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FULL ARTICLE

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Lactoperoxidase inhibition of some natural phenolic compounds: Kinetics and molecular docking studies

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Abstract

The inhibition effects of some phenolic compounds from natural products such as taxifolin, resveratrol, olivetol, cynarine, and phloretin on bovine milk lactoperoxidase (LPO) enzyme were examined. For this aim, LPO was purified by the affinity chromatography technique with a yield of 77.68% in 421.32 times. The kinetic value, K_i, was calculated from the equations obtained from drawn graphs. In order to discover inhibition mechanism of phenolic compounds, induced fit docking process was performed on the LPO receptors. The binding affinity of the compounds was calculated and at the best-scored ligand-receptor complex, residues responsible for enzyme inhibition were detected. As a result, this molecule demonstrated the potential inhibitory effect on LPO. According to the results of kinetic study. It has shown a noncompetitive inhibition effect, Phloretin's K_i value was determined by 48.89 ± 14.22 nM.

Practical applications

There are natural antimicrobial systems, such as the lactoperoxidase (E.C.1.11.1.7; LPO) system, which eliminates the harmful effects of microorganisms in milk. The chemical reactions in this system are catalyzed by the LPO. In the dairy industry, the LPO system is considered critical for the preservation of pasteurized milk, yogurt, raw milk, and cheese. The system is used for improvement the protection condition of milk at high temperatures.

KEYWORDS

bovine milk, enzyme inhibition, lactoperoxidase, molecular docking, phenolic compounds

1 | INTRODUCTION

Phenolic agents are the most important natural antioxidant compounds (Gulcin & Beydemir, 2013). They have crucial properties such as biological, pharmacological and they show strong antioxidant activity, anticancerogenic, antibacterial, anti-inflammatory, and antiviral effects (Sarikaya, Sisecioglu, Cankaya, Gulcin, & Ozdemir, 2015). Phenolics are a chemical class including a hydroxyl group (−OH) directly bounded to aromatic ring. This chemical class is biologically and pharmaceutically very reactive (Gulcin, 2009, 2010, 2011, 2012). Flavonoids are the main class of phenolics and widely dispersed in plants. They are present as a constitutive agent in plants, which response to microbial attack (Bimpilas, Tsimogiannis, Balta-Brouma, Lymperopoulou, & Oreopoulou, 2015; Topal, Gocer, et al., 2016).

Taxifolin (dihydroquercetin), which has many biological properties, is a flavonoid. Antioxidant, antiradical, anti-inflammatory, hepatoprotective, antitumor and antiglaucoma are common effects of Taxifolin (Gocer et al., 2016; Topal, Topal, et al., 2016). Resveratrol as natural antioxidant and radical scavenger had powerful influence a series of intracellular mediators (Gulcin, 2010). Also, it effectively inhibited mammalian carbonic anhydrase isoforms I-XV (Innocenti,

Gulcin, Scozzafava, & Supuran, 2010). Moreover, olivetol is used in various syntheses of tetrahydrocannabinol and had strong antioxidant and anticholinergic properties (Taslimi & Gulcin, 2018). Cynarine, which is known as 1,3-dicaffeoylquinic acid, is formed by esterification reaction. Some its known pharmacological properties are hypocholesterolemic, hepatoprotective, antioxidant, antiradical, antiviral, antibacterial, anticholinergic, and antihistamic effects (Topal, Gocer, et al., 2016). Phloretin, another type of natural phenol, known as dihydrochalcone and can be found in apple tree leaves and the apricot (Picinelli, Dapena, & Mangas, 1995). It inhibited active transportation of glucose units into cells (Idris & Donnelly, 2009).

Milk contains lactoferrin, lysozyme, immunoglobulins, and lactoperoxidase (LPO) (Haddain, Ibrahim, & Robinson, 1996; Koksal, Gulcin, & Ozdemir, 2016). Especially, LPO is a prominent milk enzyme (Sisecioglu, Cankaya, Gulcin, & Ozdemir, 2009, 2010). It catalyzes the oxidation of both organic and inorganic substrates using H_2O_2 as an oxidant agent (Kohler & Jenzer, 1989; Koksal, Kalin, Gerni, Gulcin, & Özdemir, 2017; Sisecioglu, Uguz, Cankaya, Özdemir, & Gulcin, 2011). Also, it converts bromide (Br^-) and iodide (I^-) ions to hypobromite (BrO^-), and hypoiodite (IO^-), respectively. Because of this property, LPO can be categorized as a haloperoxidase (Sisecioglu, Cankaya, Gulcin, & Ozdemir, 2012; De Wit & van Hooydonk, 1996). Also, the LPO system shows its effect by killing bacteria in milk and secretory glands in our innate immune system. Therefore, the system does not attack DNA (Sarikaya et al., 2015; White, Pruitt, & Mansson-Rahemtulla, 1983). Conversely, the LPO system may contribute to oxidative stress under some conditions (Sipe, Jordan, Hanna, & Mason, 1994). The LPO system affects bacterial cell wall (Owens, Ray, Watts, & Yancey, 1997). Bacterial inhibition is an extensively studied issue by researchers. Bacterial cytoplasmic membranes are structurally damaged; as a result some metabolic events stop (Sisecioglu et al., 2010; Koksal et al., 2017).

As it is well known, some drugs and chemical agents may be activate or inhibit to enzymes. They can have some dangerous effects because of some defects can easily occur in the metabolic pathways (Koksal, Alim, Beydemir, & Ozdemir, 2016; Koksal, Kalin, Gulcin, Ozdemir, & Atasever, 2016; Sarikaya et al., 2015).

The main goal of the study is to determine the inhibitory or activatory effects of some natural phenolics on LPO as milk enzyme. For this aim, LPO enzyme was purified using by chromatographic method from raw bovine milk, and then the inhibition types and kinetic properties were determined.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Bovine milk was commercially purchased from a local market in Erzurum. L-Tyrosine, CNBr-activated Sepharose 4B, sulfanilamide, taxifolin, resveratrol, olivetol, cynarine, and phloretin, protein assay reagents, Amberlite CG-50-NH_4^+, standard protein markers, and other chemicals were obtained from Sigma-Aldrich Co.

2.2 | LPO activity assay

The enzyme activity was measured according to the assay of Shindler and Bardsley (1975). In this method, the absorbance increases with oxidation of ABTS by H_2O_2 is measured at 412 nm.

2.3 | Protein determination

Protein concentration was calculated using the Bradford method (1976) using standard of serum albumin from bovine (Bicer et al., 2019; Burmaoglu et al., 2019; Ozaslan, Demir, Aslan, Beydemir, & Küfrevioğlu, 2018).

2.4 | Purification of LPO

The milk was centrifuged at 3,000 × g for at 4°C during 15 min. The resin of Amberlite CG50 NH_4^+ was added (30 mg/ml). The resin to which the proteins bound was washed to obtain milk homogenate, which held in chromatographic procedure to obtain the purified LPO (Koksal, Kalin, et al., 2016).

2.5 | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

After the chromatographic procedure, to control the LPO enzyme purity, SDS-PAGE was applied according to the previous procedure (Arabaci, Gülçin, & Alwasel, 2014; Topal & Gulcin, 2014). A single protein band was seen at 78 kDa.

2.6 | In vitro inhibition studies

The K_i value was determined by means of Linewaver-Burk graphs (Koksal, Alim, et al., 2016; Yigit et al., 2018).

2.7 | Molecular docking studies

The X-ray crystal structure of bovine LPO receptor (PDB: 3Q9K) complexed with phenyl isothiocyanate at 1.7 Å resolution was chosen as the best structure due to its percentile rank and downloaded from RCSB Protein Data Bank. Moreover, it has a complexed ligand in active site. The crystal structure was prepared and repaired with protein preparation wizard panel for molecular docking studies (Bayrak, Taslimi, Karaman, Gulcin, & Menzek, 2019; Turkan, Cetin, Taslimi, Karaman, & Gulcin, 2019). For this aim, bond order and charges were assigned by using chemical component dictionary, missing hydrogen atoms were added to crystal structures, and then zero bond order to metals and disulfide bonds were created between two sulfur atoms. Missing side chains were filled with the help of Prime module. After these process, amino acids were ionized by setting

pH 7.0 using Propka module and hydroxyl, Asn, Gln, and His residues were optimized using ProtAssign module. Water molecules that were formed less than three contacts with the ligand or protein were removed. Finally, geometry optimization and energy minimization have also been done using optimized potentials for liquid simulations (OPLS) force field until root-mean-square deviation was 0.30 Å for heavy atoms. 2D structures of phenolic compounds were obtained from https://pubchem.ncbi.nlm.nih.gov (PubChem) and 3D structures were then created using LigPrep module in Maestro 11.4. Correct protonation state and molecular geometries of phenolic compounds were produced by using Epik module and OPLS-2005 force field (pH 7.0 ± 2.0, Schrodinger, 2017).

2.7.1 | Active site identification

The active site of the bovine LPO receptor was predicted and analyzed with SiteMap module in Maestro 11.4. To generate binding site, prepared receptor was processed using the SiteMap module with default setting of the top-ranked potential Protein binding site, which had properties were analyzed on bases of Sitescore and Dscore and the scores were used to evaluate active site of the bovine LPO receptor. Docking hits on the receptor were evaluated the predicted active site (Bayrak et al., 2019; Schrodinger, 2009a; Turkan et al., 2019).

2.7.2 | Induced fit docking (IFD)

Induced fit docking module was used for characterization interactions between the phenolics and bovine LPO receptor. Centroid of box was generated around by selecting phenyl isothiocyanate (FTA) ligand in the active site of the bovine LPO receptor. Thereafter, side chains were automatically adjusted based on B-factor; closest residues to the ligand were tried within 3.4 Å of ligand pose in prime refinement. In the first step, docking validation was performed to show the accuracy of the abovementioned IFD methodology. The second step, phenolic compounds which inhibited competitively were docked into the bovine LPO receptor aforementioned IFD methodology. Following all steps, interaction mechanism of best-scored molecule-receptor complex was analyzed (Bayrak et al., 2019; Schrodinger, 2009b).

3 | RESULTS

LPO was purified by 421.32-fold with a yield of 77.68%. SDS-PAGE was used for the check the enzyme purity and a single protein band was found as 78 kDa (Figure 1 and Table 1). The in vitro inhibition effects of taxifolin, resveratrol, olivetol, cynarine, and phloretin on LPO were established (Figure 2). Lineweaver–Burk graphs were drawn, for determination of the inhibition types and inhibition constants (K_i) for all phenolic molecules. K_i values were found as to be 1,308.03 ± 476.7, 73.79 ± 3.8, 855.31 ± 101.1, 1,359.98 ± 521.8, and 48.89 ± 14.22, for taxifolin, resveratrol, olivetol, cynarine, and phloretin, respectively (Table 2 and Figures 2 and 3). It was observed that all phenolics showed competitive inhibition type except Phloretin, which had noncompetitive inhibition type (Figure 3). It was identified that binding sites in co-crystallized complex with SiteMap module. We calculated SiteScore and Dscore to determine whether the binding sites are active and druggable. SiteScore and Dscore of identified binding sites of the receptor detected as 1.116 and 1.064, respectively. These results convinced us that the binding site possesses active sites and druggable site properties. The active site was used to illustrate how ligands are surrounded by active sites including hydrophilic and hydrophobic.

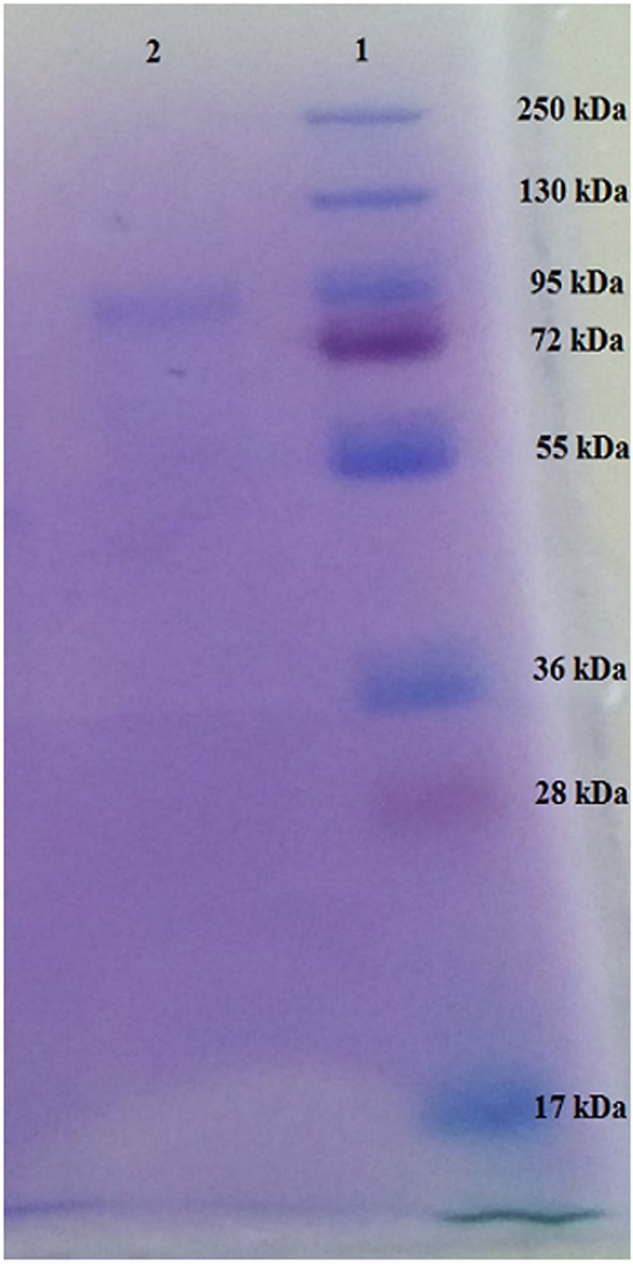

FIGURE 1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of lactoperoxidase (LPO) purified bovine milk. Column 1: Standard proteins (Line 1:260 kDa, Line 2:180 kDa, Line 3:86 kDa, Line 4:72 kDa, Line 5:66 kDa, Line 6:38 kDa, Line 7:28 kDa, and Line 8:17 kDa). Column 2: Purified LPO from bovine milk by affinity column chromatography

TABLE 1 Purification results of lactoperoxidase from bovine milk after purification steps

Purification step	Enzyme Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total enzyme activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification fold
Homogenates	1.10	55.0	16.0	880.00	66.00	0.07	100	1.00
Sepharose-4B-L-tyrosine-sulfanilamide affinity chromatography	4.70	10.0	0.164	1.64	47.00	28.65	77.68	421.32

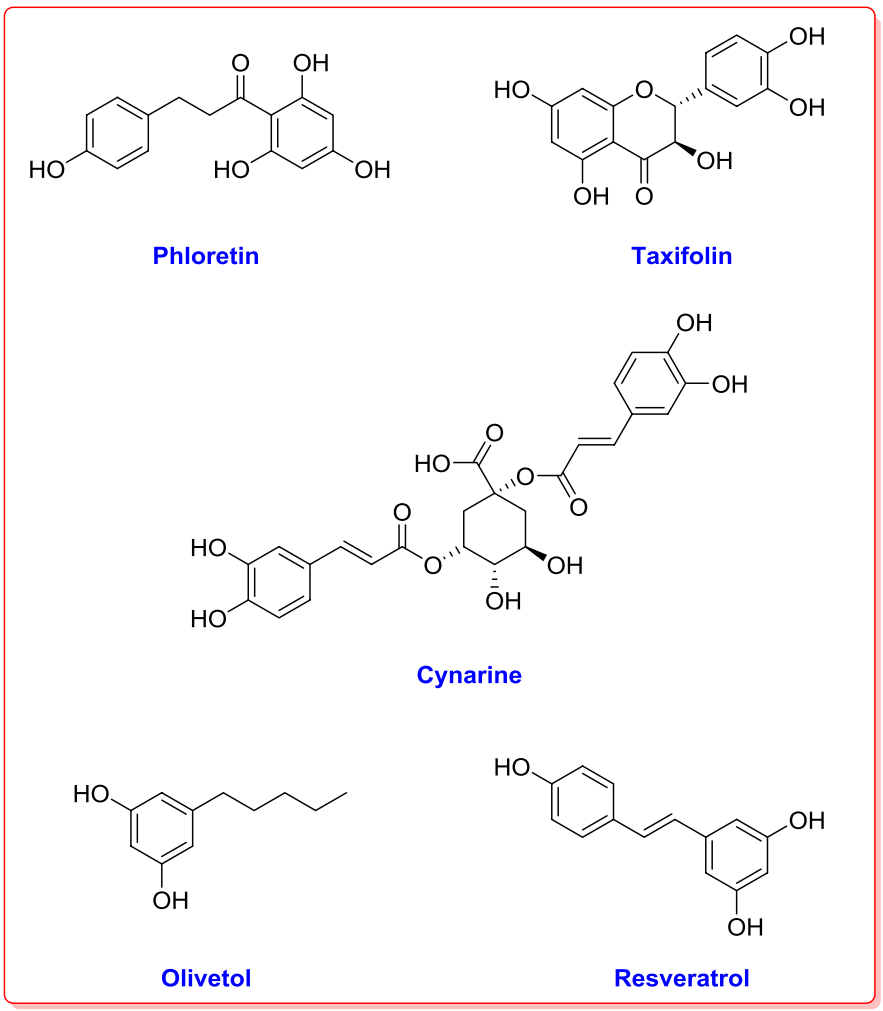

FIGURE 2 Molecular structure of phenolic compounds used in this study

TABLE 2 The K_i constants and inhibition types determined for phenolic molecules having inhibitory effects on bovine LPO

Phenolic compounds	K_i (nM)	Inhibition type
Resveratrol	73.79 ± 3.80	Competitive
Taxifolin	1,308.03 ± 476.70	Competitive
Olivetol	855.31 ± 101.10	Noncompetitive
Cynarine	1,359.98 ± 521.80	Competitive
Phloretin	48.89 ± 14.20	Noncompetitive

Induced fit docking methodology was used docking process of phenolic compounds into the active site of the bovine LPO receptor because the docking methodology provides fully flexibility ligand and receptor during docking calculation. To test reliability of the docking methodology, complexed ligand, FTA, into the crystal structure of the receptors was re-docked and binding pose of best-scored ligand was analyzed. The docking reliability test shown that re-docked and co-crystallized ligands are very closely located in the catalytic active site of the receptor as shown in Figure 4.

4 | DISCUSSION

Lactoperoxidase has different applications in many fields. It is widely used in milk and cheese industries (Barrett, Grandison, & Lewis, 1999) because of its reduction of the microflora (Reiter & Haernulv, 1984).

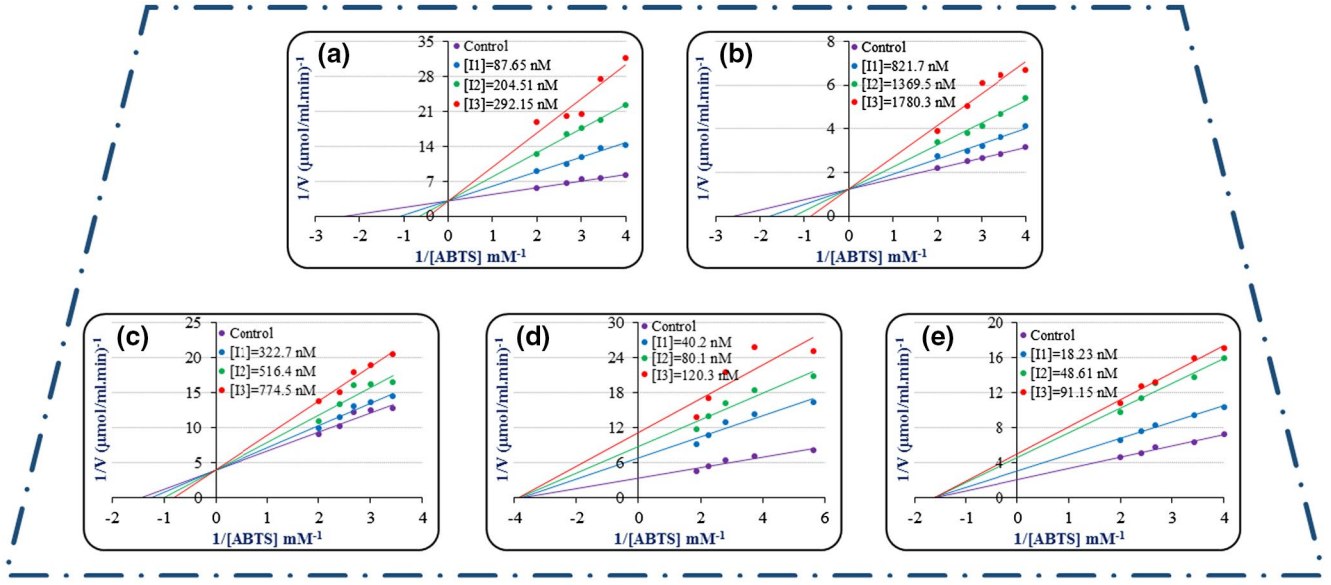

FIGURE 3 Lineweaver-Burk graph of molecules (Resveratrol (a), Taxifolin (b), Cynarine (c), Olivetol (d), and Phloretin (e)) for LPO)

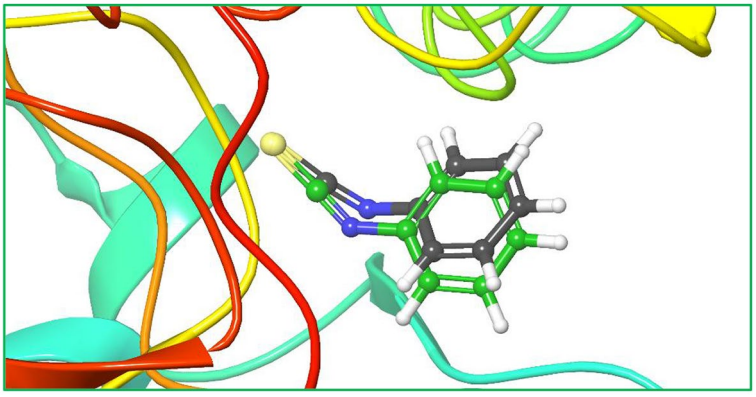

FIGURE 4 The docking reliability test. Co-crystallized ligand is represented in grey color ball and stick modeling and re-docked ligand is represented in green color ball and stick modeling

LPO has a major role in prevention of bacterial growth and this is attributed to the POD system (Jacob, Anthony, Sreekumar, & Haridas, 2000).

Phenolic or polyphenols are the most known plant secondary metabolites. It is known that there are more than 8,000 polyphenols, up to now. Natural phenols and flavonoids ranging from simple molecules (benzoic and cinnamic acid) to highly polymerized compounds (lignin, melanin, and tannins) can be composed of a subgroup, which is the most common and widely distributed. Phenolic acids are defined by having a benzene ring, a carboxyl group and one or more hydroxyl and/or methoxy groups in the molecule (Bravo, 1998). Flavonoids, which is of significant interest in the scientific arena in terms of having various usage areas and positive impacts on health recently. Flavonoids had effective antioxidant effects by their effects such as removing free radicals and chelating (Gocer et al., 2016; Harborne & Williams, 2000).

Taxifolin (dihydroqueretin) is a subclass of flavononols (Rice Evans, Miller, & Paganga, 1996). Olivetol (5-pentylresorcinol or 5-pentyl-1,3-benzenediol) is an organic compound that found in certain lichens. Cynarine is a hydroxycinnamic acid derivative and had active functional groups composed of certain plants and foods. Cynarine, 1,3-dicaffeoylquinic acid, consists of the esterification of two units of caffeic acid and one unit of quinic acid. It prevents the taste receptors and ensures that the water is sweet. It has been suggested to have certain pharmacological properties, including hypocholesterolemic, hepatoprotective, antiviral, antihistamic, and antibacterial effects (Topal, Topal, et al., 2016). Phloretin belongs to flavonoids and

TABLE 3 Binding affinity (kcal/mol) of the phenolic compounds in the catalytic active sites of LPO

Compounds	Docking score	Glide score	IFD score
Resveratrol	−7.562	−7.562	−1,356.453
Taxifolin	−7.908	−7.935	−1,354.032
Cynarine	−7.985	−7.985	−1,363.982

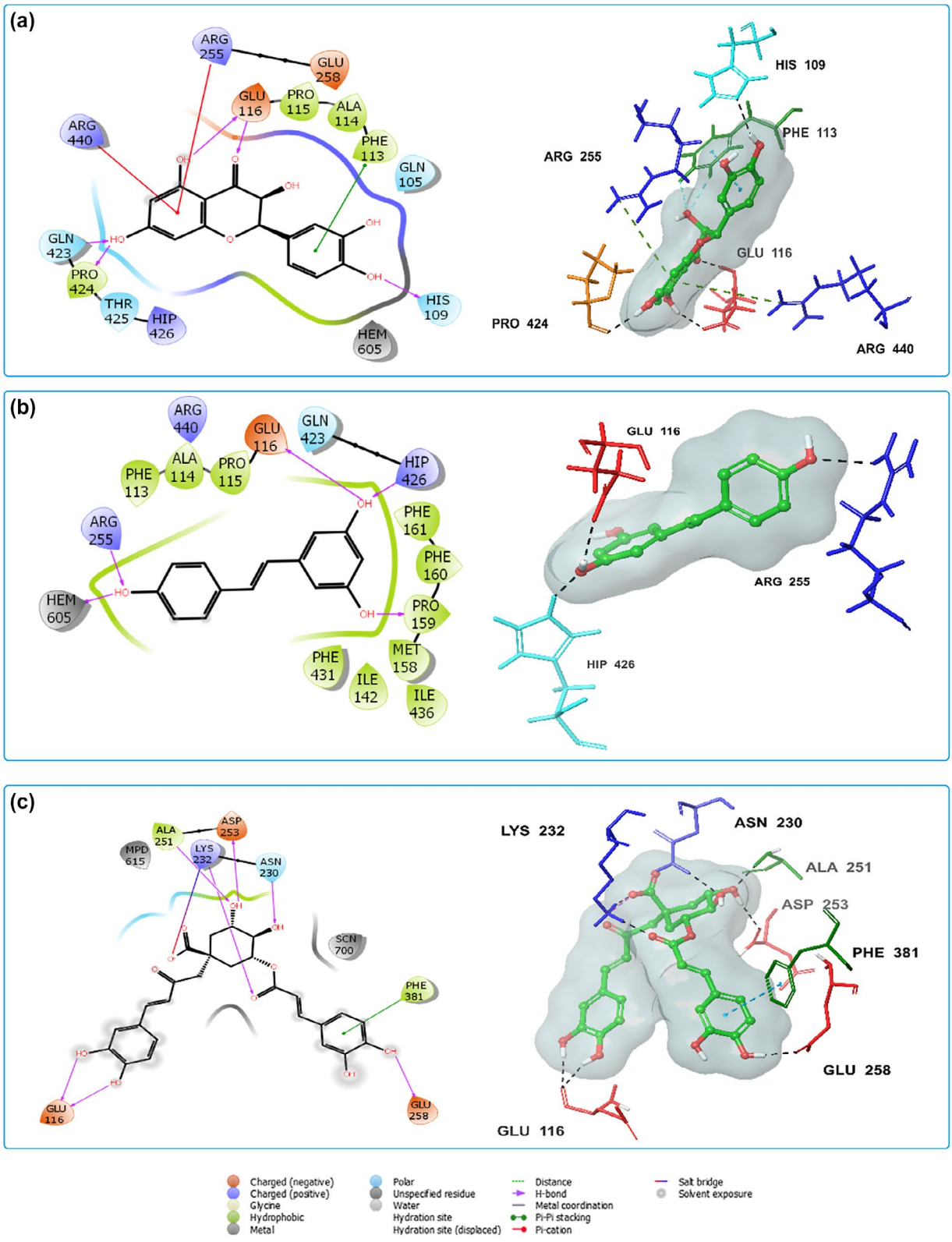

FIGURE 5 2D receptor–ligand interaction diagrams and 3D detailed interaction diagrams of (a) Taxifolin, (b) Resveratrol, and (c) Cynarine into bovine LPO receptor. The phenolic compounds are represented in ball and stick modeling which green color carbon and residues are represented in thick tube modeling. Hydrogen bond is represented with black dashed line, π interaction is represented with green dashed line, and salt bridge is represented with pink dashed line

had great interest with its beneficial effects. It is a dihydrochalcone flavonoid that shows a large spectrum of biological activities including antioxidant, antitumor, antibiosis, and antidiabetic effects (Nithiya & Udayakumar, 2016).

Up to date, various studies have been performed for investigation of the interaction between phenolics and enzymes (De Wit & van Hooydonk, 1996; Wolfson & Sumner, 1993). For instance, the inhibitory impact of some phenolic molecules including ellagic acid, ferulic acid, gallic acid, caffeic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and syringic acid has been analyzed and these molecules have been shown to have a potent inhibition effect on carbonic anhydrase I and II isoforms (Sarikaya, Gulcin, & Supuran, 2010). In another study, 6-phosphogluconate dehydrogenase (6PGD) and glucose-6-phosphate dehydrogenase (G6PD) enzymes were inhibited by some phenolics including caffeic, ferulic ellagic, and synaptic acids. However, some other phenols including p-coumaric, chlorogenic, and syringic acids have no inhibitory impact on the both 6PGD and G6PD (Adem, Comakli, Kuzu, & Demirdag, 2014).

Moreover, the impacts of tannic, 3,4-dihydroxybenzoic, 3,5-dihydroxybenzoic, chlorogenic, sinapic, 4-hydroxybenzoic, vanylic, salicylic, and 3-hydroxybenzoic acids on LPO were investigated and a strong inhibition effect was detected (Koksal, Alim, et al., 2016).

The phenolic compounds used in this study have been shown to inhibit LPO. Phloretin was shown to be the most effective inhibitor property (K_i: 48.89 ± 14.22 nM) among the phenolic compounds used in this study. While all molecules exhibited competitive inhibition, this molecule showed noncompetitive inhibition. This molecule was followed by resveratrol with the value of (K_i: 73.79 ± 3.8 nM). The cynarine molecule (K_i: 1,359.98 ± 521.8 nM) has also the value of olivetol (K_i: 855.31 ± 101.1 nM) and taxifolin (K_i: 1,308.03 ± 476.7 nM) (Table 2).

In this study, the molecular docking studies were realized for phenolic compounds which inhibited competitively in order to assess the probable binding mechanism into the LPO active site. The phenolic compounds were docked into the catalytic active site of bovine LPO receptor with reliable docking methodology and the docking calculations were examined on the basis of binding affinity, interactions diagram, and superimposition of best-scored ligands. The binding affinities are presented as Glide, Docking, and IFD scores (kcal/mol) in Table 3. According to the scores, the phenolic compounds exhibit good binding affinity against bovine LPO receptor. Also, the docking calculations are compatible with in vitro studies results in large scale.

2D and 3D interaction diagrams of best posed the phenolic compounds are analyzed as shown in Figure 5. Cynarine has the best binding affinity due to its low binding score. The compound formed hydrogen bonds with Glu116, Asn230, Lys232, Ala251, and Asp253 residues and interacted thought salt bridge with Lys232 residues (Figure 5c). Resveratrol only formed hydrogen bonds with Pro159, Glu116, Arg255, and Hip426 residues and hemoglobin (HEM605) (Figure 5b). Taxifolin formed hydrogen bonds with His109, Glu116, Gln423, and Pro424 residues, interacted through π-cation with Arg255 and Arg440 residues and formed π-π stacking with Phe113 residue. Phe113 residue also formed aromatic hydrogen bounds as seen detailed interaction diagram (Figure 5a). The finding shown that ligand interacted more active site residues has higher binding affinity against the receptor in comparison with less interacted ligands. Our docking score and receptor–ligand interaction are compatible with previous studies. Gau and co-workers (2016) has shown that luteolin, (-)-epicatechin, and (+)-pinocembrin interact with His109, Glu116, and Arg255 residues of LPO receptor. Gąsowska-Bajger and co-workers (2018) have been identified that Carbidopa has binding energy with 6.8 kcal/mol and interacted with His109, Glu116, Arg255, Gln423, Arg440 residues and the heme distal side of LPO receptor. Phenolic compounds have mostly interacted as hydrophobic and hydrophilic with His109, Glu116, and Arg255 residues. Resveratrol interacted through hydrogen bound and π-π stacking interaction with HEM605. These interactions reveal that the residues are quite important for LPO activity.

We also identified whether catalytic active site residues surrounding ligands have the hydrophobic and hydrophilic character as shown in Figure 6. The figure shows that taxifolin and cynarin were closely surrounded by hydrophilic residues. However, resveratrol was closely surrounded by both hydrophobic and hydrophilic residues.

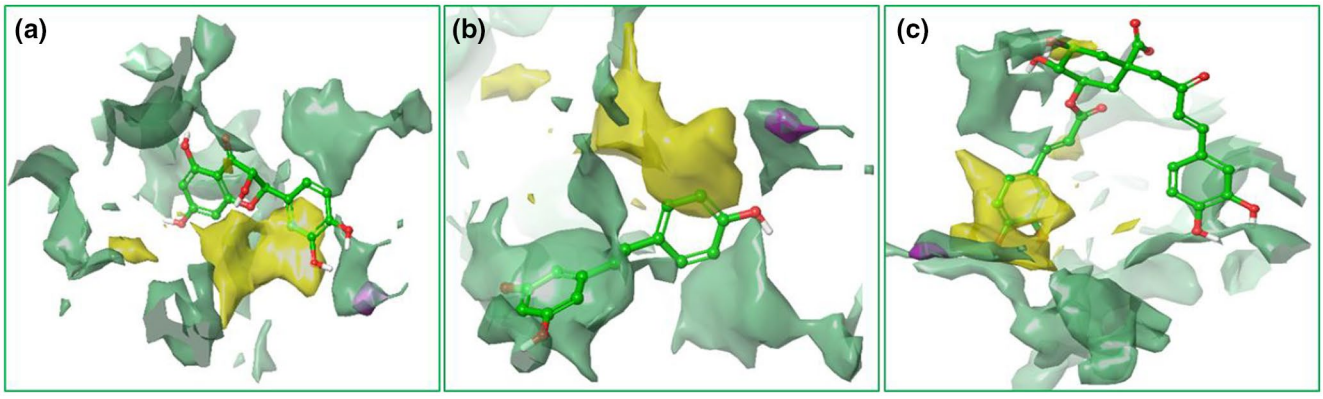

FIGURE 6 Best-scored pose of the phenolic compounds into bovine LPO receptor. (a) Taxifolin, (b) Resveratrol, and (c) Cynarine. Hydrophilic residues are represented as yellow solid surface, hydrophobic residues are presented as green solid surface, and hemoglobin site is represented as purple solid surface

5 | CONCLUSION

There is a high level of interest in the health benefits of phenolic molecules that form a significant portion of bioactive compounds. However, in some studies, diverse detrimental effects of phenolic compounds have been described. Therefore, we should be careful when using foods or drugs containing phenolic agents. This study indicates that used phenolic compounds decrease LPO activity, which is great importance in the lactation period. Therefore, LPO is of great importance in the immune system of newborns. Decreasing enzyme activity means weakening of the immune system.

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CONFLICT OF INTERESTS

The authors reported that no conflict of interest.

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