RESEARCH PAPER

Design, Synthesis, Characterization And Biological Evaluation Of Novel Pyrazole-Isonazid Hybrids As Dual-Activity Anti-Inflammatory And Anti-Microbial Agent

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Abstract
Modern chemistry focuses on the discovery of innovative chemical entities that exhibit synergistic therapeutic effects and enhanced biological activity. These compounds are designed to optimize treatment outcomes and overcome limitations. Hybrid drug design is an approach in medicine to create treatments that target multiple things and work better. This study is about creating, making, testing and evaluating a compound that combines pyrazole and isoniazid. The goal is to make a medicine that can help with inflammation and fight infections. The process to make this involved three steps. First, ethyl acetoacetate and hydrazine hydrate were mixed to create ethyl 1H- pyrazole -3-carboxylate. Then thus was changed into 1H-pyrazole- carboxylic acid. Finally, this was combined with isoniazid using a helper or linker called dicyclohexlcarbodiimide (DCC) in a liquid called dimethylformamide (DMF). The structure of the compound was checked using infrared (IR) spectroscopy. The new compound was tested for its ability to reduce inflammation and fight infections. The results showed that it worked well in both areas. In the test Human Red Blood Cell Stabilization assay the compound showed it could reduce inflammation as well as it worked better at amounts than isoniazid. In the antimicrobial test of Agar well diffusion method the compound showed it could fight infections and worked better than the INH at all tested concentrations. These results suggest that combining pyrazole with active parts like isoniazid could lead to new treatments that work better. This study provides a base for future research into these kinds of treatments. The hybrid drug design approach seems promising for creating medicines that target multiple things. The pyrazole-isoniazid hybrid compound shows potential as a treatment, for inflammation and infections. More research is needed to understand its effects and possibilities.

Keywords: Hybrid drug design, Pyrazole-isoniazid conjugate, Anti-inflammatory activity, Antimicrobial activity, HRBC membrane stabilization, Agar well diffusion, Isoniazid, ethyl acetoacetate, DCC coupling, DMF solvent.
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1. Introduction

Medicinal chemistry is an interdisciplinary field combining physical chemistry, pharmacology, microbiology, biochemistry, and computer chemistry to understand how pharmaceutical compounds interact with living systems. It focuses on discovering, designing and developing medicines through these integrated approaches. Medicinal chemistry primarily focused on organic molecules, encompassing both naturally occurring and synthetically derived compounds, which constitute a significant proportion of pharmaceuticals. In addition to these, metal based and biological therapeutics also important. Natural product come from microorganisms, marine organisms, plants and animals. They can have therapeutic or toxic effects. Historically natural products have been a source of new medicines. In the 1900s most of the medicines are come from plants, later he focuses shifted to high-throughput screening and combinatorial chemistry

Pyrazole is a kind of heterocyclic organic compound that was first discovered by Ludwig Knorr in 1883. You do not usually find pyrazole in nature. In 1959 people found a type of pyrazole called 1-pyrazolyl-alanine in watermelon seeds. The pyrazole part is a ring with five members, which includes three carbon atoms which is flat, that makes the ring very stable and two nitrogen atoms, form bond with things, that affect how the compound works in the body.

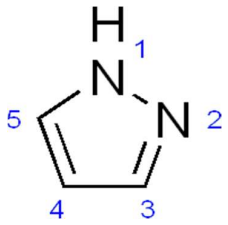


Fig 1.1: Molecular structure of pyrazole

Pyrazole exhibits some physical and chemical properties, which melts at 70oc, it appears as a colourless and forms dimer due to intermolecular hydrogen bond contributing to its high melting point. Chemically, pyrazole shows reactions like halogenation, sulphonation, nitration. Its aromaticity can be showed by multiple resonating structures, which enhance it stability and reactivity. This stability makes it very useful for making compounds. Pyrazole also exhibits nucleophilic aromatic substitution reaction which allows the introduction of various functional groups. Synthetic approaches to pyrazole include condensation reactions between ethyl acetoacetate and hydrazine to prepare pyrazolone as well as the Vilsemeier-Haack reaction to introduce chloro and formyl groups. Pyrazole can be classified into different compounds based on where hydrogen atoms are. The standard pyrazole ring is called 1H-pyrazole. There are also types of pyrazole like 2H- and 3H-pyrazoles, which are also known as pyrazolines. These compounds have a structure that is a little different from the standard pyrazole ring. The pyrazole derivatives can form bones with metals ions and that is why they are so useful. Pyrazole derivatives have been found to have different activities ,such as fighting against tuberculosis ,AIDS, malaria and cancer .they can be also be used as anxiety medicines and insecticides. some pyrazole compounds have been used to make medicine that are available in stores like floxan and difenamizole.

Pyrazole derivatives are very important because they have many different uses. They can be used to make medicines that fight against inflammation and pain. Inflammation is a process that involves many different steps, where arachidonic acid is released and converted to prostaglandin-like metabolites via COX enzymes, contributing to inflammation and pain. Researchers have made different pyrazole derivatives that have shown promise in inhibition of cancer and other various diseases. Some of these derivatives have even been shown to inhibit the growth of cancer cells. Pyrazole derivatives are also being studied for their use in making new medicines that can fight against many different diseases. For example some pyrazole derivatives have been shown to have antipyretic, anticonvulsant, and antifungal activities. All of these properties make pyrazole derivatives promising compounds, for future research and development of new medicines. Researchers have synthesized various derivatives by linking pyrimidine, carboxyhydrazide, and ferrocenyl molecule with pyrazole, demonstrating antiproliferative activity against human lung cancer cell lines and inhibiting tubulin polymerization. Manetti et al (2006) developed new pyrazole derivatives as an inhibitor of mycobacterium tuberculosis. Pyrazole derivaties have also analgesic activity, antipyretic activity anticonvulsant activity, antibacterial activity, and antifungal activity, making them promising candidates for therapeutic application and further research in medicinal chemistry.

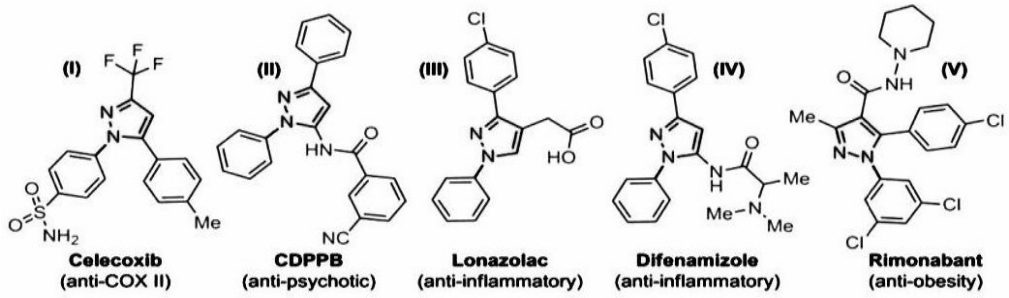


Fig 1.2: structure of pyrazole derivative

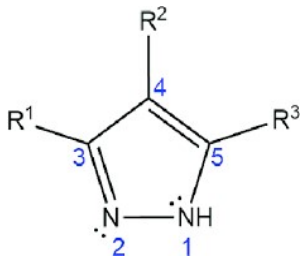


Fig 1.3: structure of pyrazole

Replacement of R1 position with alkyl group or phenyl ring enhances activity, if it has larger phenyl ring with one or two sulfone groups activity decreases. R2 position must be unsubstituted, where R3 position remain unsubstituted or substitution with phenyl group enhances activity. Replacement of cyano group or amines/heteroaromatic amines or methyl group, in R4 position increases activity. If R5 position replace with a methyl group or a hydroxyl group enhances activity.

Isoniazid is also known as isonicotic acid hydrazide, which was firstly made in 1912 by Hans Meyer and Joseph Malley. It was used for the time to treat tuberculosis in 1952 by Hoffmann-La Roche, E. R. Squibb and Bayer. Isoniazid is a type of derivative which consist of a pyridine ring and a hydrazide group. The hydrazide group is what makes isoniazid work. The pyridine ring is important for isoniazid to work against tuberculosis. The nitrogen in the ring in isoniazid helps bind to inhA enzyme. Isoniazid is an important medicine for treating tuberculosis because it is simple, stable and works very well.

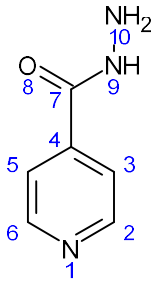


Fig 1.4: Molecular structure of isoniazid

Based on physical properties, Isoniazid is an almost white powder, odourless, slightly bitter with molecular formula of C6H7N3O and a molecular weight of 137.14 g/mol. It melts at 171-173oc and dissolves easily in water, not very well in alcohol, chloroform and ether. Isoniazid is dense with a density of 1.42 g/cm3, it also has a partition coefficient of 0.64 which means its like water. The pka value ranges from 1.8 – 3.5. Chemically, it was stable on normal storage, and can breakdown if it is exposed to light or air. It reacts with aldehydes, ketones and also form complexes with metal ions like copper and iron. When it is heated it breakdown into nitrogen oxides and carbon oxides. Isoniazid does not work well with oxidizing agents and acids which can make it break down on loss its power. Isoniazid is the choice for treating tuberculosis by inhibiting growth of mycobacterium tuberculosis. This works by isoniazid interfere with mycolic acid synthesis in the cell wall. The KatG enzyme helps to activate isoniazid, which then forms isoncotinoyl radicles, that bind with nad/nadp. it helps to produce potent antimycobacterial inhibitors. Isoniazid is not just treating tuberculosis, its derivatives have anticancer and cytotoxic properties, which means the inhibit the growth of cells. Isoniazid also have anti-inflammatory effects because it can reduce ROS level, activates PPAR expression and suppresses the NF-KB and AP-1 pathway. Some studies also shown that isoniazid -metal complexes, copper complexes inhibit RNA tumour viruses. Currently isoniazid is made using a chemical process involves using 4-cyanopyridine and hydrazine hydrate as reactants in presence of sodium hydroxide at 100oc. However, this process is hazardous and expensive. A better way to make isoniazid is, by using enzyme-catalyzed reactions with microbial lipases or triacylglycerol hydrolases. This method is safer more eco-friendly and cheaper. It can be done under mild conditions.

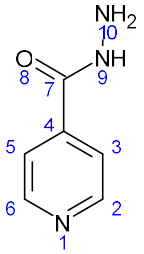


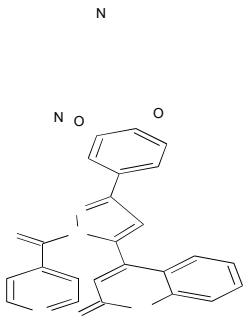
Fig i.5: Molecular structure of isoniazid

Isoniazid showed that the hydrazide group must be kept as it for the medicine to work, where pyridine ring is also very important and replacing with another ring make it less effective. Small changes like adding a methyl group to the ring or moving the nitrogen atom to a position leads no activity. The pyridine ring is necessary for the compound to inhibit tuberculosis, where nitrogen in the ring helps to bind with an enzyme. When the NH proton is replaced with an acyl group it becomes more effective and can easily pass through the microbial membrane. For the compound to show activity against mycobacteria there must be a hydroxyl group at a specific position, if something is added to that position the compound does not show any activity.

N-methyl-2-(pyridine-4-carbonyl

) hydrazine-1-carbothioamide

6-(5-chloro-2-hydroxy-4-methylphenyl)-2-(pyridine-4-carbonyl)4, 5-dihydropyridazin-3(2H)-one



4-[3-phenyl-1-(pyridine-4-carbonyl)-1*H*-pyrazol-5-yl]-2*H*-1-benzopyran- 2-

Isoniazid derivatives are interesting in medicine because they have biological activities and can be easily changed. Changing the parent isoniazid molecule, the hydrazide group allow us to add different rings that can make the compound more effective. These changes can improve antimicrobial, antiviral, and anti-inflammatory activities. Make the compound more stable and lipophilic. Creating molecules with the isoniazid structure is a good strategy to overcome drug resistance broaden therapeutic applications and show promising efficacy with acceptable toxicity. Recent studies show that modifying the isoniazid framework helps the drug pass into tissues and through the bacterial cell wall. Isoniazid has a hydrazide group that can be easily modified. This group can react with carbonyl compounds and undergo cyclization, making isoniazid a flexible starting point. Several studies indicate that isoniazid analogues, with a pyrazole moiety show anti-microbial and anti-inflammatory activities. Therefore, the present work is directed towards the synthesis of an isoniazid pyrazole hybrid, which is anticipated to possess both anti-inflammatory and antimicrobial activities.

The body has a natural response to injury and this is called inflammation. Inflammation is when the body gets pain and it tries to fix itself. This involves a lot of things happening like enzyme getting turned on special helpers getting released, fluid leaking cells moving around, then the body trying to fix the damaged tissue. Anti-inflammatory drugs mainly work on the pathway. This is a process where is a special enzyme called phospholipase, where A2 releases acid which then gets changed by another enzyme into different kinds of prostaglandins. The enzyme that does this is called cyclooxygenase or COX enzyme.

When the body is inflamed it makes a lot more of something called PGE 2. PGE 2 is a part of why we get red why it hurts and why we get a fever. It also makes things that the body uses to respond to injury work even better. PGE 2 is also made more when the body is fighting on infection because of something called IL-1. There are things that the enzyme COX makes like PGF 2 alpha, PGD 2 prostacyclin and thromboxane A 2 but they are not as important as PGE2 prostacyclin is special because it helps make the blood vessels bigger and it can make pain worse. PGE 2 prostacyclin work together to make the body red and hut when it is inflamed.

Anti-microbial agents are like medicines that can kill the bacteria. These medicines work by targeting things that the bacteria need to survive. This the way the compound inhibits the bacteria, which mainly work by the bacteria from making their cell wall or from making the things they need to survive or by breaking their cell wall. It is made up of building blocks that are linked together. Anti-microbial drugs, like D-cycloserine bacteriocin, beta-lactams and glycopeptides can inhibit the growth of bacteria.

1. Plan Of Work

2.1 Literature review

In the initial stage of work, an in-depth collection literature review was performed by gathering necessary and relevant informations on the same from authenticated scientific journals, textbooks and reliable online databases. This background checks helped in identifying the research gaps and rational design of new hybrid molecule with possible activities.

2.2 Design

Based on the knowledge gained, a new pyrazole-isoniazid hybrid molecule was designed with intension of combining the beneficial properties of both moieties into single structure.

2.3 Synthesis

The synthesis of the new hybrid was carried out using a three step chemical reaction with suitable chemical reagents and standard laboratory procedures.

2.4 Characterization

The compound was subjected to UV- Visible spectroscopic analysis to determine the product formed and get a preliminary information on its purity. The structural confirmation was obtained through IR

spectroscopic analysis and verify the presence of characteristic functional group corresponding to the pyrazole and isoniazid components in the molecule.

2.5 In- vitro biological activity evaluation

To assess the biological potential of the hybrid molecule, the in-vitro Human Red Blood Cell Stabilization method was carries out for evaluating the anti-inflammatory activity. The potential for antimicrobial activity was evaluated by using Agar Well Diffusion method.

2. Procedure

Synthesis of (1H-pyrazole-3-carbonyl) pyridine-4-carbohydrazide

Scheme 1: ethyl acetoacetate (4.8ml, 0.37mol), hydrazine hydrate (1.88ml,0.037mol) and ethanol (20ml) were added to a 250ml round bottom flask. The reaction mixture was refluxed at 60oC for 2 hours. Upon completion, the mixture was allowed to cool to room temperature and the resulting precipitate was collected by filtration. The crude solid was purified by recrystallization from ethanol to obtain ethyl-1H-pyrazole-3-carboxylate as a white crystalline product.

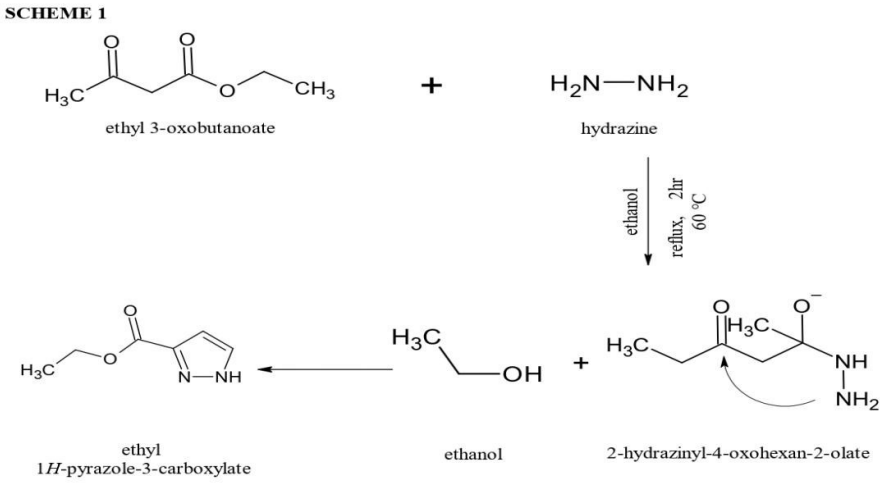


Fig.1: synthesis of ethyl-1H-pyrazole-3-carboxylate

Scheme 2: Ethyl-1H-pyrazole-3-carboxylate was dissolved in a solution of sodium hydroxide (1.6g, 0.04 mol) in water (20ml) and methanol (10ml). the mixture was refluxed at 600C for 1 hour. After completion, the mixture was cooled, adjusted the pH with dilute hydrochloric acid. The precipitated product was filtered, washed with cold water and dries to yield 1H-pyrazole-3-carboxylic acid.

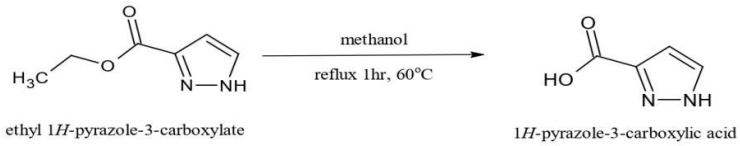


Fig .2: Hydrolysis of ethyl-1H-pyrazole-3-carboxylate

Scheme 3: 1H-pyrazole-3-carboxylic acid (0.5g, 0.004mol), dimethylformamide (DMF) (20ml), dicyclohexylcarbodiimide (DCC) (1.15g), and 4-dimethylaminopyridine (DMAP) (50g) was stirred at room temperature for 30 minutes using a magnetic stirrer to form the activated intermediate for coupling. Isoniazid (0.6g, 0.0046 mol) was dissolved in a mixture of dimethylformamide (18ml) and dichloromethane (2ml).

this mixture was added to previously activated reaction mixture and stirred at a cool temperature for 20-24hours. The precipitate formed was filtered and purified by recrystallization with ethanol and the (1H-pyrazole-3-carbonyl)-pyridine-4-carbohydrazide [IPZ-1] was obtained.

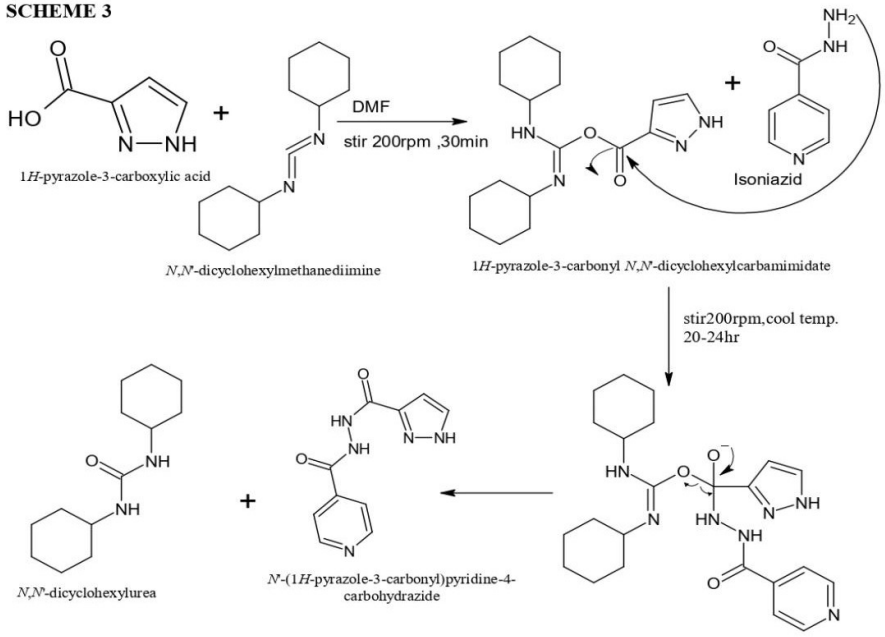


Fig 3: Coupling with isoniazid

3. Characterization

UV-Visible spectroscopy

An accurately weighed quantity of sample dissolved in suitable solvent to prepare stock solution. Appropriate dilutions were made from the stock solution. The baseline correction of the UV-visible spectrophotometer was performed using the selected solvent as blank. The sample prepared was transferred to cuvette and placed to sample compartment. The spectrum was recorded by scanning the solution over the wavelength range of 200-400nm under standard operating conditions. The wavelength corresponding to maximum absorption was recorded

IR spectroscopy

Using isopropyl alcohol, thoroughly cleaned every portion of the die to remove any dirt and dried with tissue. After proper cleaning of mortar and pestle with acetone, pat them dry with tissue. Put small amount of the sample into the mortar. Add the dried KBr into mortar and grind. Assemble the pellet press. Place the die within the opening. Transfer the ground sample into die using metal spatula. Place the die set into hydraulic press, turn the wheel to firmly secure it. Shut and pull the lever of hydraulic press to apply pressure. To extract the die, release the pressure and raise upper wheel. After removing the lower plunger, insert the die into ejector. To remove pellet from die, apply pressure. Pellet was placed in pellet holder in IR spectrophotometer and scanned in range of 4000-400cm-1.

4. In-Vitro Pharmacological Activity

Evaluation of anti-inflammatory activity

A healthy volunteer's blood was drawn and combined with an equal amount of Alsever solution (2% dextrose, 0.8% sodium citrate, 0.5% citric acid and 0.42% NaCl) and centrifuged at 3000rpm before experiment. The volunteer had not taken any NSAID for two weeks prior to experiment. A 10% suspension was prepared after washing with isosaline. Using distilled water, different concentrations of synthesized compound (10,20,30,40, and 50 microgram/ml) were prepared. 1 ml of phosphate buffer, 2ml of hyposaline and 0.5ml of HRBC suspension were added to each concentration. The sample was incubated at 370C for 30 minutes, at 3000rpm and its haemoglobin content was measured using spectrophotometry at wavelength of 560nm. 100 microgram/ml of aspirin was utilized as the reference standard and the extract was left out to create a control. The formula used;

% of haemolysis = (optical density of sample/ optical density of control) x 100

Evaluation of anti-microbial activity

Used the standard bacterial culture medium of Mueller Hinter Agar medium. Using sterile cotton swab, dip into standardized inoculum medium and streak the sterile agar plate rotating 600 each time. Allowed the inoculum to dry in agar surface for few minutes. Using a sterile cork borer or end of sterile pipette tip, make a uniform well in middle of plate. Pipette specific amount of sample to the well and keep it in room temperature for 30min. incubate the plate for 24 hours and examine the plate for clear

circular zone of inhibition around each well. The formula used;
Relative percentage inhibition = (a-b)/(c-b) x 100

Where, a- total area of inhibition of test, b- total area of inhibition of solvent, c-total area of inhibition of standard drug.

5. Result

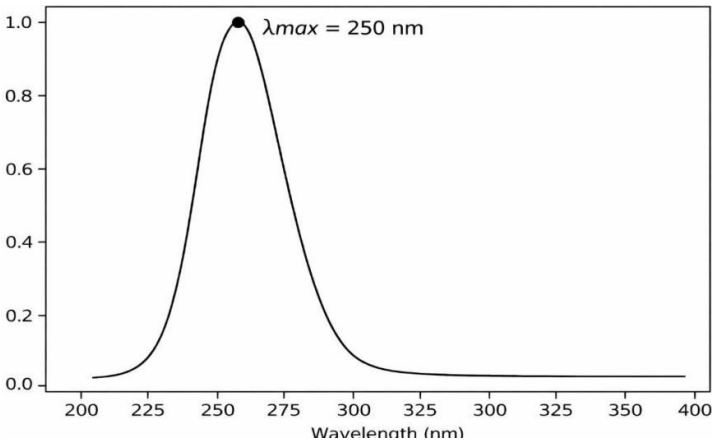


Fig 4: UV spectrum of 1H-pyrazole-3-carboxylic acid

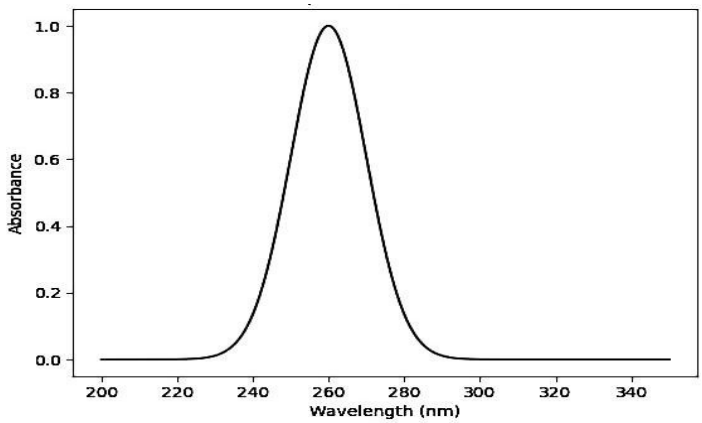


Fig 5: UV spectrum of (1H-pyrazole-3-carbonyl) pyridine-4-carbohydrazide

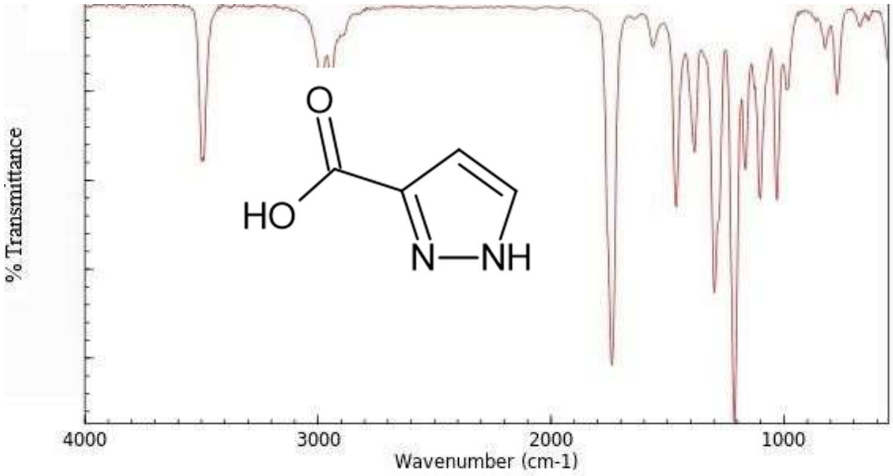


Fig 6: IR spectrum of 1H-pyrazole-3-carboxylic acid

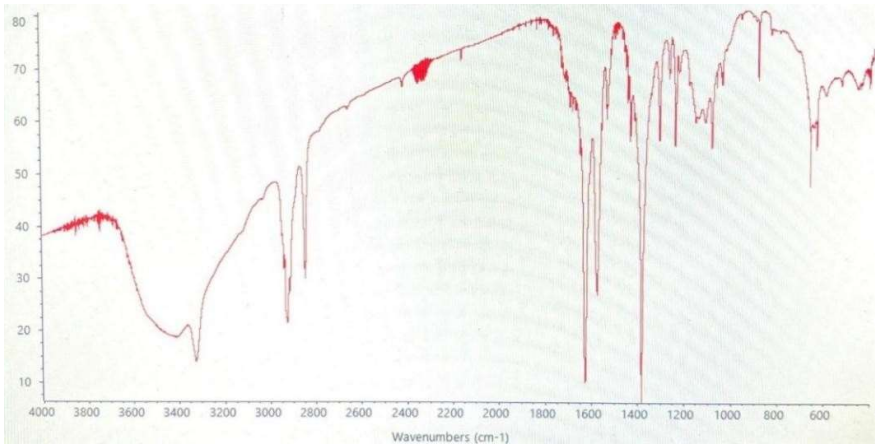


Fig 7: IR spectrum of (1H-pyrazole-3-carbonyl) pyridine-4-carbohydrazide

Table 1: Drug profile of 1H-pyrazole-3-carboxylic acid

IUPAC Name	**1H-pyrazole-3-carboxylic acid [PCA]**
Molecular formula	C4H4N2O2
Molecular weight	112.09 g/mol
Yield %	98%
Melting point	210–217 °C
Appearance	White to off-white crystalline powder
Solubility	Freely soluble in water, slightly soluble in ethanol.
Λmax	250 nm
IR	○ 3400 cm^{-1} N-H stretch ○ 3000, 2850 cm^{-1} C-H stretch ○ 1750 cm^{-1} C=O stretch ○ 1600 cm^{-1} C=N stretch

Table 2: Drug profile of (1H-pyrazole-3-carbonyl) pyridine-4-carbohydrazide

SI No.	**Concentration (µg/ml)**	**% Hemolysis of Standard (aspirin)**	**%Hemolysis of test (IPZ-1)**
1	10	32.88	38.66
2	20	30.66	36.88
3	30	26.44	36.88
4	40	23.77	31.11
5	50	20	27.11

Table 3: percentage haemolysis in anti-inflammatory activity evaluation

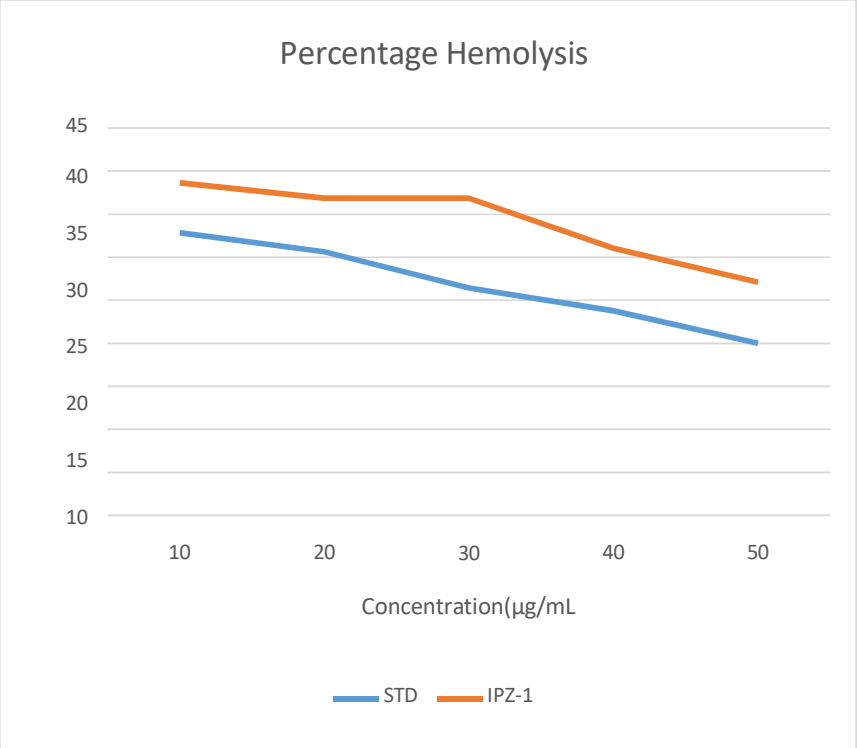


Fig 8: plot for percentage hemolysis

Table 4: Relative percentage inhibition in antimicrobial activity evaluation

Concentration (µg/mL)	Relative Percentage Inhibition(%)	
	INH	IPZ
20	10.36	27.47
40	18.59	34.93
60	26.66	39.50

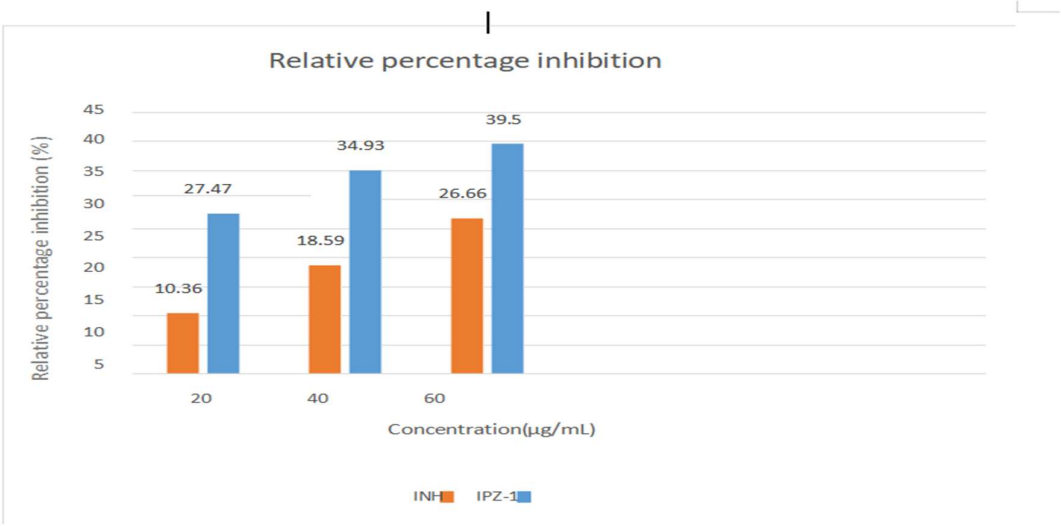


Fig 9: Relative percentage inhibition

Fig 9: Relative percentage inhibition

6. Summary

On basis of the informations from the literature review, a novel pyrazole-isoniazid hybrid was designed and successfully synthesized using standard laboratory procedure involving three step reaction sequence of cyclocondensation, hydrolysis and coupling reactions, yielding product with good yield and purity. The 1H-pyrazole-3-carboxylic acid [PCA] was synthesized by cyclocondensation reaction followed by alkaline hydrolysis with a 98% yield. It is a white to off-white crystalline powder having a molecular formula C 4H 4N 2O 2 with molecular weight of 112.09g/mol. It has a melting point ranging from 210-217 0C. It is freely soluble in water and slightly soluble in ethanol. The (1H-pyrazole-3-carbonyl) pyridine-4-carbohydrazide [IPZ-1], final hybrid product synthesized by coupling of 1H-pyrazole-3-carboxylic acid with isoniazid and obtained a 98% yield. It is a white to off-white fine powder with a molecular formula of C 10 H 9 N 5 O 2 and a molecular weight of 231.21 g/mol. It has melting point of 2800C. The characterization was carried out using the UV-Visible and IR spectroscopic analysis. The λ_{max} value obtained at 250nm and 260nm for PCA and IPZ-1, respectively. The IR spectra of PCA exhibited a broad peak around 3400cm-1 corresponding to N-H stretching. Peaks obtained at 3000 and 2850cm-1 were contributed to C-H stretching vibrations. C=O stretch seen at 1750-1 while C=N obtained at 1600cm-1. The IR spectrum for IPZ-1 showed distinct peak at 3325.2 cm -1 corresponding to N-H stretch. The absorption at 2933 and 2847 cm-1 were assigned to C-H vibrations.

The in-vitro anti-inflammatory activity of IPZ-1 evaluated using HRBC membrane stabilization method showed the percentage hemolysis rate of the IPZ-1 compared to the standard drug aspirin and the isoniazid. On comparison, it was observed that isoniazid showed higher hemolysis of 13.5% but only at low concentration while IPZ-1 showed average hemolysis of 26.75% at lower concentration. In vitro antimicrobial activity evaluated using the Agar well diffusion method showed that both INH and IPZ-1 exhibit concentration dependent inhibition of microbial growth. The difference in inhibition between the two compounds were 17.11%, 16.34% and 12.84% at 20,40 and 60 µg/mL, respectively. At all tested concentrations the IPZ-1 showed higher relative percentage inhibition compared to INH. The results of the biological evaluation reveal that the combination of isoniazid with a pyrazole moiety leads to improved activity relative to isoniazid alone. The biological response of the hybrid molecule suggests a synergistic interaction between the isoniazid and pyrazole moieties when compared to isoniazid.

Author contributions

- Elza Lalu, Fathimathul Sana K: Conceptualization; Methodology (Synthesis, Purification, Analysis); Formal Analysis; Writing- Original drat; Visualization.
- Suhana K P: Methodology (Biological Assays); Writing – Review & Editing.
- Avandhika U, Nidha Fathima T: Software Formal Analysis (ADMET prediction); Writing – Review & Editing.
- Jimmy Alex, John Thomas, Sunith D K: Conceptualization; Resources (Lab space); Supervision; Writing – Review & Editing.

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