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Pectic Polysaccharides from Walnut Kernel: Structural Characterization and Their Neuroprotective Effects

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ABSTRACT: This study systematically investigates the structural characteristics and neuroprotective effects of pectic polysaccharides isolated from defatted walnut kernels (WKP). Three fractions—WKP-W, WKP-S1, and WKP-S2—were obtained via DEAE-52 chromatography. Structural analysis revealed that WKP-S1 (15% uronic acid, 72.13 kDa) is rich in rhamnogalacturonan-I (RG-I) domains with a backbone primarily composed of $\rightarrow 4$ - β -D-GalpA-(1 $\rightarrow$ and $\rightarrow 2,4$)- α -L-Rhap-(1 $\rightarrow$). In contrast, WKP-S2 (68% uronic acid, 45.61 kDa) exhibits a hybrid homogalacturonan-RG-I (HG-RG-I) structure with partially methyl- and O-acetyl-esterified GalA residues. Bioactivity evaluation in an A β 42-expressing *Drosophila* model demonstrated that all WKP fractions ameliorated Alzheimer's disease (AD)-related symptoms, including extended lifespan, improved locomotor function, reduced A β 42 aggregation, and enhanced antioxidant defenses. Notably, WKP-S1 exhibited the most potent neuroprotective effects among the tested fractions, underscoring the critical role of RG-I domains and side-chain architecture in mediating bioactivity. Transcriptomic analysis further revealed the upregulation of neuroprotective genes (Jon25Biii, Jon99Fi, Jon99Fii) associated with serine hydrolase activity and oxidative stress mitigation. These findings highlight the structure–activity relationship of walnut pectic polysaccharides and support the potential of RG-I-enriched fractions as multi-target therapeutic candidates for AD.

Keywords: Walnut kernel; Pectic polysaccharides; Structural characterization; Neuroprotective activity; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive cognitive decline, behavioral disturbances, and memory impairment. As one of the leading causes of senile dementia, AD imposes a substantial burden on aging populations worldwide ^[1, 2]. The pathogenesis of AD is intricately associated with the abnormal accumulation of amyloid-beta (A β) and tau proteins within the brain. Among the prevailing theories, the amyloid cascade hypothesis, as the most widely accepted one, proposes

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that the A β 42 peptide, due to its strong aggregation propensity, initiates a pathological cascade by forming soluble oligomers and insoluble fibrils, thereby contributing to synaptic dysfunction, neuronal injury, and neurodegeneration [3-5]. To date, the U.S. Food and Drug Administration (FDA) has approved seven pharmacological agents for the management of AD. However, these therapies predominantly target symptomatic relief and have limited efficacy in halting or reversing disease progression. Furthermore, the use of these medications is frequently accompanied by adverse effects, including headache and gastrointestinal discomfort, highlighting the urgent need for novel, safer therapeutic strategies [6].

Pectic polysaccharides have recently drawn increasing attention for their anti-Alzheimer's disease (AD) activities, which has been summarized in Table 1. For example, pectin derived from *Polygala tenuifolia* was shown to upregulate the expression of insulin-degrading enzyme (IDE) and neprilysin (NEP), two proteases critically involved in amyloid- β (A β) degradation, thereby mitigating A β 42 accumulation [4]. Similarly, pectin from *Lycium barbarum* was reported to promote A β 42 clearance through IDE upregulation [7]. Walnut kernels have long been recognized for their neuroprotective properties, including the attenuation of memory impairment and cognitive decline [8]. Several bioactive constituents, such as polypeptides [9], walnut oil [10], and phenolic compounds [11], have been reported as primary contributors to these beneficial effects. However, the neuroprotective potential of pectic polysaccharides from walnut kernels remains largely unexplored, warranting further investigation into their structural features and bioactivity.

Table 1 Summary of pectic polysaccharides with anti-AD activity.

Pectic polysaccharides	Source	Monosaccharide composition	Structure	Model	Target mechanism	Effect	Reference
RP02-1	<i>Polygala tenuifolia</i>	Ara, Rha, GalA, Gal (63.5:8.3:8.4:19.8)	Backbone: 1,2,4- α -Rhap and 1,4- α -GalpA Branches: T-, 1,5-, 1,3,5-linked Ara; T-, 1,3-, 1,4-, 1,6- and 1,3,6-linked β -Galp	HEK293-APPsw cells	A β 42 aggregation /production	Blocks A β 42 aggregation <i>in vitro</i> ; Reduces A β 42 levels by enhancing IDE and NEP expression	[4]
LBP1C-2	<i>Lycium barbarum</i> L. fruits	Ara, Gal, Rha, GalA (49.9:33.6:8.0:8.5)	Backbone: Alternating 1,2- α -Rhap and 1,4- α -GalpA Branches: T- β -Galp, 1,3-, 1,6-, 1,3,6-linked β -Galp; T-, 1,5-, 1,3,5-linked α -Araf and T-linked β -Rhap	CHO/APP BACE1 and HEK293-APPsw cells	A β 42 production	Reduces A β 42 production and promotes A β degradation by up-regulating the ex-pression of IDE	[7]
LFA03-a	<i>Lonicera japonica</i> Thunb	Rha, Ara, Gal, GalA (18.1:25.3:36.8:19.5)	Backbone: α -L-1,2-Rhap and α -D-1,4-GalpA Branches: β -D-1,3, 1,4, 1,3,6 linked Galp and α -L-1,5-Araf	PC12 cells	A β 42 aggregation	Inhibits A β 42 aggregation <i>in vitro</i> ; Promotes neuronal differentiation	[12]

FMP-6-S4	<i>Fructus Mori (mulberry)</i>	Rha, Gal, GalA, Ara, Glc (7.25:4.62:77.66:5.62:4.85)	Backbone: 1,4-linked α -D-GalpA and 1,2-linked α -L-Rhap Branches: hexenuronic acid (HexA)	HEK293-APPsw and CHO/APP BACE1 cells	A β 42 aggregation /production	Blocks aggregation and production; Modulate APP processing resulting in the A β 42 inhibition	A β 42 and APP [13]
Modified Citrus Pectin (MCP)	Citrus peels (lemons, limes, and oranges)	n.m.	n.m.	Scopolamine-induced AD model in male Wistar rats	A β 42 production	Reduces levels, improves cognitive performance, alleviates neuroinflammation	A β 42 [14]
WAP/SWAP	<i>Cucurbita moschata</i> /Sulfated WAP derivative	GalA (99.4 %)/Sulfated GalA	Linear backbone of α -(1 $\rightarrow$ 4)-GalpA residues/Sulfated modification of WAP	In vitro binding assay	Tau aggregation	Binds to tau K18, inhibits trans-cellular diffusion (similar to heparin)/Enhanced tau-binding activity, inhibits tau fibril formation	[15]

n.m.: not mentioned.

In the present study, a pectic polysaccharide (WKP) and its two sub-fractions (WKP-S1 and WKP-S2) were extracted from defatted walnut kernels and structurally characterized using high-performance size-exclusion chromatography (HPSEC), methylation analysis, and 1D and 2D NMR spectroscopy. The anti-AD potential of WKP was assessed in a transgenic *Drosophila melanogaster* model expressing human A β 42. This study aimed to elucidate the therapeutic capacity of WKP to alleviate neurotoxicity and cognitive dysfunction by modulating A β 42 accumulation, acetylcholinesterase (AChE) activity, and cyclic adenosine monophosphate (cAMP) levels, as well as by improving climbing ability and extending lifespan. These findings underscore the potential of WKP as a multi-target candidate for the prevention and treatment of AD.

2. Materials and methods

2.1. Materials and reagents

Walnut kernels (*Juglans regia* L.) without pellicle, produced in the Shangluo region, were provided by the Xi'an Juhefu Planting Professional Cooperative and defatted by HeBei LV LEI Agricultural and Forestry Technology Co. Ltd (Hebei, China). Cellulose DE-52, papain (800 U/mg), alkaline protease (200 U/mg), and Bradford protein assay kits were obtained from the Beijing Solarbio Science and Technology Co. Ltd. (Beijing, China). Deuterium oxide (D₂O) and sodium borodeuteride (NaBD₄) were obtained from Anhui Senrise Technology Co. Ltd (Anhui, China). β -Amyloid 42 (A β 42), acetylcholinesterase (AChE), and cyclic adenosine monophosphate (cAMP) enzyme-linked immunosorbent (ELISA) kits were provided by Jiangsu Meimian Industrial Co. Ltd (Jiangsu, China). All other reagents used were of analytical grade.

2.2. Preparation and structural characterization of WKP

2.2.1. Extraction and purification of WKP

The screw-pressed defatted walnut kernels were ground through a 100-mesh sieve and extracted with distilled water (20 mL/g) at 90 °C for 4 h. The mixture was filtered through a four-layer 200-mesh nylon cloth, and the insoluble residue was re-extracted under the same conditions. The combined extracts were concentrated to half their volume using a rotary vacuum evaporator at 50 °C. The concentrate was sequentially deproteinized with alkaline protease (pH 9.0, 4 U/g, 55 °C, 4 h) and papain (pH 7.0, 15 U/g, 60 °C, 4 h). After boiling for 10 min, the hydrolysate was centrifuged at 4000 ×g for 20 min. Crude polysaccharides (WKP) were obtained from the supernatant by ethanol precipitation (3-fold volume, 4 °C overnight), followed by centrifugation, dialysis (3500 Da cut-off), and freeze-drying. The yield of WKP was calculated using Eq. (1).

$$\text{Yield (\%)} = \frac{\text{Crude polysaccharide weight (g)}}{\text{Raw material weight (g)}} \times 100\% \quad (1)$$

The fractionation of WKP was conducted using a DEAE-52 cellulose column (2.6 × 30 cm) equipped with HL-1 constant-flow pump and an automatic fraction collector (BSZ-160, Shanghai Qingpu Huxi Instrument Factory Co., Ltd., China). The WKP solution (20 mL total volume) was loaded onto the column, and eluted sequentially with deionized water, followed by 0.1, 0.3 and 0.5 M NaCl solutions at a flow rate of 1 mL/min. Subsequently, the total carbohydrate content in the eluents from the even-numbered tubes was measured by the phenol-sulfuric acid method and the elution profile was plotted. The yield of purified polysaccharide fractions was calculated according to Eq. (2). The fractions eluted with 0.3 and 0.5 M NaCl were collected, desalted and lyophilized to obtain the acidic polysaccharides WKP-S1 and WKP-S2 which were used in the present study.

$$\text{Yield (\%)} = \frac{\text{Purified polysaccharide weight (mg)}}{\text{Crude polysaccharide weight (mg)}} \times 100\% \quad (2)$$

2.2.2. Chemical composition and molecular weight determination

Protein content was measured using the Bradford assay kit ^[16]. Total carbohydrate content and uronic acid contents were determined using the phenol-sulfuric acid ^[17] and m-hydroxybiphenyl reagent methods ^[18], with glucose and galacturonic acid used as standards, respectively. The total phenolic content was analyzed using the Folin-Ciocalteu method, with gallic acid as the standard ^[19].

The molecular weight distribution was analyzed using a high-performance liquid chromatograph (HPLC) equipped with a RID-20A refractive index detector (Shimadzu, Japan), as previously described ^[20]. The analysis was performed on a Waters Ultrahydrogel column (7.8 mm × 300 mm × 10 μm, Waters, USA). The sample injection volume was 20 μL, and the elution was performed with 0.1 mol/L NaNO₃ at a flow rate of 0.6 mL/min. The column oven temperature was maintained at 40 °C for a 25-minute run. Molecular weight calibration was performed using dextran standards (10, 40, 70, 500, and 2000 kDa). Data analysis was performed using Astra software (Wyatt Technologies, USA).

2.2.3. Monosaccharide composition and FT-IR spectroscopic analysis

The monosaccharide composition of WKP fractions was determined following a modified method by Shi et al. [21]. Samples (WKP-S1 and WKP-S2) underwent a two-step acid hydrolysis: first with 0.09 M trifluoroacetic acid (TFA) at 79 °C for 90 min, followed by 2 M TFA at 120 °C for 180 min. Hydrolysates were dried using a vacuum centrifugal concentrator (Beijing JM Technology Co., Ltd., China). The released monosaccharides were analyzed using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex ICS-5000 system (Dionex Corp., USA), equipped with a CarboPac PA20 guard column (3 × 30 mm) and analytical column (3 × 150 mm). Standard solutions of GalA, GlcA, Fuc, Rha, Ara, Gal, Glc, Xyl, and Man (0.25–10 µg/mL) were used for quantification. Data were acquired and processed using Chromeleon software (Dionex Corp., USA).

The Fourier-transform infrared (FT-IR) spectra were recorded using a Nicolet IS50 FT-IR spectrometer (Thermo Fisher Scientific, USA) to characterize the functional groups. Briefly, WKP-S1 and WKP-S2 were completely dried and mixed with KBr powder at a ratio of 1:150 (w/w). The above mixture was ground into powder using an agate mortar and then pressed into transparent sheet and the UV-Visible spectra were obtained in the region of 400–4000 cm⁻¹.

2.2.4. Methylation analysis

Uronic acid reduction was performed prior to methylation^[22], as follows. The acidic polysaccharide was dissolved in deionized water and reacted with carbodiimide at pH 4.75 (maintained with 0.1 M HCl) for 1 h. The pH was then adjusted to 7.0 using 2 M HCl, followed by gradual addition of NaBH₄ over 0.5 h. After completion of the reduction reaction at pH 4.0, the sample was dialyzed against distilled water (3.5 kDa Mw cut-off) and lyophilized. Residual NaBH₄ was neutralized through three to four treatments with an acetic acid/methanol mixture (1:9, v/v) under a nitrogen stream, followed by a final methanol wash and drying under nitrogen.

The reduced polysaccharide was subjected to methylation analysis to determine glycosidic linkage patterns. The dried product was dissolved in anhydrous DMSO, and methylated by treatment with NaOH powder (2 h, 25°C) followed by addition of methyl iodide (4 h reaction). The methylated polysaccharide was extracted with dichloromethane, dehydrated through an anhydrous Na₂SO₄ column, and concentrated under a gentle nitrogen stream. Complete hydrolysis was achieved using 4 M trifluoroacetic acid (100°C, 6 h in sealed tubes). The hydrolysate was dried under nitrogen, redissolved in water containing NH₄OH, and reduced with NaBD₄ (12 h reaction). Borate byproducts were removed by repeated washing with methanol containing 5% acetic acid. The product was acetylated with acetic anhydride (2 h), and the resulting partially methylated alditol acetates (PMAAs) were analyzed by GC-MS (Agilent 6890N) equipped with an SP-2330 capillary column (30 m × 0.25 mm, 0.22 µm film thickness) using a temperature program from 160°C to 210°C at 2°C/min, then to 240°C at 5°C/min, with ion trap detection. The branching degree (DB %) of the polysaccharide was calculated using Eq. (3), as previously reported^[23].

$$DB (\%) = \frac{\text{Number of dendritic units} + \text{Number of terminal units}}{\text{Number of total units}} \times 100\% \quad (3)$$

2.2.5. NMR analysis

The 1D (^1H and ^{13}C) and 2D (COSY, HSQC, HMBC and NOESY) NMR spectra were acquired using a Bruker AVANCE NEO 600 MHz NMR spectrometer (Bruker Biospin Corporation, Canada). 30 mg sample was dissolved in deuterium oxide (D_2O , 1 mL, 99.9%) and subjected to three freeze-thaw cycles to ensure complete dissolution and equilibration. The solutions were then transferred to 5 mm NMR tubes for analysis [24]. All NMR spectra were recorded using the residual solvent signal as the internal standard (D_2O , $\delta=4.79$). The NMR data were processed by MestReNova 14.0.0 software (MestreLab Research, Santiago de Compostela, Spain).

2.3. *Drosophila* model establishment and functional assessments

2.3.1. *Drosophila* strains and culture

The *W1118* and UAS- $\text{A}\beta 42$ CyO strains were obtained from the Fungene *Drosophila* Stock Center (Qidong Fungene Biotechnology, China). Flies were reared on a basal medium (BM) containing (w/w) 3.1% sucrose, 6.3% glucose, 1.06% agar, 3.2% dry yeast, 7.7% polenta, 0.07% anhydrous calcium chloride, 0.2% potassium sorbate, and 0.15% methylparaben. The medium was replaced every three days. Flies were maintained at 25 ± 1 °C and $55 \pm 5\%$, with relative humidity under a 12 h light/dark cycle in an incubator (Ningbo Yanghui Instrument Co., Ltd, China). Global statistics show that women are more likely to suffer from Alzheimer's disease than men, researches also indicates that female fruit flies with the $\text{A}\beta 42$ gene exhibit more obvious pathological features of Alzheimer's disease [25]. Thus, female flies were selected for the subsequent experiments.

2.3.2. Experimental design

In this study, newly emerged female *Drosophila melanogaster* were collected within 24 hours of eclosion and randomly assigned to six experimental groups: a wild-type control group (Control), a transgenic $\text{A}\beta 42$ -expressing model group ($\text{A}\beta 42$), and four treatment groups fed with crude WKP ($\text{A}\beta 42$ +WKP), the WKP-W fraction ($\text{A}\beta 42$ +WKP-W), the WKP-S1 fraction ($\text{A}\beta 42$ +WKP-S1), or the WKP-S2 fraction ($\text{A}\beta 42$ +WKP-S2). Based on preliminary dose–response experiments, the optimal concentration of WKP was determined to be 1.25 mg/mL. Therefore, in all behavioral assays, biochemical analyses, and transcriptomic sequencing experiments, WKP and its fractions were administered at this final concentration by dissolving the polysaccharide in the basal culture medium. The control and $\text{A}\beta 42$ model groups received only basal culture medium without WKP supplementation.

2.3.3. Negative geotaxis climbing assay

The protocol was conducted with slight modifications as described by Ly et al. [26]. Briefly, at day 7,14 and 21, adult female flies were placed into a cylindrical tube (20 cm $\times$ 2 cm) and were gently tapped to the bottom of the climbing vials. Climbing ability was assessed by the proportion of flies that climbed above 8 cm within 12 s. Each group consisted of 80 adult flies (10 flies per tube), and climbing performance was calculated as the average percentage of flies that crossed the 8 cm marker line.

2.3.4. Survival Assay

Female flies were transferred to fresh food every three days, and mortality was recorded until all individuals had died [27]. Survival data for each group were analyzed and visualized using the log-rank test (Kaplan-Meier method), with $P < 0.05$ considered statistically significant.

2.3.5. ELISA Assays

After nitrogen blowing and freezing, the heads of the flies ($n = 30$) were resuspended in PBS ($1 \times$, pH 7.4) on ice and homogenized using a homogenizer. The samples were then centrifuged at $10,625 \times g$ for 10 min at 4°C . The supernatants were collected and used to measure the levels of A β 42 (MM-1669H1), AchE (MM-34423O1), and cAMP (MM-0876O1) in *Drosophila* brain tissue according to the protocols provided in the respective assay kits (Jiangsu Meimian Industrial Co., Ltd). The activities of superoxide dismutase (SOD) (A001-1-2), catalase (CAT) (A007-1-1) and malondialdehyde (MDA) (A003-1-2) levels were quantified in fruit fly brain homogenates using standardized SOD, CAT, and MDA assay kits, respectively, following the manufacturer's guidelines (Jian-cheng Bioengineering Institute, Nanjing, China).

2.3.6. Quantitative real-time PCR (qRT-PCR) assay

Total RNA was extracted from fly heads using a mortar pre-cooled with liquid nitrogen. Briefly, 150 heads per group were homogenized in 500 μL lysis buffer, incubated for 5 min at room temperature, and mixed with 200 μL chloroform. After centrifugation ($8000 \times g$, 10 min, $2-8^\circ\text{C}$), the aqueous phase was collected and processed with an adsorption column, including washes with ethanol and buffer ($600 \mu\text{L} \times 2$). RNA was eluted in 50 μL nuclease-free water. cDNA was synthesized using a commercial reverse transcription kit (following the manufacturer's protocol) and stored at -80°C . qPCR was performed in triplicate with gene-specific primers (Table S2), using *Gapdh1* as the internal control, and gene expression was calculated by the $2^{-\Delta\Delta\text{Ct}}$ method.

2.3.7. Total RNA extraction, library construction, and RNA sequencing

Transcriptome sequencing was performed by Uniteomics Biotechnology Co., Ltd. After behavioral tests, heads from 600 *Drosophila melanogaster* were collected. Samples were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen) and purified with the RNeasy Mini Kit (Qiagen). mRNA was enriched via poly(A) selection, fragmented (~ 300 bp), and reverse-transcribed into cDNA. Libraries were prepared using the TruSeq™ RNA Sample Prep Kit (Illumina), followed by PCR amplification and purification. Libraries were quantified (Qubit), size-verified (Bioanalyzer), and sequenced on an Illumina HiSeq platform (2×150 bp). Raw reads were filtered to remove adapters, low-quality sequences ($Q < 20$), and reads containing N bases. Clean data were assessed for Q20, Q30, and GC content.

2.4. Statistical analysis

Data were expressed as the mean $\pm$ standard deviation (SD) of triplicate experiments. Survival curves were analyzed using the Kaplan-Meier method, with statistical significance assessed by log-rank tests. Other

experimental data were evaluated by one-way ANOVA, followed by multiple comparisons for intergroup differences. A $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Structural characterization of WKP

3.1.1. Physicochemical properties and molecular weight

Walnut kernel polysaccharides (WKP) were extracted from defatted walnut kernels using hot water extraction, with an extraction rate of $0.58 \pm 0.31\%$. Subsequent purification of the crude extract was performed using DEAE-52 anion-exchange chromatography. Two distinct fractions were obtained at elution concentrations of 0.3 M NaCl (WKP-S1) and 0.5 M NaCl (WKP-S2), with corresponding yields of 15.26% and 5.68%, respectively.

The molecular weight (Mw) distributions of the WKP fractions were analyzed by HPSEC (Fig. 1A). The crude WKP displayed two distinct chromatographic peaks (Fig. 1B), indicating a heterogeneous polysaccharide composition with a broad Mw distribution ranging from approximately 3 to 500 kDa. In contrast, both WKP-S1 and WKP-S2 exhibited sharp, symmetrical peaks, consistent with narrow molecular weight distributions. The Mw values of WKP-S1 and WKP-S2 were determined to be 72.13 kDa and 45.61 kDa, respectively.

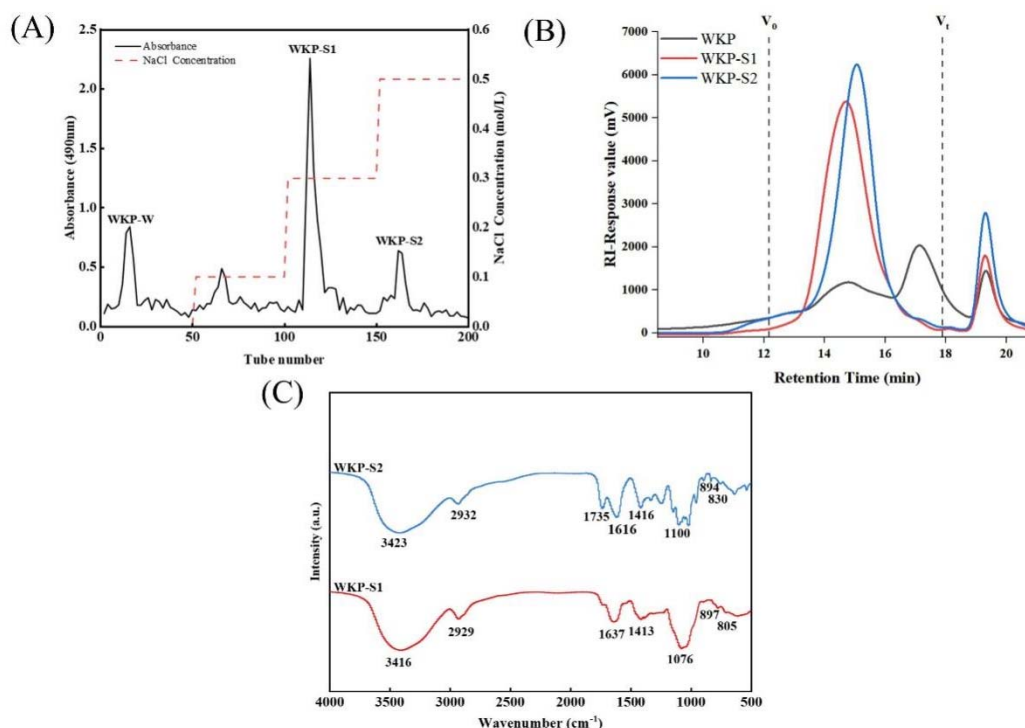


Fig. 1 Elution profile of WKP from Walnut kernel on DEAE-52 (A); High performance liquid chromatograms of WKP and its fractions WKP-S1-S2 (B); FT-IR spectra of WKP-S1 and WKP-S2 (C).

The composition of WKPs were analyzed (3). The total carbohydrate content of WKP-S1 was much higher than WKP and WKP-S2, whereas WKP-S2 demonstrated the highest uronic acid content among three fractions. The protein and total phenolic contents of WKP-S1 and WKP-S2 were both reduced after column chromatography separation and purification.

Table 2 Chemical composition of polysaccharides from walnut kernels.

Polysaccharides	Total carbohydrate content (wt%)	Uronic Acid content (wt%)	Protein content (wt%)	Total polyphenol content (wt%)
WKP	34.51±0.87	17.47±0.31	4.46±0.40	1.06±0.48
WKP-S1	62.39±0.38	15.29±0.84	0.93±0.10	0.13±0.53
WKP-S2	37.24±2.80	68.13±0.52	2.55±0.61	0.39±0.67

3.1.2. Monosaccharide composition and FT-IR analysis

Monosaccharide composition analysis revealed that both WKP-S1 and WKP-S2 were primarily composed of rhamnose, arabinose, galactose, glucose, mannose, galacturonic acid and glucuronic acid, with the molar ratios for WKP-S1 were 8.71:30.59:45.27:0.90:0.39:2.13:12.01, while those for WKP-S2 were 11.84:25.52:15.03:7.20:2.95:33.44:4.02. Notably, WKP-S1 exhibited a higher content of galactose (45.27 mol%) and arabinose (30.59 mol%) than WKP-S2, whereas WKP-S2 contained a significantly higher proportion of galacturonic acid (33.44 mol%).

As shown in Table S1, the results of monosaccharide analysis indicated that both WKP-S1 and WKP-S2 were typical pectic polysaccharides. WKP-S1 contained a higher proportion of RG-I regions (93.28 %) and no detectable homogalacturonan (HG), indicating that it was likely to have a highly branched structure with galactose and arabinose as main backbone. In contrast, a higher uronic acid content suggested that GalA might be the main backbone of WKP-S2, with a high proportion of arabinose (25.52 %), galactose (15.03 %), and rhamnose (11.84 %). Moreover, small amounts of glucose (7.20 %), mannose (2.95 %), and glucuronic acid (4.02 %) were detected in WKP-S2. In the RG-I domain, Rha is integrated into the backbone, alternating with GalA residues, whereas Gal and Ara predominantly form side chains, including arabinans, galactans, and/or arabinogalactans. GalA serves as the primary structural component of both HG and RG regions. WKP-S2 had a higher proportion of HG regions (21.60 %) and a lower proportion of RG-I (64.23 %). The contribution of the RG-I domain in pectin was further supported by the molar ratio of Rha/GalA (4.09 for WKP-S1 and 0.35 for WKP-S2). Additionally, the (Gal + Ara)/Rha ratio was significantly higher in WKP-S1 (8.71) than that in WKP-S2 (3.42), indicating a higher degree of branching within the “hairy” regions of WKP-S1.

FT-IR spectra (Fig. 1C) of WKP-S1 and WKP-S2 showed distinct peaks. Compared with WKP-S1, the distinct peak at 1735 cm⁻¹ and 1637 cm⁻¹ in the WKP-S2 fraction was associated with ester carbonyl (C=O) stretching and asymmetric stretching vibrations of uronic acid units. The degree of esterification (DE) was determined by integrating the absorbance areas of the ester carbonyl band at 1735 cm⁻¹ and the carboxylate band at 1616 cm⁻¹, following the method described by Zhang et al [28]. The calculated DE value of 28.56% for WKP-S2 indicates a moderate level of methyl esterification. In contrast, the absence of an absorption peak at 1735 cm⁻¹ for WKP-S1 suggests negligible esterification of galacturonic acid residues in this fraction.

3.1.3. Glycosidic linkages analysis

The glycosidic linkages in WKP-S1 and WKP-S2 were analyzed and summarized in Table 3, revealing 17 and 22 derivatives, respectively, indicating both WKP-S1 and WKP-S2 were branched polysaccharides, with WKP-S2 displaying a higher degree of structural complexity. Notably, both fractions shared over 10

types of glycosidic bonds, although their proportions varied. For Rha residues, the glycosidic linkages included T-Rhap, 3-Rhap, and 2, 4-Rhap in both fractions. The Ara residues predominantly appeared as T-Araf, 5-Araf, 3,5-Araf, 4-Arap, and 3-Araf, indicating the presence of both single and double-branched Ara molecules. Glc residues were mainly found as T-Glcp, 4-Glcp, 3,4-Glcp, with occasional occurrences of 4,6-Glcp, and 2,4-Glcp. Gal residues were linked as T-Galp, 4-Galp, 6-Galp, 4,6-Galp, and 3,6-Galp, as well as minor amounts of 3,4,6-Galp and 2,3,6-Galp. The presence of 4-GalpA was confirmed by the detection of the characteristic m/z 235 peak in the mass spectral fragment of 4-Galp. Since methylation analysis was performed after carboxyl reduction, the detected 1,4-linked Galp residues represent both reduced GalpA from HG/RG-I domains and neutral β -1,4-galactan side chains. The total ion chromatograms (TIC) of the partially methylated alditol acetates (PMAAs) derived from WKP-S1 and WKP-S2 are provided in Fig. S2, confirming the effective separation and reliable identification of the methylated sugar derivatives. The presence of neutral galactan was independently confirmed by NMR spectroscopy.

It should be noted that the molar ratios of GalA-derived glycosidic linkages in the methylation analysis (Table 3) were lower than the GalA content determined by monosaccharide composition (Table S1). This discrepancy is commonly encountered in methylation analysis of uronic acid-rich pectic polysaccharides and may be attributed to incomplete carboxyl reduction, partial methylation, or differential detector responses. Therefore, the structural assignment of WKP-S2 was primarily based on integrated evidence from monosaccharide composition, FT-IR, and 2D NMR spectroscopy, which collectively support the presence of highly esterified homogalacturonan domains. The structural differences between the two purified polysaccharide fractions were likely due to variations in glycosidic bonds and molar ratios, as well as differences in pectin structural domains and the degree of side-chain branching. The calculated degree of branching (DB) values were 0.46 for WKP-S1 and 0.64 for WKP-S2, indicating a high degree of sugar chain branching in both fractions.

Table 3 Methylation analysis of WKP-S1 and WPK-S2.

	Methylated Sugars	Glycosidic linkage	Molar ratios (%)	
			WKP-S1	WKP-S2
Rhamnose	2,3,4-Me ₃ -Rhap	T-Rhap	3.28	7.96
	2,4-Me ₂ -Rhap	3-Rhap	1.89	1.64
	3-Me-Rhap	2,4-Rhap	4.80	3.25
Total			9.97	12.85
Arabinose	2,3,5-Me ₃ -Araf	T-Araf	6.66	13.34
	2,5-Me ₂ -Araf	3-Araf	1.68	3.88
	2,3-Me ₂ -Araf	5-Araf	13.23	9.64
	2,3-Me ₂ -Arap	4-Arap	2.99	3.69
	2-Me-Araf	3,5-Araf	6.23	2.13
Total			30.79	32.68
Glucose	2,3,4,6-Me ₄ -Glcp	T-Glcp	0.99	2.89
	2,3,6-Me ₃ -Glcp	4-GlcpA/4-Glcp	1.91	4.25
	2,6-Me ₂ -Glcp	3,4-Glcp	6.16	-
	3,6-Me ₂ -Glcp	2,4-Glcp	-	1.51
	2,3-Me ₂ -Glcp	4,6-Glcp	3.44	0.83
Total			12.50	9.48

Galactose	2,3,4,6-Me ₄ -Galp	T-GalpA/T-Galp	10.88	7.31
	2,3,6-Me ₃ -Galp	4-GalpA/4-Galp	30.99	7.71
	2,3,4-Me ₃ -Galp	6-Galp	-	6.71
	2,6-Me ₂ -Galp	3,4-Galp	-	4.30
	4,6-Me ₂ -Galp	2,3-Galp	-	1.28
	3,6-Me ₂ -Galp	2,4-Galp	2.62	-
	2,3-Me ₂ -Galp	4,6-Galp	-	4.57
	2,4-Me ₂ -Galp	3,6-Galp	-	5.05
	2-Me-Galp	3,4,6-Galp	0.96	3.74
	4-Me-Galp	2,3,6-Galp	-	1.27
	Total		45.45	41.94
Mannose	2,4,6-Me ₃ -Manp	3-Manp	1.29	3.04
Total			1.29	3.04

–: Not detected.

3.1.4. NMR analysis

The chemical shifts of sugar residues observed in the 1D and 2D NMR spectra for WKP-S1 and WKP-S2 were assigned and summarized in Table 4. The ¹H and ¹³C NMR signals in range of δ 4.3–5.5 and δ 92–110 corresponded to anomeric protons and anomeric carbons, respectively, revealing the presence of α - and β -configurations in WKPs (Fig. 2). The weak signal observed at approximately δ 175 in the downfield region of the spectrum indicated the presence of uronic acid residues in the WKP-S2 fraction. This observation was corroborated by both monosaccharide composition analysis and methylation profiling. Furthermore, the result of uronic acid content assay provided additional evidence supporting that WKP-S2 is an acidic polysaccharide.

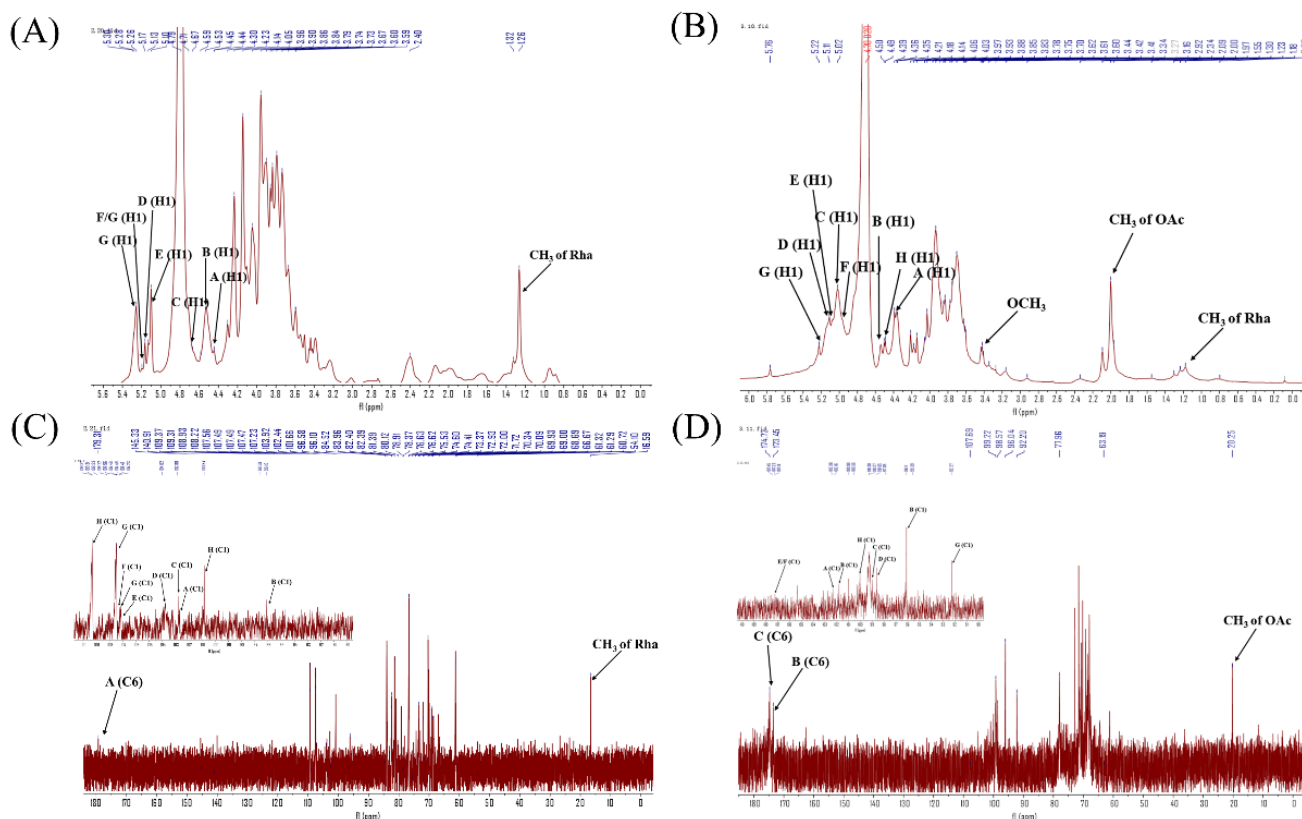


Fig. 2 ¹H-NMR spectra of WKP-S1 (A) and WKP-S2 (B); ¹³C-NMR spectra of WKP-S1 (C) and WKP-S2 (D).

Table 4 Chemical shift assignments of glycosyl residues from WKP-S1 and WKP-S2

Polysaccharid	Label	Glycosyl residues	H1	H2	H3	H4	H5	H6	H6b/	OAc
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es			C1	C2	C3	C4	C5	C6	COO Me
WKP-S1	A	→3,4)-β-D-Glcp-(1→	4.67	3.87	4.05	3.52	3.30	3.77	
		→	100.60	74.98	80.67	79.28	73.07	62.60	
	B	→4)-β-D-GalpA	4.50	3.39	3.69	4.31	4.65		
			96.08	72.73	74.41	78.37	70.34	179.31	
	C	β-D-Galp-(1→	4.46	3.58	3.78	3.96	3.86	3.64	
			102.85	73.42	74.26	70.34	73.37	62.60	
	D	→4)-β-D-Galp-(1→	4.64	3.70	3.77	4.13	3.60	3.64	
			103.49	74.34	75.06	79.02	76.52	64.12	
	E	→5)-α-L-Araf-(1→	5.09	4.07	3.93	4.15	3.80		
			107.20	82.28	76.60	83.87	68.02		
	F	→3,5)-α-L-Araf-(1→	5.03	4.15	4.06	4.22	3.95		
		→	107.52	81.26	83.85	82.15	66.73		
	G	α-L-Araf-(1→	5.01	4.06	3.92	4.04	3.56		
WKP-S2			107.20	81.32	76.65	82.42	61.63		
	H	→2,4)-α-L-Rhap-(1→	5.20	3.99	3.92	3.73	3.96	1.18	
		→	100.76	76.47	73.32	81.60	69.36	16.50	
	A	→6)-β-D-Galp-(1→	4.46	3.52	3.71	3.97	3.59	3.85	
			102.60	71.60	72.87	77.25	75.82	69.15	
	B	→4)-β-D-GalpA	4.54	3.44	3.74	4.30	4.63		
			96.09	69.69	73.08	79.13	71.70	173.45	
	C	→4)-α-D-GalpA-(1→	5.02	3.88	3.93	4.21	4.84		2.01
		→	99.22	68.06	68.79	78.15	71.24	174.09	20.25
	D	→4)-α-D-GalpA-(1→	5.11	3.74	3.93	4.45	4.71		
		→	98.57	68.25	69.14	79.12	71.12	174.51	3.74
	E	α-L-Araf-(1→	5.02	3.93	4.15	4.23	3.71		53.01
			107.02	81.18	83.97	82.02	68.43		
	F	→5)-α-L-Araf-(1→	5.08	4.03	4.12	4.23	3.69		
			107.02	81.25	84.28	83.12	68.10		
	G	→4)-α-D-GalpA	5.23	3.75	4.01	4.21	4.73		
			92.06	68.99	75.39	79.10	71.03		
	H	β-D-Galp-(1→	4.51	3.25	3.42	3.66	3.75	3.73	
			104.76	73.50	75.95	75.80	76.44	63.19	

In the HSQC spectrum (Fig. 3B and D), 8 cross-peaks were observed and were designated as residues A-H in the anomeric region in WKP-S1 and WKP-S2, respectively. Based on existing literature and methylation analysis, the signals at δ 5.09/5.08 and δ 107.20/107.02 were assigned to 5- α -L-Araf in WKP-S1 and WKP-S2, respectively. And the signals at δ 5.01/107.20 and 5.02/107.02 were attributed to α -L-Araf-(1→ in WKP-S1 and WKP-S2, respectively. While those at δ 5.03/107.52 corresponded to 3,5- α -L-Araf only found in WKP-S1. Signals corresponding to H1/C1 and H6/C6 from 2,4- α -L-Rhap were detected at δ 5.20/100.76 and δ 1.18/16.50 for WKP-S1, while the anomeric signal of α -L-Rhap-(1→ in WKP-S2 is coincident with the residual solvent peak and may be obscured in the NMR spectrum. The anomeric proton and carbon signals at δ 4.50/96.08 and δ 4.54/96.09 were assigned to →4-β-GalpA in WKP-S1 and WKP-S2, respectively, according to Shakhmatov et al [29]. In contrast, signals at δ 4.46/102.85 and δ 4.46/102.60 were assigned to β-D-Galp-(1→ and →6)-β-D-Galp-(1→ in WKP-S1 and WKP-S2, respectively. Notably, signals corresponding to →3,4)-β-D-GlcpA-(1→ at δ 4.67/100.60 was exclusively identified in WKP-S1. For WKP-S2, the presence of methyl-esterified GalpA was confirmed by the CH₃O group signal at δ 3.74/53.01, demonstrating the existence of →4)-α-D-GalpA-(1→ and was consistent with previous findings [30]. The cross-peak observed at δ 2.01/20.25 and the carbon signals at δ 174.09 confirmed the presence of *O*-acetyl groups in WKP-S2 fraction [31, 32]. In the HSQC spectrum of WKP-S1, a distinct anomeric cross-peak was

observed at δ 5.09/107.20. Analysis of the COSY spectrum revealed a series of correlated signals at δ 5.09/4.07, 4.07/3.93, 3.93/4.15, and 4.15/3.80, which were assigned to H1-H5 of the $\rightarrow 5$ - α -L-Araf-(1 $\rightarrow$ residue. In contrast, the corresponding signals in WKP-S2 were weak or not clearly distinguishable, suggesting a relative lower abundance of this structural unit. Similarly, the resonance cross-peak at δ 5.02/99.22 in the HSQC spectrum of WKP-S2, together with the sequential correlations at δ 5.02/3.88, 3.88/3.93, 3.93/4.21, and 4.21/4.84 observed in the COSY spectrum, were assigned to H1-H5 of residue C. These correlations collectively confirmed that residue C corresponds to a $\rightarrow 4$ - α -D-GalpA-(1 $\rightarrow$ unit. The remaining glycosidic proton signals were subsequently identified following the same assignment strategy based on COSY correlations (Fig. 3A and C).

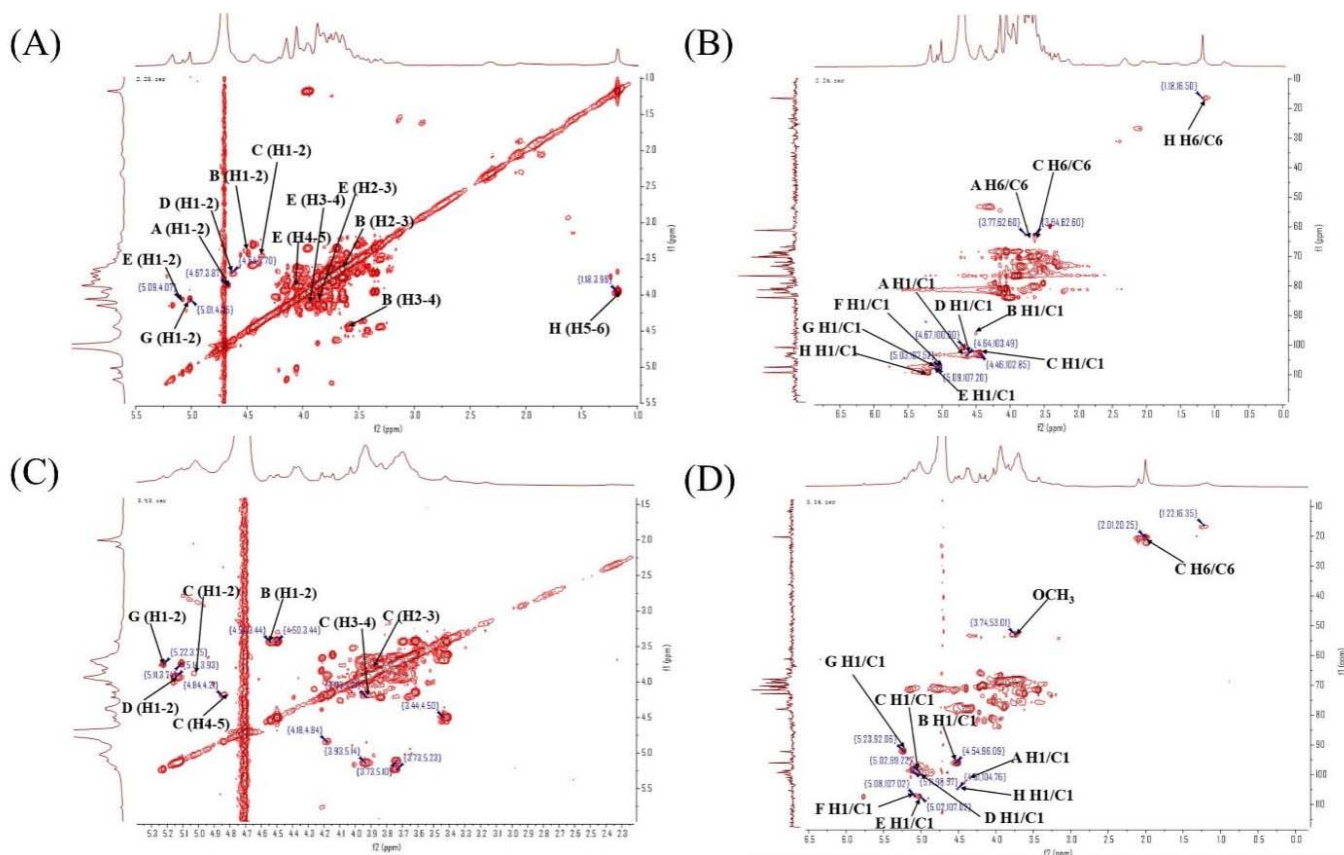


Fig. 3 (A) ^1H - ^1H COSY spectrum of WKP-S1; (B) HSQC spectrum of WKP-S1; (C) ^1H - ^1H COSY spectrum of WKP-S2; (D) HSQC spectrum of WKP-S2.

The linkage relationships among the sugar residues in WKPs were further elucidated based on the HMBC and TOCSY spectra (Fig. S1). In the HMBC spectrum of WKP-S1, the cross-peaks at δ 5.01/66.73 (G H1/F C5) and 5.01/83.85 (G H1/F C3) indicated that the anomeric proton of residue G is correlated with C5 and C3 of residue F. These correlations collectively demonstrated the presence of the $\rightarrow \alpha$ -L-Araf-(1 $\rightarrow$ 3,5)- α -L-Araf-(1 $\rightarrow$ 5)- α -L-Araf $\rightarrow$. The cross-peaks at δ 5.20/78.37 implies that the anomeric proton of residue H is correlated with C4 of residue B, indicating the linkage of $\rightarrow 2,4$ - α -L-Rhap-(1 $\rightarrow$ 4)- β -D-GalpA-(1 $\rightarrow$. Besides, the cross-peaks of C4/H1 at δ 79.02/4.64 of the 1,4- β -Galp residues in the HMBC spectrum confirmed the presence of 1,4- β -galactan segments. In the HMBC spectrum of WKP-S2, a correlation observed at δ 2.01/173.45 (OAc H/OAc C) indicated the presence of an

acetyl substituent at the 2-O and/or 3-O position(s). This assignment was further supported by the corresponding H/C signals of methoxy (CH₃O) group of $\rightarrow 4$)- α -D-GalpA-(1 $\rightarrow$ at 3.74/53.01 ppm^[33, 34]. The definitive structural assignment was established through an integrated analysis of monosaccharide composition, methylation analysis, HSQC/COSY spectral data, and corroboration with literature precedents.

3.1.5. Proposed structures of WKP fractions

Based on the combined results of monosaccharide composition, methylation analysis, and 1D/2D NMR spectroscopy, WKP-S1 and WKP-S2 were identified as two structurally distinct pectic polysaccharides derived from walnut kernels. Although both fractions exhibit typical pectic characteristics, they differ significantly in the relative abundance of rhamnogalacturonan-I (RG-I) and homogalacturonan (HG) domains, as well as in the nature and branching degree of side chains.

Taken together, WKP-S1 was identified as an RG-I-dominant pectic polysaccharide. Its backbone is primarily composed of alternating $\rightarrow 4$)- β -D-GalpA-(1 $\rightarrow$ and $\rightarrow 2,4$)- α -L-Rhap-(1 $\rightarrow$ residues, characteristic of a rhamnogalacturonan I (RG-I) structure. The side chains were found to be highly branched, mainly comprising [α -L-Araf-(1 $\rightarrow 3,5$)- α -L-Araf-(1 $\rightarrow 5$)- α -L-Araf-(1 $\rightarrow$] and [$\rightarrow 4$)- β -D-Galp-(1 $\rightarrow 4$)- β -D-Galp-(1 $\rightarrow$] segments. In contrast, referring to the structures of pectic polysaccharides reported by Shakhmatov et al^[29], Liang et al^[35] and Feng et al^[32], it is inferred that the polysaccharide contains a linear homogalacturonan (HG) backbone composed of $\rightarrow 4$)- α -D-GalpA-(1 $\rightarrow$ linkages. FT-IR and NMR results indicate significant methyl-esterification and O-acetylation of GalA residues, consistent with partially esterified HG blocks in WKP-S2. The side chains of WKP-S2 composed of arabinan and galactan, which mainly including $\rightarrow 5$)- α -L-Araf-(1 $\rightarrow$ and α -L-Araf-(1 $\rightarrow$, alongside β -D-Galp-(1 $\rightarrow 6$)- β -D-Galp-(1 $\rightarrow$ segments.

Due to the structural heterogeneity and high degree of branching in WKP-S2, direct inter-residue correlations between the side chains and the RG-I backbone could not be unambiguously established using HMBC spectroscopy. Nevertheless, the methylation analysis clearly revealed the presence of 2,4-linked Rhap residues, which serve as branching points within the RG-I domain. This diagnostic linkage pattern supports the attachment of arabinan and galactan side chains specifically at the O-4 position of the rhamnose backbone. Collectively, these data indicate that WKP-S2 adopts a highly branched RG-I structure rather than a simple alternating framework.

3.2. Neuroprotective effects of WKP on *Drosophila melanogaster*

3.2.1. WKP improves climbing ability and extends lifespan in A β 42 *Drosophila*

Behavioral dysfunction is a hallmark of AD. In *Drosophila*, locomotion and climbing abilities are primarily regulated by the mushroom body neurons^[36]. To investigate whether WKP modulates neuronal function, the A β 42 *Drosophila* model was used to assess locomotor performance. A significant decrease in climbing ability was observed in A β 42 *Drosophila* compared to wild-type W1118 controls at days 7, 14 and 21. Treatment with WKP significantly improved the climbing ability of A β 42 transgenic *Drosophila* at all time points (Fig. 4A).

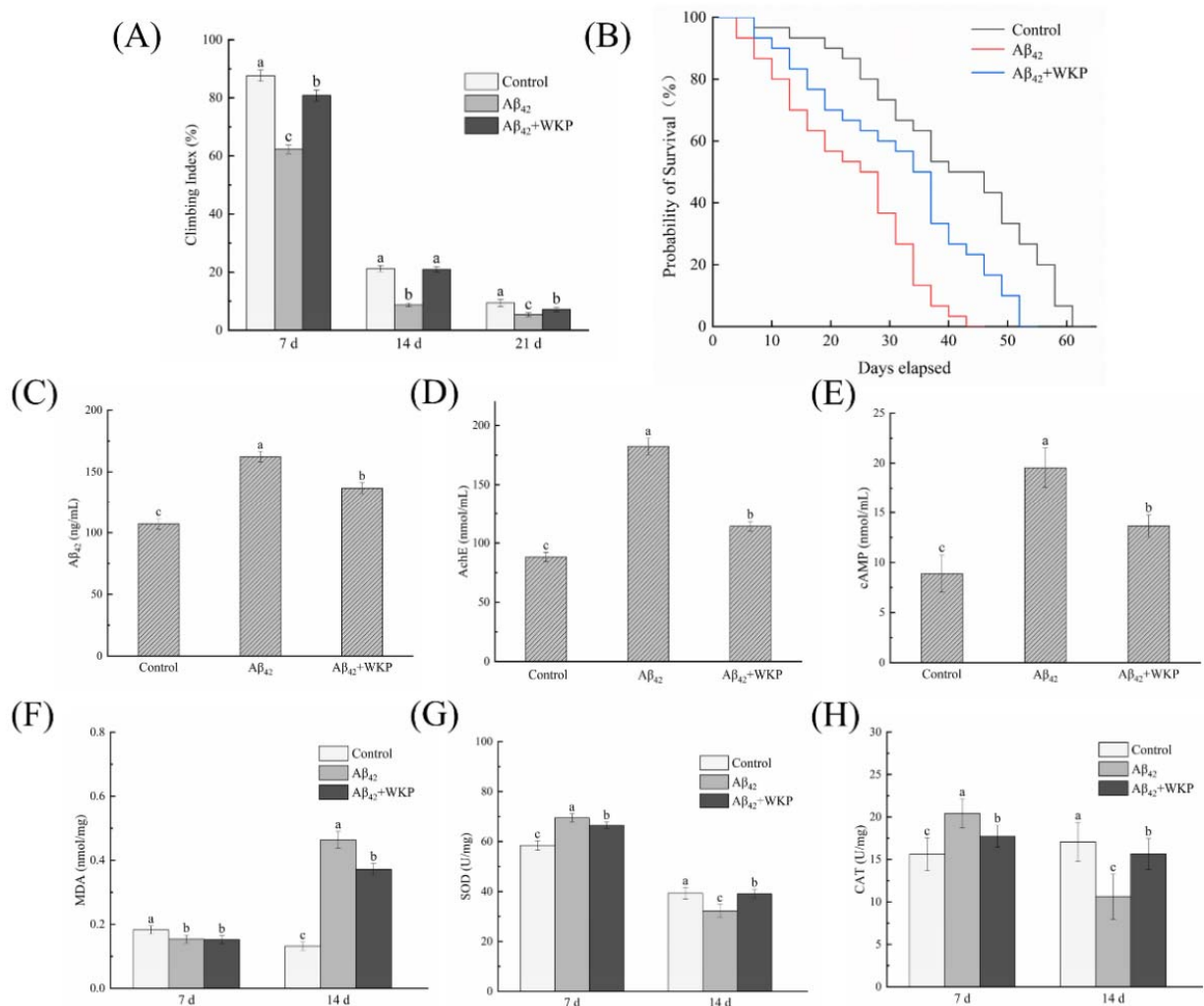


Fig. 4 (A) Effect of WKP on the climbing ability of Aβ42 transgenic flies; (B) *Drosophila* survival curves for each group; Effects of WKP on Aβ42 levels (C), AchE activity (D), cAMP content (E), MDA level (F), SOD activity (G) and CAT activity (H) in the brain of Aβ42 transgenic flies. Different letters (a-c) within a indicate significant difference ($P < 0.05$).

The pan-neuronal overexpression in the *Drosophila* AD model employed in this study has been demonstrated to significantly reduce lifespan compared to wild-type controls [37]. Survival analysis showed that Aβ42 expression significantly shortened the mean lifespan (29.80 ± 1.30 days) compared to controls (40.50 ± 0.89 days). WKP supplementation significantly extended the mean lifespan to 38.02 ± 1.25 days, a 27.58% increase (Fig. 4B, Table S3).

3.2.2. WKP reduces Aβ42 burden, modulates related biomarkers and alleviates oxidative stress

Aβ42, a peptide generated from APP cleavage, aggregates into oligomers and fibrils, causing synaptic dysfunction, neuronal toxicity, and cognitive decline. It can upregulate the expression and activity of acetylcholinesterase (AChE). This process is mediated by either glycogen synthase kinase-3β (GSK-3β) activation or adenylate cyclase (AC) inhibition, ultimately leading to reduced intracellular cAMP levels [38, 39]. The AD model group showed significantly elevated levels of Aβ42, AChE, and cAMP compared to wild-type controls (Fig. 4C–E). WKP treatment significantly attenuated Aβ42 accumulation, reduced AChE levels by 37.47% ($P < 0.0001$) and cAMP levels by 30.13% ($P < 0.01$) in fly brains compared to the untreated AD model group.

Oxidative stress contributes to AD by generating reactive oxygen species (ROS), which induce lipid peroxidation and produce malondialdehyde (MDA). Antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), neutralize ROS by converting superoxide and decomposing H_2O_2 , preventing further oxidative damage. On day 14, A β 42 transgenic flies exhibited increased MDA levels and decreased SOD and CAT activities compared to wild-type controls, indicating oxidative damage. WKP treatment resulted in a 19.83% reduction in MDA content, a 17.49% increase in SOD activity, and a 32.06% increase in CAT activity compared to the untreated A β 42 group (Fig. 4F-H).

3.2.3. Supplementation with WKP modulates the expression of five differentially expressed genes: *CG2111*, *CG3502*, *Jon25Biii*, *Jon99Fi*, and *Jon99Fii*

The clustering analysis of the nine sample groups revealed that samples within each group were closely clustered, whereas those between groups were relatively dispersed. This pattern suggests substantial intergroup differences, high intragroup reproducibility, and robust reliability of the transcriptional data, making it suitable for further analysis (Fig. 5A). To investigate the anti-aging mechanisms of WKP, we performed RNA-seq on wild-type, A β 42-expressing, and A β 42 flies treated with WKP. Differential expression analysis revealed distinct transcriptional profiles (Fig. 5B). A β 42 expression resulted in 1,174 DEGs (573 upregulated and 601 downregulated) compared with the control. Notably, WKP vs. A β 42 showed 209 DEGs (61 upregulated and 148 downregulated), suggesting a significant modulation of A β 42-induced gene expression. Venn analysis identified 106 overlapping DEGs across all comparisons (Fig. 5C–D), indicating that shared regulatory networks were influenced by both A β 42 and WKP.

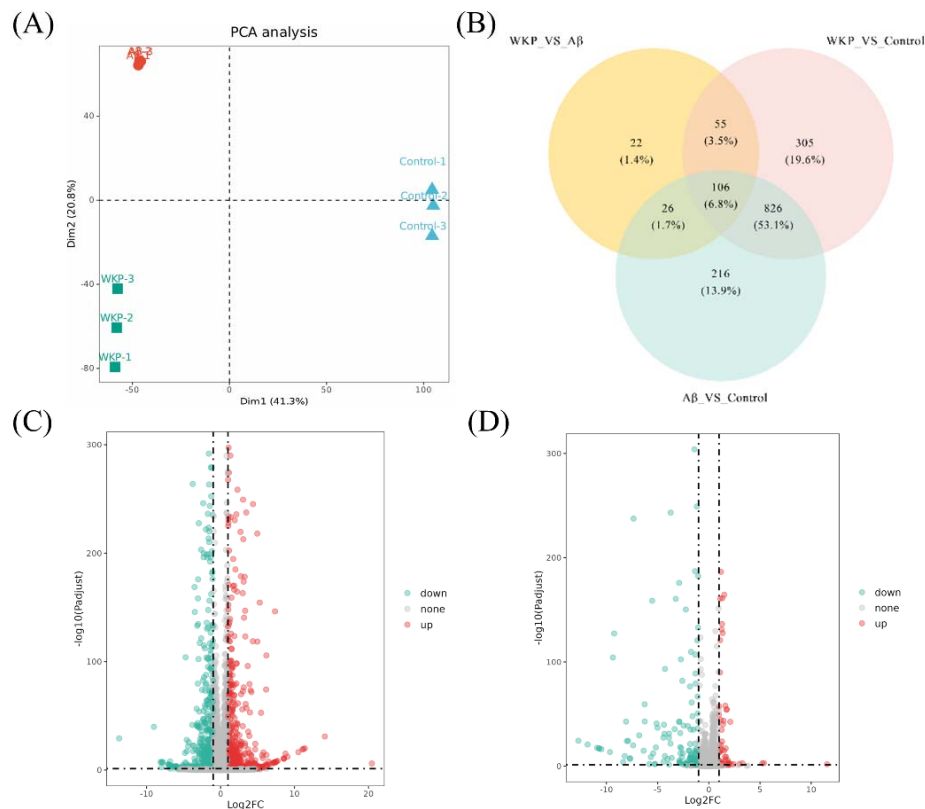


Fig. 5 (A) PCA chart; (B) Wayne diagrams; (C) Differential gene volcano map (A β vs Control); (D) Differential gene volcano map (WKP vs A β).

GO enrichment analysis ($\text{padj} < 0.05$) of WKP vs. A β_{42} DEGs revealed key functional categories (Fig. 6A–B). Upregulated genes were associated with immune responses ("defense response to Gram-positive bacteria," "humoral immune response"), extracellular localization, and metabolic activities (UDP-glycosyltransferase, carbohydrate binding). Downregulated genes were linked to proteolysis, extracellular/membrane processes, and serine hydrolase activity.

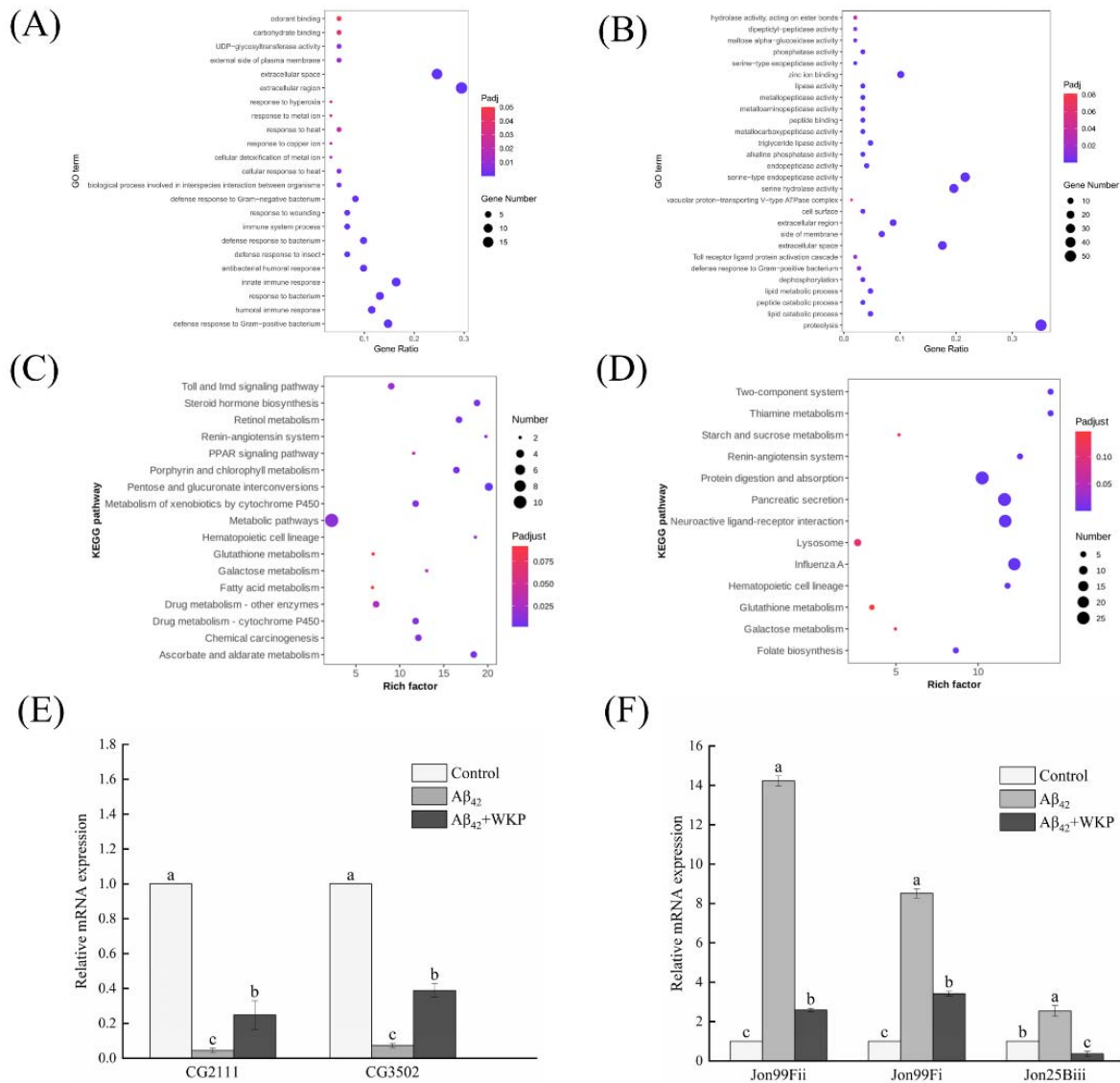


Fig. 6 Functional enrichment analysis of differentially expressed genes. (A) GO terms for upregulated genes; (B) GO terms for downregulated genes; (C) KEGG pathways for upregulated genes; (D) KEGG pathways for downregulated genes; Relative mRNA expression of upregulated genes (E) and downregulated genes (F).

KEGG pathway analysis further showed that upregulated DEGs were enriched in metabolic and detoxification pathways (steroid hormone biosynthesis, retinol metabolism, PPAR signaling, cytochrome P450 metabolism, glutathione metabolism). Conversely, downregulated DEGs were involved in protein digestion/absorption, lysosomal function, neuroactive ligand-receptor interactions, and nutrient metabolism (Fig. 6C–D).

Based on GO annotation, KEGG enrichment, and fold-change magnitude, five DEGs were selected for validation by quantitative real-time PCR (RT-PCR), ensuring both biological relevance and technical

feasibility. As shown in Fig. 6E and F, CG2111 (involved in zinc ion binding) and CG3502 (a metalloaminopeptidase) were significantly upregulated following WKP treatment. It has been reported that the interaction between zinc and tau protein can trigger the aggregation of tau, thereby contributing to the onset of AD.

Conversely, Jon25Biii, Jon99Fi, and Jon99Fii, which are implicated in serine hydrolase activity, were significantly downregulated. RT-PCR validation confirmed the RNA-seq results, demonstrating the high reliability and reproducibility of transcriptomic data. The above results confirm that the decrease in AChE (Fig. 4D, a serine hydrolase whose primary function is to degrade acetylcholine and terminate neurotransmission.) may be caused by genes related to transcriptional regulation.

3.2.4. Effects of three WKP fractions on the climbing ability, lifespan and neurotoxicity of A β 42 Transgenic Flies

As shown in Fig. 7A, among the three purified fractions of WKP, WKP-S1 demonstrated the greatest improvement in climbing performance at days 7, 14, and 21, followed by WKP-S2. This observation was consistent with the lifespan extension results. Survival analysis (Fig. 7B) showed no significant differences among groups during the initial 7 days, suggesting negligible early-stage effects. From day 8 onward, WKP-S1 produced the most pronounced effect compared to the A β 42 model group, indicating lifespan extension, confirming its superior efficacy in counteracting A β 42-induced neurodegeneration. As detailed in Table S3, WKPs significantly increased the maximum lifespan of A β 42 flies. Specifically, lifespan extensions of 47.43% for WKP-S1 were observed relative to the untreated A β 42 group, highlighting the superior longevity-enhancing effect of WKP-S1. Neurotoxicity biomarkers, including A β 42 levels, AChE activity, and cAMP levels, were assessed in the WKP fractions (Fig. 7C-E). WKP-S1 exhibited the most pronounced reduction in A β 42 levels and AChE inhibition, outperforming WKP-S2. Similarly, WKP-S1 demonstrated better suppression of cAMP levels than crude WKP and WKP-S2.

Collectively, among all the tested fractions, WKP-S1 consistently displayed the most potent neuroprotective effects, as evidenced by improved motor function, prolonged lifespan, reduced A β 42 deposition, attenuated AChE hyperactivity, and normalized cAMP signaling. These findings indicated the therapeutic potential of WKP-S1 in mitigating AD-associated neurodegeneration, this may due to its RG-I domains and branching patterns.

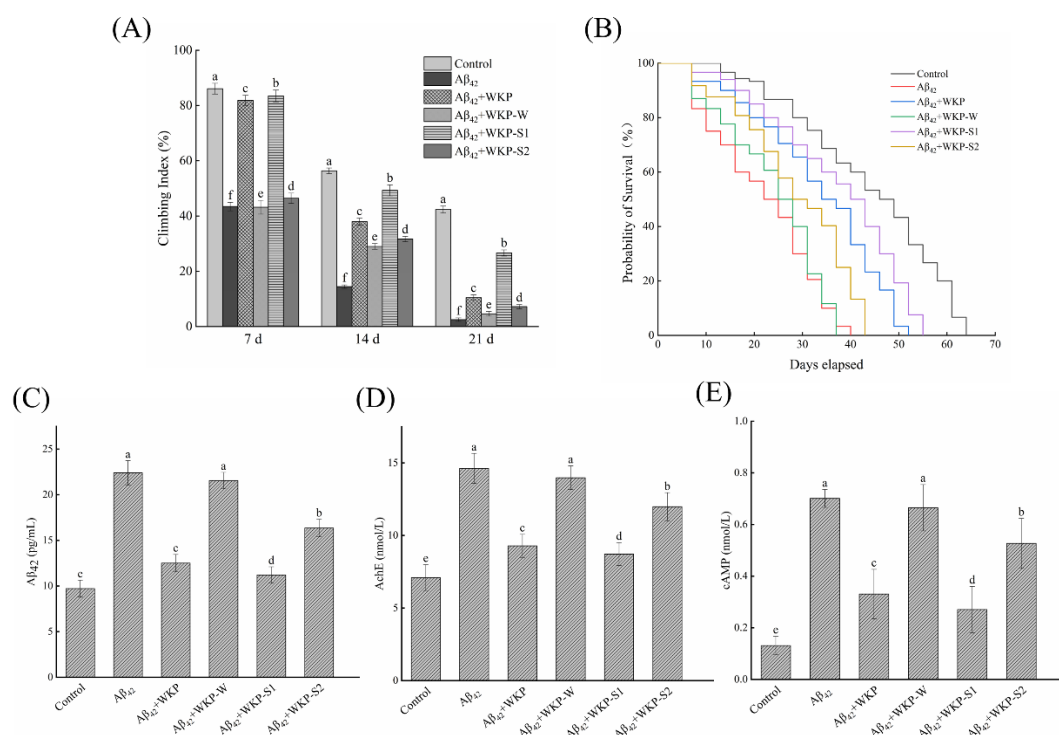


Fig. 7 (A) Effect of WKP fractions on the climbing ability of Aβ42 transgenic flies; (B) Survival curves for *Drosophila melanogaster* raised in control diet and WKP fractions consumption; Effects of WKP fractions on Aβ42 levels (C), AChE activity (D), cAMP content (E). Different letters (a-e) within a indicate significant difference ($P < 0.05$)

4. Discussion

The present study isolated and characterized two structurally distinct pectic polysaccharides, WKP-S1 and WKP-S2, from walnut kernels, and evaluated their neuroprotective effects in an Aβ42-expressing *Drosophila* AD model. Our findings demonstrate that WKP, particularly the RG-I-rich fraction WKP-S1, exerts significant therapeutic benefits against AD-like pathology.

Structural analysis revealed that WKP-S1 is an RG-I-dominant polysaccharide with highly branched arabinan and galactan side chains, while WKP-S2 is a hybrid HG-RG-I pectin with partially methyl-esterified and O-acetylated GalA residues. The structural differences likely contribute to their distinct bioactivities. RG-I domains, with their complex side chains, have been implicated in various biological activities, including immunomodulation and neuroprotection [4, 5]. The abundant arabinan and galactan side chains in WKP-S1 may facilitate interactions with biological targets, such as enzymes or receptors involved in AD pathogenesis.

In the Aβ42 *Drosophila* model, WKP treatment significantly improved locomotor performance and extended lifespan, key indicators of neurological health and aging. These effects are consistent with previous studies showing that polysaccharides from other botanical sources can improve mobility and longevity in *Drosophila* models of neurodegeneration [40, 41]. The lifespan-extending effect of WKP-S1 was particularly notable, suggesting a strong protective effect against Aβ42-induced toxicity.

At the molecular level, WKP treatment reduced the accumulation of Aβ42, a central pathological feature of AD. This effect may be attributed to the modulation of APP processing or the enhancement of Aβ clearance pathways, as reported for other pectic polysaccharides [4, 7]. Furthermore, WKP decreased AChE activity and cAMP levels. AChE hyperactivity contributes to cholinergic deficiency in AD, and its inhibition is a key

therapeutic strategy^[39]. The reduction in cAMP levels may reflect the restoration of neuronal signaling homeostasis disrupted by A β 42^[38].

WKP also demonstrated potent antioxidant activity, reducing lipid peroxidation (MDA) and enhancing the activities of SOD and CAT. Oxidative stress is a major contributor to neuronal damage in AD^[2]. The ability of WKP to bolster antioxidant defenses aligns with the known antioxidant properties of many plant polysaccharides and may be a crucial mechanism underlying its neuroprotective effects^[42].

Transcriptomic analysis provided further insights into the mechanism of action. WKP modulated the expression of genes associated with serine hydrolase activity, immune response, and oxidative stress defense. Notably, Jon25Biii, Jon99Fi, and Jon99Fii—members of the Jonah family of serine hydrolases—were significantly downregulated. Serine hydrolases constitute a large and diverse enzyme family involved in the hydrolysis of peptides, lipids, and esters, playing critical roles in neuropeptide processing, lipid signaling, and protein degradation^[43]. Their dysregulation has been increasingly implicated in neurodegenerative diseases, including Alzheimer's disease, where altered proteostasis and lipid metabolism are key pathological features^[5]. Although this association suggests a potential link between global serine hydrolase modulation and cholinergic improvement, the mechanistic relationship remains speculative. Given that Jonah family proteins are primarily implicated in digestive processes in *Drosophila*, their direct involvement in neurodegeneration has yet to be established, and their downregulation may reflect indirect metabolic effects rather than causal neuroprotection. Additionally, the upregulation of CG2111 and CG3502 indicates potential roles in metal ion homeostasis and protein degradation, respectively—processes closely associated with AD pathology^[3, 39]. However, these transcriptomic findings should be interpreted with caution and require further validation at the protein level, as well as functional characterization in the context of A β 42 toxicity.

The superior efficacy of WKP-S1 over WKP-S2 highlights the importance of structural features, specifically the RG-I domain and its branching pattern, in mediating neuroprotective effects. This structure-activity relationship is crucial for understanding the bioactivity of pectic polysaccharides and for guiding the development of polysaccharide-based nutraceuticals for AD. Nevertheless, several limitations of this study should be acknowledged. First, the transcriptomic profiling was conducted using flies treated with the crude WKP extract rather than the most bioactive fraction, WKP-S1. This was primarily due to the limited yield of the purified RG-I-rich fraction, which precluded its application in RNA-seq experiments. Consequently, although the observed transcriptional alterations—such as the modulation of genes associated with oxidative stress and serine hydrolase activity—are consistent with the pronounced phenotypic effects of WKP-S1, a direct causal relationship cannot be conclusively established. Second, the present study did not include a positive pharmacological control such as donepezil, which limits the ability to directly compare the neuroprotective potency of WKP with established AD therapeutics. Future investigations should incorporate such comparators to better contextualize the efficacy of walnut-derived pectic polysaccharides. Third, the structural elucidation of WKP-S2 was constrained by the inherent spectral limitations of NMR spectroscopy when applied to highly heterogeneous pectic polysaccharides, particularly those containing hybrid HG-RG-I

domains with multiple side chains. The signal broadening and overlapping resulting from such structural complexity compromised the quality of the HMBC spectrum, limiting the unambiguous establishment of certain inter-residue connectivities by NMR alone. Future investigations should address these limitations by (i) scaling up the purification of WKP-S1 or leveraging advanced low-input sequencing technologies to enable precise characterization of its transcriptomic signature, (ii) incorporating a positive pharmacological comparator to better contextualize the efficacy of walnut-derived pectic polysaccharides, and (iii) employing complementary structural approaches—such as enzymatic fingerprinting coupled with mass spectrometry, or isotopic labeling strategies in methylation analysis—to further refine and validate the structural models proposed for WKP-S1 and WKP-S2, thereby enabling a more definitive elucidation of their structure–activity relationships as promising pectic polysaccharide candidates for AD intervention.

5. Conclusion

Two structurally distinct pectic polysaccharides, WKP-S1 (72.13 kDa) and WKP-S2 (45.61 kDa), were isolated from defatted walnut kernels. WKP-S1 was identified as an RG-I–enriched polysaccharide featuring a backbone of $[\rightarrow 4)\text{-}\beta\text{-D-GalpA-(1}\rightarrow 2,4)\text{-}\alpha\text{-L-Rhap-(1}\rightarrow]$, together with side chains composed of $\beta\text{-1,4-galactan}$ and highly branched arabinan built from $\rightarrow 5)\text{-Araf}$ and $\rightarrow 3,5)\text{-Araf}$ units. In contrast, WKP-S2 exhibited a hybrid HG–RG-I architecture containing linear $\rightarrow 4)\text{-}\alpha\text{-D-GalpA}$ segments with partial methyl- and O-acetyl-esterification, and side chains consisting mainly of $\rightarrow 6)\text{-Galp}$ and low-branched arabinose residues. Biological assessments in A β 42-expressing *Drosophila* demonstrated that WKP fractions, particularly WKP-S1, exert significant neuroprotective effects. WKP administration extended lifespan, improved locomotor performance, decreased A β 42 burden, and alleviated oxidative stress, in part by modulating antioxidant enzyme activities (SOD, CAT) and suppressing MDA generation. Transcriptomic profiling further indicated that genes associated with oxidative defense and serine-hydrolase–mediated pathways may be involved in these protective mechanisms. Collectively, this work provides a theoretical basis for the neuroprotective potential of WKPs.

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.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material. More data underlying this article will be shared on reasonable request to the corresponding author.

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