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Identification of novel bioactive proteins and their produced oligopeptides from *Torreya grandis* nuts using proteomic based prediction

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ABSTRACT

Torreya grandis nut is a chief functional food in China consumed for centuries. Besides its rich protein composition, increasing studies are now focusing on *T. grandis* functional proteins that have not yet identified. In this study, liquid chromatography coupled with mass spectrometry detection of smaller and major proteins, revealed that the major peptide was 36935.00 Da. Proteome sequencing annotated 142 proteins in total. Bioactive proteins such as defensin 4 was annotated and its anti-microbial function was verified. Finally, functional oligopeptides were predicted by searching sequences of digested peptides in databases. Ten group of oligopeptides were suggested to exhibit antioxidant, Angiotensin-converting enzyme inhibition, anti-inflammatory. The predicted antioxidant activity was experimentally validated. It is interesting that a peptide GYCVSDNN digested from defensin 4 showed antioxidant activity. This study reports novel functional peptides from *T. grandis* nuts that have not been isolated and/or included as functional ingredients in nutraceuticals and in food industry.

1. Introduction

Human body function is mainly governed by proteins, which are an excellent source of biologically active peptides called bioactive peptides (BP). BP are protein fragments, usually 2–20 amino acids long linked by peptide bonds, already encoded as amino acid sequences, but inactive when encrypted in the parent protein. Once released after proteolytic processes, BP may act as signaling molecules in multicellular organisms (e.g. hormones, drug) since they have special receptors that introduce several physiological functions, which highlight their importance in food and nutrition (Iwaniak & Minkiewicz, 2008). Thus, BP is one of the interesting topic of research in food industry and researchers are developing studies to implement food based peptides with different bioactivity. These BP undergo different biochemical pathway and control blood pressure, electrolyte balance etc. and are more activated than original peptides being produced by fermentation or enzyme hydrolysis Based on the functions of BP, they are categorized as antimicrobial, antioxidant, anti-hypertensive, mineral binding peptide and opioid (Balgir & Sharma, 2017).

Agro-food based Industry produces 190 million tons of by-products every year worldwide. These by-products include raw materials, pomaces, leaves, seeds, shells, brans, oilseed cakes and molasses etc. These by-products need recycling and disposition. They can be valorized to gain profit depending on their nature and amount. These Agro food based products contain carbohydrates, lipids and bioactive compounds like phenols, fibers, alkaloids and pigments. Proper recovery of such by-products can bring better global food sustainability, good environment and developed economy (Gençdağ, Görgüç, & Yılmaz, 2021; Görgüç et al., 2020; Kumar, Duhan, & Duhan, 2018). Oil cakes, which are produced after extraction of oil from seed nuts, are major by-products.

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They are an important source of proteins that could be utilized by food industry for the development of BP (Görgüç et al., 2020). Previous researches on BP from various seed nuts include pecan (Hu et al., 2018), corn gluten (Zhuang et al., 2013), walnut (Kong et al., 2021), rapeseed (Zhou et al., 2016), peony seed (Zhang et al., 2019), flaxseed (Silva et al., 2016) and *Torreya grandis* (abbreviated *T. grandis*) (Luo et al., 2021).

T. grandis belongs to the family of Cepahlotaxaceae (Quan et al., 2021) which is a coniferous tree of economic value, that is mostly grown in Zhejiang, Anhui and Jiangxi Province of China. It is also grown in USA (Florida), as well as in some areas in Japan and Korea (Kang & Tang, 1995; Muhammad Saeed, Deng, Parveen, Rongji, Ahmad, & Yuhong, 2007). It has dioecious flowers, that matures with fruit nut seeds (Luo et al., 2021). (Feng, Cui, Xiao, Tian, Yi, & Ma, 2011) reported the yield of *Torreya* seeds in Zhejiang province is 1000–1500 tons/year that is 3–4 time higher than in 1990 which was about 350 tons/year. The price of the seeds were increased 40–80 RMB and 100–150 RMB/kg. Such figures shows the better economic perspective and market value of *Torreya* seeds.

The seed nuts of *T. grandis* has high nutritional and medicinal value. *T. grandis* seed contains bioactive compounds like tocopherol, sterols and polyphenols, several vitamins like nicotinic and folic acid and some minerals like magnesium, calcium, zinc and selenium (Ni et al., 2015). Several bioactive properties of *T. grandis* seed nuts include antioxidant, anti-inflammatory, antiatherosclerotic, antiviral, and antifungal, which could be used as a traditional medicine. *T. grandis* contains about 50–60 % of oil (dry mass weight), with enriched unsaturated fatty acids like linoleic and oleic acids (Dong et al., 2014). The highly nutritious and health benefits of *T. grandis* seed nuts are encouraging its consumption in China (Ni et al., 2015). The protein content of *T. grandis* nut meal ranges from 10.3 % to 16.4 %, with amino acid content of 118.1 g/Kg (he et al., 2016; J. Wang et al., 2017).

Considering that there is a growing demand for the development of BP from natural source that could be employed as functional ingredient in food and neutraceuticals, *T. grandis* meal could be a good source for BP production. However, lesser or no studies are focusing on the utilization of *T. grandis* nut meal, which is a growing challenge to the food industry. In this point of view, the present research was designed to study the structural characterization and proteomic profile of *T. grandis* nut for the development of functional BP that could be beneficial for human health and Industry. Our study provided the first results for the development of BP from *T. grandis,* which could be used as functional ingredient in food and other commercial products (e.g. cosmetics). We hope that using such approaches could help in the adding value of *T. grandis* (by-product) in future.

2. Materials and methods

2.1. Materials and chemicals

The cracked *T. grandis* nuts were harvested from a commercial garden in Xinchang, Zhejiang province, China, in September 2021 (about 525 days after floret). The seed nuts with shell were transported to the lab on the same day within 4 h, and the shell were removed by hand. Then, the nuts oil was cold-pressed by an oil press (Qingdao AUCMA Consumer Electric Co., Ltd.) and cakes were collected.

n-hexane and ethanol used for protein extraction were obtained from (Shanghai Macklin biochemical Co., Ltd. 1288 Canggong Rd, Shanghai chemical industry park, Shanghai 400–623-8666), bovine serum albumin (BSA) (Thermo scientific USA), methanol (MeOH, HPLC grade), acetontrile (ACN, HPLC grade), trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (CHCA) were purchased from Sigma, USA. Guanidine (Sigma), urea (Bio-Rad), tris-base (Bio-Rad), dithiothreitol DTT (Bio-Rad), IAA (Iodoacetamide) (Sigma), zeba spin column (Pierce), trypsin (Promega), chymotrypsin (Sigma), Glu-C, LysC (Wako), coomassie brilliant blue (R250) (Macklin AR CAS:6104–59-2). All other reagents and chemicals were of analytical grade.

2.2. Protein extraction from defatted T. Grandis nuts flour

Protein extraction was done according to the already published method (Yu, et al., 2017), with slight modification. After husking the nuts (*T. grandis* Merrillii) were milled into flour. The flour was defatted by mixing it with *n*-hexane/ethanol (9:1 v/v) in a 1:3 ratio (w/v) at room temperature. The mixture was continuously stirred for 2 h, further separated by an aspirator filter pump (Zhenzhou great wall scientific industry and Co. Ltd.). The filtrate was discarded and residual pellet was air dried at 25 °C in a laminar air flow(Shanghai Shengdai Laboratory Equipment Co. Ltd.) until free of solvent odor. The dried powder was considered as the defatted *T. grandis* flour (500 g).

The defatted *T. grandis* flour was dispersed in distilled water at a ratio of 1:10 (w/v) with stirring at 30 °C for 2 h. The mixture was centrifuged at 10,000 *g* for 20 min at 20 °C, and the precipitate was redissolved in water at a ratio of 1:5 (w/v) and repeatedly centrifuged at 10,000 rpm for 20 min at 20 °C. The two supernatants were mixed and pH was adjusted to pH 4.0 using 2 M HCl to precipitate the protein isoelectrically. The mixture was then centrifuged at 10,000 *g* for 20 min at 4 °C. The precipitates were redissolved and neutralized with 2 M NaOH and further dissolved and stirred in distilled water for 1 h. The protein extract was freeze dried, vacuum packed and stored at −80 °C for further use (Quan et al., 2021). The protein content of the mixture was determined using Bovine serum albumin kit (Thermo scientific).

2.3. Determination of the whole cell proteins in T. Grandis using SDS-PAGE

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on a discontinuous buffered system according to a method of Laemmli using 12 % resolving gel and 4 % stacking gel. The protein sample (12 mg/mL) was diluted with a sample loading buffer (1:1) containing 5 % (v/v) β-mercaptoethanol (β-ME) (Laemmli, 1970). The samples were heated at 95 °C for 5 min prior to electrophoresis by loading 20 μL samples in each lane. The current was adjusted to 180 Ma and the gel was stained with 0.1 % Coomassie Brilliant blue (R-250) for 2 h then de-stained using a de-colour solution (methanol/acetic acid/water, 20/30/350) for 8 h.

2.4. Protein separation of T. Grandis using high performance liquid chromatography (HPLC)

HPLC analysis were performed according to the method (Xia et al., 2019) with slight modifications. The supernatant of *T. grandis* nuts protein extract was filtered through 0.22 μm filter membrane prior to HPLC analysis. An Alliance system (Waters e2695 Separations Module and Waters 2998 PDA Detector) was used for the HPLC, operated on a Sepax GP-C18 column (4.6×250 mm, 5 μm).

The operating conditions were as follows: mobile phase was methanol (A) and water (B) at the following gradient elution program: 0–30 min, 10–90 % A; 30–35 min, stay 90 % A; 35–36 min, 90–10 % A; 36–66 min (the re-equilibration time), stay 10 % A. The flow rate was 1 mL/min and column temperature were maintained at 25 °C. The injection volume was 10 μL, and the elution was monitored at 214 nm.

2.5. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis of T. Grandis protein extract

Mass spectrometry analysis was performed using a MALDI time-of-flight instrument (Auflex, Bruker MS/MS, Germany) with a Nd: YAG laser (355 nm), operating in a positive-ion linear mode using a 19-kV acceleration voltage. The conditions for MALDI-TOF-MS include a first layer of matrix solution prepared with α-ciano-hydroxy-cinnamic acid (CHCA) at 10 mg/mL in methanol and dropped on the plate. The protein

sample (protein extract section 2.2) was mixed with the second matrix (prepared with CHCA at 10 mg/mL in ACN/0.1 % TFA (30/70, v/v) at a volume ratio of 1:1. 2 μL of the prepared mixtures were subsequently dropped to the top of the first layer of matrix. Finally, 2 μL of 0.1 % TFA (Trifluoroacetic acid) aqueous solution was dropped over the sample and carefully drained to remove excessive salts in each sample after 10 s. The pre-prepared protein samples were air-dried for the downstream MS measurements. Before analysis in the mass spectrometer, the MALDI plates were finally irradiated using UV lamp inside a biosecurity cabinet for at least 20 min to prevent any source of contamination in the mass spectrometer. All mass spectra were collected in positive ion mode throughout experiments. MALDI-MS fid files (Bruker) were converted to txt files with flex Analysis (version 3.3.80.0). All spectra were subsequently processed with baseline correction using the TopHat algorithm. No smoothing procedures were applied for analysis.

2.6. Peptides identification by ultra-performance liquid chromatography electron spray ionization quadrupole time of flight mass spectrometry/ mass spectrometry (UPLC-ESI-Q-TOF MS/MS)

The protein extract of *T. grandis* was first diluted and acidified with 1 % fluroacetic acid (FA). The protein extract was separated using an ultra-high- performance liquid chromatography system (Acquity UPLC I-Class) (Waters Corporation). The liquid phase A was a 0.1 % fluoroacetic acid aqueous solution, and the B liquid was 0.1 % fluoroacetic acid acetonitrile solution, the column was equilibrated with A solution. The protein extract was first loaded by an autosampler and separated using a chromatographic column C4 column (ACQUITY UPLC BEH300 C4 2.1×50 mm, 1.7 μm). The speed flow rate was at 0.3 mL/min with a detection wavelength at 280 nm, and a column temperature set at 80 °C (Shanghai Applied protein technology Co. ltd). Eluted peaks were detected using XevoG2-XS Q-TOF mass spectrometer (High resolution mass spectrometer XevoG2-XS QTOF (Waters Corporation). The length of the reaction was 10 min with a detection of positive ion precursor scan ranges from 500 to 4000 *m/z*. The original data was analyzed by UNIFI (1.8.2, Waters) Software processing.

2.7. Identification of functional proteins from T. Grandis nuts using liquid chromatography mass spectrometry (LC-MS/MS) and proteome sequencing

T. grandis protein extract was analyzed by label-free tandem mass tag and LC-MS/MS (nanoLC-QE Thermo Fisher) (Shanghai Applied protein Technology Co., Ltd.). The LC-MS/MS data were done to analyze the qualitative identification of the target protein and polypeptide molecules.

2.7.1. Trypsin digestion

The *T. grandis* protein/polypeptide were first digested with endo-proteinase (Trypsin) and then the digested protein samples were analyzed by LC-MS/MS (nanoLC-QE). A mass spectrometry matching software such as MASCOT was used for peptide identification of peptide sequences.

Enzymatic hydrolysis of the test product: After reduction and alkylation of the protein sample, Trypsin (mass ratio 1:50) was added, and the enzyme was hydrolyzed at 37 °C for 20 h. After enzymatic hydrolysis, the solutions were heated and kept at 85 °C for 15 min to inactivate the enzyme. Then, the solution was centrifuged at 5000 *g* for 15 min at 4 °C, the supernatant was collected and treated by an ultrafiltration centrifugal tube (Millipore), lyophilized, dissolved in 0.1 % fluoroacetic acid solution, and stored at −20 °C for later use.

2.7.2. LC-MS/MS analysis

Mass spectrometry conditions: Solution A is an aqueous solution of 0.1 % formic acid, and solution B is an aqueous solution of 0.1 % formic acid in acetonitrile (acetonitrile is 84 %). Column (Zeba Spin column (Pierce) was equilibrated with 95 % of the solution A, then, the protein sample was loaded onto the Trap column by the autosampler.

Mass spectrometry data acquisition: The mass-to-charge ratios of peptide fragments were collected as follows: 20 fragments were collected after each full scan spectrum (MS2 scan).

Data analysis: Proteome Discoverer 1.4 software was used to search and identified the peptide sequences in database.

2.8. Anti-microbial assay

Bacteria-*Bacillus subtilis* (ATCC6633), *E. coli* DH5α (ATCC69925), *Staphylococcus aureus* (ATCC2592), and *Pseudomonas aeruginosa* (ATCC9027) and fungus-*Monilia albican* (ATCC10231) and *Saccharomyces cerevisiae* (ATCC9763) were obtained from the American Type Culture Collection. *B. subtilis*, *S. aureus*, and *P. aeruginosa* were cultured in nutrient broth medium, *E. coli* DH5α cells were cultured in Luria-Bertani medium, *M. albican* were cultured in Martin modified medium, and *S. cerevisiae* was cultured in Sabouraud dextrose and yeast extract medium.

To determine the anti-microbial activity of defensin 4, stock cultures of microbes (in −80 °C) were grown in medium for 18 h at 37 °C, diluted to OD600 = 0.2, and then added to a 96-well plate at 100 μL/well. Each microbe was treated with defensin 4 at a final concentration of 0, 0.1, 1, 10, or 100 mg/mL. Absorbance was measured at 630 nm in a Mustikcan FC scanning multiwell spectrophotometer (Thermo Scientific, USA). Ampicillin (Sigma, USA) at a final concentration of 50 ng/mL was used as a positive control. The experiments were repeated three times.

2.9. Anti-oxidative assay

T. grandis protein extract and hydrolyzed peptides (digested with trypsin because our predicted functional peptide using proteome sequencing were from digested peptide) from *T. grandis* nut were diluted at 0.05 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1 mg/ml and 5 mg/ml. The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{+}$), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Fe^{2+} chelating activities were performed using Total Antioxidant Capacity (*T*-AOC) Assay Kit (ABTS) (Sangon Biotech, China), Antioxidant Capacity (*T*-AOC) Assay Kit (DPPH) (Sangon Biotech, China) and Total Antioxidant Capacity (*T*-AOC) Assay Kit, respectively, following the manufacturer's instruction. All of the absorbance was assessed using a microplate reader (BioTek, the USA).

2.10. Experimental data processing and statistical analysis

All experiments were done in triplicate. The data are expressed as a mean ± of standard deviation and presented in bar graphs. Differences were analyzed using one-way Analysis of Variance (ANOVA) with Tukey's multiple comparisons and $P < 0.05$ was considered statistically significant. Statistical analysis were performed using Graph Pad Prism 8.0 (La Jolla CA, USA).

3. Results and discussion

3.1. Identification of T. grandis proteins using SDS-PAGE and HPLC

In order to investigate the functional properties of *T. grandis* nut peptides, we did structural characterization using analytical techniques. Further we prepared a proteome data base for functional oligopeptides groups that could be screened, isolated and beneficial for human health and industry.

After aqueous extraction of protein from *T. grandis* seed nuts showed that the protein content is 12 mg/mL. The protein content of seed nuts usually rose high after defatting process. The high ash content showed the presence of several minerals especially Na^{+} (added during the precipitation) after several treatments. Dialysis could be used to remove

excessive salts inorder to improve protein extract. But different proteins and their contents have special advantages and applications. Our results of protein extraction from *T. grandis* nuts suggested that it is better to extract protein using aqueous solution and acid precipitation that could help in economic facilitation of Industrial processing of *T. grandis* proteins (Yu et al., 2017).

The results of SDS-PAGE of *T. grandis* protein extract were shown in Fig. 1 A. Accordingly, the major protein band occurs between 31 and 37 kDa and 20–21 kDa. These major bands emphasize that these proteins could exist as seed storage proteins in *T. grandis* nuts. Several other bands were also seen. For instance, the main band that was more obvious is near 14.4 kDa. Such a low molecular weight band showed that these proteins are minor in *T. grandis* nuts, and depend on the type of method used for protein separation. Different seed varieties of *T. grandis* nut shows different protein pattern. The difference in molecular weight confirms that they belong to different categories. SDS-PAGE is considered a better method for protein separation inorder to differentiate between multiple categories of *T. grandis*. A light band with nearly 10 kDa were also seen. Results showed that SDS-PAGE could be used for identification of different *T. grandis* varieties. The protein fraction with high band intensities was not changed, which showed that the structural stability of the major proteins in *T. grandis* nuts was high. Disulfide bonding also contributed to the stabilization of protein. The separation of peptides with small disulfide bond could be different in denatured state. Our results are consistent with (Yu et al., 2017) who used two different varieties of *T. grandis* in their study.

The high-performance liquid chromatography (HPLC) chromatogram of *T. grandis* nuts protein extract is shown in Fig. 1 B. Twenty peaks, with retention time ranging from 4 to 13 min were observed at 214 nm. The larger peptide peaks had a retention time from 9 to 10 min and smaller peaks of peptides were eluted from 4 to 8 min and then above 10 min were relatively small. Our method of protein separation by HPLC was in accordance with (Xia et al., 2019) who reported 18 different peaks. The protein extract of *T. grandis* was further subjected to mass spectrometry analysis combined with proteome profiling. Liu *et al* mentioned that HPLC is one of the most common method of separation and purification of peptides (Liu et al., 2013) besides several other chromatographic techniques like consecutive column chromatography A single separation cannot achieve the demand of highly pure peptides. Therefore, different other factors are also considered for separation of peptides to achieve better results (Wang et al., 2014).

3.2. *Identification of T. grandis peptides using matrix-assisted laser desorption/ionization time -of- flight mass spectrometry (MALDI-TOF-MS) and ultra-performance liquid chromatography electron spray ionization quadrupole time of flight mass spectrometry/mass spectrometry (UPLC-ESI-Q-TOF MS/MS)*

In order to investigate the functional of bioactive peptides from seed nuts, it is important to know the structure and function of the peptide. For this reason, structural analysis and identification is important. The standard way for structural identification of bioactive peptide i.e, Edman sequencing was time consuming and relatively insensitive. Mass spectrometry is considered as a better way for peptides identification through the investigation of their amino acid sequencing using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), quadrupole time-of-flight mass spectrometry (Q-TOF-MS), or liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Gu et al., 2014; Zheng, Li, & Ding, 2016).

Peptide identification by MALDI-TOF generates single protonated ions and signal to noise ratio (S/N) even in the low femtomole range. A spectrum pull may be observed when there is a presence of sodium (+22.99) and potassium (+39.10) in the sample. Reflector- TOF has improved mass accuracy (±0.5 to 1 Da), but a loss in signal from 5 to 10 folds may be observed, especially for larger peptides. Extraction delay can do better MALDI spectra quality, a lower chemical noise, reduction of background matrix and reduction of ion flight times on laser intensity. Consequently, the mass resolution and accuracy could be achieved even

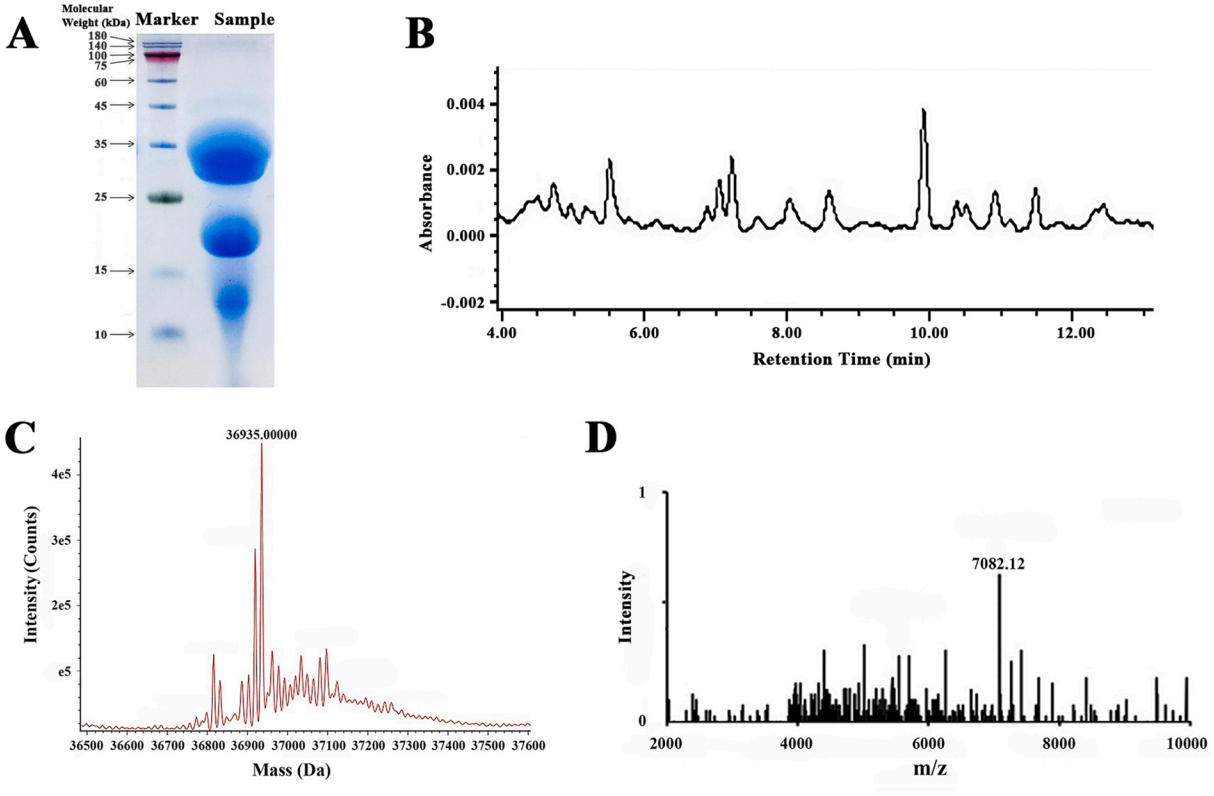

Fig. 1.

when the peaks are small (Dave et al., 2011).

Results of SDS-PAGE and HPLC have emphasized a certain complexity of *T. grandis* nut proteins. In order to further characterize the proteins extract, Q-TOF and MALDI-TOF mass spectrometry analysis were employed. 17 proteins were detected, with the most abundant having a relative molecular mass of 36935.00 Da using Q-TOF, in accordance with SDS-PAGE results (Fig. 1A). MS spectra of proteins detected with Q-TOF are shown in Fig. 1C. This result provided more details about the *T. grandis* nut extract. Wang *et al* identified a novel Angiotensin-converting enzyme (ACE) inhibitory peptide EPNGLLLPQY by UPLC-ESI-Q-TOF-MS/MS from Walnut, that was derived from the seed storage protein. They proposed that, seed storage protein with a molecular weight 1143.43 Da from Walnut could release bioactive peptides by enzymatic digestion with pepsin and trypsin. (Wang et al., 2018). Their method of protein identification is similar to our study, therefore we assumed that *T. grandis* protein with a molecular weight of 36935.00 Da could be a seed storage protein, which may have a variety of functions for the whole tree. This protein could be studied *in vivo* and *in vitro* for its functional bioactive properties.

In order to identify the proteins with the smaller molecular weights, as revealed using SDS-PAGE, MALDI-TOF mass spectrometry was employed. The peptide masses ranged between 2,000–10,000 Da, with more than 30 peaks. Some of the spots showed better peptide signals as shown in an expanded view in Fig. 1D. De-isotoping and centroiding of the spectrum were done, so that the monoisotopic masses are included in peptide mass finger printing (PMF), suggesting that the monoisotopic masses detected in MALDI-TOF-MS were essential for the structural analysis of *T. grandis* nuts proteins. These monoisotopic masses can be used as standard in MALDI-TOF-MS for future study. Liu *et al* studied the purification and identification of ACE (angiotensin I-converting enzyme) from walnut proteins. They used MALDI-TOF-MS for the identification of ACE inhibitory peptides, and found that the molecular mass of the inhibitory peptide, which they named enneapeptide, was 1033.42 Da (Liu et al., 2013). Our method for the structural characterization of *T. grandis* proteins is similar to their study. Shi *et al* studied the molecular mechanism of a peanut derived ACE inhibitory peptide, and used MALDI-TOF-MS for structural analysis of the peptide. They found a molecular weight of the peanut peptide at 808.8 Da, near to the theoretical value, which has been estimated at 807.7 Da (Shi et al., 2014). The two studies clearly showed that MALDI-TOF-MS could be used as a better approach for identification of bioactive inhibitory peptides. Liu *et al* identified the molecular weight and amino acid sequence of three different ACE-inhibitory peptides from wild pine nut peptide fractions, YLLK (Tyr-Leu-Leu-Lys), YLVPH (Tyr-Leu-Val-Pro-His) and YRLD (Tyr-Arg-Leu-Asp) by MALDI-TOF/TOF-MS. The three peptides, which had Tyr, Leu, Lys, Val and Pro in their amino acids sequences, exhibited a good ACE inhibitory activity (Liu et al., 2018).

3.3. Proteomic sequencing of T. grandis protein fraction and functional proteins

T. grandis protein/polypeptide extract was digested with trypsin, and analyzed using LC-MS/MS (nanoLC-QE) generated a total of 348 peptides and 142 distinct accumulated proteins (listed in Supplementary file S1 and Supplementary file S2).

Many studies have been done on the seed storage protein of soybean that releases different bioactive peptides with variety of physiological functions. However limited information is available on the bioactive peptide from other seed storage protein, since most of the studies have focused on single bioactivity. The biological activity of every peptide is different depending on the protein source, the enzyme used and the method of processing. By doing hydrolysis of the one single seed storage protein using different enzymes will release peptides with multiple bioactivity. *In vivo* and animal studies are going on for the investigation of bioactivities of peptides derived from seed storage proteins. However less human clinical studies have been done, and there is less understanding of the *in vivo* availability of such bioactive peptide in human digestive system from food derived source. More research could be done to explore the commonly used grains and oil seeds to see whether they release bioactive peptides. Some identified bioactive peptide are reactive as they have low molecular weight, but the exact mechanism of their activity is not clear (Marambe & Wanasundara, 2012).

The most abundant proteins included are 4 vicilin-like storage protein, and a bark protein (Table 1). Amongst annotated proteins, storage protein, especially vicilin-like antimicrobial peptide and β-conglutins, were the important functional proteins in *T. grandis* nuts. β-conglutin proteins are seed storage proteins, which exhibit anti-inflammatory properties, they also help in prevention and treatment of diabetes (Lima-Cabello et al., 2016). In a cell line model, olive seed β-conglutins exerted anti-inflammatory activity in the Pancreatic cell line (PANC-1) induced inflammation model, and whether same effect be observed in *T. grandis* nut has yet to be examined for the prevention and treatment of inflammatory-related diseases. One of the most important functional protein predicted in *T. grandis* nuts was plant defensin that exhibit antibacterial, anti-tumor, in addition to fungicidal properties (Lima-Cabello et al., 2016).

Sequence alignment of defensin protein from *T. grandis* nuts showed similarity in Pinus nut proteins (Fig. 2A) with several difference in amino acid sequences (Fig. 2B). Although the 3D structure of defensin proteins from *T. grandis* nuts is similar to most of defensin comprised of α-helix and 3 β-pleated sheets (Fig. 2C). To verify the function of *T. grandis* nuts defensin, we fusion expressed its recombinant protein (Fig. 2D). *B. subtilis*, *E. coli* DH5α, *S. aureus*, and *P. aeruginosa*, *M. albican*

Table 1
Top 20 proteins annotated from total peptides after trypsin digestion and sequence matching.

Accession	Peptides	Molecular Weight	Abundance	Annotation
107000_c0_g2	30	37.6	54×10^{9}	Vicilin-like storage protein
111713_c2_g2	11	14	28×10^{9}	Bark protein-like protein
106331_c2_g8	7	12.9	18×10^{9}	2S seed storage protein
105006_c1_g1	25	23.5	11×10^{9}	Vicilin-like storage protein
105006_c1_g2	14	23.9	94×10^{8}	Vicilin-like storage protein
118302_c3_g2	5	11.5	60×10^{8}	2S seed storage protein
110991_c1_g1	7	11.1	48×10^{8}	Conglutin beta 2
112970_c3_g2	4	14.5	44×10^{8}	2S seed storage protein
102495_c1_g1	5	13.4	25×10^{8}	2S seed storage protein
119119_c0_g4	9	21.6	24×10^{8}	Uncharacterized protein
114850_c4_g2	4	11.6	17×10^{8}	Bark storage protein A
110283_c1_g1	9	12.2	12×10^{8}	Bark protein-like protein
113223_c2_g1	6	12	12×10^{8}	Bark storage protein A
108551_c3_g1	6	11	71×10^{8}	Bark protein-like protein
111068_c3_g1	6	16.8	21×10^{8}	Vicilin-like antimicrobial peptides 2–2
104874_c1_g3	13	13.8	20×10^{7}	Vicilin-like storage protein
123793_c1_g1	7	13.5	19×10^{8}	Uncharacterized protein
102580_c2_g2	6	44.5	99×10^{7}	Actin
112154_c0_g3	6	23.3	54×10^{8}	Uncharacterized protein
107925_c1_g1	7	11.9	20×10^{6}	Uncharacterized protein

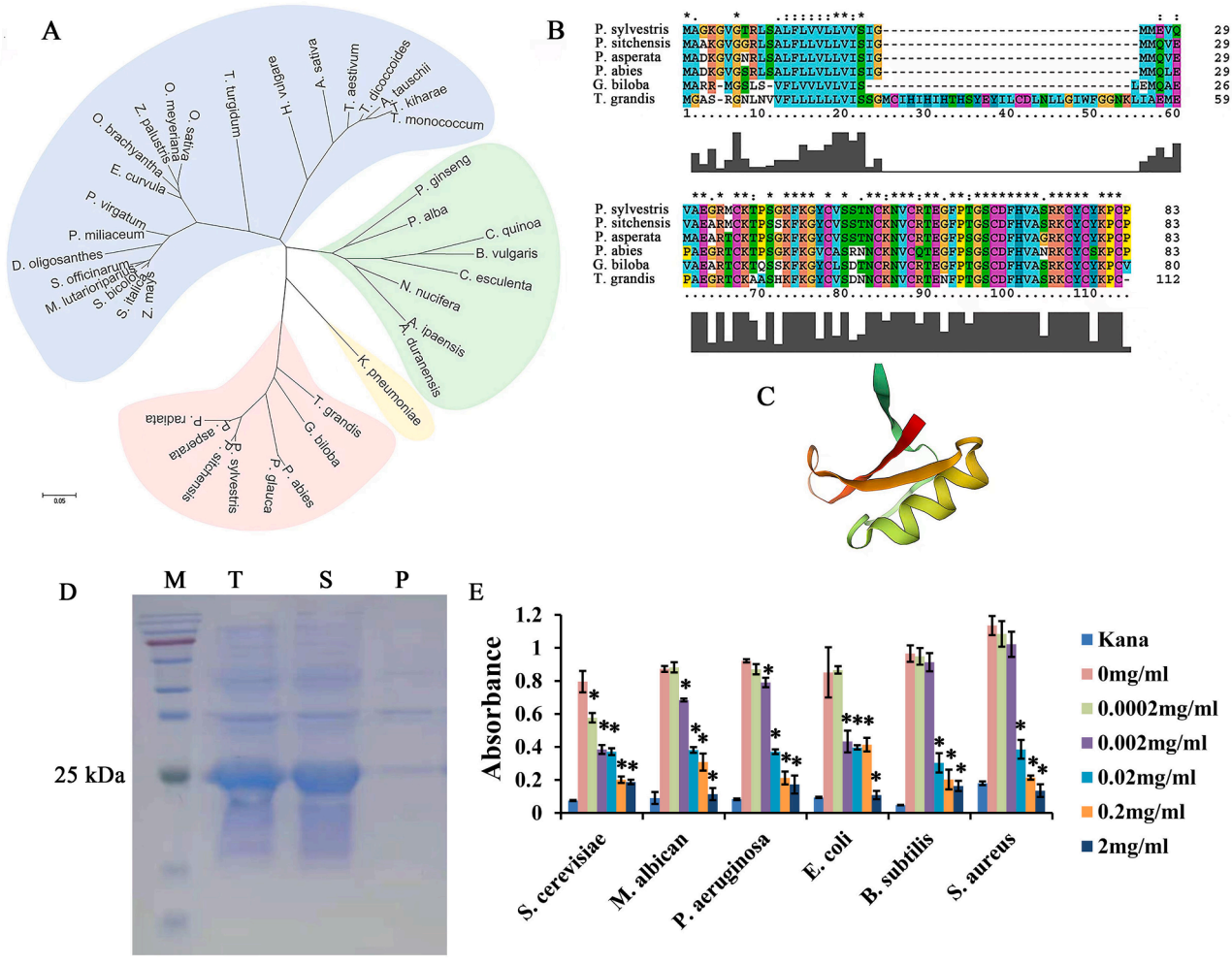

Fig. 2.

and *S. cerevisiae* was used to verify the activity of *T. grandis* nuts defensin, respectively. Results showed that all of the proliferation of 6 microbes were inhibited by *T. grandis* nuts defensin 4 significantly (Fig. 2E). In order to better explain the functional properties of defensin 4 and other detected peptides, we further determined their amino acid sequences post digestion with trypsin. Particularly, sequences of the digested peptides from defensin 4, Conglutin β-2 and Vicilin-like antimicrobial peptides 2–2 and some other *T. grandis* nut proteins are mentioned in Supplementary File S3.

3.4. Functional oligopeptides prediction in T. grandis protein fraction

Based on the sequencing *T. grandis* nut peptides, the sequence of digested peptides was searched in a BIOPEP database. The results showed 76 oligopeptides belong to 10 functional groups were annotated (Table 2). 27 antioxidative oligopeptides from *T. grandis* nuts proteins were included, which matched with its antioxidative activity previously reported (Luo et al., 2021). Novel functional groups of oligopeptides in *T. grandis* nuts identified in this study included ACE inhibiting, anti-inflammatory, immunomodulating oligopeptides.

ACE inhibiting oligopeptides have been reported in management of several diseases including cardiovascular disease, type 2 diabetes, increased blood pressure, increased level of triglycerides in the blood, and obesity (Chakrabarti & Wu, 2015; Girgih et al., 2013). "34 ACE inhibiting oligopeptides have been identified in our study (Supplementary table S1)."A peptide, IPP (Ile-Pro-Pro), reported with anti-inflammatory activity (Chakrabarti et al., 2015) was also identified in *T. grandis* nuts digested peptides for the first time.

Abundance profile determined by relative level of each oligopeptide showed higher abundance of ACE inhibitor peptides compared to the levels of other functional peptides i.e, pancreatic lipase inhibitor, renin inhibitor (Fig. 3). These results suggested that *T. grandis* nuts cardiovascular disease effects should be explored, along with ACE inhibition. Further exploration would better explain whether consumption of *T. grandis* nuts could be associated with a lower risk of Cardio vascular disease (CVD) mortality in humans (Quan et al., 2021; Zielińska et al., 2020). In addition, our results showed several potential antioxidative peptides in *T. grandis* nuts (Fig. 3).

3.5. Evaluation of the antioxidant activity of T. grandis nut peptides

A BP with antioxidant activity that could neutralize Reactive oxygen species (ROS) prevent lipid peroxidation and protect structure and function of cell and mitochondria, will contribute to delay aging and protect against many adverse health effects (Ren et al., 2018). The antioxidant BP may also posses other benefits, such as better water solubility, least foaming, safety, broad application etc. Therefore, antioxidant BP has gained market value for development and utilization. The mechanism of antioxidant BP is closely related to its structure (amino acids composition, their sequence, conformation, molecular weight, etc.) at molecular level (Gu et al., 2015). Antioxidant BP may be active at different levels, by stabilizing ROS, transferring electron to free radical compounds, chelation of transition metals like iron and copper, deactivation of singlet oxygen, etc. Durand et al mentioned that BP

Table 2
Functional peptides predicted from *T. grandis* nut digested peptides.

Proteins	peptide sequences	Activity	peptide numbers
Bark protein-like protein, Actin, Peroxygenase 2, Serpin-ZX, Probable glutathione S-transferase parC, Unknown, Unknown, Unknown, Non-specific lipid-transfer protein-like protein, Unknown, Bark storage protein A, Serpin-ZX, Unknown, Cytochrome *c* oxidase subunit 1, Unknown, Unknown, Superoxide dismutase, Unknown, Unknown, Chitin-binding, Protein strictosidine synthase-like 4, Vicilin-like seed storage protein At2g28490, Vicilin-like antimicrobial peptides 2–2, Non-specific lipid-transfer protein, Desiccation-related protein PCC13-62, Lactoylglutathione lyase, Probable E3 ubiquitin-protein ligase HIP1, Unknown, Bark storage protein A, MD-2-related lipid-recognition protein ROSY1, Unknown, Unknown, Mannose-specific lectin, Unknown	YGLF, MYPGIA, VNP, LNF, LEF, SDGS, FQPSF, HQG, ALP, LEE, GQP, VQV, LVQ, ITF, EAP, LEK, GVY, YPG, VTR, VGP, SGP, QGP, KGP, TLS, PPP, GTG, MAP, LGV, RVR, VVR, VAPEEHPV, LALPA, LPL, LVLL	ACE inhibitor	34
Chitin-binding, Subtilisin-like protease SBT1.8	GPR, PGP	Antithrombotic	2
Non-specific lipid-transfer protein-like protein At2g13820, Mannose-specific lectin	LALPA, LPL	Renin inhibitor	2
Actin	VAPEEHPV	Pancreatic lipase inhibitor	1
MD-2-related lipid-recognition protein ROSY1	PPG	Dipeptidyl peptidase IV inhibitor	1
4-hydroxy-4-methyl-2-oxoglutarate aldolase, Chitin-binding, Bark protein-like protein, Myosin heavy chain, thaumatin-like protein, Unknown	YGG, YYP, YGLF, EAE, GVM, KEEAE	Immunomodulating	6
Chitin-binding, Unknown, Bark storage protein A, Ricin B-like lectin R40G3, Superoxide dismutase, Vicilin-like storage protein, Unknown, Unknown, Subtilisin-like protease SBT1.7, Mannose-specific lectin, Unknown, MD-2-related lipid-recognition protein ROSY1, Unknown, Unknown, Unknown, Methylmalonate-semialdehyde dehydrogenase, Actin, 4-hydroxy-4-methyl-2-oxoglutarate aldolase, Bark storage protein A, Unknown, Desiccation-related protein PCC13-62-like protein, Unknown, Protein fizzy-related 2, Unknown, Non-specific lipid-transfer protein-like protein At2g13820, Unknown, Bark protein-like protein, defensin 4	EYY, YIY, YLY, KHH, LHE, LHI, LHN, LWF, LWG, LWS, PHY, VKV, GGE, EAK, TFE, NHAV, TDY, LLR, DHG, GAA, GPP, ACQ, VYV, VAPEEHPV, LALPA, LPL, YGLF, GYCVSDNNCK	Antioxidative	27
Polyubiquitin 11	IPP	Anti inflammatory	1
Desiccation-related protein PCC13-62, Ricin B-like lectin R40G3	PPPA, PPAP	Dipeptidyl carboxypeptidase inhibitor	2
Bark protein-like protein	YGLF	Opioid agonist	1

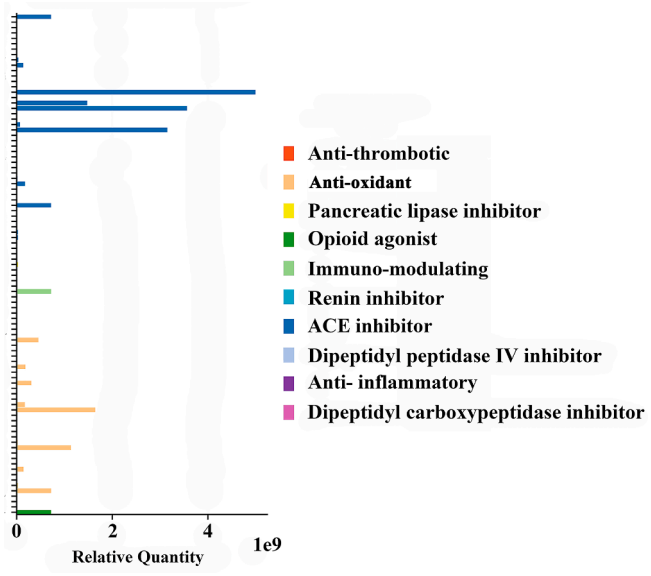

Fig. 3.

together with parent protein shows antioxidant activity through different ways by radical scavenging, metal chelation, electron or hydrogen transfer reduction and aldehyde quenching (Durand et al., 2021).

T. grandis has been already recognized for its antioxidant activity. However, less information (Quan et al., 2021) is available on the anti-oxidant activity of *T. grandis* nut peptides. According to the proteome sequencing results in this study, anti-oxidative oligopeptides has been predicted after digestion with trypsin. Thus, we performed anti-oxidative assay to study the activity of *T. grandis* nuts oligopeptides. The antioxidative activity was evaluated with $ABTS^{+}$ assay, DPPH radical scavenging, and Fe^{2+} chelating, as shown in Fig. 4(A–C). Interestingly, the hydrolyzed peptides (digested with trypsin) showed a higher antioxidant activity than *T. grandis* protein extract, especially in DPPH scavenging and Fe^{2+} chelating. Our results indicated that the hydrolyzed fraction contains peptides that may act as antioxidant, in

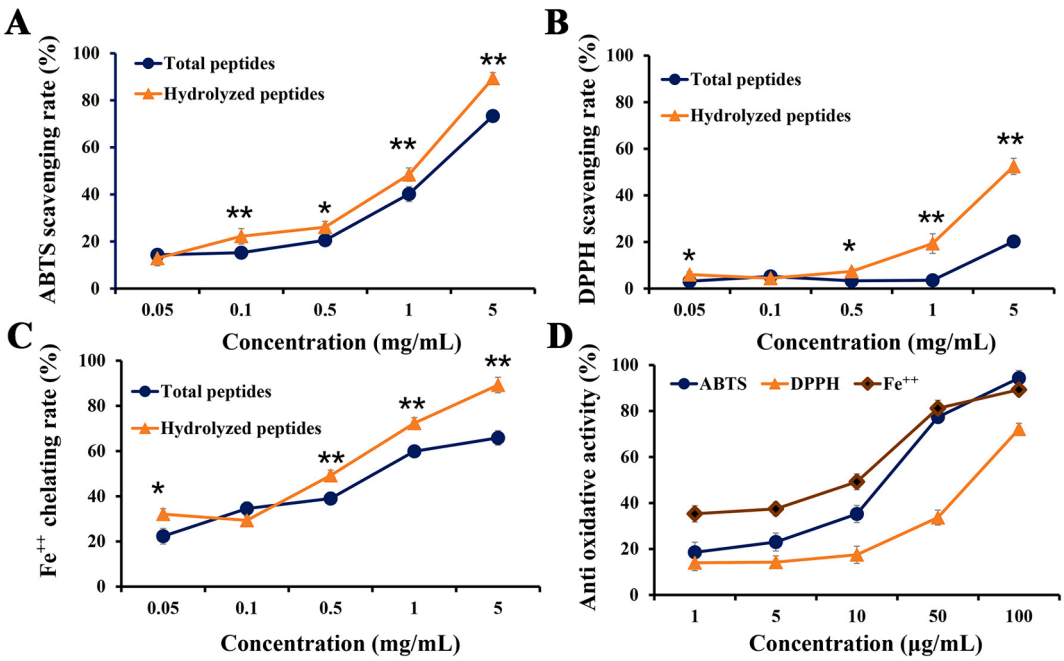

Fig. 4.

accordance to the results of functional oligopeptides prediction of proteome.

Quan *et al* studied antioxidant activity of *T. grandis* protein and its hydrolysates, with five proteases, using $ABTS^{+}$, DPPH, hydroxyl, and superoxide radical scavenging, along with the metal chelation activity. They concluded that $ABTS^{+}$ and DPPH radical scavenging capacities, and metal chelating ability, were increased in a concentration dependent manner. Our results are almost similar, since we have also predicted that both total peptides and hydrolyzed peptides had dose-dependent anti-oxidative activity (Quan et al., 2021). Luo *et al* also studied the antioxidant activity of *T. grandis* meal protein hydrolysate. They concluded that the BP antioxidant activity is dependent to the amino acid composition, the molecular weight and also different physical properties. They also proposed that the radical scavenging activity ($ABTS^{+}$, DPPH) and Fe^{+2} chelation activity assays are not sufficient to estimate the antioxidant potential of a better peptide (Luo et al., 2021).

Our results provide a basis for utilization of *T. grandis* meal for development of natural products. More interestingly, we find a peptide with amino acid sequence GYCVSDNNC, which was digested from *T. grandis* nut defensin 4 by trypsin, showed antioxidant activity by ABTS, DPPH and Fe^{2+} assay (Fig. 4D). This shows that some functional proteins as a food source when absorbed in gut are changed to functional peptides with better function.

4. Conclusion

In order to draw the attention of *T. grandis* nut to be effectively use in nutraceuticals and in food industry, the structural characterization of the proteins and the BP profiling were investigated. Proteome sequencing was done to reveal functional peptides distribution in *T. grandis* nut. A total of 348 peptides were found and belonged to 142 proteins. Further, 10 groups of functional oligopeptides related to ACE inhibiting, anti-inflammatory, immunomodulating were annotated. More importantly, we reported a functional protein, defensin 4, with activity in anti-microbial, could digested to an antioxidant peptide: GYCVSDNNC, these results give us new concepts in protein-source foods production. The current study has comprehensively predicted the functional bioactive peptides using proteome sequencing.

Our study reported the BP profiling in *T. grandis* nuts for potential health benefits that have to be tested using *in vitro* and *in vivo* bioassays. Further studies could be done on isolation, purification, the structure of these functional peptides using nano-HPLC, Nuclear Magnetic resonance (NMR) and X-ray crystallography along with *invivo* study to explore the potential use of *T. grandis* to be used as dietary supplement in food and as traditional medicine.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134843.

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