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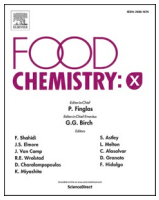

Several natural phytochemicals from Chinese traditional fermented food-pickled *Raphanus sativus L.*: Purification and characterization

Yunxuan Hu, Xiaoze Liu, Xiuna Wu, Zhengxiao Zhang, Daren Wu, Chaoxiang Chen, Wenjin Su, Lingyu Zhang, Jian Li [*], Hui-Min David Wang [*]

[a] *College of Food and Biological Engineering, Jimei University, Xiamen 361021, China*
[b] *Fujian Provincial Engineering Technology Research Center of Marine Functional Food, Xiamen 361021, China*
[c] *Graduate Institute of Biomedical Engineering, National Chung Hsing University, Taichung 402, Taiwan*

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β-Sitosterol (PubChem CID222284)
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α-Linolenic (PubChem CID5280934)
1-Monopalmitin (PubChem CID14900)
Chaenomic acid A (PubChem CID102339344)

ABSTRACT

In this study, we aimed to isolate and identify the bioactive compounds from 5-year pickled radish. The pickled radish was extracted with methanol or ethyl acetate. Sephadex LH-20, normal phase and reverse phase silica gel column chromatography were used for separation and purification, combined with thin layer chromatography (TLC), high performance liquid chromatography (HPLC), electrospray mass spectrometry (ESI-MS), nuclear magnetic resonance spectroscopy (NMR) technology for structural identification. The results showed that 6 compounds were separated and purified from methanol and ethyl acetate extracts of 5-year-old pickled radish. The structures were identified as 5-hydroxymethylfurfural, β-sitosterol, β-sitosterol-3-O-glucose glycosides, α-linolenic acid, 1-monopalmitin and chaenomic acid A. Using molecular docking, it was determined that β-sitosterol and its derivative β-sitosterol-3-O-glucose glycosides have high affinity for five antioxidant enzymes, and there were multiple hydrogen bonds between them. These results indicated that pickled radishes might be used as an important source of natural chemical substances.

Introduction

Pickled radish is a kind of pickled food, which is favored by people because of its unique flavor, rich nutrition and long shelf life. Locally, pickled radish is made from high-quality fresh radish, salted with traditional sea salt and naturally fermented for many years. Pickled radishes can even be stored for decades in southern Fujian of China. Since this ancient, pickling is a traditional cooking technique that is popular all over the world (Chakraborty & Roy, 2018). The fresh food is treated by dehydration and salt, and then put in a closed container through anaerobic fermentation to get the pickled food (Sawada et al., 2021). During salting and fermentation, complex biochemical reactions, such as changes in nutrient content, microbial composition and product texture, will occur in radishes (Cheigh, & Park, 1994). According to related folk applications, the pickled radish in the region has a variety of potential dietary healing properties. However, the nutritional benefits of pickled radish are only known to be folklore. Previously we have published studies in which we analysed the chemical fractions of local pickled radishes in different years. The results of the experiment revealed that the content of specific chemical components of pickled radish varied from year to year and that there was a correlation between the content and the year. Also, after spectral peak identification and matching, the study revealed that the chemical fractions of 5 year old pickled radish were richer and more reproducible across batches compared to fresh white radish (Xiong et al., 2016). Therefore, the more researchful 5 year old pickled radish was chosen for the next analysis.

More and more evidences showed that pickled radish had a variety of health benefits and biological activities, including antibacterial, anti-atherosclerosis and lipid-lowering (Manivannan et al., 2019; Terras et al., 1992). Previous studies have shown that antihypertensive factors such as polyphenols, arginine, and α-linolenic acid contained in salted radish can reduce heart rate and systolic blood pressure of spontaneously hypertensive rats by inhibiting sympathetic nerve activity and ACE activity (Kumakura et al., 2017). In addition, another report

* Corresponding authors.
E-mail addresses: lijian2013@jmu.edu.cn (J. Li), davidw@dargon.nchu.edu.tw (H.-M.D. Wang).

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showed that the consumption of radish leaves ethyl acetate extract may improve the hypertensive state of rats by increasing serum NO concentration, fecal Na+ concentration and antioxidant enzyme activity (Chung et al., 2012). In our previous research, it was determined that several phenolic compounds in pickled radish have antibacterial and antioxidant effects (Li et al., 2020). In another study, pickled radish phenolics could reduce lipid levels in high-fat mice by regulating the intestinal flora (Li et al., 2021). Therefore, as a high-quality source of natural active chemical substances, the biologically active factors contained in pickled radishes need to be further explored.

The oxidation reaction will inevitably lead to accelerated food spoilage and deterioration during storage. Some harmful by-products, such as ROS and so on, will be overproduced under oxidation conditions (He et al., 2017). Therefore, effective antioxidants have become the key to food preservation. Many evidences prove that the inhibition of some key enzymes can improve the adverse effects of oxidative stress. For example, inhibition of NADPH oxidase (Gao et al., 2019), Cytochrome p450 (He et al., 2017), Hematopoetic cell kinase (Kong et al., 2020), Xanthine oxidase (Bergamini et al., 2009), Myeloperoxidase (Aratani, 2018) and other enzyme proteins plays a positive role in improving oxidative stress. Molecular docking helps to screen potential enzyme inhibitors and has a strong advantage in the leading link of drug development. Studying the interaction between small molecules and target enzymes is helpful to the design and development of lead drugs.

In this study, we selected 5-year-old pickled radishes as the research object, and extracted and analyzed the active compounds with the aim of providing a useful reference for the nutritional development of pickled radishes. Sephadex LH-20 column chromatography, positive phase silica gel column chromatography and reversed phase C18 (RP-C18) silica gel column chromatography was used to separate and purify methanol and ethyl acetate components. The structures of the compounds were further identified by thin layer chromatography (TLC), ultraviolet detection (UV), nuclear magnetic resonance spectroscopy (NMR) and electrospray ionization mass spectrometry (ESI-MS). In addition, the binding degree of six pickled radish compounds to some enzymes involved in oxidative stress was studied by molecular docking, and their interaction patterns were analyzed. Therefore, considering the importance of pickled radish to traditional Chinese pickled products, these findings help to provide further insight into the nutritional benefits of pickled radish.

Materials and methods

Materials

Five-year-old pickled radishes were purchased from Zhangpu market in 2015 in Fujian Province, and samples were prepared from fresh white radish root. Different batches of test samples from the region were purchased and tested for this trial to ensure that the chemical composition of each batch was consistent. Methanol, ethyl acetate and deuterated methanol are all chromatographic grade reagents (≥99 %), chloroform is a pure reagent for analysis (≥97 %), purchased from Guangdong Guanghua Technology Co., ltd. The high-precision analytical balance used in the test (accuracy: 0.0001 g) was purchased from Mettler Toledo Technology (China) Co., ltd. The metrological class of glassware used (500 ml rotary evaporation flask, 500 ml measuring cylinder, 10 ml pipette, 1L filtration flask, 1L Separatory funnel) were purchased from Fuzhou North Glass Experimental Instrument Co., ltd. Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden); Reverse phase Silica gel 60 RP-C18 was purchased from Biotage Company (Sweden); Normal-phase chromatography Silica gel P60 was purchased from SiliCycle (Canada); Precoated silica gel GF 254 plates was purchased from Qingdao Marine Chemical Factory (Qingdao, China).

Preparation of crude extract

The 5-year pickled radish were sliced and dried in an oven at 45 ℃ to constant weight. Samples were mixed with 10-fold volumes of methanol followed by crushing. The homogenate was extracted by stirring at 40 ℃ for 6 h, and the entire extraction procedures were repeated twice. After filtration, the supernatants were combined and evaporated to obtain the methanol crude extracts of pickled radish. Furthermore, the ethyl acetate extracts were prepared. Pickled radish powder was mixed with 10-fold volumes of ethyl acetate, and the other steps were the same as above.

Separation and purification of crude extract

Dissolve the crude extract sample with methanol, Sephadex LH-20 column and Silica gel 60 RP-C18 column were used for preliminary separation. The collected components were tracked by thin layer chromatography (TLC) and the same target point were combined. The compounds were further isolated and purified by normal-phase chromatography Silica gel P60. The purified compounds were stored at 4 ℃.

Analysis of the mass spectrometry

The separated and purified compound was dissolved in 0.1 % formic acid–methanol solution and the mass spectrum was measured. The ionization source is an electrospray ionization (ESI) source or an atmospheric pressure chemical ionization (APCI) source. Mass spectrometry was obtained by Xevo™ G2 Q Tof (Waters MS Technologies, Manchester, UK). It is a quadrupole and orthogonal acceleration time-of-flight tandem mass spectrometer.

Analysis of nuclear magnetic resonance analysis (NMR)

The compounds dissolved with deuterated reagents and moved into nuclear magnetic tube, the sample was measured on AVANCE III HD 400 MHz Nuclear magnetic resonance (NMR, Bruker Biospin GmbH, Karlsruhe, Germany) in deuterated reagents. All the samples were identified by ^{1}H NMR, ^{13}C NMR. Chemical shift values (δ) were given in parts per million (ppm) and coupling constant (J) in Hz. Signal multiplicities were described as follows: s (singlet), d (doublet), dd (doublet of doublets), t (triplet), m (multiplet), br s (broad singlet). The data were processed by phase correction and baseline correction using MestReNova software.

Analysis of high performance liquid chromatography (HPLC)

The purified compounds were dissolved into appropriate concentrations and analyzed by Ultimate 3000 Analytical high performance liquid chromatograph (HPLC, Thermo Company) under the same conditions. Chromatographic conditions: HPLC was used to perform gradient elution, methanol (A) 0.1 % formic acid water (B) system as the mobile phase. The flow rate was 1 ml/ min, the injection volume was 10 μL, keep the column temperature at 35 ℃ and use multiple wavelengths for detection. Elution gradient: 0–2 min, 10 % A and 90 % B; 2–10 min, 25 % A and 75 % B; 10–15 min, 25 % A and 75 % B; 15–20 min, 50 % A and 50 % B; 20–25 min, 75 % A and 25 % B; 25–35 min, 100 % A and 0 % B; 35–45 min, 100 % A and 0 % B; 45–50 min, 10 % A and 90 % B.

Molecular docking

Autodock Vina software was used to conduct docking experiments on pickled radish compounds and enzymes related to oxidation reaction, and explain their interactions. Protein crystal structure was downloaded from RCSB Protein Data Bank (https://www.RCSB PDB). Namely NADPH oxidase (PDB ID: 2CDU), Cytochrome p450 (1OG5), Hematopoetic cell kinase (PDB ID: 2HCK), Xanthine oxidase (PDB ID: 3DBJ),

Myeloperoxidase (PDB ID: 1DNU). Ten ligand 3D structures were mapped by Chem3D Ultra 8.0. The ligand and receptor were optimized by sybyl-X 2.0 Tripos field molecular mechanics program Mnimize, Loadeding the Gasteiger-Huckel charge, the Power energy gradient method was used for optimization, and a stable conformation was obtained after optimization. Delete the water molecules in the protein before docking, and Assign hydrogen atoms and Gasteiger charges to the PDB file. The Grid Box containing the active pocket was defined as: 60A* 60A* 60A. The grid spacing was 0.375 A. Keep the default values for other parameters and select the optimal conformation for bonding analysis.

Results

Determination of detection wavelength

An ultraviolet spectrophotometer was used to scan the whole wavelength of the 5-year pickled radish, and the scanning wavelength ranged from 190 nm to 600 nm. The scanning results showed that the sample has larger absorption peaks at 225 nm and 274 nm. Therefore, 225 nm and 274 nm were selected as the detection wavelengths for HPLC.

Chemical structure identification

Six compounds were obtained by separation of the crude extracts. The compounds were characterized by mass spectrometry, ^{1}H NMR, and ^{13}C NMR.

Purified components of methanol extract

Compound **1** was a light-yellow needle-like solid, which was easily soluble in water and more polar organic solvents. The mass spectrometry revealed that ESI (+)-MS: $m/z = 127$ $[M + H + H_2O]^+$ and ESI-MS^2: 109 $[M + H]^+$, and the molecular weight was 126. To judge from its mass spectrum, compound **1** probably contained –OH. ^{1}H NMR spectra showed 5 groups of subsidiar signals, included an aldehyde proton (δ_H 9.61) and two heterocyclic aforementers (δ_H 6.53 and δ_H 7.23. Moreover, the ^{13}C NMR spectrum showed that 6 groups of carbon signals, which revealed an aldehyde carbon (δ_C 177.8) and four heterocyclic aromatic hydrocarbon (δ_C 160.6, δ_C 110.1, δ_C 122.7 and δ_C 152.6). In ^{1}H NMR and ^{13}C NMR spectrum, δ_H 6.53 (1H, d, $J = 3.2$ Hz) and δ_C 110.1 showed feature signals of furan (**Table 1**). After data analysis and comparison with the literature, it was identified as 5-hydroxymethylfurfural (5-HMF), the molecular formula is $C_6H_6O_2$ (Dibenedetto et al., 2016).

Purified components of ethyl acetate methanol extract

Compound **2** was a transparent needle-like compound, which was easily soluble in dimethyl sulfoxide (DMSO) and insoluble in water. The mass spectrometry revealed that ACPI (+)-MS: $[M-H_2O + H]^+ = 397$. The results inferred that the molecular weight of compound **2** was 414. The ^{1}H NMR spectra of compound **2** indicated a doublet of olefinic

Table 1
^{1}H NMR and ^{13}C NMR Spectral Data of Compound **1** to **3** (^{1}H 400 MHz, ^{13}C 100 MHz, TMS, δ ppm).

	5-HMF (**1**)			β-sitosterol (**2**)			β-sitosterol-3-O-glucose glycosides (**3**)		
Solvent	$CDCl_3$			$CDCl_3$			DMSO-d_6		
Position	^{13}C	^{1}H	Multiplicity; *J* (Hz)	^{13}C	^{1}H	Multiplicity; *J* (Hz)	^{13}C	^{1}H	Multiplicity; *J* (Hz)
1	57.8	4.73	s	37.3	—		37.3	—	
2	160.6	—		31.6	—		29.8	—	
3	110.1	6.53	d; 3.2	71.8	3.55	m	77.4	—	
4	122.7	7.23	d; 3.2	42.4	—		overlapped	—	
5	152.6	—		140.9	—		140.9	—	
6	177.8	9.61	s	121.7	5.38	d; 5.0	121.7	5.27	d; 4.0
	—	3.71	s						
7				31.6	—		31.9	—	
8				31.6	—		31.9	—	
9				50.1	—		50.1	—	
10				36.5	—		36.7	—	
11				21.1	—		21.1	—	
12				39.8	—		39.8	—	
13				42.3	—		42.3	—	
14				56.7	—		56.6	—	
15				24.3	—		24.3	—	
16				28.3	—		28.2	—	
17				56.1	—		55.9	—	
18				11.9	0.71	s	12.1	0.67	s
19				19.4	1.03	s	19.5	0.97	s
20				36.1	—		36.0	—	
21				18.8	0.95	d; 6.5	19.1	0.89	d; 5.6
22				33.0	—		34.0	—	
23				26.2	—		26.2	—	
24				45.8	—		45.9	—	
25				29.2	—		29.3	—	
26				19.8	0.84	d; 7.0	19.6	0.80	m
27				19.0	0.86	d; 7.0	19.4	0.81	m
28				23.1	—		23.1	—	
29				12.0	0.87	t; 7.4	12.2	0.83	m
Hexose									
1′							101.2	4.16	d; 4.0
2′							73.9	2.95	m
3′							77.1	3.06	m
4′							70.6	3.00	m
5′							77.3	2.97	m
6′							61.6	3.40, 3.57	m,m

proton at δ_H 5.38 (1H, d, J = 5.0 Hz, H-6), a signal of oxymethine proton at δ_H 3.55 (1H, m, H-3). There were two methyl single chains at δ_H 0.71 (3H, s, H-18) and δ_H 1.03 (3H, s, H-19), which were characteristic of hydroxyl sterols of the mother nucleus for Δ^5-3β-. Compound **2** had δ_H 0.95 (3H, d, J = 6.5 Hz, H-21), δ_H 0.84 (3H, d, J = 7.0 Hz, H-26) and δ_H 0.86 (3H, d, J = 7.0 Hz, H-27), appeared of three methyl doublets, and a methyl triplet at δ_H 0.87 (3H, t, J = 7.5 Hz, H-29). In the ^{13}C NMR spectra of compound **2**, three -C- groups were seen at δ_C 140.9 (C-5), δ_C 42.3 (C-13) and δ_C 36.5 (C-10); nine –CH- groups were observed at δ_C 121.7 (C-6), δ_C 71.8 (C-3), δ_C 56.1 (C-17), δ_C 56.7 (C-14), δ_C 50.1 (C-9), δ_C 45.8 (C-24), δ_C 36.1 (C-20), δ_C 31.6 (C-8) and δ_C 29.2 (C-25); eleven –CH_2- groups were observed at δ_C 42.4 (C-4), δ_C 39.8 (C-12), δ_C 33.0 (C-22), δ_C 31.6 (C-2), δ_C 37.3 (C-1), δ_C 31.6 (C-7), δ_C 26.2 (C-23), δ_C 24.3 (C-15), δ_C 28.3 (C-16), δ_C 23.1 (C-28) and δ_C 21.1 (C-11); and six –CH_3- groups were observed at δ_C 19.4 (C-19), δ_C 11.9 (C-18), δ_C 18.8 (C-21), δ_C 19.8 (C-26), δ_C 19.0 (C-27) and δ_C 12.0 (C-29) (Table 1). Based on the above information, after identification and comparison with the literature, the compound **2** was determined to be β-sitosterol, and the molecular formula was $C_{29}H_5O$ (Li et al., 2008).

Compound **3** was obtained as a white powder with poor water solubility, readily dissolved in a mixture of dimethyl sulfoxide (DMSO) and chloroform. The ACPI (+)-MS result was $[M-C_6H_{11}O_6^+]^+ = 397$, and the molecular weight of compound **3** was 576. Compared ^{1}H NMR and ^{13}C NMR spectrum between compound **2** and compound **3**, the results were similar except for the signal of more group of *hexa*-carbon in compound 3. The compound **3** might be inferred to be sugar derivatives of β-sitosterol. In the ^{13}C NMR spectrum, δ_C 101.2 (C-1′), δ_C 73.9 (C-2′), δ_C 77.1 (C-3′), δ_C 70.6 (C-4′), δ_C 77.3 (C-5′), δ_C 61.6 (C-6′) were signals of glucose, it indicated that the sugar attached to the compound **3** was glucose (Table 1). According to the results of the above data analysis, it was finally determined that the compound was β-sitosterol-3-O-glucoside, and the molecular formula was $C_{35}H_{60}O_6$ (Lee et al., 2002).

Compound **4** was a light-yellow oily compound, soluble in small polar organic solvents, but insoluble in water. The mass spectrometry revealed that ESI (+)–MS: m/z = 279 $[M+H]^+$, M = 278; ESI-MS^2: m/z = 261 $[M-H_2O+H]^+$, m/z = 243 $[M-2H_2O+H]^+$, m/z = 223 $[M-55+H]^+$. As indicated by the ^{1}H NMR and ^{13}C NMR results, the multiplet signal at δ_H 5.37 (6H, m, H-9, 10, 12, 13, 15, 16) represented olefinic unsaturated protons, and the δ_C 129.7 (C-9), δ_C 126.8 (C-10), δ_C 127.8 (C-12), δ_C 127.7 (C-13), δ_C 127.4 (C-15), δ_C 131.2 (C-16) appeared, which were correlated to carbons. A typical triplet signal at the δ_H 0.97 (3H, t, J = 7.3 Hz, CH_3-18) demonstrated three protons, and the carbon at δ_C 13.3 represented a terminal methyl (omega-3, CH_3-18) group. A multiplet signal at δ_H 2.09 (4H, m, CH_2-8, 17) assigned to four protons correlated with the carbon at δ_C 26.7(CH_2-8), and δ_C 20.1 (CH_2-17) indicated two methylene groups. A triplet signal in δ_H 2.82 (4H, t, J = 6.2 Hz, CH_2-11, 14) showed four protons correlated with the carbons at δ_C 25.1 and δ_C 25.0 in two individual methylene (CH_2-11, 14) groups. An intense singlet at δ_H 1.32 (8H, s, H-4, 5, 6, 7) showed eight protons correlated to the carbons at δ_C 28.7–29.3 of four methylene (CH_2-4, 5, 6, 7) groups. A multiplet at δ_H 1.61 (2H, m, H-3) attributed to protons correlated directly with a carbon at δ_C 24.6 (CH_2-3). A triplet signal at δ_H 2.28 (2H, t, J = 7.0 Hz, H-2) was assigned to the protons correlated to a carbon at δ_C 33.4 in the methylene (CH_2-2) group, respectively. The δ_C 174.6 (COOH-1) was a typical carboxyl carbon signal (Table 2). After data analysis, it was identified that compound **4** was α-linolenic acid, and the molecular formula was $C_{18}H_{30}O_2$ (Li et al., 2010).

Compound **5** was a light buttery compound, and easily soluble in organic solvents. The mass spectrometry revealed that ESI (+)-MS: m/z = 331 $[M+H]^+$, m/z = 312 $[M-H_2O]^+$, m/z = 353 $[M+Na]^+$, and the molecular weight was 330. As indicated by the ^{1}H NMR and ^{13}C NMR data of compound **5**, the typical triplet signal at the δ_H 0.88 (3H, t, J = 8.0 Hz, CH_3-16) was three protons, and the carbon correlated to δ_C 14.1, which represented a terminal methyl (omega-3, CH_3-16) group. An intense singlet at δ_H 1.28 (26H, s, H-4–15) showed eight protons correlated to the carbons at δ_C 22.7–31.9 of four methylene (CH_2-4–15) groups. A multiplet at δ_H 1.62 (2H, m, H-3) attributed to protons correlated directly with a carbon at δ_C 24.9 (CH_2-3). A triplet signal at δ_H 2.35 (2H, t, J = 6.5 Hz, H-2) was assigned to the protons correlated to a carbon at δ_C 34.2 in the methylene (CH_2-2) group, respectively. The δ_C 174.4 (COOH-1) was a typical carboxyl carbon signal. The carbon values of δ_C 65.2 (CH_2-1′), δ_C 70.3 (CH-2′), and δ_C 63.3 (CH_2-3′) shift to the low field, which was guessed to be the effect of –OH group (Table 2). Combining the physical and chemical properties and data analysis, compound **5** was 1-monopalmitin and the molecular formula was $C_{19}H_{38}O_4$.

Table 2
^{1}H NMR and ^{13}C NMR Spectral Data of Compound **4** to **6** (^{1}H 400 MHz, ^{13}C 100 MHz, TMS, δ ppm).

	α-linolenic acid **(4)**			1-monopalmitin **(5)**			chaenomic acid A **(6)**		
Solvent	CD_3OD			$CDCl_3$			$CDCl_3$		
Position	^{13}C	^{1}H	Multiplicity; J (Hz)	^{13}C	^{1}H	Multiplicity; J (Hz)	^{13}C	^{1}H	Multiplicity; J (Hz)
1	174.6	—		174.4	—		176.4	—	
2	33.4	2.28	t; 7.0	34.2	2.35	t; 6.5	33.6	2.31	t; 6.5
3	24.6	1.61	m	24.9	1.62	m	25.5	1.59	m
4	28.7	1.32	br s	29.1	1.28	br s	28.9	1.38	br s
5	28.8	1.32	br s	29.3	1.28	br s	29.2	1.38	br s
6	28.9	1.32	br s	29.4	1.28	br s	28.9	1.38	br s
7	29.3	1.32	br s	29.5	1.28	br s	24.7	1.38	br s
8	26.7	2.09	m	29.6	1.28	br s	36.9	1.55	m
9	129.7	5.37	m	29.7	1.28	br s	71.6	4.15	q; 12
10	126.8	5.37	m	29.6	1.28	br s	134.6	5.73	m
11	25.1	2.82	t; 6.2	29.5	1.28	br s	129.4	5.73	m
12	127.8	5.37	m	29.4	1.28	br s	74.6	3.95	m
13	127.7	5.37	m	29.3	1.28	br s	74.6	3.95	m
14	25.0	2.82	t; 6.2	31.9	1.28	br s	129.6	5.73	m
15	127.4	5.37	m	22.7	1.28	br s	135.1	5.73	m
16	131.2	5.37	m	14.1	0.88	t; 8.0	74.3	4.15	m
17	20.1	2.09	m				29.3	1.55	m
18	13.3	0.97	t; 7.3				13.0	0.94	t; 7.4
							—	5.35	–
1′				65.2	4.18	dd			
2′				70.3	5.36, 3.70	m, dd; 4,12			
3′				63.3	3.94, 3.60	m, dd; 4,12			

Compound **6** was a light-yellow solid, and soluble in water. The mass spectrometry results showed that ESI (+)-MS: m/z = 367 [M + Na] $^{+}$, 299 [M−COOH] $^{+}$, and the molecular weight was 344. As indicated by the ^{1}H NMR and ^{13}C NMR data of compound **6**, the multiplet signal at the δ_H 5.73 (4H, m, H-10,11,14,15) represented olefinic unsaturated protons, and carbons correlated to δ_C 134.6 (C-10), δ_C 129.4 (C-11), δ_C 129.6 (C-14) and δ_C 135.1 (C-15) were appeared. A typical triplet signal at the δ_H 0.94 (3H, t, J = 7.3 Hz, CH_3-18) demonstrated three protons correlated to the carbon at δ_C 13.0 represented a terminal methyl (omega-3, CH_3-18) group. A multiplet signal at δ_H 1.55 (4H, m, CH_2-8, 17) assigned to four protons correlated with the carbon at δ_C 36.9(CH_2-8) and δ_C 29.3 (CH_2-17) indicated two methylene groups. The two a multiplet at δ_H 4.15 (2H, m, H-9, 16) and δ_H 3.95 (2H, m, H-12, 13) correlated to δ_C 71.6 (C-9), δ_C 74.3 (C-16) and δ_C 74.6 (C-12, 13), which connected to the –OH group, respectively. An intense singlet at δ_H 1.38 (8H, s, H-4, 5, 6, 7) showed eight protons correlated to the carbons at δ_C 24.7–29.2 of four methylene (CH_2-4, 5, 6, 7) groups. A multiplet at δ_H 1.59 (2H, m, H-3) attributed to protons correlated directly with a carbon at δ_C 25.5 (CH_2-3). A triplet signal at δ_H 2.31 (2H, t, J = 6.5 Hz, H-2) was assigned to the protons correlated to a carbon at δ_C 33.6 in the methylene (CH_2-2) group, respectively. The δ_C 176.4 (COOH-1) was a typical carboxyl carbon signal (**Table 2**). After data analysis, it was identified that compound **6** was chaenomic acid A, and the molecular formula was $C_{18}H_{32}O_6$ (Kim et al., 2014).

Comparison of HPLC results

Whereas high performance liquid chromatography (HPLC) is an

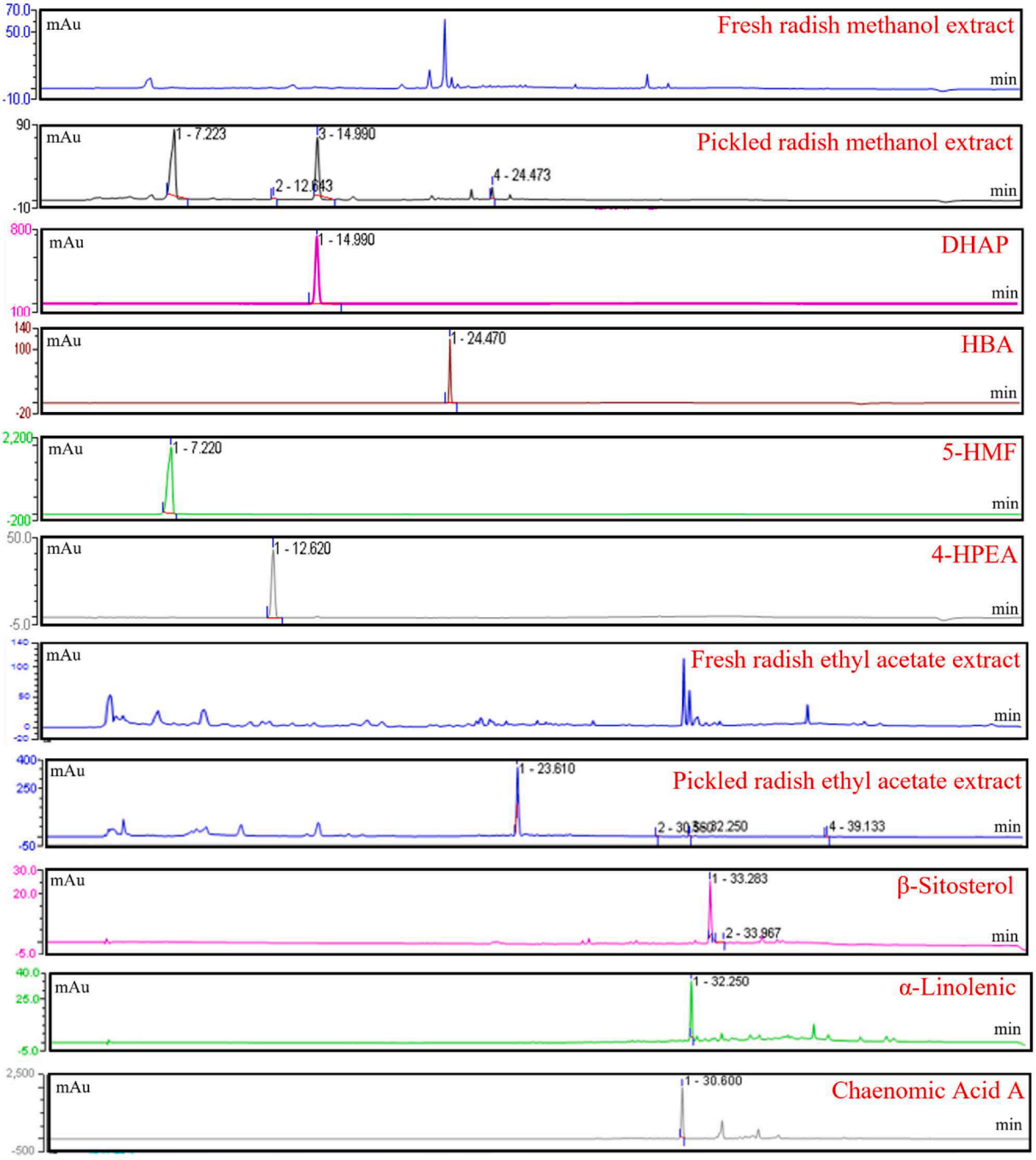

Fig. 1. Comparison of HPLC Chromatogram of Methanol Extract and Ethyl Acetate Extract.

efficient separation and identification technology with high precision and good reproducibility. 6 compounds were obtained from 5-year of pickled radish through separation, purification and structural identification. The methanol component and ethyl acetate component of pickled radish were analyzed by HPLC (methanol extract was detected at 296 nm wavelength, ethyl acetate extract was detected at 256 nm wavelength). The comparative explanation of the methanol and ethyl acetate components was shown in Fig. 1. Except β-sitosterol-3-O-glucose glycosides and 1-monopalmitin, other compounds were detected. The methanol components of fresh radishes and pickled radishes were different, and 5-HMF was only detected in pickled radishes, but not in fresh white radish. It was speculated that 5-HMF was a new compound produced by pickling fresh white radish. In the ethyl acetate extraction of pickled radish, the content of β-sitosterol, α-linolenic acid and chaenomic acid A were lower than those of fresh radish (Figs. 1 and 2).

Computational docking studies

Through molecular docking, the compounds in pickled radish that bind most favorably to the enzyme proteins related to oxidation reaction were determined, and the conformation with the lowest binding energy was selected among the 9 results of docking output. As shown in Fig. 3, better combination of β-sitosterol-3-O-glucose glycosides with NADPH oxidase, Hematopoetic cell kinase and Myeloperoxidase. The binding energy was: −8.9, −8.2, −10 kcal/mol. β-sitosterol has a better combination with Cytochrome p450 and Xanthine oxidase. The binding energy was: −9.3, −8.1 kcal/mol. Further analyze the interaction between them, such as Fig. 4**A**. The glucoside structure of β-sitosterol-3-O-glucose glycosides has affinity for the inside of the pocket. The hydroxyl group on its glycoside forms hydrogen bonds with ASP-179, TYR-188 and CYS-242 of NADPH oxidase, with bond lengths of 2.7. Å, 2.1 Å, 3.0 Å. The hydroxyl groups on β-sitosterol-3-O-glucose glycosides form hydrogen bonds with TYR-90, GLN-161 and SER-248 of Hematopoetic cell kinase. The bond lengths were: 2.6 Å, 1.5 Å, and 2.0 Å, respectively. In addition, the hydroxyl group of the glycoside structure tends to form hydrogen bonds with ASP-94, ASP-98, and THR-100 located in the Myeloperoxidase pocket. The bond lengths were: 2.7 Å, 1.8 Å, 2.2 Å, and 3.3 Å, respectively. The hydroxyl end of β-Sitosterol was located inside the hydrophobic pocket of Cytochrome p450 and Xanthine oxidase. A hydrogen bond with a bond length of 1.8 Å was formed between the H atom on the hydroxyl group and the GLY-296 of Cytochrome p450. The hydroxyl of β-sitosterol and ASP-740 and HIS-741 of Xanthine oxidase form hydrogen bonds with bond lengths of 2.9 Å and 2.0 Å, respectively. In addition, the pockets of the five enzyme proteins have multiple amino acid residues and π-π conjugates between the compounds (Fig. 4**B**).

Discussion

The emergence of fermented food not only provides people with a new diet choice, but also brings a lot of nutritional benefits. More and more people realize that fermented food has many benefits, such as improving food safety, providing nutritional factors, and unique flavor and taste (Licandro et al., 2020). For example, soy sauce and vinegar, as traditional fermented foods with a long history, have produced rich nutritional factors that are beneficial to the human body during the production process (Budak et al., 2014; Li et al., 2016). Pickled radish is a kind of fermented food popular all over the world. As a potential source of bioactive substances, their nutritional value has been widely concerned. However, reports on its nutritional content are scarce. In our previous research, we have stated that the three polyphenol compounds identified from pickled radish are the key to lasting freshness (Li et al., 2020). In this study, we used column chromatography to separate and purify 6 compounds from methanol extracts and ethyl acetate extracts of 5-year pickled radish. The resolved picture of the above compounds has been obtained through a combination of thin layer chromatography (TLC), HPLC method, ESI-MS and NMR technology. Through these methods, we identified six bioactive components of pickled radish, including 5-hydroxymethylfurfural (**1**); phytosterol: β-sitosterol (**2**) and its derivative β-sitosterol-3-O-glucose glycosides (**3**); Two fatty acids: α-linolenic acid (**4**) and 1-monopalmitin (**5**); chaenomic acid A (**6**). Among them, all other compounds except α-linolenic acid were discovered from radish for the first time.

5-hydroxymethylfurfural (5-HMF) is an intermediate product of Maillard reaction, and it is also a common indicator for measuring

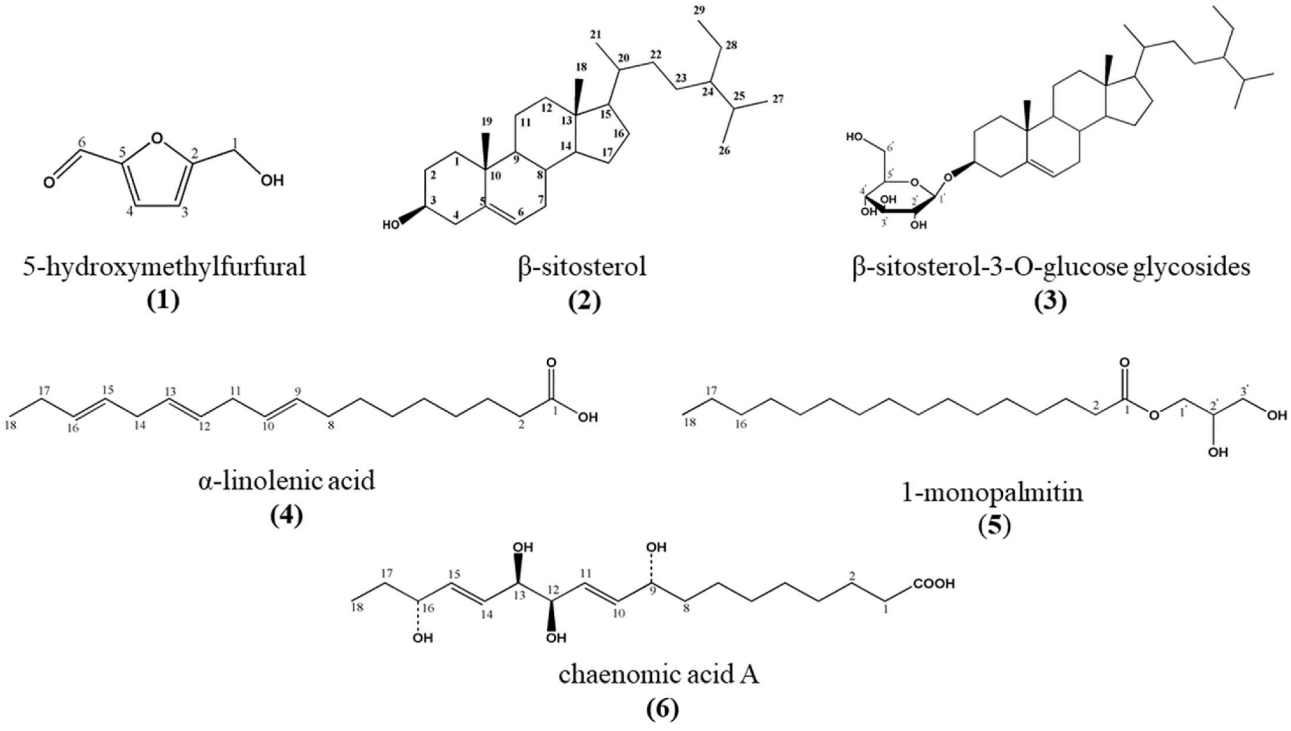

Fig. 2. Structures of 6 Compounds Extracted from Pickled Radish.

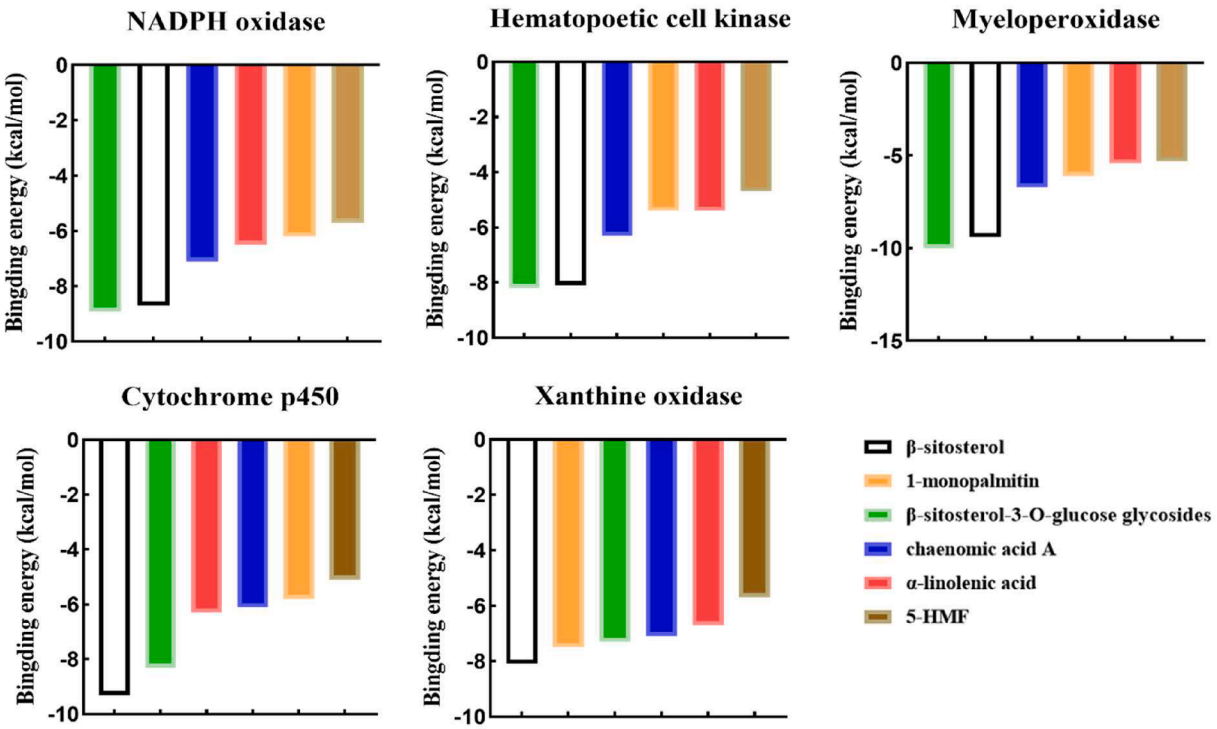

Fig. 3. Calculation of binding energy between pickled radish compound and protein.

browning index in the field of food inspection. The radish contains sugar and organic acid. During the pickling process, with the loss of water, the pH decreases, and the sugars in it are easily dehydrated under the above conditions to produce 5-HMF. Numerous biologically active functions of 5-HMF have been confirmed. For example, 5-HMF could effectively ameliorate alcohol-induced liver injury (Li et al., 2015), against acute hypobaric hypoxia (Li et al., 2011) and alleviate LPS-induced RAW 267.4 cells inflammatory response (Kong et al., 2019).In a recent report, 5-HMF effectively isolated and identified from *Symplocos chinensisf*. Pilosa Ohwi was shown to be effective against nerve pain (Kim et al., 2021). However, it has been reported that the safety of 5-HMF is controversial, but as a common bioactive factor in fermented foods, 5-HMF deserves further study (Severin et al., 2010). Phytosterols are a class of bioactive molecules found in plants. More and more evidences show that plant sterols have a variety of nutritional benefits. Whereas, the body cannot produce sterols on its own, people need to get them from their diet. As the most common phytosterol in People's Daily diet, β-sitosterol is widely found in *Rubiaceae*, *Cruciferae*, *Araceae* and other plants. A recent study showed that β-sitosterol injections significantly improved chronic anxiety in naive Stressed mice (Kim et al., 2021).In addition, β-sitosterol has been reported to have antitumor (Cao et al., 2020), anti-inflammatory (Zhang et al., 2020), lipid-lowering (Navarro Del Hierro et al., 2021) and bacteriostatic activities (Evangelina et al., 2021). A glycosidic bond is formed between the –OH of β-sitosterol C-3 and glucose. The resulting β-sitosterol-3-O-glucose glycosides is also commonly found in many medicinal plants. β-sitosterol-3-O-glucose glycosides and β-sitosterol were often isolated and identified together from some fruits, vegetables, plants and Chinese herbal medicines with biological activity (Hussain et al., 2020; Kayo et al., 2020; Khan et al., 2020).

In addition, two fatty acid compounds were found in this study. α-Linolenic acid (**4**) has been shown to be present in radishes in previous studies. α-linolenic acid (ALA) is a kind of plant-derived polyunsaturated fatty acid (PUFA), which is essential fatty acid for human body and has been proved to have a positive effect on the prevention of cardiovascular disease (Stivala et al., 2013; Stivala et al., 2020). In recent years, new evidence has shown that dietary supplementation of ALA could inhibit thrombosis through intestinal flora (Saeedi Saravi et al., 2021). 1-monopalmitin (**5**) is a kind of 1-monopalmitin based on palmitoyl, and formed by the esterification of palmitic acid and glyceride. 1-monopalmitin has been identified in a variety of plant extracts and has been shown to have antiviral and antibacterial biological activity (Cheng et al., 2020; Nipun et al., 2021; Wang et al., 2020). In addition, 1-monopalmitin has been reported to be a metabolic marker for Parkinson's disease and lipid synthesis (Ke et al., 2020; Shah et al., 2019). Finally, we determined that the component (**6**) was chaenomic acid A. As a new oxylipins, chaenomic acid A has been reported to have strong anti-neuroinflammatory activity (Kim et al., 2014). In particular, methanol extract and ethyl acetate extract were compared by HPLC. We found that the chemical composition of pickled radishes in 5 years has changed compared with that of fresh radishes, but the types of compounds have not changed significantly. After pickling fresh radishes, the moisture decreases and the acidity increases. Among them, the content of β-sitosterol, α-linolenic acid and chaenomic acid A is reduced, and 5-HMF is a new product of radish after pickling. In addition, the three phenolic compounds 2, 6-dihydroxyacetophenone (DHAP), 4-hydroxyphenyl alcohol (4-HPEA) and 4-hydroxybenzaldehyde (HBA) that have been reported in our previous research were shown in the spectral image (Li et al., 2020). Among them, HBA already exists in fresh radishes, while DHAP and 4-HPEA were both found in pickled radishes.

In addition, β-sitosterol and its derivative β -sitosterol-d-glucopyranoside has a high affinity for several key enzymes involved in oxidative stress through molecular docking. There were multiple hydrogen bonds between the hydroxyl group of β-sitosterol and the glycoside structure of β-sitosterol-3-O-glucose glycosides and the inner hydrophobic pocket of the protein, this was the key to their effective anchoring. β-sitosterol has previously been shown to slow CCl_4-induced oxidative stress and liver damage (Devaraj et al., 2020). It was also reported that β-sitosterol relieves alcohol-induced oxidative stress by increasing the activity of antioxidant enzymes (Chen et al., 2020). It was clear that β-sitosterol and its derivatives β-sitosterol-3-O-glucose glycosides may be involved in preventing the deterioration caused by over-oxidation of pickled

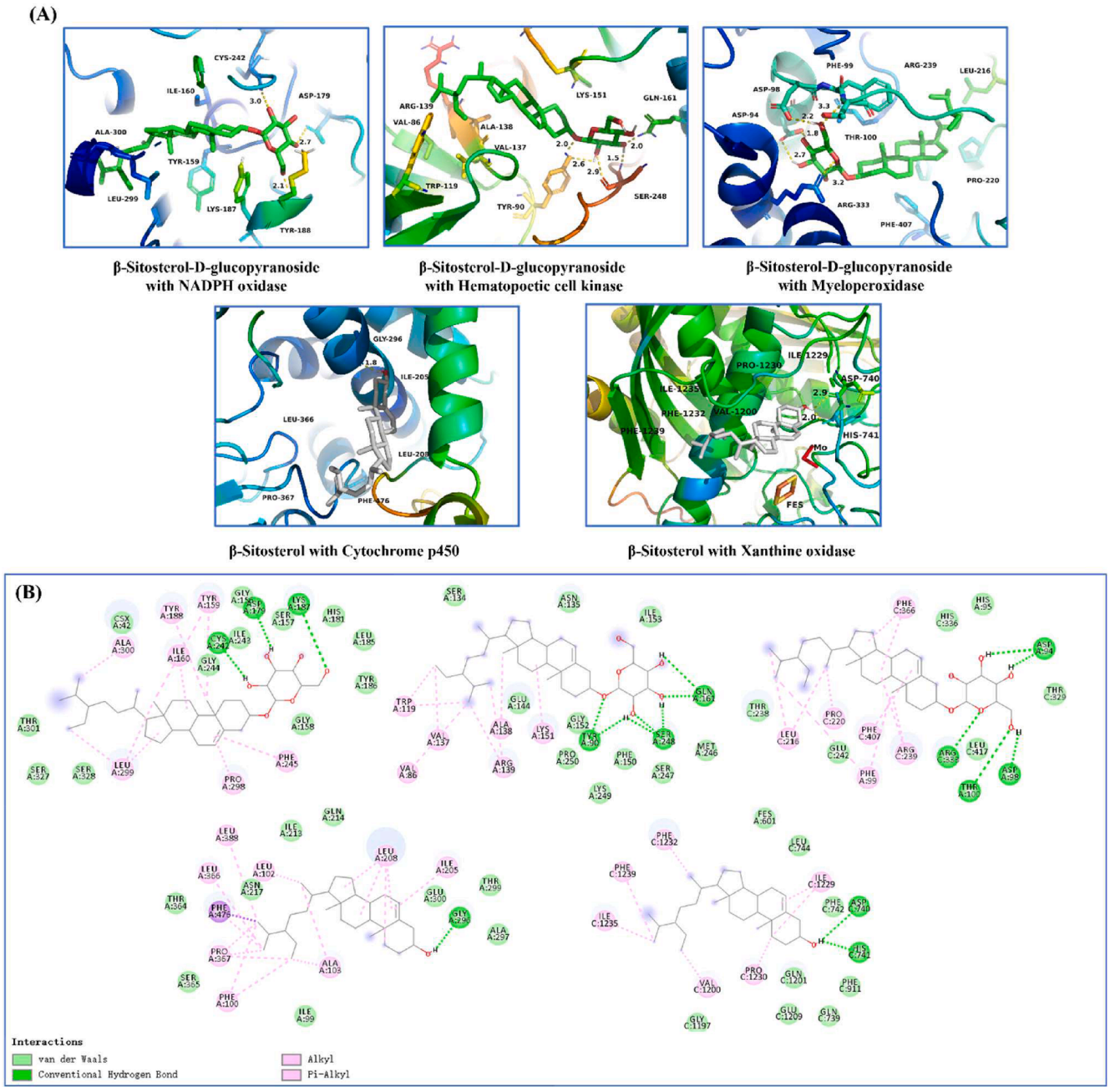

Fig. 4. Interaction patterns between pickled radish compounds and proteins.

radish, which has potential antioxidant development value.

Conclusion

In this study, we identified 6 compounds of 5-year pickled radish, which were: 5-hydroxymethylfurfural (**1**); phytosterol: β-sitosterol (**2**) and its derivative β-sitosterol-3-O-glucose glycosides (**3**), as well as two fatty acids: α-linolenic acid (**4**) and 1-monopalmitin (**5**), and chaenomic acid A (**6**). These bioactive compounds were commonly found in many plants, but α-linolenic acid was found in pickled radish for the first time. Molecular docking analysis revealed that β-sitosterol and β-sitosterol-3-O-glucose glycosides have high affinity for multiple key enzymes involved in oxidation reactions. With this report, we hope to provide a scientific basis for the Chemical composition and nutritional benefits of pickled radish. Considering pickled radish is a kind of pickled food popular in the world, it is very important to reveal its nutritional composition.

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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