CHAPTER 4

MATERIALS AND METHODS

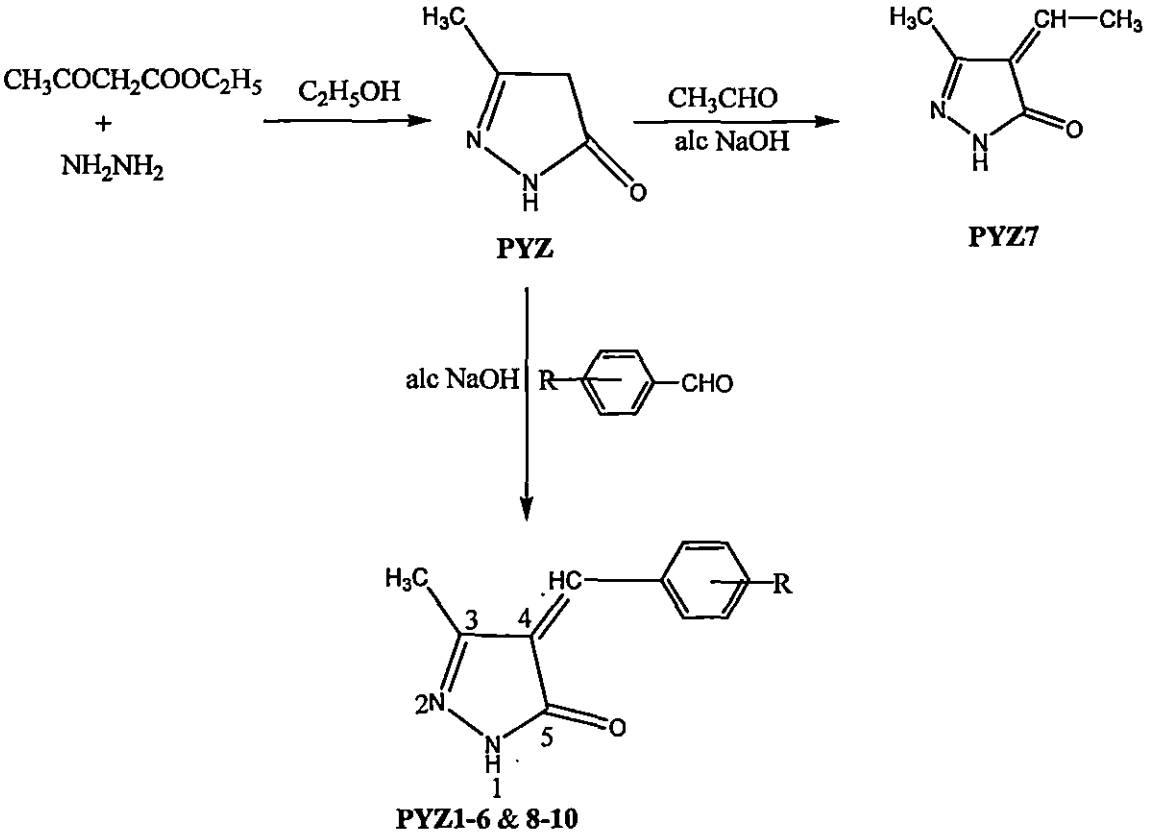
4. MATERIALS AND METHODS

4.1. Materials

4.1.1. Chemicals used for synthesis

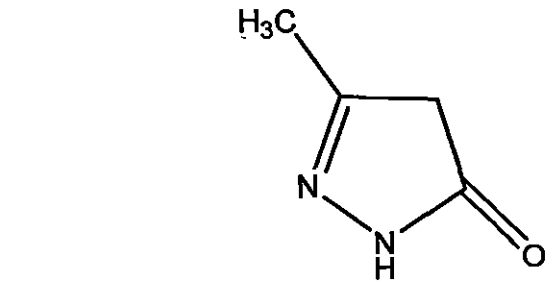
Ehyl acetoacetate, hydrazine hydrate, absolute ethanol, sodium hydroxide and various substituted aromatic aldehyde, were obtained from S. D Fine Chemicals, Mumbai, India.

4.2 Scheme of synthesis

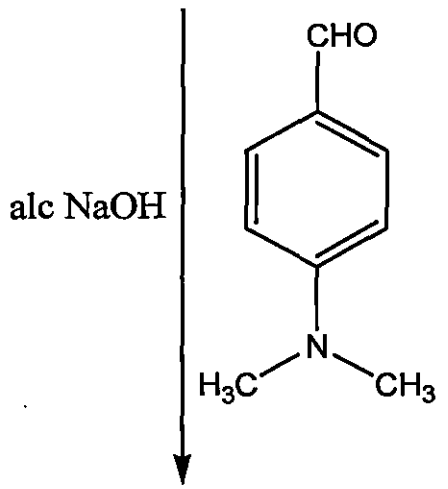


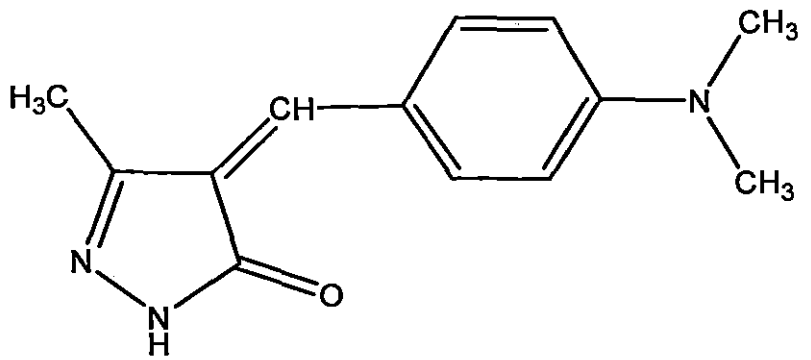
Compounds	R	Compounds	R
PYZ1	$-N(CH_3)_2$	PYZ 6	H
PYZ 2	$C_6H_5CH=CH-$	PYZ 8	4—OMe
PYZ 3	$-OCH_3(_{2',3})$	PYZ 9	3-OCH_3
PYZ 4	3-NO_2	PYZ 10	2-OH
PYZ 5	3-Cl		

4.2.1. Scheme of synthesis of 4-[4-N dimethyl aminobenzylidine] -3-methyl pyrazolin-5(4H) - one (PYZ1)



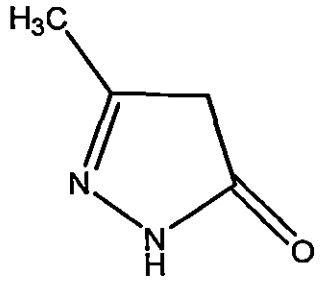
PYZ



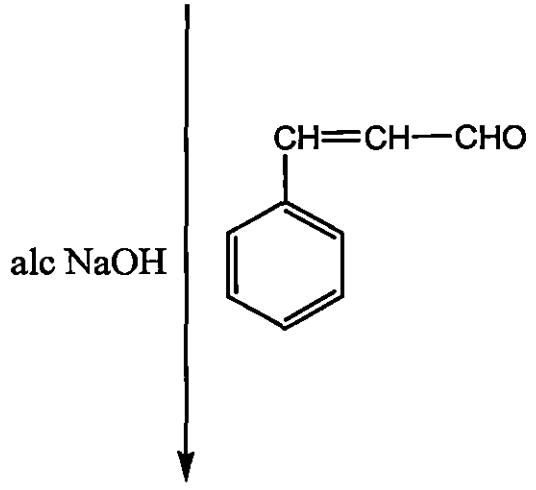


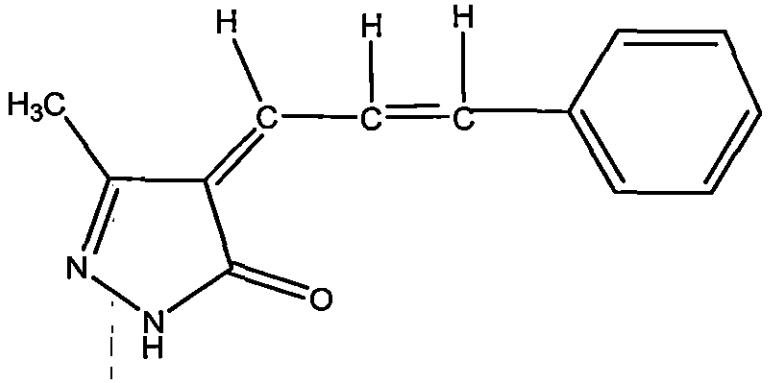
PYZ1

4.2.2 Scheme of synthesis of 4-[phenyl allylidene]-3-methyl pyrazolin-5(4H) – one (PYZ2)



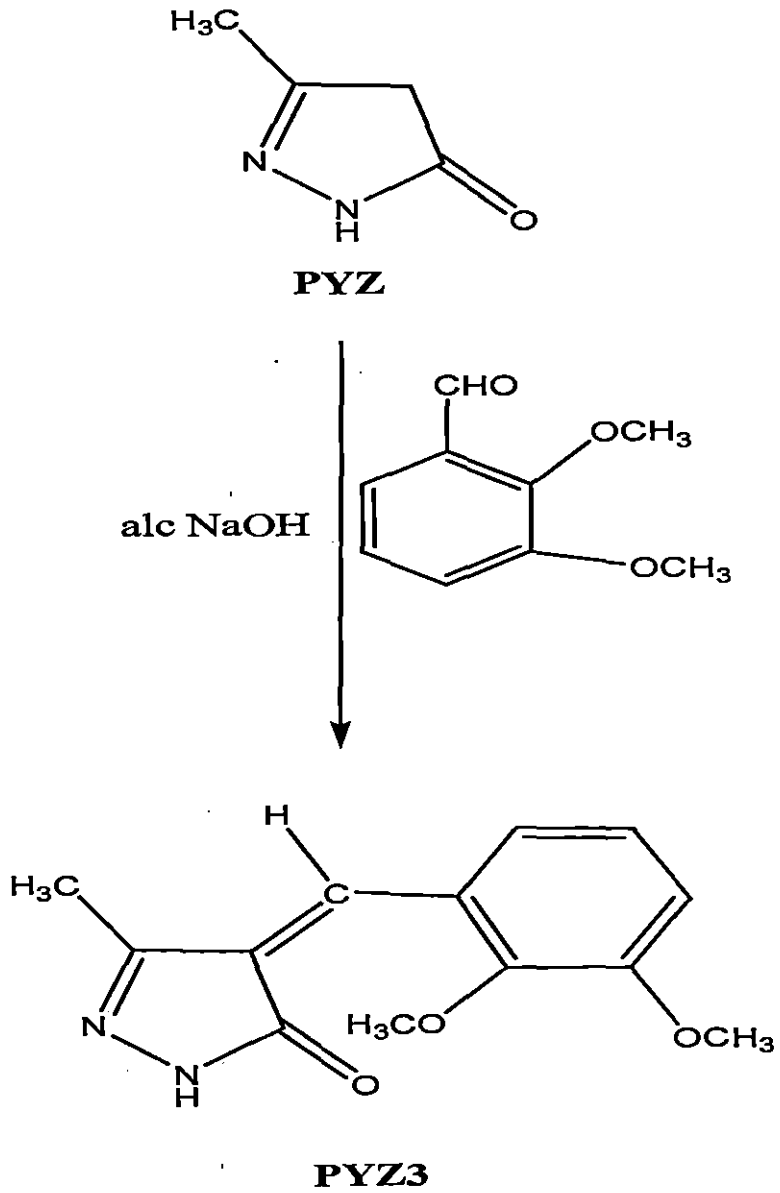
PYZ





PYZ2

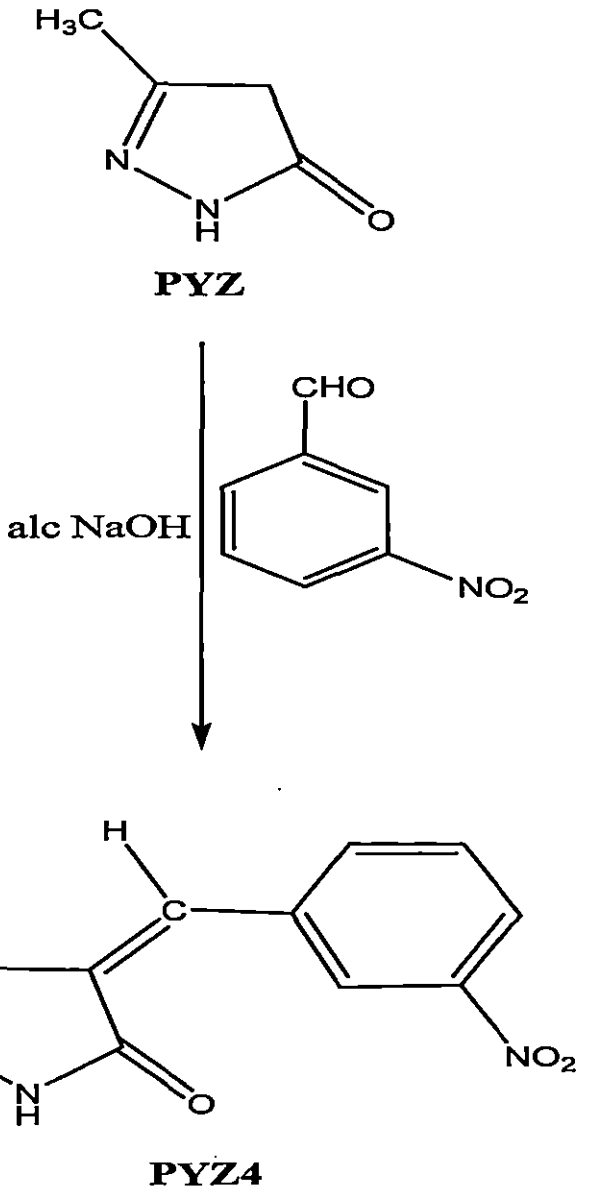
4.2.3. Scheme of synthesis of 4[2, 3- dimethoxy benzylidine]-3-methyl pyrazolin-5(4H) - one (PYZ3)



PYZ

PYZ3

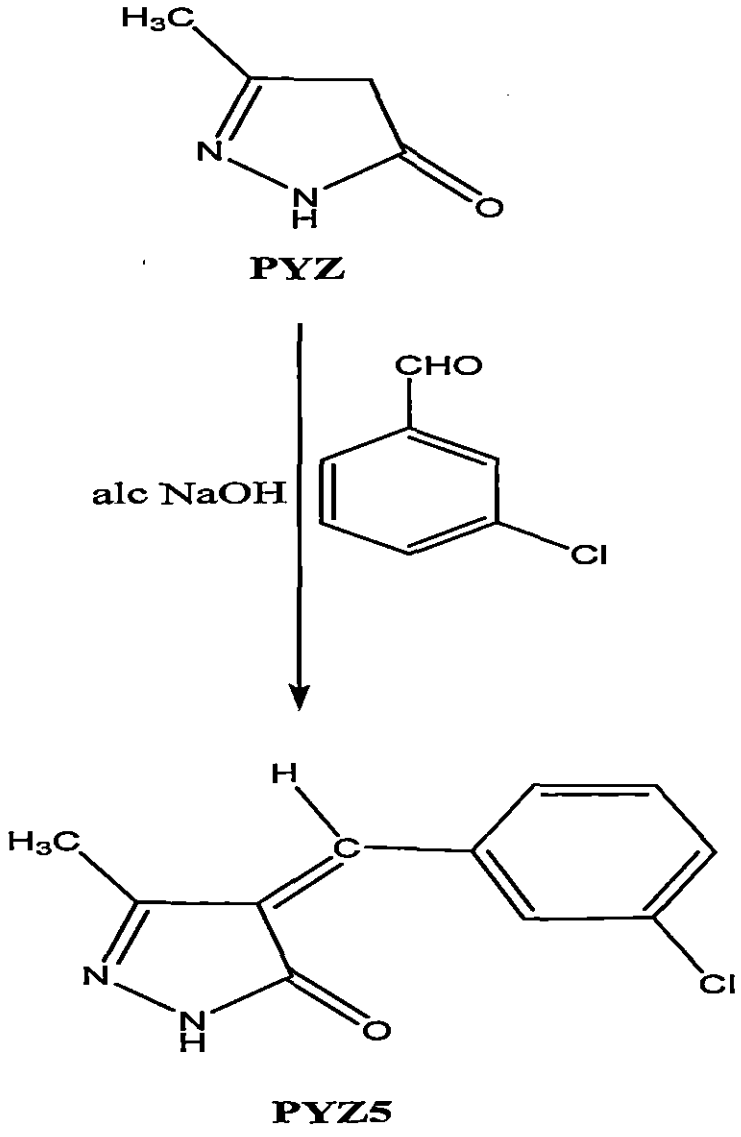
4.2.4. Scheme of synthesis of 4-[3-nitro benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ4)



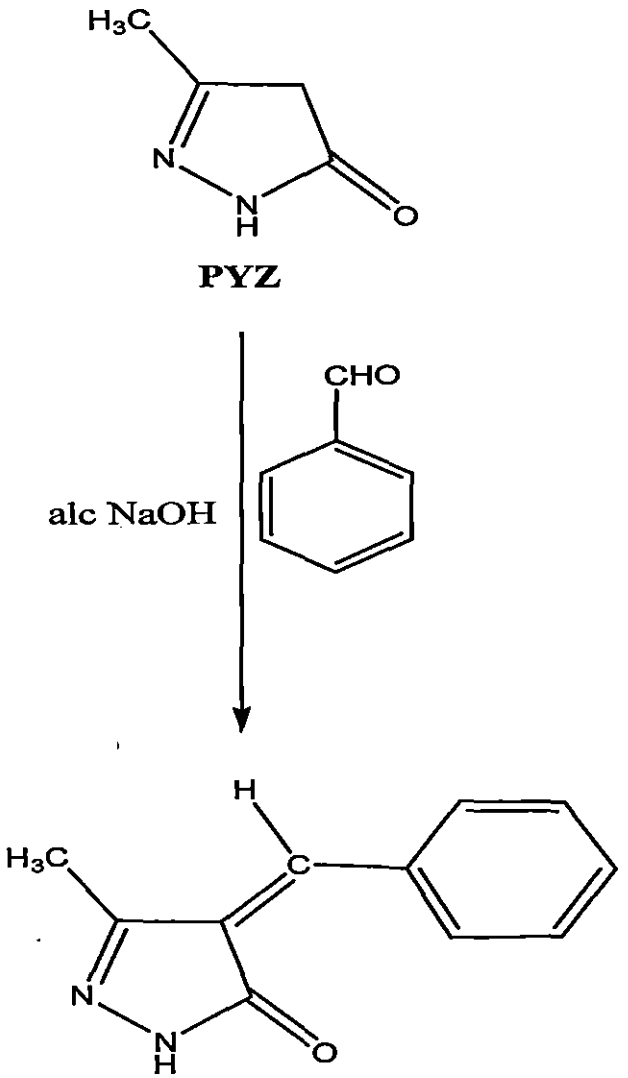
PYZ

PYZ4

4.2.5. Scheme of synthesis of 4-[3-chloro benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ5)



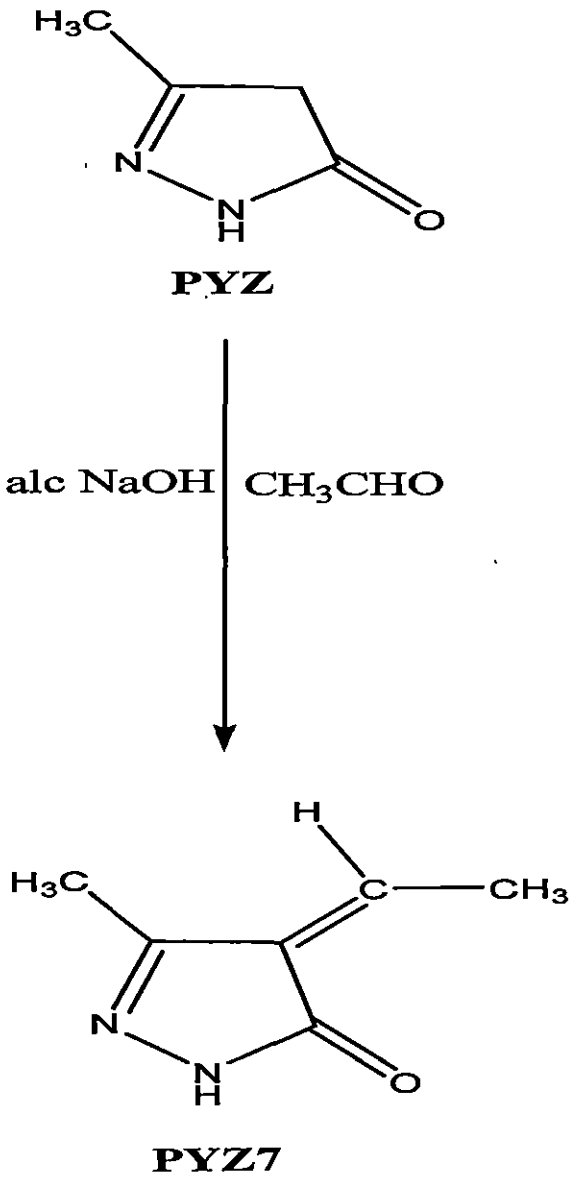
4.2.6. Scheme of synthesis of 4-[benzylidine]-3-methyl pyrazolin-5(4H)-one (PYZ6)



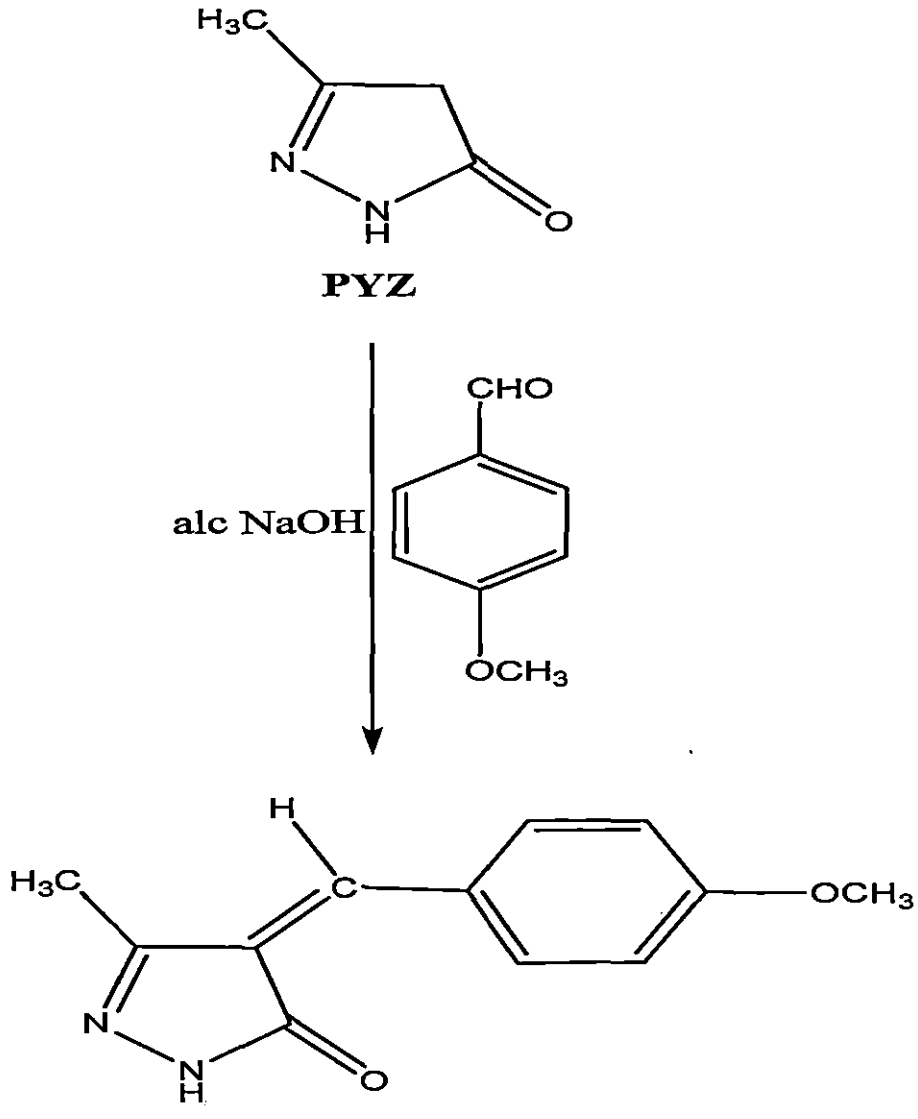
PYZ

PYZ6

4.2.7. Scheme of synthesis of 4-[acetylidine]-3-methyl pyrazolin-5(4H)- one (PYZ7)



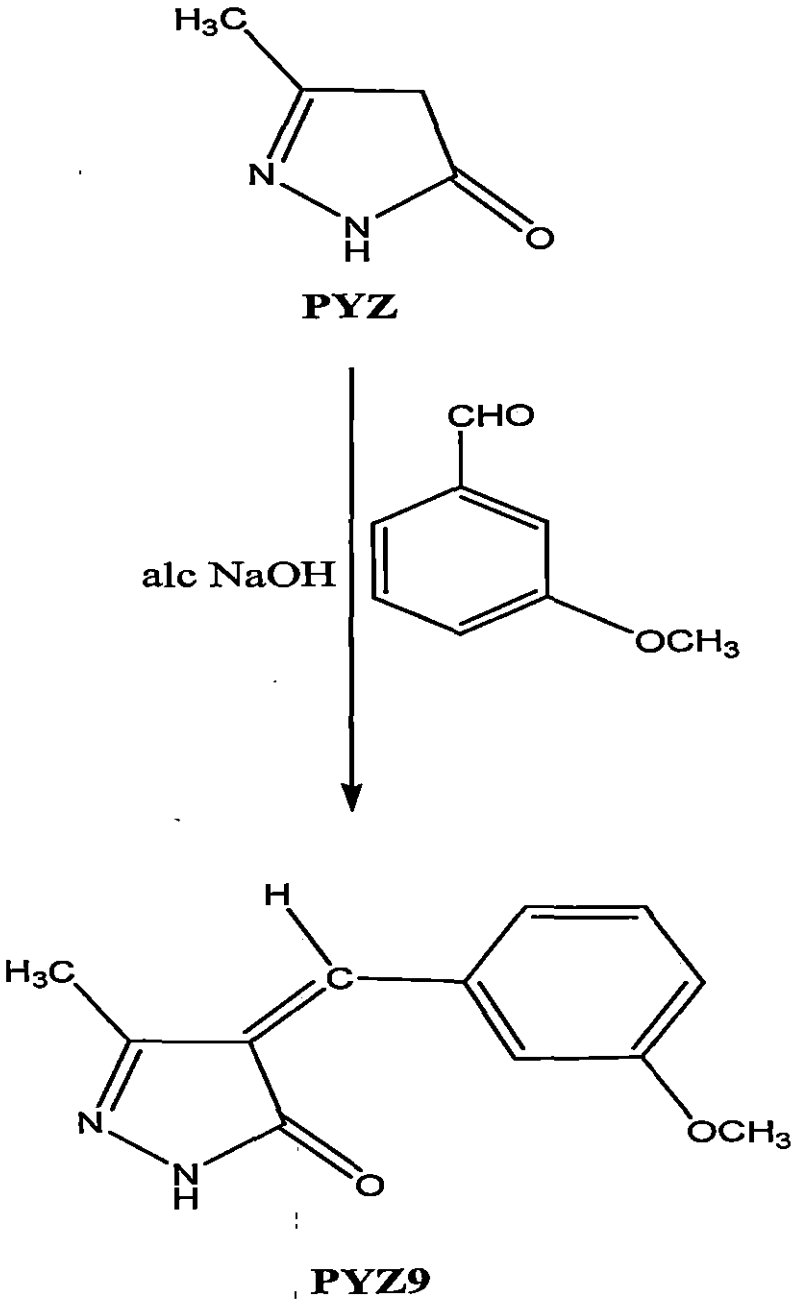
4.2.8. Scheme of synthesis of 4-[4-methoxy benzylidine]-3-methyl pyrazolin-5(4H) -one (PYZ8)



PYZ

PYZ8

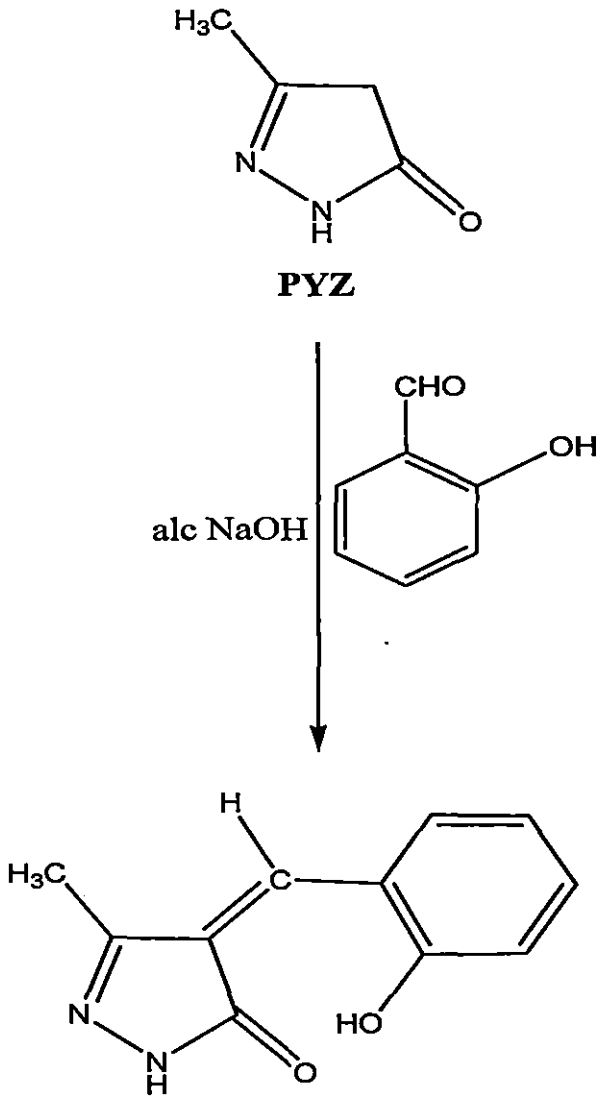
4.2.9. Scheme of synthesis of 4-[3-methoxy benzylidine]-3-methyl pyrazolin-5(4H) -one (PYZ9)



PYZ

PYZ9

4.2.10 .Scheme of synthesis of 4-[2-hydroxy benzylidine]-3-methyl pyrazolin-5(4H) – one (PYZ10)



PYZ

PYZ10

The compound code, various substituted group and I.U.P.A.C nomenclature are summarised in the following table.

Table.4.2.11. Substituted groups in the synthesized compounds

Sl No	Compound code	-R	I.U.P.A.C.name
1	PYZ1	$N(CH_3)_2$	4-[4-N dimethyl amino benzylidine]-3-methyl pyrazolin-5(4H)- one
2	PYZ2	phenyl allyl	4-[phenyl allylidene]-3-methyl pyrazolin-5(4H)- one
3	PYZ3	2-OCH_3 3- OCH_3	4-[2,3- dimethoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
4	PYZ4	3-NO_2	4-[3-nitro benzylidine]-3-methyl pyrazolin-5(4H)- one
5	PYZ5	3-Cl	4-[3-chloro benzylidine]-3-methyl pyrazolin-5(4H)- one
6	PYZ6	-H	4-[benzylidine]-3-methyl pyrazolin-5(4H)- one
7	PYZ7	-CH_3	4-[acetylidine]-3-methyl pyrazolin-5(4H)- one
8	PYZ8	4-OCH_3	4-[4-methoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
9	PYZ9	3-OCH_3	4-[3-methoxy benzylidine]-3-methyl pyrazolin-5(4H)- one
10	PYZ10	2-OH	4-[2-hydroxy benzylidine]-3-methyl pyrazoline-5(4H)- one

4.3. Methods

4.3.1. Synthetic procedure

4.3.1.1. Preparation of 3-methyl pyrazol-5-one (PYZ)

65g (0.5mol) of freshly distilled ethylacetoacetate was taken in a 250ml two necked round bottomed flask fitted with reflux condenser and was stirred with magnetic stirrer. 25g (0.5mol) of hydrazine hydrate in 40ml absolute ethanol was added drop wise from a dropping funnel. The temperature of the reaction mixture was maintained at about 60°C. A crystalline deposit was separated after stirring for 1 hour and then the reaction mixture was cooled in an ice bath to complete the crystallization. The crystals were separated by filtration under suction pump and dried in air. The crystals were recrystallised from alcohol. m.p 222°C, yield 42gm (89%).

4.3.1.2. Synthesis of 4-[4-N,N dimethyl aminobenzylidine]-3-methyl pyrazolin-5-one (PYZ1)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of N, N-dimethyl amino benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Dark Brick Red powder , mp 236-238 °C, %yield 65%, TLC (Ethanol & Water 1:1) R_f 0.91, λmax(nm) ethanol 466 IR (KBr, cm^{-1}): 3147(N-H), 1666(C=O), 1616(C=C), 2829[C–H(CH_3)], 1367 (C=N), 974, 819, 769, 516 (C–H Ar) ^{1}H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 6.80-8.61 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.11 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z 229,* , $[M]^{+\cdot}$, *230* $[M+1]^{+\cdot}$, *231* $[M+2]^+$ Anal Calcd for $C_{13}H_{15}N_3O$: C, 68.12 ;H, 6.55 ;N, 18.34 ;Found: C68.01, ;H6.25, ;N 18.00.

4.3.1.3. Synthesis of 4-[phenyl allylidene]-3-methyl pyrazolin-5- one (PYZ2)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of cinnamaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Dark yellow powder, mp 168-170°C, % yield 72 % , TLC (Ethanol & Water 1:1) R_f 0.85, λmax (nm) ethanol 268 IR (KBr, cm^{-1}): 2953 (N-H str.), 3070 (C-H str., Ar-H), 2833 (C-H str., CH_3), 1600 (C=O str.), 1364 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 212 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 213 Anal Calcd for $C_{13}H_{12}N_2O$: C, 73.58 ;H, 5.66 ;N, 13.20 ;Found: C 73.05, ;H 5.01, ;N 13.21,.

4.3.1.4. Synthesis of 4-[2,3-dimethoxy benzylidine]-3-methyl pyrazolin-5-one (PYZ3)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3, 4 dimethoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

White crystalline powder, mp 208-210°C, % yield 68%, TLC (Ethanol& Water 1:1) R_f 0.92, λmax (nm) ethanol 231 IR (KBr, cm^{-1}): 3196 (N-H str.), 3070 (C-H str., Ar-H), 2937 (C-H str., CH_3), 1585 (C=O str.), 1363 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H,

CH_3). MS : *m/z* 246 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 247.83 Anal Calcd for $C_{13}H_{14}N_2O_3$: C, 63.41; H, 5.69; N, 11.38; Found: C, 62.98; H 5.56, N 11.23.

4.3.1.5. Synthesis of 4-[3-nitro benzylidine]-3-methyl pyrazolin-5-one (PYZ4)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3- nitro benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale yellow coloured powder, mp 180-182°C, % yield 56 % , TLC (Ethanol & Water 1:1) R_f 0.94, λmax (nm) ethanol 253 : IR (KBr, cm^{-1}): 2831 (N-H str.), 3070 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1631 (C=O str.), 1635 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 231 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 232 Anal Calcd for $C_{11}H_9N_3O_3$: C, 57.14; H, 3.89; N, 18.18; Found: C 56.91, ;H 3.65, ;N 18.00,

4.3.1.6. Synthesis of 4-[3-chloro benzylidine]-3-methyl pyrazolin-5-one (PYZ5)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 3- chloro benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale pinkish white powder, mp 216-218°C, % yield 84 % , TLC (Ethanol & Water 1:1) R_f 0.87, λmax (nm) ethanol 300: IR (KBr, cm^{-1}): 3070 (N-H str.), (C-H str., Ar-H), 2715 (C-H str., CH_3), 1697 (C=O str.), 1363 (C=N str.); ^1H NMR ($CDCl_3$): δ 8.25 (s, 1H, NH), 7.31-7.66 (m, 4H, Ar-H), 6.11 (s, 1H, =CH-Ar), 3.29 (s, 6H, $N(CH_3)_2$), 1.93 (s, 3H, CH_3). MS : *m/z* 221 $[M]^+$ Anal Calcd for $C_{11}H_9N_2OCl$: C, 59.73; H, 4.07; N, 12.66; Found: C 59.38; H, 4.57 ;N, 12.98.

4.3.1.7. Synthesis of 4-[benzylidine]-3-methyl pyrazolin-5- one (PYZ6)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale yellow white powder, mp 240-242°C, % yield 61 % , TLC (Ethanol & Water 1:1) R_f 0.89, λmax (nm) ethanol 249: IR (KBr, cm^{-1}): 3176 (N-H str.), 3070 (C-H str., Ar-H), 2831 (C-H str., CH_3), 1604 (C=O str.), 1491 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 18.36 (CH_3), 48.4-48.99 (C3 of pyrazolone), 49.27-49.84 (C4 of pyrazolone), 58.32 (C5), 143 (olefinic C), 126-129 (C Ar). MS : *m/z* 186 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 187, $[M+2]^{+\cdot}$, 188, Anal Calcd for $C_{11}H_{10}N_2O$: C, 70.96; H, 5.37; N, 15.04; Found: C 70.86, ;H 5.65, ;N 15.14,

4.3.1.8. Synthesis of 4-[acetylidine]-3-methyl pyrazolin-5-one (PYZ7)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of acetaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during

which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

white powder, mp 258-260°C, % yield 75 % ,TLC (Ethanol &Water 1:1) R_f 0.88, λmax (nm) ethanol 437: IR (KBr, cm^{-1}): 3352 (N-H str.), 3070 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1610 (C=O str.), 1452 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 48.14 (CH_3), 48.42 (C3 of pyrazolone), 48.76 (C4 of pyrazolone), 48.99 (C5), 49.27(olefinic C), 49.56-49.84 (C6 of pyrazolone). MS : *m/z* 124 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 125, $[M+2]^{+\cdot}$, 126, Anal Calcd for $C_6H_8N_2O$: C, 56.25; H, 6.25; N, 15.05; Found: C 56.04, ;H 6.12, ;N 15.68,

4.3.1.9. Synthesis of 4-[4-methoxy benzylidine]-3-methyl pyrazolin-5- one (PYZ8)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 4-methoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Yellow powder, mp 258-260°C, % yield 66 %, TLC (Ethanol &Water 1:1) R_f 0.96, λmax (nm) ethanol 284: IR (KBr, cm^{-1}): 3070 (N-H str.), 2883 (C-H str., Ar-H), 2713 (C-H str., CH_3), 1680 (C=O str.), 1510 (C=N str.). ^{13}C NMR ($CDCl_3$): δ 10.63 (CH_3), 48.5-48.71 (C3 of pyrazolone), 49.00-49.57 (C4 of pyrazolone), 33.41 (C5), 128-133 (olefinic C), 55.62-56.20 (OMe), 144-145 (C Ar). MS : *m/z* 216 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 217 Anal Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.55; N, 12.96; Found: C 66.10, ;H 5.14, ;N 12.46.

4.3.1.10. Synthesis of 4-[3-methoxy benzylidine]-3-methyl pyrazolin-5-one (PYZ9)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02

moles of 3- methoxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

Pale orange powder, mp 170-172°C, % yield 57 % , TLC (Ethanol & Water 1:1) R_f 0.83, λmax (nm) ethanol 314: IR (KBr, cm^{-1}): 3070 (N-H str.), 2883 (C-H str., Ar-H), 2715 (C-H str., CH_3), 1600 (C=O str.), 1489(C=N str.); ^{13}C NMR ($CDCl_3$): δ 10.36 (CH_3), 48.15-48.72 (C3 of pyrazolone), 49.00-49.57 (C4 of pyrazolone), 34.17 (C5), 142.83-145.16 (olefinic C), 55.51-55.60 (OMe), 112-130 (C Ar). MS : *m/z* 216 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 217, $[M+2]^{+\cdot}$, 218. Anal Calcd for $C_{12}H_{12}N_2O_2$: C, 66.65; H, 5.55; N, 12.96; Found: C 66.47, ;H 5.68, ;N 12.86,

4.3.1.11. Synthesis of 4-[2-hydroxy benzylidine]-3-methyl pyrazolin-5-one (PYZ10)

1.2g (0.02 mole) of pure 3-methyl pyrazol-5-one was taken in a 100 ml flat bottom flask, 50 ml of freshly prepared 20% solution of alcoholic sodium hydroxide was added. The mixture was stirred with magnetic stirrer for 30 minutes at room temperature. Then 0.02 moles of 2-hydroxy benzaldehyde was added slowly to the reaction mixture. The mixture was stirred magnetically for 8 hours; the reaction was monitored by TLC on silica gel using ethanol: water (1:1). Then the mixture was poured into crushed ice and neutralized with dilute hydrochloric acid. It was then allowed to stand for 1 hr at room temperature during which the crystals were separated. The crystals were filtered by using Buchner funnel and dried in air. The crude product was recrystallised from hot alcohol and dried in vacuum desiccators.

orange powder, mp 212-214°C, % yield 79 %, TLC (Ethanol & Water (1:1) R_f 0.82, λmax (nm) ethanol 249: IR (KBr, cm^{-1}): 3070 (N-H str.), 2831 (C-H str., Ar-H), 2362 (C-H str., CH_3), 1511 (C=O str.), 1604 (C=N str.); ^{13}C NMR ($CDCl_3$): δ 10.84 (CH_3), 48.15-48.71 (C3 of pyrazolone), 49.00-49.48 (C4 of pyrazolone), 28.90 (C5), 105.79 (olefinic C), 115.60-130.83 (C Ar). MS : *m/z* 202 $[M]^{+\cdot}$, $[M+1]^{+\cdot}$, 203, $[M+2]^{+\cdot}$, 204 Anal Calcd for $C_{11}H_{10}N_2O_2$: C, 65.34; H, 4.95; N, 13.85; Found: C 64.90; H, 4.48; N, 13.44.

4.4. Pharmacological studies

4.4.1. Animals

Swiss albino mice (CF-1 strain or Swiss (18-25 g) and rats (Sprague-Dawley, 100-150 g) were used as an experimental animals. They were housed under standard conditions of temperature (24±1°C), relative humidity (65±10%) and 12 light/dark cycle environment. During the study period, guidelines of CPCSEA (IAEC) were followed for the maintenance of animals and the experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) No: HPI/09/60/IAEC/0075.

4.4.2. Chemicals and drugs

Isoproterenol hydrochloride (ISO) was purchased from (Sigma Chemical Co. St. Louis, MO, USA). Sodium chloride, magnesium chloride, adenosine triphosphate and trichloroacetic acid (TCA) were obtained from (Himedia Laboratories Private Ltd. Mumbai, India). Nitroblue tetrazolium (NBT), phenazine methosulphate and nicotinamide adenine dinucleotide (NAD) were purchased from Sisco Research Laboratories Ltd. Mumbai, India. Thiobarbituric acid (TBA), 2, 4-dinitrophenyl hydrazine (DNPH), 1, 1, 3, 3-tetramethoxy propane (TMP), 5, 5 dithiobis 2-nitrobenzoic acid (DTNB) and 4-HNE (4- Hydroxy- 2- Nonenal) were obtained from Sigma Chemicals, USA. The other chemicals used in this study were of analytical grade unless otherwise stated.

4.4.3. Test compounds

Pyrazolone derivatives investigated in the present study were synthesized and characterized in Department of Pharmaceutical Chemistry, Himalayan Pharmacy Institute, Sikkim, India.

4.4.4. Biochemical diagnostic Kits

The diagnostics kit of Total Cholesterol, Total Protein, HDL, VLDL, LDL, TG, AST (SGOT), and ALT (SGPT) and were purchased from Span Diagnostics Ltd., Surat, India.

4.4.5. Acute toxicity study (Determination of LD_{50})

It is conducted to determine the median lethal dose (LD_{50}) i.e. the dose which will kill 50% of the animals of a particular species. In addition, such studies may also indicate the probable target organ of the chemical and its specific toxic effect. It provides guidance on the doses to be used in more prolonged studies.

Up and Down Method by Organization for Economic Co-operation and Development (OECD) guidelines for the testing of chemicals are periodically reviewed in the light of scientific progress or changing assessment practices. The concept of up and down testing approach was first described by Dixon and Mood. In 1985; Bruce proposed to use Up and Down procedure (UDP) for the determination of acute toxicity of chemicals. There exist several variations of the Up and Down experimental design for estimating an LD_{50}.The method permits estimation of LD_{50} with a confidence interval and the result allow a substance to be ranked and classified according to the Globally Harmonised system for the classification of chemicals, which cause acute toxicity. The method is easiest to apply to materials that produce death within one or two days. The method would not be practical to use when considerably delayed death (five days or more) can be expected. A limit test can be used efficiently to identify chemicals that are likely to have low toxicity.

The limit test is a sequential test that uses a maximum of 5 animals. A test dose of up to 2000, or exceptionally 5000mg/kg may be used. The procedure for testing at 2000 and 5000mg/kg are slightly different. The main test consists of single ordered dose progression in which animals are dosed, one at a time, at 48 hour intervals. The first animal receives a dose a step below the level of the best estimate of the LD_{50}. If the animal survives, the dose for the next animal is increased to a factor of 3/2 of original dose; if it dies, the dose for the next animal is decreased by a similar dose progression. Each animal should be observed carefully for up to 48 hours before making decision on whether and how much to dose the next animal. Dosing is stopped when one of these criteria is satisfied, at which time an estimate of LD_{50} and a confidence interval are calculated for the test based on the status of all animals at termination.

4.4.6. Experimental Animals

Fifty (50) adult male albino rats (10 groups, 5 in each) 9 - 10 weeks old, weighing between 84 and 200 g were procured from the laboratory animal house of the Himalayan Pharmacy Institute. The rats were kept in metal cage, fed with commercial standard growers feed and water *ad libitum* and maintained under standard laboratory condition prior to the procedure. During the study period, guidelines of CPCSEA (IAEC) were followed for the maintenance of animals and the experimental protocol was approved by Institutional Animals Ethics Committee (IAEC) No: HPI/09/60/IAEC/0075.

4.4.7. Method of Acute toxicity study

The limit dose test, up and down procedure as revised by Dixon (1965, 1991) was used to evaluate the acute oral toxicity of Pyrazolone derivatives in adult male rats. Five (5) adult male rats were randomly selected for the experiment. They were marked and housed individually in cages in the laboratory for 7 days to allow for acclimatization to the laboratory conditions. The rats were fasted overnight but allowed free access to water prior to dosing on each occasion. A rat from Group I was picked, weighed and dosed orally with a limit dose 2000 mg/kg body weight of the freshly prepared pyrazolone derivative (PYZ1). Another animal from the same group was given the same dose of the pyrazolone derivative (PYZ1) until all the animals in the group were fed with the same dose of PYZ1.

Each animal was observed each time for instant death and then watched for the successive 24 h for the short-term outcome and finally for the next 24 hours for any sign toxic effects. Similarly all the groups were tested with rest of the pyrazolone derivatives. But after 48 hours, there was no death on rats due to any derivative. Hence the same procedure was repeated with 5000 mg/kg b.w. of all the pyrazolone derivatives. All the animals were observed for next 48 hours for any sign of toxic effects.

4.4.9. Experimental Procedure for Cardio Protective Study by Chemical Methods

Seventy two male rats were completely randomized into twelve groups of six animals in each group. Group 1: normal control (distilled water p.o.), Group 2: ISO-treated (5.5 mg and 8.5 mg/ kg, s.c.) at an interval of 24 h for two days. Groups 3 to 12 were administered with 10 mg/kg body weight/ day p.o. of pyrazolone derivatives (PYZ1-PYZ10) for 5 days followed by ISO treatment at an interval of 24 h for two days. 24 hours after the second injection of ISO, the rats were sacrificed by ether anaesthetization and the heart was dissected out. The neck area was quickly cleared of fur to expose the jugular vein. The vein, after being slightly displaced, was sharply cut with sterile surgical blade and an aliquot (5 ml) of the blood was collected and centrifuged at 10000 rpm for 5 minutes. The serum was carefully aspirated with a Pasteur pipette into sample bottles for biochemical analysis.

Blood (2ml) was aspirated from the left ventricle, collected in a heparinised vial, centrifuged at 1000 rpm for 30 min and the plasma stored at -20 °C for estimation of plasma LDH, total cholesterol, triglycerides, total protein and creatinine kinase using the

ready to use kits supplied by Span Diagnostics Ltd., Kolkata, India. The heart was removed and stored in liquid nitrogen for biochemical estimation. The heart was washed with ice-cold saline, and the homogenate prepared in 0.1M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and the supernatant was used for the assay of superoxide dismutase (SOD) and catalase (CAT).

4.4.9.1. Estimation of reduced glutathione (GSH)

Reduced glutathione (GSH) was determined by the method of (Ellman, 1959). To 0.1ml of tissue homogenate 2.4 ml of 0.02M EDTA solution was added and kept on ice bath for 10 min. Then 2 ml of distilled water and 0.5 ml of 50% TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 rpm for 15 min. 1 ml of supernatant was taken and 2 ml of Tris-HCl buffer was added. Then 0.05 ml of 5, 5 dithiobis 2-nitrobenzoic acid (DTNB) solution (Ellman's reagent) was added and vortexed thoroughly. The absorbance was read within 2-3min after the addition of DTNB at 412 nm against a reagent blank. The Absorbance values were compared with standard curve, generated from known GSH. The amount of GSH was expressed as µg/mg wet tissue.

4.4.9.2. Estimation of superoxide dismutase (SOD)

SOD activity was estimated by the method described by (Kakkar et al., 1984). The reaction mixture contains 0.1ml of supernatant, 1.2ml of sodium pyrophosphate buffer(pH 8.3, 0.052M), 0.1ml of phenazine methosulphate (186µM), 0.3ml of nitro blue tetrazolium, 300µM, 0.2ml of NADH(Nicotinamide adenine dinucleotide reduced disodium salt, 750µM). The reaction was started by addition of NADH. After incubation at 30^0C for 90 sec, the reaction was stopped by the addition of 0.1ml of glacial acetic acid. The reaction mixture was stirred vigorously with 4.0ml of n-butanol. The mixture was allowed to stand for 10 min, centrifuged and the butanol layer was separated. The colour intensity of the chromogen in the butanol was measured at 560nm by spectrophotometrically and the concentration of SOD was expressed as units/mg of protein.

4.4.9. 3. Estimation of catalase (CAT)

Catalase activity was assayed by the method of Beers and Sizer 1952. The catalase activity was assayed by adding 0.1ml of homogenates to 1.4ml of freshly prepared 13.2 mM H_2O_2 IN 0.05 M K_2HPO_4 (pH 7.0)(0.15 ml of 30% H_2O_2 per 100 ml). Procedures in which the H_2O_2 was added last to initiate the reaction caused a loss of enzyme activity. The solution was mixed, and a loss of absorbance was determined at 240 nm by using UV-spectrophotometer for 1 to 3 min. The units of catalase were calculated as IU/min/mg of tissue.

4.4.9. 5. Estimation of total protein

Protein concentration was estimated according to the method of Lowery *et al.*, 1951. Standard dilution of bovine serum albumin of 25, 50, 75 and 100µg/ml were prepared and appropriate serial dilution of the serum sample to be measured were also prepared. 1.0ml of each of the above was placed in to separate tubes. 100µl of 72% w/v trichloroacetic acid was added to each tube. The tubes were centrifuged for 15 minutes at 3000 rpm and the supernatant were discarded. Then 1ml of water was added to each tube to dissolve the pellet. 1.0ml of water was also added to a new tube (including blank), vortexed and allowed to set for 10min. After that, 500µl Folin-ciocalteu was added to each tube, vortexed and allowed to set for 30 minutes and read spectrophotometrically at 750 nm. Data were expressed as mg/ml.

4.4.9. 6. Estimation of lactate dehydrogenase (LDH)

Lactate dehydrogenase activity was estimated in serum by the method of Tietz, 1977. The method uses NADH and pyruvate as substrates. To 10µL of the sample, 1000µl of the reagent containing substrate was added and the coloured complex was measured spectrophotometrically at 340nm. Data were expressed as IU/L.

4.4.9. 7. Estimation of total cholesterol

Total cholesterol was estimated by the method of Wybenga *et al.*, 1970. To 10µL of the serum sample, 1000µL of the reagent containing ferric perchlorate, ethyl acetate and sulphuric acid was added incubated for 10 min at 37^0C to form a colored complex which was measured spectrophotometric ally at 540nm. Data were expressed as mg/dL.

4.4.9.8. Estimation of total triglycerides

Total triglycerides were estimated by the method of Trinder, 1969. To 10µl of the serum sample, 1000µL of the working reagent containing the enzyme was added and incubated for 10 min to form a coloured complex which was measured spectrophotometrically at 505 nm. Data were expressed as mg/dL.

4.4.9.9. Estimation of creatine kinase

Creatine kinase was estimated by the method of Szasz, 1978. To 50 µL of the serum sample, 1050 µL of the working reagent containing the antibody was added. The rate of NADH formation was measured at 340 nm. CK activity was expressed as U/L.

4.4.9.10. Estimation of SGPT and SGOT

Serum transaminase (SGPT and SGOT) was estimated by the method of Reitman and Frankel, 1957. Each Substrate (0.5 ml) [either α L-alanine (200mM) or L-aspartate (200mM)] with 2mM α-ketoglutarate] was incubated for 5 min at 37°C in a water bath. Serum (0.1 ml) was then added and the volume was adjusted to 1ml with sodium phosphate buffer (pH 7.4). The reaction mixture was incubated for exactly 30 min and 60 min for SGPT and SGOT, respectively. Then to the reaction mixture, 0.5 ml 2, 4- dinitro phenyl hydrazine 1 mM was added and left for another 30 min at room temperature. Finally the colour developed by addition of 5ml NaOH (0.4N) and the colour complex formed was read at 505 nm. Data were expressed as U/L.

4.4.9. 11. Estimation of ALT

Serum alkaline phosphatase (ALT) was determined by the method of Kind and King, 1954. The buffered substrate pH 10 (reagent1) was prepared by the following method: 1.09 g of disodium phenyl phosphate was dissolved in water and the volume was made up to 500 ml. It was heated up to the point of boiling and cooled. Little amount of chloroform was added and kept in refrigerator. This was designated as solution I.

8.18 g of anhydrous sodium carbonate and 1.69 mg of sodium bicarbonate were dissolved in water and volume made up to 500ml .This was designated as solution II.

Finally equal volume of solution I and II were mixed and pH adjusted to 10 to get reagent 1. Four test tubes were taken for control, blank, standard and test samples. In each of the test tube, 0.5ml of reagent 1 was taken. In blank test tube 1.55ml distilled water was added and in rest of the tubes 1.5 ml of water was added, shaken well for

uniform mixing and incubated for 3 min at 37°C. Then 0.05 ml serum was added to the experimental test tube and 0.05 ml of phenol standard 10% mg (reagent 3) was added to the standard test tube. These were mixed well and incubated for 15 min at 37°C.Then 1ml p-Nitrophenyl phosphate 10 µM (reagent-2) was added to each of the test tubes. Lastly, 0.05 ml of serum was added to the control tube. The content of the tubes were mixed well and optical density of the samples were read at 510nm. Data were expressed as U/L.

4.4.10. *In vitro* antioxidant activity of pyrazolone derivatives

4.4.10.1. *In vitro* myocardial ischemic-reperfusion injury by using Langendorff's apparatus

Seventy two male rats were completely randomized into twelve groups of six animals in each group. Group 1: normal control (K-H buffer only), Group 2: Ischemia induced control. Groups 3 to 12 were administered with 10 mg/kg body weight/ subjected to 5 min flow + 9 min. no-flow + 12 min. reperfusion. After 48 hours the rats were anaesthetized with ether, the chest opened and the heart along with one cm of ascending aorta attached was quickly removed and dipped in ice-cold saline. The hearts were then mounted on Langendorff's apparatus and perfused with Krebs's Hensleit(K-H) buffer (NaCl (120 mM), $NaHCO_3$(25 mM), $MgSO_4$(1.2 mM), KH_2PO_4 (1.2 mM), $CaCl_2$(1.2 mM), and glucose (11 mM) at a constant pressure of 60-70mm Hg at 37 °C, and aerated with a mixture of O_2 (95%) and CO_2 (5%). Following an initial period of 5 min of stabilization, the flow was stopped for 9 min (ischemia) followed by perfusion with K-H buffer for 12 minutes (reperfusion). Then the heart was removed from the apparatus and subjected for the biochemical estimation of TBARS and 4-HNE.

4.4.10.2. Estimation of Thiobarbituric acid reactive substances (TBARS)

TBARS activity in the myocardium was determined by a modified version of the method described by Okhawa *et.al.* 1979. Hearts were homogenized in 10% trichloroacetic acid at 4°C. 0.2 ml homogenate was pippeted in to a test tube followed by the addition of 0.2 ml of 8.1% sodiumdodecyl sulphate (SDS), 1.5 ml of 20% acetic acid (pH 3.5) and 1.5 ml of 0.8% TBA. All tubes were boiled for 60min at 90°C and then cooled on ice. 1.0 ml double distilled water and 5ml of n-Butanol: pyridine (15:1v/v) mixture was added to the tubes and centrifuged at 4000 rpm for 10 minutes. The absorbance of developed colour

in organic layer was measured at 532 nm. TBARS activity was determined, from the standard curve of TBA adduct formation when various concentration of commercially available 1, 1, 3, 3-tetramethoxypropane. The concentration of malonaldehyde (MDA) was expressed in nM.

4.4.10.3. Estimation of 4- Hydroxy- 2- Nonenal (4-HNE) (Esterbauer et al., 1941)

The heart homogenate was prepared as per the procedure mentioned above. 2ml of filtrate was taken in a 13x100mm glass tube with cap. 1ml of DNPH was added to all the tubes containing heart homogenate, mixed thoroughly and set aside for 1h to react with 4-HNE. The formed adduct of 4-HNE and DNPH was extracted by hexane, which was evaporated under argon at 40°C. After cooling, 2ml of methanol was added to all the samples and the absorbance was measured at 350 nm in the spectrophotometer. The quantity of 4-HNE was calculated by linear regression analysis. The concentration of 4-HNE present in myocardial tissues was expressed in nM.

4.4.10. 4. Analgesic activity study

It was measured (D'Amour and Smith, 1941). The tips of tail of animals were individually placed on radiant heat source at constant temperature 55±1°C and the reaction of the animals, like flicking of the tail was noted. Male albino rats of 12 groups (6 in each group) were taken for study. First group was kept as control, second as standard and rest as test groups for different synthesized compounds. Test drugs **1-10** at a dose of 100 mg/kg p.o were administered. Diclofenac 10 mg/kg p.o was used as standard drug. The tail-flick latency was assessed by analgesiometer (Techno, India). Basal reaction time to radiant heat was taken for rat. The rat which responded within 2-3 seconds only considered for studies. After administration, the reaction time was noted at 1h, 2h, 3h and 4 h time interval of the above mentioned groups. The cut-off reaction time was fixed at 10 sec to avoid tissue damage. The observations were made and data obtained were statistically analyzed and results are summarized in table 4.9.

4.4.10.5. Anti-inflammatory activity study

It was studied by inducing paw edema (Winter et al., 1962). Male albino rats of 12 groups (6 in each group) were taken for study. Group one was kept as control, group two was treated with standard drug aspirin 100mg/kg p.o and the remaining groups were administered with test drugs **(1-10)** at a dose of 100 mg/kg p.o. A mark was made on left

paws just beyond tibio-tarsal junction (knee joint) of each animal of all groups, so that each time the paw was dipped in the water column of digital paw edema meter (520-R, IITC Life Science, USA) up to the fixed mark made on left paws to ensure constant paw volume. Carrageenan (1%, 0.1 mL) (Sigma-Aldrich, Milan, Italy) was injected subcutaneously into the plantar surface of the rat hind paw 1hr after the oral administration of the test compound. After the administration of carrageenan solution, the paw volume of control, standard and test groups were noted at 1h, 2h, 3h and 4h time interval. The percentage of inhibition was calculated by applying New bould formula (New bould, 1963).

4.4.10. 6.Antipyretic activity study

This activity was performed on rabbits of either sex according to the reported method described by Lu et al. (2004). An aliquot of 3ml/kg of 10% Brewer's yeast suspension was subcutaneously injected into the rabbit back. The temperature was monitored by means of thermometer inserted at least 10 cm into the rectum and recorded by calibrated thermometers. An animal was excluded from the study if the baseline temperature was not within the range of 39.2 °C to 39.8°C. After 5 hour, animals showing at least an increase of 1°C of rectal temperature were selected for the experiment. The animals were administered with standard paracetamol (100mg/kg p.o) and all pyrazolone derivatives (400mg/kg) orally. The rectal temperature was measured at 0, 1, 2 and 3 hour after treatment.

4.4.11. Histopathological studies

Histopathological processing of liver and heart was carried out after a proper wash with cold isotonic saline solution and slices were prepared. The liver and heart sections were fixed in10% (v/v) formalin solution. After dehydration pieces of liver and heart were embedded in paraffin wax, cut into 4–6 mm thick sections, and stained using haematoxylin and eosin. They were then examined under a microscope for histo architecture changes. The following morphological criteria (Zingarelli *et al.*, 1998) were used to assess the histopathological damage: interstitial edema and focal necrosis; diffuse myocardial cell swelling and necrosis; necrosis with the presence of contraction bands, neutrophil infiltration and the capillaries were compressed; and wide spread necrosis

with the presence of contraction bands, neutrophil infiltration, capillaries compressing and hemorrhage. The observed histopathological changes were shown in the figure 4.60.

4.4.12. Statistical analysis

The data were expressed as mean ± standard error of mean (S.E.M.). The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnett's test with the help of GraphPad Prism 3 statistical software. P-values <0.05 were considered as significant.

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