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Synthesis and Anti-inflammatory Activities of 4-Arylidene-2-phenyloxazol-5(4*H*)-one Derivatives

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In this study, four different 4-arylidene-2-phenyloxazol-5(4*H*)-ones (**5a-d**), were synthesized *via* the Erlenmeyer-Plöchl reaction and characterized by FT-IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic techniques. Evaluation of their *in vitro* anti-inflammatory activities using the heat-induced human red blood cell (HRBC) membrane stabilization assay revealed concentration dependent inhibitory effects. The IC_{50} values of **5a**, **5b**, **5c** and **5d** were reported as 4.65 ± 0.22 mM, 7.34 ± 0.28 mM, 5.23 ± 0.18 mM and 1.96 ± 0.09 mM, respectively. O-Acetyl salicylic acid as standard showed its IC_{50} value at 6.41 ± 0.18 mM. Docking studies against human cyclooxygenase (COX) 1 and 2 revealed affinities of the compounds to binding cavities of COX enzymes. Particularly, compound **5d** exhibited remarkable activity in both HRBC membrane stabilization and the formation of hydrogen bonds with binding cavities. This suggests a potential correlation between the number of hydroxyl groups of oxazolone derivatives and the enhancement of anti-inflammatory activity.

Keywords: Oxazolones, Anti-inflammatory, Erlenmeyer-Plöchl reaction, HRBC membrane stabilization.

INTRODUCTION

Inflammation is a complex biological response initiated by biological systems themselves in response to various stimuli. It originates from the metabolism of arachidonic acid, involving three enzymatic pathways *viz*. the cyclooxygenase (COX) pathway, the lipoxygenase (LOX) pathway and the monooxygenase cytochrome P450 pathway [1]. The COX pathway leads to the production of prostaglandin H_2 (PGH_2), which is the pivotal step in prostaglandin (PG) formation [2]. The COX pathway encompasses two isoforms: the constitutively expressed COX-1 isoform and the inducible COX-2 isoform. These isoforms catalyze identical reactions but produce different prostaglandins and thromboxanes [3]. Prostaglandins derived from COX-1 mainly play roles in maintaining cellular and tissue homeostasis and normal physiological functions [4]. Conversely, prostaglandins derived from COX-2 are associated with fever, pain and inflammation in the body [5]. However, COX-1 has also been implicated in the inflammatory process [3].

Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly prescribed to treat fever, inflammation and autoimmune diseases such as rheumatoid arthritis and osteoarthritis [6]. Selective NSAIDs predominately inhibit the COX-2 isoform, whereas non-selective ones inhibit both COX isoforms [7,8]. However, their use is limited due to side effects resulting from impacts on molecules beyond their intended targets. Common side effects include osteoporosis, glaucoma, diabetes, abdo-minal obesity, cataracts, skin atrophy, avascular necrosis and infection, growth retardation and hypertension [9,10].

Given the limitations of NSAIDs, there is a growing interest in multifunctional compounds with diverse biological activities for treating complex diseases such as type 1 diabetes, multiple sclerosis and rheumatoid arthritis [1,5]. In the recent years, heterocyclic compounds have earned a prominent attention due to their diversified biologically and medically important uses [11]. Several natural and synthetic heterocyclic compounds serve as potential scaffolds and can be found in more than 90% of novel drugs [5]. Therefore, the research interest on these compounds in medicinal chemistry and biochemistry have been intensified in the recent past [11]. Oxazolones are heterocyclic compounds that exhibit numerous biological activities. These five membered heterocyclic compounds contain

oxygen and nitrogen as hetero atoms. The C-2 and C-4 positions of oxazolones are particularly explored for biological activities such as antimicrobial, antidiabetic, anti-inflammatory, analgesic, antidepressant, anticancer, anti-HIV, tyrosinase inhibiting, anticonvulsant and anti-obesity activities [12-15].

In this study, we focused on synthesizing various derivatives of 4-arylidene-2-phenyl-oxazol-5(4*H*)-ones, wherein the position of hydroxyl group (-OH) attached to 4-arylidene moiety was systematically altered, using the Erlenmeyer-Plöchl reaction reaction. Hydroxyl groups are a prevalent functional group in organic compounds and facilitate hydrogen bonding [16]. The presence of hydroxyl groups in molecules can impart specific properties that are often relevant in the context of anti-inflammatory agents [17]. Moreover, the anti-inflammatory activities of novel oxa-zolone derivatives were also against heat-induced human red blood cell (HRBC) membrane stabilization, alongside conducting *in silico* assessments against human COX-1 and COX-2.

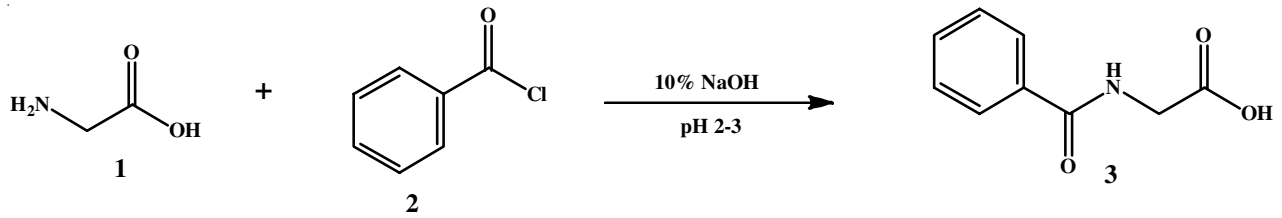
EXPERIMENTAL

Chemicals for synthesis were purchased from Sigma- Aldrich Company, Sisco Research Laboratories Pvt. Ltd., Loba Chemie Pvt. Ltd. and Fluka Chemika. The progress of the reactions was monitored by using TLC 0.2 mm thickness aluminum sheets pre-coated with silica gel. Melting points of all compounds were determined by the open capillary method using Afon® DMP200 melting point device. Fourier transform infrared spectra were recorded on Horizon ABB-MB 3000ATR spectrometer. High resolution mass spectrometry data were obtained using Agilent 6230 spectrometer, Agilent 1200 series system consisting of G13793 degasser, G1312B binary pump and G1367C auto sampler was used as the injector. Methanol with 0.1% formic acid at a flow rate of 0.2 mL/min was used in the mobile phase. ^{1}H and ^{13}C NMR of all the compounds were recorded on Bruker 400 MHz NMR spectrometer using DMSO as an internal reference.

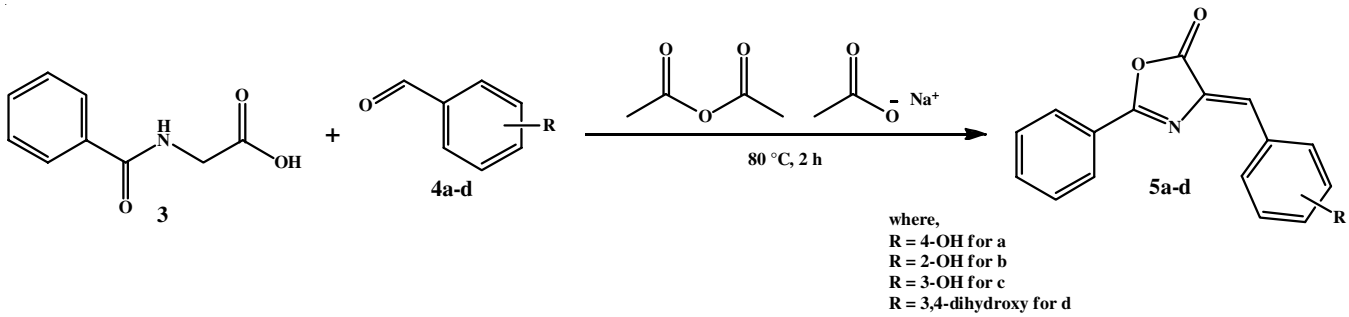
Synthesis of hippuric acid (benzoyl glycine): Hippuric acid (**3**) was synthesized according to a previously described method (**Scheme-I**) [12]. A 5.0 g of glycine (**2**) was dissolved in 10 mL of 10 % NaOH solution and then kept in ice cold water followed by dropwise addition of 10.8 mL of benzoyl chloride while stirring. After the addition of benzoyl chloride, the pH of the reaction mixture was adjusted to 2-3 using conc. HCl and a white precipitate was obtained. Yield: 9.67 g, 85.58 %; m.p.: 187.0-189.0 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3331.40 (N-H *str.*), 3012.20 (carboxylic O-H *str.*), 1734.08 (-C=O *str.*); ^{1}H NMR (400 MHz, DMSO) δ ppm: 8.82 (s, 1H, -NH), 7.95 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.87 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.58 (dd, *J* = 32.0, 9.4 Hz, 1H, Ar-H), 7.51-7.43 (t, 2H, Ar-H), 3.93 (d, *J* = 5.9 Hz, 2H, -CH_2-). ^{13}C NMR (101 MHz, DMSO) δ ppm: 171.2 (C(=O)OH), 166.4 (C(=O)N), 131.3-127.2 (Ar-C), 41.2 (C-N). HRMS *m/z* $[M+H]^+$ calcd; $C_9H_{10}NO_3^+$, 180.0655, found 180.0663.

Synthesis of oxazolone derivatives (5a-d): Different 4-arylidene-2-phenyloxazol-5(4*H*)-ones (**5a-d**) were synthesized through Erlenmeyer-Plöchl reaction according to the method described earlier with slight modifications (**Scheme-II**) [18]. Hippuric acid (benzoyl glycine) (**3**), acetic anhydride, sodium acetate and corresponding hydroxybenzaldehyde (**4a-d**) were placed into a round bottom flask in a 1:4:2:1 molar ratio. Then the reaction mixture was heated with continuous stirring at 80 ºC for 2 h. The progress of the reaction was monitored by performing TLC. Finally, the reaction mixture was cooled and precipitated in crushed ice.

4-(4-Hydroxybenzylidene)-2-phenyloxazol-5(4*H*)-one (5a): Pale yellow crystalline solid, yield: 0.79 g, 54%, m.p.: 171-173 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3378.29 (O-H *str.*), 3078.59 (aromatic C-H *str.*), 1790.27 (-C=O *str.*), 1653.20 (-C=N *str.*), 1556.20 (C=C *str.*); ^{1}H NMR (400 MHz, DMSO) δ ppm: 8.37



Scheme-I: Reaction scheme for synthesis of hippuric acid (**3**) by using glycine (**1**) and benzoyl chloride (**2**)



Scheme-II: Reaction scheme for synthesis of 4-arylidene-2-phenyloxazol-5(4*H*)-one derivatives (**5a-d**) by using hippuric acid (**3**) and hydroxybenzaldehydes (**4a-d**)

(d, *J* = 8.7 Hz, 2H, Ar-H), 8.15 (dd, *J* = 7.4 Hz, 2H, Ar-H), 7.74 (t, *J* = 7.3 Hz, 1H, Ar-H), 7.65 (m, *J* = 7.7 Hz, 2H, Ar-H), 7.39 (s, 1H, -CH=), 7.32 (d, *J* = 8.6 Hz, 2H, Ar-H), 2.31 (s, 1H, -OH); ^{13}C NMR (101 MHz, DMSO) δ ppm: 168.8 (C=O), 163.1 (C=N), 152.4 (C-OH), 133.7 (C=C), 133.6-125.1 (Ar-C), 122.5 (-CH=); HRMS *m/z* $[M+H]^+$ calcd; $C_{16}H_{12}NO_3^+$, 266.0812, found 266.0801.

4-(2-Hydroxybenzylidene)-2-phenyloxazol-5(4*H*)-one (5b): Yellow crystalline solid, yield: 0.18 g, 24%, m.p.: 142-144 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3356.33 (O-H *str.*), 3074.85 (aromatic C-H *str.*), 1790.94 (-C=O *str.*), 1652.13 (-C=N *str.*), 1598.57 (C=C *str.*); ^{1}H NMR (400 MHz, DMSO) δ ppm: 8.64 (s, 1H, -CH=), 8.15 (d, *J* = 7.5 Hz, 1H, Ar-H), 7.97 (d, *J* = 7.5 Hz, 2H, Ar-H), 7.79 (d, *J* = 6.1 Hz, 1H, Ar-H), 7.69-7.61 (dd, 2H, Ar-H), 7.56 (dd, *J* = 9.8, 4.9 Hz, 2H, Ar-H), 7.39 (t, *J* = 7.5 Hz, 1H, Ar-H), 2.41 (s, 1H, -OH); ^{13}C NMR (101 MHz, DMSO) δ ppm: 169.1 (C=O), 163.9 (C=N), 150.2 (C-OH), 133.4 (C=C), 132.2-125.0 (Ar-C), 124.1 (-CH=); HRMS *m/z* $[M+H]^+$ calcd; $C_{16}H_{12}NO_3^+$, 266.0812, found 266.0820.

4-(3-Hydroxybenzylidene)-2-phenyloxazol-5(4*H*)-one (5c): Yellow crystalline solid, yield: 1.30 g, 88%, m.p.: 164-166 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3372.52 (O-H *str.*), 3078.16 (aromatic C-H *str.*), 1789.80 (-C=O *str.*), 1650.94 (-C=N *str.*), 1558.36 (C=C *str.*); ^{1}H NMR (400 MHz, DMSO) δ ppm: 8.20-8.14 (dd, 2H, Ar-H), 8.11 (dd, *J* = 18.0, 1.3 Hz, 2H, Ar-H), 7.74 (t, *J* = 7.4 Hz, 1H, Ar-H), 7.65 (m, *J* = 7.9 Hz, 2H, Ar-H), 7.58 (t, *J* = 8.0 Hz, 1H, Ar-H), 7.37 (s, 1H, -CH=), 7.29 (d, *J* = 8.1 Hz, 1H), 2.34 (s, 1H, -OH); ^{13}C NMR (101 MHz, DMSO) δ ppm: 169.1 (C=O), 163.5 (C=N), 150.7 (C-OH), 134.7 (C=C), 133.8-125.0 (Ar-C), 124.7 (-CH=); HRMS *m/z* $[M+H]^+$ calcd; $C_{16}H_{12}NO_3^+$, 266.0812, found 266.0815.

4-(3,4-Dihydroxybenzylidene)-2-phenyloxazol-5(4*H*)-one (5d): Pale yellow crystalline solid, yield: 0.36 g, 89%, m.p.: 128-130 ºC. FTIR (KBr, ν_{max}, cm^{-1}): 3375.14 (O-H *str.*), 3075.24 (aromatic C-H *str.*), 1791.91 (-C=O *str.*), 1654.41 (-C=N *str.*), 1556.46 (C=C *str.*); ^{1}H NMR (400 MHz, DMSO) δ ppm: 8.22 (dd, *J* = 10.7, 1.8 Hz, 2H, Ar-H), 8.17-8.09 (d, 2H, Ar-H), 7.74 (t, *J* = 7.4 Hz, 1H, Ar-H), 7.65 (m, *J* = 7.6 Hz, 2H, Ar-H), 7.46 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.37 (s, 1H, -CH=), 2.35 (s, 1H, -OH), 2.32 (s, 1H, -OH); ^{13}C NMR (101 MHz, DMSO) δ ppm: 168.1 (C=O), 163.5 (C=N), 143.9 (C-OH), 142.2 (C-OH), 133.8 (C=C), 133.7-125.0 (Ar-C), 124.2 (-CH=); HRMS *m/z* $(M-H)^-$ calcd; $C_{16}H_{10}NO_4^-$, 280.0615, found 280.0604.

Evaluation of *in vitro* anti-inflammatory activity

HRBC membrane stabilization method: The assay was carried out as per the method described in Leelaprakash & Dass with slight modifications [19]. Preparation of compounds at different concentrations: First, 200 mM stock solutions from each compound were prep-ared in DMSO solvent. Then they were further diluted to obtain 20 mM solutions with normal saline. Double dilution series were prepared from 20 mM solution to achieve concentrations of 10 mM, 5 mM, 2.5 mM, 1.25 mM, 0.625 mM and 0.3125 mM.

Blood collection, preparation and storage: Human blood was collected from a healthy volunteer who had been absent from taking any anti-inflammatory drug for 2 weeks before the blood collection. Prior to experiment, it is essential to secure the donor's written informed consent and obtain approval from the relevant ethics committee. Fresh human blood (5 mL) was collected into two 2.5 mL K_3-EDTA tubes and then, the tubes were centrifuged) at 3000 rpm for 20 min at 4 ºC. The supernatant of each tube was discarded carefully and cells were washed three times using equal amounts of normal saline for three times until the supernatant become colourless. Finally, the supernatant was discarded before storing the packed cells in the freezer at −20 ºC. Prepared cells were utilized for the assay within 7 days.

Assay: Prepared blood cells were reconstituted to prepare 10 % v/v HRBC suspension with normal saline. The HRBC suspension (100 µL) was added into 1 mL of different concentrations (20 mM, 10 mM, 5 mM, 2.5 mM, 1.25 mM, 0.625 mM and 0.3125 mM) of samples in Eppendorf tubes. For the control, HRBC suspension was added into 1 mL of normal saline. After addition, all the reaction mixtures were gently mixed and the tubes were placed in the hot water bath for 30 min at 56 ºC. Then the tubes were taken out and cooled under running tap water. All the tubes were centrifuged at 3000 rpm for 15 min at 4 ºC and the absorbance of the supernatants was measured at 560 nm by using the microplate reader (Thermo-Fisher Scientific Co. 1510-01360). O-Acetyl salicylic acid with different concentrations (20 mM, 10 mM, 5 mM, 2.5 mM, 1.25 mM, 0.625 mM and 0.3125 mM) was used as standard drug.

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Statistical analysis: All values of HRBC membrane stabilization assay were expressed as mean percentages ± standard error of the median (SEM). The IC_{50} values of the assay were obtained by non-linear curve fit analysis method using GraphPad Prism (version 9.5.1 (733), GraphPad software, Boston, MA, USA) software.

Evaluation of *in silico* anti-inflammatory activity: Reported data showed that COX-1 and COX-2 enzymes have identical molecular weights and ligand binding sites. However, their inhibitors have exhibited different binding modes due to the sequence homology of amino acids close to 65%. Replacement of ILE523 with smaller VAL523 and the changes of TYR355 conformations allow the formation of a hydrophobic pocket in COX-2. Position 523 has been recognized as the main cluster differentiation between COX-1 and COX-2 [20].

Selection of co-crystallized COX structures: The complete high-resolution structures of human COX-1 (PDB ID: 6Y3C) and human COX-2 (PDB ID: 5F1A), co-crystallized with salicylic acid were selected for molecular docking. Literature showed binding cavity of COX-1 enzymes contain ARG120, SER353, TYR355, TYR385, ILE523 and SER530 amino acid residues. However, an additional binding pocket has been created in COX-2 due to the replacement of ILE523 by VAL523. This additional binding cavity has LEU352, SER353, TYR355, PHE518 and VAL523 amino acids [20].

Molecular docking: The macromolecules, COX-1 (PDB ID: 6Y3C) and COX-2 (PDB ID: 5F1A) were retrieved from RCSB protein data bank, were prepared by removing water

TABLE-1
INHIBITORY PERCENTAGES OF HRBC MEMBRANE STABILIZATION ASSAY AGAINST OXAZOLONES (**5a-d**) AND O-ACETYL SALICYLIC ACID; VALUES GIVEN AS MEAN ± SEM; n = 3

Concentration (mM)	**5a**	**5b**	**5c**	**5d**	O-Acetyl salicylic
20	68.29 ± 0.25	61.37 ± 0.54	64.71 ± 0.53	71.30 ± 0.76	67.54 ± 0.62
10	66.83 ± 0.75	59.39 ± 0.42	62.61 ± 1.07	69.55 ± 0.51	59.78 ± 0.66
5	54.23 ± 1.39	47.63 ± 0.64	51.99 ± 0.53	66.04 ± 0.32	47.24 ± 0.97
2.5	30.92 ± 0.69	27.57 ± 0.56	35.54 ± 0.66	54.00 ± 1.67	32.58 ± 0.69
1.25	29.92 ± 0.75	24.35 ± 0.49	32.79 ± 0.37	44.39 ± 0.64	20.30 ± 1.12
0.625	28.30 ± 0.75	23.85 ± 0.53	22.94 ± 0.85	38.65 ± 0.89	17.67 ± 0.72
0.3125	17.45 ± 0.81	20.51 ± 0.34	14.58 ± 0.87	23.32 ± 0.39	12.65 ± 0.82

molecules, hetero atoms and additional chains. Active sites of both enzymes were explored with the help of reported data and by using BIOVIA Discovery Studio Visualizer (version 19.1.0.18287, BIOVIA, Dassault Systèmes, San Diego, CA, USA).

Simultaneously, 2D structures of ligands were drawn and converted into 3D structures by using ChemDraw Professional and Chem3D Ultra (version 22.0.0.22, Perkin-Elmer Informatics, Waltham, MA, USA) Energy minimization of the ligands were done using Avogadro version 1.2.0 software by choosing MMFF94s as the force field and conjugate gradient option.

Molecular docking between compounds (ligands) and COX enzymes was performed using Hermes GOLD version 5.3 software. All synthesized compounds (ligands) along with O-acetyl salicylic acid (aspirin) were docked into the pre-defined binding cavities of COX enzymes one at a time. Gold fitness values were obtained for the best ranking pose by analyzing 10 poses of each ligand using the ChemPLP score function. Upon the completion of docking, solutions were studied by using BIOVIA Discovery Studio Visualizer (version 19.1.0.18287, BIOVIA, Dassault Systèmes, San Diego, CA, USA) software.

RESULTS AND DISCUSSION

***In vitro* evaluation of anti-inflammatory activity:** Inflammation is usually characterized by swelling, redness, heating, pain and loss of function at the tissue level [21]. Lysosomes perform a vital and multifaceted function in the realms of immunity and inflammation. Their involvement includes overseeing autophagy, managing the release of cytokines through inflammasomes and regulating the sphingolipid metabolism [22]. Essentially, maintaining healthy lysosomes is crucial for a standard host response to infections and the preservation of a typical inflammatory reaction. When lysosomes are impaired, it results in irregular autophagy, increased inflammation activation and compromized infection control [2]. Stabilization of lysosomal membrane is therefore important in the management of inflammation [23]. In this case, HRBC membrane is a good model to mimic lysosomal membrane in the laboratory experiments [24]. Thus, we employed HRBC membrane stabilization assay to evaluate *in vitro* anti-inflam-matory activity.

In this work, the results showed the inhibitory activity of all compounds (**5a-d**) and O-acetyl salicylic acid (aspirin) in a concentration dependent manner (Fig. 1). At the highest concen-tration (20 mM), compound **5d** showed the highest inhibitory activity of 71.30 ± 0.76%, while compound **5b**

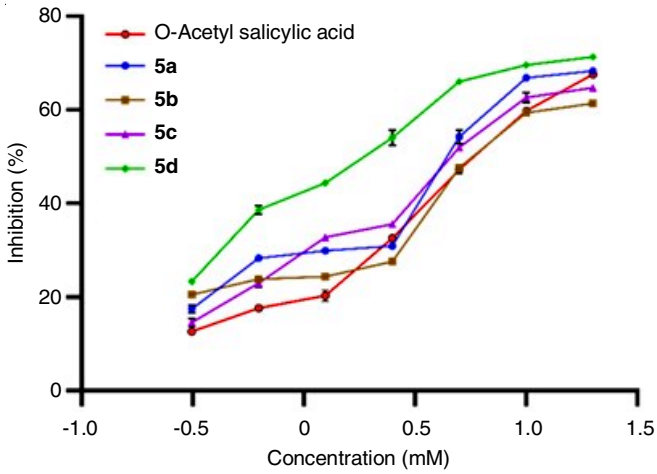


Fig. 1. Effect of oxazolone derivatives (**5a-d**) and O-acetyl salicylic acid on HRBC membrane stabilization; values expressed as mean ± SEM; n = 3

showed the lowest inhibitory activity of 61.37 ± 0.54%. At the lowest concentration of 0.3125 mM, the lowest inhibitory activity of 12.65 ± 0.82% were reported for the standard, O-acetyl salicylic acid while all the synthesized compounds showed compara-tively higher activities (Table-1).

The IC_{50} value or the half-maximal inhibitory concentration suggests the concentration of drug or inhibitor, which exhibits 50% inhibitory activity. It is a widely used informative measure of the efficacy of the tested compounds in bioassays. The results showed the lowest IC_{50} value of 1.96 ± 0.09 mM for the compound **5d**. Furthermore, the highest IC_{50} value was reported for compound **5b** while the second highest value was reported for O-acetyl salicylic acid (Table-2). Therefore, compounds **5a**, **5c** and **5d** were more effective than O-acetyl salicylic acid against the heat-induced membrane stabilization assay.

The *in vitro* study further revealed that all the tested compounds are potential HRBC membrane stabilizers. They must

TABLE-2
IC_{50} VALUES OF **5a-d** COMPOUNDS AND O-ACETYL SALICYLIC ACID AGAINST HRBC MEMBRANE STABILIZATION ASSAY; VALUES EXPRESSED AS MEAN ± SEM, n = 3

Compound	IC_{50} (mM)
5a	4.65 ± 0.22
5b	7.34 ± 0.28
5c	5.23 ± 0.18
5d	1.96 ± 0.09
O-Acetyl salicylic acid	6.41 ± 0.18

possess the capability of protecting lysosomal membrane against the triggering of inflammation. Thus, it can be concluded that these synthesized compounds are potential anti-inflammatory candidates by stabilizing the lysosomal membrane. Since the results have indicated that compound **5d** is the most effective candidate for HRBC membrane stabilization, it can be inferred that there might be a positive correlation between the number of substituted hydroxyl groups and the potential for exerting anti-inflammatory effects of these compounds. Additionally, compound **5a** exhibited a lower IC_{50} value compared to two other compounds **5b** and **5c**. These compounds contain only one hydroxyl group, positioned differently on the benzene ring. Hence, the position of the hydroxyl group may account for the varying activities of these compounds.

***In silico* study of anti-inflammatory activity:** COX-1 and COX-2 inhibition may trigger different complications within the body. Though, COX-2 is the enzyme mainly linked with inflammation, it is well known that NSAIDs are providing the relief from the symptoms of pain and inflammation by blocking both COX-1 and COX-2 isoforms [25]. The inhibition of COX-1 is associated with gastrointestinal related side effects while the inhibition of COX-2 is associated with cardiovascular side effects. These cardiovascular problems are arising due to hindering prostacyclin while not blocking thromboxane A2 [6]. Prostacyclin which is accountable for preventing platelet aggregation in the blood, being hindered by COX-1 inhibition [25]. Therefore, the ideal anti-inflammatory agents should be selective COX-2 inhibitors but not specific COX-2 inhibitors [6].

Thus, *in silico* study approach was employed to predict the possible binding modes and interactions between the crystal structure of human COX enzymes and the oxazolone ligands. Chemical Piecewise Linear Potential (CHEMPLP) was the scoring function used in GOLD software to derive individual's GOLD score. It unveils the higher the score, the stronger the binding affinity between the target and a ligand molecule [26]. The best Gold.PLP.fitness scores for all four compounds have been reported against the standard (Table-3). It illustrated an increased binding affinity of COX enzymes with oxazolone derivatives than O-acetyl salicylic acid.

The resulted protein-ligand interactions based on the best binding pose of compounds **5a-d** and the standard compound were visualized by using BIOVIA Discovery Studio Visualizer software. O-Acetyl salicylic acid (aspirin) was used as standard in this study and currently used in anti-inflammatory therapies worldwide. In case of COX-1, compounds **5c** and **5d** showed a tendency for the occurrence of hydrogen bonds between the hydroxyl groups and the binding pocket (Fig. 2). Compound **5d** showed three hydrogen bonds with TYR355 and SER530 amino acids while compound **5c** showed only two hydrogen bonds with TYR385 and SER530 and in gaining their stabilization. Moreover, TYR355 and ARG120 were the two amino acids in the binding pocket, involving in the formation of hydrogen bonds with O-acetyl salicylic acid. Meanwhile, compounds **5a** and **5b** had not participated in forming hydrogen bonds (Fig. 3).

In addition, docking studies for human COX-2 showed that all the synthesized compounds **5a-d** including the standard forming hydrogen bonds with the binding cavity (Figs. 2-4). The highest number of hydrogen bonds were shown for compound **5d** (Fig. 2), which enabled both hydroxyl groups of aryl group to form four hydrogen bonds with TYR355 and ARG120 in the binding cavity. Compound **5b** showed two hydrogen bonds in their stabilization with TYR355 and ARG120, while the other compounds **5a** and **5c** showed only a one hydrogen bond with the binding cavity. O-Acetyl salicylic acid achieved its stabilization with two hydrogen bonds between SER530 and ALA527. However, all the hydroxyl groups of **5a-d** compounds and the standard showcased the possibility of forming hydrogen bonds.

Thus, the results of *in silico* study indicated all oxazolone derivatives (**5a-d**) potentially possess inhibitory effects on both COX-1 and COX-2 enzymes. As it was noticed that O-acetyl salicylic acid showed slightly lower scores compared to oxazolone compounds, suggesting that they may be more useful COX-1 and COX-2 inhibitors than the standard. In spite of this, it is possible that the presence of hydrogen bonding should be taken into consideration. As hydrogen bonds can play a crucial role in molecular recognition processes, many biological macromolecules rely heavily on hydrogen bonding interactions to recognize and bind to their ligands with high specificity. Therefore, the standard drug may exhibit higher specificity to COX enzymes compared to some of the compounds due to its ability to form two hydrogen bonds with each binding cavity. However, compound **5d** may stand out as the most effective COX inhibitor among all compounds as it showed the tendency to form the highest number of hydrogen bonds between the two hydroxyl groups derived from protocatechuic aldehyde and each COX enzyme.

Hence, both studies unveiled that these compounds could become promising anti-inflammatory agents through COX inhibition and lysosomal membrane stabilization. Nevertheless, it appears that the number of hydroxyl groups may contribute to exhibiting higher *in vitro* and *in silico* anti-inflammatory activities. Therefore, further research will be necessary to explore the anti-inflammatory mechanisms in depth and to develop these compounds as potential candidates for anti-inflammatory therapy.

Conclusion

Our pursuit of potent anti-inflammatory compounds in the modern world has led us to focus on synthetic compounds

TABLE-3
Gold.PLP.fitness SCORES AGAINST HUMAN COX-1 (PDB ID 6Y3C) AND COX-2 (PDB ID 5F1A)

	5a	**5b**	**5c**	**5d**	O-Acetyl salicylic acid
Human COX-1	48.26	56.81	63.95	50.06	41.05
Human COX-2	51.82	54.33	55.58	53.30	45.06

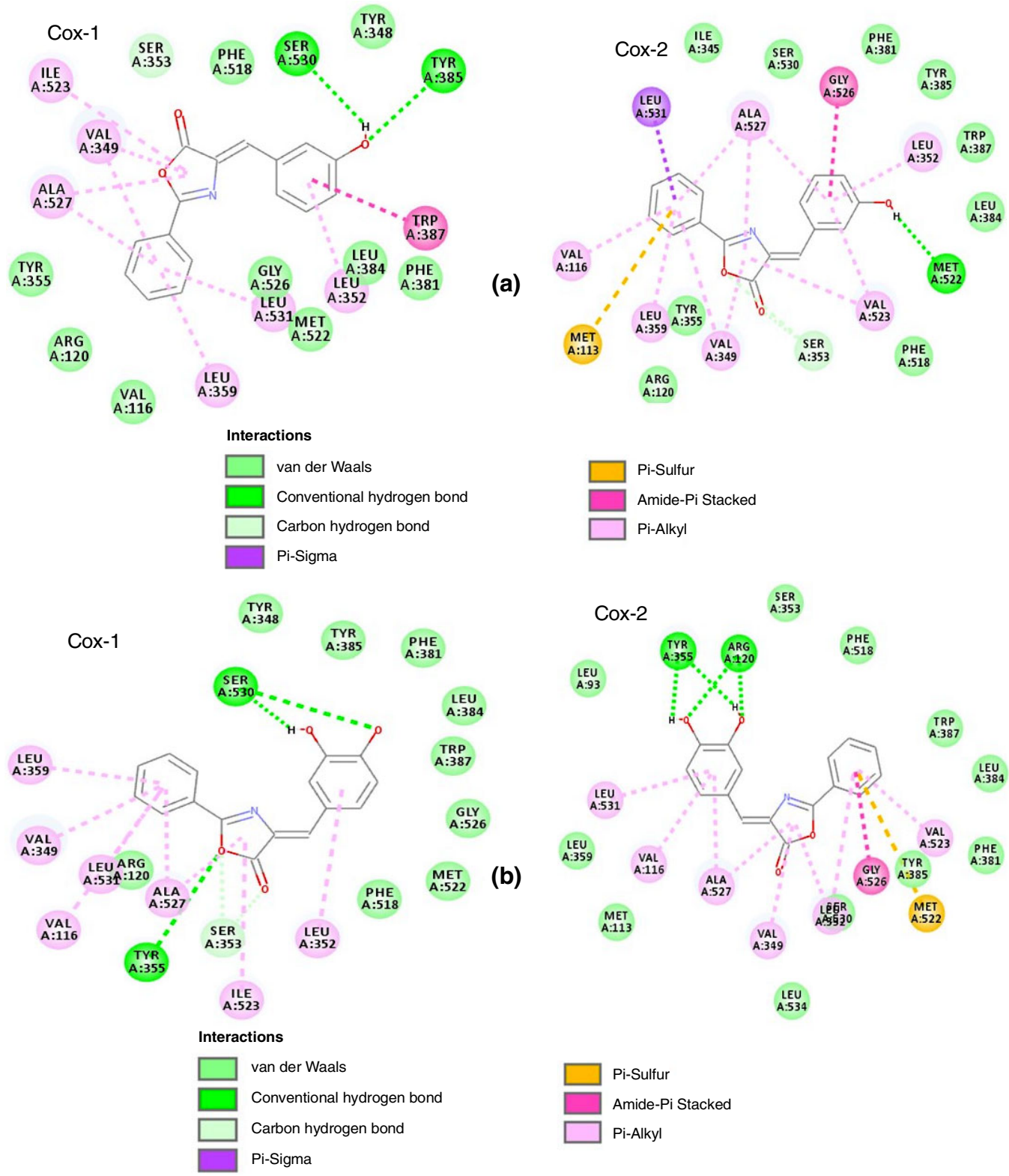


Fig. 2. 2D interaction diagrams for compounds (a) **5c** and (b) **5d** compound with COX-1 and COX-2

due to their favourable side effect profiles. Among these, oxazolones have emerged as promising entities in current research. Through Erlenmeyer-Plöchl reaction, few oxazolone derivatives (**5a-d**) were synthesized, characterized and evaluated their anti-inflammatory activity using both *in vitro* and *in silico* methods. *In vitro* evaluations were focused on HRBC membrane stabilization activity under heat induced conditions. It demonstrated anti-inflammatory effects, where the inhibition of RBC membrane breakdown mirrored the inhibition of lysosomal membrane breakdown in a realistic scenario. As indicated by their respective IC_{50} values, compound **5d** emerged as the most potent anti-inflammatory compound. This enhanced activity could be attri-

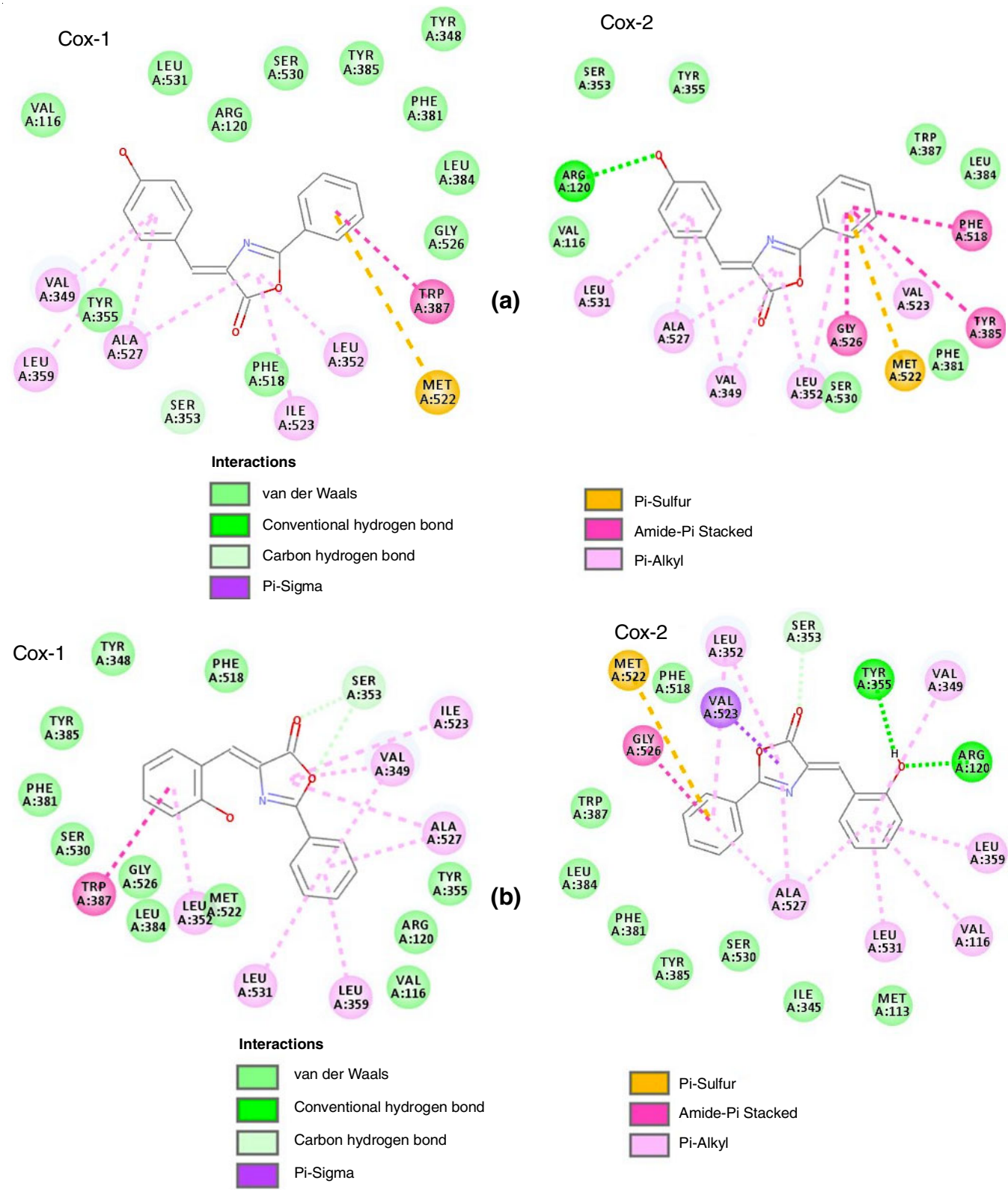


Fig. 3. 2D interaction diagrams for compounds (a) **5a** and (b) **5b** compound with COX-1 and COX-2

buted to the presence of two free hydroxyl groups on the benzene ring. All the compounds exhibited lower IC_{50} values compared to the standard drug except compound **5b** suggesting their promising efficacy against inflammation. The *in silico* study revealed the characteristic shared by all oxazolone derivatives **5a-d** of binding capabilities to both COX-1 and COX-2 enzymes. The hydrogen bond formation with the binding cavity may increase their stabilities with binding clefts of COX enzymes. In this case, hydrogen bond formation between the ligands, the binding cavity of COX enzymes, were considered crucial

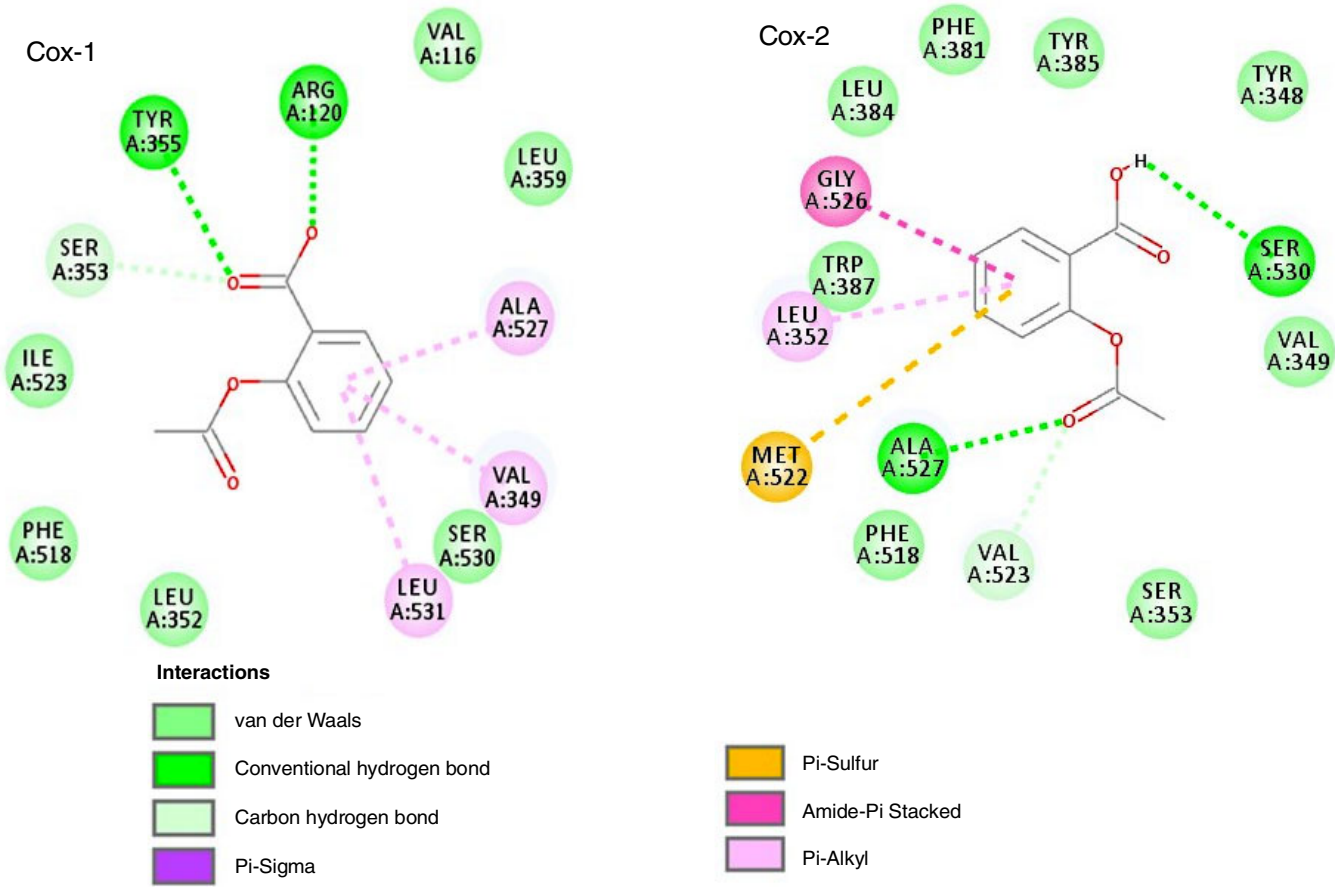


Fig. 4. 2D interaction diagrams for *o*-acetyl salicylic acid against COX-1 and COX-2

for the recognition of target sites. Thus, compound **5d** was exceptional due to its effective hydrogen bond formation with the binding cavities of each enzyme. The present findings suggest these compounds possess possible anti-inflammatory activities *via* lysosomal membrane stabilization and COX inhibition. We also emphasize the necessity for further investigations in the future to unveil therapeutic potential of these oxazolone derivatives to develop them as novel therapeutic drugs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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