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Synthesis and Anti-inflammatory Tests of 2-Hydroxy-N-(Pyridine-2-yl)Benzamide Mannich Base Substituted Morpholine and N-methyl piperazine

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Abstract: Salicylic acid is a naturally active substance known to have anti-inflammatory and antioxidant activity, but it has side effects in the gastrointestinal tract. The modification of its carboxylic groups into amide derivatives can be a solution to overcome its weakness. This study synthesized salicylamide analogs, 2-hydroxy-N-(pyridine-2-yl)benzamide (**1**) and, its Mannich base derivatives. The synthesized compounds (**2a-b**) showed anti-inflammatory activity based on an in-vitro anti-inflammatory activity test using the inhibition protein denaturation method. The IC50 obtained was in the range of 0.121-0.145 mM. The potency was lower than piroxicam used as a standard compound (IC_{50} = 0.0073 mM). The molecular docking result shows that the ratio of COX-2/COX-1 binding affinity and ligand interaction of all synthesized compounds were not COX-2 selective.

Keywords: Mannich base; Salicylamide analogs; Anti-inflammatory; Molecular docking

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs that are often prescribed with broad efficacy as analgesics and antipyretics (Lou & Zhu, 2016). Anti-inflammatory drugs still have side effects on the gastrointestinal tract. To improve anti-inflammatory potency of these drugs while reducing the side effects of NSAIDs is by modifying the carboxylic groups into esters or amides development (Muhi-Eldeen et al., 2009; Razzak, A. Abdul, Nadeem Hussan & Qaseer, 2017; Ullah et al., 2016).

The Food and Drug Administration's drug database reveals the significance of approximately 75% of unique small-molecule drugs containing heterocyclic nitrogenous structures in the design and engineering of pharmaceutical drug discovery (Kerru et al., 2020). The introduction of the Mannich base group is a method of modifying the structure of a compound by incorporating nitrogen into the starting compound structure to enhance its biological activity (Rahmawati et al., 2020; Rani & Ravindranath, 2016; Roman, 2015). The Mannich base substitution research have been widely published. Research from asymmetric mono-carbonyl analogs of curcumin Mannich base of N-methyl piperazine has result higher in vitro anti-inflammatory activity than its parent compound, and diclofenac sodium as a standard. One of study about structural modifications of vanillic acid Mannich bases substituted dimethylamine, diethylamine, morpholine and N-methyl piperazine, showed better antioxidant activity than the parent compound, even the 5-(pyrrolidine-1-yl-methyl)-vanillic acid compound, was able to scavenge free radicals better than quercetin as a standard (Hayun et al., 2020; Rahmawati et al., 2020).

The synthesis and biological activity of various Mannich base salicylamide derivatives has not been widely reported. In this research compound of 2-hydroxy-N-(pyridine-2-yl) benzamide, and its Mannich base derivatives substituted morpholine and N-methyl piperazine are synthesized. The synthesized compounds were then tested for their anti-inflammatory activity using protein denaturation inhibition and molecular docking to cyclooxygenase receptor enzymes (COX-1

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and COX-2). Modification of the Mannich base-substituted 2-hydroxy-N-(pyridine-2-yl) benzamide derivatives is expected to become a new drug design with better anti-inflammatory activity.

Method

The tools used in this study include a spectrophotometer UV-Vis (Shimadzu UV-Vis 1601), spectrophotometer infrared (Shimadzu FTIR 8400S), ^{13}C-NMR spectrophotometer (JEOL-ECZ 125 MHz 500R), LC-MS/MS ESI mode (+) (Advion-AVANT™), melting point analog model SMP11 (Stuart Scientific), analytical balance (Sartorius), magnetic stirrer (IKA C-MAG HS7), TLC vessel (CAMAG), and glassware.

The ingredients used include salicylic acid, dicyclohexyl carbodiimide (DCC), dimethylamino pyridine (DMAP), 2-aminopyridine, morpholine, N-methyl piperazine, formaldehyde, piroxicam (PT. Kimia Farma), chemical reagents and other solvents obtained from Merck® distributors .

Molecular dockinghardware are Macbook Air 2012 Intel Core i5 Operating System Catalina and a desktop computer HP 10 Pro Intel® Core™ i5-2400 CPU @3.10 GHz Random Access Memory 4 GB and an operating system Microsoft Windows 8.1 Pro 64-bit. The software used includes AutoDock Tools 4.2, MarvinSketch 5.2, PyMOL 4.6, UCSF Chimera 1.16, Biovia Discovery Studio 21.1.0, PyRx 0.9.9, and Open Babel 2.4.1. COX-1 target macromolecule (PDB ID: 1EQG) (Ugwu et al., 2018) and COX-2 (PDB ID: 5KIR) (Aqeel et al., 2020) downloaded from the Protein Data Bank page http://www.rcsb.org/pdb.

Synthesis of 2-hydroxy-N-(pyridine-2-yl)benzamide (compound **1**). Some salicylic acid (2 mmol), 2-aminopyridine (2.2 mmol), DCC (2.2 mmol), and DMAP (0.2 mmol) were dissolved in 5 mL pyridine each in a separate container, then mixed. The mixture was refluxed at 55°C with constant stirring and the reaction was monitored hourly with TLC (mobile phase hexane-ethyl acetate - 2:3). After the reaction was completed, the mixture was separated from the dicyclohexylurea precipitate by filtration, the filtrate was acidified with 2% HCl to a pH of 5.5-6.0, and extracted with ethyl acetate. The organic layer was washed with 1% NaOH solution to neutral pH 7 and dried over anhydrous $MgSO_4$ (Salahuddin et al., 2013). The filtrate was concentrated, extracted with dichloromethane, and washed with cold methanol.

Synthesis of Mannich base derivatives (Compounds **2a-b**). The synthesis method refers to the reaction for the formation of the amino-alkylation of vanillic acid which has been reported previously (Hayun et al., 2020). An amount of compound **1** (20 mmol) was dissolved in tetrahydrofuran (18 mL). In a separate flask, the formaldehyde (80 mmol) and the corresponding secondary amine (80 mmol) were homogenized for 30 minutes in an ice bath, then the two solutions were mixed in the reflux flask at 50-60°C assisted by constant stirring. The reaction was monitored every 30 minutes until completed using TLC, then continued with stirring at room temperature ($\pm$ 25°C) for approximately 24 hours. The solution was stored in a refrigerator at <$\pm$10°C for at least 12 hours, evaporate the solvent, and washed the residue using a combination of ethyl acetate – petroleum ether 1:1 to obtain pure compounds **2a-b**.

The Anti-inflammatory evaluation was carried out based on a reported heat-induced albumin denaturation inhibition protocol with some modifications, using piroxicam and salicylic acid as standard (Hayun et al., 2019). The test solution mixture consisted of 1 ml of standard solution or test compound in methanol, in various concentrations, and 9 ml of Bovine Serum Albumin (BSA) solution (0.5% w/v, pH 6.3) made in Tris buffer solution. Shaking was carried out at room temperature 25°C for 15 minutes, followed by heating for 10 minutes in a water bath at 70 ± 2°C, then cooled to room temperature ($\pm$ 25°C). Then the absorbance was measured at λ 660 nm using a spectrophotometer. Each test compound and the standard were carried out three times (in triplicate). Control solutions were prepared as above but without the test compound. Percentage of protein denaturation inhibition using Equation 1:

$$\% \text{ Inhibition} = \frac{\text{Negative control uptake} - \text{uptake of test solution}}{\text{Negative control uptake}} \times 100 \quad (1)$$

The IC_{50} value was calculated by linear regression analysis of the relationship between the Log concentration of the test solution and the % Inhibition.

Molecular docking COX-1 and COX-2 receptors were represented by target macromolecules 5KIR and 1EQG downloaded from www.pdb.org. Isolate their native ligand from each macromolecules that bind to cocrystals (Rouzer & Marnett, 2020). The chemical structure of synthesized compounds were drawn using MarvinSketch then converted into three-dimensional ligands using Open Babel, and downloaded in *.pdb format. The positive control compound for piroxicam was downloaded from the pubchem.ncbi.nlm.nih.gov. Optimization of the molecular docking method is carried out by re-docking the native ligand molecule to analyze for a 3D conformation. The grid box size and coordinates must comprise the binding pocket of target protein (Padmini et al., 2021). The validity of the docking method was shown from the root mean square deviation (RMSD) value of the alignment of the 3D conformation of the native ligand. Evaluation of the RMSD value at the binding sites found and the parameters used are considered valid if the RMSD value ≤ 2Å (Suherman et al., 2020). Each ligand docking will have binding energy (ΔG, kcal/mol), binding pose in active site , and the types of interaction bonds with amino acid residues.

Result and Discussion

The 2-hydroxy-N-(pyridine-2-yl)benzamide (**1**) and its Mannich base derivatives (**2a-b**) were synthesized in two steps. The first step was the amidation reaction of salicylic acid with 2-aminopyridine and the second step was the Mannich reaction of compound **1** with formaldehyde and the corresponding secondary amine, as shown in Figure 1. The structure of the synthesized compound was confirmed using FTIR, ^{13}C-NMR, and LCMS/MS.

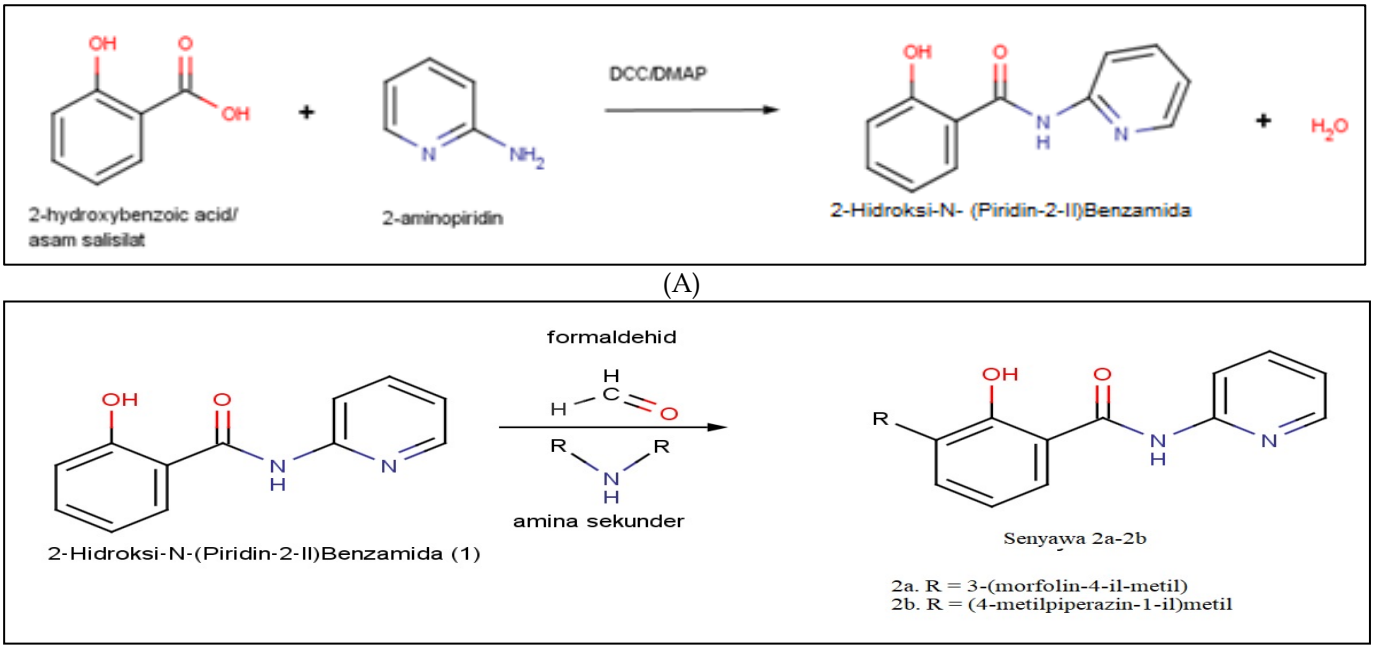


Figure 1. Reaction Scheme of 2-Hydroxy-N-(Pyridine-2-Il)Benzamide (**1**) (A) and its Mannich Base Derivatives (**2a-b**) (B)

Characterization results and spectral data for structure confirmation are shown in Table 1. In the infrared spectrum of compound **1**, bands appear at wave number (cm^{-1}) 3,489 (Ar-OH/phenolic), 3,429 (secondary NH), and 1,749 (C=O amide), while in the spectrum of the Mannich base derivatives (**2a-b**) the phenolic band is no longer visible, indicating that Mannich base substitution has substituted in the phenol group. In addition, there is also a sharp and strong band at 3,200-3,000 (secondary NH), and 1,000-1,250 (aliphatic C-N). The carbon NMR spectrum data of compounds **2a-b** produced a specific chemical shift with the appearance of methylene groups at δ 49.050-52.074 ppm, indicating the formation of methylene groups attached to amines. The methylene group bonded to electronegative atoms such as oxygen and nitrogen has a signal in the δ 30-80 ppm region because it is deshielded compared to those attached to other carbon atoms(Mohrig et al., 2010).

A downfield chemical shift is seen for secondary alkyl signals C14-C17 in compound **2a** at δ 52.909 and δ 67.086 ppm, compound **2b** at δ 34.422 and δ 34.038ppm, because the carbon atoms are affected by the electronegativity properties of the oxygen and nitrogen atoms of the morpholine and piperazine groups. Further structural confirmation is supported by LCMS/MS results data showing full agreement with the expected structure.

The anti-inflammatory activity was analyzed based on the inhibition of heat-induced protein denaturation, as proven in several existing anti-inflammatory drugs. This method can be a measurable parameter for an anti-inflammatory candidate compound that modulates protein stabilization to treat inflammation (Hayun et al., 2019). Measurement of protein denaturation inhibition was observed by a decrease in absorbance atλ 660 nm after heating the albumin solution, which is the optical density range of aggregation of albumin protein (Sharma, 2010). The results of the potential anti-inflammatory activity of the synthetic compound were 17 times lower than piroxicam as a standard compound, which only required 0.0073 mM to inhibit 50% protein denaturation in 0.5% Bovine Serum Albumin (BSA) solution, compared to the test compound requiring a minimum of up to 17 times concentration of piroxicam, around up to range of 0.121-0.145 mM to inhibit 50% of the BSA protein (Table 2).

Table 1. Characterization and Spectrum Data

Compound	**1**	**2a** (Mannich Base Morpholine)	**2b** (Mannich's base N-Methyl Piperazine)
Characterization	Yellowish white powder, yield 52.27%, m,p,: 208-209°C	White powder, yield 14.59%, m,p,: 220-221°C	White powder, yield 23.56%, m,p,: 203-205°C
FTIR (cm^{-1})	3,500-3,200 O-H 3,489 N-H 3,429 C=C 2,931 C-H 1,749 C=O 1,541 C-C	3,327.32 N-H 1,575.89 C=C 2,852.81 C-H 1,627.97 C=O 1,454.18 C-C	3,323.46 N-H 1,527.67 C=C 2,852.81 C-H 1,726.35C=O 1,446.66 C-C
^{13}C-NMR, σ (ppm)	111.90 (1C); 114.50 (1C); 126.50 (1C); 124.30 (1C) 135.00 (1C); 156.70 (1C) 124.10 (1C); 132.90 (1C) 122.30 (1C); 151.00 (1C) 159.70 (1C, phenol); 163.00 (1C, amide)	111.90 (1C); 126.50 (1C); 124.30 (1C); 135.00 (1C) 132.90 (1C); 151.00 (1C) 114.50 (1C); 124.10 (1C) 122.30 (1C); 156.70 (1C) 159.70 (1C, phenol); 163.00 (1C, amide); 52.00(H2C-N); 52.90 (2C), 67.00 (2C)	118.10 (1C); 124.30 (1C); 126.50 (1C); 133.00 (1C) 130.60 (1C); 151.10 (1C) 116.60 (1C); 122.30 (1C) 134.70 (1C); 133.00 (1C) 157.00 (1C, phenol); 163.20 (1C, amide); 49.20 (H2C-N); 34.40 (4C), 25.60 (1C),
Mass $[M+H]^+$ (m/z)	Found: 215.10, neutral mass calculation $C_{12}H_{10}N_2O_2$ = 214,22; Mass Error = 0.80	Found: 315.10, neutral mass calculation $C_{17}H_{19}N_3O_3$ = 313.35; Mass Error = 1.75	Found: 328.20, neutral mass calculation $C_{18}H_{22}N_4O_2$ = 326.39; Mass Error = 1.81

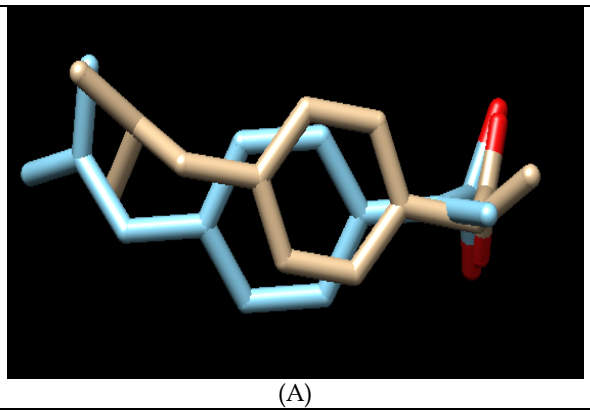
The results of this study are different from previous studies on cyclovalone derivatives, where the substitution several Mannich bases was able to improve the anti-inflammatory activity of the parent compound, which was probably due to the presence of a methoxy group in the ortho position to the hydroxyl group which revealed a significant contribution to the anti-inflammatory activity, which was limited the parent compound benzamide synthesized in this research (Putri et al., 2018).

Table 2. Anti-Inflammatory Activity Test Results

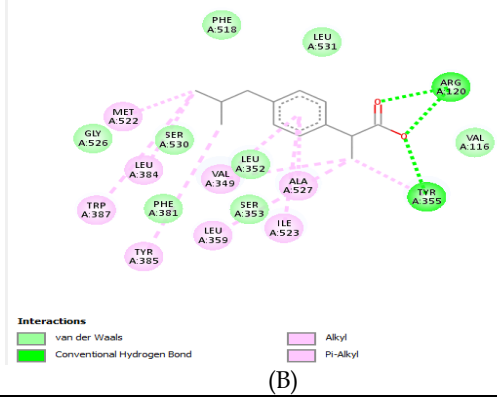
Compound	IC_{50} (mM)	Ratio COX-2/ COX-1
Salicylic acid	0.003 ± 0.110	-
Piroxicam	0.007 ± 0.050	0.990
1	0.121 ± 0.670	0.910
2a	0.134 ± 0.930	0.960
2b	0.145 ± 0.750	0.910

Molecular docking studies have become a scientific approach to studying the interactions between organic compounds and macromolecules, which involve predicting the conformation and orientation of the ligand (pose) in a targeted binding site to obtain structural modeling along with fairly accurate activity predictions (Pishawikar & More, 2017). The interaction of the ligand with the receptor will tend to be in the lowest energy state. The lower binding energy (ΔG) will become more stable the conformation of ligand-protein complex. This mechanism simulates the pharmacokinetics process and biological effects of drugs in the body (Beny et al., 2020).

Re-docking result of ibuprofen as a native ligand in 1EQG enzym has ΔG value= -8.49 kcal/mol and RMSD 0.82 Å. The ligand-receptor interaction has ten active sites, Tyr355 and 385, Arg120, Val349, Leu359 and 384, Met522, Ile523, Ala527, and Trp387, while the piroxicam ligand binding result has ΔG = -8.67 kcal/mol and interaction with amino acid residues Gln203 and Trp387 to form hydrogen bonds and three pi-alkyl bonds with residues Ala199, Leu390 and Val291 to form hydrophobic bonds. Compounds **1, 2a,** and **2b** have the same interaction in amino acid residues Trp387 and Gln203 to form hydrogen and hydrophobic bonds, respectively (Figure 2).



(A)



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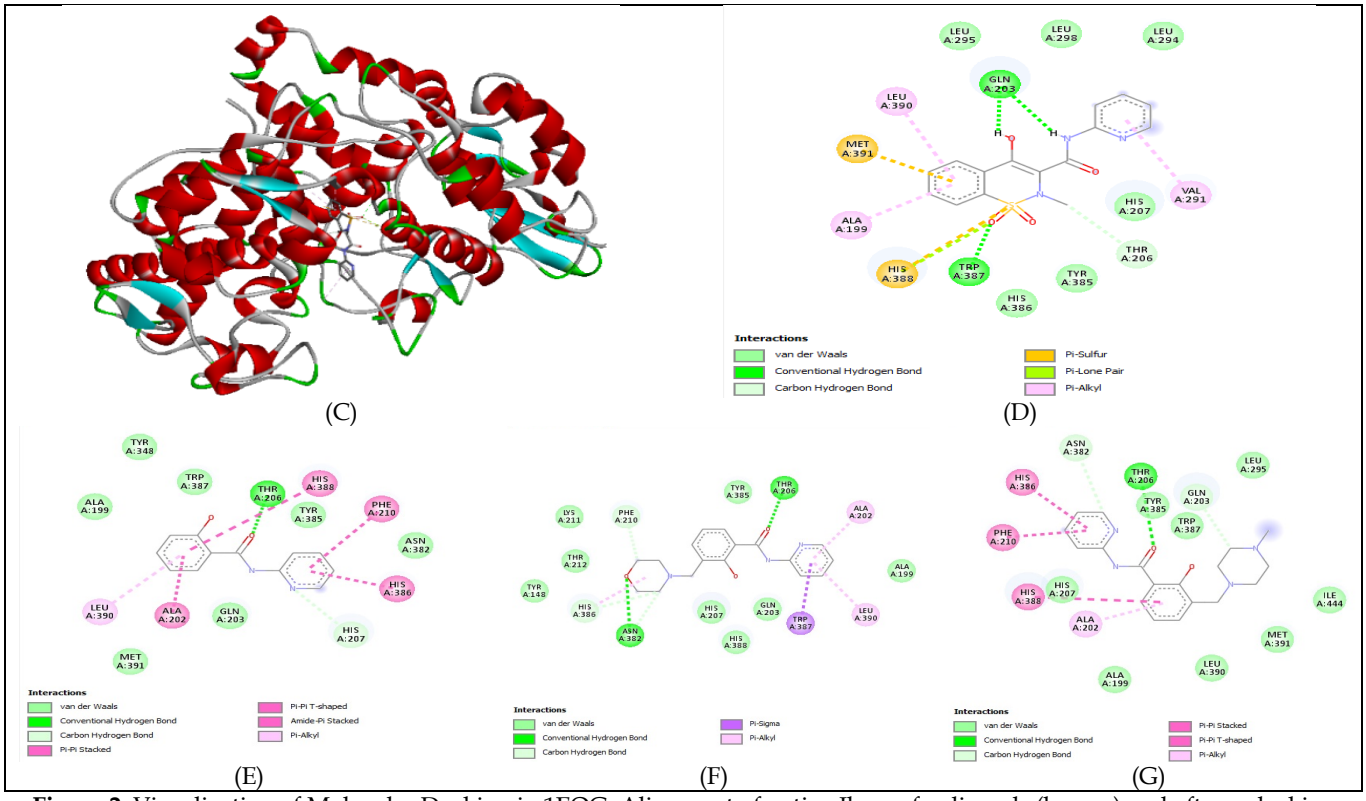

Figure 2. Visualization of Molecular Docking in 1EQG. Alignment of native Ibuprofen ligands (brown) and after redocking (blue) (A), Interactions of Ibuprofen ligands (B), Interactions of Piroxicam (C) & (D) ligands, Interactions of ligands of Compound **1** (E), Compound **2a** (F), and Compound **2b**(G)

Based on the molecular docking data result, it can be predicted that the ligand of the test compounds can bind in the active site of 1EQG receptor (Table 3).

Table 3. Molecular Docking of Receptor 1EQG (COX-1)

Compound	Affinity Energy (kcal/mol)	Amino Acid Residues	Bond Type
Ibuprofen (natural ligand)	-8.49	TYR355	Hydrogen
		ARG120	Hydrogen
		VAL349	Hydrophobic
		LEU359	Hydrophobic
Piroxicam (standard)	-8.67	GLN203	Hydrogen
		TRP387	Hydrogen
		VAL291	Hydrophobic
		ALA199	Hydrophobic
1	-8.08	THR206	Hydrogen
		HIS207	Hydrogen
		GLN203	Hydrophobic
		ALA202	Hydrophobic
2a	-8.64	THR206	Hydrogen
		ASN382	Hydrogen
		TRP387	Hydrophobic
		ALA202	Hydrophobic
2b	-8.41	THR206	Hydrogen
		GLN203	Hydrogen
		ALA202	Hydrophobic
		HIS386	Hydrophobic

Tabel 4. Molecular Docking of Receptor 5KIR (COX-2)

Compound	Affinity Energy (kcal/mol)	Amino Acid Residues	Bond Type
Rofecoxib (a natural ligand)	-9.65	ARG513	Hydrogen
		SER530	Hydrogen
		VAL349	Hydrophobic
		ALA527	Hydrophobic
Piroxicam (standard)	-8.62	ALA527	Hydrogen
		GLY526	Hydrogen
		LEU531	Hydrophobic
		VAL349	Hydrophobic
1	-7.39	TRP387	Hydrogen
		ALA199	Hydrophobic
		LEU391	Hydrophobic
		TYR385	Hydrogen
2a	-8.30	THR212	Hydrogen
		ALA202	Hydrophobic
		HIS386	Hydrophobic
		TYR385	Hydrogen
2b	-8.17	HIS388	Hydrogen
		LEU390	Hydrophobic
		ALA202	Hydrophobic

The active sites of cyclooxygenase (COX) enzymes are amino acid residues Arg120, Val349, Ser353, Tyr355, Ile523 (COX-1), Ala-527 and Val523 (COX-2) (Rouzer & Marnett, 2020). The valine residue is relatively smaller on COX-2 forms a hydrophobic area, where the isoleucine residue on COX-1 is sterically more blocked,

so that selective COX-2 ligands are specifically known to have interactions with the Val523 residue (Liu et al., 2009).

Re-docking of rofecoxib as a native ligand in the 5KIR enzyme has ΔG value = -9.65 kcal/mol and an RMSD of 0.05. RMSD value < 2 Å indicates the validity of the method is good. The ligand-receptor interaction has seven active sites, Val349 and 523, Arg513, Leu531 and 352, Ala527 and Ser530, while the piroxicam ligand (ΔG = -8.62 kcal/mol) has the same interaction on the amino acid residue Ala527 to form hydrogen bonds, the pi-sigma bond in Leu531 and the pi-alkyl in Val349 form a hydrophobic bond. Based on the molecular docking data result, it can be predicted that the ligand of the test compounds can bind in the active site of the 5KIR receptor (Table 4), but do not have a specific interaction with amino acid residue Val523 (Figure 3).

The binding energy ratio data on COX-2/COX-1 was obtained in the range of 0.91-0.96. These results indicate that all synthesized compounds are predicted not to have good selectivity for COX-2. The association of COX-2 inhibition is considered more beneficial, in addition to reducing side effects on the gastrointestinal tract, it also influences many processes, especially in various stages of carcinogenesis (Streppa et al., 2002).

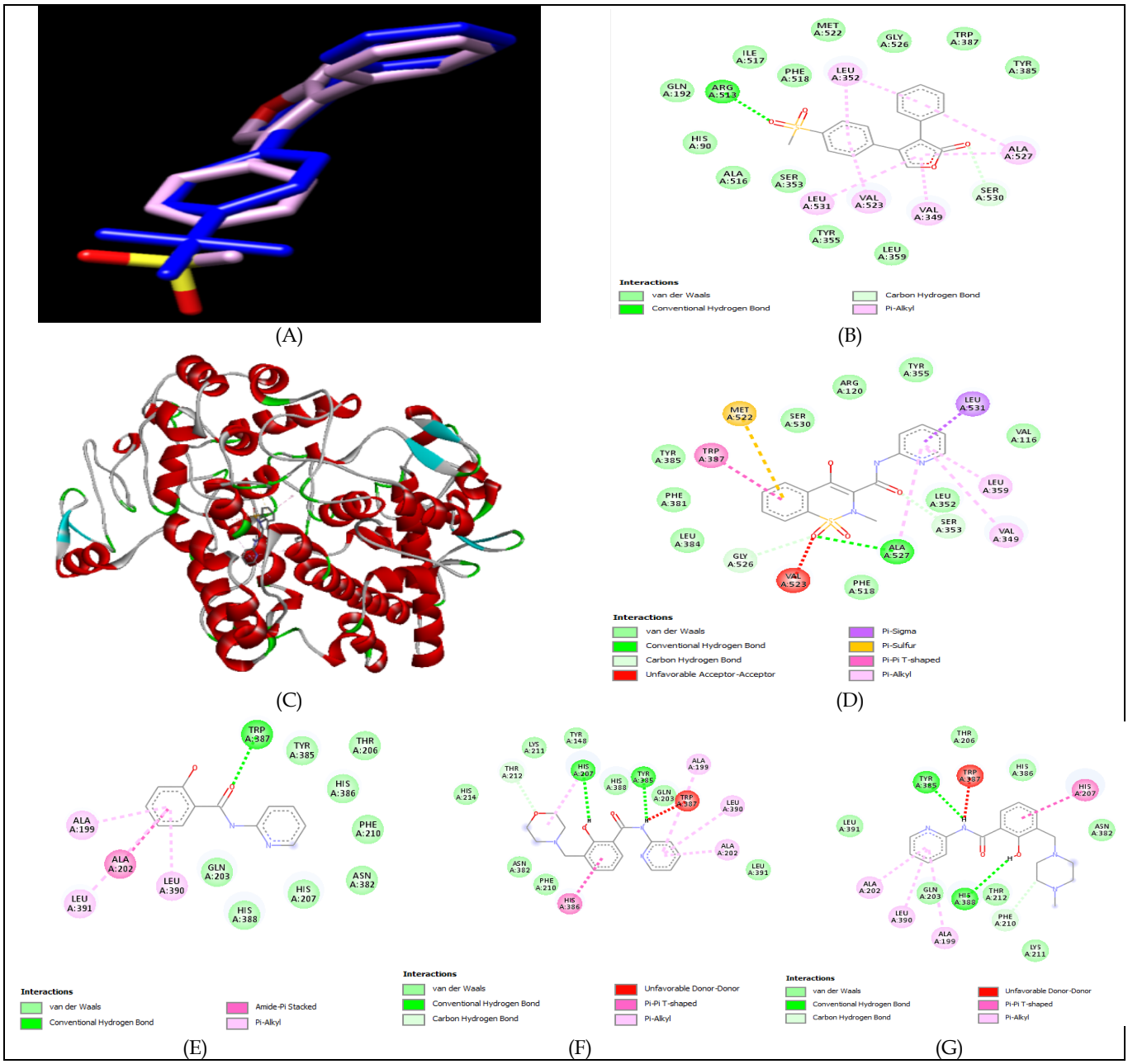

Figure 3. Visualization of Molecular Docking in 5KIR. Rofecoxib native ligand alignment (blue) and after redocking (magenta) (A), Rofecoxib ligand interactions (B), Piroxicam (C) & (D) ligand interactions, Compound **1** (E) ligand interactions, Compound **2a** (F), and Compound **2b** (G)

Conclusion

The compounds of 2-Hydroxy-N-(pyridine-2-yl)benzamide series and their Mannich base derivatives have been successfully synthesized. The anti-inflammatory activity result was still low potential, with IC_{50} range = 0.121-0.145 mM. Molecular docking studies show that all synthesized compounds bind at the active site of receptors COX-1 (PDB ID:1EQG) and COX-2 (PDB ID:5KIR) but are non-selective.

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