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In Vitro Anti-Inflammatory Assays of Synthesized acetophenone

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ABSTRACT:-

At 1600µg/ml, the greatest percentage of denaturation of egg albumin was inhibited by synthesized acetophenone (89.16±0.38%). At the greatest concentration, the synthesized acetophenone showed a significant effect of obstructing protein denaturation in a dose-dependent manner. It was investigated whether the synthesized acetophenone could keep BSA from becoming denaturated. Synthesized acetophenone demonstrated the greatest percentage denaturation of BSA to be 76.59±0.56% at 1600µg/ml. The RBC membrane was stabilized by the synthesized acetophenone in a dose-dependent manner, with the maximum stability occurring at 1600 µg/ml. At 1600 µg/ml, the greatest percentage inhibition of RBC membrane lysis (91.15±0.31%) was observed, in contrast to DS (90.01±0.02%) and piroxicam (80.34±0.29%). The highest and lowest levels of trypsinase activity hindrance were seen at 1600µg/ml and 50µg/ml, respectively, for the synthesized acetophenone. The greatest percentage of trypsinase activity suppression was found to be 83.94±0.24.

KEY WORDS:-

Trypsinase, isosaline, Cycloxygenase, Lipoxygenase, Anti-Inflammatory, Rheumatoid Arthritis.

INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune disease that progresses over time and causes chronic, disabling synovitis and synovial inflammation. The disease is characterized by significant bone and cartilage destruction, leading to severe joint damage and decreased functionality (Jeon et al. 2019, Lee et al. 2007). This disease can spread swiftly through an individual and impact multiple body regions that become inflamed or excruciatingly painful. It primarily affects the elderly, but it can also affect those with immune system failure or

degenerative bone disease (Muruganathan et al. 2013). Swelling, stiffness, discomfort, and a decrease in or loss of joint function are associated with this condition, which can also arise from the immune system targeting the synovial membrane (Muruganathan et al. 2013). Numerous inflammatory mediators, such as tumor necrosis factor (TNF-α), interleukin-1β, interleukin-6, nitric oxide (NO), prostaglandins, reactive oxygen species (ROS), platelet-activating factor, leukotrienes, enzymes (lipoxygenases, cyclooxygenases (COX-1 and COX-2), and phospholipases), play a crucial role in bone destruction and inflammation of the synovial membrane during the onset and progression of rheumatoid arthritis (Feldmann et al. 1996, Irem et al. 2018).

MATERIAL AND METHOD

1. **Inhibition of Protein Denaturation: -** With a few minor adjustments, the methodology from Padmanabhan and Jangle (2012) and Elias and Rao (1988) was applied to assess the synthesized acetophenone anti-inflammatory properties.

   A. **Protein denaturation assay using egg's albumin**: - The assay was carried out using the procedure that Pavithra et al., 2018 previously detailed. 0.2 ml of egg albumin, 2.8 ml of PBS (pH 6.4), and 2 ml of synthesized acetophenone at concentrations of 50, 100, 200, 400, 800, and 1600µg/ml made up the 5 ml reaction mixture. Instead of synthesized acetphenone, piroxicam/diclofenac sodium (DS) was present in the standard solution. In all of the in vitro anti-arthritic studies, the control solution comprised DW and 5% DMSO in place of the synthesized acetophenones for n-hexane and butanol. These solutions were heated for five minutes at 70o C after being incubated for 0.25 hours at 37o C. Following a room temperature cooling period, the absorbance of the solutions was determined at 660 nm. The experiment was run three times, and the percentage of denaturation of the protein was determined.

$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

   B. **Protein denaturation assay using BSA**:- The experiment was carried out by according to the earlier Naz et al., 2017 protocol description. 0.5 ml of the test control solution in this assay included 0.05 ml of DW and 0.45 ml of BSA. Product control included 0.5 milliliters of synthesized acetophenone. The test solution (0.5 ml) contained 0.05 ml of synthesized acetophenone at concentrations of 50, 100, 200, 400, 800, and 1600 µg/ml and 0.45 ml of BSA. Instead of synthetic acetophenone,

0.05ml of piroxicam/DS was added to the normal solution (0.5ml). All of these solutions were heated to 60 degrees Celsius for three minutes after being incubated for 20 minutes at a pH of 6.3. Each tube was filled with 2.5 ml of pH 6.3 PBS after it had cooled to room temperature, and the absorbance was gauged at 660 nm. Three assay runs were conducted, and the % inhibition of BSA denaturation was calculated.

C. **Human red blood cell membrane (HRBC) stabilization assay:-** Three milliliters of blood were obtained for this assay from a healthy volunteer who hadn't taken an NSAID in the previous two weeks. To separate packed cells, blood was combined with Alsever's solution and centrifuged for 15 minutes at 3000 rpm. Three washes with isosaline solution (0.85% w/v NaCl, pH 7.2) were performed on packed cells. Isosaline solution was used to create packed cell suspension (10% v/v), which was employed right away (Ghavipour et al. 2017). 1 milliliter of PBS (pH 7.2), 2 milliliters of hypotonic saline solution (0.36% w/v NaCl), 0.5 milliliters of manufactured acetophenone in various concentrations (similar to the protein denaturation assay), and 0.5 milliliters of blood suspension made up the test solution. Piroxicam/DS was present in the standard solution, but 2 milliliters of DW or DMSO were used in the control solution instead of synthetic acetophenone. After 0.5 hours of incubation at 37° C, each solution was centrifuged at 3000 rpm. At 560 nm, the absorbance of the supernatant in each tube was measured. The test was run three times, and the mean value was determined. It was determined what proportion of HRBC was stabilized.

D. **Proteinase inhibition assay:-** The assay was carried out by using the procedure that Naz et al., 2017 had previously outlined. 60µl of trypsin (0.6µg), 1 ml of generated acetophenone (at quantities comparable to the protein denaturation assay), and 1 ml of tris HCl buffer (25 mM pH 7.4) made up the 2 ml sample solution used in this assay. Whereas the standard solution contained piroxicam/DS in place of the synthesized acetophenone, the blank solution contained tris HCl buffer. After five minutes of incubation at 37° C, 1 milliliter of casein (0.8% w/v) was added to each solution. Solutions were incubated for 20 minutes once more. To halt the reaction, 2 milliliters of 70% v/v perchloric acid was added. Centrifugation was then performed for five minutes at 5000 rpm. At 280 nm, the supernatant's absorbance was measured. The test was carried out thrice and the percentage inhibition of proteinase enzyme was calculated.

2. **Assay of Cyclooxygenase and 5-Lipoxygenase Inhibition**

Lymphocyte Culture Preparation

Human peripheral lymphocytes were cultured in RPMI 1640 (HIMEDIA) supplemented with inactivated fetal calf serum, penicillin, and streptomycin; phytohemagglutinin (HIMEDIA) was employed to stimulate cell proliferation. Following filtering (with 0.2 micron cellulose acetate, Sartorios), 1 × 106 cells/ml of plasma was added, and the culture was cultured for 72 hours. Next, 1 µl of lipopolysaccharide was added, activating the culture once more for 24 hours. Following a 24-hour incubation period, synthesized acetophenone and piroxicam were added at final concentrations of 100, 200, 500, and 1000 µg/ml. The mixture was then centrifuged for 10 minutes at 6000 rpm to facilitate sedimentation. Following the extraction of the supernatant, 50 µl of cell lysis buffer was added, and the combination underwent another centrifugation at 6000 rpm for ten minutes. The anti-inflammatory test was performed according to the method used by Viji and Helen, 2008.

A. **Assay of Cyclooxygenase:-** For the experiments, a combination of hemoglobin, glutathione, tris-HCl buffer, and enzyme was utilized. Arachidonic acid and TCA (10% in 1N HCl, 0.2 ml) were added, and the mixture was then incubated for 20 minutes at 37°C. After adding the TBA (0.2 ml), the mixture was boiled in boiling water for 20 minutes. After cooling, it was centrifuged at 1000 rpm for 3 minutes, and the COX activity at 632 nm was measured in the supernatant (Viji and Helen, 2008).
B. **Assay of 5-Lipoxygenase:-** After dissolving and pipetting linoleic acid (70 mg) and an equal weight of interpolation in 4 ml of nonoxygenated water, sodium hydroxide (0.5 N) and oxygen-free water (25 mL) were added. After being split into tiny 0.5 ml parts, the final solution was washed with nitrogen and frozen. The reaction was carried out using a quartz cuvette with an optical path of 1 cm and a temperature of 25°C. A combination of sodium linoleate (0.2 ml), enzyme (50 ml), and tris buffer (2.75 ml, pH 7.4) was used to perform the OD measurement at 234 nm [6]. The % inhibition was calculated using the following formula:

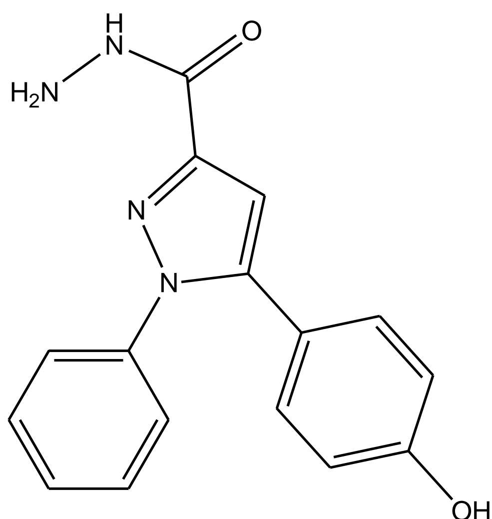
$$\text{Percentage inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

RESULT AND DISCUSSION

Different Heterocyclic Pyrazole derivatives were synthesized using substituted acetophenone.

Amongst the various synthesized derivatives one compound ***5-(4-hydroxyphenyl)-1-phenyl-***

1H-pyrazole-3-carbohydrazide showed appreciable anti-inflammatory response during *In vitro* testing. This compound was synthesized with the help of starting material 4-hydroxy-acetophenone.



5-(4-hydroxyphenyl)-1-phenyl-1*H*-pyrazole-3-carbohydrazide

At 1600µg/ml, the greatest percentage of denaturation of egg albumin was inhibited by synthesized acetophenone (89.16±0.38%). As shown in (figure 1), the produced acetophenone inhibited protein denaturation in a dose-dependent manner, with the greatest effect being shown at the highest dosage. It was examined if the artificial acetophenone could prevent BSA from becoming denaturated. Figure 2 illustrates the maximum percentage denaturation of BSA, as determined by synthetic acetophenone, which is 76.59±0.56% at 1600µg/ml. The RBC membrane was stabilized by the synthesized acetophenone in a dose-dependent manner, with the maximum stability occurring at 1600 µg/ml. Figure 3 illustrates the greatest percentage inhibition of RBC membrane lysis (91.15±0.31%) at 1600 µg/ml when compared to DS (90.01±0.02%) and piroxicam (80.34±0.29%). The highest and lowest levels of trypsinase activity hindrance were seen at 1600µg/ml and 50µg/ml, respectively, for the synthesized acetophenone. The greatest percentage of trypsinase activity suppression was found to be 83.94±0.24. Figure 4 included a discussion of the outcomes. Mean ± S.D. is used to present the results. Tukey's test was performed after two-way analysis of variance (ANOVA) was used to statistically examine the values. The results were deemed significant (p~0.05) when compared to DS and piroxicam. Cyclooxygenase activity analysis revealed how produced acetophenone affected prostaglandin synthesis. According to the findings, piroxicam and generated acetophenone strongly ($p < 0.001$) inhibit cyclooxygenase activity at 1600 µg/ml, with 80.96% and 94.83%,

respectively, as shown in figure 5. The impact of the produced acetophenone on leukotriene synthesis was investigated by measuring the activity of 5-lipoxygenase. Figure 6 demonstrates that piroxicam and manufactured acetophenone both significantly reduce the activity of 5-lipoxygenase ($p < 0.001$), with 79.36% and 93.48%, respectively.

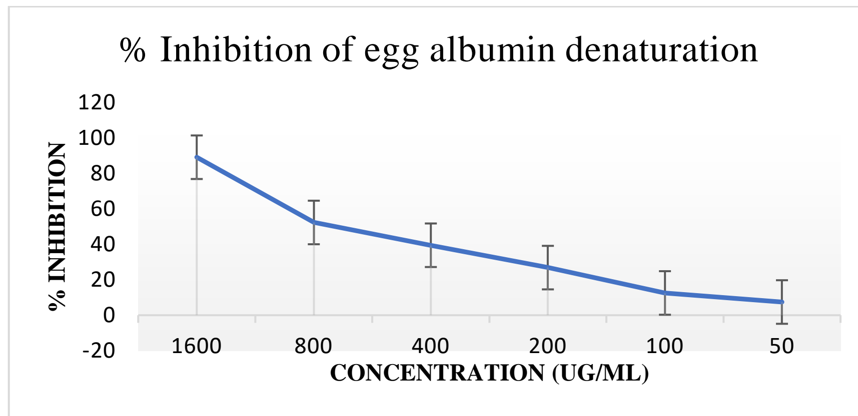


Figure 1:- In vitro anti-arthritic potential activity against egg albumin denaturation

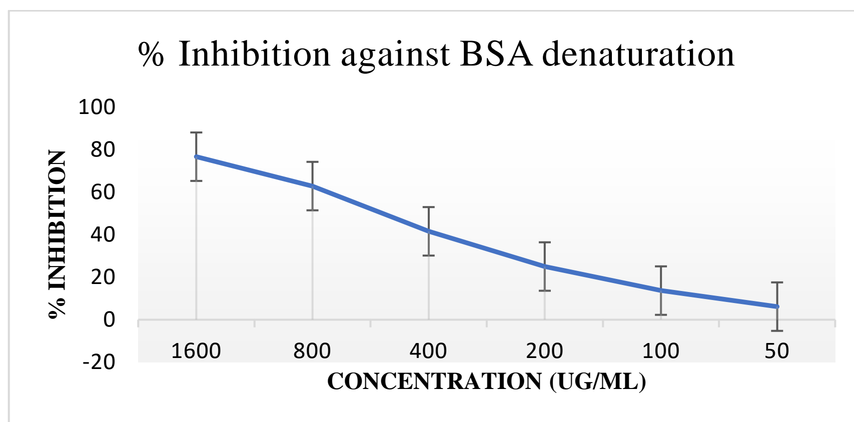


Figure 2:- In vitro anti-arthritic potential activity against BSA denaturation

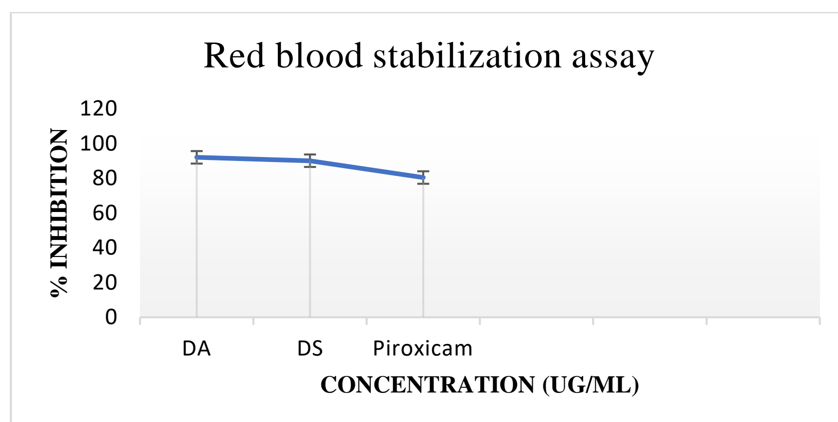


Figure 3:- In vitro anti-arthritic potential activity against Red blood stabilization assay

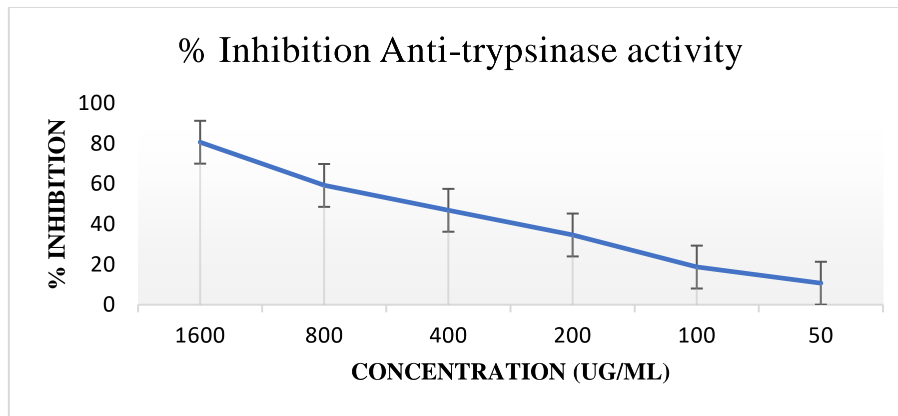


Figure 4:- In vitro anti-arthritic potential activity against Red blood stabilization assay

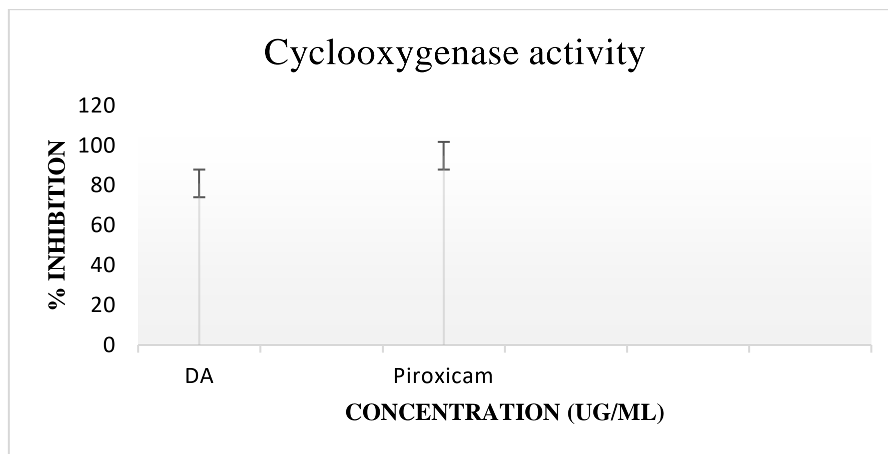


Figure 5:- In vitro anti-arthritic potential cyclo-oxygenase activity

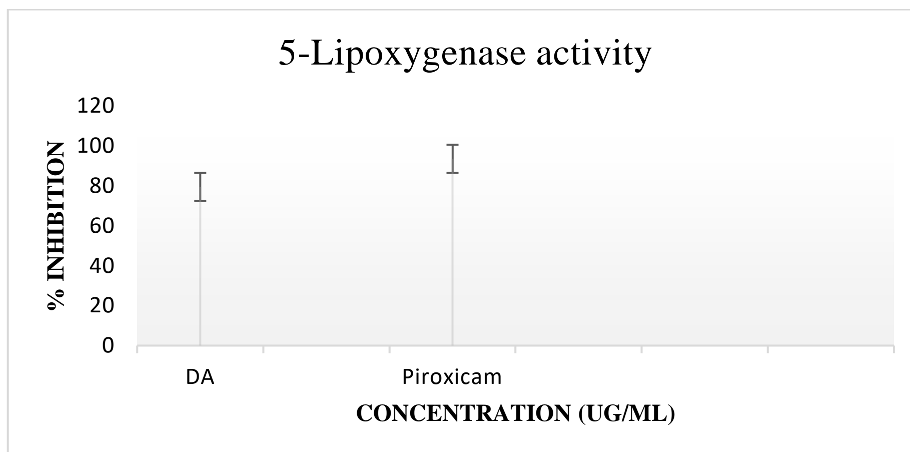


Figure 6:- In vitro anti-arthritic potential 5-Lipoxygenase activity

CONCLUSION

Denaturation of proteins brought on by heat and chemical exposure causes stress and triggers the production of autoantigens that damage the cartilage and synovial membrane of joints. In a dose-dependent way, synthesized acetophenone inhibited the denaturation of egg albumin and BSA at 50, 100, 200, 400, 800, and 1600µg/ml. In the event of arthritis, phospholipase A2 and proteases leak out of lysosomal membrane lysosomes. The membranes of RBCs resemble

lysosomes. Consequently, substances that stabilize the RBC membrane under stress may also be able to stabilize the endogenous lysosomal membrane. In in vitro studies, the synthesized acetophenone showed the strongest anti-arthritic potential.

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