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Research Article

IN-VITRO INHIBITION STUDIES ON ENDOGENOUS PROTEOLYSIS OF LIVER HOMOGENATE IN PRESENCE OF SYNTHESIZED PYRAZOLINES

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ABSTRACT

1-substituted 3,5-diaryl-2-pyrazolines were synthesized in a two step reaction. In the first step, prop-2-en-1-ones were synthesized using Claisen–Schmidt reaction of substituted benzaldehydes with the acetophenone. These chalcones thus obtained were allowed to react with hydrazine hydrate or phenylhydrazine in acetic acid or ethanol to afford 1-substituted 3,5-diaryl-2-pyrazolines in high yields. The structure of the compounds was confirmed on the basis of IR & ^{1}H-NMR spectrum. In the present work, we have evaluated *in-vitro* effects of substituted pyrazolines on endogenous proteolysis in liver homogenate.

Keywords: Chalcones, Inhibition studies, 1-substituted 3,5-diaryl-2-pyrazolines, Endogenous proteolysis.

INTRODUCTION

Pyrazolines are well known five membered heterocyclic compounds containing nitrogen. These compounds are known to posses diverse pharmacological activities, due to which significant attention has been focused on this class of heterocyclic compounds. Various biological activities possessed by pyrazolines includes antibacterial[1], antifungal[2], antiviral[3], antiparasitic[4], antitubercular[5], insecticidal agents[6], antipyretic[7], diuretic[8], antidiabetic[9], tranquillizing[10], muscle relaxant[11], psychoanaleptic[12], anticonvulsant[13], antihypertensive[14] and antidepressant[15]. Some of these compounds have also showen anti-inflammatory[16], anaesthetic[17], immunosuppressive[18] and analgesic[19-20]properties. They have also been found to be Nitric oxide synthase (NOS) inhibitors and had shown Cannabinoid CB1 receptor antagonist's[21]activity. In recent decades, the problems of multi-drug resistant microorganism have reached an alarming stage in many countries around the world. A potential approach to overcome the resistance problem is to design pioneering agents with a different mode of action, so that no cross resistance with the present therapeuticals can occur. The review of the literature shows that the pyrazoline nucleus is quite stable and has inspired chemists to utilize these stable fragments in bioactive moieties to synthesize new compounds possessing biological activities. Changes in their structure have offered a high degree of diversity that has proven useful for the development of new therapeutic agents having improved potency and lesser toxicity. Encouraged by these observations it was envisaged to synthesize series of pyrazoline derivatives and to investigate their possible endogenous proteolytic activity in liver.

MATERIALS AND METHODS

General Procedure

Melting points were determined in open capillary tubes and are uncorrected. All the chemicals and solvents used were of laboratory grade. IR spectra (KBr, cm-1) were recorded on a Perkin-Elmer spectrometer. ^{1}H NMR spectra was recorded on Brucker 300 MHz NMR spectrometer (chemical shifts in δ ppm) using TMS as an internal standard. The purity of the compounds was ascertained by thin layer chromatography on aluminium plates percoated with silica gel G (Merck) in various solvent systems using iodine vapors as detecting agent or by irradiation with ultraviolet lights (254 nm). ELISA plate reader was used for measuring absorbance in the visible range. The Spectrofuge was used for centrifugation purpose.

Preparation of liver homogenate

Goat liver was purchased freshly from the local slaughter house. The fresh goat liver was first washed with cold isotonic saline solution. The tissue was then homogenized in 0.1M sodium acetate buffer pH 5.5 containing 0.2M NaCl in a mixer-cum-blender to obtain 10% (w/v) homogenate. It was then stored at 4^{o}C.

Protein estimation

The acid soluble proteins were quantitated in the supernatant using Bradford method[22].

Assay for proteolytic activity

The proteolysis was carried out at pH 5.0 at 37^{o}C using 0.1 M acetate buffer as the incubation medium. The homogenate was mixed with the buffer at this pH was incubated at 37^{o}C for 3 h and 24 h. The reaction was stopped by the addition of TCA and the resulting solution was centrifuged to precipitate proteins. The acid soluble proteins were quantitated in the supernatant using Bradford method[22]. The experiment was conducted in triplicate and the results are presented in table.

General Procedure for the synthesis of 1, 3, 5 - triphenyl pyrazolines

A mixture of chalcone (0.01moles) and phenylhydrazine (0.02 moles) was refluxed for 6 hr in absolute ethanol (50 mL). The solution was left for cooling at room temperature and the solid formed was filtered off, washed with water, dried and crystallized from absolute ethanol[23].

1, 3, 5- triphenyl-2-pyrazoline (1a): Yield 79.43%; m.p.132°C; IR (KBr, cm-^{1}): 3032 (-C=CH stretching of aromatic ring), 1659(-C=N stretching), 1497(-C=C- stretching of aromatic ring); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.13-3.21(1H, dd, -CH_a-, *J* = 7.2, 17.1Hz), 3.82-3.92(1H, dd, -CH_b-, *J* = 12.3, 17.1 Hz), 5.26- 5.33(1H, dd, -CH_x, *J* = 7.2, 12.3 Hz), 6.78-7.76 (10H, m, Ar-H).

5-(-2'-chlorophenyl)-1, 3-diphenyl-2-pyrazoline (1b): Yield 37.17%; m.p.130°C; IR (KBr, cm-^{1}): 3020(-C=CH stretching of aromatic ring), 1622(-C=N stretching), 1589(-C=C- stretching of aromatic ring), 748(-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.04 -3.12(1H, dd, -CH_a-, *J* = 6.9, 16.8 Hz), 3.94 - 4.08 (1H, dd, -CH_b-, *J* =14.7, 16.8 Hz), 5.64 - 5.70(1H, dd, -CH_x, *J* = 6.9, 14.7 Hz), 6.83-8.02(9H, m, Ar-H).

5-(-3'-chlorophenyl)-1, 3-diphenyl-2-pyrazoline (1c): Yield 89.75%; m.p. 102°C; IR (KBr, cm-^{1}): 3020(-C=CH stretching of aromatic ring), 1620(-C=N stretching), 1589(-C=C- stretching of aromatic ring), 784(-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.02 - 3.13(1H, dd, -CH_a-, *J* = 6.9, 17.1 Hz), 3.82-4.02(1H, dd, -CH_b-, *J* = 12.3, 17.1Hz), 5.25 - 5.38(1H, dd, -CH_x, *J* = 6.9, 12.3Hz), 6.80-7.75(9H, m, Ar-H).

5-(-4'-chlorophenyl)-1, 3-diphenyl-2-pyrazoline (1d): Yield 95.61%; m.p. 134 °C; IR (KBr, cm-^{1}): 3032(-C=CH stretching of aromatic ring), 1620(-C=N stretching),1580(-C=C- stretching of aromatic ring), 774 (-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.08-3.16(1H, dd, -CH_a-, *J* =7.2, 17.1Hz), 3.81-4.02(1H, dd, -CH_b-, *J* =12.6, 17.1Hz), 5.24-5.30(1H, dd, -CH_x, *J* =7.2, 12.6 Hz), 6.80-7.75(9H, m, Ar-H).

5-(-2'-methoxyphenyl)-1, 3-diphenyl-2-pyrazoline (1e): Yield 77.61%; m.p. 125-128 °C; IR (KBr, cm-1): 3032(-C=CH stretching of aromatic ring), 1620(-C=N stretching), 1556(-C=C- stretching of aromatic ring), 1126(-C- OCH_3 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.97(s,3H, -OCH_3), 2.99-3.07(1H, dd, -CH_a-, *J* = 7.2, 17.4 Hz), 3.68-3.87 (1H, dd, -CH_b-, *J* = 12.6 , 17.4 Hz), 5.58-5.65(1H, dd, -CH_x, *J* =7.2, 12.6 Hz), 6.76-7.76 (9H, m, Ar-H).

5-(-3'-methoxyphenyl)-1, 3-diphenyl-2-pyrazoline (1f): Yield 80.69%; m.p.98 °C; IR (KBr, cm-1): 3020(-C=CH stretching of aromatic ring), 1659(-C=N stretching), 1589(-C=C- stretching of aromatic ring), 1126(-C- OCH_3 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.80(s,3H, -OCH_3), 3.13-3.21(1H, dd, -CH_a-, *J* = 7.5, 17.1Hz), 3.80-4.02 (1H, dd, -CH_b-, *J* =12.6, 17.1Hz), 5.21-5.28 (1H, dd, -CH_x, *J* =7.5, 12.6 Hz), 6.78-7.75 (9H, m, Ar-H).

5-(-4'-methoxyphenyl)-1, 3-diphenyl-2-pyrazoline (1g): Yield 93.20%; m.p. 116-118 °C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 1638 (-C=N stretching), 1556 (-C=C- stretching of aromatic ring), (-C- OCH_3); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.82(s,3H, -OCH_3), 3.09-3.18(1H, dd, -CH_a-, *J* =7.2, 16.8 Hz), 3.83-4.03(1H, dd, -CH_b-, *J* = 13.0, 16.8 Hz), 5.22-5.29 (1H, dd, -CH_x, *J* = 7.2, 13.0 Hz), 6.77-7.78(9H, m, Ar-H).

5-(-2'-nitrophenyl)-1, 3-diphenyl-2-pyrazoline (1h): Yield 53.42%; m.p.136-138°C; IR (KBr, cm-1): 3032(-C=CH stretching of aromatic ring), 1652(-C=N stretching), 1575(-C=C- stretching of aromatic ring),1350,1528(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.15-3.23(1H, dd, -CH_a-, *J* =6.6, 17.7 Hz), 4.10-4.31(1H, dd, -CH_b-, *J* = 12.3, 17.7 Hz), 5.86-5.89(1H, dd, -CH_x, *J* =6.6, 12.3 Hz), 6.77-7.78(4H, m, Ar-H).

5-(-3'-nitrophenyl)-1, 3-diphenyl-2-pyrazoline (1i): Yield 70.67%; m.p. 86-88 °C; IR (KBr, cm-1): 3020(-C=CH stretching of aromatic ring), 1614(-C=N stretching),1590(-C=C- stretching of aromatic ring),1350,1528(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.12-3.20(1H, dd, -CH_a-, *J* = 7.2 ,17.1Hz), 3.90-4.35(1H, dd, -CH_b-, *J* =12.3, 17.1Hz), 5.37-5.48(1H, dd, -CH_x, *J* = 7.2, 12.3 Hz), 6.82-7.83(9H, m, Ar-H).

5-(-4'-nitrophenyl)-1, 3-diphenyl-2-pyrazoline (1j): Yield 88.95%; m.p. 98-100°C; IR (KBr, cm-1): 3025(-C=CH stretching of aromatic ring), 1622(-C=N stretching), 1575(-C=C- stretching of aromatic ring), 1350,1528(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.10-3.22(1H, dd, -CH_a-, *J* = 6.9, 16.8Hz), 3.88-3.98(1H, dd, -CH_b-, *J* = 12.3, 16.8 Hz), 5.30- 5.40 (1H, dd, -CH_x, *J* =6.9, 12.3 Hz), 6.87-7.79(4H, m, Ar-H).

General Procedure for the synthesis of 3, 5 - diphenyl pyrazolines: A mixture of chalcone (0.01 mol) and hydrazine hydrate (0.02 mole) was heated at reflux for 6 hr in absolute ethanol (50 mL). The solution was left to cool at room temperature and the solid formed was filtered off, washed with water, dried and crystallized from absolute ethanol.

5-(-4'-chlorophenyl)-3-phenyl-2-pyrazoline (2a): Yield 76.80%; m.p. 112 °C; IR (KBr, cm-1): 3063 (-C=CH stretching of aromatic ring), 3315(-NH stretching),1682 (-C=N stretching), 1597 (-C=C-stretching of aromatic ring), 748(-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.58-2.75 (1H, dd, -CH_a-, *J* =6.3, 16.2Hz), 3.54-3.63(1H, dd, -CH_b-, *J* = 10.8, 16.2 Hz), 5.08-5.16(1H, dd, -CH_x, *J* = 6.3, 10.8Hz), 7.23-7.63(11H, m, Ar-H).

5-(-4'-methoxyphenyl)-3-phenyl-2-pyrazoline (2b): Yield 66.87%; m.p. 116°C; IR (KBr, cm-1): 3024(-C=CH stretching of aromatic ring), 3340(-NH stretching), 1605(-C=N stretching), 1504(-C=C- stretching of aromatic ring), 1242(-C-OCH_3 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 3.75(s, 3H, -OCH_3), 3.15-3.23(1H, dd, -CH_a-, *J* = 5.2, 17.7 Hz),3.72-3.81(1H, dd, -CH_b-, *J* =12.2, 17.7Hz), 5.56-5.62(1H, dd, -CH_x, *J* =5.2, 12.2 Hz), 6.86-7.80(11H, m, Ar-H).

5-(-2'-nitrophenyl)-3-phenyl-2-pyrazoline (2c): Yield 67.78%; m.p.116-118 °C; IR (KBr, cm-1): 3025(-C=CH stretching of aromatic ring), 3325(-NH stretching),1597(-C=N stretching), 1512(-C=C-stretching of aromatic ring), 1342,1512(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.48-2.81(1H, dd, -CH_a-, *J* =5.3, 17.9 Hz), 3.50-3.59(1H, dd, -CH_b-, *J* = 11.0, 17.9 Hz), 5.86- 5.89 (1H, dd, -CH_x, *J* = 5.3, 11.0 Hz),7.31-8.26(11H, m, Ar-H).

5-(- 3'-nitrophenyl)-3-phenyl-2-pyrazoline (2d): Yield 70.87%; m.p. 110-112°C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 3294(-NH stretching),1597(-C=N stretching), 1528(-C=C-stretching of aromatic ring), 1342,1528(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.50-2.94(1H, dd, -CH_a-, *J* =7.3, 16.5 Hz), 3.51-3.60(1H, dd, -CH_b-, *J* =11.8, 16.5 Hz), 4.99- 5.07(1H, dd, -CH_x, *J* = 7.3, 11.8 Hz), 7.32-8.25(11H, m, Ar-H).

5-(- 4'-nitrophenyl)-3-phenyl-2-pyrazoline (2e): Yield 87.56%; m.p.138-140°C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 3325(-NH stretching),1597(-C=N stretching), 1512(-C=C-stretching of aromatic ring), 1342,1512(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.50-2.91(1H, dd, -CH_a-, *J* = 5.4, 16.5 Hz), 3.51-3.60(1H, dd, -CH_b-, *J* = 10.8, 16.5 Hz), 4.97-5.05 (1H, dd, -CH_x, *J* = 5.4, 10.8 Hz), 7.30-8.24(11H, m, Ar-H).

General Procedure for the synthesis of N-acetyl-3,5-diphenyl pyrazolines

A mixture of chalcone (0.01moles), hydrazine hydrate (0.02moles) in acetic acid (40 ml) was refluxed for 3 hours then poured into ice cold water. The precipitate was separated by filtration, washed free of acid and crystallized from methanol to afford 2-pyrazolines, dried and recrystalized from ethanol.

N- acetyl -3,5 - diphenyl pyrazolines: Yield 77.90%; m.p. 120-122°C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 2938 (-C-H stretching of –$COCH_3$ group), 1643 (-C=O stretching of -$COCH_3$ group), 1520(-C=C- stretching of aromatic ring); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.45(3H, -$COCH_3$), 3.15-3.22(1H, dd, -CH_a, J=4.5, 17.4 Hz), 3.73-3.83(1H, dd, -CH_b, J= 12.0,17.4 Hz), 5.59-5.64(1H, dd, -CH_x, J=4.5, 12.0 Hz), 7.73-7.77(10H, m, Ar-H).

N-acetyl 5-(-2'-chlorophenyl)-3-phenyl-2-pyrazoline (3a): Yield 75.34%; m.p. 127-128 °C; IR (KBr, cm-1): 3023 (-C=CH stretching of aromatic ring), 2948 (-C-H stretching of –$COCH_3$ group) , 1666 (-C=O stretching of – $COCH_3$ group), 1520 (-C=C- stretching of aromatic ring), 774 (-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.51 (3H, s, -$COCH_3$), 3.04-3.12(1H, dd, -CH_a, J=5.1, 18.0 Hz), 3.82-3.92(1H, dd, -CH_b-, *J* = 11.7, 18.0 Hz), 5.91-5.97(1H, dd, -CH_x, J=5.1, 11.7 Hz), 7.06-7.76 (9H, m, Ar-H).

N-acetyl 5-(-3'-chlorophenyl)-3-phenyl-2-pyrazoline (3b): Yield 80.12%; m.p. 114-116 °C; IR (KBr, cm-1): 3022 (-C=CH stretching of aromatic ring), 2940 (-C-H stretching of –$COCH_3$ group) , 1651 (-C=O stretching of – $COCH_3$ group), 1566 (-C=C- stretching of aromatic ring), 764 (-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.45(3H, s, -$COCH_3$), 3.12-3.19 (1H, dd, -CH_a, J=4.8,17.7 Hz), 3.73-3.83(1H, dd, -CH_b-, *J* = 12.0,17.7 Hz), 5.55-5.60(1H, dd, -CH_x, J=4.8, 12.0 Hz), 7.13-7.77 (9H, m, Ar-H).

N-acetyl 5-(-4'-chlorophenyl)-3-phenyl-2-pyrazoline (3c): Yield 90.23%; m.p. 108-110°C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 2948 (-C-H stretching of –$COCH_3$ group) , 1643(-C=O stretching of – $COCH_3$ group), 1589 (-C=C- stretching of aromatic ring), 779(-C-Cl stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.43(3H, s, -$COCH_3$), 3.11-3.19 (1H, dd, -CH_a, J=4.5, 17.7 Hz), 3.73-3.82(1H, dd, -CH_b-, *J* = 12.0, 17.7 Hz), 5.55-5.60(1H, dd, -CH_x, J=4.5, 12.0 Hz),7.18-7.77(9H, m, Ar-H).

N-acetyl 5-(-2'-methoxyphenyl)-3-phenyl-2-pyrazoline (3d): Yield 86.52%; m.p.128-130°C; IR (KBr, cm-1): 3022 (-C=CH stretching of aromatic ring), 2940(-C-H stretching of –$COCH_3$ group), 1651(-C=O stretching of – $COCH_3$ group), 1597(-C=C- stretching of aromatic ring), 1149(-C-OCH_3 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.49 (3H, s, -$COCH_3$), 3.87 (3H, s, -OCH_3), 3.02-3.09(1H, dd, -CH_a-, *J* =4.5, 17.7 Hz), 3.69-3.79(1H, dd, -CH_b-, *J* =11.7, 17.7 Hz), 5.84-5.90 (1H, dd, -CH_x, *J* =4.5, 11.7 Hz), 6.88-7.76(9H, m, Ar-H).

N-acetyl 5-(-3'-methoxyphenyl)-3-phenyl-2-pyrazoline (3e): Yield 74.98%; m.p.106-107°C; IR (KBr, cm-1): 3025 (-C=CH stretching of aromatic ring), 2938 (-C-H stretching of –$COCH_3$ group), 1651(-C=O stretching of – $COCH_3$ group), 1597(-C=C-stretching of aromatic ring), 1165(-C-OCH_3 stretching); ^{1}H-NMR (300

MHz, CDCl3, δ ppm): 2.49(3H, s, -$COCH_3$), 3.87(3H, s, -OCH_3), 3.02-3.09(1H, dd, -CH_a-, *J* = 4.5, 17.7 Hz), 3.69-3.75(1H, dd, -CH_b-, *J* =12.0, 17.7 Hz), 5.84-5.90 (1H, dd, -CH_x, *J* =4.5, 12.0 Hz), 6.88-7.76 (4H, m, Ar-H).

N-acetyl 5-(-4'-methoxyphenyl)-3-phenyl-2-pyrazoline (3f): Yield 88.67%; m.p.107-108°C; IR (KBr, cm-1): 3055 (-C=CH stretching of aromatic ring), 2932 (-C-H stretching of –$COCH_3$ group), 1643(-C=O stretching of – $COCH_3$ group), 1598(-C=C- stretching of aromatic ring), 1142 (-C- OCH_3 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.42(3H, s, -$COCH_3$), 3.75(3H, s, -OCH_3), 3.14-3.22(1H, dd, -CH_a-, *J* = 4.8, 17.7 Hz), 3.70-3.79(1H, dd, -CH_b-, *J* =12.0,17.7 Hz), 5.54-5.60 (1H, dd, -CH_x, *J* = 4.8, 12.0 Hz), 6.84-7.78(9H, m, Ar-H).

N-acetyl 5-(-2'-nitrophenyl)-3-phenyl-2-pyrazoline (3g): Yield 58.32 %; m.p.108-109°C; IR (KBr, cm-1): 3022 (-C=CH stretching of aromatic ring), 2932 (-C-H stretching of –$COCH_3$ group) , 1666 (-C=O stretching of – $COCH_3$ group), 1520(-C=C- stretching of aromatic ring), 1335,1520(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.45(3H, s, -$COCH_3$), 3.13-3.21 (1H, dd, -CH_a, J=5.4,18.3 Hz), 4.06-4.12(1H, dd, -CH_b-, *J* = 12.0,18.3 Hz), 6.13- 6.19(1H, dd, -CH_x, J=5.4, 12.0 Hz),7.02-8.04 (9H, m, Ar-H).

N-acetyl 5-(-3'-nitrophenyl)-3-phenyl-2-pyrazoline (3h): Yield 77.98 %; m.p. 157-158°C; IR (KBr, cm-1): 3055 (-C=CH stretching of aromatic ring), 2932 (-C-H stretching of –$COCH_3$ group) , 1643 (-C=O stretching of – $COCH_3$ group), 1528(-C=C- stretching of aromatic ring), 1342,1528(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.47(3H, s, -$COCH_3$), 3.14-3.22(1H, dd, -CH_a, J=5.1,17.7 Hz), 3.82-3.92(1H, dd, -CH_b-, *J* =12.0,17.7 Hz), 5.66-5.72(1H, dd, -CH_x, J= 5.1, 12.0Hz), 7.28-8.12(9H, m, Ar-H).

N-acetyl 5-(-4'-nitrophenyl)-3-phenyl-2-pyrazoline (3i): Yield 87.45 %; m.p. 164-165 °C; IR (KBr, cm-1): 3055 (-C=CH stretching of aromatic ring), 2932 (-C-H stretching of –$COCH_3$ group) , 1643 (-C=O stretching of – $COCH_3$ group), 1597(-C=C- stretching of aromatic ring), 1342,1512(-C-NO_2 stretching); ^{1}H-NMR (300 MHz, CDCl3, δ ppm): 2.46(3H, s, -COCH3), 3.13-3.21(1H, dd, -CH_a, J=5.1,17.7 Hz), 3.80-3.90 (1H, dd, -CH_b-, *J* =12.0, 17.7 Hz), 5.65-5.70(1H, dd, -CH_x, J= 5.1, 12.0 Hz),7.28-8.23(9H, m, Ar-H).

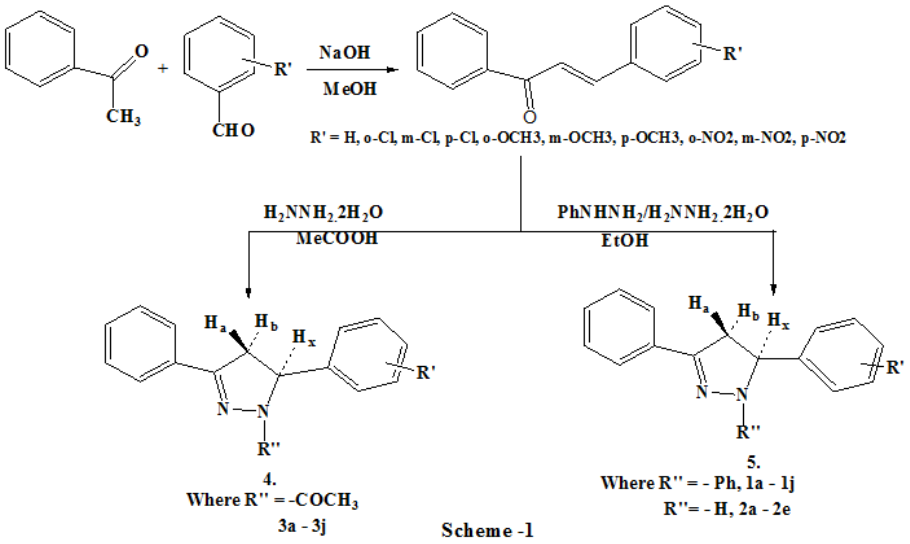
RESULTS AND DISCUSSION

Chalcones were prepared easily using Claisen–Schmidt reaction from substituted aldehydes and ketones, in presence of alkali NaOH and methanol as solvent. Chalcones were allowed to react with hydrazine hydrate or phenylhydrazine in hot acetic acid to afford 1-substituted 3, 5-diaryl-2- pyrazolines in high yields. In presence of acetic acid and hydrazine hydrate *N*-Acetylation of all 2-pyrazolines occurred whereas with phenyl hydrazine, N-phenylpyrazolines were obtained. To obtain diphenyl pyrazolines, reaction was carried out in presence of alcohol and in absence of acetic acid. During the synthetic studies, it was found that diphenyl pyrazolines were difficult to be isolated in pure form probably due to oxidation and formation of side products and therefore only few diphenyl pyrazolines which were synthesized in pure form were selected further for endogenous proteolytic studies. In contrast, triphenyl pyrazoline and *N*-acetyl derivatives of 1- substituted 2-pyrazolines are quite stable compounds and can be used further for biological and pharmaceutical trials without the risk of undesirable decomposition.

All the synthesized pyrazolines (scheme-1) show a characteristic IR absorption peak.The structure elucidation of compounds (1a-1j, 2a-2e and 3a-3j) was based on the spectral data (IR& ^{1}H NMR). The IR spectra showed mainly stretching bands at 1580-1610, 1450-1600 and 3000-3100 cm-1 assigned to (C=N), aromatic (C=C) and (C-H) functionalities, respectively. Particularly,the derivatives 1b-1d, 2a and 3b-3d showed typical absorption bands at 700–750 cm-1 due to their C-Cl stretching vibrations. The derivatives 1e-1g, 2b and 3e-3g exhibited a band at 1050–1300 cm-1 due to their C-OCH_3 stretching vibrations. The derivatives 1h-1j, 2c-2e and 3h-3j demonstrated typical absorption bands at 1335-1365 and 1510-1550 cm-1 due to symmetric and asymmertic NO_2 stretching vibrations. In acetyl pyrazolines -C=O stretching bands of – $COCH_3$ group is observed at 1643-1666 cm-1.

^{1}H-NMR spectra of these compounds generally exhibit an ABX pattern for the presence of two diastereotopic methylene protons at C-4 and one single stereogenic proton at the C-5 positions. Thus, the 4'-H_a and 4'-H_b appear each one as a double-doublet at δ= 3.01–3.21 ppm (J_{ab}, J_{ax}) and δ= 3.68–4.35 ppm (J_{ab}, J_{bx}) respectively while 5'-H appears as a double-doublet at δ= 5.21–5.89 ppm (J_{bx}, J_{ax}) where(J_{ab} = 16.2 – 18.0 Hz, J_{ax} = 4.5 – 7.2 Hz, J_{bx} = 10.8 – 12.6 Hz). The NH protons of compounds were observed which appears as a singlet at δ = 7.19 ppm. In the ^{1}H-NMR spectra of 1-acetyl-2-pyrazolines, a singlet signal at around 2.4 ppm refers to the presence of an *N*-acetyl group.

The proteases are ubiquitous in nature and account for approximately 2% of the genes in most organisms, second in number only to transcription factors[24]. Because of this, proteases have long been the subject of intensive research[25].Their use is gaining more and more attention because of their involvement in oncology, inflammatory conditions, blood rheology control and immune regulation etc[26]. At present, an estimated 5-10 % of all pharmaceutical targets are proteases[27]. Precise control of proteolytic processes is essential for appropriate functioning of cells and whole organisms[28]. In our previous work[29-31]we have reported the effect of substituted hydrazonesand aryl semicarbazones on endogenous proteolysis.



Scheme -1

The focus of proteolytic studies can result into two fold information. One is to investigate pyrazolines as potential inhibitors to proteases, which can be utilized further for therapeutic purposes in various tissue degenerative disorders where proteases are found in elevated levels. The second is to correlate anyside effects produced by potential therapeutic agents having this structural moiety by evaluating their effect on proteases playing significant role in important biological processes. In the present study, we have evaluated the effects of pyrazolines on endogenous protein substrates at pH 5.0. We have restricted our study at this pH as the major proteases, which are active at this pH are cysteine proteases[29]. Literature study also suggests that cysteine proteases are active at pH 5.0 where as serine proteases are active at pH 3.5[32]. Cysteine proteases having a cysteine residue at the active site and belong to papain super family has a catalytic triad i.e. Histidine, Aspartic acid and Cysteine and are the key factors in the pathogenesis[33-35] of cancer invasion, arthritis, osteoporosis etc. The effect of synthesized pyrazolines on proteolysis of endogenous protein substrates was observed at pH 5.0 (Fig. 1, Fig. 2 and Fig. 3). The proteolytic studies on endogenous protein substrates were conducted for 3.0 hr and 24.0 hr. The experiment was conducted in triplicate and the results are presented in table 1.

Table 1: Effect of Substituted Pyrazolineson Endogenous Protein Hydrolysis in Liver Homogenate

S. No.	Compound Name	3h		24 h	
		M.D.±S.M.D.	% Residual Activity	M.D.±S.M.D.	% Residual Activity
	Control	**0.156 ±0.0153**		**0.228±0.0254**	
1.	1, 3, 5- triphenyl-2-pyrazoline (1a)	0.057 ±0.0016	36.54 ±1.02	0.112±0.0302	49.12 ±13.2
2.	5-(-2'-chlorophenyl)-1,3-diphenyl-2-pyrazoline (1b)	0.0 01±0.0054	00.64 ±3.46	0.056±0.0050	24.56 ±2.19
3.	5-(-3'-chlorophenyl)-1,3-diphenyl-2-pyrazoline (1c)	0.0 27±0.0130	17.31 ±8.33	0.150±0.0058	65.79 ±2.54
4.	5-(-4'-chlorophenyl)-1,3-diphenyl-2-pyrazoline (1d)	0.006 ±0.0110	03.85 ±7.05	0.068±0.0320	29.82 ±14.0
5.	5-(-2'-methoxyphenyl) -1,3-diphenyl-2-pyrazoline (1e)	0.043 ±0.0121	27.56 ±7.75	0.100±0.0170	43.85 ±7.45
6.	5-(-3'-methoxy phenyl)-1,3-diphenyl-2-pyrazoline (1f)	0.026 ±0.0154	16.67±9.87	0.038±0.0188	16.67 ±8.24
7.	5-(-4'-methoxy phenyl)-1,3-diphenyl-2-pyrazoline (1g)	0.027 ±0.0001	17.30±0.064	0.046±0.0024	20.17 ±1.05
8.	5-(-2'-nitrophenyl)-1,3-diphenyl-2-pyrazoline (1h)	0.002 ±0.0070	01.28 ±4.48	0.070±0.0064	30.70 ±2.80
9.	5-(-3'-nitrophenyl)-1,3-diphenyl-2-pyrazoline (1i)	0.004 ±0.0023	02.56 ±1.47	0.116±0.0110	50.88 ±4.82
10.	5-(-4'-nitrophenyl)-1,3-diphenyl-2-pyrazoline (1j)	0.012 ±0.0024	07.69 ±1.53	0.006±0.0006	02.63 ±0.26
	Control	0.158 ±0.0006		0.270±0.0306	
11.	5-(-4'-chlorophenyl)-3-phenyl-2-pyrazoline (2a)	0.097 ±0.0015	61.39 ±0.95	0.208±0.0306	77.0 ±11.33
12.	5-(-4'-methoxy phenyl)-3-phenyl-2-pyrazoline (2b)	0.138 ±0.0006	87.34 ±0.38	0.282±0.0080	104.4 ±2.96
13.	5-(-2'-nitrophenyl)-3- phenyl - 2 -pyrazoline (2c)	0.085 ±0.0040	56.67 ±2.53	0.228±0.0304	84.44±11.25
14.	5-(-3'-nitrophenyl)-3- phenyl-2-pyrazoline (2d)	0.028 ±0.0015	17.72 ±0.95	0.142 ± 0.026	52.60 ±9.62
15.	5-(-4'-nitrophenyl)-3- phenyl-2-pyrazoline (2e)	0.056 ±0.0032	35.44 ±2.02	0.048±0.0246	17.77 ±9.10
	Control	0.071 ±0.0148		0.146±0.0304	
16.	N-acetyl-3,5-diphenyl pyrazolines (3a)	0.036 ±0.0058	50.70 ±0.17	0.102±0.0084	69.86 ±5.75
17.	N-acetyl-5-(-2'-chloro phenyl) - 3 -phenyl-2-pyrazoline (3b)	0.004 ±0.0019	05.63 ±2.67	0.136±0.0048	93.15 ±3.28
18.	N-acetyl-5-(-3'-chloro phenyl) - 3 -phenyl-2-pyrazoline (3c)	0.001 ±0.0025	01.40 ±3.52	0.062±0.0090	42.46 ±6.16
19.	N-acetyl-5-(-4'-chloro phenyl) - 3 -phenyl-2-pyrazoline (3d)	0.034 ±0.0073	47.89±10.28	0.124±0.0134	84.93 ±9.17
20.	N-acetyl-5-(-2'-methoxyphenyl)-3-phenyl-2-pyrazoline (3e)	0.015 ±0.0012	21.73 ±1.69	0.096±0.0042	65.75 ±2.87
21.	N-acetyl-5-(-3'-methoxyphenyl)-3-phenyl-2-pyrazoline (3f)	0.005 ±0.0058	07.04 ±8.16	0.144±0.0032	98.63 ±2.19
22.	N-acetyl-5-(-4'-methoxyphenyl)-3-phenyl-2-pyrazoline (3g)	0.008± 0.0034	10.95 ±4.78	0.028±0.0038	19.72 ±2.60
23.	N-acetyl-5-(-2'-nitro phenyl)-3-phenyl-2-pyrazoline (3h)	0.004± 0.0022	05.63 ±3.09	0.072±0.0072	49.31 ±4.93
24.	N-acetyl-5-(-3'-nitro phenyl)-3-phenyl-2-pyrazoline (3i)	0.020 ±0.0023	28.16 ±3.23	0.056±0.0096	38.36 ±6.57
25.	N-acetyl-5-(-4'-nitro phenyl)-3-phenyl-2-pyrazoline (3j)	0.030± 0.0069	42.25 ±9.71	0.002±0.0018	01.37 ±1.23

The TCA soluble peptides were estimated at 630 nm using Bradford method[22] and the results are the mean and S.M.D. of the experiment conducted in triplicate and is calculated as protease activity in 0.1% liver homogenate. The % residual activity is calculated w.r.t. control where no compound was added but an equivalent amount of solvent was present.

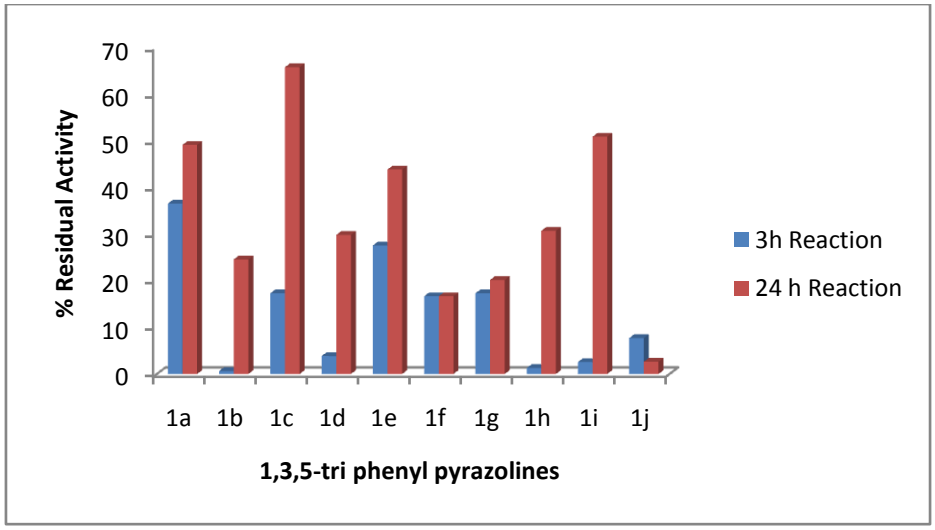


Fig. 1: Effect of 1,3,5- triphenyl pyrazolines(1a-1j) on the endogenous proteolytic activity.

The data in each bar **(1a-1j)** represents the % Residual Activity in presence of individual compound w.r.t. control taken as 100.

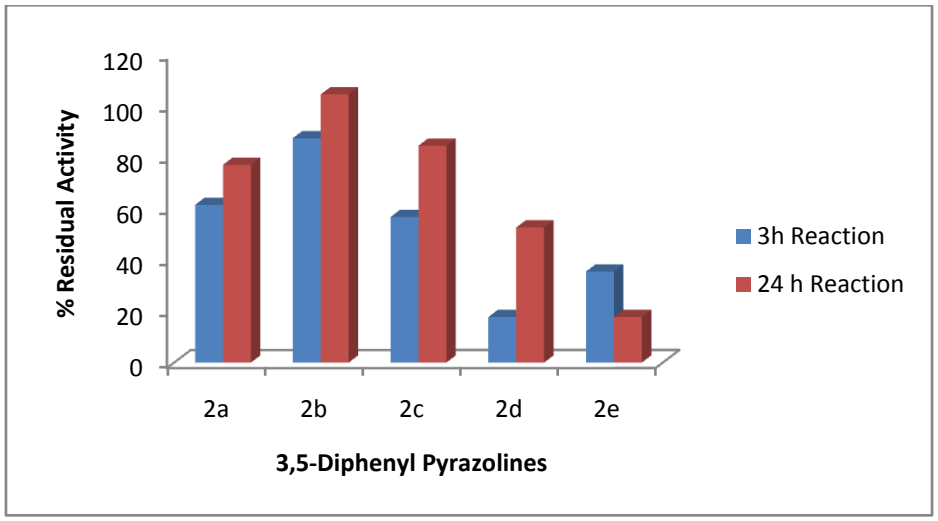


Fig. 2: Effect of 3,5- diphenyl pyrazolines(2a-2e) on the endogenous proteolytic activity.

The data in each bar **(2a-2e)** represents the % Residual Activity in presence of individual compound w.r.t. control taken as 100.

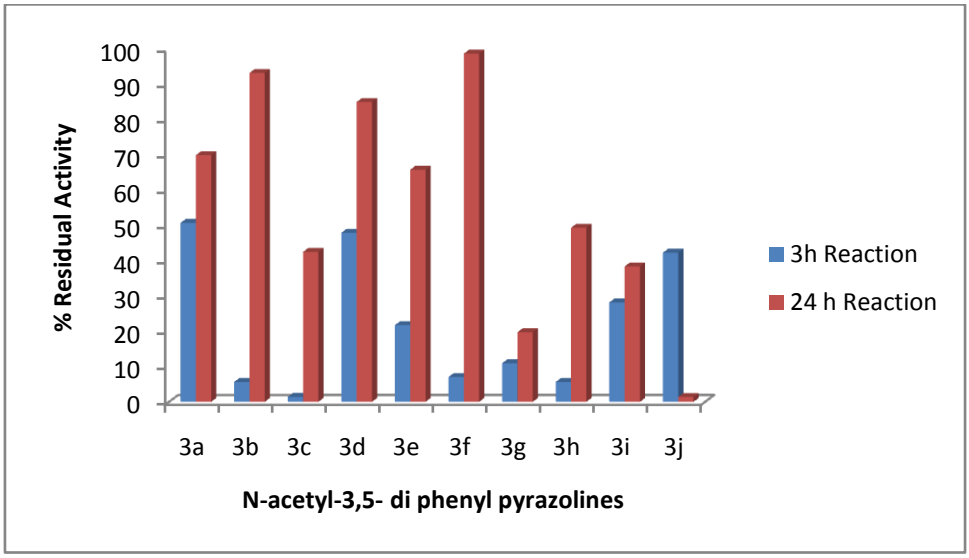


Fig. 3: Effect of N-acetyl-3,5-diphenyl pyrazolines(3a-3j)on the endogenous proteolytic activity.

The data in each bar **(3a-3j)** represents the % Residual Activity in presence of individual compound w.r.t. control taken as 100.

In phenyl pyrazolines, maximum inhibition was observed in 5-(-2'-chlorophenyl)-1,3-diphenyl-2-pyrazoline (1b) at 3 hr reaction and 5-(-4'-nitrophenyl)-1,3-diphenyl-2-pyrazoline (1j) at 24 hr reaction. In the case of Simple 3,5-diphenyl pyrazolines, 5-(-3'-nitrophenyl)-3-phenyl-2-pyrazoline (2d) was found more inhibitory at 3 hr whereas 5-(-4'-nitrophenyl)-3-phenyl-2-pyrazoline (2e) showed maximum inhibition at 24 hr reaction. In acetyl pyrazolines, N-acetyl-5-(-3'-chloro phenyl) -3- phenyl-2-pyrazoline (3c) showed maximum inhibition at 3 hr reaction while at 24 hr incubation, N-acetyl-5-(-4'-nitrophenyl)-3-phenyl-2-pyrazoline (3j) showed maximum inhibition.

It may be mentioned here that the enzyme preparation contain many proteases. To establish a general line of structure activity relationship is very difficult in this present study but certainly some trends could be visualized. One observation is that in most of the cases, inhibition was more at 3.0 hr and less at 24.0 hr. The reason may be that binding of proteases with pyrazolines might have been reversed with time. In the intial stages, binding is more and results in high inhibition and activity is regenerated with time resulting in lesser or no inhibition after 24.0 hr reaction time. It may also be interpreted that with time some enzymes might have been activated which are less sensitive towards pyrazolines.

Secondly, we have observed that this reversal of inhibition at 3 and 24 hr is increased in case of *p*-nitro substituents. It suggests that either due to strong electron withdrawing nature or due to interaction with nitro group, there might have been irreversible binding of compounds with proteases whereas in case of other substituents, the binding of pyrazolines with proteases might have been reversed with time. Another observation was that N-substituted pyrazolines were more inhibitory than their precursors.

In general, di & triphenylpyrazoline and acetyl pyrazoline derivatives have been found to exhibit inhibition to endogenous proteolysis. The results suggest that pyrazolines inhibit endogenous proteolytic activity appreciably. In some cases ~100% inhibition is achieved at 1mM concentration. The data reported in this paper indicate that pyrazolines act as inhibitors to proteases active at pH 5.0. and inhibits endogenous protein hydrolysis significantly. This may prove to be a helpful guide in studying their effect on different enzymes and provides a case history for relating the enzyme inhibition studies with pyrazolines for various pharmacological purposes.

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