Original Research Article

Synthesis of (E)-N'-(pyridine-2-ylmethylene) picolino hydrazide with promising antimicrobial and anti-inflammatory activities along with their *in-silico* studies

ABSTRACT

In recent years, there has been a significant amount of interest in hydrazide-hydrazone compounds as a result of their extensive use in the pharmaceutical industry. In this study, the new compound *(E)-N'-(pyridin-2-ylmethylene) picolino hydrazide*, with the formula $C_{20}H_{19}N_3O_2$, was synthesised and confirmed using different spectroscopic techniques. The newly synthesised compound was tested for anti-inflammatory and antibacterial properties *in vitro*. The results of an *in vitro* anti-inflammatory activity test using the denaturation of bovine serum albumin as the measurement kit revealed a highly effective inhibitory effect. In addition, the results of the antimicrobial test showed that the compound demonstrated a maximum zone of inhibition (18.2 mm) against the *Staphylococcus aureus* strain at a higher concentration (32 µg/ml) of the tested sample (32 g/ml), which is greater than the standard drug (ciprofloxacin: 17.5 mm). The antifungal activity of (*E)-N'-(pyridin-2-ylmethylene) picolinohydrazide* indicated a maximum inhibition zone of 9.2 mm at a higher concentration (32 µg/ml) of the tested sample, which is comparable to that of the standard drug (ketoconazole: 9.0 mm). The anti-inflammatory activity performed by the carrageenan-induced paw edema method also gave a promising result. Molecular docking analysis revealed that the active compounds had a higher docking score and interacted effectively with the target protein. In silico molecular docking, results revealed the COX-5 and LL-37 (residues 17–29) proteins mainly depends on H-bonds between the pyridine groups of the synthesized ligand and the catalytic residues of the binding site, π-cation/anion interactions, (iii) π-alkyl interactions, and formation of carbon-hydrogen bond, π- π bond that stabilizes the interaction with the active site. Among the analogs of the hydrazine hydride class, the newly synthesized compound: *(E)-N'-(pyridin-2-ylmethylene) picolino hydrazide* proved to be a vital bioactive molecule.

Keywords: Hydrazide-hydrazone, Antimicrobial, Anti-inflammatory, Docking

1. Introduction

Inflammation can be induced by a variety of factors, including bacterial and viral infections, allergies, irritants, toxic substances, and injury to any tissue in the body (Zabrodskii). Inflammation can be generated from the outside (exogenously) or the inside (endogenously) (Gallucci and Matzinger; Lon, Liu, and Jusko). This may also involve trauma, surgery, autoimmunity, adult respiratory distress syndrome, prolonged exposure to industrial toxins, and reoxygenation damage (Weigand et al.). The inflammatory response can be sped up by molecules that are readily available in the body, such as histamine, prostaglandins, leukotrienes, oxygen- and nitrogen-based free radicals, serotonin, bradykinin, and interleukins. An excessive inflammatory response can increase the risk of having a stroke or developing heart disease, as well as lupus, cancer, tissue injury, physiological de-compensation, organ damage, and even mortality (Ward and Lentsch). Researchers in the field of medicinal chemistry face a problem when it comes to the hunt for more potent anti-inflammatory drugs, because of the toxic chemical involved with the inflammation process. Aspirin, indomethacin, flufenamic acid, ibuprofen, and a host of other anti-inflammatory medications are acidic in nature (Whitehouse; John R Vane and Renia M Botting; John R Vane and Regina M Botting). One of the most important classes of medications for treating inflammation is known as non-steroidal anti-inflammatory drugs (NSAIDs). These drugs work by reducing inflammation in the body by inhibiting an enzyme called cyclooxygenase (COX 5), which is involved in the production of prostaglandins (Kovala-Demertzi; Brideau et al.).

In the process of organic synthesis, hydrazines and the derivatives of these compounds make up an important class of substances that have been utilized in a variety of diverse applications (Rollas, Gulerman and Erdeniz; Gürsoy et al.). Hydrazines have traditionally been employed as reagents for the derivatization and analysis of carbonyl compounds. In recent years, the N-N bond has emerged as a key structural motif in numerous bioactive drugs. A growing number of N-N bond-containing heterocycles and peptidomimetics have found their way into commercial use as therapeutic medicines. Particularly, N-N bond-containing heterocycles and peptidomimetics are increasingly utilized as medicinal and agricultural agents in the industry (Vicini et al.; Mamolo et al.). In addition, hydrazide-hydrazones have gained prominence due to their various biological applications,

which include antibacterial (Alaa, El-Subbagh and Kunieda), anti-HIV (Metobo et al.), anticancer (Kamat et al.), anti-inflammatory, antioxidant, and anticoagulant (de Candia et al.; Chaban et al.) activities.

Infections caused by microorganisms have become one of the primary causes of death in recent decades (Roukiatou Traoré, Emmanuel Sampo and Savadogo; Lauretta). Researchers are actively looking for antimicrobial candidates that are more potent and effective against bacteria that are both drug-sensitive and drug-resistant to overcome current challenges. BrunoLeal et al.(Leal et al.), Jacques et al. (Bompart et al.), Rakha et al. (Rakha et al.), and Altundas et al. (Altundas, Ayvaz and Logoglu) found numerous antibacterial candidates including pyridine. The pyridine nucleus has been regarded as one of the most potent bioactive scaffolds in many biological systems based on the reported literature.

Various anti-inflammatory and antibacterial drugs containing pyridine have also been identified in the literature. For instance, Abdelmonsef et al., (Abdelmonsef et al.), Santosh et al.,(Undare et al.), and Santhisudha et al., (Sarva et al.) reported on anti-inflammatory compounds derived from pyridine. Huan et al., (Du et al.), Yusufzai et al.,(KhanYusufzai et al.) Desai et al., (Desai et al.) Mathew (Bijo et al.) and Zahra, et al.(Najafi et al.) have discovered antibacterial agents. In light of these findings, we also sought to synthesize bioactive scaffolds consisting of hydrazides connected to picolinic-containing pyridine. The newly synthesized compound such as: *(E)-N'-(pyridin-2-ylmethylene) picolino hydrazide* have been characterized and confirmed by different spectroscopic technics. In addition, *in vitro* antibacterial and *in vivo* anti-inflammatory properties, as well as *in silico* molecular docking investigations, were methodically covered here.

2. Methods

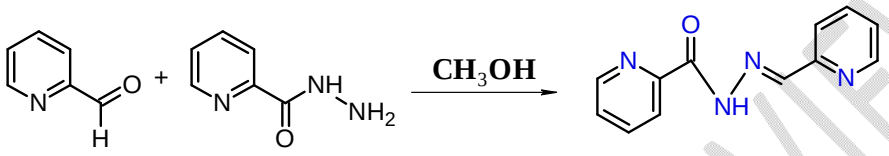
2.1. Chemicals, solvents, and instrumentations

All of the chemicals and solvents used in this study were acquired from Hi-Media laboratory, India, and Spectrochem Pvt. Ltd., India, and was utilized untreated. The reactions were conducted according to standard protocol. In an open capillary tube, the uncorrected melting points of newly synthesized compounds were determined. Thin-layer chromatography (TLC, silica gel 60 F254) was used to evaluate the reaction's progress and the purity of the result. The solvent mixture of ethyl acetate and hexane was used as the mobile phase, and it was monitored at ~254 nm under UV light. Shimadzu-IR was used to record FTIR spectra. The Bruker-400 spectrometer was used to record ^{1}H NMR spectra,

using DMSO-d6 as a solvent and TMS as an internal standard (the chemical shift in δ ppm and coupling constants (J) were represented in parts per million (ppm) and Hz, respectively).

2.2. Synthesis of (E)-N'-(pyridine – 2 – ylmethylene) picolinohydrazide.

The picolinohydrazide (0.137g, 1.0 mM) and picolinaldehyde (0.107g, 1.0 mM) are dissolved in methanol and refluxed for 2 hrs. This reaction mixture was poured into ice-cold distilled water and kept for 30 mins at room temperature. The precipitate formed was filtered and air dried and re-crystallized using THF. Yield:74.00%.



N'-[(*E*)-(pyridin-2-yl)methylidene]pyridine-2-carbohydrazide

(74% Yield)

Scheme 1. Synthetic Pathway of the *(E)-N'-(pyridine – 2 – ylmethylene) picolinohydrazide*

White solid; Yield: 74.00%; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.415-7.449 (1H, ddd, *J* = 7.2, 4.8, 1.2 Hz), 7.680-7.714 (1H, ddd, *J* = 8.0, 3.2, 1.6 Hz), 7.876-7.918 (1H ddd, *J* = 15.6, 7.6, 1.2 Hz), 7.993-8.013 (d, *J* = 8.0, Hz), 8.054-8.097 (1H, ddd, *J*= 15.6, 8.0, 2.0 Hz), 8.144-8.164 (d, *J* = 8.0 Hz), 8.620-8.631 (d, *J* = 4.4 Hz), 8.697 (1H, S), 8.738-8.749(1H d *J*=4.4), 12.506 (1H S).

2.3. In vitro antimicrobial activity

Human pathogens such as *Staphylococcus aureus* (ATCC 25923), *Escherichia Coli* (ATCC 25922), and *Candida albicans* (ATCC 22972) were used for the microbial studies. The clinical isolates were obtained from the National Chemical Laboratories in Pune, India. The antimicrobial activity of all samples was evaluated on Muller Hinton agar (MHA) for bacteria and sterile Saboraud dextrose broth (SDB) for the fungi using the disc diffusion method (Bharadwaj et al.). The discs were prepared by punching sterile Whatman filter paper with a sterile punching machine. To determine antimicrobial activity, each standard antibiotic disc was dipped in DMSO containing synthesized organic compound at the following concentrations: 4, 8, 16, and 32 µg/ml of test compounds was made by adding 10 µl of different concentration and allowed to dry (RT for 30 min). The MHB and SDB tubes with

and without test samples were used as the control. A standard antibacterial drug (ciprofloxacin) and an antifungal drug (ketoconazole) were used for comparison. The bacterial and fungal strains were added to the nutrient broth and incubated on a rotary shaker at 200 rpm and 37ºC for 24 hours. Post incubation, the optical density at 600nm was adjusted to get an inoculum size of approximately 107 CFU/ml and used for the assay. On MHA plates, the turbidity of broth cultures was compared to that of standard 0.5 McFarland solutions, along with a standard antibiotic disc and a prepared disc containing different amounts of test samples. After 24 hours at 37°C, digital calipers were used to measure the inhibitory zones (Mitutoyo Rochester, New York). Three experiments were conducted, and the average zone diameter was determined.

2.4 Details animal grouping and in vivo anti-inflammatory activity

Male Swiss albino mice of weight 100 to 120 g were considered for the in vivo anti-inflammatory studies. With sufficient water, standard *ab libitum* diet was employed for about 7 days prior to the experiments. The animals were allowed for fasting overnight at the start of the experiments. Animal Ethical committee guidelines from Chromed Biosciences Pvt. Ltd. Hirehalli Industrial Area, Tumkur with the clearance number CBPL-IAEC-014/07/2022 were followed and the good care was taken for the animals.

For *in vivo* anti-inflammatory activity, carrageenan induced paw edema method. The animals were divided into six groups with six animals each in a group. Group I was administered with only DM water (10 ml/kg p.o/day). Group II was administered with standard drug indomethacin (10 mg/kg). Group III to Group VI animals were administered with synthesised *(E)-N'-(pyridine – 2 - ylmethylene) picolinohydrazide* (10, 25, 50 and 100 mg/kg, respectively). Using Plethysmometer, initial paw volume was measured and then the drugs were induced accordingly. The drug was administered and after 30 min, 1 % of carrageenan (25 µl) was injected in saline into the sub-plantar region of the right hind paws. The volume of paw was then recorded at 1, 2, 4, 6, and 24 h. The final edema was calculated using initial and final paw volumes at each time points.

2.5 Statistical Analysis

SPSS version 20.0 were utilized for the statistical analysis. The studies were run in triplicate and the findings were represented as mean standard deviation (MSD) by one-way ANOVA. Turkey's Multiple comparisons were utilized to compare standard and synthesized

compounds. Using Pearson's correlation analysis, $p < 0.01$ was considered statistically significant.

2.6. Molecular docking simulation

The structure of proteins cyclooxygenase-5 (COX-5; PDB code: 1EQG) and human antimicrobial peptide LL-37(residues 17-29) was collected from Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (Adams et al.). Energy minimization of ligands was accomplished in Open Babel (version 2.4.0) (O'Boyle et al.). The docking approach was validated by re-docking the native ligands into the binding pockets of the corresponding proteins. The docking of the target protein-ligand was performed using Auto-Dock Vina (version 1.1.2) (Trott and Olson). Docking simulations were performed with a grid whose centers were positioned at the active site of the individual proteins and whose dimensions were 25,00, 25,00, and 25.00 Å. Discovery Studio Client 2019 has examined the interactions between ligands and macromolecular targets. In this docking technique, several ligand conformations were generated, and the final energy refinement of the ligand posture took place. The Dock score of the best poses docked into the target protein was determined for the test compound.

3. Results and Discussion

3.1. Chemistry

Scheme 1 illustrates the complete synthetic approach for the target compound. Scheme 1 also shows the structure and yield of the synthesized compound. Initially, picolinohydrazide (0.137g, 1.0 mM) and picolinaldehyde (0.107g, 1.0 mM) are dissolved in methanol and refluxed for 2 hrs. This reaction mixture was poured into ice-cold distilled water and kept for 30 mins at room temperature. The precipitate formed was filtered in Whatman filter paper No 41 and air dried and re-crystallized using THF. Yield:74.00%. The formation of the novel compound was confirmed by ^{1}H NMR (Fig. 1). The formation of -CH=N was confirmed by a singlet at δ 8 ppm.

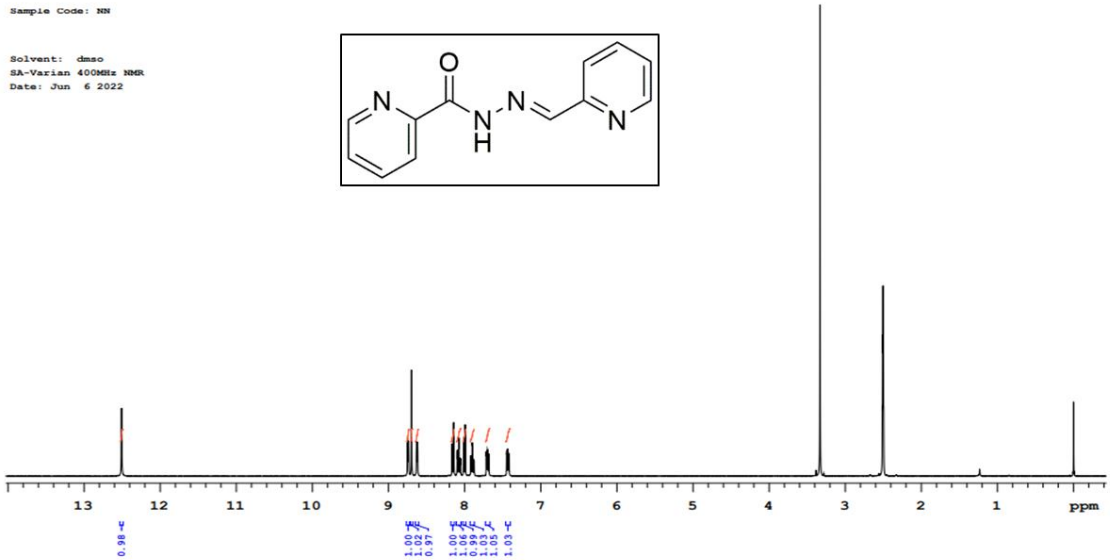


Fig. 1: ^{1}H NMR spectrum of (*(E)-N'-(pyridine - 2 - ylmethylene) picolinohydrazide*)

3.2. Antimicrobial Activity

The antimicrobial activity of the newly synthesized hydrazine hydride: (*(E)-N'-(pyridine - 2 - ylmethylene) picolinohydrazide*) was examined by the disc diffusion method (Bharadwaj et al.). Bacterial strains, namely Staphylococcus aureus (ATCC 25923), E. coli (ATCC 25922), and a fungus strain, viz. The Candida albicans (ATCC 22972) species was employed in this study. The control is DMSO, while the standards for bacterial and fungal strains are ciprofloxacin and ketoconazole, respectively. Table 1 shows the zone of inhibition results of the novel compound against both tested microbial strains. Fig. 2 depicts typical images of kirby-bauer disk diffusion technique. *(E)-N'- (pyridin- 2- ylmethylene) picolinohydrazide's* antibacterial activity reveals a maximum zone of inhibition (18.2 mm) at higher concentrations of the tested sample (32 g/ml) against *Staphylococcus aureus* strain, which demonstrated a maximum zone of inhibition than the standard (ciprofloxacin: 17.5 mm). On the other hand, *E. coli* did not exhibit any zone of inhibition even when exposed to a higher dosage of 32 µg/ml. The inclusion of an NH group on the aromatic ring or the occurrence of the heterocyclic moiety in the synthesized compound causes greater antimicrobial activity (Kamat et al.).

A fungus strain known as *Candida albicans* was utilized. The growth of the fungus was carried out in a Sabouraud dextrose agar (SDA) medium, and testing was carried out in a Sabouraud dextrose broth (SDB) media. The cultures and the viable count were performed using the same method that was used in the antibacterial tests, except for the temperature, which should be kept at 28 ± 1°C for approximately 72 hours. For the antifungal experiments, the same concentration of the test chemical, solvent (DMSO), and standard (ketoconazole) as was employed in the prior preparations was utilized. The antifungal activity of synthesized *(E)-N'- (pyridine – 2 – ylmethylene) picolinohydrazide* demonstrated a maximal zone of inhibition at a higher concentration of the tested sample, i.e., 9.2 mm zone of inhibition, which is almost comparable to that of the standard (ketoconazole: 9.0 millimeters). Table 1 presents the results of measurements made on the antifungal activity of *(E)-N'-(pyridine – 2 - ylmethylene) picolinohydrazide*. Fig. 2 depicts typical images of kirby-bauer disk diffusion technique. Similar behavior of antimicrobial activity was reported for pyridine-based hydrazide compounds in the literature (Rollas, Gulerman and Erdeniz; Vicini et al.; Halli, Sumathi and Kinni).

Table 1: Antimicrobial activity (zone of inhibition) of as-synthesized compound by disc diffusion method

Organisms name	Concentration (µg/ml)	Mean ZOI (mm)
S.aureus	32	18.2
E.coli	32	No zone of inhibition
C. albicans	32	9.5

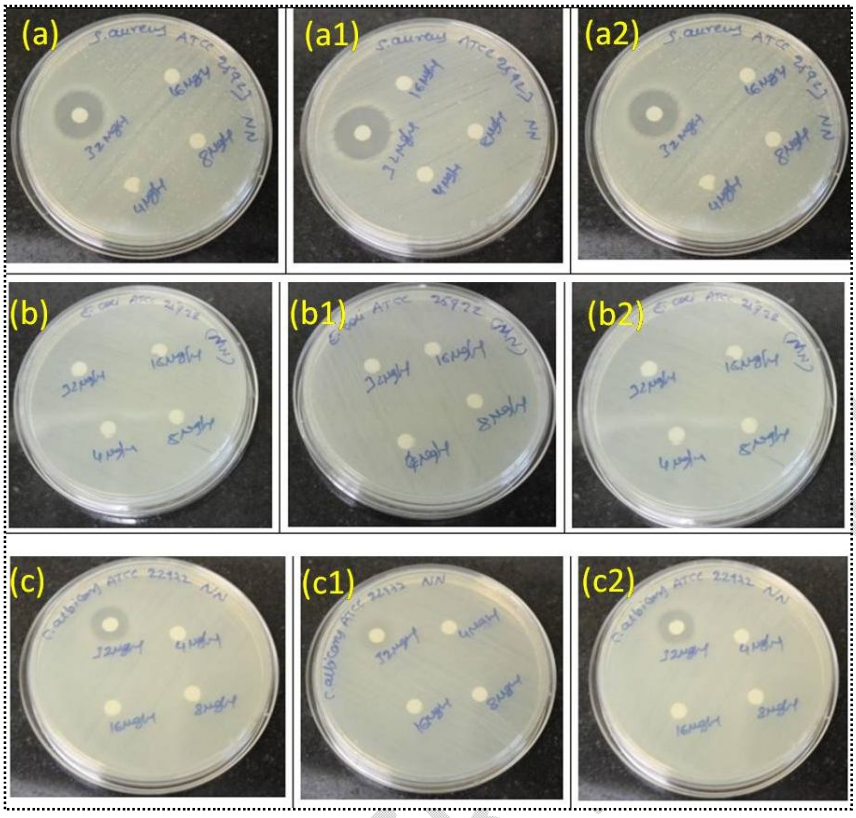


Fig. 2: Photographic images of antimicrobial activities by zone of inhibition of (*E)-N'-(pyridin-2-ylmethylene) picolinohydrazide*); viz., *S. aureus (a-a2); E. coli(b-b2) and C. albicans (c-c2)*

3.3 In vivo anti-inflammatory activity

The synthesised *(E)-N'-(pyridin-2-ylmethylene) picolinohydrazide*) was coded as NN. The results are provided in Fig. 3. A progressive increment of paw thickness was observed and was greater for the control group at four hours indicating the inflammation induced by 1 % carrageenan. However, with the increase in the dosage of the drug, the paw edema decreased indicating the anti-inflammatory activity at 4 hours. The edema further decreased with increasing 24 hours and remained almost equal. At 4 hours the control group showed 0.36 ml of paw edema, group II with control drug showed 0.23 ml, whereas, the groups III to VI, with increased concentrations of drug showed average of 0.35, 0.31, 0.28 and 0.26 ml of paw edema, respectively, confirming their anti-inflammatory activity.

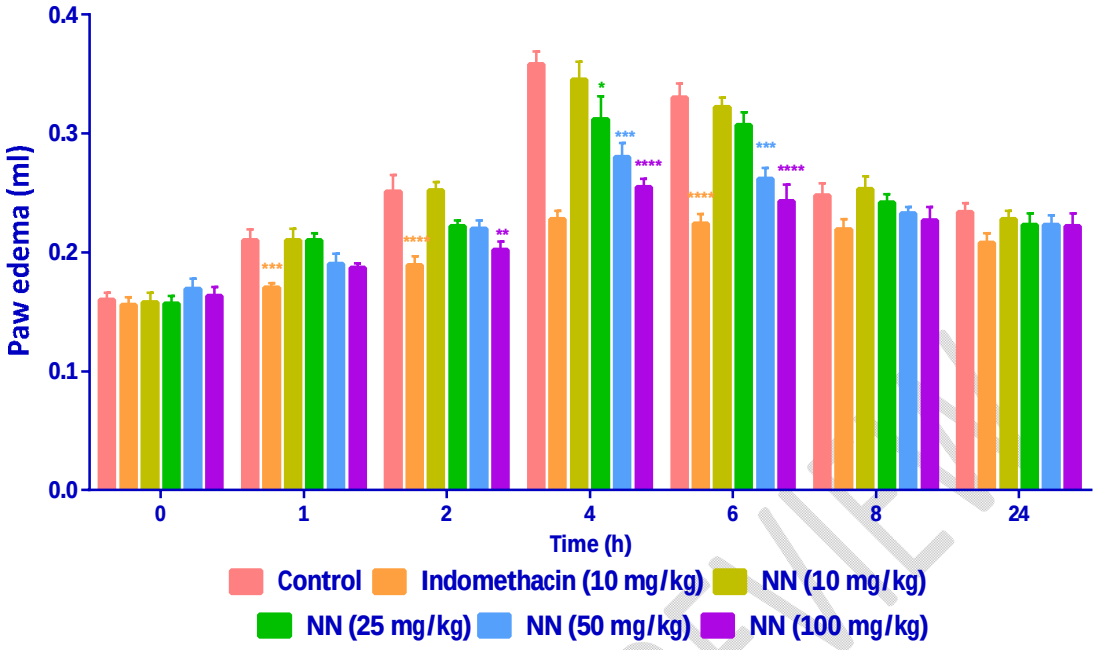


Fig. 3: Anti-inflammatory activity of *(E)-N'-(pyridin-2-ylmethylene) picolinohydrazide*) coded as NN, by carrageenan induced paw edema method.

3.4. Molecular Docking Studies

The docking study was performed using Autodock Vina (version 1.1.2), which is a flexible docking program, that can take into consideration the flexibility of the ligand as well as the flexibility of the active site residues of COX 5 (Trott and Olson). Docking tests were carried out to evaluate the potential binding manner of the significantly active chemical against the COX-5 (PDB code: 1EQG) proteins, which served as the target of the investigation. Before beginning the docking tests on the test compound, the root means square deviation (RMSD) of the X-ray pose and re-docked pose of the co-crystallized ligand in the target protein were analyzed. These data were also obtained in the protein data bank (www. rcsb.org). The fact that the RMSD was calculated to be 0.20 Å suggested that the docking methodology may be trusted for the docking experiments of the test chemical. After careful inspection of the co-crystal ligands (3D and 2D) poses (Fig. 4), it was found that celecoxib revealed four hydrogen bond interactions within the target, with the maximum docking score being -4.8 kcal/mol and the energy is 60.10 kcal/mol. The following amino acid residues actively engaged in the formation of hydrogen bonds between celecoxib and amino acid residues such as ALA-443, ARG-185, and PHE-504. In addition to the hydrogen bond, celecoxib interacted with the amino acid residues through two aromatic bonds. The

interaction between the pyridine moiety of celecoxib and the pyrazole part of celecoxib was taking place through ASP-393. In addition, the active compound also exhibited four aromatic bond interactions with the amino acid residue ILE: 442, ASP: 393, PHE: 404, and ALA: 443 through the pyridine moiety, as shown in Fig 5 and 6. The compound exhibited a docking score of −4.3 kcal/mol and docking energy of −42.10 kcal/mol, which is comparatively less than those of the standard drug celecoxib. Relationships between the structure and the activity of the newly synthesised hydrazine hydride: *((E)-N'-(pyridine – 2 - ylmethylene) picolinohydrazide)* (possible inhibitors of COX 5), a docking study demonstrated that the inhibitory activity of *((E)-N'-(pyridine - 2 - ylmethylene) picolinohydrazide)* towards COX 5 is dependent on (i) hydrogen bonds between the pyridine groups of the synthesized ligands and the catalytic residues of the binding site, (ii) πcation/anion interactions, (iii) π-alkyl interactions, and (iv) formation of carbon-hydrogen bond, (v) π- π bond that stabilizes the interaction with the active site.

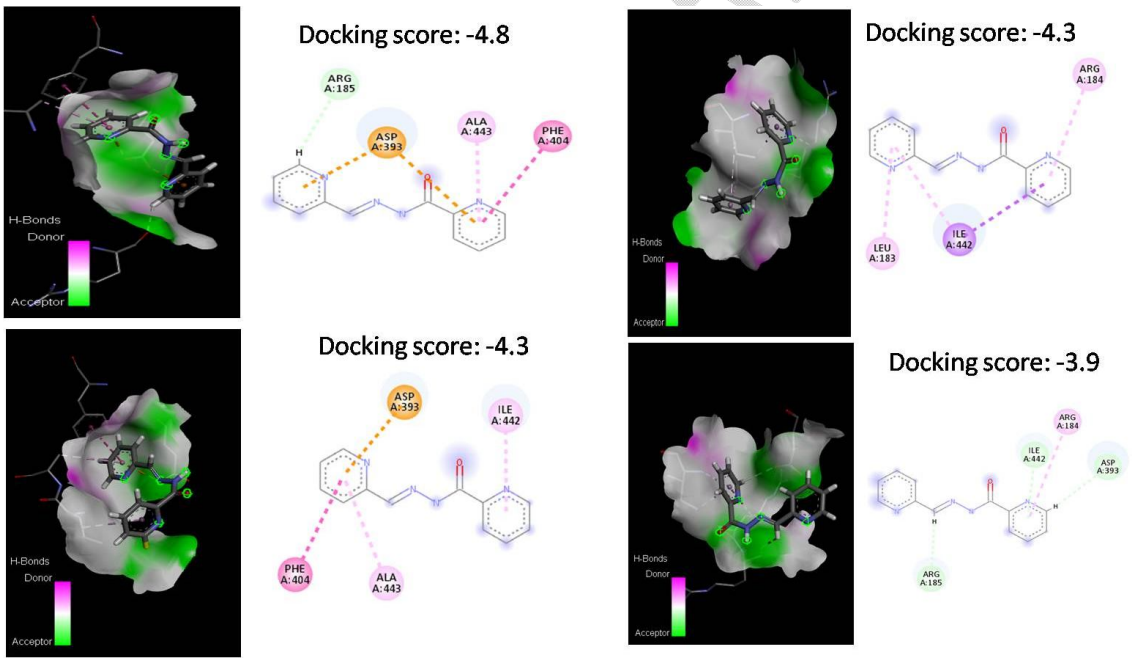


Fig. 4. COX 5 - hormone interactions; the hormone is stabilized mainly by Amide (i) hydrogen bonds between the pyridine groups of the synthesized ligands and the catalytic residues of the binding site, (ii) πcation/anion interactions, (iii) π-alkyl interactions, and (iv) formation of carbon-hydrogen bond, (v) π- π bond that stabilizes the interaction with the active site.

The potent inhibitory activity of the synthesized compound: *(E)-N'- (pyridine – 2 - ylmethylene)picolinohydrazide)* as new antimicrobial agents prompted us to study the docking of these derivatives inside the active sites of the human LL 37 (17-29) protein. The human LL-37 (hLL-37), also known for its ability to self-assemble, is a cleavage product of the hCAP-18 protein. It is recognized to play a vital part in the body's first line of defense against microbial infections (Xhindoli et al.). LL-37, also known as hLL-37, has a role in replication and transcription, and it catalyzes the formation of negative supercoils in the circular DNA of microbes. Additionally, LL-37 (hLL-37) is a known target for antimicrobial drugs because its inhibition results in the death of microbes (Xhindoli et al.).

In our attempt to explain the activity of the above-synthesized compound, we docked within the active site of LL-37 (hLL-37) protein (residues 17-29). The binding pocket of the enzyme includes the following residues LYS 2, Arg7, and Arg3. Docking studies revealed that the compound showed two H-bonds between the pyridine groups of the synthesized ligands and the catalytic residues of the binding site, πcation/anion interactions, π-alkyl interactions, and formation of carbon-hydrogen bond and π- π bond that stabilizes the interaction with the active site (Fig. 5). Docking results indicated that LYS 2, Arg7, and Arg3 play important roles in the activity. The explanation of the biological activity of the new compound may be attributed to the possibility of inhibition of these three amino acids.

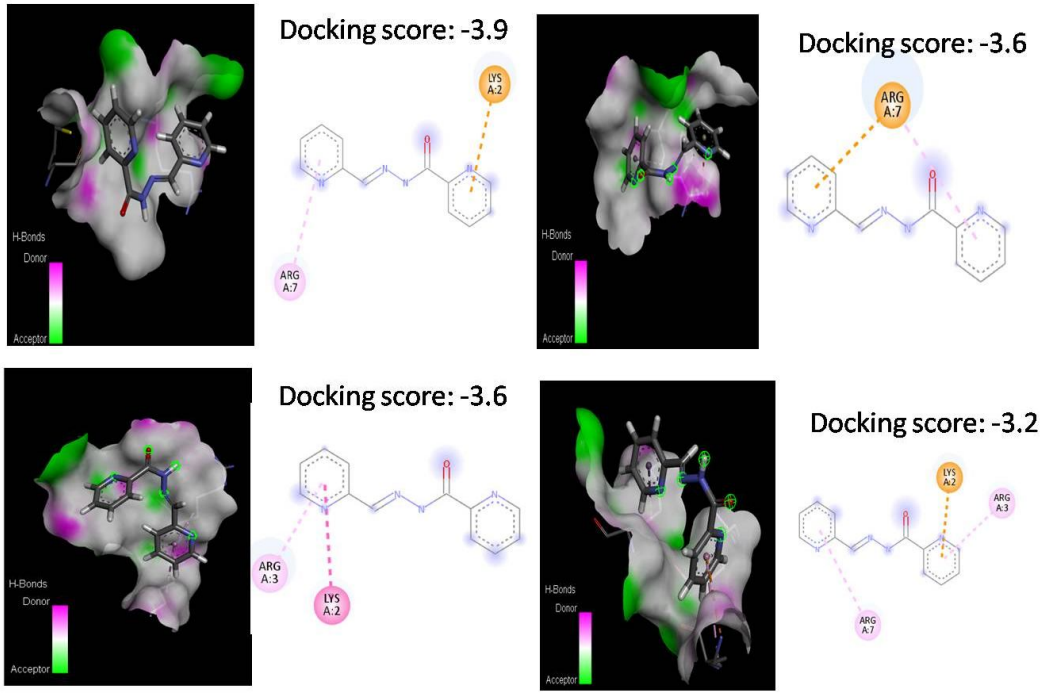


Fig. 5. LL 37 (17-29) - hormone interactions; the hormone is stabilized mainly by Amide (i) hydrogen bonds between the pyridine groups of the synthesized ligands and the catalytic residues of the binding site, (ii) πcation/anion interactions, (iii) π-alkyl interactions, and (iv) formation of carbon-hydrogen bond, (v) π- π bond that stabilizes the interaction with the active site.

4. Conclusion

In the present study, we synthesized a novel anti-inflammatory and antimicrobial agent: *(E)-N'-(pyridine - 2- ylmethylene) picolinohydrazide*. The in vitro anti-inflammatory study of the synthesized compound revealed that are significant inhibitors among the other series of hydrazine hydrides reported in the literature. The *in vitro* antibacterial experiment revealed that the synthesized compound is effective at a higher dose (32 g/ml) against *Staphylococcus aureus* (zone of inhibition 19.5 mm) and *Candida albicans* (zone of inhibition 9.5 mm) strains. The anti-inflammatory activity was confirmed by the reduced paw edema at 4 hours in the groups administered with the synthesized drug. *In silico* molecular docking study also evaluates the efficiency of interactions with target molecules. Based on our findings, we may conclude that this is a promising path for the development of new antibacterial and anti-inflammatory drugs.

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Abbreviations

NSAIDs: Non-steroidal Anti-inflammatory Drugs; Cyclooxygenase 5: COX 5; Thin-layer chromatography:TLC; DMSO: Dimethyl Sulfoxide; NMR : Nuclear Magnetic Resonance; Ppm: Parts per million; SDB: Saboraud dextrose broth (SDB); RT : Room Temperature; MSD: Mean Standard Deviation; RCSB PDB : Structural Bioinformatics Protein Data Bank; THF: Tetrahydrofuran, SDA: Sabouraud dextrose agar