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In vitro and *in silico* evaluation of antioxidant capacity of protein hydrolysates from chia seed by-product from the oil industry

Evaluación *in vitro* e *in silico* de la capacidad antioxidante de los hidrolizados proteicos del subproducto de la semilla de chia de la industria del aceite

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Technological innovation: Give high added value to chia seed by-product for the benefit of human health.

Industrial application area: decreasing the oil industry residue and its application as a functional ingredient.

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Resumen

El residuo agroindustrial proveniente de la industria del aceite de la semilla de chía tiene alto contenido proteico y podrían hidrolizarse para la obtención de péptidos con capacidad antioxidante. El objetivo del presente trabajo fue obtener un hidrolizado proteico a partir del residuo de la chía y caracterizar su biofuncionalidad *in vitro* e *in silico*. La capacidad antioxidante del hidrolizado de proteína de chía (HPC) por donación de átomos de hidrógeno y electrones se midió mediante el método de captación de radical DPPH y $ABTS^{+}$. El poder antioxidante reductor férrico se midió mediante FRAP. Finalmente, se realizó el acoplamiento molecular de péptidos derivados de HPC con un componente del complejo NADPH oxidasa (productor de especies reactivas de oxígeno en el cuerpo). La capacidad antioxidante de HPC (5 mg/mL) determinada por captación del radical DPPH fue de 35.60 ±4.14% y por captación del radical $ABTS^{+}$ fue de 30.02 ±2.17%. El poder antioxidante reductor medido por FRAP fue de 0.05mM Equivalentes de Trolox/mg de HPC. Según el análisis *in silico*, los péptidos presentes en el HPC interactuaron con los aminoácidos de p47phox (proteína de NADPH oxidasa) con la energía de unión más baja de -7.4 kcal/mol. El HPC mostró capacidad antioxidante como donante de átomos de hidrógeno y de electrones. Además, los péptidos presentes en HPC tuvieron potencial de inhibir la actividad de NADPH oxidasa al

interactuar con su componente p47phox. De esta manera, los HPC mostraron potencial de proteger contra el daño oxidativo en macromoléculas y la muerte celular acelerada *in silico*.

Palabras clave: Capacidad antioxidante, hidrolizados, péptidos, residuo de chía.

Abstract

Agro-industrial waste from the oil industry derived from chia has high protein content and could be hydrolyzed to obtain bioactive peptides with antioxidant capacity. This study aimed to obtain protein hydrolysates from chia seed by-product and characterize their functionality *in vitro* and *in silico*. The antioxidant capacity of the chia protein hydrolysate (CPH) by donating hydrogen atoms and electrons was measured by DPPH and $ABTS^{+}$ radical scavenging method. The ferric reducing antioxidant power was measured by FRAP. Finally, molecular docking of peptides derived from CPH was performed with a component of NADPH oxidase complex (producer of reactive oxygen species in the body). The antioxidant capacity of CPH (5 mg/mL) determined by DPPH radical scavenging was 35.60 ± 4.14% and by ABTS radical scavenging was 30.02 ± 2.17%. The reducing antioxidant power measured by FRAP was equivalent to 0.05 mM Trolox Equivalents/mg CPH. According to *in silico* analysis, the peptides present in the CPH interacted with amino acids of p47phox (protein of NADPH oxidase) with the lowest binding energy of -7.4 kcal/mol. The CPH showed antioxidant capacity as hydrogen and electrons donors. Furthermore, the peptides present in CPH exhibited the potential to inhibit NADPH oxidase complex activity by interacting with its p47phox component. In this way, CPH has the potential to protect against oxidative damage in macromolecules and accelerated cellular death.

Keywords: Antioxidant capacity, chia by-product, hydrolysates, peptides.

1. Introduction

Chia is an oilseed that belongs to the Labiatae family. Beyond the excellent lipid profile, chia seed has a higher amount of protein (19-23%) than grains traditionally used in Mexico, besides, it also has 18 of the 20 amino acids that build proteins (Sandoval-Oliveros & Paredes-López, 2013). The production, consumption, and demand of chia in Mexico and the world have increased in recent years. It is a source of oil with high levels of polyunsaturated fatty acids and phenolic compounds. World production has grown rapidly, an example is Nicaragua, where chia production went from 230,040 tons in 2013, to 8,281,440 tons in 2014 (López et al., 2017). Chia imports to the European Union in 2017-2018 were 17,500 tons, of which Paraguay ranks first in exporting 5,417 tons, and Mexico only exported 1,500 tons (Eurostat, 2018).

However, the industrial importance of chia seed has focused on its oil, generating significant amounts of residues rich in dietary fiber and protein, with great potential to be used as a source of bioactive peptides (Capitani et al., 2012). Biological activities have been reported that present in chia, soy, and bean with antioxidant capacity, the inhibitory activity of the angiotensin-converting enzyme, and lipid regulation (Mojica et al., 2017; Orona-Tamayo et al., 2015; San Pablo-Osorio et al., 2019; Torres et al., 2006).

Peptides with antioxidant activity have been relevant since they have the potential to help in the prevention and treatment of chronic degenerative and epidemiological non-

communicable diseases such as cardiovascular, cerebrovascular, cancer or diabetes since there is evidence *in vitro* tests in which peptides act by inhibiting the oxidation of free radicals (Zou et al., 2016).

Free radicals contribute to various physiological alterations in humans, causing disease. The mechanism of action of some antioxidants is to break the oxidation chain of lipid radicals by donating hydrogen atoms or electrons, or both, to produce antioxidant radicals that protect lipid molecules (Apak et al., 2016).

In this way, the initiation of free radical oxidation is inhibited when it reacts with a lipid radical; or diffusion when reacting with peroxyl, alkoxy, or lipid radicals (Apak et al., 2016). Some methods for measuring antioxidant activity by this mechanism are DPPH and $ABTS^{+}$ radical scavenging, the FRAP method is used to evaluate the reducing capacity of peptides by donating their electrons to reduce Fe 3^{+} to Fe 2^{+}. Another mechanism of antioxidant capacity is inhibiting pro-oxidant enzymes such as xanthine oxidase, NADPH oxidase, etc., this type of enzyme produces reactive oxygen species (ROS) (Zou et al., 2016). The multi-subunit NADPH oxidase complex plays a very important role in host defense to attack microbial infection by producing reactive oxygen species (Diebold et al., 2015). In this case, ROS production is generated from NADPH oxidase, which must be activated by binding the cytoplasmic complex p40-p47-p67-phox with heterodimeric flavocytochrome p22-gp91phox that is bound to the membrane. In this way, p47phox interacts with the cytoplasmic tail of p22phox, forming the active membrane-bound enzyme complex (Groemping et al., 2003). The amount of ROS in the body can be balanced by the action of antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase, however, an excess of ROS caused by external conditions such as unhealthy food, contamination, ultraviolet light, among others, can cause inflammation states that activate NADPH oxidase (Apak et al., 2016; Diebold et al., 2015; Zou et al., 2016). One way to evaluate the inhibition of this protein is by *in silico* method, calculating the free binding energy between the peptides present in the CPH and p47phox by molecular docking.

Molecular docking is a computational method used to theorize a binding model between two molecules, it is widely used in the pharmaceutical industry to develop new drugs. It calculates the free energy of binding that exists between two molecules, generally, it is a small molecule (Ligand) and a macromolecule (Protein). When the binding free energy becomes lower, the binding force between the protein and the ligand increases, indicating that the evaluated protein's inhibition could be carried out (Prieto-Martínez et al., 2018). Therefore, this work aimed to evaluate the antioxidant capacity of the peptides derived from chia seed by-product *in vitro* and *in silico*.

2. Materials and Methods

Chia seed meal by-product was donated by Grupo Chia Omega de México. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, methanol solution, pepsin from porcine gastric mucosa, and sodium chloride were purchased from Sigma Aldrich (St Louis, MO, USA). The spectrophotometer used was Multiskan GO and the centrifuge used was Thermo sorvall legend XTR refrigerated centrifuge (Thermo Scientific).

2.1. Chia protein hydrolysate (CPH)

Chia protein hydrolysates were obtained according to San Pablo-Osorio et al. (2019). Briefly, first, chia protein isolates were obtained with the solubilization of proteins at pH 11.2 and their subsequent precipitation at pH 4.5. Second, the CPH was obtained from chia protein isolate by enzymatic hydrolysis with pepsin for 1 hour in 0.03 M NaCl, and the reaction was stopped by heat shock (85-90°C). And finally, after the heat shock, it was centrifuged, and the supernatant was stored at -8°C until analysis.

2.2. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (ABTS)

This technique is based on the discoloration of the $ABTS^{+}$ radical due to the donation of hydrogens or electrons offered by antioxidants. The $ABTS^{+}$ radical is a chromophore having an absorbance at 734 nm generated by the oxidation of ABTS (2,2'-azino-bis- (ammonium 3-ethyl benzothiazoline-6-sulfonate) with potassium persulfate (Re et al., 1999). It was carried out according to Orona-Tamayo et al. (2015) with some modifications. Briefly, a solution of 7 mM ABTS and 2.45 mM potassium persulfate was prepared in distilled water and left stirring for 16-20 hours in dark conditions for its total oxidation. Subsequently, the $ABTS^{+}$ radical solution was diluted with water until obtaining an absorbance of 0.7 at a wavelength of 734 nm. 16.5 mg of CPH was diluted in 1 mL of distilled water, and dilutions were made, water was used as a blank. 20 µL of the sample were placed in a 96-well microplate and 180 µL of the $ABTS^{+}$ radical solution was added, it was left incubate for 3 minutes, the absorbance was read at 734 nm, and the antioxidant capacity of the samples was calculated according to the following equation:

$$\%\boldsymbol{Antioxidant\ capacity}: \left(\left(\frac{\boldsymbol{A-A1}}{\boldsymbol{A}}\right) * \mathbf{100}\right)$$

Where A= absorbance of the blank and A1= absorbance of the sample.

2.3. Radical scavenging activity 2,2-diphenyl-1-picryl-hydrazil (DPPH)

DPPH is a free radical that, when solubilized in methanol, has an absorbance of 517 nm. When DPPH radical meets a proton donor molecule such as antioxidants, the DPPH radical is eliminated, and absorbance is reduced. This radical scavenging test is fast and reproducible, which is why it is widely used to predict the antioxidant activities of compounds. It was carried out according to Zhang et al. (2008) with some modifications. Briefly, a methanolic solution was prepared in an 80:20 ratio (methanol-water) that was used to prepare a 5.98 µM DPPH radical solution, subsequently, 16.5 mg of CPH were weighed and diluted in 1 mL of distilled water, dilutions were made in the following concentrations: 5, 2.5, 1.25, 0.625 and 0.3125 mg/mL, water was used as a blank. 20 µL of the sample was placed in a 96-well microplate and 180 µL of DPPH radical solution was added, it was left to incubate for 30 min and the absorbance was read at 517 nm, whole experiment was carried out in dark conditions. The antioxidant capacity was calculated with the following equation:

$$\%\boldsymbol{Antioxidant\ capacity}: \left(\left(\frac{\boldsymbol{A-A1}}{\boldsymbol{A}}\right) * \mathbf{100}\right)$$

Where A= absorbance of the blank and A1= absorbance of the sample.

2.4. Ferric reducing antioxidant power (FRAP assay)

FRAP assay was performed as previously described by Benzie & Strain (1999). The

experiment was carried out at 37°C under conditions of pH 3.6. In FRAP assay, the reducers (antioxidants) in the sample reduce the Fe (III)/tripyridyltriazine complex, present in stoichiometric excess, to the blue ferrous form, with an increase in absorbance at 593 nm. ΔA is proportional to the combined (total) ferrous reducing antioxidant power (FRAP value) of the antioxidants in the sample. Final results were expressed as millimolar (mM) Trolox equivalents (TE) per gram on a dry basis of chia protein hydrolysate (mM TE/mg HPC).

2.5. Molecular docking

In silico analysis of the sequence of five peptides obtained by San Pablo-Osorio et al. (2019) present in CPH with p47phox, component of an enzymatic complex of NADPH oxidase. The molecular interactions between peptide sequences and catalytic site of p47phox were predicted using molecular docking. The crystal structure of p47phox (1OV3) with a resolution of 1.80 Å, was obtained from Protein Data Bank (http://www.rcsb.org) (Lafarga et al., 2016). Peptides were drawn in Marvin Sketch Software version 17.29.0 and transformed to the PDB extension in Discovery Studio Visualizer software version 17.2.0.16349. Flexible torques, loads, and grid size were assigned using Autodock tools (Morris et al., 2009). Docking calculations were made using AutoDock Vina (Olson & Trott, 2009), and coupling with the lowest binding energy was selected for visualization in Discovery Studio Visualizer Software (Luna-Vital et al., 2017; 2016).

2.6. Statistical analysis

Statistical analysis was done using Statgraphics Centurion Software version 16.1. To determine if the mean of results were different, a Tukey ANOVA test was carried out, with a significance level of $\alpha = 0.05$. Those groups among which $P < 0.05$ were considered different.

3. Results and Discussion

3.1. 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity (ABTS)

Table 1 shows the different concentrations of CPH that were evaluated to measure antioxidant capacity by ABTS radical scavenging. CPH at a concentration of 5 mg/mL had 30% antioxidant capacity. The highest concentrations of CPH were evaluated from 0.65 mg/mL to 26 mg/mL, however, when graphing the data, it presents a logarithmic trend and doesn´t have differences between values of the hydrolysate at a concentration of 6 mg/mL and 26 mg/mL with 40.61% (data not shown). Antioxidant capacity has been reported by ABTS radical scavenging from other vegetable protein hydrolysates such as rice with 35% (Zhang et al., 2020), also from peanuts with 31%, soy with 20%, and whey protein with 21% (Jin et al., 2020) at the same concentration of 5 mg/mL. Due to the above, the use of emulsions of this type of hydrolysates with curcumin to increase its antioxidant capacity has also been reported (Jin et al., 2020; Li et al., 2019). However, a higher antioxidant capacity has also been reported in poultry skin hydrolysates with 50% at 1.10 mg/mL (Nadalian et al., 2019), also in mungbean hydrolysates with 70%. Nevertheless, CPH presents antioxidant capacity similar to other vegetable protein hydrolysates by ABTS radical scavenging.

Table 1. Percentage of inhibition of ABTS radical of CPH at different concentrations.

ABTS radical scavenging	
mg/mL	**(%)**
5	30.02 ± 2.17[a]
2.5	15.69 ± 1.31[b]
1.25	8.57 ± 0.81[c]
0.625	4.46 ± 1.62[d]
0.312	3.09 ± 1.33[d]

**The values shown correspond to the mean ± standard deviation. [a-d] Different letters indicate the significant difference with 95 % confidence. Data are the average of 3 determinations.

3.2. Radical scavenging activity 2,2-diphenyl-1-picryl-hydrazil-hydrate (DPPH)

The different concentrations of CPH are shown in Table 2. To solubilize DPPH radical it is necessary to use a methanol solution which causes precipitation of proteins (Wessel & Flügge, 1984), however, it was possible to measure the antioxidant capacity by this method up to 5 mg/mL concentration of CPH with a value of 35.6%. Peptides with antioxidant capacity of animal and vegetable origin have been reported, Nadalian et al. (2019) reported peptides from hydrolyzed poultry skin with 50% of antioxidant capacity by DPPH radical scavenging at 2.80 ± 0.37 mg/mL, also peptides from plant origin such as rice proteins with 50% at 5 mg/mL (Zhang et al., 2020), lower antioxidant capacity was also reported with sorghum hydrolysates kafirin, 20.43% at the same concentration (5 mg/mL) (Xu et al., 2019). The antioxidant capacity of peanut hydrolyzates with 32%, of soy hydrolyzates with 31%, and of whey hydrolyzates with 24% had also been reported at the same concentration of 5 mg/mL (Jin et al., 2020). Low molecular weight peptides are generally associated with higher antioxidant activity in contrast to larger peptides this can be attributed to possible steric hindrance due to the size and increased peptide repulsion of the larger peptides (Nwachukwu & Aluko, 2019). In this case, peptides present in CPH with molecular weights of 4-12 kDa were reported in previous work (San Pablo-Osorio et al., 2019) because of that the size of peptides could be influencing the antioxidant capacity found by these methods, notwithstanding, CPH has antioxidant capacity compared to other vegetable protein hydrolysates.

Table 2. Percentage of inhibition of DPPH radical of CPH at different concentrations.

DPPH radical scavenging	
mg/mL	**(%)**
5	35.60 ± 4.14[a]
2.5	18.62 ± 3.65[b]
1.25	10.86 ± 4.72[bc]
0.625	4.53 ± 1.43[c]
0.312	2.58 ± 1.03[c]

**The values shown correspond to the mean ± standard deviation. [a-c] Different letters indicate the significant difference with 95% confidence. Data are the average of 3 determinations.

3.3. Ferric reducing antioxidant power (FRAP assay)

Calibration curves with Trolox as positive control and CPH were performed to establish a ratio of Trolox equivalents (mM TE) per mg of CPH. The behavior of the curves was linear, that is, as the concentration increases, the reducing antioxidant power increases (Figure 1). CPH presented high ferric reducing antioxidant power with 0.05 mM TE/mg CPH, compared to a red algae protein hydrolysate that has 0.1132 mM TE/g dry base (Pimentel et al., 2020) and also with a

grain residue hydrolysate used in a brewery that had 0.28mM TE/g dry base (Connolly et al., 2019). Therefore, CPH has a greater antioxidant capacity as a ferric reducer compared to other hydrolysates of plant origin. This method is also based on donating electrons present in peptides but measures them in different ways and gives us a better vision of the antioxidant capacity of CPH.

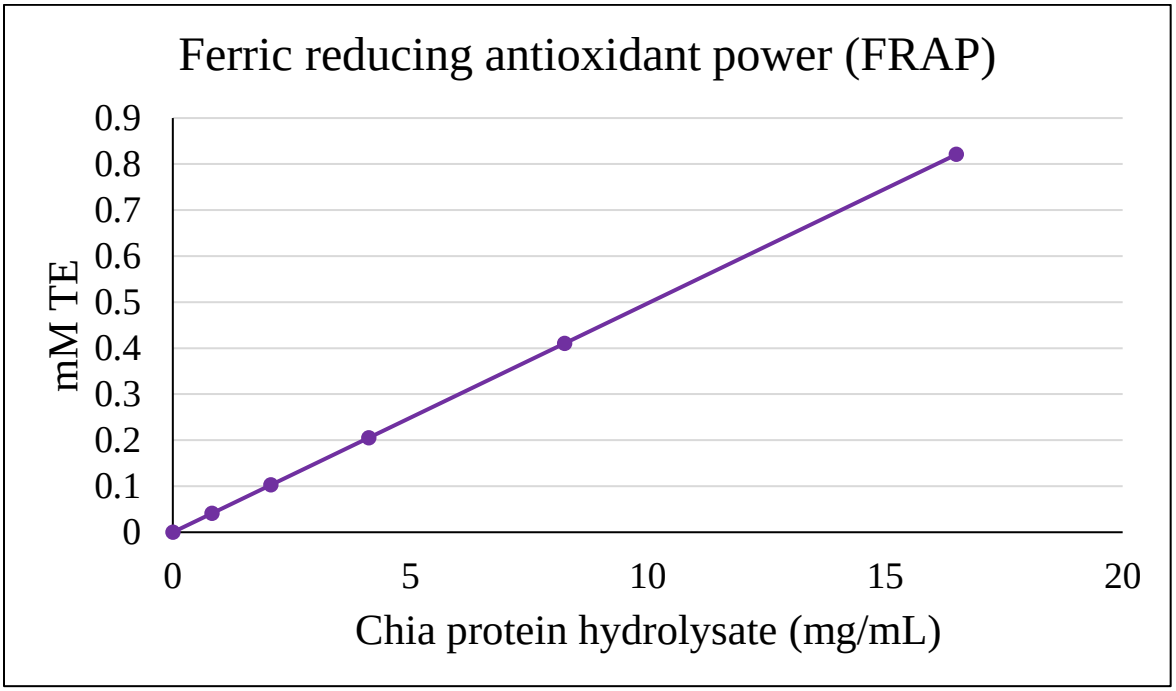

Figure 1. Ferric reducing power, mM Trolox equivalents of CPH at different concentrations.

3.4. Molecular docking

Molecular docking was made with five sequences reported by San Pablo-Osorio et al. (2019) present in CPH (Table 3) with p47phox subunit of NADPH oxidase complex resulting in low binding energy values increasing the binding strength between CPH peptides and p47phox. PGLTIGDTIPNL was a peptide with lower binding energy (-7.4 kcal/mol), even lower than Ebselen, a known inhibitor of p47phox (Solbak et al., 2020), however, the five peptides could interact with amino acids that interact with the p22phox subunit with binding energies in the range -6.6 to -7.4 kcal/mol. The amino acids of p47phox that interact with p22phox are Trp263B, Trp193A, Pro206B, Tyr167A, Pro276B, Tyr279B, Tyr274B, Phe209B, Trp204B and Ala165A (Macías-Pérez et al., 2013; Ogura et al., 2006), it was important peptides could interact with any of these amino acids. Table 3 shows types of bonds, distance, and amino acids with which each of the peptides interacts. Figure 2 shows the interaction of known inhibitor of p47phox, Ebselen with important amino acids to inhibit its interaction with p22phox, and thus avoid the activation of NADPH oxidase complex is represented, in the same way in Figure 3 interaction of PGLTIGDTIPNL (peptide present in CPH) with p47phox is represented. Although all the peptides were able to interact with p47phox, IVSPLAGRL, LIVSPLAGRL, and PGLTIGDTIPNL were the peptides that interact with Trp263 (p47phox) with three different bonds in that amino acid, thus increasing the potential to inhibit the binding of p47phox with p22phox.

Table 3. Molecular docking of 5 sequences peptides (San Pablo-Osorio et al., 2019) with the p47phox subunit of NADPH oxidase.

Peptide	Predicted binding energy kcal/mol	Bond (Å)			
		Hydrogen-bonds	Carbon-hydrogen bonds	Hydrophobic bonds	Pi-bond
IVSPLAGRL	-6.6	Trp263B (4.83), Ser208B (4.28)-(3.98)		Trp263B (6.51)-(4.58)-(3.89), Tyr274B (4.33), Pro212B (5.53)	
LIVSPLAGRL	-6.8	Trp263B (5.69), Ser208B (4.20)-(4.23), Asp261B (4.23), Leu260B (3.91)	Asp217B (5.29)	Trp193A (3.76), Trp263B (4.17)-(4.00), Tyr274B (4.12)	
LSLPNYHPNPRL	-7.2	Glu223B (5.08), Leu210B (6.98), Leu260B (3.99), Ser208B (3.94)	Tyr274B (3.93), His257B (3.87), Leu260B (4.82)	Tyr274B (7.06), Leu259B (4.70), Phe209B (2.64), Tyr167A (4.29), Ala207B (4.29), Val265B (6.69)	Glu223B (6.44)
PGLTIGDTIPNL	-7.4	Trp193A (4.84), Tyr237B (6.10), Trp263B (5.09), Asp261B (5.63), Tyr274B (5.92)	Gly192A (4.46)	Phe209B (5.51), Ile164A (5.76), Trp193A (4.16)-(5.98)-(6.65)	
TAQEPTIRF	-6.7	Asp261B (3.68), Leu260B (4.24), Ser208B (4.84), Tyr274B (5.90)		Ile164A (5.01), Tyr274B (4.39)	
Ebselen	-6.9			Trp263B (6.05)-(6.28), Trp193A (5.07), Pro206B (6.53), Pro276B (5.31)	Trp263B (6.28)

(**A°**): Distance between amino acids of the peptide and p47phox. p47phox amino acids that interacted with two or more amino acids in the peptides are marked with a dash (-). **A:** Alanine; **R:** Arginine; **N:** Asparagine; **D:** Aspartate; **C:** Cysteine; **Q:** Glutamine; **G:** Glycine; **H:** Histidine; **I:** Isoleucine; **L:** Leucine; **K:** Lysine; **M:** Methionine; **F:** Phenylalanine; **P:** Proline; **S:** Serine; **T:** Threonine; **Y:** Tyrosine; **V:** Valine and **E:** Glutamic acid.

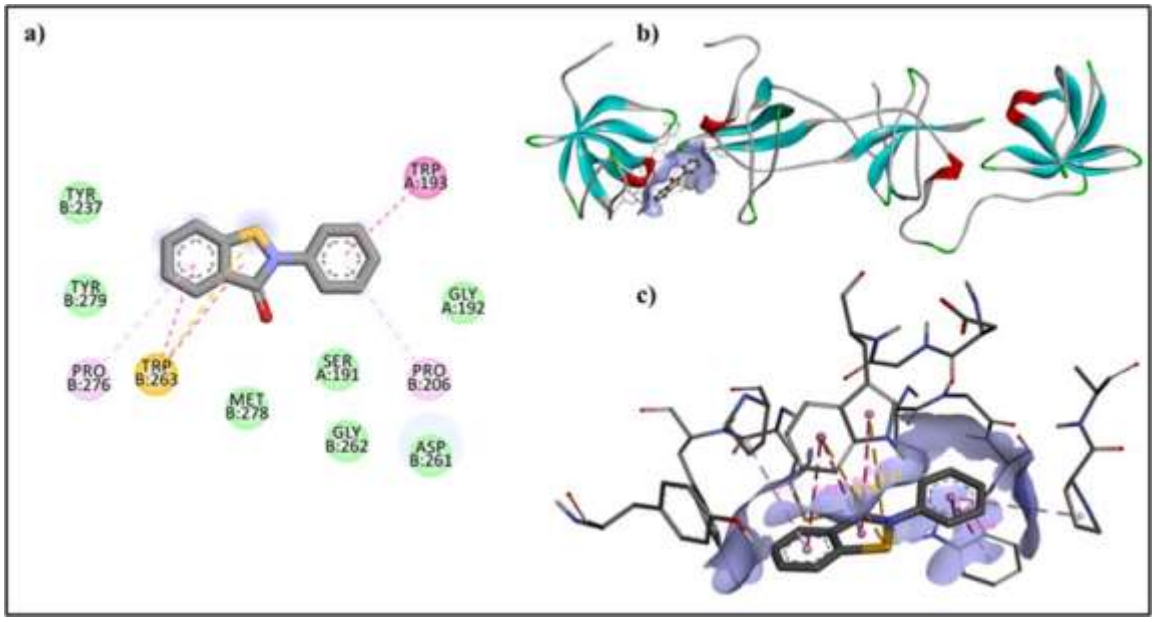

Figure 2. Molecular docking scheme exemplifying the p47phox and Ebselen interactions. (a) Potential interactions with amino acid residues of p47phox; (b) example of p47phox with Ebselen and (c) the identified pocket of p47phox interacts with Ebselen determined by molecular docking.

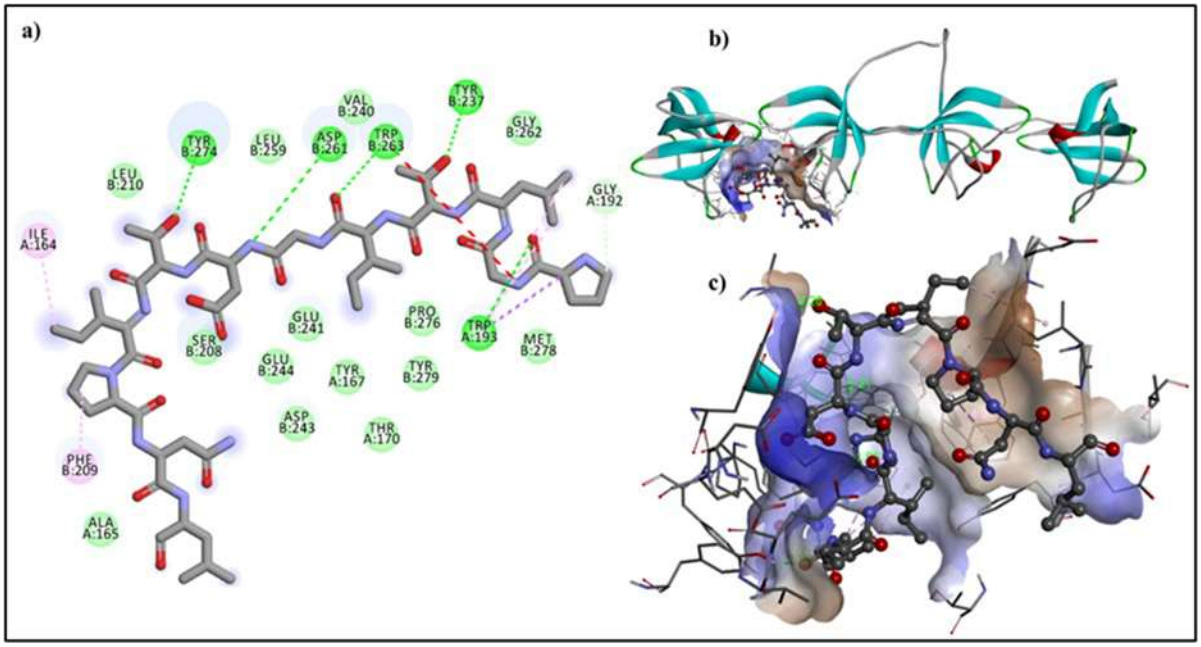

Figure 3. Molecular docking scheme that exemplifies the p47phox and PGLTIGDTIPNL interactions. (a) Potential interactions with amino acid residues of p47phox; (b) Example of p47phox with PGLTIGDTIPNL and (c) the identified pocket of p47phox interacts with PGLTIGDTIPNL determined by molecular docking.

4. Conclusions

The chia protein hydrolysate has the antioxidant capacity as a donor of hydrogen atoms, electrons, and iron-reducing antioxidant power. The peptides present in the chia protein hydrolysate have the potential to interact with the p47phox component of NADPH oxidase complex, thus inhibiting its activity according to molecular docking that was carried out. Chia protein hydrolysate has the potential to protect against oxidative damage in lipids, proteins,

DNA, tissue damage, and accelerated cell death. Therefore, the chia seed could be fully exploited using its by-product that is generated once the chia oil is extracted by pressing, giving it high added value.

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