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Inhibition Effects of Phenolic Compounds on Human Serum Paraoxonase-1 Enzyme

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ABSTRACT: Metabolic processes in living organisms are closely related to the catalytic activity of enzymes. Inhibition or induction of enzymes leads to toxicities and metabolic interactions. This study aims to contribute to the growing drug design field by studying PON1-phenolic compound interactions. For this purpose, the paraoxonase-1 enzyme was purified from fresh human serum by using rapid and different chromatographic techniques. Additionally, it was investigated the inhibitory effects of some phenolic substances on the PON1 and was found that the purified enzyme had the molecular weight of 43 kDa and the specific activity of 3945.15 EU mg^{-1}. These compounds showed potent inhibition against PON1, especially homovanillic acid exhibited a significant inhibition profile against PON1 with an IC_{50} value of 13.84±0.08 mM. K_i constants were 6.10±0.26 mM for homovanillic acid and 16.96±0.76 mM for phloridzin dihydrate. Homovanillic acid had competitive inhibition while the phloridzin dihydrate inhibited the PON1 as non-competitive. Also, molecular docking computations were performed by using the Glide XP mode. Glide energy of the homovanillic acid determined to be -23.95 kcal mol^{-1}.

Keywords: Paraoxonase, HDL, chromatography, inhibition, phenolic compound, molecular docking.

Fenolik Bileşiklerin İnsan Serum Paraoksonaz-1 Enzimi Üzerindeki İnhibisyon Etkileri

ÖZET: Canlı organizmalardaki metabolik süreçler, enzimlerin katalitik aktivitesi ile yakından ilişkilidir. Enzimlerin inhibisyonu veya indüksiyonu toksisiteye ve metabolik etkileşimlere yol açar. Bu çalışma, PON1-fenolik madde etkileşimlerini inceleyerek büyüyen ilaç tasarım alanına katkıda bulunmayı amaçlamaktadır. Bu amaçla, paraoksonaz-1 enzimi, hızlı ve farklı kromatografik teknikler kullanılarak taze insan serumundan saflaştırılmıştır. Ek olarak, bazı fenolik bileşiklerin PON1 üzerindeki inhibisyon etkileri araştırılmış ve saflaştırılan enzimin 3945.15 EU mg^{-1} protein spesifik aktiviteye ve 43 kDa molekül ağırlığına sahip olduğu bulunmuştur. Bu bileşikler PON1'e karşı güçlü inhibisyon gösterdiler, özellikle homovanilik asit 13.84±0.08 mM'lık bir IC_{50} değeri ile PON1'e karşı önemli bir inhibisyon profili sergiledi. K_i sabitleri homovanillik asit için 6.10±0.26 mM ve phloridzin dihidrat için 16.96±0.76 mM idi. Homovanilik asit yarışmalı inhibisyona sahipken, phloridzin dihidrat PON1'i yarışmasız olarak inhibe etmiştir. Ayrıca, moleküler yerleştirme hesaplamaları Glide XP modu kullanılarak gerçekleştirilmiştir. Homovanilik asitin glide enerjisi -23.95 kcal mol^{-1} olarak belirlenmiştir.

Anahtar kelimeler: Paraoksonaz, HDL, kromatografi, inhibisyon, fenolik madde, moleküler yerleştirme

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INTRODUCTION

HDL-associated paraoxonase (organophosphate hydrolase; PON1) is responsible for reducing the oxidation of LDL by metabolizing oxidized phospholipids. PON1 is also accountable for most of the anti-inflammatory, and antioxidative effects of HDL (Mackness et al., 1993; Teiber et al., 2018). Cardiovascular diseases are highly related to low HDL. It has been shown that treatments for increasing plasma HDL cholesterol in humans especially support regression of cardiovascular diseases and reduced the progression of atherosclerosis. HDL also has acts such as the inhibition of proteinases in the plaque protecting regular endothelial function (Kontush and Chapman, 2006). All functions of HDL which subscribe to its protective effects act directly attenuating risk of coronary heart diseases. The reverse transfer of cholesterol from cells and the easy transport of macrophages in the artery wall are the antiatherogenic properties of HDL.

PON, which is a calcium-dependent esterase, has the ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. PON1 detoxifies various nerve agents and also hydrolyzes metabolites of a number of other insecticides (Costa et al., 1999; Manco et al., 2018). Additionally, PON1 is an esterase which has a molecular mass of approximately 43-45 kDa and consists of 354 amino acids (Furlong et al., 1989; Hassett et al., 1991). Decreasing PON1 activity has been reported to be a potent risk factor for coronary events rather than genetic polymorphisms. PON1 is the most dominant antioxidant enzyme, among some other HDL-associated proteins such as apolipoprotein A1, platelet-activating factor acetyltransferase, and lecithin-cholesterol acyltransferase (Mackness and Durrington, 1995).

Phenolic compounds are a critical group produced by plants as secondary metabolites. Plants synthesize phenolic substances to defend against pathogens, protection against ultraviolet light and to regulate metabolic activities (Velderrain-Rodríguez et al., 2014). Phenolic substances are classified according to with respect to attached substituents and the number of phenolic rings (Manach et al., 2005). Also, the position and number of these components affect biological function, significantly (Fraga et al., 2010). It is determined that dietary components, including phenolic compounds, influence drug metabolizing enzymes and can cause important toxicological results (Middleton et al., 2000; Surh, 1998). The significance of these compounds is rising, because of the increasing frequency of various diseases such as coronary artery disease, cancer, Alzheimer's disease, and diabetes, which are develop linked to their eating habits in recent times (Gutiérrez-Grijalva et al., 2016; Kuzu et al., 2018).

Homovanillic acid belongs to the class of phenolic acids known as hydroxyphenylacetic acids and is a strong radical scavenger (Tuck and Hayball, 2002). Phloridzin dihydrate (Phloretin 2'-glucoside), a natural anti-diabetic molecule, is the flavonoid glucoside as a member of dihydrochalcone family (Wang et al., 2007). This flavonoid has different biological activities (Masumoto et al., 2009).

Metabolic processes in living organisms are closely related to the catalytic activity of enzymes. Changes in enzyme activity can cause various diseases. Thus, compounds which inhibit enzyme activity must be beneficial therapeutic agents (Copeland, 2004; Copeland, 2013; Robertson, 2005). This study set out to the *in vitro* effects of some phenolic substances, such as homovanillic acid and phloridzin dihydrate (Figure 1) on PON1 enzyme activity. For this reason, PON1 was purified from human serum by using simple and rapid methods, easily. Moreover, to support the experimental results, molecular docking studies also were performed.

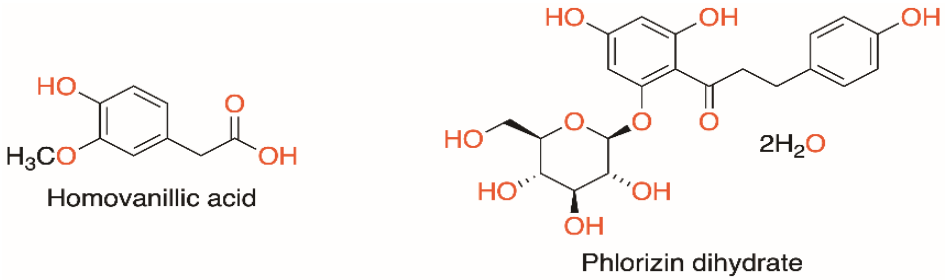


Figure 1. The molecular structures of the homovanillic acid and phloridzin dehydrate

MATERIALS AND METHODS

General Information

The chromatography media, including IEX and SEC resins, and all chemical agents were purchased from Sigma-Aldrich Chemie. The chemical substances used for preparing the test solutions were of analytical grade. Human serum samples were provided from Research Hospital of Erzincan Binali Yıldırım University (Erzincan, Turkey).

Paraoxonase Activity Determination

The process of paraoxonase enzyme activity was carried out as in our previous research, spectrophotometrically (Türkeş et al., 2014). Enzymatic activity was computed by using the molar absorptivity coefficient (ε=18,290 $M^{-1}cm^{-1}$) of p-nitrophenol at 412 nm (Figure 2) (Renault et al., 2006). The EU is the amount of the enzyme that catalyzes the reaction of 1 micromole of substrate per minute under standard conditions at 25°C (Mackness and Durrington, 1995).

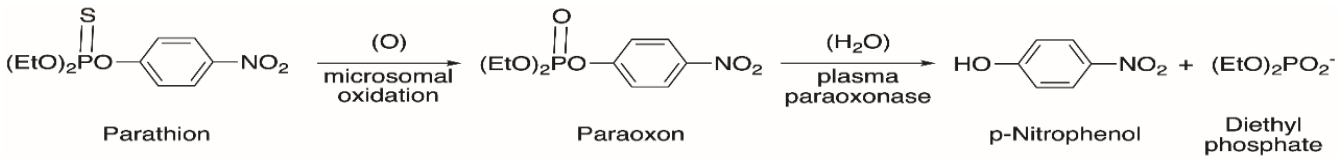


Figure 2. The reaction for which paraoxonase enzyme was named (Furlong, 2008)

Ammonium Sulfate Precipitation of Proteins

Ammonium sulfate method (60-80%) was used in for precipitation of enzymes by pulling water molecules away according to the procedure used as our previous papers (Akbaba et al., 2013). The precipitate obtained after the precipitation of approx. 30 mL of fresh human serum was centrifuged at 12,500x*g* for 30 minutes and dissolved in phosphate buffer solution at 100 mM, pH 7.0 (Sinan et al., 2006).

Ion Exchange Chromatography (IEX)

The enzyme precipitate, which had been dialyzed in the presence of 1 mM Na-phosphate buffer (pH 7.0) at 4°C, was loaded onto IEX column (DEAE anion exchanger, separation range 30 to 100 kDa for A-50), which had been equilibrated with binding buffer (100 mM Na-phosphate buffer, pH 7.0). The column (30 mm × 30 cm in dimensions) was washed with the same buffer and then elution was achieved using an increasing salt gradient of 0-1.5 M NaCl. The qualitative protein analysis result and paraoxonase activity were recorded for each tube (Türkeş et al., 2013).

Size Exclusion Chromatography (SEC)

Size exclusion chromatography (i.e., gel filtration, GF) was performed after ion exchange

chromatography since the buffer composition doesn't affect the final separation. Eluates obtaining from the IEX column were loaded onto the SEC column (separation range 4 to 150 kDa for Sephadex G-100) which is 20 mm × 60 cm in dimensions which had been equilibrated with binding buffer (100 mM Na-phosphate buffer, pH 7.0). Desorption was performed with the same buffer, isocratically (Alim and Beydemir, 2016). The effluents were analyzed for both qualitative protein analysis and paraoxonase activity.

Quantitative Protein Determination

After mixing, the mixture (i.e., Bradford reagent with the protein sample) changed to blue color and a change of the absorption from 465 nm to 595 nm occurred. Bovine serum albumin was utilized as the standard in Bradford Protein Assay, a spectrophotometric method (Bradford, 1976; Aslan et al, 2018).

SDS-PAGE Study

The molecular weight, purity, and subunit composition of the enzyme were controlled with Laemmli SDS-PAGE (3-8%) technique, an analytical method (Laemmli, 1970; Türkeş et al., 2016). Prestained protein standard (size range from 10 to 180 kDa) was used as the molecular mass standard.

Inhibition Studies

For the specification of the inhibition impacts of homovanillic acid and phloridzin dihydrate on the PON enzyme, the Activity%-[Phenolic compound] graphs were drawn (Türkeş et al., 2015). The IC_{50} values were calculated from these graphs for homovanillic acid and phloridzin dihydrate. Lineweaver-Burk ($(EU\ ml^{-1})^{-1}$-$[Paraoxon]^{-1}$) curves were utilized to determine inhibition parameters, such as inhibition type, K_i, and Vmax (Lineweaver and Burk, 1934).

Molecular Docking Analysis

The docking study of homovanillic acid was carried out using Schrödinger-Glide. PON1 (PDB ID: 3SRE), which was taken from PDB (www.rscb.org), was prepared utilizing the protein preparation wizard (Greenwood et al., 2010; Shelley et al., 2007). The ligand file was acquired from the NCBI PubChem database (CID 1738 for homovanillic acid). Then, LigPrep was used to prepare the ligand and the structure was optimized utilizing the OPLS3e force field (Sastry et al., 2013). Also, 2-hydroxyquinoline (2-HQ) was used as a reference inhibitor agent for 3SRE. The ligand docking was done flexibly using extra precision (XP) mode of Glide module (Friesner et al., 2004; Halgren et al., 2004) (Glide, Schrödinger 2018-4, LLC, New York, NY, 2018).

Table 1. Purification steps of the paraoxonase-1

Purification steps	Activity ($EU\ ml^{-1}$)	Total volume (ml)	Protein ($mg\ ml^{-1}$)	Total protein (mg)	Total activity (EU)	Specific activity ($EU\ mg^{-1}$)	Yield (%)	Purification fold
Serum	105.3	30	6.40	192.00	3160.20	16.46	100.00	1.00
Ammonium sulfate precipitation	103.2	20	4.60	92.00	2063.06	22.42	65.28	1.36
Ion exchange chromatography	72.9	15	0.20	3.00	1093.49	364.50	34.60	22.15
Size exclusion chromatography	67.1	10	0.02	0.17	670.68	3945.15	21.22	239.69

RESULT AND DISCUSSION

All of the disease cases are caused by dysregulated enzyme activities (Copeland, 2004). In some tissues with diseases, the *in vivo* or *in vitro* measuring of enzyme activity, and then imaging with inhibition of drug practice is

utilized as a pharmacokinetic, and pharmacodynamic marker. There are many enzymes in xenobiotic metabolism, and the most significant ones are paraoxonases, cholinesterases, and carboxylesterases (Klaassen and Watkins, 1996). Recently, some research has been conducted on PON1, which has been known to play an important role in the development of coronary heart diseases. However, there are only a few scientific data concerning the effects of chemical agents on PON1 activity in the scientific literature. Therefore, this study provides an important contribution to the researches on PON1-drug interactions.

Table 2. IC_{50} values and K_i constants for the compounds

Inhibitor	IC_{50} (mM)	K_i (mM)	Inhibition type
Homovanillic acid	13.84 ± 0.08	6.10 ± 0.26	Competitive
Phloridzin dihydrate	14.62 ± 0.24	16.96 ± 0.76	Non-competitive

The assay results were presented as mean ± standard deviation.

In the previous study, for illustrate, scientists investigated the effects of some metals on PON1 activity. They found that Pb^{2+}, Cr^{2+}, Fe^{2+}, and Zn^{2+} were effective inhibitors for PON1, and IC_{50} values were 0.838, 1.991, 3.960, and 7.410 mM, respectively, and K_i constants were in the range of 0.639-4.604 mM. In the same study, they also identified that Pb^{2+} and Fe^{2+}, and Zn^{2+} showed competitive inhibition; Cr^{2+}, non-competitive; and Zn^{2+}, uncompetitive inhibition at the end of the research (Ekinci and Beydemir, 2010). In a study on PON1 activity was reviewed the effects of various pesticides, such as dichlorvos, imidacloprid, lambda cyhalothrin, and fenoxaprop-p-ethyl. As a result of the study, IC_{50} values were found to be 0.0103, 0.0690, 0.1570, and 0.2000 μM, respectively (Cebeci et al., 2014).

Table 3. XP Glide docking results for homovanillic acid

Inhibitor	PubChem CID	Glide GScore (kcal mol^{-1})	ΔG vdW (kcal mol^{-1})	ΔG Coulomb (kcal mol^{-1})	Glide energy (kcal mol^{-1})	Glide model (kcal mol^{-1})
Homovanillic acid	153921	-3.29	-15.48	-8.47	-23.95	-28.15

In our previous study, it was investigated *in vitro* inhibitory effects of some bromophenols, which are between the natural organohalogen agents, on PON1 activity. IC_{50} values of these bromophenols were found ranging between 0.123 and 1.212 mM (Akbaba et al., 2013). Additionally, in our previous studies, we researched that the *in vitro* effects of various nucleoside analogues (e.g., 5-fluorouracil, and acyclovir, and gemcitabine hydrochloride) (Türkeş et al., 2013), certain calcium-channel antagonists including, amlodipine besylate, isradipine, nifedipine, and nitrendipine (Türkeş et al., 2014), some antibacterial drugs, such as, ceftizoxime sodium, cefotaxime sodium, cefepime hydrochloride, levofloxacin hemihydrate, and moxifloxacin hydrochloride (Türkeş et al., 2015), and diverse antineoplastic

drugs, (bevacizumab, and palonosetron hydrochloride) (Türkeş et al., 2016) on enzyme activity of human serum PON1. According to the findings of these researches, some chemical compounds, such as metals and pesticides, have shown inhibition effect on PON1 at very low concentrations and exhibited several types of inhibition. In addition, even though most of the drugs used were in therapeutic doses, they strongly inhibited PON1.

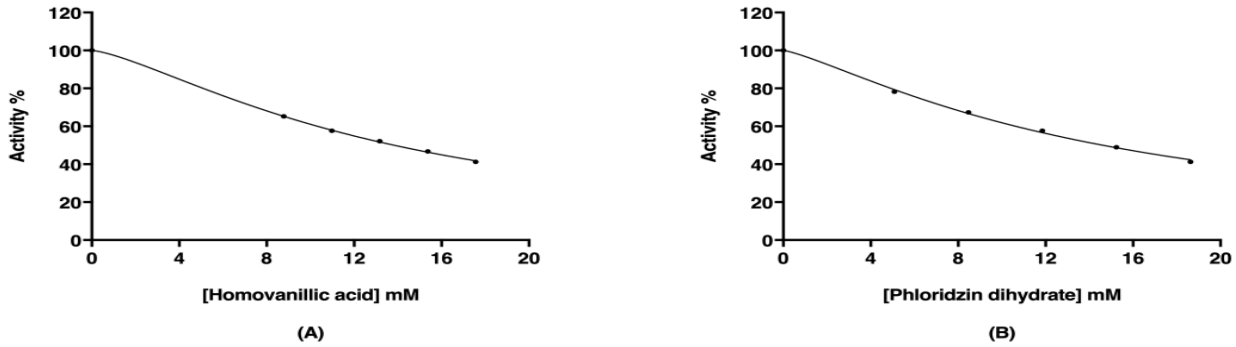


Figure 3. *In vitro* effect of phenolic compounds

In this study, it was utilized the similar procedure used by our group in previous studies and purified human serum PON1 in three simple and rapid steps. The purification resulted in as a 21.22% yield, 3945.15 EU mg^{-1} proteins specific activity, and approx. 239-fold (Table 1). Single protein band was obtained for this enzyme and the molecular weight (MW) of the enzyme was computed as 43 kDa by SDS-PAGE. These results match those found in earlier studies (Alim and Beydemir, 2016; Alim et al., 2017; Alım et al., 2018; Beydemir and Demir, 2017; Ekinci and Beydemir, 2009; Ekinci et al., 2010; Pla et al., 2007; Sinan et al., 2006).

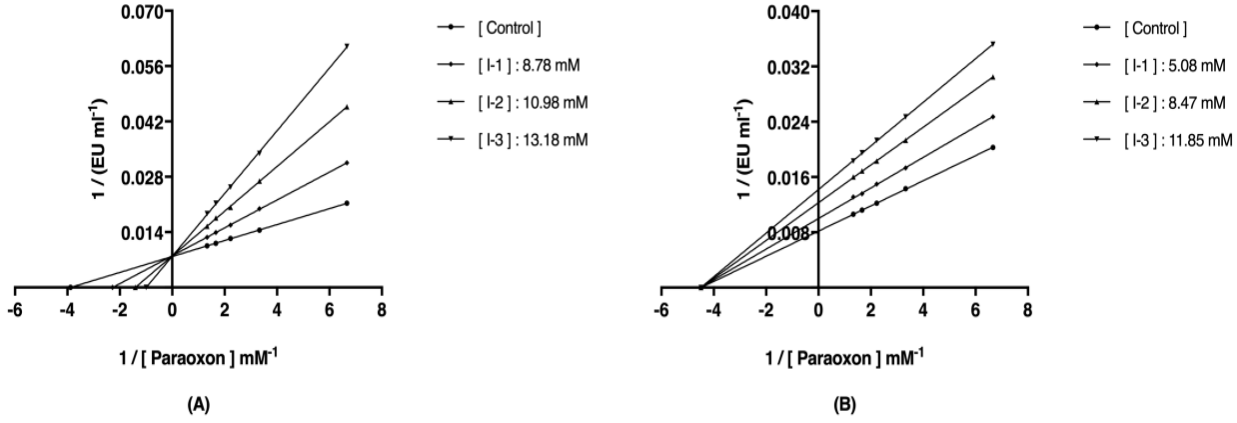
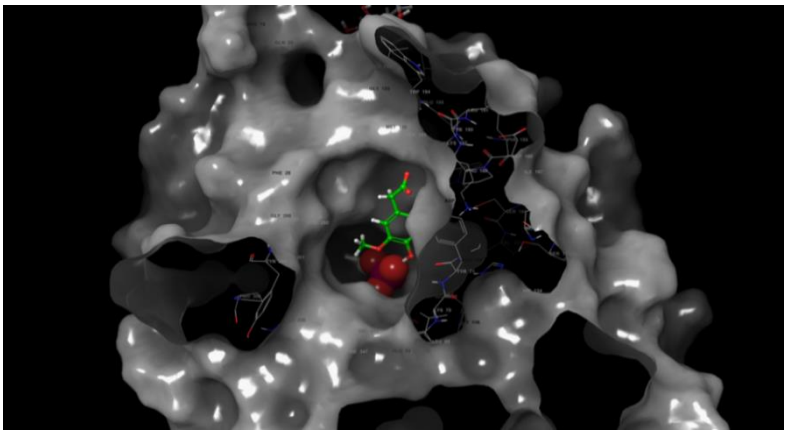


Figure 4. Lineweaver-Burk curves. (A) Homovanillic acid and (B) Phloridzin dehydrate

In the same study, we also focused on the *in vitro* inhibition role of some phenolic substances including homovanillic acid and phloridzin dihydrate on PON1 enzyme activity. IC_{50} values were 13.84±0.08 mM for homovanillic acid, and 14.62±0.24 mM for

phloridzin dihydrate (Table 2 and Figure 3). K_i constants for homovanillic acid, and phloridzin dihydrate were calculated to be 6.10±0.26 mM and 16.96±0.76 mM, respectively (Table 2, Figure 4). These results suggest that homovanillic acid interacts binding with the residues in the PON1's active site and shows competitive inhibition. To verify experimental results, molecular docking studies were implemented by using the Glide software. Molecular docking result of homovanillic acid conducted using Glide-XP protocol is depicted in Table 3. Homovanillic acid displayed Glide XP score of -3.29 kcal mol^{-1}, Glide energy of -23.95 kcal mol^{-1} and a Glide emodel score of -28.15 kcal mol^{-1}. Residues Tyr71, His184, $PO_4$1358, and Phe222 were found strongly interacting with the homovanillic acid (Figure 5). Binding energies in the 3SRE-homovanillic acid interaction has shown to fit best the ligand binds with PON1 and supported obtained results during biological activities analyses. Also, since the inhibition mechanism of phloridzin dihydrate has non-competitive, this compound could be linked to anywhere except the active site of the enzyme.



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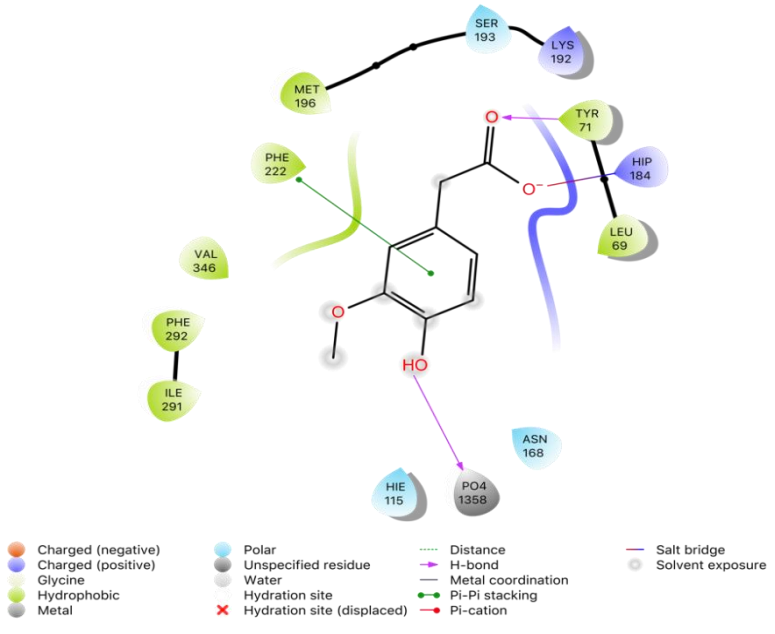

b

Figure 5. (a) Ligand interaction of homovanillic acid with 3SRE, (b) Glide docking image of homovanillic acid with 3SRE

CONCLUSION

In this study, PON1 was purified having a specific activity of 3945.15 EU mg^{-1} proteins, with a yield of 21.22% and 239-fold and determined that homovanillic acid and phloridzin dihydrate are potent inhibitors for PON1 enzyme. The most striking result to emerge from the data evaluation was that phenolic compounds concentrations used in the calculation of the IC_{50} values and K_i constants in the assays were at the low levels. The findings of this research have a number of major implications for future practice.

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