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Evaluation Of Pyrazoline-Bearing Primary Sulfonamides as Potent Inhibitors of Lactoperoxidase

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Pirazolin Taşıyan Primer Sülfonamidlerin Potansiyel Laktoperoksidaz İnhibitörü Olarak Değerlendirilmesi

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Abstract
Lactoperoxidase (LPO E.C. 1.11.1.7) is a glycoprotein belonging to the class of oxidoreductases and constitutes a notable member of the peroxidase enzyme family. The biological significance of LPO is attributed to its role as a natural defense system against microbial infection. In the current study, to examine the inhibitory effects of 8 different polyfluoro-substituted pyrazoline-type primary sulfonamide derivative compounds on LPO enzyme activity, LPO enzyme was extracted from buffalo milk and purified using Sepharose 4B-etilendiamin-4-tiyoüreido-benzensülfonamid affinity column, resulting in a 2212.45-fold purification, a 19.31 % yield, and a specific activity of 4955.9 EU/mg. K_i values for the active inhibitors were determined to be between 1.02±0.53-6.67±2.94 µM, while their IC_{50} values were in the range of 0.144±0.03-0.798±0.07 µM. The IC_{50} value and K_i values for the most effective inhibitor, compound **3**, were calculated as 0.144±0.03 and 6.06±4.06 µM, respectively, and it was found that this compound inhibited LPO enzyme activity non-competitively.

***Keywords:** Lactoperoxidase; Buffalo Milk; Pyrazoline; Sulfonamide; Fluorine; Enzyme İnhibition*

Öz
Laktoperoksidaz (LPO E.C. 1.11.1.7) peroksidaz enzim ailesinin önemli bir üyesi olan, oksidoredüktaz sınıfı bir glikoproteindir. LPO' nun biyolojik önemi mikroorganizma istilasına karşı doğal bir koruma sistemi oluşturmasıdır. Bu çalışmada 8 farklı polifloro-sübstitüe pirazolin tipi sülfonamid türevi bileşiklerin LPO enzim aktivitesi üzerine inhibisyon etkilerini incelemek için öncelikle LPO enzimi manda sütünden Sepharose 4B-etilendiamin-4-tiyoüreido-benzensülfonamid afinite kolonu ile 19,31% verim ve 4955,9 EU/mg spesifik aktivite ile 2212,45 kat saflaştırılmıştır. LPO enzimi üzerine inhibisyon etkisi gösteren moleküllerin IC_{50} değerleri 0.144±0.03-0.798±0.07 µM, iken K_i değerleri 1.02±0.53-6.67±2.94 µM aralığında belirlenmiştir. En etkili inhibitör olan bileşik **3** için IC_{50} değeri ve K_i değerleri sırasıyla 0.144±0.03 ve 0.06±4.06 µM olarak hesaplanmıştır ve bu bileşiğin LPO enzim aktivitesini rekabetçi olmayan olarak inhibe ettiği bulunmuştur.

***Anahtar Kelimeler:** Laktoperoksidaz; Manda Sütü; Pirazolin; Sülfonamid; Florin; Enzim İnhibisyonu*

1. Introduction

Lactoperoxidase (LPO E.C. 1.11.1.7) is a glycosylated, heme-carrying protein found in body fluids such as tears, saliva, milk, and the lining fluids of airways. The primary physiological function of LPO in mammalian organisms is the oxidation of thiocyanate to hypothiocyanate using hydrogen peroxide. Since hypothiocyanate has bactericidal properties, LPO is a crucial part of innate host defense (Fweja, *et al*. 2007). Acting as a natural defense against the assault of microorganisms makes the LPO a crucial part of innate host defense. These antimicrobial properties give LPO a significant role in various fields. For instance, antimicrobial activity of LPO protects the newborn babies against pathogens found in their digestive system. It is reported that another notable benefit of LPO is the inhibition the biofilm formation in saliva (Rajesh *et al.* 1995; Urtasun *et al*. 2017). It is also reported that LPO protects the animal cells from the damaging effects of peroxidation by breaking down various carcinogens (Magacz *et al*. 2019; Nishioka, *et al*. 1981). Another study proposed that the LPO/I_2/H_2O_2 system may contribute to protecting the respiratory tract from viruses (Fischer *et al*. 2011). The absence of LPO system accompanied with a high incidence of neuroinflammation which affected multiple system and led to tissue injury like brain pathology, vasculitis, and tumors according to an investigation conducted on middle-aged mice with a deleted LPO gene (Yamakaze and Lu 2021). On the other hand, LPO enzymes were suggested as a potent biomarker for Parkinson's disease since the concentration of cerebrospinal fluid LPO increases among patients with idiopathic Parkinson's

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disease compared to controls (Smith, *et al.* 2022). All these qualities have made LPO versatile enough to be used in a wide spectrum of industries including dairy, cosmetics, pharmaceuticals, veterinary, and agriculture.

The sulfonamides are a group of synthetic antibacterial agents bearing $–SO_2NH_2$ in their structure. Despite their initial discovery decades ago as a chemotherapeutic substance for antibacterial properties, sulfonamides have continued attraction due to their newly discovered pharmacological properties, such as antibacterial, anti-carbonic anhydrase, anti-obesity, diuretic, hypoglycemic, anti-thyroid, anti-tumor, and anti-neuropathic pain properties, make sulfonamides an important class of medications (Congiu *et al.* 2015; Köksal *et al.* 2017; Otten 1986). Sulfonamides show the potential to selectively inhibit carbonic anhydrase (CA) isozymes Petrou *et al*. 2016; Yamali *et al*. 2020), glutamate carboxypeptidase II (Blank *et al.* 2011), acetylcholine esterase enzymes (Yamali *et al.* 2020), and also LPO enzymes, as evidenced by multiple studies (Kavaz *et al.* 2022; Li *et al*. 2019; Morita *et al.* 2011; Ozdemir, *et al*. 2002). In addition, it has been reported that some recently synthesized polyfluoro-substituted pyrazoline type primary sulfonamide derivatives exhibit nanomolar-level inhibitory effects for both carbonic anhydrase enzyme and acetylcholine esterase enzyme (Yamali *et al.* 2020). It is well known that while some drugs or chemical agents inhibit/activate various enzymes, they may have the opposite effect on others. As a result of such undesirable effects, some disruptions may occur in important metabolic pathways in the organism (Köksal and Alim 2020; Sharma *et al*. 2013).

This study set out to investigate the impact of these primary sulfonamides on LPO, an enzyme of significance in the innate immune response and industrial applications. To this end, LPO was purified from buffalo milk using the Sepharose 4B-etilen diamin-4-tiyoureido-benzenesulfonamide affinity column and IC_{50} values, K_i constants and types of inhibition were determined for each molecule.

2. Materials and Methods

2.1 Chemicals

Buffalo milk was commercially purchased from a local market in Balikesir. 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Cyanogen bromide (CNBr), Sepharose 4B, and other chemicals were obtained from Sigma-Aldrich Co. Polyfluoro-substituted pyrazoline derivative sulfonamides (1-8) used in this study (See the figure 1) were previously synthesized by Yamali *et al* (2020).

2.2 LPO Purification

To get skimmed milk, raw buffalo milk was centrifuged at 14000 rpm for 30 minutes at 4°C. Ammonium sulfate precipitation at 60% saturation was applied to the supernatant, following the centrifugation at 14000 rpm for 45 minutes at 4°C. The obtained precipitate was dissolved in the 10 mM Na_2HPO_4 pH 6.8 buffer to apply in the last stage of purification. The homogenate was passed through the Sepharose 4B-ethylenediamine-4-thioureido-benzenesulfonamide affinity column to get purified LPO enzyme, and then the pure enzyme was obtained with the elution buffer (1 M NaCl / 25 mM Na_2HPO_4, pH 6.8) (Bozdag *et al*. 2015; Sipal Beste 2016).

2.3 LPO activity assay

LPO enzyme activity was determined according to the assay of Shindler and Bardsley (1975) using 2,2'-azino-bis (3-etilbenztiazolin–6-sulfonic acid) (ABTS) as a substrate. This method based on the absorbance increases with oxidation ABTS substrate by H_2O_2 is measured at 412 nm. One LPO enzyme unit (EU) is described as the quantity of LPO that catalyzes the oxidation of l μmol of substrate (ABTS) per min at 20°C (Rajesh *et al*. 1995b).

2.4 Protein determination

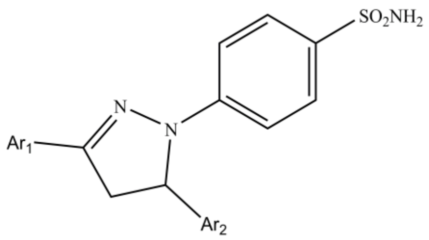
Bradford method (1976) was used to calculate protein concentration. Bovine serum albumin was used as standard protein (Bradford 1976).

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

Following the purification of lactoperoxidase enzyme by affinity chromatography, the purity of the enzyme was controlled by running discontinuous sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) using the method found by Laemelli at two different acrylamide concentrations: stacking gel 3% and separation gel 10% (Laemmli 1975).

2.5 Determination of IC_{50} and K_i values

The inhibitory effect (IC_{50}) of sulfonamide compounds on the LPO enzyme was examined from the graph drawn by working at different inhibitor concentrations against a constant concentration of ABTS substrate under optimum conditions. Following the collection of the IC_{50} value, Lineweaver-Burk plots were created using the values obtained at three different sulfonamide and five different substrate concentrations. Finally, the K_i value and inhibition type of sulfonamide were calculated using the graphs. At least three repetitions of inhibition studies were carried out (Dedeoglu and Guler 2009).



Compounds

	Ar_1	Ar_2
1-	2, 5-Difluorophenyl	2, 3-Dimethoxyphenyl
2-	2, 5-Difluorophenyl	2, 4-Dimethoxyphenyl
3-	2, 5-Difluorophenyl	2, 5-Dimethoxyphenyl
4-	2, 5-Difluorophenyl	3, 4-Dimethoxyphenyl
5-	3, 5-Difluorophenyl	2, 3-Dimethoxyphenyl
6-	3, 5-Difluorophenyl	2, 4-Dimethoxyphenyl
7-	3, 5-Difluorophenyl	2, 5-Dimethoxyphenyl
8-	3, 5-Difluorophenyl	3, 4-Dimethoxyphenyl

Figure 1. Chemical structures of polyfluorosubstituted pyrazoline-type primary sulfonamide derivative compounds used in the study.

3. Results and Discussions

LPO, a heme-containing glycosylated enzyme has a role as an antimicrobial agent in mammalian fluids such as saliva, tears, milk. LPO is a component of the host's natural defense mechanism against invasive microorganisms and is present in the secretions of mammals' mammary, salivary, and lacrimal glands (Gnanesh Kumar, Reddy, and Kottekad 2018; Seifu, *et al.* 2005). Using thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2), the hemoprotein lactoperoxidase catalysis the formation of hypothiocyanous acid (HOSCN) which is an antimicrobial compound also thought to be linked to carcinogenesis (Jahanbakhsh *et al.* 2020). Due to its natural antimicrobial properties, LPO is the most significant enzyme for the dairy industry and has numerous clinical applications for the prevention of dental caries (Bafort *et al.* 2014; Welk *et al.* 2021). The LPO enzyme not only has antimicrobial properties, but it is also a potent defense mechanism against oxidative stress (Jahanbakhsh *et al.* 2020; Magacz *et al.* 2019). Understanding the circumstances and molecules that cause the LPO enzyme, which performs such vital tasks, to be inhibited or activated is crucial for both the health of living things and the manufacturing of industrial products. Because of this, the majority of research has concentrated on purification studies that use less funding to purify the LPO enzyme. Despite several LPO purification strategies reported in the literature, affinity chromatography is the most preferred, because of its high enzyme yield from multiple sources (Borzouee *et al*. 2016; Kavaz *et al.* 2022; Köksal, Usanmaz, *et al*. 2017; Rajesh *et al.* 1995; Urtasun *et al.* 2017). In the study, LPO, a versatile enzyme, was purified by using Sepharose-4B-L-ethylenediamine-4-isothiocyanate benzene sulfonamide affinity column from buffalo milk, which is accessible and where the enzyme is abundant. LPO enzyme was isolated 2212.5 fold from buffalo milk by affinity gel with 4955.9 EU/mg specific activity and a yield of 19.31% as shown in Table 1. SDS-PAGE electrophoresis was performed to check the purity of the purified LPO enzyme. Purified lactoperoxidase showed a single band at a position corresponding to molecular weight of approximately 80 kDa as mentioned in the literature (Figure 2) (Atasever *et al.* 2013; Bayrak 2015; Koksal *et al.* 2016). Ki and IC_{50} values are often used to compare the relative potency of inhibitors. The inhibitor-enzyme binding affinity is described by the K_i value (the dissociation constant), whereas the total inhibitor concentration needed to attain 50% inhibition is known as the IC_{50}. Smaller K_i values usually indicate tighter binding (Aykul and Martinez-Hackert 2016). However, the half-maximal inhibitory concentration, or IC_{50}, is the most pertinent in vitro metric used to assess a molecule's, ligand's, or other pharmacologic agent's therapeutic potential. It indicates the amount of a specific pharmacologic agent that is required to inhibit a given biological activity by half, and the lowest IC_{50} values point out the strongest inhibitor against the enzyme investigated (Burlingham and Widlanski 2003).

Table 1. Purification steps for LPO Enzyme purified by Sepharose 4B-etilen diamin-4-tiyoureido-benzenesulfonamide affinity column

Purification step	Total Volume (ml)	Activity (EU/mL)	Total Activity (EU/mL)	Protein (mg/mL)	Total Protein (mg)	Specific Activity (EU/mg)	Yield %	Purification fold
Crude homogenate	150	1050.5	157575	468.05	70207.5	2.24	100	-
Affinity column	12	2535.8	30429.6	0.512	6.14	4955.9	19.31	2212.45

The K_i and IC_{50} values are the most suitable criteria for demonstrating inhibitory effects. Therefore, to examine the effects of pyrazoline derivative primary sulfonamide molecules (**1-8**), which have important potential as pharmacological agents, the LPO enzyme purified from buffalo milk, K_i and IC_{50} values were determined for each molecule (Table 2). The results are stated as IC_{50} (The concentration required for 50% inhibition, µM) and K_i (inhibition constant, µM) values, and the lowest values point out the strongest inhibitor against the enzyme investigated. K_i and IC_{50} graphs of compound **3**, which is the strongest inhibitory effect on LPO, are given in Figure 3 demonstrating an example.

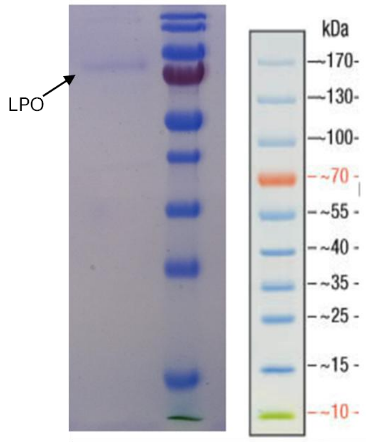


Figure 2. SDS-polyacrylamide gel electrophoresis image of LPO enzyme purified by affinity chromatography. The first row is purified LPO enzyme, and the second is protein standard.

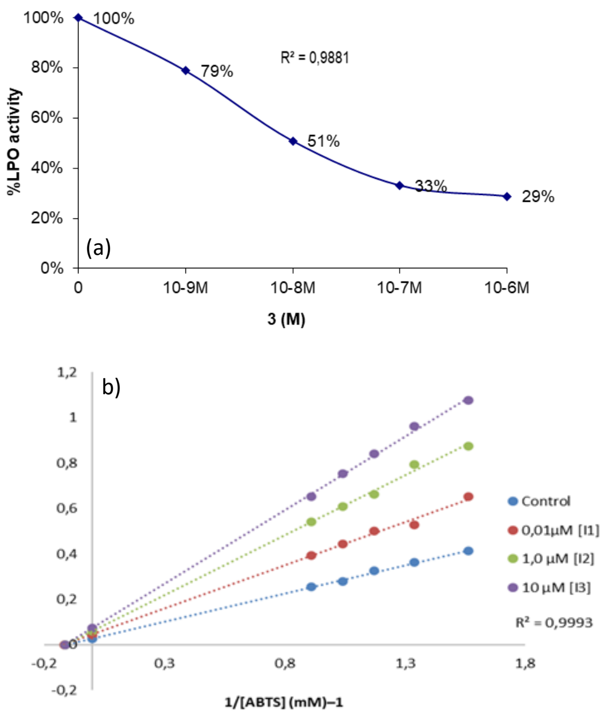


Figure 3. Graphs for the determination of IC_{50} (a) and K_i (b) constants of Compound 3 possessing the most potent inhibitory activity against the LPO enzyme.

According to data obtained, sulfonamides **1-6** inhibited the LPO enzyme at the micromolar level, however, compounds **7** and **8** had no inhibitory effect on the enzyme's activity in the concentration range tested. K_i values of sulphonamides obtained from Lineweaver-Burk graphs were range of 1.02±0.53-6.67±2.94 µM. In the state of the 2,5-difluorophenyl derivatives (**1-4**) inhibitory effects against LPO, the IC_{50} of the compounds 0.144±0.03-0.798±0.07 µM, while their K_i values were observed in the range of 1.02±0.53-6.67±2.94µM µM. When the IC_{50} values of compounds **1** and **3** were compared, it was seen that the inhibition potency increased approximately 6-fold when passing from compound **1** to **3**. This occurred when the methoxy group changed from position 3 to position 5, highlighting the importance of the position of the substituent on the ring in inhibition. Some inhibitors may bind to a site other than the active site of the enzyme or to the enzyme-substrate complex, causing noncompetitive or uncompetitive inhibition. Compounds **1** and **2** inhibited the enzyme activity through a competitive inhibition mechanism, whereas derivatives **3** and **4** exhibited non-competitive uncompetitive inhibition, respectively. When considering 3,5-difluorophenyl derivatives, IC_{50} values of compound **5**, and **6**, were 0.578±0.02 and 0.458±0.04 µM, respectively, whereas K_i values were 2.22±9.05 and 2.08±6.58 µM, respectively. By using an uncompetitive mechanism, both compounds inhibited LPO enzyme activity. Some inhibitor molecules may bind to the enzyme's active site, resulting in competitive inhibition; other inhibitor molecules may bind to the enzyme-substrate complex or a site other than the enzyme's active site, resulting in noncompetitive or uncompetitive inhibition (Koksal 2021). Inhibitory pathways for LPO could involve inhibiting heme iron interactions with the substrate or blocking the binding site of the substrate (Caro-Ramírez et al. 2024).

In light of this, it may be concluded that compound **3** may inhibit the enzyme by attaching to a location rather than the enzyme's active site, whereas effect by binding to the complex between the enzyme and the substrate. These possibilities suggest that there are various ways in which the pyrazoline sulfonamide skeleton can interact with the LPO active site. Compound **3** had the strongest inhibitory activity among the **8** compounds, with an IC_{50} of 0,.144±0.03 µM and a K_i value of 6.06±4.06 µM, according to the Table 2.

The inhibitory effect of acetyl-L-carnitine (ALC) molecule on lactoperoxidase (LPO) enzyme was investigated in a study where LPO was selected as a model enzyme since its resemblance to thyroid peroxidase (TPO) and its availability in pure form. Results obtained showed that ALC inhibited LPO activity, which supported its potential as a TPO inhibitor (Caro-Ramírez et al. 2024). A study by Korkmaz et al. examined the possible inhibitory effects of several anthraquinone derivatives that are known for their coloring qualities on the lactoperoxidase (LPO) enzyme. The findings obtained within the scope of the study demonstrated that the anthraquinone derivatives examined showed a competitive inhibition mechanism with K_i values ranging from 0.4964 ± 0.042–2.0907 ± 0.1044 µM (Korkmaz et al. 2025). In a study investigating the *in vitro* inhibition kinetics of carnosol and its derivatives, a diterpenoid found in rosemary (Rosmarinus officinalis), on lactoperoxidase (LPO) enzyme. According to the obtained data, the studied compounds generally exhibited noncompetitive inhibition, except for 12-methylcarnosic acid, which displayed a different inhibition model with the K_i values of the studied molecules varying between 34.64 ± 1.93 and 392.9 ± 59.3 µM (Köksal et al. 2024).

According to a study examining the effects of various thiophene-2-sulfonamide derivatives on the lactoperoxidase (LPO) enzyme's *in vitro* activity purified from bovine milk, 5-(2-thienylthio)thiophene-2-sulfonamide compound demonstrated the most potent inhibitory effect among the compounds examined, with an IC_{50} of 3.4 nM and a Ki value of 2 ± 0.6 nM (Koksal 2021). The *in vitro* inhibitory effects of celecoxib derivatives with pyrazole-linked sulfonamide groups on the lactoperoxidase (LPO) enzyme were examined in the study conducted by Bayrak et al. It was determined that an aromatic pyrazole derivative containing 2,3-dimethoxyphenyl functional groups (compound **1**) exhibited the strongest inhibitory effect on the LPO enzyme purified by affinity chromatography, with a Ki value of 3.2±0.7 nM, indicating it was a noncompetitive inhibitor (Bayrak et al. 2024).

In addition to the effects of these eight pyrazoline derivative sulfonamides synthesized by Yamali *et al*. on acetylcholine esterase and carbonic anhydrase I and II enzymes were previously examined. According to the findings, the IC_{50} and K_i values of these eight compounds for the acetylcholine esterase (AChE) enzyme and CA isoenzymes were observed in the nanomolar range (Yamali *et al*., 2020). 2,5-difluorophenyl derivatives (**1–4**) were identified as more potent inhibitors for both LPO and AChE compared to 3,5-difluorophenyl derivatives (**5–8**).

The 2,5-Difluorophenyl derivative compound **4** (IC_{50} = 6.36 nM) showed the most potent inhibitory activity on the AChE enzyme compared to other derivatives. On the other hand, compound 3 (IC_{50} = 3.77 nM) and compound **8** (IC_{50} = 4.45 nM) displayed a greater degree of inhibitory activity against the isoenzymes hCA I and hCA II, respectively. In the view of LPO, Compound **3** (0.144±0.03 µM) demonstrated the most potent LPO inhibition activity, whereas compound **7** and compound **8** showed no discernible inhibitory effect at this concentration range (Yamali et al. 2020). According to the data presented above, promising LPO inhibitory activity is demonstrated by structures like pyrazoline-sulfonamide, anthraquinone, carnosol, and thiophene-sulfonamide.

Table 2. Concentrations, K_i values, and types of inhibition of sulfonamide derivatives, 1-8 compounds, causing 50% inhibition of LPO enzyme using ABST substrate.

	Compounds	IC_{50} (µM)	K_i (µM)	Inhibition Type
1	4-(3-(2,5-Difluorophenyl)-5-(2,3-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.798±0.07	4.8±0.274	Competitive
2	4-(3-(2,5-Difluorophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.277±0.06	6.67±2.94	Competitive
3	4-(3-(2,5-Difluorophenyl)-5-(2,5-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.144±0.03	6.06±4.06	Noncompetitive
4	4-(3-(2,5-Diphorophenyl)-5-(3,4-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.704±0.02	1.02±0.53	Uncompetitive
5	4-(3-(3,5-Difluorophenyl)-5-(2,3-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.578±0.02	2.22±9.05	Uncompetitive
6	4-(3-(3,5-Difluorophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	0.458±0.04	2.08±658	Uncompetitive
7	4-(3-(3,5-Difluorophenyl)-5-(2,5-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	Nd.	-	-
8	4-(3-(3,5-Difluorophenyl)-5-(3,4-dimethoxyphenyl)-4,5-dihydropyrazol-1-yl) benzenesulfonamide	Nd.	-	-

4. Conclusion

Here we reported the effects of polyfluoro-substituted pyrazoline-type sulfonamides on the LPO enzyme activity. According to results, 6 of the 8 compounds showed micromolar inhibition levels towards the LPO enzyme. The results indicated that compound **3** had the strongest inhibitory potency with the lowest K_i value compared to the others. Sulfonamide derivatives are promising compounds with distinct biological activities. However, the fact that compounds **7** and **8** did not show any inhibitory effect for LPO enzyme, whereas compound **8** showed a strong inhibitory effect on hCA II isoenzyme, will provide ideas for the synthesis of new compounds that do not inhibit the activity of LPO enzyme, which have antimicrobial effects in the discovery of new pharmaceutical agents to be synthesized. Lactoperoxidase (LPO) is a promising enzyme with a wide range of biological activities that can be utilized in various applications, spanning the food industry to medicine. Furthermore, because of exposure to the substances that inhibit the LPO enzyme, the role of lactoperoxidase in newborns' antimicrobial defense may have a negative impact on the immune system. For these reasons, such information is crucial when designing new compounds for therapeutic use and when organizing preclinical research.

Declaration of Ethical Standard
The authors declare that they comply with all ethical standards.

Credit Authorship Contribution Statement
Author-1: Conceptualization, investigation, methodology and software, visualization and writing, Supervision – original draft.

Author-2: Investigation, Funding acquisition
Author-3: Investigation, methodology, and software, visualization and writing
Author-4: Investigation, methodology

Declaration of Competing Interest
The authors have no conflicts of interest to declare regarding the content of this article.

Data Availability Statement
The authors declare that the main data supporting the findings of this work are available within the article. Datasets are available on request. The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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