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Evaluation of anti-arthritic activity of aripiprazole through multi models: new potential of an old drug.

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ABSTRACT

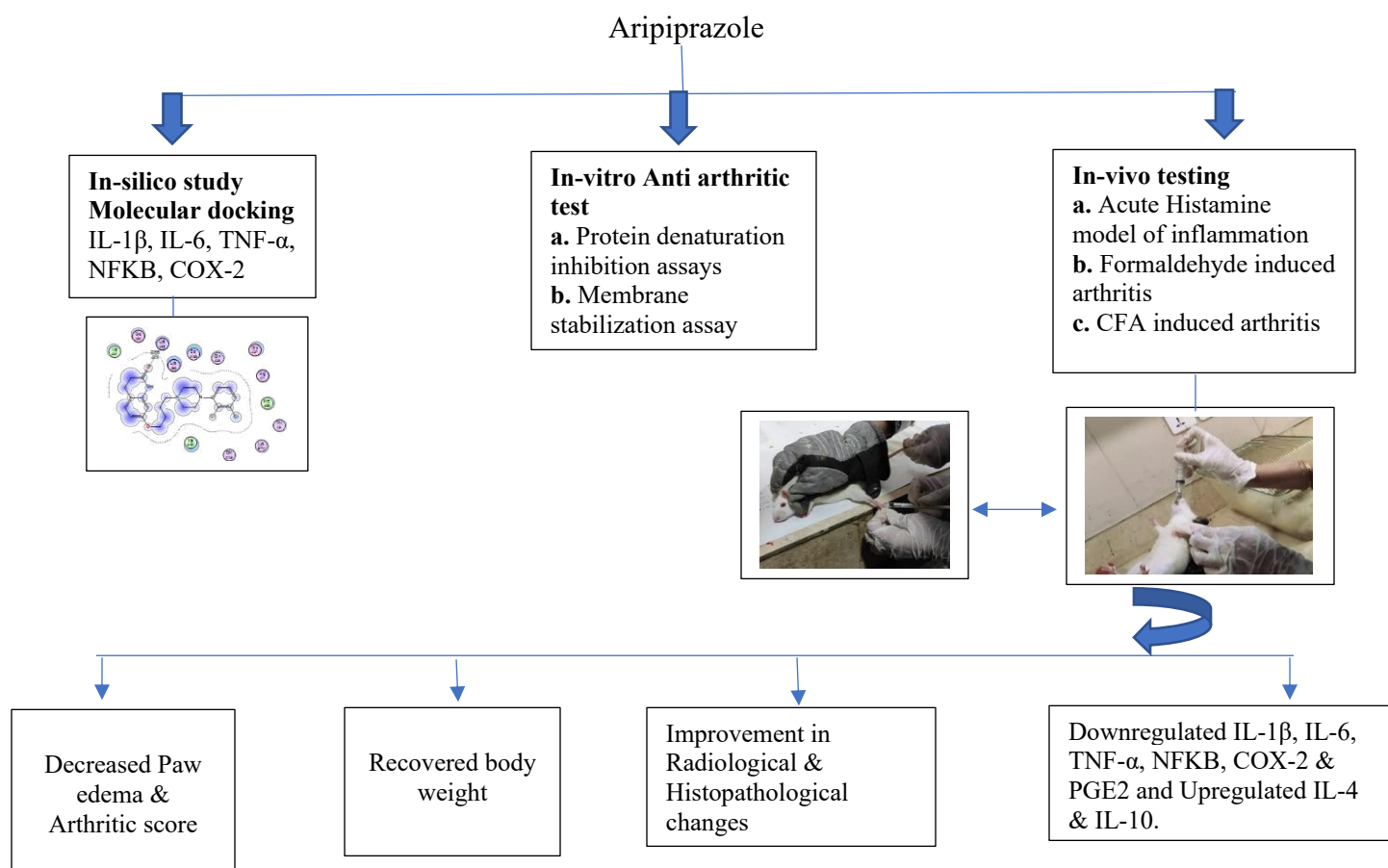
Aim of study: This study was conducted to investigate the antiarthritic effect of three different doses of aripiprazole based on its previously reported anti-inflammatory activity.

Material and methods: Aripiprazole, an atypical antipsychotic, has been used in the present study in animal models. Histamine model was used to verify the documented property of aripiprazole to treat inflammation. *In-silico* evaluation included molecular docking that was done by MOE-Dock of Chemical Computing Group. *In-vitro* studies involved heat evoked BSA protein degradation and egg albumin degradation and maintenance of human RBCs membrane test, whereas *in-vivo* studies encompassed, formaldehyde evoked arthritis & CFA evoked arthritis. Acute histamine model was carried out for four hours. Formaldehyde provoked arthritic disease in Sprague Dawley rats was studied to appraise antiarthritic action of three respective doses of aripiprazole at 5 mg/kg, 10 mg/kg & 15 mg/kg oral dose for 10 days while the time period for study of same activity was twenty eight days with Complete Freund adjuvant and diameter of rat hind paw, arthritic index, body weight, radiological and histopathological investigation of ankles joints were performed. Variety of inflammation causing bio-markers (interleukin-1 β , interleukin-6, nuclear factor-K β , tumor necrosis factor- α , Cox-2, interleukin-4, interleukin -10 and PGE2) had been investigated by real time-polymerase chain reaction and ELISA.

Results: Aripiprazole significantly suppressed paw edema in acute inflammatory model of histamine. Molecular docking analysis disclosed that aripiprazole shows binding interactions with TNF- α , IL6, IL-1 β , NF- κ B and Cox-2. It was evaluated in this study that aripiprazole showed inhibition of protein degradation and stability of human red blood cell membrane in concentration gradient manner, demonstrating maximum activity at concentration of 6400 microgram/ml. Similarly, considerable(****p<0.0001) prevention in hind paw edema and

arthritic score had been shown at dose of 15mg/kg in arthritic models that was interestingly greater than piroxicam. Aripiprazole had recovered body weight alteration as well. Radiographic and histopathological investigation displayed no substantial architectural variations in joints of rats who were given treatment. Inflammatory genes IL-1 β , TNF- α , IL-6, Cox-2, NF-K β and PGE2 were down regulated together with remarkable rise in expression of interleukin-4 & interleukin -10 in treated animals had been revealed by aripiprazole.

Conclusion: Above stated results strongly claim that aripiprazole is an effective antiarthritic therapy as verified through molecular docking, in-vitro and in-vivo assessment & via down regulating proinflammatory markers and up regulation of anti-inflammatory cytokines and can be thought to be an appropriate option to repurpose the drug as anti-arthritic agent.



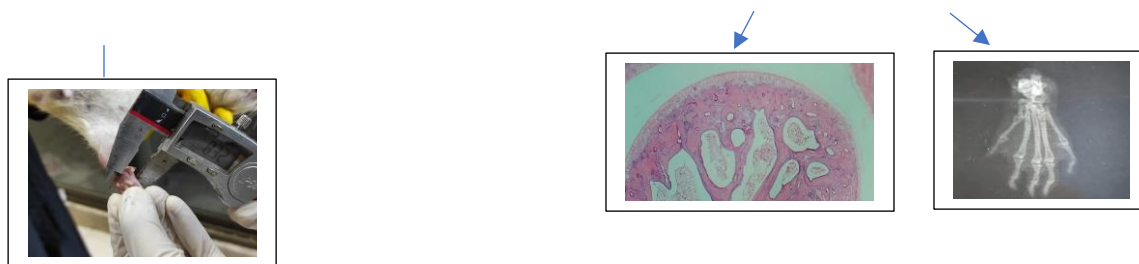


Fig 1: Graphical abstract of present study

1. INTRODUCTION

RA is a long-lasting & autogenerated disease categorized via destruction of bones & joints, pain and inflammation of joints. The etiology of rheumatoid arthritis is unknown as the detailed mechanism of this disease is still under investigation.(Walsmith et al., 2002). It is a common inflammatory disorder affecting major population. The objectives of RA therapy are to stop joint destruction, bone erosion and diminish pain. Most commonly used therapies are NSAIDs, glucocorticoids, anti-TNF- α agents, IL-receptors antagonists, immunosuppressive drugs and (DMARD) disease modifying antirheumatic drugs such as methotrexate, hydroxychloroquine, sulfasalazine, azathioprine & infliximab. But all these therapies are not only correlated with adverse effects, immunosuppression but also with the high expense associated with target specific monoclonal antibody therapy necessitating a dire need of such therapy that has enormous capability to alleviate arthritis with least adverse events in an affordable price range.

Aripiprazole is second generation(atypical), partial agonist of dopaminergic D2 receptors approved by FDA in November 2002 to treat schizophrenia. Its better safety profile is evocative of its use in other disorders such as to avert weight gain related to other antipsychotic or antimanic drugs, for primary psychotic diseases, dementia.(Centorrino et al., 2005). Aripiprazole also shows clinical effectiveness against mania and type 1 bipolar syndrome, behavioral disorders related to autism, tic syndromes, aggressive and disruptive behavioral disease.(Masa'il Greenaway et al., 2009).

Aripiprazole also exhibits anti-inflammatory properties via intensifying anti-inflammatory signaling at cellular and molecular levels. It evidently suppresses the level of interleukin-1 β , interleukin -6, interferon- β , tumor necrosis factor- α , interleukin-12, interleukin-23 with augmentation in the levels of interleukin-10.(Gholampour et al., 2017; Sobiš et al., 2015). From

recently reported studies it is obvious that aripiprazole constrains the mRNA expression of inflammation causing genes including COX-2, inducible NO synthase (iNOS), and TNF- α .(Yoo et al., 2018). Hence considering the actions of aripiprazole in down regulation of inflammatory pathways it was worthwhile to investigate anti-arthritis property of aripiprazole using *in-vitro* and *in-vivo* models. Choosing aripiprazole in this research is based on therapeutic efficacy of aripiprazole in carrageenan induced inflammation in animal model.(Adedayo et al., 2017) & its previously reported study suggesting its anti-inflammatory role in humans with schizophrenia showing that aripiprazole obstructs the inflammation provoking signaling of microglial tissues in schizophrenia. (Yoo et al., 2018).

2. MATERIAL AND METHODS

2.1. Chemicals

Chemicals employed in this research are bovine serum albumin BSA (Sigma-Aldrich,USA), potassium dihydrogen phosphate (Merck, Germany), Sodium chloride (Merck, Germany), disodium hydrogen phosphate (Merck, Germany), Aripiprazole (Wilshire Laboratories(private) LTD Pakistan, Histamine (Sigma-Aldrich,USA), Formaldehyde (Sigma-Aldrich, USA), CFA (Sigma-Aldrich, USA), Piroxicam (Medley Pharmaceutical LTD, Wah Cantt), citric acid (Sigma-Aldrich, USA), sodium citrate (Sigma-Aldrich, USA), D-glucose (Sigma-Aldrich, USA), DMSO (Sigma-Aldrich, USA), Tween 80 (Sigma-Aldrich, USA), cDNA synthesis kit (Zokeyo kit), PCR kit, Syber green Master mix (RT- PCR) (Wizbio), Trizole (Bioshop, Bioworld), PCR Loading Dye (ROX dye), DNA Ladder (Thermo-scientific, America), RNase free water (Thermo-scientific, America), PGE2 ELISA kit (Bioassay Technology Laboratories).

2.2. Animals

Sprague-Dawley rats of both sexes (140–300 g), young and healthy rats had been placed in animal light regulator house (twelve hour/well-lit/shady cycle) under standard condition of temperature (25°C) and 60% relative humidity and were given food and water *ad libitum*. Animals were treated & handled following the principle guidelines of Animal ethics committee at Department of Pharmacology, Punjab university college of pharmacy, University of Punjab. (Approval No.D/105/FIMS).

2.3. Paw edema induced with histamine-acute model of inflammation

Rats had been grouped into 6 groups having three rats in every group. 3 ml/kg of distilled water was given to normal control group (Group I). Group 2 was designated as disease control group, provided with histamine injection only. Group 3-Standard group was given piroxicam (10 mg/kg) orally. While remaining three groups, group 4,5,6 were given 5mg/kg, 10mg/kg, 15mg/kg of aripiprazole respectively. After thirty min, every rat was given injection of 0.1ml (1%) histamine at sub-planter area. Paw diameter was measured with Vernier caliper at 0 min & at one hourly interval for four hours. (Abbas & Ansari, 2021) Percentage inhibition had been estimated by the formula.

$$\% \text{ inhibition of edema} = \frac{C-T}{C} \times 100$$

where, T and C are paw diameter of treated and diseased rats.(Qasim et al., 2020; Shabbir et al., 2018)

2.4. In silico study-Molecular docking

Molecular docking analysis was conducted to assure anti-arthritis property of aripiprazole through molecular operating environment (MOE-Dock) of Chemical Computing Group Inc. version 2015.10. Three dimensional structures of interleukin-1 (protein data bank ID: 1ITB), tumor necrosis factor-(protein data bank ID: 2AZ5), nuclear factor-KB (protein data bank ID: 1SVC), and interleukin-6 (protein data bank ID: 1N26) had been repossessed through Protein Data Bank. Aripiprazole structure was retrieved from Pubchem. Missing atoms and ions were checked in protein file. On the protein, hydrogen and fractional charges were given & file was saved for docking. Drug was docked via MMFF94 force field (MOE 2013.08), which kept the bulky atoms constant till RMSD activity of 0.05kcal mol⁻¹ had been achieved. Assessment of reactive sites using MOE's Site Finder tool. Active site is a massive space covering co-crystalline ligands. Each target's binding pocket was docked with drug and top binding positions were selected for further study. Docked compound had been used to estimate the docked connections at active position of protein.

2.5. Egg albumin degradation suppression

5ml test solution had been composed by adding 0.2ml hen's egg albumin in 2.8ml phosphate buffer (pH 6.4) and 2ml of different concentrations (100-6400 µg/ml) of aripiprazole and piroxicam, separately. Same amount of distilled water was used as control. Test solutions were

incubated for fifteen minutes at $37 \pm 2^{\circ}\text{C}$ and then for five minutes at 70°C . After cooling, spectrophotometer was used to determine absorbance at 660 nm. Percentage suppression in albumin degradation had been appraised by the formula.(Bashir et al., 2020)

$$\% \text{ inhibition} = 100 \times [(A)/(B)-1]$$

Where A is absorbance of test sample and B is absorbance of control.

2.6. Suppression of bovine serum albumin degradation

Anti-arthritis activity of aripiprazole was evaluated via bovine serum albumin degradation preservation test. (Bashir et al., 2020). Several concentrations (100, 200, 400, 800, 1600, 3200, 6400 $\mu\text{g/ml}$) of aripiprazole and piroxicam (standard treatment) had been composed. 0.5ml Test control mixture contained 5% w/v aqueous solution of BSA 0.45 ml and distilled water 0.05 ml. Similarly, 0.5 ml product control contained test solution 0.05 ml and distilled water 0.45 ml. Test solution of 0.5ml comprised BSA 0.45 ml together with 0.05ml of test solution of aripiprazole. Standard solution 0.5ml prepared by taking piroxicam 0.05 ml and BSA 0.45 ml. These reaction mixtures were preserved at 6.3 pH then incubated at 37°C for twenty min. After this reaction mixtures were heated at 57°C for thirty minutes.

2.5ml of phosphate buffer was poured in these solutions. Spectrophotometer had been used to assess absorbance at 660 nm and percentage preservation of BSA degradation had been estimated using formula.(Alamgeer et al., 2019; Bashir et al., 2020).

$$\% \text{ Inhibition} = 100 - [(A - B) / C] \times 100$$

Where A is absorbance of Test solution, B is absorbance of product control & C is absorbance of test control.

2.7. Human red blood cell membrane stability test

5ml blood of physically fit human had been collected and similar amount of Alsever's solution was added and then solution was centrifugated at 3,000 rpm. Packed cells at the bottommost had been separated & washed using normal saline and a 10% suspension was prepared using normal saline. 4ml test solution comprising 2ml hypotonic saline, 1ml of phosphate buffer, 0.5 mL of test samples of aripiprazole at various concentrations (100, 200, 400, 800, 1600, 3200, 6400 $\mu\text{g/ml}$) and 10% v/v HRBCs (0.5ml) were prepared. Also, test control having composition similar to test solution excluding the several concentrations of test samples was prepared.

Standard solution contained similar constituents as test solution excepting that it has piroxicam in place of test samples of aripiprazole. These mixtures had been incubated for thirty minutes at 37°C & then centrifugated at 3000 rpm. Spectrophotometric absorbance had been determined at 560 nm. Percentage stability from lysis was figured out by the formula.(Bashir et al., 2020)

$$\% \text{ inhibition} = 100 - [(A)/(B) \times 100].$$

Where A is optical density of sample and B is optical density of control.

2.8. Formaldehyde-triggered arthritis

Rats had been grouped into different groups having three to six rats in every group. 3 ml/kg of distilled water was given to normal control group (Group I), Group II-disease control group had administered 0.1ml sub-planter injection of 2% formaldehyde. Group III-standard group had been given 10 mg/kg of piroxicam whereas the other 3 groups, group IV, V & VI were given 5mg/kg, 10mg/kg, 15 mg/kg of aripiprazole correspondingly. Drugs were given orally to the rats. Oral route was used for administration to rats. At first day, after thirty minutes of orally administrating drugs to rats, non-immunological acute arthritis had been incited via sub-planter injection of 2% (0.1 ml) formaldehyde, the injection was repeated at third day. Administration of drugs had been continued for ten days. Arthritic disease had been estimated via determination of rise in diameter of hind paws for ten days through vernier calliper. Percentage suppression in paw edema had been assessed though the formula.

$$\% \text{ inhibition of edema} = C - T / C \times 100$$

where, T and C are the paw diameter of treated and diseased rats.(Qasim et al., 2020; Shabbir et al., 2018)

2.9. Complete Freund's Adjuvant evoked arthritis

Sprague Dawley rats had been grouped into six groups having three to six rats. 3 ml/kg of distilled water was given to normal control group (Group I). Whereas, group II was designated as the diseased control group and was given vehicle only. Group III had been provided 10 mg/kg/day piroxicam via per oral route. Group IV, V and VI were given 5mg, 10mg, 15mg aripiprazole correspondingly. Arthritis was evoked by injecting 0.1 ml CFA in sub-plantar area of hind paw at day zero. The drugs had been continued for twenty eight days. Clinically arthritis was assessed through investigation of different parameters. Throughout the period of 28 days of CFA model, rat body weights were assessed every 7th day(Cheng et al., 2015). Diameter of hind

paws were measured using a vernier scale on day 3, 7, 14, 21, 28. (Gomaa et al., 2009; Hasan et al., 2015). Percentage suppression in paw edema was evaluated by the formula.

$$\% \text{ inhibition of edema} = C-T/C \times 100$$

where, T and C are the paw diameter of treated and diseased rats. (Qasim et al., 2020; Shabbir et al., 2018). Animals had been observed on 0, 7, 14, 21 and 28 days to investigate the severity of arthritis through macroscopic score system. (Qasim et al., 2020). Arthritic index had been estimated by addition of score for every paw as normal paw; 1=redness of toe; 2=redness and inflammation of paws; 3: inflammation of ankles; 4= inflammation of the full leg and inability to move it. (Gomaa et al., 2009). On twenty eighth day animals had been sacrificed after anesthesia to collect blood samples for Elisa test to estimate levels of PGE2 and to assess mRNA level of IL-1 β , IL-6, NF-K β , TNF- α , Cox-2, IL-4, IL-1 through PCR. (Shabbir et al., 2016). Hind paws of these animals had separated for histopathological and radiological examination. (Alamgeer et al., 2017; Cheng et al., 2015).

2.10. Radiological and Histopathological investigation

At 28th day of CFA model ankle joints of rats were removed for radiology and histopathology of normal control, diseased and treated rats in order to evaluate the degree of complications of RA.

2.11. Enzyme-linked immunosorbent assay (ELISA) for PGE2

Elisa test for PGE2 and was performed with serum in accordance with kit manufacturer's guidelines (Bioassay Technology Laboratory). Samples had been operated and optical density was estimated by a microplate reader set to 450nm.

2.12. Estimation of mRNA levels of IL-1 β , IL-6, NF-K β , TNF- α , Cox-2, IL-4, IL-10

TRIzol methodology was applied for extraction of RNA from blood samples and Nanodrop reader was employed to measure it. cDNA synthesis was performed according to the kit manufacturer's guidelines. (Zokeyo kit, Korea). RNA was transformed into primary strand of complementary DNA (cDNA). Amplification of cDNA was done through gene particular primers with SYBR Green (2 $\times$) PCR master mix (WizPure). Bio-Rad (thermocycler) system was used to measure CT value which was further used to calculate concentration of genes. (Lim et al., 2017; Shabbir et al., 2016)

2.12. Statistical Analysis

Graph-pad prism software version 8.0.1(244) had been used for statistical computation of results. Analysis was done through two-way analysis of variance (ANOVA) and one-way ANOVA to compare the various groups. Whole data had been presented as mean $\pm$ Standard Deviation (Mean $\pm$ SD). $p < 0.05$ was decided to be as limit of significance.

3. Results

3.1. Inhibitory effect of aripiprazole in histamine induced inflammatory model.

Anti-inflammatory property of aripiprazole was revealed via acute-histamine model of inflammation. Histamine administration in the diseased control animals showed significant increase in paw diameter. While the hind paw assessment completed at 4th hour demonstrated that administration of 15 mg/kg of aripiprazole showed significant ($p < 0.0001$) prevention of paw edema showing percentage inhibition of (56.10 %) and diameter of 3.13mm (± 0.046) in comparison to the disease control rats showing diameter of 7.13mm (± 0.064), whereas the reference drug offered significant ($p < 0.0001$) percentage protection against paw swelling of (55.82%) and the paw diameter of 3.15mm (± 0.052) as presented in figure 2.

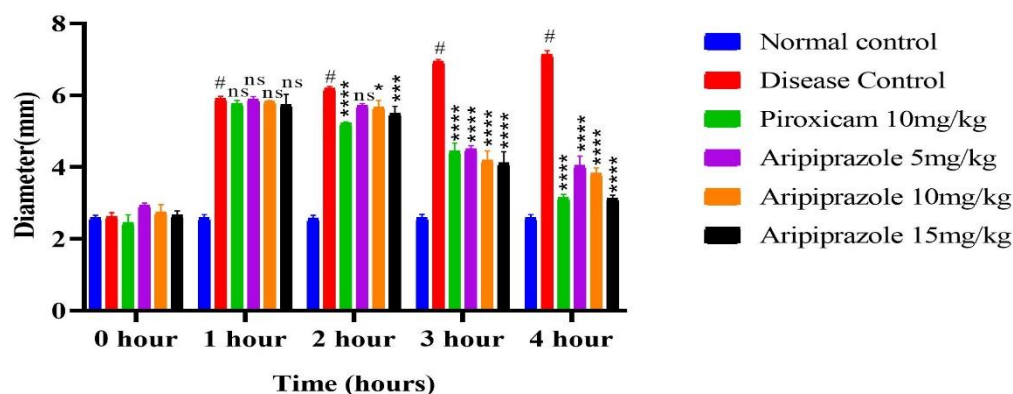


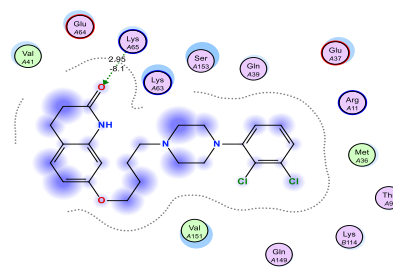
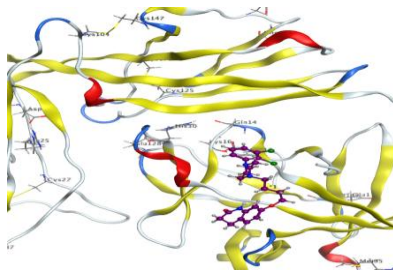
Figure 2: Paw diameter in histamine induced acute model shown by aripiprazole and piroxicam in a dose dependent manner. Values are specified as Mean $\pm$ SEM. Where **** $p < 0.0001$, *** $p < 0.001$, * $p < 0.05$ while ns is nonsignificant, in comparison with disease control group and # is $p < 0.0001$ as compare to normal control group, performed by two-way ANOVA test following Bonferroni analysis.

3.2. Molecular docking result

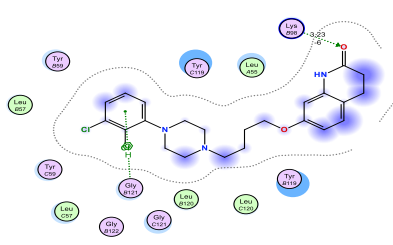
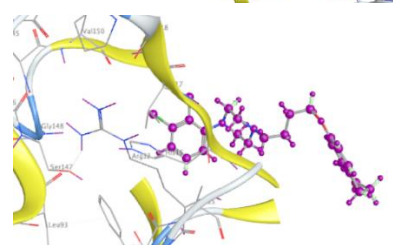
Three dimensional structures of pro-inflammatory parameters including IL-1 (Protein data bank ID: 1ITB), TNF- (Protein data bank ID: 2AZ5), NF-KB (Protein data bank ID: 1SVC), and IL-6

(Protein data bank ID: 1N26) were reclaimed from protein data bank. Multiple sites of aripiprazole-target complex were identified to forecast the predilection for binding sites and pattern of ligand-target communication from which the top most binding positions were selected for further assessment. Aripiprazole presented powerful binding interaction with target proteins that is verified through docking analysis as shown in figure 3.

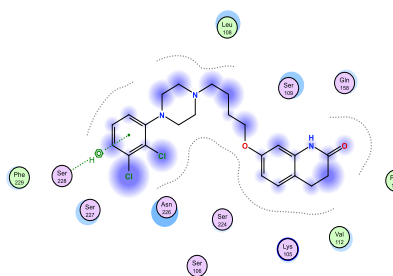
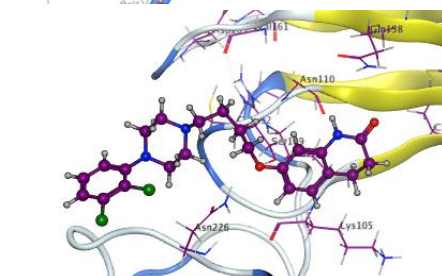
Interleukin-1 beta



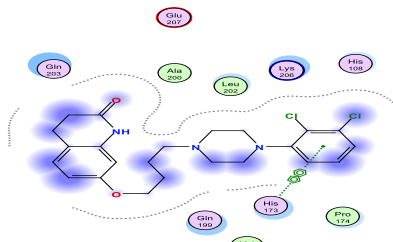
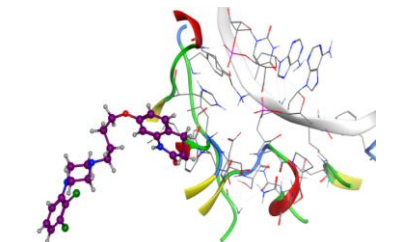
TNF-alpha



IL-6



NF-KB



Cox-2

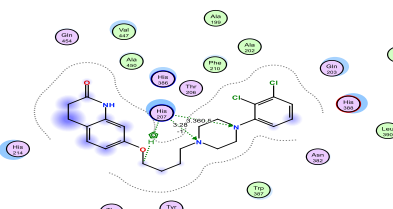
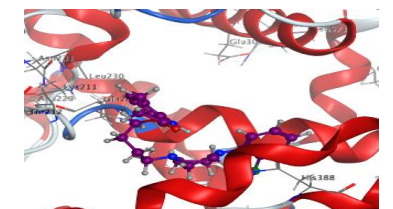


Figure 3: Three-dimensional ligand communication depiction of aripiprazole. Aripiprazole with IL-1 β , TNF-alpha, IL-6, NF-kB, Cox-2 complexes.

3.3. Inhibitory effect of aripiprazole against heat provoked egg albumin denaturation.

Inhibitory results of aripiprazole against degradation of egg albumin protein triggered by heat are portrayed in figure 4(a). Aripiprazole and standard drug piroxicam displayed concentration gradient suppression of albumin degradation. Aripiprazole at the highest concentration of 6400 μ g/ml presented (613.33%) percentage suppression of albumin degradation that is interestingly greater than the percentage inhibition demonstrated by reference drug piroxicam (603.84%) at the equivalent concentration as shown in figure 4(a).

3.4. Inhibitory results of aripiprazole against heat induced BSA denaturation

Aripiprazole at various concentrations delivered marked shielding effect in a concentration gradient pattern against degradation of bovine serum albumin triggered by high temperature as revealed in figure 4(b). Findings obtained represented a highest percentage inhibition of (88.02%) at the highest concentration of 6400 μ /ml that is prominently greater than the inhibition revealed by the piroxicam (79.16%). Consequently, aripiprazole had been proven to be an effective drug in providing protection against degradation of BSA protein.

3.5. Protective impact of aripiprazole on membrane of RBCs

Aripiprazole provided protection to red blood cell membrane from rupturing caused by hypotonicity in a concentration dependent pattern as shown in figure 4(c). The maximum protection (84.98%) had been revealed at the highest concentration of 6400 μ g/ml of aripiprazole and the results of the present investigation are comparable to the results of reference drug showing (83.26%) of stabilization of red blood cells membrane. Hence, this confirms that aripiprazole can depicts antiarthritic property through the stabilization and maintenance of red blood cells.

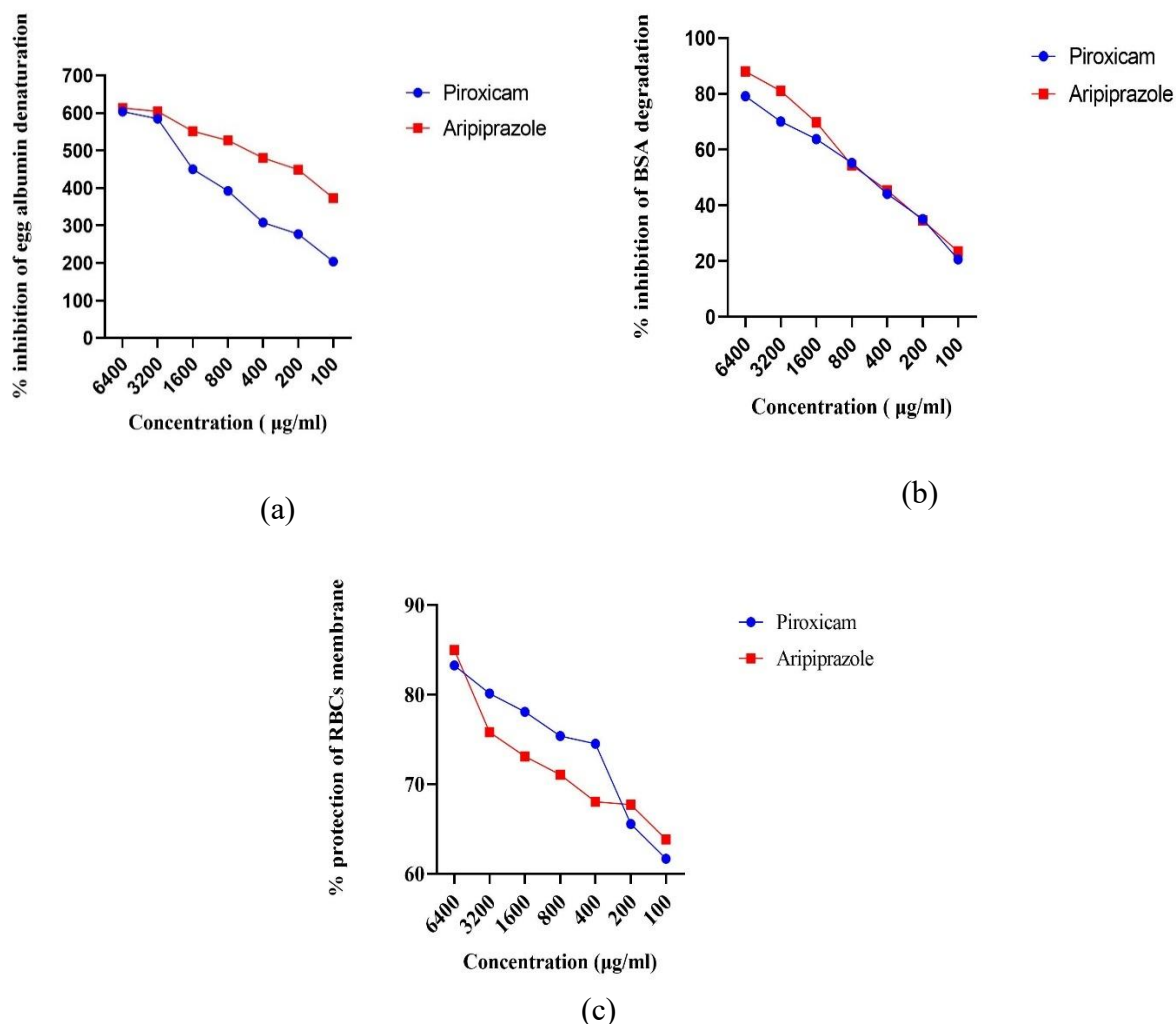


Figure 4: Percentage inhibition of aripiprazole and piroxicam against thermally induced denaturation of egg albumin protein (a), bovine serum protein (b) and stabilization of HRBCCs membrane (c) in a concentration gradient manner. Values are specified as Mean $\pm$ SEM (n=7).

3.6. Defensive role of Aripiprazole in formaldehyde induced arthritis

Antiarthritic potential of aripiprazole had been observed through formaldehyde evoked arthritic disease in animals. Administration of injection formaldehyde in disease control animals showed significant increase 8.26mm (± 0.066) in diameter of hind paws. In contrast, piroxicam delivered significant ($****p < 0.0001$) prevention of rise in diameter showing (61.62%) percentage protection from swelling of paw and diameter of 3.17mm (± 0.137). Similarly, aripiprazole suppressed increase in paw diameter in dose dependent manner. 15 mg/kg of aripiprazole showed significant ($****p < 0.0001$) suppression of in paw edema showing percentage inhibition (64.14%) and paw diameter of 2.94mm (± 0.044) comparative to the disease control rats as depicted in figure 5. Body weight alteration observed at regular interval in formaldehyde model for rats of all treatment groups showed continuous decrease in body weights of rats of arthritic

control group who had provided no treatment. On the other hand, aripiprazole treated group and piroxicam group depicted prevention in fall in body weight of rats as presented in figure 5.

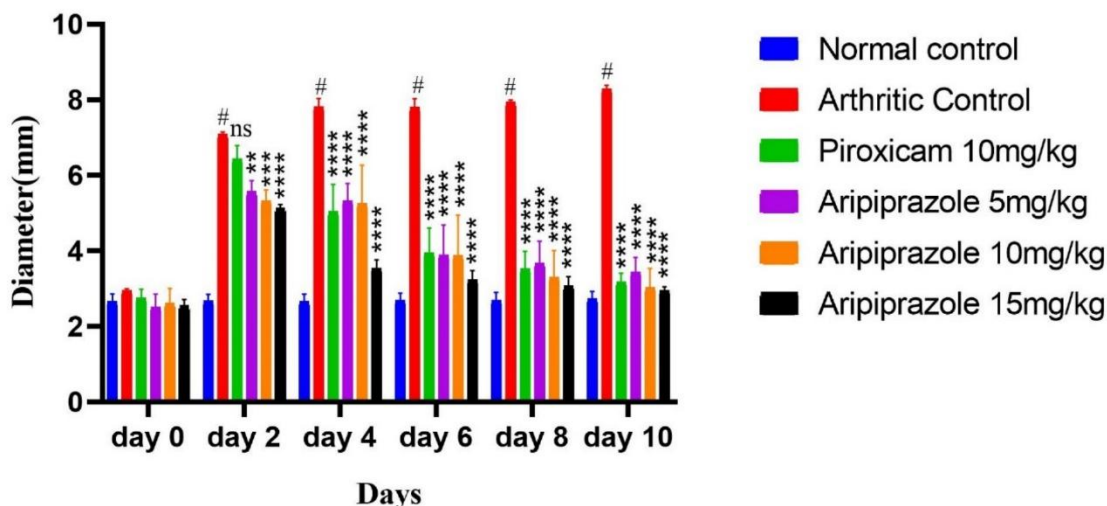


Figure 5: Paw diameter of animals administered with aripiprazole and piroxicam in formaldehyde provoked arthritic model in concentration gradient pattern. Values are specified as Mean $\pm$ SEM. Where****p < 0.0001, ***p < 0.001, **p < 0.01, ns is nonsignificant, in comparison with disease control group and # is p < 0.0001 as compare to normal control group, performed by two-way ANOVA test following Bonferroni analysis.

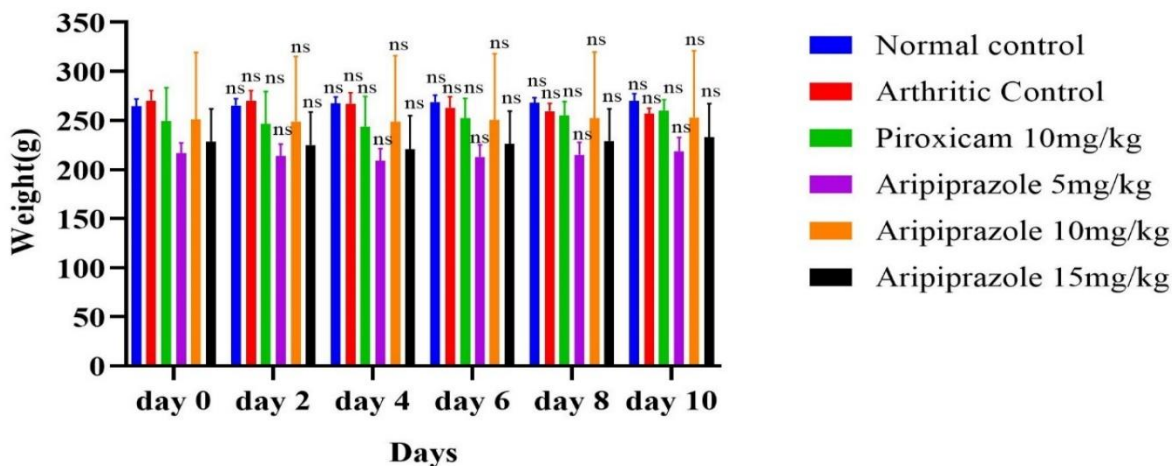


Figure 6: Impact of aripiprazole and piroxicam on rat body weight alteration in Formaldehyde provoked arthritic disease. Values are specified as Mean $\pm$ SEM. Where ns is non-significant, when weight of each group at different days is compared with weight at day 0, by two-way ANOVA test following Bonferroni analysis.

3.7. Protective impact of aripiprazole on physical parameters of CFA induced arthritis.

Rats divided in different groups were investigated for evaluating complications of disease through assessment of paw diameter, arthritic index and body weight alteration. The hind paw assessment carried out on day 28 exhibited that disease control rats were showing significantly increased paw diameter of 11.90mm (± 0.168), on contrary, the treatment with piroxicam delivered significant ($****p < 0.0001$) inhibition of paw edema 3.95mm (± 0.124) and percentage inhibition of diameter of rat paws (66.80%). Likewise, treatment with aripiprazole has markedly inhibited paw edema in a concentration gradient manner. Administration of 15 mg/kg of aripiprazole presented significant ($****p < 0.0001$) suppression in paw edema showing percentage inhibition (70.58%) that was interestingly greater than piroxicam and paw diameter of 3.5mm (± 0.105) as presented in figure 7. Similarly, in disease control group the maximum arthritic score of 4 (± 0.00) revealed that edema has spread from ipsilateral to contralateral limbs. On the other hand, rats who had been given standard drug piroxicam had shown significant ($****p < 0.0001$) decrease in arthritic score 1.3 (± 0.333). In the same way, aripiprazole 15mg/kg has significantly ($****p < 0.0001$) reduced arthritic score with suppression of edema confirmed by arthritic score 1 (± 0.00) recorded on 28th day of model in contrast to disease control rats as shown in figure 8. In the same way findings obtained after 28 days of CFA model showed the continuous decline in rat body weight of disease control group. However, marked suppression in fall in body weight of rats had been documented in piroxicam treated and aripiprazole treated animals at day 28 as depicted by figure 9.

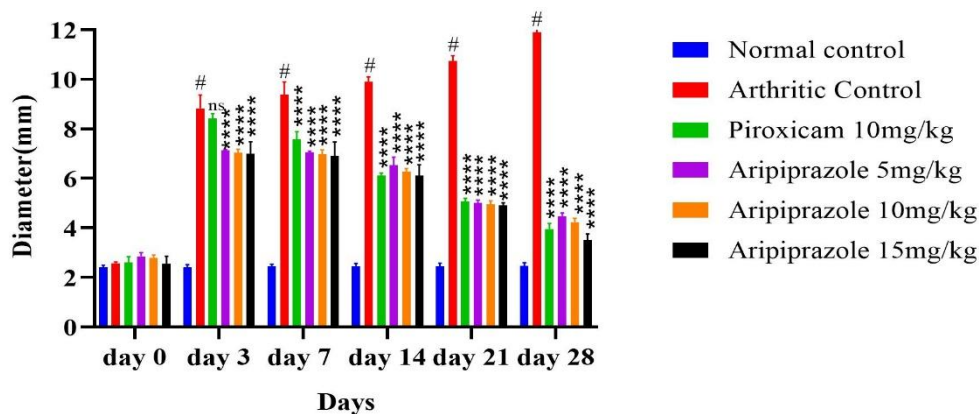


Figure 7: Paw diameter of rats treated with aripiprazole in CFA provoked arthritis. Values are specified as Mean $\pm$ SEM. Where $****p < 0.0001$, ns is nonsignificant, in comparison with

disease control group and # is $p < 0.0001$ as compare to normal control group, performed by two-way ANOVA test following Bonferroni analysis.

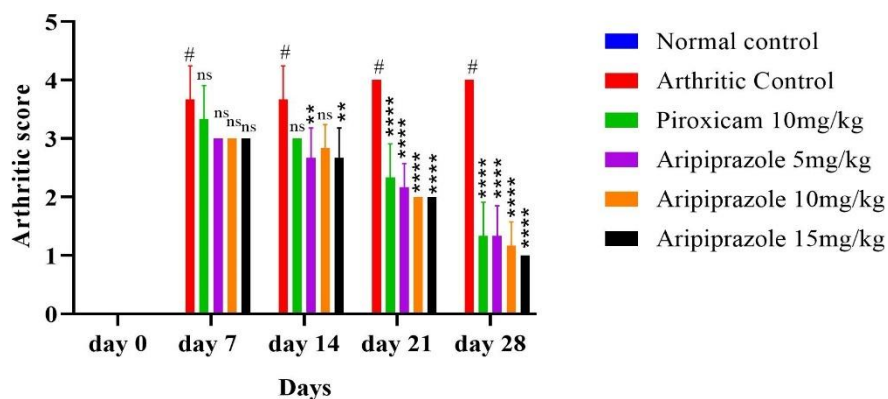


Figure 8: Action of Aripiprazole on arthritic score in CFA evoked arthritic model. Values are stated as Mean $\pm$ SEM. Where **** $p < 0.0001$, ** $p < 0.01$, ns is nonsignificant, in comparison with disease control group and # is $p < 0.0001$ as compare to normal control group, performed by two-way ANOVA test following Bonferroni analysis.

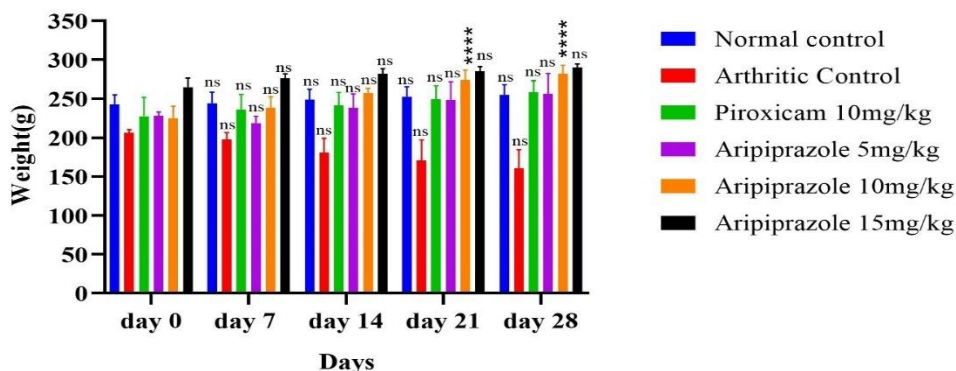


Figure 9: Impact of aripiprazole in body weight alteration in rats in complete Freund adjuvant provoked arthritic rats. Values are stated as Mean $\pm$ SEM. Where, **** $p < 0.0001$, ns is nonsignificant when weight of each group at different days is compared with weight at day 0 by two-way ANOVA test following Bonferroni analysis.

3.8. Defensive impact of aripiprazole on radiologic changes in CFA model.

X-ray investigation of normal control animals exposed no inflammation of ankle joints, regular bone structure and cartilage appearance. Whereas, radiographic assessment in arthritic control animals revealed soft tissue edema, contraction of joint spacing and bone destruction in ankle joints. In contrast, aripiprazole prevented soft tissue inflammation, joint spacing contraction and erosion of bone. Similarly, suppression in soft tissue edema, erosion of bones, decline in ankle joint spacing and joint fluctuations had observed in animals who were given treatment with reference drug piroxicam as shown in figure 10.

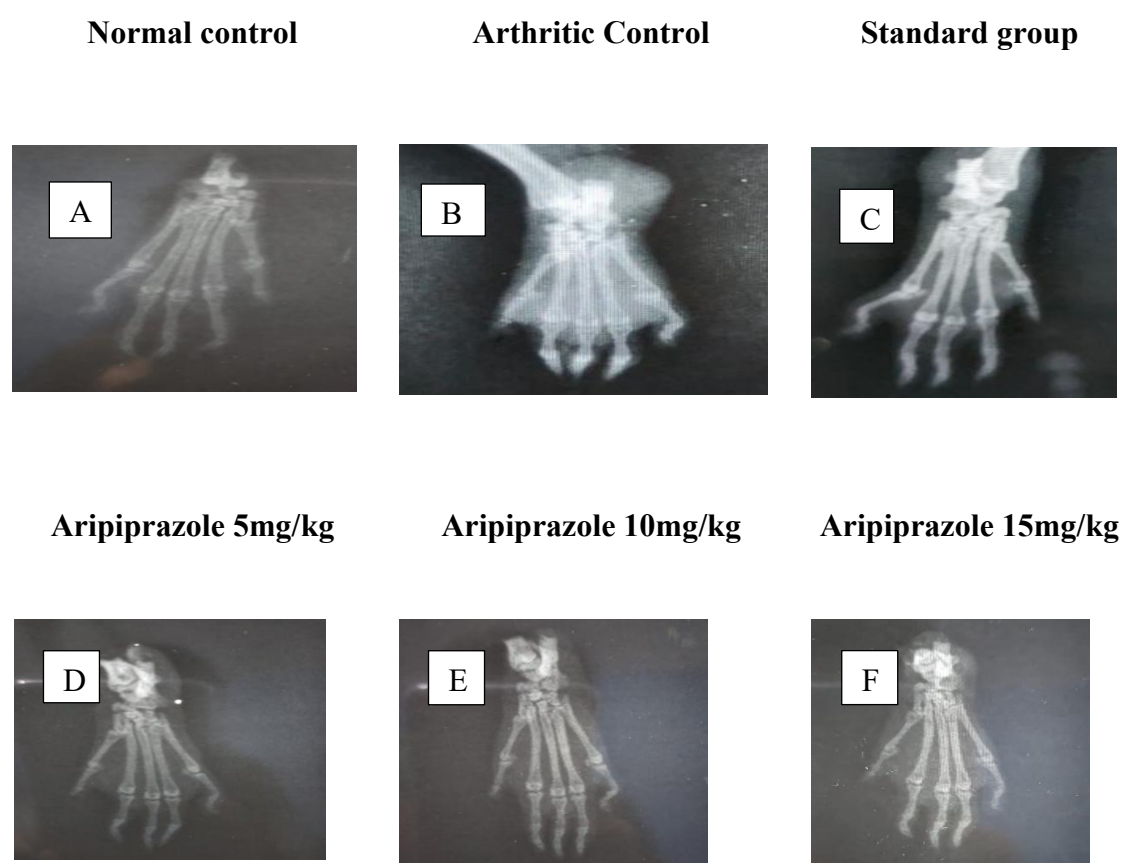


Figure 10: Radiologic examination of hind paw of animals of different treatment groups in complete Freund adjuvant provoked arthritic model.

3.9. Protective effect of aripiprazole on histopathology in CFA provoked arthritic rats

Histological examination of tissue slices of ankle joint was done at 28th day, after the completion of CFA model. Normal rats had integral histopathological structure of synovium and cartilage tissue. However, arthritic control animals presented cartilage damage, invasion of inflammatory

cells with development of granuloma, obvious pannus development, distorted synovial tissue line, collection of collagen fibers and erosion in cartilage and bone and lymphocytes accumulation as portrayed in figure 11. But, reference drug piroxicam (10 mg/kg) presented minor cartilage destruction, moderate invasion of inflammatory cells and intact synovium line & restriction of hyperplasia. Aripiprazole (5mg/kg) also displayed moderate cartilage damage and erosion, deposition of collagen fiber, less disturbed synovial lining. While, aripiprazole (10mg/kg) showed minor cartilage degradation, some inflammatory cells, less lymphocytes accumulation. In the same way, treatment with aripiprazole (15mg/kg) displayed substantial protection against cartilage injury, intact synovial lining, few inflammatory cells with no pannus development, preserved morphology almost similar to normal joint structure of hind paws.

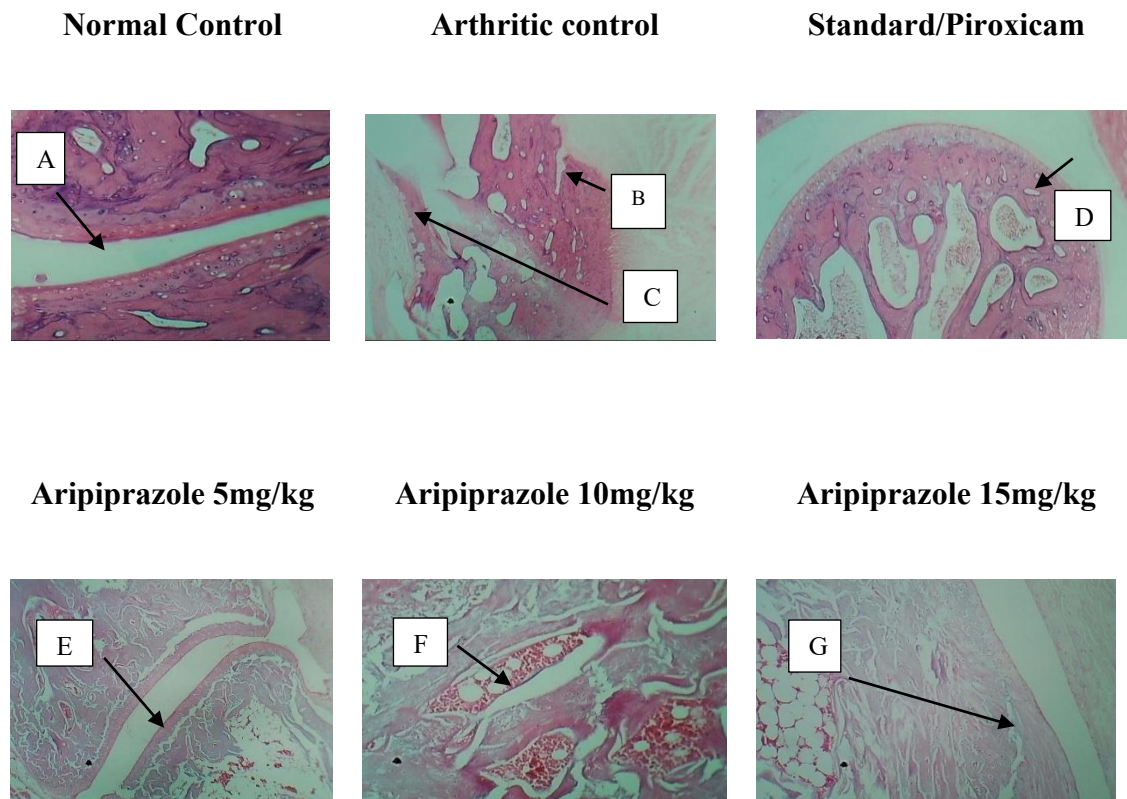


Figure 11: Impact of aripiprazole on histological assessment of rats in complete Freund adjuvant provoked arthritic model. Slides of normal control group rats did not show cartilage erosion, pannus formation and has intact synovial lining (A). Severe erosion with damaged synovial lining (B) and severe pannus formation as well as hyperplasia (C) was seen in arthritic control group (B-C). Piroxicam treated animals showed less erosion and intact synovial lining (D).

Aripiprazole exhibited minimum erosion and infiltration of lymphocytes, preserved morphology of synovial joint. (E-G).

3.10. Downregulation of PGE2 by aripiprazole through Elisa testing.

PGE2 level of different treatment groups had been observed through Elisa test. A considerably raised levels of PGE2 381.222 ng/ml (± 1.888) was detected in arthritic control animals. However, significant ($****p < 0.0001$) reduction in level of PGE2 as 241.694 ng/ml (± 8.505) in piroxicam group, and significant ($****p < 0.0001$) decrease of 222.055 ng/ml (± 4.760) was detected at highest dose of aripiprazole 15mg/kg comparative to disease control animals as presented in figure 12. Hence, aripiprazole considerably reduced PGE2 level in dose dependent manner and this reduction was unusually greater than piroxicam reduction of PGE2.

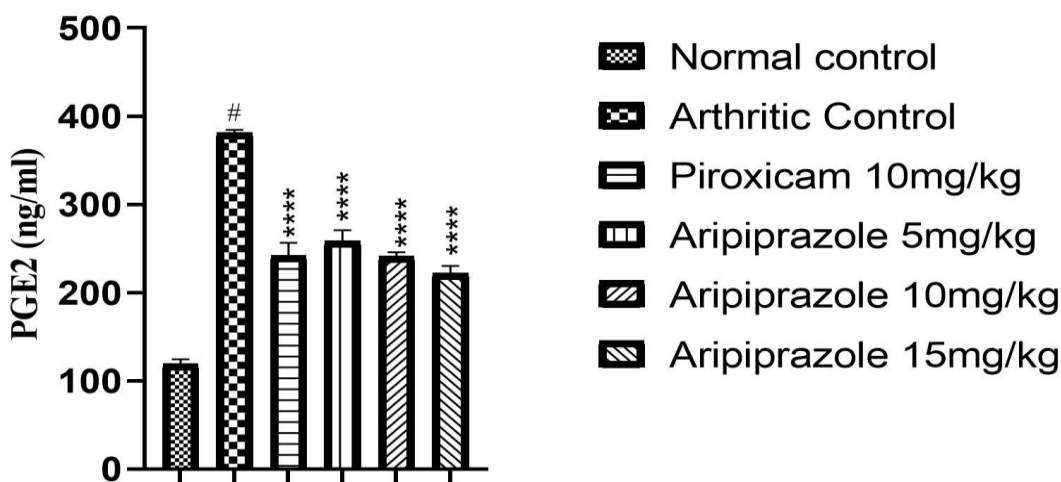
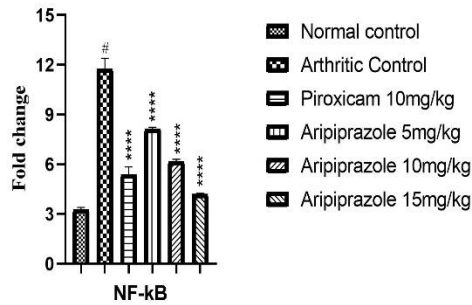


Figure 12: Impact of aripiprazole at three different doses on expression of PGE2 through ELISA. Values are stated as Mean $\pm$ SEM (n-3). Where, $****p < 0.0001$ as compare to disease control group & # is $p < 0.0001$ as compare to normal control group, performed by one-way ANOVA test following Dunnett analysis.

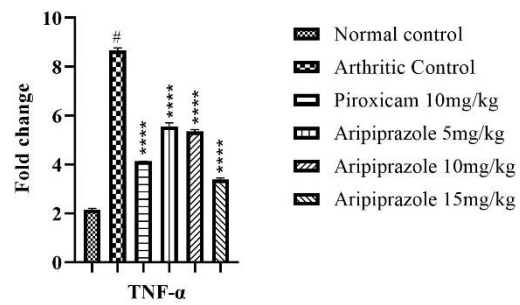
3.11. Downregulation of mRNA level of NF- κ B, IL-1 β , TNF- α , IL-6, Cox-2, and augmentation of IL-10 and IL-4 by aripiprazole in PCR analysis.

Blood samples of various treatment groups were subjected to gene expression analysis of NF- κ B, IL-1 β , TNF- α , IL-6, Cox-2, IL-10 and IL-4 through real time PCR test. Level of NF- κ B, IL-1 β , TNF- α , IL-6, Cox-2 were considerably increased in arthritic control group with a remarkable decline in IL-4 & IL-10 expression in diseased control animals. Whereas, a significant ($****p < 0.0001$) downregulation of fold change of NF- κ B, IL-1 β , TNF- α , IL-6, Cox-2 in dose dependent manner was shown by aripiprazole as well as standard drug piroxicam, the impact of

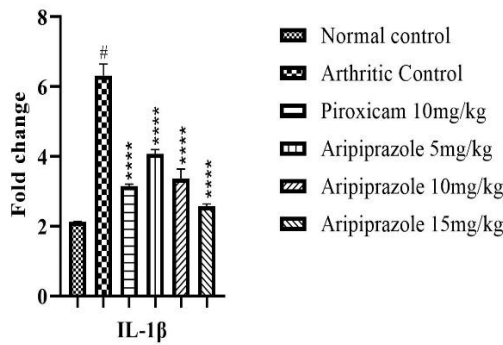
which is amusingly lower than aripiprazole. And significant (**** $p < 0.0001$) upregulation of IL-10 and IL-4 was exhibited in dose dependent manner by aripiprazole and piroxicam comparative to disease control group as shown in figure 12 (a-g).



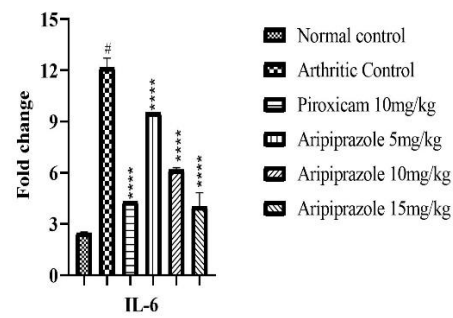
(a)



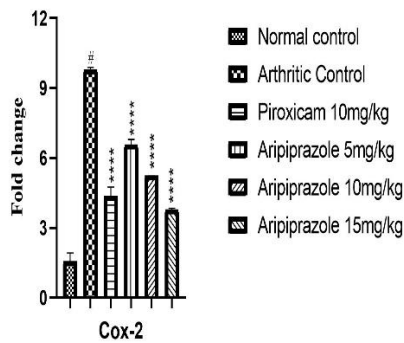
(b)



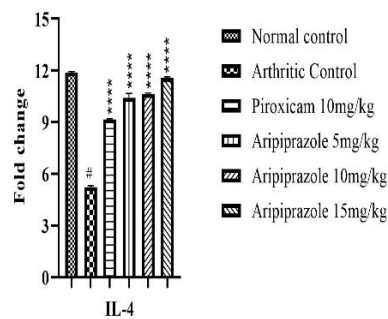
(c)



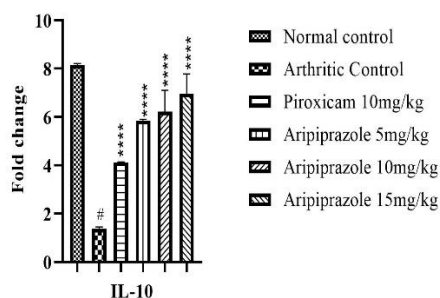
(d)



(f)



(g)



(h)

Figure 12: Impact of aripiprazole on mRNA expression of NF- κ B (a), TNF- α (b), IL-1 β (c), IL-6 (d), Cox-2 (e), IL-4 (f), IL-10 (g) through real time polymerase chain reaction. Values are stated as Mean $\pm$ SEM. (n-3). Where, ****p < 0.0001 comparative to disease control group and # is p < 0.0001 as compare to normal control group, performed by one-way ANOVA test following Dunnett analysis.

4. DISCUSSION

RA is a complex, auto-immune disease generally manifested as joint inflammation & deterioration. The exact pathogenesis of arthritis is still under investigation creating more complications and progressive deterioration of joints, bones and cartilage. In RA the levels of inflammatory mediators including interleukin-6, tumor necrosis factor- α , interleukin-1 β are raised while there is significant reduction in level of anti-inflammatory markers such as IL-4 & IL-10.(Kim et al., 2016). Despite the availability of wide range of treatment options for RA, still there is a need to discover appropriate therapy to overcome the adverse reactions associated with drugs that are used in management of arthritis in cost effective range. Aripiprazole-anti psychotic drug was previously reported to have anti-inflammatory activity so this study evaluated its role as an anti-arthritic in order to give another chance to the drug through repurposing.

The results of the present study conducted depicted a dose dependent anti-inflammatory & anti arthritic potential of aripiprazole. Anti-inflammatory activity of aripiprazole was appraised through acute model of histamine that was further verified through in silico study-molecular docking. In-vitro & in-vivo tests were used to reassure its anti-arthritic potential.

Current study findings showed that aripiprazole considerably inhibited the edema caused by histamine. On contact with toxic agents, mast cells degranulation occurs and expression of histamine resulting in dilation of vessels and intensification of capillary permeability, recruitment of eosinophils and mast cells, inflammatory cells relocation, adhesion molecule

generation, cytokines & chemokines expression, chemotaxis, immunomodulation, enhances vascular penetrability and edema resulting in augmentation of histamine interceded immune response causing inflammatory response manifested as synovial tissue inflammation and erosion & deformity of articular cartilage. All these proceedings ultimately lead to inflammatory action. Aripiprazole remarkably prevented the inflammatory edematous condition which confirmed its role as an anti-inflammatory.(Abbas, Ansari, et al., 2021)

In-silico study-molecular docking is considered to be an important means for forecasting several binding positions for protein ligand connections. Hence this approach can be used in order to evaluate molecular bonding & the information about the appropriate interaction spot as well. Diverse binding connections, depending upon space among the atoms of amino acid and ligand can be investigated as well.(Qasim et al., 2020). So, anti-arthritic potential of aripiprazole was further verified through in-silico study-molecular docking and interaction of aripiprazole with interleukin-1 β , tumor necrosis factor-alpha, interleukin-6, nuclear factor-kB, Cyclooxygenase-2 were investigated showing that aripiprazole has strong binding affinity towards these targets. Aripiprazole displayed strong binding interaction with aforesaid targets of inflammatory pathways offering a theoretical confirmation for its anti-arthritic potential.

There is significant protein denaturation in RA through auto-antigens production. Main mechanism behind protein degradation is modification in disulfide bridges, hydrogen bonds, hydrophobic bonds and electrostatic interaction in protein molecules(Uttra et al., 2017). Many drugs inhibit heat evoked protein degradation in a concentration gradient pattern and can be considered as candidate to assess anti-arthritic potential..(Gautam et al., 2013). Assessment from present study showed remarkable inhibitory action of aripiprazole on egg albumin and bovine serum protein degradation. Hence, this drug has the potential to alleviate rheumatoid arthritis and its progress via decreasing the protein denaturation-key parameter of arthritis via reducing production of auto-antigens.

HRBC membrane is considered to have similar membrane as that of lysosomal membrane. Lysosomal membrane rupturing is caused by hypotonicity with release of lysosomal constituents together with phospholipase-A2 and hence expression of inflammation stimulating markers. Consequently, stabilization of membrane of red blood cells prevents release of markers of inflammation. So suppression of rupturing of human red blood cells prompted by hypotonicity can be linked with anti-arthritic feature of this agent.(Hasan et al., 2015) A significant protection

of red blood cells membrane breakdown provoked by hypotonic solution was resulted with aripiprazole in a dose dependent pattern comparable with piroxicam. Present study results stability of membrane as another possible anti-arthritic mechanism of aripiprazole.

Formaldehyde-stimulated arthritis is most commonly employed model of arthritis and is reliable in vivo model for evaluation of anti-arthritic activity as it shows similarity with human arthritis(Udavant et al., 2012). Formaldehyde provokes arthritis by protein degradation at site of injection stimulating inflammation in response to these degraded proteins. (Ben et al., 2016). Also, formaldehyde provoked arthritis has been reported to be two phasic process encompassing neurogenic stage and then tissue mediated reaction stage(Nair et al., 2012). The primary edematous phase is related to expression of kinins, histamine and serotonin while, secondary edematous phase could be associated with expression of prostaglandin like substances resulted because of type 3 hypersensitivity reaction.(Owoyele et al., 2011; Shaikh, 2011). Improvement in paw edema by aripiprazole in formaldehyde provoked arthritis model can be considered as a consequence of suppression of these inflammatory mediators. Maximal reduction in edema had been observed at the dose of 15mg/kg. Additionally, as aripiprazole repressed primary as well as secondary stage of inflammatory response it could be postulated that it is acting centrally to impart anti-arthritic action.

Weight loss is ascribed as an extra-articular property related to arthritis may be because of muscle degeneration provoked via protein degradation or lesser food consumption as a result of high expression of leptin after instigation of arthritis with formaldehyde. (Qasim et al., 2020).Aripiprazole prevented this weight loss as detected in diseased control group thereby preventing cachexia occurring because of arthritic disease.

CFA contains strains of temperature slayed *Mycobacterium tuberculosis* in liquid paraffin which invokes cell-mediated immunity resulting in generation of antibodies and protein degradation.(Kim et al., 2016). Adjuvant provoked arthritis encourages T-cell mediated instantaneous inflammatory response called as primary abrasions appearing in three to five days of CFA administration because of its sensitivity towards cartilage proteoglycans, heat shock proteins and natural flora of intestine. Later on, secondary abrasions appearing following a gap of eleven to twelve days, manifest as swelling of non-injected spots because of the expression of tumor necrosis factor-alpha, interleukin-12, interleukin-6, interferon- γ & some other chemokines. Late synovitis stage resulted in permanent bone deterioration.(Hasan et al.,

2015). Aripiprazole in concentration gradient manner prohibited rat paw edema in contrast to diseased control rats. It also declined arthritic index via reducing swelling and erythema along with constraining progress of secondary abrasions i.e., inflammatory abrasions on non-injected spots because of their anti-inflammatory and antiarthritic attributes. Findings of current analysis depicted that aripiprazole significantly prohibited weight loss in contrast to diseased control rats. Radiologic fluctuations in RA are beneficial tools for diagnosis & assessing the advancement and complexity of the disorder. Radiographic study presented soft tissue edema, contraction of joint spacing and bone resorption in joints of the diseased control animals. On the other hand, markedly repressed soft tissue edema, space tightening and bone erosion by protecting against joints obliteration in animals who were administered with piroxicam and aripiprazole in dose dependent pattern because of downregulation of nuclear factor-kB, tumor necrosis factor-alpha, interleukin-1 β , interleukin-6 that contribute towards structural damage.(Hasan et al., 2019).

Histopathological examination of all the animals showed the diseased control animals demonstrated the specific characteristics of arthritis as cartilage and bone destruction with infiltration of inflammatory cells and pannus formation. (Zhu et al., 2014). Aripiprazole(15mg/kg) & standard group conserved the histopathological fluctuations in relation to diseased control rats because of inhibition of the infiltration of inflammatory cells and suppression of architectural changes caused by inflammatory markers such as tumor necrosis factor-alpha, interleukin-12, interleukin -6, interferon- γ , nuclear factor-kB and other chemokines. Arthritis comprises T cell-intermediated immune feedback which excites expression of an arrangement of inflammation provoking cytokines and encourages generation of antibody resulting in gradual joint deterioration. Tumor necrosis factor an autocrine promotor and paracrine stimulating agent, incites the appearance of genes of interleukin-1 and interleukin-6, NF- kB, nitric oxide Cox-2 and prostaglandin-E2. Hence, inflammation stimulating cytokines such as IL-1, IL-6, and TNF-alpha are imperative in commencement of arthritic disorder prompting generation of matrix metalloproteinases and osteoclasts stimulation, vascular dilation, fluid extravasation, angiogenic signaling resulting in resorption of bones observable from cartilage degeneration. Hence, blockage of these cytokines markers is efficacious in control of chronic inflammation.(Kim et al., 2016). Aripiprazole has expressively suppressed the levels of these inflammation provoking cytokines genes including nuclear factor-kB, interleukin-1 β , tumor necrosis factor-alpha, interleukin-6, cyclooxygenase-2 and prostaglandin-E2 with

upregulation of interleukin-4 that elevates the anti-inflammatory contribution of Th2 cells and interleukin-10 genes arresting pro-inflammatory role of Th1 cells.(Yoo et al., 2018). And these outcomes are apparent from findings of Elisa and PCR testing.

5. CONCLUSION

In a nutshell, aripiprazole can be considered as an efficacious anti-arthritic drug; though comprehensive mechanistic assessment is necessary to present the precise mechanism of action in order to introduce the drug in clinical trials. Findings obtained in this research drew attention to repurpose the aripiprazole for arthritic disorder since this approach of discovering the drug would assist in saving time and expense and will be more successful to prevent arthritic disorders.

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