SYNTHESIS AND CHARACTERIZATION OF PYRAZOLIDINE DERIVATIEVES AND ITS PHARMCOLOGICAL ACTIVITY

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ABSTRACT

By synthesizing pyrazolidine derivatives with 1-benzoyl-3-methyl-1H-pyrazol-5(4H)-ONE, which interacts with different benzaldehydes, six novel pyrazolidine compounds (PZ1 to PZ6) were produced.With the help of mass spectrometry, 1H-NMR spectroscopy, and IR spectroscopy, the novel synthesised derivatives were characterized.

The selected derivatives of the title compounds (PZ6, PZ5) are examined for their anti-arthritic activity using an in-vitro anti-arthritis activity was performed by egg albumin protein denaturation and bovine serum protein denaturation method with diclofenac sodium as a reference standard, and an invitro antibacterial activity was performed by cup plate method or cylinder plate method with gentamicin as reference standard, the selected derivatives of the title compo The experiment's findings indicate that pyrazolidine derivatives have stronger antibacterial and anti-arthritic effects.

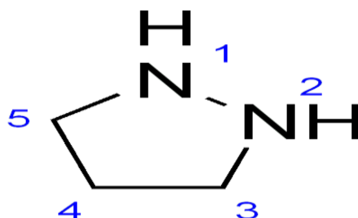
KEYWORDS: Diclofenac, gentamicin, pyrazolidine, anti-arthritic, anti-bacterial action.

INTRODUCTION:

PRAZOLIDINE:

is. Three carbon atoms and two nitrogen atoms make up the five-membered ring of the heterocyclic compound pyrazolidine. Given that it is a saturated pyrazole derivative, there are no double bonds in the ring structure.[1]

Due to its diverse properties and possible uses, pyrazolidines are a key class of molecules in organic chemistry and pharmaceutical research[2.] Because of their diverse structural makeup, pyrazolidines can undergo a wide range of alterations and functional group substitutions, which can change both their chemical and biological characteristics.There are various ways to make pyrazolidines, including cyclization processes with the right precursors or by altering existing pyrazole derivatives. With the use of these synthetic techniques, a variety of pyrazolidine derivatives with various chemical structures and characteristics can be produced.[3]



` Fig 1. Pyrazolidine

Materials and methods:

Synthesized chemicals are weighed precisely using a weighing balance. One capillary tube was used to determine the melting point of newly synthesized compounds using a melting point equipment. Using TLC plates that had been coated with silica gel, the reaction was observed. After the reaction was complete, the crystals were recrystallized using a suitable solvent, and the purity of the new chemical was assessed using TLC plates. The plates were then run in a solvent system such benzene and acetone (7:3), and the results were observed in a UV chamber. Newly synthesized compounds are then subjected to IR spectral analys

EXPERIMENTALMETHODOLOGY:

STEP-1 Synthesis of benzohydrazide:

The hydrazine hydrate [80% 3mol] and benzoic acid [1mol] were ground together for 3 to 5 minutes in a mortar and pestle. then after ten minutes of digestion, the reaction mixture solidified. The solid mass was crystallised from ethanol to produce hydrazides, and the reaction's completeness was verified using thin layer chromatography[4].

Step-2 Preparation of intermediate:

The procedure described earlier was used to create the chemical. In a conical flask, 0.1 mol of ethanol was taken, and 20 ml of hydrazine hydrate in ethanol was added drop by drop while stirring. When the crystalline solid was created, the temperature stayed at 60 °C. To complete the crystallisation, the reaction mixture was agitated for an additional hour at room temperature[5].

Step-3 Preparation of pyrazolidine derivatives:

0.01M of above derivative compound was taken, added a 20ml 0f N,N- dimethyl formamide and 25 ml of ethanol. The above mixture was stirred for half an hour by using magnetic stirrer. After that mixture kept in ice both a solid mass obtained the mixture was poured into churched ice.Filter the product and dried at room temperature. Recrystallized with ethanol[6].

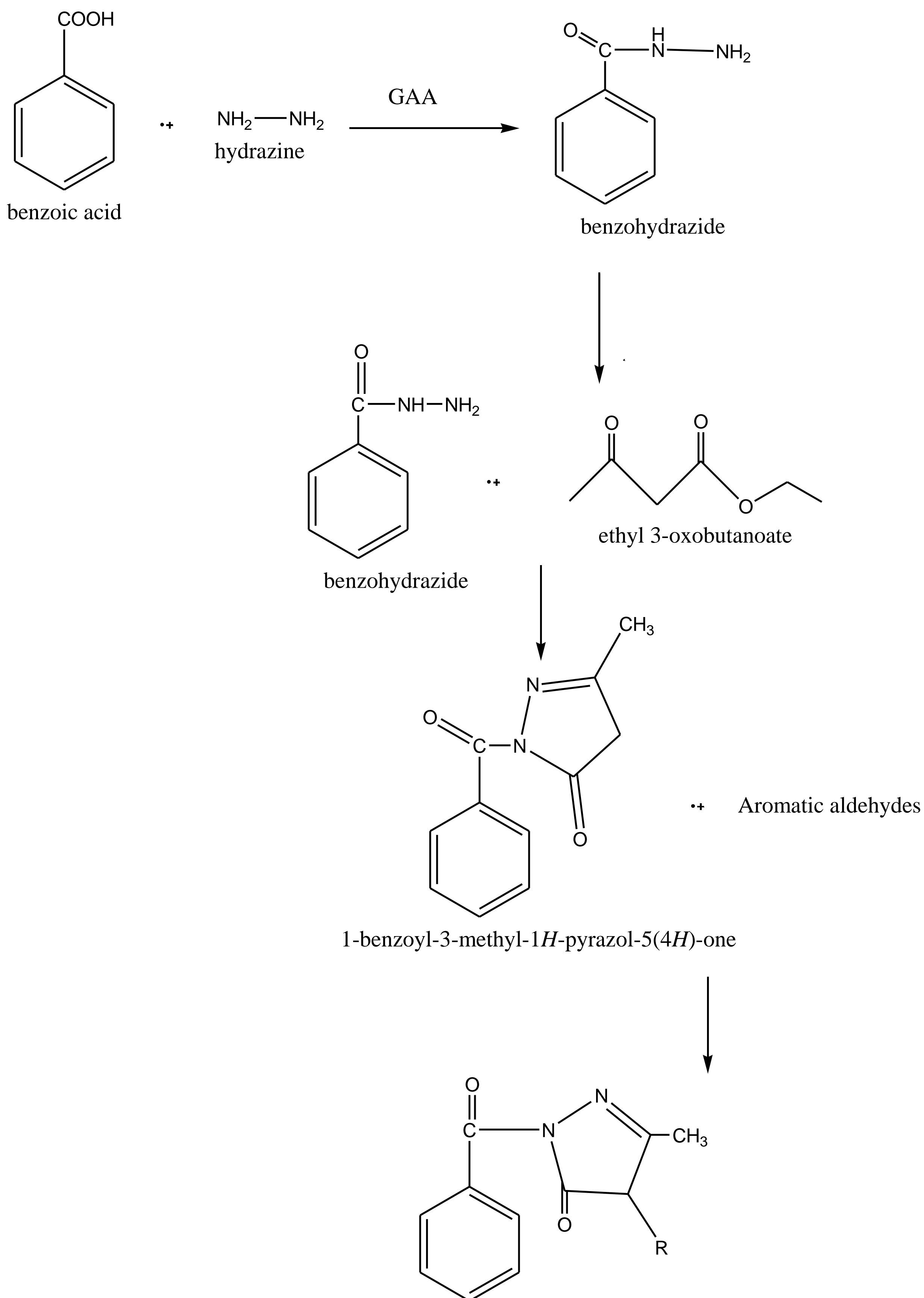


Fig 2. Scheme for pyrazolidine derivatives

IN VITRO ANTI ARTHRITIC ACTIVITY:
1. Bovine Serum Protein Denaturation Method:
Preparation of Reagents

0.5% Bovine Serum Albumin (BSA): Dissolved 500mg of BSA in 100 ml of water.

Phosphate Buffer Saline P^H 6.3: Dissolved 8 g of sodium chloride (NaCl), 0.2 g of potassium chloride (KCl), 1.44 g of disodium hydrogen phosphate (Na_2HPO_4), 0.24 g of potassium dihydrogen phosphate (KH_2PO_4) in 800 ml distilled water. The P^H was adjusted to 6.3 by using 1N HCl and make up the volume to 100 ml with distilled water[7].

Method:

Test solution (0.5%) is made up of 0.05 ml of test solution at various concentrations and 0.45 ml of bovine serum albumin (0.5% w/v aqueous solution)[8].

0.45 ml of bovine serum albumin (0.5% w/v aqueous solution) and 0.05 ml of distilled water make up the test control solution (0.5 ml)[9].

0.05 ml of the test solution and 0.45 ml of distilled water make up the product control (0.5 ml)[10].

0.45 ml of bovine serum albumin (0.5% w/v aqueous solution) and 0.05 ml of diclofenac sodium in varying concentrations make up the standard solution (0.5 ml)[11].

Materials:

Bovine Serum Albumin, Egg Albumin, phenazine derivatives(Test), Diclofenac sodium (Standard drug), Phosphate buffer solution (P^H 6.3), Distilled water.

Procedure:

Diclofenac sodium (10,50,100,250,500 g/ml), the standard medication, and 0.05 ml of various concentrations of the test medicines (10,50,100,250,500 g/ml) were each taken, and 0.45 ml (0.45% w/v BSA) were combined. The temperature was raised to retain the samples at 570C for 3 minutes after the samples had been incubated at 370C for 20 minutes. Add 2.5 ml of phosphate buffer to the aforementioned solution once it has cooled. At 225 nm, a UV-Visible

Spectrophotometer was used to determine the absorbance. Protein denaturation at 100% is represented by the control. Diclofenac sodium results were contrasted with the findings. One can compute the percentage inhibition of protein denaturation using the formula[12].

Percentage inhibition = 100 - (OD of test - OD of product control) /OD of control × 100

2 Egg Albumin Protein Denaturation Method:

The reaction mixture (5ml) consisted of 0.2 ml of Egg Albumin (from fresh hen's egg), 2.8 ml of Phosphate-buffered saline (PBS, P^{H}-6.4) and 2 ml of varying concentrations (10,50,100,250,500mg/ml) of drug. A similar volume of double distilled water served as the control. Next, the mixtures were incubated at 37^{0}C± 2^{0}C in a BOD incubator for 15 minutes and then heated at 70^{0}C for 5 minutes. After cooling, their absorbance was measured at 660 nm by using the vehicle as a blank. Diclofenac Sodium in the concentrations of 10,50,100,250,500 μg/ml was used as a reference drug and treated similarly for the determination of absorbance[13].

The percentage inhibition of protein denaturation was calculated by using the following formula:

Percentage inhibition = 100× [Vt / V c – 1]

Where,

Vt = absorbance of the test sample

Vc = absorbance of control

Each experiment was done in triplicate and the average was taken

The extract concentrations for 50% inhibition (IC 50) was determined by the dose- response curve.

IC50 = concentration / %inhibition x 50% of population

IN VITRO ANTI BACTERIAL ACTIVITY:

CUPPLATE METHOD OR CYLINDER PLATE METHOD:

This method depends on the diffusion of an antibiotic from a vertical cavity or cylinder , throw the solidified agar layer in a petri plate the growth of test micro-organism is inhibited Entirely in a circular area or zone around the cavity of cylinder containing a solution of antibiotic.

Requirements and materials

gram positive bacteria : pneumococci Gram negative bacteria : klebsiella , Petri plate , Conical flask , glass rod , burner , pH paper , inoculums loop , heating mantle , volumetric flask , hot air owen , auto clave , weighing balance.

Chemicals :

Agar-Agar, Beef extract, Peptone, Sodium chloride (NaCl), Hydrochloride (HCl), Sodiumhydroxide (NaOH),distilled water.

Procedure:

Nutrient Agar is a general purpose, nutrient medium used for the cultivation of microbes supporting growth of a wide range of non-fastidious organisms. Nutrient agar is popular because it can grow a variety of types of bacteria and fungi, and contains many nutrients needed for the bacterial growth[14].

Composition of Nutrient

0.5% peptone	It is an enzyme digest of animal protein . peptone is the principal source of organic nitrogen for the growing bacteria .
0.3 % beef extract / yeast extract	It is the water – soluble substance which aid in bacterial growth , such as vitamins , carbohydrates , organic nitrogen compounds and salts .

1.5% agar	It is the solidifying agent .
0.5% NaCl	The presence of sodium chloride in nutrient agar maintains a salt concentration in the medium that is similar to the cytoplasm of the microorganism .
Distilled water	Water is essential for the growth and reproduction of micro – organisms and also provides the medium through which various nutrients can be transported.
pH (7.4)	Stable pH is important requirement for optimum microbial growth in culture media .

Preparation of nutrient agar

1. Suspend 28g of nutrient agar powder in 1 litre of distilled water .
2. heat this mixture while stirring to fully dissolve all components .
3. Autoclave the dissolved mixture 121 degrees Celsius for 15 minutes .
4. once the nutrient agar has been autoclaved, allow it to cool but not solidify .
5. pour nutrient agar into each plate and leave plates on the sterile surface until the agar has solidified .
6. replace the lid of each petri dish and store the plates in a refrigerator .

Nutrient Broth

Nutrient Broth is a medium widely used for the culture of undemanding microorganisms. It is recommended in many standardized methods of analysis of foods, dairy products, water and other products. Nutrient Broth has the same formulation as Nutrient Agar, only agar has been omitted (which causes the medium to solidify at room temperature)[15]. It is made from a mixture of Tryptone and meat extract which helps in the growth of microorganisms. Sodium chloride is intended for maintaining osmotic pressure[16].

Procedure:

*Add 13g to 15g of nutritious broth powder in 1L of distilled water.

*Mix and dissolve completely

*Sterilize by autoclaving at 121 °C for 15 minutes.

Microbial culture by streak plate method:

1. Sterilize the inoculating loop in the Bunsen burner by putting the loop into the flame until it is red hot. Allow it to cool.
2. Pick an isolated colony from the agar plate culture and spread it over the first quadrant (approximately 1/4 of the plate) using close parallel streaks or insert your loop into the tube/culture bottle and remove some in oculum. You don't need a hugechunk[17].
3. Immediately streak the inoculating loop very gently over a quarter of the plate Using a back-and-forth motion.
4. Flame the loop again and allow it to cool. Going back to the edge of area 1 that you just streaked, extend the streaks into the second quarter of the plate (area 2).
5. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area 2), extend the streaks into the third quarter of the plate (area 3)[18].
6. Flame the loop again and allow it to cool. Going back to the area that you just streaked (area3), extend the streaks into the centre fourth of the plate (area 4)[19].
7. Flame your loop once more.

Standard Solution:

Peptones	10g
Beef extract	1g
Yeast extract	2g
Sodium chloride	5g
pH Final	6.8±0.2at25° C

To make a stock solution, dissolve a reasonable amount of the USP Preference Standard for the provided antibiotic in the solvents, or, if appropriate, the whole contents of a vial of the standard, and then diluted to the specified concentration. Store at 2-8* and use within the time frame mentioned. Prepare five or more test dilutions from the stock solution on the day of the assay, each of which increases in concentration sequentially and typically has a 1:1.25 concentration ratio.Utilize the final diluents, chosen so that the concentration is at the median[20].

Sample drug derivatives dilutions:

1. Prepare the stock solutions of drug derivatives i.e. (vanillin, salicylicbenzaldehyde, para-di methyl amino Benzaldehyde, para- chlorobenzaldehyde, cinnamaldehyde, benzaldehyde) by weighing each about 20mg accurately and transfer into 20ml of solvents i.e.(ethanol, chloroform, acetone) respectively[21].
2. From each stock solutions take about 1ml, 2ml, 3ml, 4ml, and dissolve each in 20mlwith distilled water respectively (10µg/ml, 20µg/ml,30µg/ml,40µg/ml,)[22].

General procedure:

1. Sterilize glass wears with the help hot air oven at 170 c about 30 min before tp start the process.

2. Now prepare required amount agar –agar medium and sterilize in autoclave at 121 c and solidify it.

3. Now from pure culture of gram positive and gram negative bacteria take in oculum and streak over petri plates having agar medium and incubate for 48 hours.

4. The cultured bacteria transferred into nutrient broth 2 to 3 hours and allow it to growth until the turbidity appears[23.]

5. Appears another freshly agar-agar medium and nutrient broth to it before it gets to solidify.

6.Transfer all of the contents into petri plates, allowing them to solidify and form into cups or cylinders. Next, fill each petri plate with various drug derivative dilution concentrations, and set each one in an incubator for roughly 24 hours.

7. A zone of antibiotic activity inhibitions can be noticed with regard to the individual concentrations after 24 hours[24.]

8. Calculate the diameter by dividing it by the respective inhibition zone's R/2 value.

Note : Absence of turbidity indicative negative control , if the turbidity appears then it said to be positive control.

RESULTS AND DISCUSSION

Characterization:

Compound(PZ_1):

IR (KBr in cm^{-1}): C=O stretch 1782.06, C-C Stretch 1611.37, Aromatic 1820.2, C-H Stretch 1303.4, N-N Stretch 1271.16

NMR chemical shift (δ, ppm): 2.2 (S,1H, CH, ArCH),7.2-7.8 (M, Ar CH,14H),8,4 (S, NH,1H)

Mass: Base peak 79.20000, molecular ion peak 306.71

Compound ($PZ2_2$):

IR (KBr in cm^{-1}): N-H Stretch 3319.3, C-C Stretch 1142.2, C=O Stretch 1543.1, N-N Stretch 1286.1,

NMR chemical shift (δ, ppm): 1.3 (S, CH,1H, Ar CH), 7.3-7.8 (M, Ar-H, 12H),8.6 (S,1H, NH)

Mass: Base peak 384.00, Molecular ion peak 334.29

Compound (PZ_3):

IR (KBr in cm^{-1}): N-H Stretch 3819.3, C=O Stretch 1722.2**,** C-C Stretch 1643.1, C-F Stretch2792.1,

NMR chemical shift (δ, ppm): 1.3 (S,1H, CH),1 (solvent peak),7.3-7.8 (M,12H), 7(S,1H, NH)

Mass: Base peak 79.30000, Molecular ion peak 336.33

Compound (PZ_4):

IR (KBr in cm^{-1}): Aromatic2602.3, C=O Stretch 1642.2 **,**C-CHO Stretch 743.1, N-H Stretch 3316.1 ,

NMR chemical shift (δ, ppm): 1(S,1H, CH) , 5.5 (S,OH, 1H), 7-7.7 (M, Ar-H,12H), 8.6 (S,NH, 1H, Ar-CH).

Mass: Base peak 79.2500, Molecular ion peak 334.27

Compound (PZ_5):

IR (KBr in cm^{-1}): N-H Stretch 3302.3, C=O Stretch 1712.2, C-O Stretch1323.1, N-N Stretch 1246.1, Aromatic C-H Stretch3022.6

Compound ($_6$):

IR (KBr in cm^{-1}): N-H Stretch 3349.3, C=O Stretch 1622.2, N=N Stretch1216.1,C-C Stretch 1641.31

RESULTSANDDISCUSSION:

IN VITRO ANTI-ARTHRITIC ACTIVITY:

The anti-arthritic activities of the synthesized derivatives were evaluated bybovine serum protein denaturation and egg albumin protein denaturation method against the standard, diclofenac. The results of this method were represented in the Table.

S.no	Concentration (µg/ml)	Absorbance (nm)	% inhibition
1.	10	0.107±0.005	69.6±0.811
2.	50	0.115±0.0023	81.6±3.457
3.	100	0.119±0.0012	89.3±1.891
4.	250	0.122±0.0015	93.0±2.325
5.	500	0.123±0.0015	94.3±2.325

Table no 1 : *Anti-arthritic activity of diclofenac by Egg albumin denaturation method*

Con (µg/ml)	Pz_1		Pz_2		Pz_3		Pz_4		Pz_5		Pz_6	
	Abs	%Inh	Abs	%Inh	Abs	%Inh	Abs	%Inh	Abs	%Inh	Abs	%Inh
10	0.070±0.006	12.8±2.61	0.318±0.236	28.5±2.51	0.074±0.002	17.4±2.71	0.088±0.022	40.1±3.69	0.086±0.003	36.4±3.99`	0.075±0.003	36.43±3.99
50	0.072±0.0024	18.4±2.08	0.092±0.002	45.9±3.29	0.076±0.002	20.0±3.21	0.095±0.003	50.7±4.20	0.093±0.003	47.0±4.30	0.080±0.001	46.0±4.30
100	0.085±0.002	34.8±3.29	0.095±0.002	50.8±2.39	0.086±0.003	35.9±4.70	0.108±0.002	70.8±3.83	0.113±0.003	82.1±4.41	0.090±0.001	81.8±4.41
250	0.097±0.001	53.9±3.99	0.109±0.004	73.50±6.99	0.093±0.002	47.0±3.21	0.118±0.003	87.8±4.70	0.118±0.001	87.7±1.03	0.098±0.002	87.2.7±1.03
500	0.103±0.001	63.4±2.41	0.115±0.004	81.4±5.03	0.102±0.102	61.3±3.46	0.119±0.003	89.3±4.61	0.122±0.001	93.6±1.84	0.112±0.003	93.5±1.84

Table no 2: Anti-arthritic activity of test compounds by Egg albumin denaturation method

Bovine Serum Albumin method:

S.no	Concentration (µg/ml)	Absorbance (nm)	% inhibition
1.	10	0.107±0.005	69.6±0.811
2.	50	0.115±0.0023	81.6±3.457
3.	100	0.119±0.0012	89.3±1.891
4.	250	0.122±0.0015	93.0±2.325
5.	500	0.123±0.0015	94.3±2.325

***Table no 3*:** Anti-arthritic activity of diclofenac by Bovin serum albumin method

Con (µg/ ml)	Pz_1		Pz_2		Pz_3		Pz_4		Pz_5		Pz_6	
	Abs	**%Inh**	**Abs**	**%Inh**	**Abs**	**%Inh**	**Abs**	**%Inh**	**Abs**	**%Inh**	**Abs**	**%Inh**
10	**0.786±0.003**	**27.5±0.578**	**0.641±0.006**	**31.9±0.742**	**0.784±0.006**	**25.2±0.17**	**0.389±0.006**	**41.6±5.58**	**0.444±0.003**	**45.6±5.58`**	**0.424±0.002**	**49.3±0.636**
50	**0.727±0.004**	**43.6±0.186**	**0.582±0.006**	**43.1±2.40**	**0.717±0.006**	**37.9±0.945**	**0.387±0.001**	**59.4±0.186**	**0.326±0.003**	**65.0±0.78**	**0386±0.001**	**67.0±2.636**
100	**0.599±0.004**	**49.4±0.26**	**0.447±0.006**	**50.5±2.85**	**0.627±0.006**	**43.40±0.379**	**0.292±0.006**	**69.6±1.31**	**0.311±0.001**	**71.5±0.36**	**0.308±0.001**	**71.9±0.233**
250	**0.409±0.001**	**53.2±0.12**	**0.388±0.001**	**69.7±0.463**	**0.409±0.005**	**46.9±0.18**	**0.277±0.015**	**72.±2.96**	**0.261±0.001**	**82.2±0.233**	**0.261±0.001**	**82.1±0.33**
500	**0.299±0.001**	**67.5±0.306**	**0.309±0.001**	**72.2±0.285**	**0.419±0.007**	**51.3±0.14**	**0.198±0.001**	**91.1±0.120**	**0.224±0.001**	**93.2±1.43**	**0.247±0.004**	**84.5±1.06**

***Table no 4*:** Anti-arthritic activity of test compounds by Bovin serum albumin method

In vitro anti -bacterial activity:

The anti-bacterialactivitiy of the synthesized derivatives were evaluated by cup plate method (or) cylinder plate method against the standard, gentamicin. The results of this method were represented in the Table.

s.no	Compound code	Zone of inhibition(diameter=R/2)								Positive control		Negative control
		Gram+ve				Gram-ve				+ve	-ve	
		10	20	30	40	10	20	30	40			
1	Pz1	1.5	1.2	1.1	0.9	1.7	1.8	1.0	0.8			
2	Pz2	2.3	1.8	1.7	1.1	1.6	1.9	1.1	0.7			
3	Pz3	1.6	1.7	1.0	0.6	1.2	1.1	1.3	0.8			
4	Pz4	1.2	1.3	1.0	0.8	1.2	1.0	0.9	0.5			
5	Pz5	0.8	0.7	0.5	0.4	1.1	0.8	0.7	0.7			
6	Pz6	1.2	1.0	0.8	1.1	1.3	1.2	0.9	0.9			
7	gentamicin	1.8	1.7	1.0	0.9	1.6	1.3	1.5	0.7			

Table no 5**:** Anti-bacterial activity of test compounds by cup plate method

Discusion:

In-vitro anti arthritic activity

In-vitro anti inflammatory activity was performed by egg serum albumin and bovine serum albumin method against the standard diclofenac drug. All the titled compounds (PZ_1-PZ_6) were evaluated for *in-vitro* anti inflammatory activity. The effect of the synthesized titled compounds was tested with different concentrations (10, 50, 100,250and 500ug/ml). All derivatives were able to shows anti-inflamatory activity. The most effective was PZ_4, PZ_6 shows more potent activity. The results were tabulated in table no.2 The order of anti-inflammatory activity of synthesized compounds as follows.

Egg albumin method

$PZ_4 > PZ_5 > PZ_6 > PZ_2 > PZ_3 PZ_1$

Serum albumin method

$PZ_6 > PZ_5 > PZ_4 > PZ_2 > PZ_1 > PZ_3$

In-vitro anti-bacterial activity

The anti-bacterial activitiy of the synthesized derivatives were evaluated by cup plate method (or) cylinder plate method against the standard, gentamicin. The results revealed that PZ_5 shows more potent activity against gram positive and gram negative bacteria. The order of anti-bacterial activity of synthesized derivatives as follows.

$PZ_5 > PZ_6 > PZ_4 > PZ_1 > PZ_3 > PZ_2$

CONCLUSION

Six new substituted pyrazolidine derivatives were created using a straightforward three-step technique. All derivatives were then characterised and tested for in-vitro anti inflammatory and in vitro anti-bacterial activity.

The results of anti-inflammatory revealed that in egg albumin method PZ_4 and in serum albumin method PZ_6 shows potent activity against standard diclofenac drug. In anti- bacterial activity PZ_5 shows more activity against gram positive and gram negative bacteria.

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