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Research Article

SYNTHESIS, CHARACTERIZATION AND IN VITRO ANTI-INFLAMMATORY ACTIVITY OF NOVEL PYRAZOLINE DERIVATIVES

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ABSTRACT:

The objective of the present investigation to synthesize novel pyrazoline derivatives and assess the anti-inflammatory activity of the compounds. The pyrazoline derivatives (XI-XV) were synthesized in four sequential steps involving synthesis of dihyropyrimidine nucleus, followed by hydrazination of the carboethoxy group. The various chalcone molecules were prepared in the third step via claisen Schmidt condensation and finally the chalcone were cyclo-condensed to yield the pyrimidine-pyrazolinex compounds (XI-XV). The anti-inflammatory action of the synthesized compounds was determined using inhibition of albumin denaturation and inhibition of protease activity models. The products were soluble in chloroform and were obtained in 61-67% yield. In the IR spectrum, stretching bands of C-H (300-2840 cm-1), C=S (1818-1705 cm-1), C-C (1300-800 cm-1), C=N (1612-1602 cm-1) and C-S (950-850 cm-1) were observed in each compound. Bending vibration of N-H (1650-1580 cm-1) and C-N (1250-1020 cm-1) were also visible. The 1H-NMR spectra revealed protons of amine, methyl, acetyl and aromatic protons along with methylene of pyrazoline. All the compounds exhibited dose dependent inhibition of albumin denaturation with 6d having the highest capacity to cause the inhibition (67.2 %) at the concentration of 500µg/mL. The antiprotease action was also dose dependent and 6d at 500µg/mL was able to inhibit (48.3 %) of protease activity. The type of substitution on the terminal ring was found to affect the anti-inflammatory action in both the models.

Keywords: Pyrazoline, anti-inflammatory, anti-protease, albumin, in vitro

INTRODUCTION

Inflammation is normal and necessary protective response to the harmful stimuli such as infectious agents, antigen-antibody reactions, thermal, chemical, physical agents, and ischemia [1]. Rheumatoid arthritis and degenerative arthritis are the major inflammatory diseases affecting people worldwide [2]. Heterocyclic compounds have significant importance in the field of medicinal chemistry due to their diverse range of biological potentials [3]. Pyrazoline is a five-membered heterocyclic scaffold that has two adjacent nitrogen atoms inside the ring, which makes it basic in nature [4]. The pyrazoline scaffold plays a vital role in heterocyclic chemistry as a fundamental building block in both organic and medicinal chemistry. Many pharmacologically active molecules contain pyrazolines, which exhibit a wide range of potential pharmacological activities, such as sulfinpyrazone (uricosuric), oxyphenbutazone (anti-inflammatory), as well as aminophenazone, phenazone, and methampyrone (analgesic and antipyretic) [5-11]. By changing and modifying their structures, pyrazoline derivatives have undergone a great deal of variety. Likewise, it will help researchers design and develop new drugs that will work better and safer. Previously attempts have been made to prepare a few 2-pyrazoline derivatives as potential anti-inflammatory agents acting by cyclooxygenase inhibition. Pyrimidine based compounds have been long researched for various pharmaocological potentials. Considering the vast pharmacological potential of these two scaffolds, it was hypothesized that linking of these two might be able to render potential compounds for management of inflammation.

Material and Methods

All the synthesized compounds were characterized for melting point, solubility, yield and elucidation of the structure [12]. The structure elucidation was performed by spectroscopic analysis (NMR, Mass and IR). A few novel pyrimidine containing pyrazoline derivatives were synthesized using the steps reported by Mishra et al [13], Padhy et al [14] and Tok et al [15] (Scheme 1).

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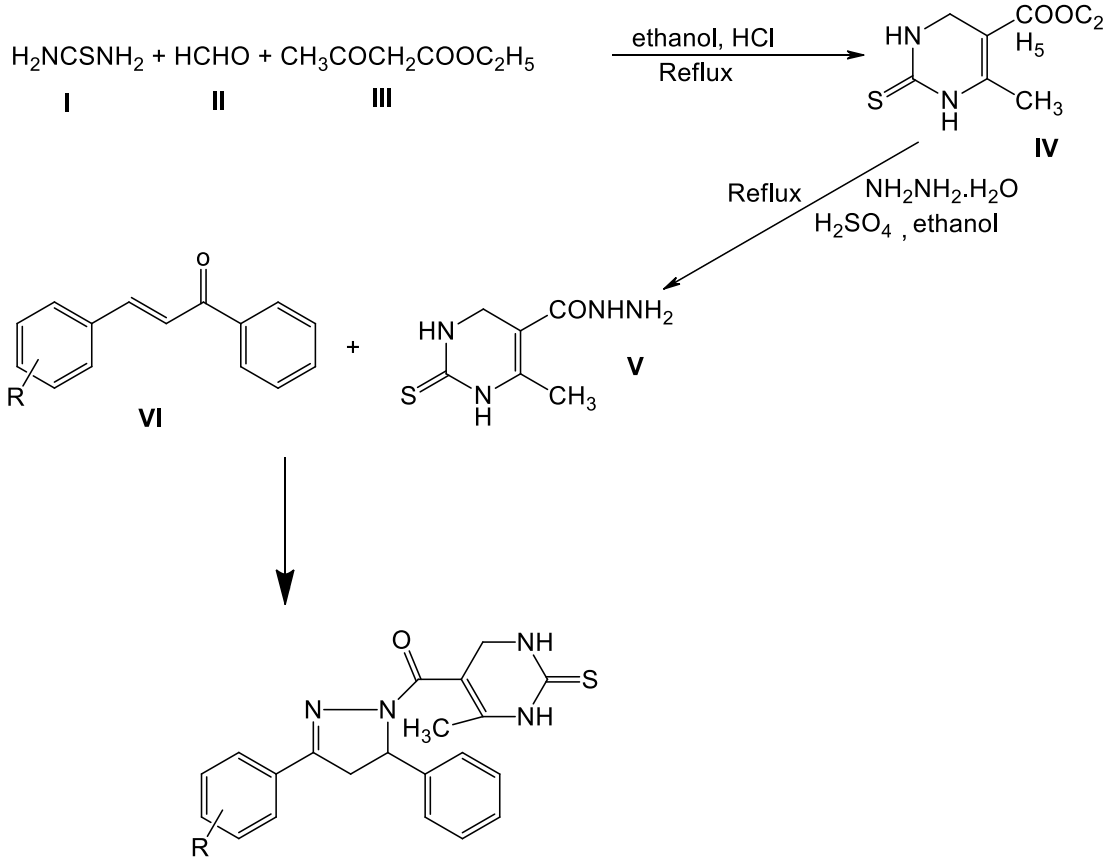

Scheme 1. Synthetic pathway

The synthesis has been achieved into 4 steps involving synthesis of dihydropyrimidine in the first step, followed by is hydrazination in the second step; the third step involved the synthesis of chalcone derivatives and finally cyclization to form pyrazoline in the fourth step.

Step 1. Synthesis of ethyl 6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate

The synthesis of 3, 4-dihydropyrimidine was performed by using benzaldehyde, thiourea and ethylacetoacetate according to method of Biginelli condensation [16]. 0.2 moles of thiourea (**I)**, 0.75 moles of ethylacetoacetate (**III)** and 0.2 moles of benzaldehyde (**II)** were mixed in 18 mL of ethanol. Catalytic amount of concentrated hydrochloric acid (5 drops) was added to the mixture and the mixture was refluxed until the completion of the reaction (approximately 4 hours). On cooling, a solid separated which was filtered and recrystallized using ethanol to give the product **IV.** Completion of the reaction was monitored by TLC. This method is an acid catalyzed multi-component reaction of an aldehyde, a β-keto ester and thiourea in presence of ethanol with a catalytic amount of concentrated hydrochloric acid at reflux temperature.

Step 2. Synthesis of 6-methyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbohydrazide

The hydrazide derivative of the dihydropyrimidine-2-thione was synthesized by the treatment of the product of step 1 by hydrazine hydrate in presence of ethanol with catalytic amount of concentrated sulfuric acid. To 0.1 mole of the product **IV** in 20 mL ethanol, 0.1 mole of hydrazine hydrate was added. To the mixture, catalytic amount of concentrated sulfuric acid was added. The mixture was refluxed until the completion of the reaction (approximately 2 hours). On cooling, a solid separated, which was recrystallized from ethanol to give the product **V.** The reaction proceeds by the way of ammonolysis of esters. The mechanism involves nucleophilic attack on the electron deficient carbon atom of the ethoxy group, $-OC_2H_5$, by the base, hydrazine hydrate. The alkoxy group gets replaced by $–NHNH_2$ to yield the product, hydrazide derivative.

Step 3. Synthesis of chalcone derivatives

A few chalcone compounds were prepared using acetophenone and substituted benzaldehydes in acetic acid and catalytic amount of sulfuric acid using Claisen-Schmidt condensation. To a solution of corresponding aldehyde (0.0054 mol) and acetophenone (0.0054 mol) in acetic acid (10 mL) was added conc. H_2SO_4 (0.3 mL) and stirred at 10-20°C until the completion of reaction (≈72 h). The precipitate was filtered off and recrystallized from acetic acid. The Claisen-Schmidt condensation for C-C bond formation in the synthesis of title compounds is usually carried out in presence of strong bases in alcoholic solution. The first step of the base catalyzed condensation involves the nucleophilic addition of the carbanion derived from the acetophenone to the carbonyl-carbon of the aromatic aldehyde. Dehydration of the hydroxyketone to form the conjugated unsaturated carbonyl compound occurs spontaneously.

Step 4. Synthesis of pyrazoline molecules (XI-XV)

The pyrazoline compounds were prepared by cyclization reaction of the carbohydrazide pyrimidine with the prepared chalcone compounds. To a solution of 0.001 mol chalcone derivative in 15 mL of acetic acid was added 0.002 mol of the carohydrazide pyrimidine and refluxed for 6 hours. The reaction mixture was monitored by thin layer chromatography, then poured onto ice, washed with distilled water, filtered and crystallized from ethanol.

Anti-inflammatory activity

The synthesized molecules were individually dissolved in dimethyl sulfoxide (DMSO) and appropriately diluted to prepare solutions of 100, 200, 300, 400 and 500 µg/mL concentration. Briefly, 10 mg of the synthesized molecule was dissolved in 10 mL DMSO to obtain stock solution of 1 mg/mL. From the stock solution 0.5, 1.0, 1.5, 2.0 and 2.5 mL were pipetted out in separate volumetric flasks and the volume of each flask was made upto 5 mL using DMSO resulting in solutions of 100, 200, 300, 400 and 500 µg/mL concentration.

Inhibition of albumin denaturation

A solution of PBS was prepared by dissolving an accurately weighed quantity of 8 g NaCl, 0.2 g KCl, 1.44 g disodium hydrogen phosphate and 0.24 g potassium dihydrogen phosphate in deionized water to produce 1 L of solution. The technique of inhibition of albumin denaturation reported previously [17,18] was used with slight modifications. A solution of 1% BSA in deionized water was prepared for the test. It was prepared by dissolving 0.1g BSA in 10 mL of deionized water. The reaction vessel was filled with 200 µL of BSA, 1400 µL of PBS and 1000 µL of the test solutions. Ibuprofen solution (10 µg/mL) was used in the positive control and distilled water was used in the negative control vessels instead of test solution.

The reaction mixtures were incubated at 37°C for 15 min and then heated at 70°C for 5 min. The mixtures were then allowed to cool to room temperature and the absorbance of constituent of each vessel were analyzed in UV-Visible spectrophotometer at 660 nm. The inhibition of percent denaturation of albumin was determined using the following formula:

$$\% \text{ Denaturation inhibition} = (1 - D/C) \times 100\%$$

Where D is the absorbance reading of the test sample, and C is the absorbance reading without test sample (negative control).

Antiprotease action method

The technique of antiprotease action reported by Oyedepo *et al* [19] and Sakat *et al* [20] was used with slight modifications. The reaction mixture was prepared with 0.06 mg trypsin, 1 mL 20 mM Tris-HCl buffer (pH 7.0) and 1 mL test sample of different concentrations (100 - 500 µg/mL). The mixture was incubated at 37°C for 5 min followed by the addition of 1 mL of 0.8% w/v solution of casein in water. The mixture was incubated additionally for 20 min. In order to stop the reaction, 2 mL of 70% perchloric acid was added to the mixture. The turbid suspension obtained after the reaction was centrifuged and the absorbance of the supernatant was recorded at 210 nm against buffer as blank. The percentage inhibition of protease inhibitory activity was calculated by the following formula:

$$\text{Percentage inhibition} = (\text{Abs control} - \text{Abs sample}) \times 100 / \text{Abs control}$$

Results and Discussion

The synthesis of all the compounds was achieved using the scheme depicted in scheme 1. The synthesized conjugates were characterized by determining the practical yield, melting point, solubility and spectral studies. All the compounds were soluble in chloroform. The confirmation of the structures of the compounds was done by ^{1}H-NMR, mass and IR spectral study. The stretching vibrations for amine, carbonyl, imine, and bending vibrations for C-N ring deformation were present in the FT-IR spectra of the conjugates. In the ^{1}HNMR spectra the peaks at chemical shift value of 8.73 and 5.68 corresponding to the proton of pyrimidine nitrogen (N-H), 2.13 corresponding to the proton of methyl group and 7.1 to 7.6 corresponding to the protons of the aromatic rings were present in all the conjugates.

(3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl)(6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methanone, XI

Yield: 61%; Color: Yellow; Rf: 0.48; M.P: 261-263°C; ^{1}HNMR ($CDCl_3$, δ ppm) – 8.53, 6.58 (N-H, pyrimidine), 7.28 to 7.67 (C-H, aromatic), 5.49 (C-H, pyrimidine), 5.52 (C-H, imidazole), 3.48 (C-H, methylene), 2.13 (C-H, methyl); FT-IR (cm^{-1}) – 3413.55 cm^{-1} (N-H), 1713.59 cm^{-1} (C=S), 1453.91 cm^{-1} (C=N), 1158.84 cm^{-1} (C-N) and bending vibrations at around 812.82 cm^{-1} (C-N); m/e: 452.1

(3-(4-chlorophenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)(6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methanone, XII

Yield: 63%; Color: Dark-Yellow; Rf: 0.59; M.P: 241-243°C; ^{1}HNMR ($CDCl_3$, δ ppm) – 8.53, 6.58 (N-H, pyrimidine), 7.27 to 7.67 (C-H, aromatic), 5.49 (C-H, pyrimidine), 5.52 (C-H, imidazole), 3.87 (C-H, methylene), 2.13 (C-H, methyl); FT-IR (cm^{-1}) – 3365.77 cm^{-1} (N-H), 1693.08 cm^{-1} (C=S), 1141.19 cm^{-1} (C-N) and bending vibrations at around 815.82 cm^{-1} (C-N), and 683.73 (C-Cl); m/e: 486.2

(6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)(3-(4-nitrophenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)methanone, XIII

Yield: 61%; Color: Yellow; Rf: 0.61; M.P: 225-227°C; ^{1}HNMR ($CDCl_3$, δ ppm) – 8.73, 6.58 (N-H, pyrimidine), 8.18 (C-H, adjacent to NO_2), 7.28 to 7.90 (C-H, aromatic), 5.49 (C-H, pyrimidine), 5.53 (C-H, imidazole), 3.87 (C-H, methylene), 2.13 (C-H, methyl); FT-IR (cm^{-1}) – 3342.99 cm^{-1} (N-H), 1690.78 cm^{-1} (C=S), 1478.91 (C=N), 1157.26 cm^{-1} (C-N) and bending vibrations at around 809.11 cm^{-1} (C-N); m/e: 497.1

(3-(4-hydroxyphenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)(6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)methanone, XIV

Yield: 64%; Color: Yellow; Rf: 0.61; M.P: 258-260°C; ^{1}HNMR ($CDCl_3$, δ ppm) – 8.73, 6.58 (N-H, pyrimidine), 6.85 to 7.61 (C-H, aromatic), 5.49 (C-H, pyrimidine), 5.52 (C-H, imidazole), 3.48 (C-H, methylene), 2.13 (C-H, methyl); FT-IR (cm^{-1}) – 3733.02 cm^{-1} (O-H), 3555.01 cm^{-1} (N-H), 1704.43 cm^{-1} (C=S), 1461.38 (C=N), 1134.02 cm^{-1} (C-N) and bending vibrations at around 820.13 cm^{-1} (C-N); m/e: 468.1

(6-methyl-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidin-5-yl)(5-phenyl-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone, XV

Yield: 67%; Color: Dark-Yellow; Rf: 0.64; M.P: 258-260°C; ^{1}HNMR ($CDCl_3$, δ ppm) – 8.73, 6.58 (N-H, pyrimidine), 7.19 to 7.58 (C-H, aromatic), 5.49 (C-H, pyrimidine), 5.53 (C-H, imidazole), 3.87 (C-H, methylene), 2.31 (C-H, methyl of benzene), 2.13 (C-H, methyl of pyrimidine); FT-IR (cm^{-1}) – 3340.46 cm^{-1} (N-H), 1699.89 cm^{-1} (C=S), 1480.66 (C=N), 1157.71 cm^{-1} (C-N) and bending vibrations at around 812.47 cm^{-1} (C-N); m/e: 466.1

Anti-inflammatory action

The anti-inflammatory action of the synthesized compounds was evaluated using two of the well established *in vitro* methods viz., protease inhibition activity and inhibition of albumin denaturation. The results are presented in table 1 and 2 respectively.

Table 1. Inhibition of albumin denaturation by test compounds

Treatment	Inhibition of albumin denaturation (%)					
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	10 µg/mL
6a	7.1	12.3	22.8	30.4	40.7	ND
6b	5.5	10.6	17.8	22.5	29.1	ND
6c	4.2	17.3	31.1	41.4	50.6	ND
6d	15.9	25.7	35.2	54.9	67.2	ND
6e	14.1	11.6	20.4	28.4	38.1	ND
Ibuprofen	ND	ND	ND	ND	ND	51.5

ND-Not Determined

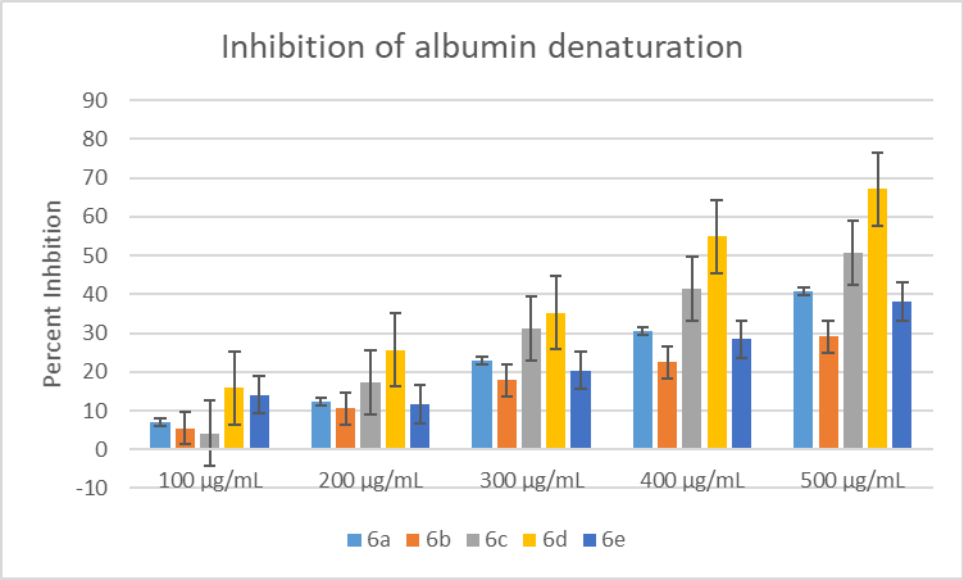

Figure 1. Inhibition of albumin denaturation

Table 2. Percent inhibition of protease action by test compounds

Treatment	Inhibition of Protease Action (%)					
	10 µg/mL	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL
Ibuprofen	50.7	ND	ND	ND	ND	ND
6a	ND	7.3	14.1	19.6	23.4	33.7
6b	ND	5.6	8.9	12.3	21.2	24.3
6c	ND	5.1	7.3	11.5	17.4	21.6
6d	ND	10.4	17.5	22.1	34.8	48.3
6e	ND	9.6	16.1	20.8	30.2	44.7

ND-Not Determined

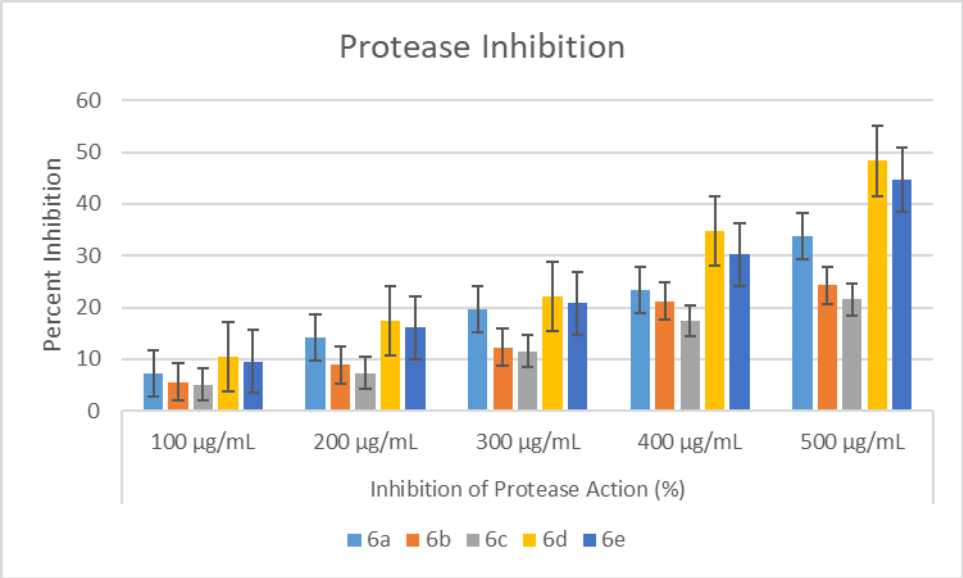

Figure 2. Inhibition of protease action

The pyrazoline derivatives (**XI-XV**) were synthesized in four sequential steps involving synthesis of dihyropyrimidine nucleus, followed by hydrazination of the carboethoxy group. The various chalcone molecules were prepared in the third step via claisen Schmidt condensation and finally the chalcone were cyclo-condensed to yield the pyrimidine-pyrazolinex compounds (**XI-XV**)

In the IR spectrum, stretching bands of C-H (300-2840 cm^{-1}), C=S (1818-1705 cm^{-1}), C-C (1300-800 cm^{-1}), C=N (1612-1602 cm^{-1}) and C-S (950-850 cm^{-1}) were observed in each compound. Bending vibration of N-H (1650-1580 cm^{-1}) and C-N (1250-1020 cm^{-1}) were also visible. The NMR spectra revealed protons of amine, methyl, acetyl and aromatic protons along with methylene of pyrazoline.

The anti-inflammatory activity was determined using the albumin denaturation method and antiprotease method. Protein denaturation has been significantly correlated with the occurrence of the inflammatory response and may lead to various inflammatory diseases including arthritis. It has been said that tissue injury might be due to denaturation of the protein constituents of cells or of intercellular substance. Hence, the ability of the test compounds to inhibit the denaturation of protein signifies obvious potential for anti-inflammatory activity. It has also been reported that leukocytes protease have an important role in the development of tissue damage during inflammatory reactions and significant level of protection could be provided by protease inhibitors. Hence the inhibition of protease action by test compounds signifies its role as anti-inflammatory molecules.

All the compounds exhibited dose dependent inhibition of albumin denaturation with **6d** having the highest capacity to cause the inhibition (67.2 %) at the concentration of 500µg/mL. The antiprotease action was also dose dependent and **6d** at 500µg/mL was able to inhibit (48.3 %) of protease activity. The type of substitution on the terminal ring was found to affect the anti-inflammatory action in both the models. The absence of any substituent was found to be better that substitution electron withdrawing effects (butyl, 6e). On the other hand increasing the electron donating capacity of the substituent was found to increase the inhibition of inflammatory enzymes. The order of activity was 6d>6e>6a>6b>6c.

CONCLUSION

The primary objective of the work was synthesize novel pyrazoline derivatives for anti-inflammatory activity. The objective was achieved by attaching the pyrimidine nucleus to the pyrazoline ring and assess their anti-inflammatory action using *in vitro* models of protein denaturation. The compounds were able to assess significant anti-inflammatory action.

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