ORIGINAL SCIENTIFIC ARTICLE

ANTIOXIDATIVE ACTIVITY OF SOME S-ALKYL DERIVATIVES OF THIOSALICYLIC ACID. *IN VIVO* AND *IN SILICO* APPROACH

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ABSTRACT

This study examined the effects of S-alkyl derivatives acute administration on local redox status and interaction between tested compounds and antioxidant enzymes via molecular docking studies. This study included 88 male Wistar albino rats divided into three experimental groups, receiving different S-alkyl derivatives per os in three different doses (10 mg/kg, 15 mg/kg, and 20 mg/kg) and two control groups, CMC - rats treated with 1% carboxymethyl cellulose and indomethacin group (IND) – rats treated with indomethacin (10 mg/kg). Carrageenan-induced paw edema model was used for evaluation of local antioxidant potential of the investigated S-alkyl derivatives. After finishing the experimental protocol, carrageenan-induced edema feet of each animal were collected and homogenized. From isolated supernatant pro-oxidative parameters ($O_2^{\cdot-}$, NO_2^-, and TBARS) and antioxidant enzymes activity (SOD, CAT, and GSH) were spectrophotometrically measured. Molecular docking studies were performed in AutoDock Vina software. The levels of pro-oxidative parameters were significantly decreased in tissue of rats treated with S-alkyl derivatives, while dose dependent manner in TBARS reduction was observed in L3 groups ($p<0.05$). Moreover, tested compounds exposed antioxidant activity due to enhanced CAT activity compared to untreated rats while the most prominent changes in GSH activity was observed after acute administration of L3 in the highest dose ($p<0.05$). According to molecular docking parameters, derivative L3 exhibited the highest binding affinity towards antioxidant enzymes. Obtained in vivo and in silico results suggest the high antioxidative potential of L3 and its beneficial effect on redox balance recovery in state of increased inflammation.

Keywords: *S-alkyl derivatives, carrageenan, oxidative stress, rat, molecular docking .*

INTRODUCTION

Prolonged exposure to elevated levels of pro-oxidants may cause structural and functional impairments of various cellular biomolecules leading to changes in gene expression (1-3). For this reason, molecules with antioxidant properties that counteract oxidative stress have become one of the most studied compounds in the research community (4-6). Antioxidants can provide protection against molecular damage by neutralizing free radicals and reactive oxygen species (ROS) (7,8). A particular focus of research has been directed at medicinal plants with antioxidant activity and their potential to treat or prevent certain human disorders in which oxidative stress appears to be one of the causes (9,10).

It is well known that inflammation can induce the formation of various ROS such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) (11). These ROS continuously generate lipid peroxidation in tissues, attacking unsaturated fatty acids (12,13). It is also well established that oxidative stress plays a very important role in the pathogenesis of many inflammatory diseases such as, rheumatoid arthritis, Alzheimer's disease, ankylosing spondylitis, type 2 diabetes, asthma, and cancer (2,3,12,14). On the other hand, aerobic organisms possess numerous antioxidant protective systems including ascorbate, α-tocopherol, glutathione (GSH), and enzymes such as superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPx), as well as various proteins like peroxiredoxins and thioredoxins (15-17).

Considering the effect of non-steroidal anti-inflammatory drugs (NSAIDs) on redox status, we can find quite controversial data in the literature. Namely, NSAIDs can act as antioxidants and pro-oxidants in different investigated systems depending on the applied doses and agents for the induction of oxidative stress (18). NSAIDs can generate ROS in numerous biological systems, through different enzymes from the nicotinamide adenine dinucleotide (NADPH) oxidase group, lipoxygenase, nitric oxide synthase, xanthine oxidoreductase, cytochrome P450 and cyclooxygenases (19). Therefore, it is assumed that oxidative stress is involved in the mechanism of NSAIDs side effects occurrence including gastrointestinal toxicity, nephrotoxicity, and hepatotoxicity (20-22). On the other hand, numerous laboratory studies reported an antioxidant activity of NSAIDs (23-25). Dabhi and coworkers showed that ibuprofen achieves antioxidant, anti-inflammatory, and non-ulcerogenic activity on atherosclerotic animals (26). In the study of Dokmeci and collaborators, the protective effect of ibuprofen was confirmed in an experimental model of testicular torsion/detorsion that occurs as a result of antioxidant and anti-inflammatory effect of the drug (27).

Chemically, S-alkyl derivatives of thiosalicylic acid belong to the derivatives of hydroxybenzoic acid, specifically, salicylic acid. These derivatives including methyl salicylate, acetylsalicylic acid, diflunisal, mesalazine, olsalazine are widely used for the treatment of various inflammatory diseases (28,29). S-alkyl derivatives of thiosalicylic acid were synthesized in alkylation reaction of thiosalicylic acid with corresponding alkyl halide based on previously published procedure (30). These mercaptobenzoic acid derivatives were also used as ligands in the synthesis of various biologically active coordination compounds of the copper, palladium, and zinc (31-33).

The biological activity of these derivatives was examined *in vitro* in previously published study (32), whereby these molecules exhibit moderate and dose-dependent cytotoxic effects on human colon HCT-116 cell line. In addition, certain S-alkyl derivatives exhibited moderate and selective antibacterial activity, as well as weak antifungal activity (33). Also, structurally similar S-alkenyl derivatives of thiosalicylic acid demonstrated mild antibacterial activity against Gram-positive bacteria, weak activity towards Gram-negative bacteria, as well as weak antifungal effect (34). Lastly, binuclear complexes of copper(II) with S-alkyl derivatives of thiosalicylic acid showed weak antioxidative properties in DPPH and reducing power assay (35).

The molecular docking analysis represents well established computational platform in identification of small molecule inhibitors against various molecular targets (36). These *in silico* studies can efficiently predict the interaction between ligands and target proteins and provide more precise insight into the ligand-protein binding interactions (37).

Up to date, no study has evaluated *in vivo* antioxidant capacity of S-alkyl derivatives of thiosalicylic acid and their effect on local oxidative stress. Thus, this study aimed to examine the effect of S-alkyl derivatives acute administration on the local redox status parameters of Wistar Albino rats. Additionally, molecular docking studies were performed with the aim to confirm the interaction between tested compounds and antioxidant enzymes involved in molecular mechanisms of antioxidative activity.

MATERIALS AND METHODS

Ethical approval

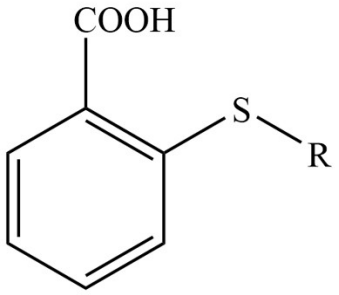
The experimental protocol of the current study was approved by the Ethics Committee for Experimental Animal Well Being of the Faculty of Medical Sciences of the University of Kragujevac, Serbia (No: 01-4505, approval date: 22 April 2019). All experimental procedures were performed according to the Guidelines for the Care and Use of Laboratory Animals.

Drug and Chemicals

All chemicals used for reagents and solutions preparation (indomethacin, carrageenan, trichloroacetic acid (TCA), sodium hydroxide, thiobarbituric acid (TBA), sulfonylic acid, phosphoric acid, N-(1-naphthyl)-ethylenediamine-dihydrochloride (NEDA), ammonium chloride, sodium tetraborate, tris(hydroxymethyl)aminomethane, ethylenediaminetetraacetic acid disodium salt dehydrate, gelatin, nitro-blue-

tetrazolium (NBT), sodium chloride, metaphosphoric acid, disodium phosphate, 5,5-dithiobis-6,2-nitrobenzoic acid (DTNB), sodium citrate, L-Glutathione reduced (GSH), epinephrine, sodium carbonate) were obtained from Sigma Aldrich (St. Louis, Missouri, United States). Investigated compounds, S-alkyl derivatives of thiosalicylic acid (**Fig. 1**), were synthesized in the laboratory for Pharmaceutical Chemistry of Faculty of Medical sciences, University of Kragujevac, according to the previously published synthetic procedure (30).

Figure 1. General structural formula of investigated compounds. R = methyl-(L1), ethyl-(L2), propyl-(L3)



Experimental Animals

A total of 88 healthy male Wistar albino rats were included in this study (8 weeks old, body weight 180±20g). The animals were obtained from the Military Medical Academy, Belgrade, Serbia and acclimatized for two weeks in the vivarium of the Faculty of Medical Sciences, University of Kragujevac, Serbia. Rats were housed under controlled environmental conditions (room temperature 22±2°C, 12h light/dark cycle) and had free access to standard food and water (*ad libitum*).

Rats were randomly divided into three experimental and two control groups (8 animals per group). The experimental groups included rats that received different S-alkyl derivatives *per os* in three different doses (10 mg/kg, 15 mg/kg, and 20 mg/kg) dissolved in 1% carboxymethyl cellulose (CMC) sixty minutes before inducing inflammation and were further divided into three subgroups according to the applied dose of S-alkyl derivatives (8 animals per subgroup). Control groups involved 1% CMC- rats were treated *per os* with 1% CMC sixty minutes before inducing inflammation and indomethacin group – rats were treated *per os* with indomethacin (10 mg/kg) dissolved in 1% CMC sixty minutes before inducing inflammation. The experimental and control groups are divided as follows:

- CMC - control group of animals treated with 1% CMC
- IND - positive control of animals treated with indomethacin in dose of 10 mg/kg
- L1-10 - animals treated with S-methyl derivate in dose of 10 mg/kg
- L1-15 - animals treated with S-methyl derivate in dose of 15 mg/kg
- L1-20 - animals treated with S-methyl derivate in dose of 20 mg/kg
- L2-10 - animals treated with S-ethyl derivate in dose of 10 mg/kg
- L2-15 - animals treated with S-ethyl derivate in dose of 15 mg/kg
- L2-20 - animals treated with S-ethyl derivate in dose of 20 mg/kg
- L3-10 - animals treated with S-propyl derivate in dose of 10 mg/kg
- L3-15 - animals treated with S-propyl derivate in dose of 15 mg/kg
- L3-20 - animals treated with S-propyl derivate in dose of 20 mg/kg

The dose of standard NSAID, indomethacin, was selected based on previously published studies (38-40). On the other hand, the applied doses of S-alkyl derivatives were calculated using equation that converts the human equivalent dose into the animal dose (41). For that purpose, we used human equivalent dose of indomethacin (100 mg) and converted it in the appropriate animal dose.

Evaluation of antioxidant activity

In order to evaluated local antioxidant potential of the investigated S-alkyl derivatives, carrageenan-induced paw edema model was used in this study. Inflammation was induced by an intraplantar injection of 1 mL of 0.5% carrageenan in 0.9% saline in the left hind paw. One hour before carrageenan injection all tested compounds were administrated *per os* in a single volume of 0.3 ml per rat (42).

Five hours after inflammation induction, all animals were anesthetized with intraperitoneal injection of mixture of anaesthetics (ketamine/xylazine - 100/10 mg/kg of body weight) and sacrificed by decapitation. The carrageenan-induced edema feet of each animal were collected, weighed, and homogenized by using electrical homogenizer (*Omni-Prep™ multi-sample homogenizer, Omni International, GA, United States*). 0.1 g of tissue sample was homogenized in four volumes of phosphate buffer (PBS; pH=7.4) on ice and centrifuged for 15 min at 15,000 rpm in order to isolate clear supernatant for further biochemical analysis (43).

Oxidative stress parameters

From paw tissue homogenate samples following pro-oxidative parameters were measured: superoxide anion radical ($O_2^{-\cdot}$), nitrites (NO_2^-), and index of lipid peroxidation measured as thiobarbituric acid (TBA) reactive substances (TBARS). We also determined the activity of reduced glutathione (GSH) as non-enzymatic antioxidants as well as the activity levels of the enzymatic defense system including catalase (CAT) and superoxide dismutase (SOD). All of these parameters regarding redox state were determined spectrophotometrically (*Shimadzu UV-1800UV-VIS spectrophotometer, Japan*).

Determination of pro-oxidative parameters

The level of $O_2^{-\cdot}$ was measured using the Nitro Blue Tetrazolium (NBT) reaction in TRIS buffer with tissue sample (supernatant) at a wavelength of 530 nm (44).

In order to indirectly assessed nitric oxide (NO) level, nitrites were measured using Griess's reagent. The isolated animal samples were precipitated with 30% sulfo-salicylic acid, vortexed, and centrifuged at 3,000 rpm for supernatant isolation. Equal volumes of Griess's reagent and supernatant were incubated and measured at 543 nm (45).

Using 1% TBA in 0.05 M NaOH we estimated the index of lipid peroxidation measured as TBARS in animal's tissue samples. The samples were incubated at 100°C for 15 min and measured at 530 nm (46).

Determination of antioxidative activity

The protocol for the evaluation of GSH activity is based on the determination of the formation of 5-thio-2-nitrobenzoic acid (TNB) at 420 nm. Exactly, GSH is oxidized by 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB) to GSSG and TNB, while GSSG is then reduced to GSH by glutathione reductase (GR) (47).

For CAT determination prepared homogenate samples, CAT buffer, and 10 mM H_2O_2 were used and measured spectrophotometrically at 360 nm (48).

The SOD activity was evaluated by mixture of homogenate samples with carbonate buffer and epinephrine and measurement at 470 nm (49).

Ligand preparation for molecular docking

Investigated molecules were drawn in ChemDraw Ultra 7.0. Their energy optimization was performed in Chem3D Ultra 7.0 (50) using AM1 semiempirical quantum method. Molecules were prepared for molecular docking analysis in AutoDockTools 1.5.6 (51), whereby Gasteiger changes were added and rotatable bonds were defined.

Selection and preparation of target macromolecules for molecular docking

The complete crystal structures of antioxidant enzymes, human erythrocyte catalase (PDB ID: 1DGF) (52) and human glutathione peroxidase 1 (PDB ID: 2F8A) were downloaded from the Protein data bank (https://www.rcsb.org/). The target macromolecules were prepared in BIOVIA Discovery Studio Visualizer 2021 (53). All docking calculations were performed on chain A of target enzymes. Preparation of macromolecules was utilized by adding Kollman charges and hydrogen atoms in AutoDockTools 1.5.6.

Molecular docking methodology

Semi-flexible docking protocol was executed in AutoDock Vina software (54) with the default scoring function. Protein residues were defined as rigid, while bonds of tested compounds were set as rotatable. Blind molecular docking studies were performed on the target proteins, using maximum defined grid box with dimensions 126 × 126 × 126 points and grid spacing of 0.375 Å. The grid box center for x, y, and z coordinates was defined as follows: 20.945, 60.993, and 58.326 for CAT, and 4.661, 16.264, and 29.083 for GPx. The three-dimensional view of binding interactions, as well as the docked binding poses of the investigated compounds with the lowest binding energy were visualized using Pymol 2.5.5 (55).

Molecular docking simulations were carried out to examine the category, type, total number of non-covalent binding interactions, and free binding energy (ΔG). Equilibrium binding constant (K_b) is calculated from free binding energy value according to the following equation, $\Delta G = -RT\ln K_b$, where T is a temperature of 298 K, R is a gas constant with the value of $1.9872036 \cdot 10^{-3}$ kcal $K^{-1}mol^{-1}$, while K_b represents the equilibrium binding constant.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8 (Version for Windows, GraphPad Software, La Jolla California, USA). All data are presented as the mean ± standard deviation (SD). The normality of data distribution was analyzed by Shapiro-Wilk test. Data were analyzed using a one-way ANOVA (Repeated measured ANOVA) and the post hoc Bonferroni test for multiple comparisons. p value <0.05 was observed statistically significant.

RESULTS

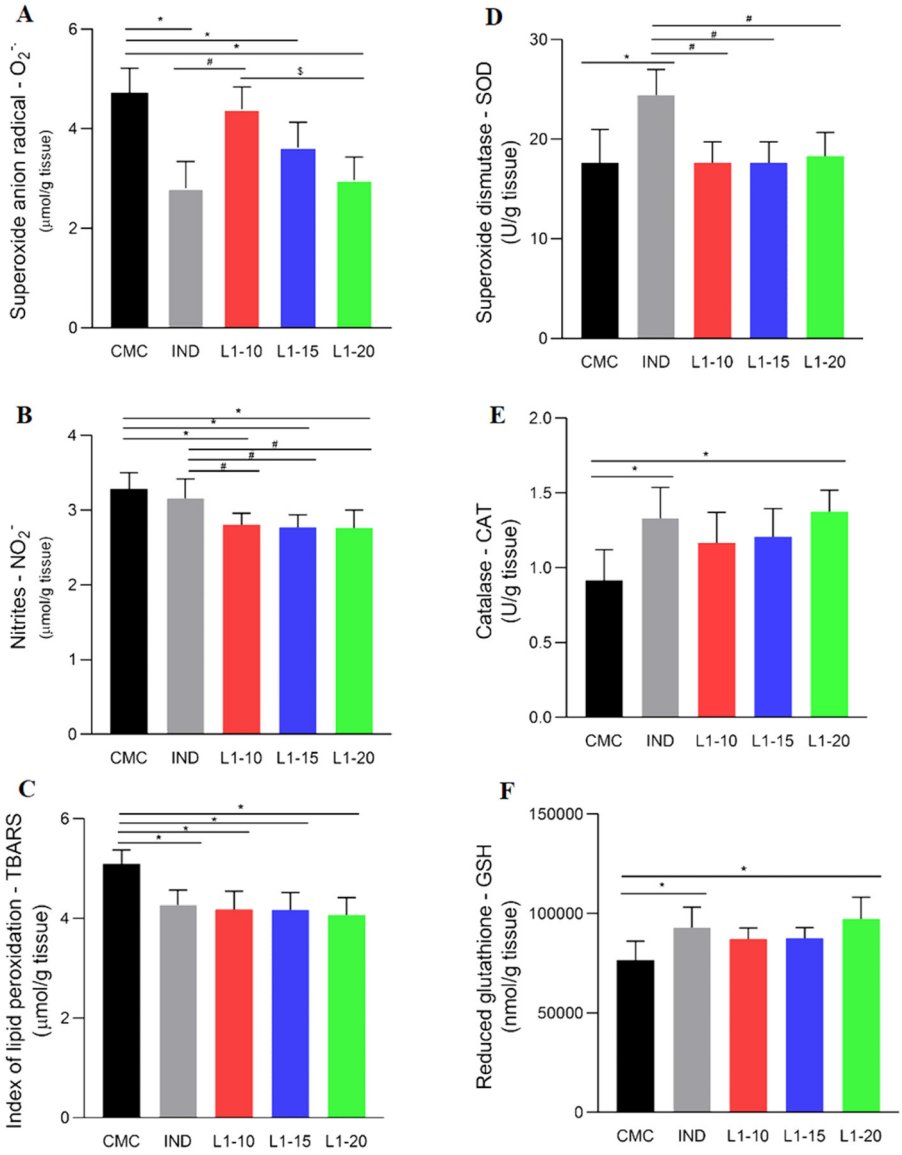
Effect of L1 acute administration on local redox state

Carrageenan injection was found to disturb redox status in rat's paw tissue as confirmed with significantly higher $O_2^{-\cdot}$ and TBARS levels ($p<0.05$) coupled with reduced antioxidants activities ($p<0.05$) in CMC group compared to rats treated with indomethacin as standard NSAID (**Fig. 2**). However, administration of L1 in all tested doses led to markedly reduced pro-oxidant parameters (NO_2^- and TBARS) compared to CMC group (**Fig. 2B and Fig. 2C**). An analysis of O_2^- level revealed that both the highest and medium doses of L1 decreased release of this pro-oxidant compared to CMC group ($p<0.05$) while the lowest dose was not effective ($p>0.05$) (**Fig. 2A**). Additionally, the greatest potential for pro-oxidative parameters reduction was found in the IND group, while none of the applied doses of L1 managed to lead to statistically lower O_2^-, NO_2^-, and TBARS values compared to IND group of animals (**Fig. 2A-C**).

The rats from IND group showed the highest level of antioxidant parameters which is reflected in the higher SOD, CAT, and GSH activity compared to CMC group ($p<0.05$) (**Fig. 2D-F**). Moreover, SOD activity was significantly increased in IND group compared to L1 treated groups (**Fig. 2D**), but no prominent changes were observed in CAT and GSH activity between these groups (**Fig. 2E, F**). Although

dose-dependent treatment with L1 did not improve antioxidant parameters compared to IND rats ($p>0.05$), we found significantly higher CAT and GSH levels in rats treated with the highest dose of L1 compared to CMC group ($p<0.05$) (**Fig. 2E, F**).

Figure 2. Effect of applied L1 derivative on the redox state.



(A) Superoxide anion radical - O2.; (B) nitrites - NO2-; (C) index of lipid peroxidation - TBARS; (D) Superoxide dismutase - SOD; (E) catalase – CAT and (F) reduced glutathione - GSH. CMC - control group of animals treated with 1% CMC, IND - positive control of animals treated with indomethacin in dose of 10 mg/kg, L1-10 - animals treated with S-methyl derivate in dose of 10 mg/kg, L1-15 - animals treated with S-methyl derivate in dose of 15 mg/kg, L1-20 - animals treated with S-methyl derivate in dose of 20 mg/kg. Data are presented as means ± standard deviation. Statistical significance at the level $p < 0.05$: *compared to CMC; #compared to IND; $compared to experimental group.

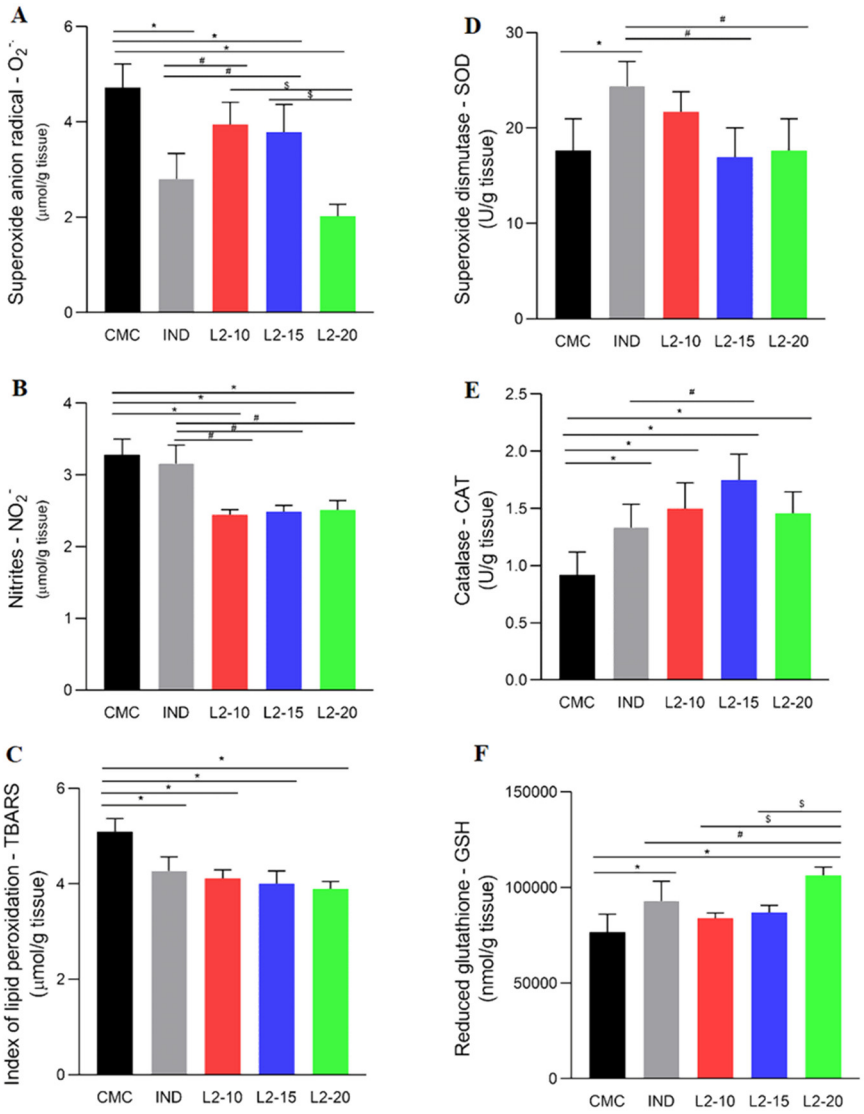
Effect of L2 acute administration on local redox state

Acute administration of L2 derivatives in all investigated doses resulted in lower release of pro-oxidants, NO_2^- and TBARS compared to CMC group ($p<0.05$) (**Fig. 3B, C**), except of O_2^- which values were decreased only in L2-15 and L2-20 groups compared to untreated rats (**Fig. 3A**). Although the highest dose of L2 showed the lowest O_2^- level compared to the other investigated doses ($p<0.05$) (**Fig. 3A**), none of the applied treatment led to significantly lower release of this pro-oxidative marker relative to IND group ($p>0.05$) (**Fig. 3A**). In addition to O_2^-, results of this study demonstrate prominent changes in NO_2^- level after acute administration of all tested L2 doses indicating lower level of this pro-oxidants

compared to IND group ($p<0.05$) (**Fig. 3B**). On the other hand, no statistical differences were observed in TBARS value between L2 groups and animals treated with IND ($p>0.05$) (**Fig. 3C**).

While acute L2 administration in all tested doses did not lead to improvement in SOD activity ($p>0.05$) (**Fig. 3D**), CAT levels were significantly higher in group of rats treated with L2 derivatives compared to CMC group ($p<0.05$) (**Fig. 3E**). Moreover, increase in CAT activity in L2-15 group was superior compared to IND group ($p<0.05$) (**Fig. 3E**). When it comes to GSH values, the activity of this antioxidant was significantly increased in group of animals treated with the highest L2 dose in comparison to L2-10 and L2-15 groups, as well as to control groups of animals ($p<0.05$) (**Fig. 3D**).

Figure 3. Effect of applied L2 derivative on the redox state.



(**A**) Superoxide anion radical - O_2^-; (**B**) nitrites - NO_2^-; (**C**) index of lipid peroxidation - TBARS; (**D**) Superoxide dismutase - SOD; (**E**) catalase – CAT and (**F**) reduced glutathione - GSH. CMC - control group of animals treated with 1% CMC, IND - positive control of animals treated with indomethacin in dose of 10 mg/kg, L2-10 - animals treated with S-ethyl derivate in dose of 10 mg/kg, L2-15 - animals treated with S-ethyl derivate in dose of 15 mg/kg, L2-20 - animals treated with S-ethyl derivate in dose of 20 mg/kg. Data are presented as means ± standard deviation. Statistical significance at the level $p < 0.05$: *compared to CMC; $^{\#}$compared to IND; $^{\$}$compared to experimental group.

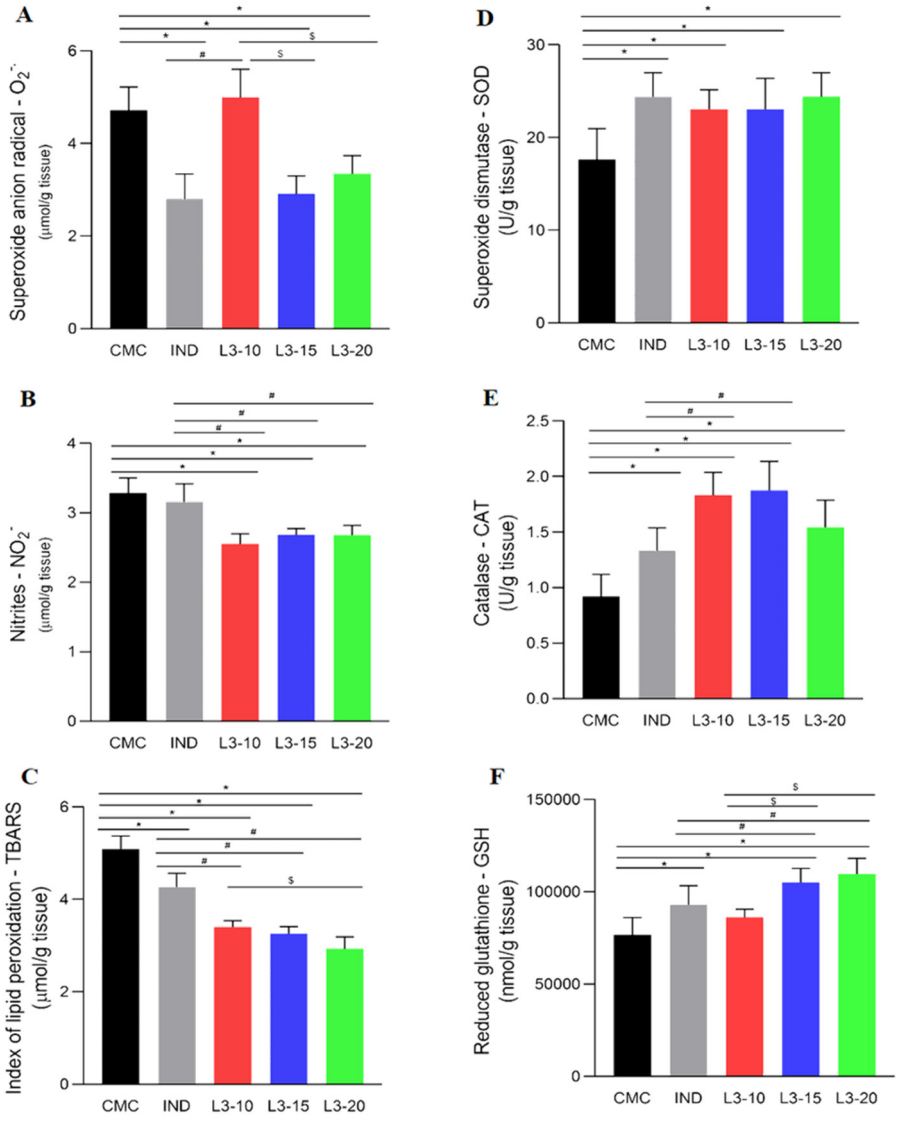
Effect of L3 acute administration on local redox state

The rats treated with medium and the highest dose of L3 significantly reduced O_2^- level compared to CMC ($p<0.05$), but with no statistical differences relative to IND group ($p>0.05$) (**Fig. 4A**). However, the lowest dose was not

effective in reduction of this pro-oxidants (p>0.05) (**Fig. 4A**). On the other hand, the levels of both NO_2^- and TBARS were markedly reduced experimental groups compared to IND as well as to CMC group (p<0.05) (**Fig. 4B, C**). Although no dose-dependent manner was noticed investigating effect of L3 derivative on NO_2^- level (p>0.05) (**Fig. 4B**), the highest dose of L3 derivative led to the most prominent decrease in TBARS value compared to other tested doses (p<0.05) (**Fig. 4C**).

Antioxidant systems were significantly improved after L3 derivatives administration in all tested doses reflected in increased SOD, CAT, and GSH activity in experimental and IND groups compared to CMC (p<0.05) (**Fig. 4D-F**). While there were no statistical differences in SOD activity between L3 and IND groups (p>0.05) (**Fig. 4D**), CAT activity was significantly improved in L3-10 and L3-15 groups compared to IND (p<0.05) (**Fig. 4E**). Additionally, GSH level was significantly improved in L3-15 and L3-20 group of rats compared to IND and L3-10 groups (p<0.05), while the lowest dose of L3 derivative was not superior in GSH activity compared to IND group (p>0.05) (**Fig. 4F**).

Figure 4. Effect of applied L3 derivative on the redox state.



(A) Superoxide anion radical - O2.; (B) nitrites - NO2-; (C) index of lipid peroxidation - TBARS; (D) Superoxide dismutase - SOD; (E) catalase – CAT and (F) reduced glutathione - GSH. CMC - control group of animals treated with 1% CMC, IND - positive control of animals treated with indomethacin in dose of 10 mg/kg, L3-10 - animals treated with S-propyl derivate in dose of 10 mg/kg, L3-15 - animals treated with S-propyl derivate in dose of 15 mg/kg, L3-20 - animals treated with S-propyl derivate in dose of 20 mg/kg. Data are presented as means ± standard deviation. Statistical significance at the level p < 0.05: *compared to CMC; #compared to IND; $compared to experimental group.

Molecular docking analysis

To confirm the molecular interaction between tested S-alkyl derivatives of thiosalycilic acid and antioxidant enzymes, this *in silico* study examined the molecular docking of tested compounds into the active sites of CAT and GPx. The binding affinity was assessed using following variables: free binding energy (ΔG), equilibrium binding constant (K_b), as well as the number, category, and type of non-covalent binding interactions. Values of the free binding energy and equilibrium binding constant for the best-docked binding poses of investigated compounds are listed in Table 1. A lower value of ΔG and a higher value of K_b indicate a stronger interaction of the tested compounds with antioxidant enzymes.

Table 1. Molecular docking parameters for the best-docked binding poses of tested compounds in interaction with antioxidant enzymes.

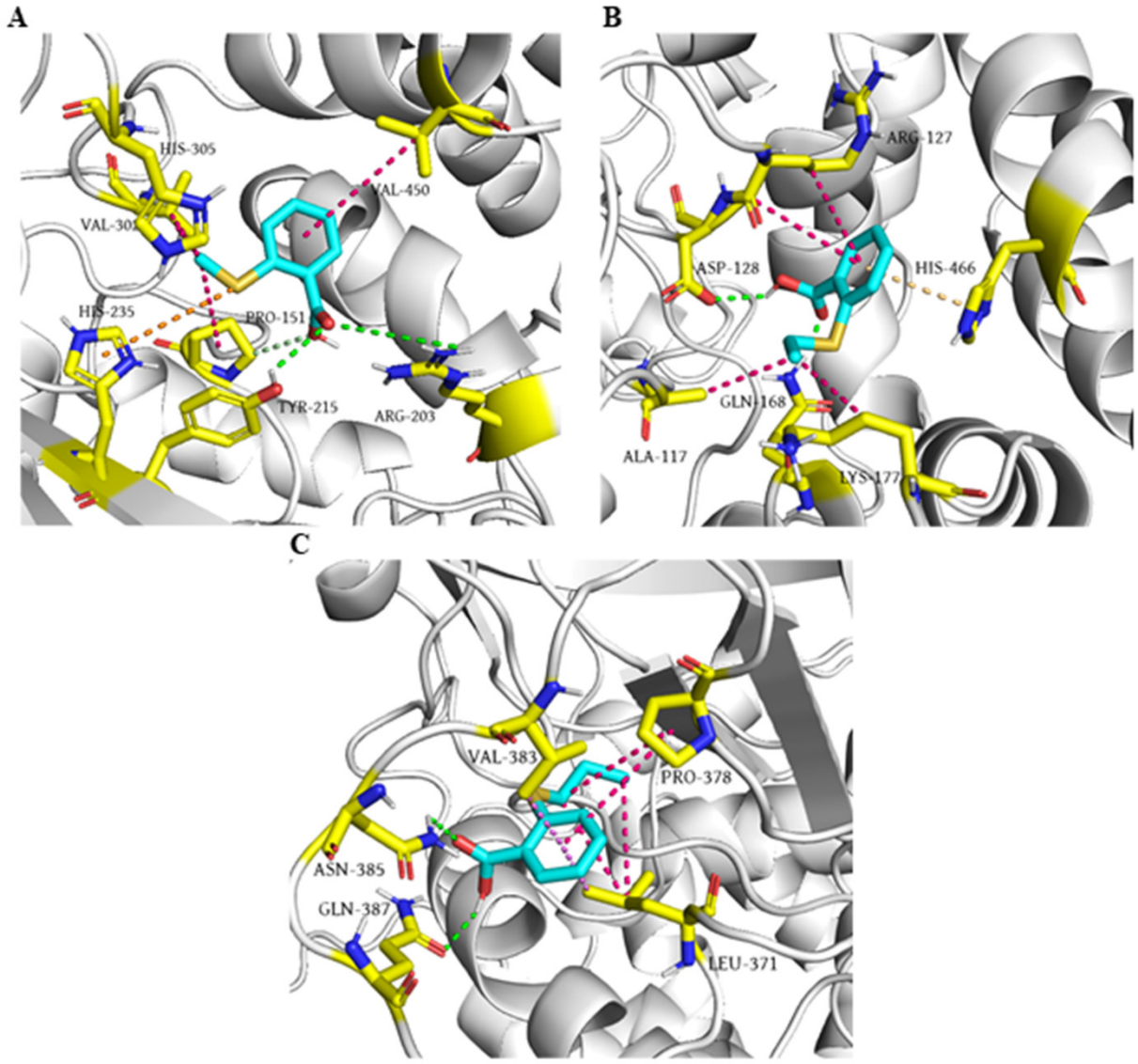
Ligand	Target enzyme	Free binding energy (kJ/mol)	Equilibrium binding constant, K_b (M^{-1})
L1	Catalase	- 19.2464	$2.3651 \cdot 10^3$
	Glutathione peroxidase	-15.4808	$5.1732 \cdot 10^2$
L2	Catalase	- 20.0832	$3.3155 \cdot 10^3$
	Glutathione peroxidase	-17.1544	$1.0165 \cdot 10^3$
L3	Catalase	-21.3384	$5.5029 \cdot 10^3$
	Glutathione peroxidase	-17.9912	$1.4250 \cdot 10^3$

During molecular docking of L1 into the CAT active site, tested molecule bound to the target enzyme with a free binding energy value of -19.2464 kJ/mol and an equilibrium binding constant of $2.3651 \cdot 10^3$ M^{-1}. The interaction of L1 and CAT is characterized by the formation of two conventional hydrogen bonds. The carbonyl oxygen atom of L1 carboxyl group (hydrogen bond acceptor) forms a hydrogen bond with phenolic group of TYR215 (hydrogen bond donor) and a hydrogen bond with a guanidine group of residue ARG203 (hydrogen bond donor). Hydroxyl oxygen atom of L1 carboxyl group forms a weak C-H hydrogen bond with pyrrolidine ring of residue PRO151, whereas methylthio group from the side chain of L1 forms π-sulfur interaction with imidazole ring of HIS235 residue. In addition, carbon atom of methylthio group is involved in the formation of multiple hydrophobic interactions with residues PRO151 (alkyl), VAL302 (alkyl), and HIS305 (π-alkyl). Finally, the benzene ring of L1 establishes a π-alkyl interaction with isopropyl group of VAL450 (**Fig. 5A**).

L2 exhibited slightly higher binding affinity for CAT compared to L1, with a docking score (-20.0832 kJ/mol) and an equilibrium binding constant ($3.3155 \cdot 10^3$ M^{-1}). Similarly as L1, carbonyl oxygen atom of L2 carboxyl group (hydrogen bond acceptor) interacts with amide group of GLN128 side chain (hydrogen bond donor). On the other hand, hydroxyl oxygen atom of L2 carboxyl group (hydrogen bond donor) establishes a hydrogen bond with carbonyl oxygen atom of ASP128 carboxyl group (hydrogen bond acceptor). Nitrogen atom of HIS466 imidazole ring forms an electrostatic π-cation interaction with benzene core of L2. The benzene ring of L2 is also involved in the formation of amide-π interaction with a peptide bond between residues ARG127 and ASP128, and in the formation of π-alkyl interaction with ARG127. Additionally, carbon atoms of ethylthio group establishes two hydrophobic alkyl interactions with side chains of residues ALA117 and LYS177 (**Fig. 5B**).

L3 formed even nine binding interactions into the active site of CAT and exhibited the highest binding affinity, as evident from the values of free binding energy (-21.3384kJ/mol) and equilibrium binding constant ($5.5029 \cdot 10^3$ M^{-1}). Carboxyl group of L3 through hydroxyl oxygen atom (hydrogen bond donor) interacts with amide group of GLN387 (hydrogen bond acceptor), while the carbonyl oxygen atom of L3 carboxyl group (hydrogen bond acceptor) forms a hydrogen bond with amide group of ASN385 (hydrogen bond donor). Benzene ring of L3 significantly contributes to the high binding affinity of this molecule towards CAT by formation of three hydrophobic interactions with residues LEU371, VAL383 (two π-σ interaction) and PRO378 (π-alkyl interaction). In addition, propylthio group of L3 side chain participates in the ligand-protein interaction by forming four hydrophobic interactions of alkyl type with residues PRO378 (two) and LEU371 (two) (**Fig. 5C**).

Figure 5. Molecular docking of L1 (**A**), L2 (**B**), and L3 (**C**) into the active site of catalase.



Conventional hydrogen bonds (green dashed lines), C-H hydrogen bonds (pale green dashed lines), π-σ hydrophobic interactions (violet dashed lines), other types of hydrophobic interactions (hot pink dashed lines), π-sulfur interactions (orange dashed lines), and electrostatic interactions (light orange dashed lines) are shown within three-dimensional depiction of binding contacts.

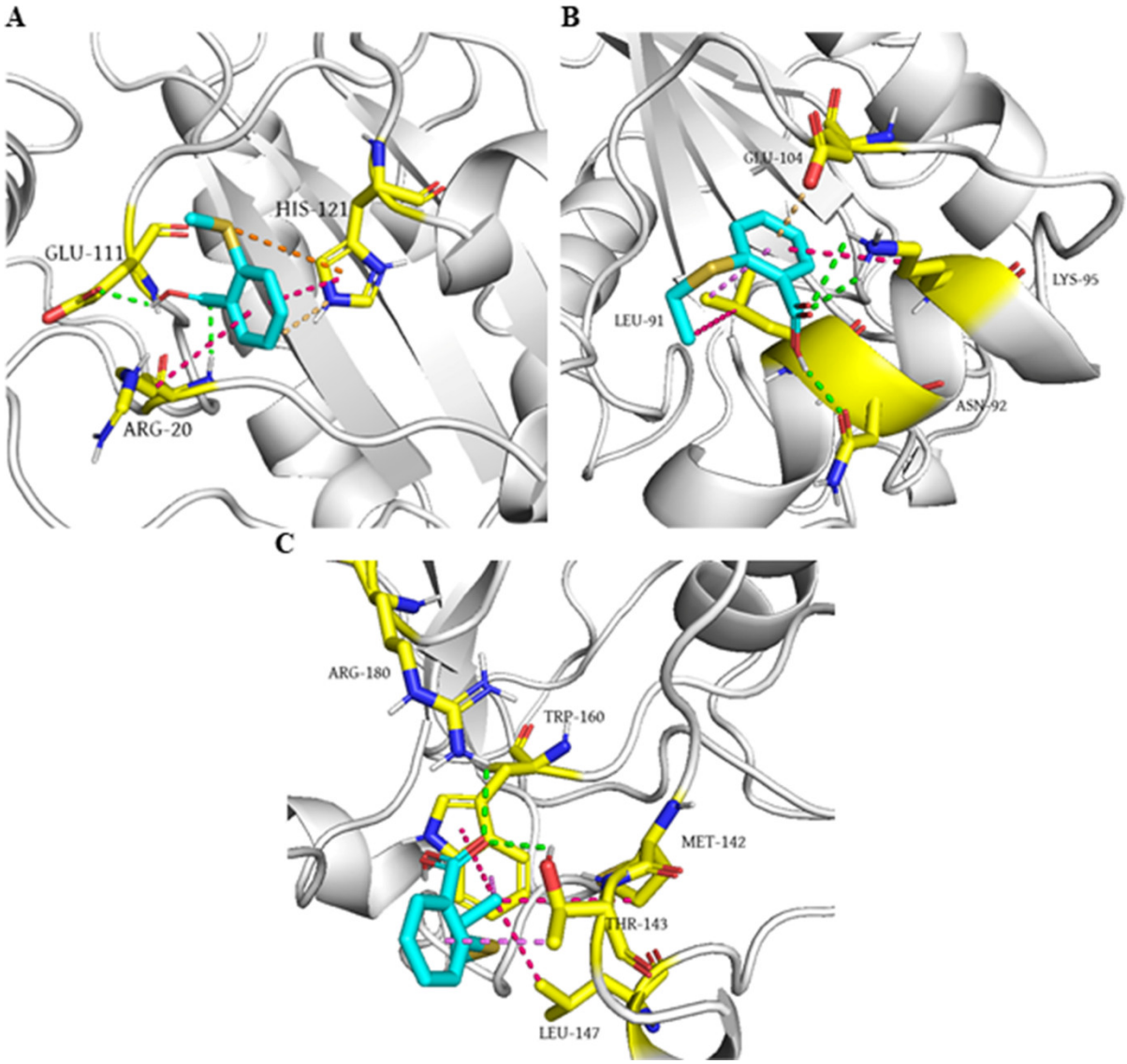
When L1 was docked into the active site of GPx, it achieved the highest free binding energy (-15.4808 kJ/mol) with the lowest value of the equilibrium binding constant ($5.1732 \cdot 10^2$ M^{-1}) compared to the other two tested molecules. Carboxyl group of L1 forms two conventional hydrogen bonds. Namely, carbonyl oxygen atom (hydrogen bond acceptor) interacts with guanidine group of ARG20 (hydrogen bond donor), while hydrogen atom of the same group is bound to carbonyl oxygen of GLU111 side chain (hydrogen bond acceptor). Only one electrostatic interaction is formed between benzene ring's π-electrons of L1 and imidazole nitrogen of HIS121. The same amino acid establishes π-π type of hydrophobic interaction with aromatic nucleus of tested ligand. Benzene ring of L1 forms one more hydrophobic contact with carbon atom of ARG20 side chain (**Fig. 6A**).

L2 bound into the binding pocket of GPx with a free binding energy value of -17.1544 kJ/mol and an equilibrium binding constant of $1.0165 \cdot 10^3$ M^{-1}. Carbonyl oxygen atom of carboxyl group achieves two conventional hydrogen bonds simultaneously with primary amino group of LYS95 (hydrogen bonds donor), whereas hydroxyl part of the same group interacts as proton donor with carbonyl oxygen of ASN92. Benzene ring of L2 forms π-σ and π-alkyl interactions with hydrocarbon groups of LEU91 and LYS95, respectively. A single π-anion interaction is established between the π-electrons of the L2 aromatic core and the

carboxylate anion of GLU104 residue. Finally, carbon atom of ethylthio group forms alkyl type of hydrophobic interaction with LEU91 (**Fig. 6B**).

The most stable complex with Gpx was formed by L3 with a free binding energy value of -17.9912 kJ/mol and an equilibrium binding constant of 1.4250 $\cdot 10^3$ M^{-1}. Hydroxyl group of THR143 and guanidine group of ARG180 as proton donors simultaneously establish two hydrogen bonds with carbonyl oxygen of ligand's carboxyl group. The indole bicyclic system of the residue TRP160 forms two hydrophobic interactions of π-alkyl and π-σ type with the carbon atom of L3 propylthio group. The same carbon atom of ligand forms two alkyl contacts with LEU147 and MET142. Benzene ring of L3 achieves the only remaining hydrophobic interaction (π-σ type) with the hydrocarbon part of the THR143 side chain (**Fig. 6C**).

Figure 6. Molecular docking of L1 (**A**), L2 (**B**), and L3 (**C**) into the active site of glutathione peroxidase.



Conventional hydrogen bonds (green dashed lines), C-H hydrogen bonds (pale green dashed lines), π-σ hydrophobic interactions (violet dashed lines), other types of hydrophobic interactions (hot pink dashed lines), π-sulfur interactions (orange dashed lines), and electrostatic interactions (light orange dashed lines) are shown within three-dimensional depiction of binding contacts.

DISCUSSION

Numerous NSADs are widely prescribed for the treatment of many inflammatory diseases, but the use of these drugs is sometimes restricted due to different adverse reactions. However, the data showed that an excessive amount of ROS in cells leads to induction of oxidative stress, and in the case when oxidative stress is out of control many dangerous consequences for cells may occur among which inflammation stands out as the most significant. Namely, it was reported that increased production of free radicals at the inflammatory site is the principal cause of tissue damage caused by the presence of different inflammatory disorders. Due to this finding, it can be assumed that the control of oxidative stress is a promising strategy in reducing the occurrence of numerous inflammatory diseases (56). To avoid side effects of NSAIDs many efforts have been made regarding development of new compounds to prevent ROS production and consequent inflammation (57). In this study, we investigated the potential of different S-alkyl derivatives of thiosalicylic acid in dose-dependent manner on oxidative stress in case of acute inflammation caused by single carrageenan injection. The results of our study demonstrated high potential of S-alkyl derivatives to prevent free radicals' formation and also to increase the activity of antioxidant defense system. To further elucidate the molecular mechanisms involved in antioxidative activity of tested compounds, we performed the binding affinity assessment of S-alkyl derivatives towards selected antioxidant enzymes using an *in silico* approach. For that purpose, we conducted molecular docking studies with aim to confirm the molecular interaction between tested compounds and antioxidant enzymes and to obtain precise insight into the binding interactions involved in the formation of the tested ligand-enzyme complex.

Previously published studies have shown significant role of ROS production in the inflammatory reaction highlighting $OH^{\cdot}$, H_2O_2, and $O_2^{\cdot-}$ as key factors for both the initiation and progression of cellular damage (58). In the present study, acute administration of S-alkyl derivatives led to prominent change in $O_2^{\cdot-}$ value after carrageenan-induced inflammatory state reducing the release of this pro-oxidative markers in rat paw tissue. Among tested derivatives, L1 and L2 showed dose-dependent manner in reduction of this parameter (**Fig. 2A; Fig. 3A**), while the L3-15 stood out as the most effective dose of S-propyl thiosalicylic acid derivative (**Fig. 4A**). However, the strongest potential in $O_2^{\cdot-}$ reduction was observed in IND rats compared to all tested doses of S-alkyl derivatives. These results can be explained due to possible function of NSAIDs to eliminate toxic oxygen radicals such as $O_2^{\cdot-}$ and $OH^{\cdot}$ either by themselves or by formation of metal complexes *in vivo* (59). Additionally, carrageenan injection is closely associated with release of different mediators including nitric oxide (60) which is confirmed by results obtained in our study showing elevated NO_2^- level in CMC group of rats. Also, we demonstrated strong potential of all tested S-alkyl derivatives to significantly reduce the level of this marker not only compared to CMC but also with positive control group of rats (**Fig. 2-4B**). Measurement of NO_2^- is useful indicator for determination of NO level as an important signaling molecule with multiple crucial roles in different systems but also in inflammatory response. Namely, NO is free uncharged radical and due to unpaired electron it can easily reacts with $O_2^{\cdot-}$ to form extremely toxic peroxynitrite that interacts directly with DNA, proteins, lipids, and induces oxidative stress (61). Thus, one of the proposed mechanism by which our tested S-alkyl derivatives prevent oxidative stress in state of acute inflammation may be assigned to their potential to prevent peroxynitrite formation due to reduction in $O_2^{\cdot-}$ and NO_2^- levels. Moreover, the generation of ROS is closely linked with malondialdehyde (MDA) production, a thiobarbituric acid reactive substances, as a product of lipid peroxidation (21). In our study, the level of MDA is measured as TBARS in subplantar tissue of rats and the results showed significantly higher TBARS value in CMC group compared to the other groups, providing a piece of evidence for inflammation-induced oxidative stress (**Fig. 2-4C**). Also, we observed markedly reduced TBARS level in rats treated with indomethacin as well as with examined S-alkyl derivatives in all tested doses compared to untreated rats. However, L3 seems to have higher potential to reduce release of this pro-oxidant compared to positive IND control as this S-alkyl derivative achieved the lowest TBARS level in all tested doses compared to standard applied NSAIDs (**Fig. 4C**). These findings are linked with that earlier obtained indicating significantly increased MDA level after carrageenan injection but also the effect of indomethacin pretreatment to attenuate increased MDA level (62). Moreover, our results are in agreement with results obtained by *Zoubair et al.* indicating scavenging activity of ibuprofen via reduction in TBARS level thus restoring the balance of redox reactions in mice treated with this NSAID (24). Since that ROS generation releases MDA from plasma membrane, tissues accumulation of MDA may be used for the determination of degree of oxidative stress and inflammation. In that sense, we can assume that our tested compound achieved antioxidant properties due to reduced TBARS tissue content and consequent reduced inflammation state in rat tissue.

As we above-mentioned, in state of oxidative stress the cellular antioxidant capacity is suppressed and excessive ROS production occurs. The first line defense antioxidants include SOD, CAT, and GSH, taking the main place in the overall antioxidant defense system (63). Under physiological condition, low amounts of $O_2^{\cdot-}$ is converted to H_2O_2 as a less toxic radical, by the action of SOD. Acute administration of indomethacin, as a standard NSAID, succeeded to increase SOD activity compared to untreated rats thus improving redox state in rat paw tissue. However, the most important finding of our study concerns the potential of all tested doses of S-propyl alkyl derivate to significantly improve SOD activity compared to CMC group, reaching the same SOD level as standard NSAID, indomethacin (**Fig. 4D**). Therefore, increased SOD level in L3 groups of rats may be the reason for the significantly decreased TBARS levels in these groups due to suppression of this pro-oxidative molecule by the activation of SOD as enzyme of antioxidant defense system. The same results were published by *Emad et al.* who reported

significantly reduced lipid peroxidation and increased antioxidant activity in rats treated with diclofenac sodium (64). We also compared the potential of different S-alkyl derivatives to improve CAT and GSH activity in rat subplantar tissue followed by carrageenan-induced inflammation. Our findings confirmed great potential of all tested compounds to improve CAT activity compared to untreated rats, but medium dose of L2 as well as the lowest and medium doses of L3 showed higher antioxidant potential compared to IND group of rats (**Fig. 2E; Fig. 3E**). Molecular docking results strongly support the findings obtained in *in vivo* assay concerning CAT activity and confirms the highest antioxidant potential of L3 in comparison to other investigated compounds. Namely, results of CAT activity are in good agreement with the free binding energies obtained in molecular docking study. *In silico* results indicate that binding affinity of S-alkyl derivatives towards CAT increases in a sequence L1<L2<L3. Accordingly, the forming of the most stable L3-CAT complex can be explained by the formation of the highest number of non-covalent hydrophobic interactions between ligand and protein (seven). Benzene ring and propylthio group of L3 are predominantly involved in the formation of these contacts (**Fig. 5C**). Despite the high binding affinity of L3 for CAT, this molecule exhibited a significantly higher value of free binding energy (-21.3384 kJ/mol) compared to *S*- and *R*-naringenin (-42.2584, and -45.1872 kJ/mol, respectively) (65). Also, in the same study enantiomers of neringenin formed hydrogen bonds with residues SER120 and ARG112 in the active site of CAT in contrast to our tested compounds (L1: TYR215, ARG203; L2: ASP128; L3: ASN385, GLN387), which suggests that S-alkyl derivatives of thiosalicylic acid bind to the CAT with different binding orientation.

On the other hand, there were a differences in GSH activity between examined S-alkyl derivatives. While only the highest dose of L1 improved this antioxidant activity compared to untreated rats but still without significant differences compared to IND (**Fig. 2F**), rats from L2-20 group reached higher GSH activity compared to those treated with indomethacin as well as other doses of this derivative (**Fig. 3F**). However, L3 showed the greatest potential to increase GSH activity especially in medium and the highest doses thus reaching the values higher that IND group of rats (**Fig. 4F**). The antioxidant potential of L3 through the improvement of GSH activity is indirectly confirmed by molecular docking studies. Specifically, it is well known that GPx enzyme catalyzes the conversion of GSH to its oxidized form GSSH. However, GSH can be regenerated from GSSG via enzyme glutathione reductase (GR) (66). Molecular docking results indicate that L3 interacts with GPx achieving the lowest value of free binding energy and the highest value of an equilibrium binding constant, which indirectly confirms the best antioxidant potential of L3 and its effect on increase of GSH levels. Amino acid residues in the active site of GPx, ARG20 and GLU11, ASN92 and CYS95, THR143 and ARG180 are hydrogen-bonded in the molecular interaction with L1, L2, L3, respectively (**Fig. 6C**). For comparison, *R*-neringenin established three hydrogen bonds with residues GLN82 and ARG98, indicating the completely distinct binding mode. In spite of the high binding affinity of L3 for GPx, this derivative demonstrated a notable higher value of free binding energy (-17.9912 kJ/mol) compared to S- and R-naringenin (-29.7064 kJ/mol) (65). The high antioxidant potential of S-alkyl derivatives documented in the present study is in correlation with earlier published data indicating ability of subacute treatment with diclofenac sodium to improve CAT and GSH activity thus serving as possible agent for alleviation of stress-induced changes (64). *De La Cruz* et al. obtained similar results demonstrating increased GPx activity in rats' tissue treated with salicylic and acetylsalicylic acids with reduced potential to decrease GSH level compared to control group of rats (67).

CONCLUSIONS

In summary, this study indicate that S-alkyl derivatives can scavenge ROS and improve antioxidant defense system thus preventing oxidative stress in tissue damaged by inflammation. The results of *in vivo* study showed similar potential of tested compound to indomethacin in reduction of pro-oxidative markers. However, L3 derivative stood out as the most potent in increase of antioxidant enzyme activity which is reflected in higher GSH activity compared to indomethacin administration. In addition, molecular docking results confirm high antioxidant potential of L3, due to a strong binding affinity of this molecule towards key antioxidant enzymes.

In vivo results along with *in silico* analysis suggest the therapeutic potential of L3 to treat or prevent inflammatory disorders in which oxidative is one of the causes. However, further research is necessary to clearly reveal proposed mechanism in the basis of their beneficial effects on redox balance recovery.

A significant limitation of the present study was the relatively small set of investigated compounds that limits our conclusions regarding the antioxidative activity of S-alkyl derivatives of thiosalycilic acid. On the other hand, conducted semi-flexible docking protocol implies conformational rigidity of the protein residues, which is also a limitation of this study. Finally, within *in silico* analysis, impact of water molecules in the active sites of targeted molecules was not investigated, which is a frequent shortcoming of molecular docking studies. To overcame this issue molecular dynamic simulation studies should be performed in future research.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this manuscript.

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