm. The suspension was heated at 95 °C for 15 min and chilled with water to develop a pink color. The samples were then centrifuged at 5000 ×*g* for 10 min and the absorbance of the supernatant was read at 532 nm using a UV/visible spectrophotometer. A calibration curve of 1, 1, 3, 3 - tetramethoxypropane was applied at varying concentrations ranging from 0 to 12 ppm. The TBARS values were displayed as mg malonaldehyde (MDA) equivalents per kg sample.

2.9. Statistical analysis

Significant differences in the means were tested by one-way analysis of variance (ANOVA) using IBM Statistics Software version 26 (SPSS INC, Chicago, IL, USA) at $p < 0.05$. Duncan's multiple range test was applied for comparison of the means. All of the experiments were performed in triplicate.

3. Results and discussion

3.1. Interactions between pea protein and polyphenols from Tasmanian pepper berry and pepper leaf

Total phenolic content, anthocyanin content, and free amino group content were evaluated to investigate the bindings of polyphenols with pea protein molecules (Fig. 1). Total phenolic content of the pea protein (PP) ranged from 4.19 μg/mL to 15.59 μg/mL, and incorporation of polyphenols from Tasmanian pepper leaf (L) and berry (B) significantly enhanced the total phenolic content of pea protein. The total phenolic contents of the pea protein-Tasmanian pepper leaf polyphenol complex (PL) (334.94–445.92 μg/mL) were significantly higher than the pea protein-Tasmanian pepper berry polyphenol complex (PB) (72.89–153.03 μg/mL) at all pH, indicating stronger binding capacity of pea protein with phenolic acids and flavonols in Tasmanian pepper leaf than anthocyanins in Tasmanian pepper berry. Polyphenols with lower molecular weights tend to bind strongly to proteins (Kim et al., 2024). Phenolic acids and flavonols such as chlorogenic acid, quercetin, and *p*-coumaric acid, which are more prominent in the PL, have relatively smaller molecular weights (354.30 g/mol, 302.24 g/mol, and 164.05 g/mol, respectively) than anthocyanins (595.50 g/mol for cyanidin 3-rutinoside and 484.83 g/mol for cyanidin 3-glucoside), which are more prominent in the pea protein-Tasmanian pepper berry polyphenol complex (PB) and may yield higher degree of complexation with pea protein (Konczak et al., 2010).

Total phenolic contents of the PB at pH 3 was significantly higher than the PB at other pH range, suggesting that anthocyanins in Tasmanian pepper berry were more stable in acidic conditions, leading to stronger binding affinity to proteins (Liu et al., 2018). The PB exhibited significantly higher anthocyanin content compared to those of the PP and PL at all pH (Fig. 1B), confirming that anthocyanins are a prominent polyphenol class from Tasmanian pepper berry (Konczak et al., 2010).

Free amino group content was measured to confirm whether the covalent linkage between protein and polyphenol was formed (Zhou et al., 2020). Free amino group content in the PP was decreased with increasing pH (Fig. 1C). The free amino group content in the PL and PB was pH dependent. At pH 3, the PL did not show significant difference in the free amino group to PP, while that of the PB was decreased. In contrast, the PL and PB showed a trend of the increase in free amino group at pH 7 and 9, showing hydrogen bonding between hydroxyl groups in polyphenols and amino groups of proteins (Liu et al., 2021). This indicates that the number and the position of hydroxyl groups

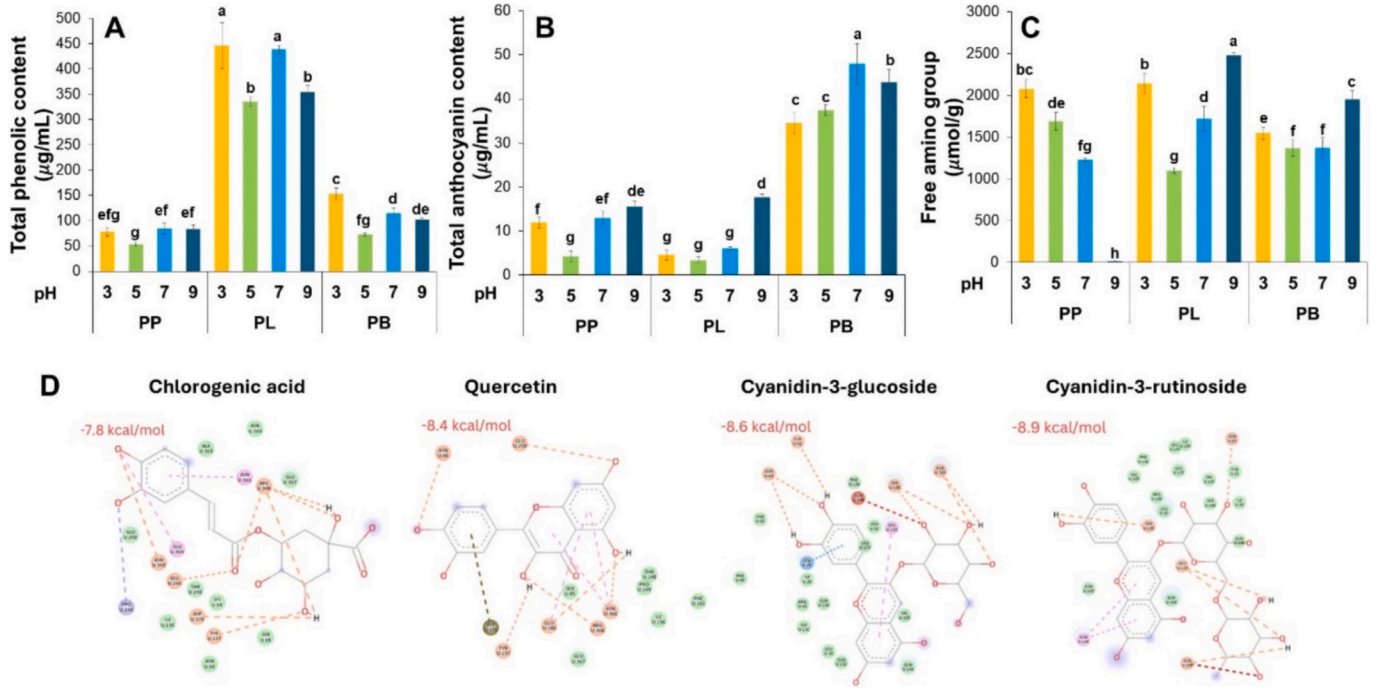

Fig. 1. Total phenolic content (A), total anthocyanins (B), free amino group (C), and molecular docking (D) of pea protein-polyphenol complex. The binding mode of vicilin, one of pea protein fractions, with major polyphenols from Tasmanian pepper leaf (quercetin and chlorogenic acid) and berry (cyanidin-3-rutinoside and cyanidin-3-glucoside) is shown in Fig. 1D. The bindings of other pea protein fractions (legumin and convicilin) with polyphenols at pH 7 are shown in Fig. S1.

influence on the reactivity of the polyphenols and them modification of protein structure by inducing non-covalent bonding.

The interactions between three distinct pea protein fragment receptors (vicilin, legumin, and convicilin) and four selected polyphenols (chlorogenic acid, quercetin, cyanidin 3-rutinoside, and cyanidin 3-glucoside) were investigated to understand how varying protonation states would influence the stability of these interactions (Fig. 1D & Fig. S1). The pea protein receptors and polyphenols were subjected to docking simulations under different pH conditions (3,7, and 9). Interactions at pH 7 demonstrated significantly greater stability compared to those at other pH levels, with the affinity in the range of 7.6–9.6 kcal/mol according to Autodock Vina. Polyphenols had stable interactions via hydrogen bonding and non-covalent links (hydrophobic and electrostatic interactions) with pea proteins, especially with vicilin due to high flexibility in its structure. Quercetin exhibited π-cation/anion interaction with vicilin and convicilin, which suggests better interactions between the amino acid side chains of a receptor and the presence of aromatic rings on ligand (Fig. 1D).

3.2. Structural properties

Structural properties of pea protein-polyphenol complex were investigated by secondary structure quantification by MMS (Table 1), zeta potential and surface hydrophobicity (Fig. 2). The absolute zeta potential value of the PL and PB at pH 3 was lower than the PP, suggesting lower colloidal stability upon polyphenol incorporation (Fig. 2A). On the other hand, the PL and PB showed significantly higher absolute value in zeta potential (−34.5 ± 1.3 mV and − 33.8 ± 1.0 mV, respectively) at pH 9 compared to the PP. The increase in absolute zeta potential values is attributed to the fact that polyphenols change the secondary structure of pea protein, resulting in the reduction of the exposure of the positive charge groups and the increase in the negative charge groups of the pea protein (Li, Wang, et al., 2020).

Table 1
Secondary structure of pea protein upon incorporation of polyphenol quantified by microfluidic modulation spectroscopy.

		Secondary structure content (%)			
		α-helix	β-sheet	β-turn	unordered
pH 3	PP	13.61 ± 3.40^{d}	46.40 ± 2.45^{cde}	21.14 ± 1.80^{bcd}	18.85 ± 2.97^{ab}
	PL	18.78 ± 0.87^{b}	51.24 ± 0.63^{ab}	11.27 ± 0.15^{f}	18.70 ± 0.89^{ab}
	PB	19.30 ± 1.37^{a}	40.48 ± 1.37^{fg}	19.51 ± 2.63^{cde}	20.71 ± 2.14^{a}
pH 5	PP	17.85 ± 2.74^{bc}	42.62 ± 0.88^{ef}	22.20 ± 2.39^{bcd}	17.99 ± 0.11^{ab}
	PL	15.93 ± 4.25^{bc}	48.51 ± 1.78^{abc}	19.34 ± 4.48^{cde}	16.22 ± 3.05^{ab}
	PB	14.50 ± 2.64^{bc}	47.90 ± 1.29^{bc}	23.12 ± 2.67^{bc}	14.49 ± 4.51^{b}
pH 7	PP	14.14 ± 2.66^{c}	43.72 ± 0.23^{def}	25.61 ± 2.84^{ab}	16.52 ± 1.20^{ab}
	PL	18.51 ± 1.92^{b}	38.64 ± 2.18^{g}	27.65 ± 2.33^{a}	15.20 ± 1.81^{b}
	PB	20.45 ± 1.70^{a}	40.15 ± 3.37^{fg}	22.87 ± 2.58^{bc}	16.53 ± 2.37^{ab}
pH 9	PP	17.29 ± 2.33^{bc}	52.18 ± 0.33^{a}	15.68 ± 0.31^{e}	14.84 ± 2.08^{b}
	PL	19.50 ± 2.35^{a}	47.92 ± 3.82^{bc}	18.36 ± 0.54^{cde}	14.22 ± 1.83^{b}
	PB	20.82 ± 2.19^{a}	46.81 ± 3.26^{cd}	17.88 ± 3.24^{de}	14.49 ± 2.25^{b}

Surface hydrophobicity significantly decreased in the PL and PB (1742.00 ± 207.59 and 806.67 ± 50.05) at pH 3 compared to PP (9892.00 ± 224.03), potentially due to protein aggregation caused by polyphenol addition (Fig. 2B). At pH 7, the PL showed a decrease in the surface hydrophobicity, while no significant difference between the PB and PP was found. This suggests protein aggregation was caused by adding phenolic acids and flavonols in the PL, but no effect on the protein hydrophobicity from anthocyanins in PB at pH 7. The decrease of surface hydrophobicity in the pea protein upon adding phenolic acids was previously reported due to a high hydroxyl content of phenolic acids, making pea protein-polyphenol complex more hydrophilic (Hao et al., 2022; Liu et al., 2021). Conversely, the surface hydrophobicity of the PL at pH 9 was significantly higher than that of the PP, suggesting unfolding of pea protein with phenolic acids addition at pH 9.

The secondary structure as quantified by MMS indicated that the PP consisted of a high amount of β-sheet (42.6–52.2 %) and a low amount of α-helix (13.6–17.9 %) (Table 1), that are commonly seen in pulse proteins (Hao et al., 2022). A shift from β-turn to β-sheet is observed in the PP at pH 3 and 9 from pH 7, suggesting higher water molecule hydration ability (Kim, Wang, Ma, et al., 2023). The secondary structure of the pea protein-polyphenol complex was dependent on the types of polyphenols and pH levels. In the PL at pH 3, an increase in α-helix and β-sheet and a decrease in β-turn were seen compared to the PP, while β-sheet was shifted to β-turn at pH 7 and 9. This implies that incorporation of phenolic acids and flavonols led to more ordered structure of pea protein at pH 3, but phenolic acids and flavonols at pH 7 and 9 make internal structure of pea protein looser and more disordered (Dai et al., 2022; Li, Wang, et al., 2020). The PB showed consistent results in all pH, showing an increase in α-helix and the decrease in β-sheet and β-turn. This trend toward an ordered structure of proteins with anthocyanin addition is found elsewhere due to hydrophobic interaction between hydrophobic areas of the proteins and anthocyanins (Li, Zhao, et al., 2020).

3.3. Morphology

The incorporation of polyphenols to pea protein solution influences their macroscopic properties (Fig. 3). The PP were stable at all pH except pH 5 showing multimodal distribution in the particle size. The D50 values significantly vary across the pH levels, from 12.53 ± 0.55 μm, 5.71 ± 1.50 μm, to 1.66 ± 0.16 μm at pH 3, 7, and 9, respectively (Fig. S2). Increasing pH from neutral to alkaline condition leads to the reduction of particle size due to acid-base reaction within amino acids increasing the contact area between water and protein (Akkam et al., 2021). Pea protein precipitated at pH 5, near the isoelectric point, and in this case polyphenol incorporation did not prevent pea protein from precipitation. Except at pH 5, the PP maintained excellent colloidal stability with polyphenol addition. The PL showed brown coloration influenced by chlorogenic acid and quercetin in Tasmanian pepper leaf with increasing pH. This appears to be the browning of phenolic compounds from Tasmanian pepper leaf in alkaline conditions that promote deprotonation of hydroxyl groups in polyphenols, making them more reactive and susceptible to oxidation, resulting in the formation of quinones while interacting with pea proteins (Cabral et al., 2022). Similarly, a pink color was developed in the PB at pH 3, while the color shifted to blue at high pH, indicating less stability and degradation of anthocyanins caused by structural breakdown in alkaline conditions. Anthocyanins, rich in Tasmanian pepper berry, are sensitive to the acidity and alkalinity due to the molecular structure transformations from flavylium cation in acidic condition to quinonoidal base near pH 7, and chalcone at pH 8–9 (Tang et al., 2019).

Significant changes in the particle size distribution were observed upon pea protein-polyphenol complex formation (Fig. 3B). An adverse effect of polyphenol addition was seen in the PL, causing an increase in particle size. On the other hand, the particle size was decreased in the PB at pH 7 and 9 despite the increase in particle size at pH 3. Transmission electron microscopic (TEM) images at pH 7 revealed that small and

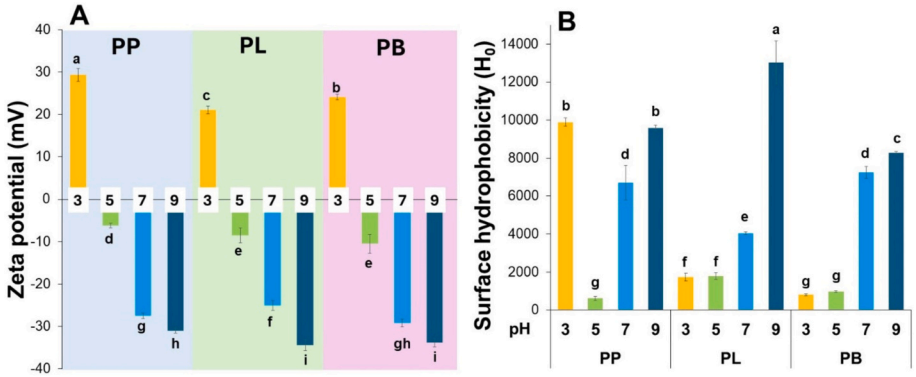

Fig. 2. Zeta potential (A) and surface hydrophobicity (B) of pea protein-polyphenol complex.

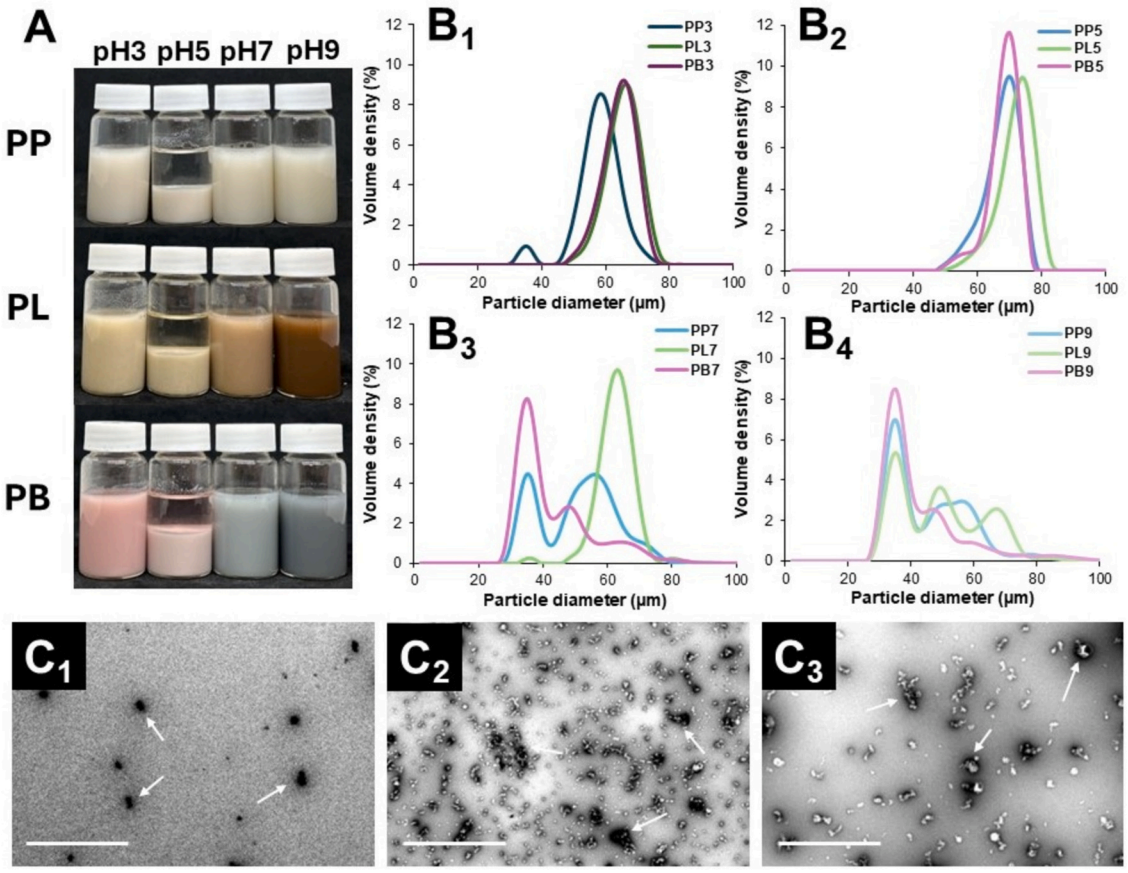

Fig. 3. Macroscopic image (A), particle size distribution (B), and transmission electron microscopic (C) image of pea protein-polyphenol complex. Particle size distribution of pea protein-polyphenol complex at pH 3 (B_1); at pH 5 (B_2); at pH 7 (B_3); at pH 9 (B_4). Transmission electron microscopy of pea protein (PP) at pH 7 (C_1); pea protein-polyphenol from Tasmanian pepper leaf complex (PL) at pH 7 (C_2); pea protein-polyphenol from Tasmanian pepper berry complex (PB) at pH 7 (C_3) with arrows pointing pea protein particles. The scale bars represent 500 nm.

spherical pea protein particles were aggregated with the inclusion of polyphenol (rich in phenolic acids and flavonols) from Tasmanian pepper leaf, while pea protein particles appeared to co-exist with thermodynamic compatibility and/or show weak interactions with anthocyanins from Tasmanian pepper berry (Fig. 1C). Diaz et al. (2020) also found that the incorporation of polyphenols from cranberry and blueberry juices did not alter particle size or protein aggregation of rice protein, suggesting weak interactions between proteins and anthocyanins. Polyphenols can act as ligands connecting proteins and protein-polyphenol complexes, and the presence of phenolic acids and flavonols, due to their structures compared to those of anthocyanins, may have saturated the binding sites of proteins, resulting in large aggregates (Zhang et al., 2025). In addition, Shpigelman et al. (2010) found that adding flavonol to heated protein solution causes turbid appearance of the solution due to the formation of large visible aggregates. This is consistent with the phenomenon observed here where the addition of phenolic compounds that are rich in flavonol to the thermally denatured commercial pea protein caused particle aggregation.

3.4. Antioxidant capacity

The antioxidant capacity of pea protein-polyphenol complex was

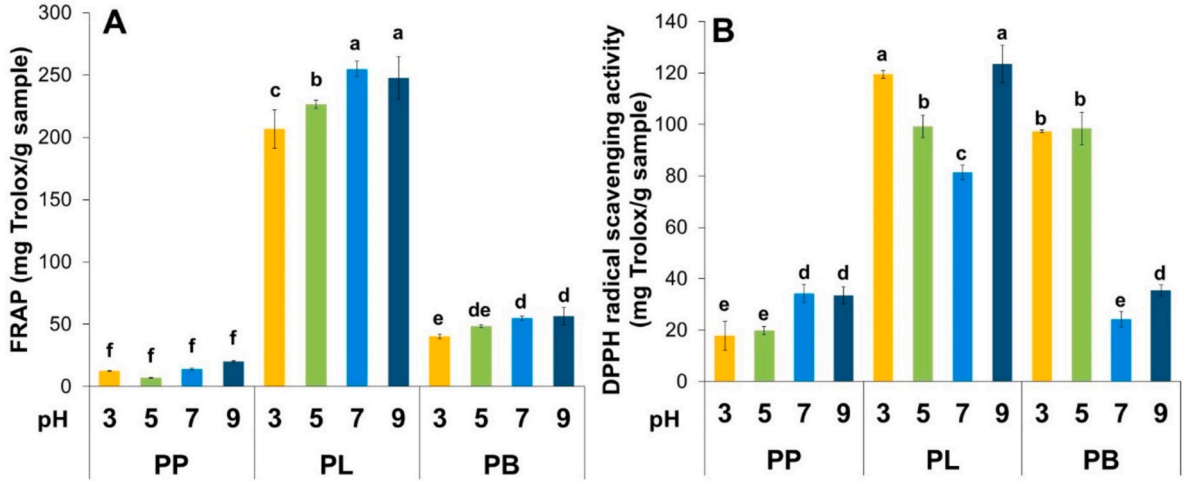

Fig. 4. FRAP (A) and DPPH radical scavenging activity (B) of pea protein-polyphenol complex.

evaluated by FRAP and DPPH assays (Fig. 4). Overall, the incorporation of polyphenols improved the FRAP and DPPH values compared to the PP. The PP showed 7.21–20.24 mg Trolox/g sample at all pH in the FRAP test. The FRAP values of the PL were 206.83–255.00 mg Trolox/g sample at all pH, significantly higher than the PB ranging from 40.55 to 55.14 mg Trolox/g sample. This trend correlates with the total phenolic content, where the total phenolic contents and FRAP values are 4–5 times higher in the PL than the PB (Fig. 1A). The DPPH results showed a similar tendency to the FRAP values, except the PB at pH 3 and 5 showing notably higher DPPH levels compared to FRAP results. The presence of hydroxyl groups in the polyphenols is known to be responsible for antioxidant capacity (de Araújo et al., 2021). In our case, the introduction of different polyphenols greatly contributed to antioxidant activity of pea protein-polyphenol complex with larger number of phenolic hydroxyl groups in the PL (Liu et al., 2021).

3.5. Physical stability of oil-in-water emulsions

Flaxseed oil-in-water emulsions using pea protein-polyphenol complexes were prepared at pH 3, 7, and 9. Samples at pH 5 were excluded due to the precipitation, resulting in immediate phase separation of the emulsions. Physical stability of the emulsions was evaluated by droplet size distribution, microstructure, and creaming index (Fig. 5 & Fig. S2). Macroscopic images of the emulsions are shown after a week of storage (left image of Fig. 5). At pH 3, the PP did not show phase separation, while the PL and PB had creaming, indicating that polyphenol incorporation adversely affected the physical stability of the emulsions. Moreover, the droplet size of the PL and PB was larger than the PP at pH 3, suggesting the polyphenol addition in acidic condition is not advantageous for droplet stability of the emulsions. Despite the physical stability of the PP at pH 3, oil droplets were not completely covered by proteins, showing a partially collapsed structure. Upon the polyphenol

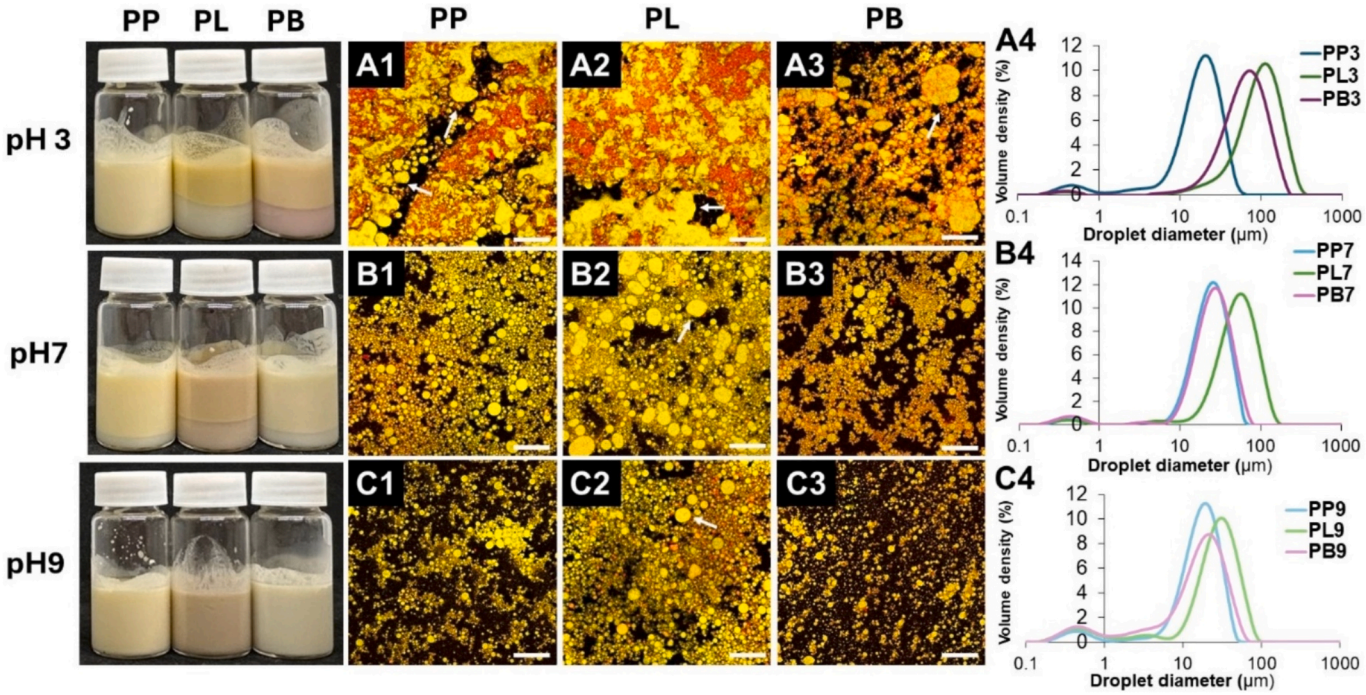

Fig. 5. Physical stability of flaxseed oil-in-water emulsions stabilized by pea protein-polyphenol complex. (*left*) Macroscopic image of the emulsions after 7-day storage, (*middle*) microstructure of the emulsions (red indicates protein and yellow indicates oil), (*right*) droplet size distribution of the emulsions. The scale bars represent 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

addition, the PL showed a completely collapsed structure, whereas the large droplets with coalescence and flocculation were observed in the PB. Polyphenol addition at pH 3 caused protein aggregation with increase in α-helix content indicating more ordered structure (Table 1 & Fig. 3), resulting in the decrease of surface charge and surface hydrophobicity (Fig. 2). This indicates that the repulsive forces between droplets are less effective with decreased surface charge and less hydrophobic surface of pea protein upon polyphenol addition hinders the adsorption to oil phase that negatively impact the emulsifying properties (Kim, Wang, & Selomulya, 2020; Li, Wang, et al., 2020.

At pH 7, creaming was observed in all samples, with the PP and PB showing creaming index of 15.97 ± 0.36 % and 19.62 ± 0.89 %, respectively, while the PL showed much higher creaming of 41.40 ± 4.23 % (Fig. S2). Similarly, the droplet size distributions of PP and PB were nearly identical, whereas the droplet size of PL was significantly larger at pH 7. Phenolic acids and flavonols from Tasmanian pepper leaf at pH 7 likely increased the particle size and reduced the surface hydrophobicity, consistent with TEM images showing more aggregated proteins (Fig. 2&3). In contrast, anthocyanins from Tasmanian pepper berry appeared to reduce the particle size without affecting the surface hydrophobicity and surface charge, leading to the similar emulsifying properties of the PP due to relatively weaker interactions between protein and polyphenol (Fig. 2&3). Upon polyphenol addition, although same trend of an increase in α-helix and a decrease in β-sheet was observed in both PL and PB at pH 7, the decrease of β-sheet, indicating amphiphilicity, was greater when adding polyphenols from Tasmanian pepper leaf (Kim, Wang, Vongsvivut, et al., 2023). Phenolic acids and flavonols in Tasmanian pepper leaf induced protein to more folded structure and less amphiphilic, resulting in large droplets and instability of emulsions due to difficulty in reducing interfacial tension at oil/water interface.

At pH 9, all emulsions were stable after a week of storage. The droplet size of all pH 9 emulsions was significantly reduced compared to those at pH 3 and 7, showing relatively stable droplets in the microstructural images (Fig. 6. C1—6C3). However, the PL exhibited significantly larger droplets than PP and PB, suggesting the negative impact from phenolic acids and flavonols regardless of pH.

3.6. Oxidative stability of oil-in-water emulsions

The oxidative stability of the emulsions was evaluated by measuring hydroperoxide value and TBARS value for 2 weeks of storage (Fig. 6). The same trend was shown in peroxide value and TBARS value of all emulsion samples. Interestingly, the oxidative stability of the emulsions did not correlate to its physical stability, rather it was correspondent to the total phenolic content of pea protein-polyphenol complex (Fig. 1). At pH 3, the PP showed the highest oxidation followed by those at pH 7 and 9. At pH 3 and 7, the PL and PB significantly enhanced the oxidative stability with the polyphenols from Tasmanian pepper leaf and berry, which demonstrated more effective protection with polyphenols from Tasmanian pepper leaf than those of Tasmanian pepper berry, aligning with the total phenolic content and antioxidant capacity (Fig. 1&4). At pH 3, the oxidative stability of the PP sharply decreased from D0 (0.11 ± 0.01 mmol/kg oil and 0.23 ± 0.04 mg MDA/kg) to D14 (0.70 ± 0.03 mmol/kg oil and 2.68 ± 0.16 mg MDA/kg), whereas the PL and PB showed significantly lower peroxide and TBARS values on D14 (0.42–0.45 mmol/kg oil and 0.56–0.76 mg MDA/kg, respectively), indicating substantial prevention of linolenic acid in flaxseed oil from oxidation by polyphenol addition. Similarly, at pH 7, the PP had peroxide and TBARS values of 0.38 ± 0.01 mmol/kg oil and 1.54 ± 0.04 mg MDA/kg on D14, while the PL and PB showed significantly lower values of 0.33–0.35 mmol/kg oil and 0.46–0.72 mg MDA/kg, respectively.

At pH 9, no significant difference was observed between samples in the beginning of the storage. However, the PL and PB unexpectedly showed higher peroxide and TBARS values (0.18–0.19 mmol/kg oil and 0.44–0.55 mg MDA/kg, respectively) compared to the PP (0.16 ± 0.05 mmol/kg oil and 0.27 ± 0.02 mg MDA/kg, respectively) on D7, considering that PL had a significant high total phenolic content and antioxidant capacity. This trend continued through D14. The PL was most effective in preventing oxidation at pH 3 followed by pH 7, and least effective at pH 9, corresponding to its total phenolic content (Fig. 1), suggesting that phenolic acids and flavonols in the PL are more effective in oxidation under acidic conditions. Similarly, the PB showed more effectiveness in preventing oxidation at pH 3 than at pH 7, due to the stability of anthocyanins in acidic conditions, correspondent to its higher total phenolic content and antioxidant capacity at pH 3 (Fig. 1&4). The inferior oxidative stability of the emulsions at pH 9 with polyphenol addition can be following reasons. The formation of stable droplets by protein is thought to be more important in the prevention of lipid oxidation rather than antioxidant capacity of protein-polyphenol complex. Moreover, phenolic compounds are generally unstable in

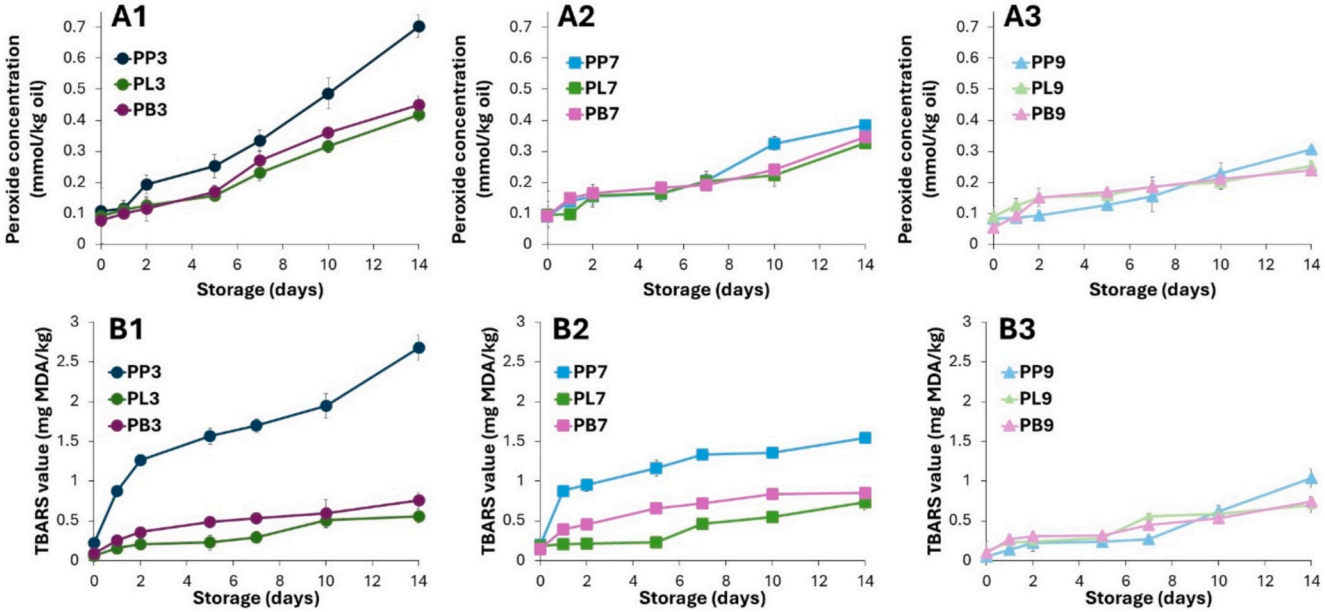

Fig. 6. Changes in the peroxide (A) and TBARS (B) values of flaxseed oil-in-water emulsions stabilized by pea protein-polyphenol complex for 14-day storage.

alkaline conditions, and oxidation of both polyphenols from Tasmanian pepper leaf and berry was observed to form quinones at pH 9 as appeared by discoloration (Fig. 3). Therefore the possibility of their degradation affecting the TBARS values in the PL and PB at pH 9 is seen (Pasquet et al., 2024). In addition, polyphenols can act as prooxidants depending on the location of the proteins in emulsions that are largely affected by pH (Tian et al., 2022). Interfacial concentration of pea protein at pH 9 is generally lower than pH 7, and therefore proteins located in the aqueous phase at pH 9 may have pulled the transition metals away from the droplet surfaces (Liang & Tang, 2013).

4. Conclusion

This study presents the structure and antioxidant capacity of pea protein according to the addition of two types of polyphenols from *Tasmannia lanceolata* and their impact on physical and oxidative stability of flaxseed oil-in-water emulsions. Overall, polyphenol incorporation enhanced the oxidative stability of pea protein-stabilized emulsions. However, we observed different effects of polyphenols according to their classification on the bindings and structure of pea protein. Polyphenols from Tasmanian pepper leaf, rich in phenolic acids and flavonols, exhibited stronger bindings with pea protein than the polyphenols from Tasmanian pepper berry, rich in anthocyanins. However, protein aggregation was observed at all pH levels upon addition of phenolic acids from Tasmanian pepper leaf. In contrast, anthocyanins from Tasmanian pepper berry induced protein aggregation under acidic conditions but co-existed at neutral and alkaline pH levels. Incorporation of phenolic acids and flavonols negatively impacted the physical stability of oil-in-water emulsions with larger droplets, while anthocyanins had little effect on their physical stability except at pH 3. Under acidic and neutral conditions, polyphenol addition contributed to retarding the oxidation of flaxseed oil, with phenolic acids and flavonols from Tasmanian pepper leaf showing greater effectiveness due to their strong bindings with pea protein. At pH 9, emulsions exhibited high physical and oxidative stability regardless of polyphenol addition. The results provide insights into non-covalent interactions between pea protein and different types of polyphenols in relation to antioxidant and emulsifying ability with potential use of Tasmanian pepper leaf and berry as new functional ingredients.

CRediT authorship contribution statement

Woojeong Kim: Writing – original draft, Methodology, Investigation, Conceptualization. **Muhammad Bin Zia:** Writing – original draft, Investigation. **Rishi Ravindra Naik:** Writing – original draft, Investigation. **Kacie K.H.Y. Ho:** Writing – review & editing, Validation, Resources. **Cordelia Selomulya:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing 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.

Printed version of figures in black-and-white. No color in print is required.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141589.

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