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Anti-Inflammatory, Anti-Arthritic Activity of Cobalt Derivative of 3-Methoxysalicylaldehyde-2-AminoBenzoylhydrazone in Rats

Imad Uddin MD[1*], Firasat Ali[2], Parvinder Singh[2], Chandrashekhar VM[2], Gudasi KB[3], Badiger DS[3]

[1]Deptartment of Pharmacology, Pulla Reddy Institute of Pharmacy, Annaram, Jinnaram, SangaReddy, Telangana-502313
[2]Department of Pharmacology, HSK College of Pharmacy, Bagalkot–587101, Karnataka, India
[3]Department of Chemistry, Karnataka University, Pavate Nagar, Dharwad 580003, Karnataka, India

***Corresponding author e-mail:** imadpharma111@gmail.com

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ABSTRACT

This study was piloted to screen anti-inflammatory, anti-arthritic effect of Cobalt (Co) derivative of 3-methoxysalicylaldehyde-2-aminobenzoylhydrazone (AQF). Acute oral toxicity study was conducted according to guidelines OECD-425. Widely accepted carrageenan model was adopted in this study to evaluate Anti-inflammatory effect of Co-AQF. Freunds Complete Adjuvant (FCA) was used for screening anti-arthritic effect and was evaluated by paw edema volume, paw width, paw height, Development of Arthritis (DOA), vascular permeability, release of histamine from blood, Erythrocyte Sedimentation Rate (ESR), Radiographic Analysis and Histopathological assessment. Reference standard Diclofenac Sodium (DS) and Co-AQF have shown significant decrease in all Arthritis evaluating parameters as compared to control group. Pathological changes and histopathological abnormalities which occurred in control group animals were treated with DS and Co-AQF. Hence, based on the results Co-AQF was recognized as anti-arthritic compound and further it can be elucidated at molecular level to establish its potency.

Keywords: Carrageenan, Arthritis, FCA, paw volume, vascular permeability.

INTRODUCTION

Struggle to develop an orally effective, less toxic drug is principal goal of modern research to alleviate the burden of arthritis [1]. Additionally, to cytokines other significant mediators accountable in inflammation are IL-1, IL-6, TNF-α, PG and NO [2]. Carrageenan, a polysaccharide induces inflammation, serving as putative model to assess acute inflammation [3]. For evaluation of chronic inflammation, FCA model is pre-eminent tool [4]. Orthodoxly, remedies for arthritis are with disheartening profile of side effects [5]. Quinazolin-4-(3H)-one derivatives are deliberated as attention-grabbing moieties since they possess anti-tumour [6], anti-HIV [7], selective oestrogen beta modulator [8], anti-inflammatory [9], anti-bacterial [10], and antidepressant [11] activities. Therefore, present study was steered to assess anti-inflammatory, anti-arthritic effect of Co-AQF.

MATERIALS AND METHODS

Synthesis and Characterization of Co-AQF

Condensation of 3-methoxysalicylaldehyde with 2-Aminobenzoylhydrazone yielded 3-methoxy salicylaldehyde-2-aminobenzoylhydrazone. Metal complexes of AQF like Ni-AQF, Co-AQF, Cu-AQF,

Mn-AQF were Synthesized, characterized by thermal, NMR, UV-Vis, IR, and Mass spectroscopic methods and evaluated for anti-microbial activity [12]. In 2014, we also publicized anti-arthritic action of Ni-AQF [13].

Acute toxicity study

Female swiss-mice (25-30g) were given limit doses of 2000mg/kg and 550mg/kg according to OECD guidelines-425 and observed for 4hrs and then up to 14days. 100% mortality was occurred with higher limit dose. AOT software was used to calculate $LD_{50.}$ According to the results of acute toxicity study the doses of 5mg/kg Body Weight (BW) and 10mg/kg BW were chosen for the experiment.

Animals

Female Sprague-Dawley (SD) rats (150-200gm) were obtained from central animal house of HSK College of Pharmacy, Bagalkot maintained with standard husbandry conditions (Temp. 22-28^{0}C; Relative Humidity 65±10%) for 12hr dark and 12hr light cycle respectively in standard propylene cages. The animals were fed with standard food (Pranav Agro Industries, Sangli and Maharashtra) and water *ad libitum*. Experiments were conducted in accordance with Institutional Animals Ethics Committee (HSKCOP/IAEC, Clear/2010-11/1-14).

Chemicals

FCA (Sigma-Aldrich, Chemical Co. USA.), Trichloroacetic acid (TCA), 2-thiobarbituric acid (TBA), 5-5'-dithiobis (2-nitrobenzoic acid), (±) epinephrine, histamine, *O*-phthaladehyde, Evans blue (SD Fine-Chemicals, Boisar), DS (gift sample from *Empree* medicaments Ltd. Belgaum, Karnataka). All other chemicals, reagents and kits were procured are research grade.

Instruments

Instruments used are Digital Plethysmometer (7140UGO Basile, Italy), Refrigerator centrifuge (MPW-350R, Korea), UV spectrophotometer (UV-1601, Shimadzu corporation, Kyoto, Japan), X-ray machine (Multiphos10, version1.0, Model no. DX-300, Pune, India), Flourimeter (CL-53, Elico Ltd, Hyderabad, India), Autoanalyser, Remi centrifuge and others.

Effect of Co-AQF on Carrageenan induced acute inflammation

In this model, animals were divided into four groups of six each and starved overnight. Group I served as control, receiving vehicle (10%v/v tween80; p.o.). Whereas Group II, III and IV received DS (5mg/kg; p.o.), Co-AQF 5mg/kg and 10mg/kg respectively. 1 hr after drug administration all groups were challenged with 0.1ml of Carrageenan solution (1.0%w/v NS) [14]. Oedema induced was measured with Digital Plethysmometer at 0, 0.5, 1, 2, 3 and 5hr [15]. Percentage inhibition of oedema was calculated as, Percentage (%) inhibition = (Vc-Vt / Vc) x 100. Where, Vt = mean volume of each treated group, Vc = mean volume of control group

Induction of Adjuvant Arthritis (AA) and treatments

To assess Co-AQF efficacy, female SD rats (150-200g) were divided into 4 groups with 8 in each. On day zero, 0.1ml of FCA injected s.p to all animals for inducing arthritis [16]. Group I taken as Control receiving 10%v/v tween80; p.o., Group II, III and IV received DS (5mg/kg; p.o.), Co-AQF 5mg/kg and 10mg/kg respectively, all these treatments are from 1st to 28thday.

Effect of Co-AQF on FCA induced paw oedema, paw width and paw height

On 0th, 7th, 14th, 21st and 28thday paw oedema volume measured by using Digital Plethysmometer. Whereas paw width and height by caliper ruler. Percentage inhibition of paw oedema volume was calculated as mentioned above [17].

Effect of Co-AQF on Development of Arthritis (DOA) against FCA induced AA

On the testing days as mentioned above two blinded observers assessed DOA by using a three point scale as follows, 0= normal joint; 1= slight inflammation and redness; 2= severe erythema and swelling affecting the entire paw with inhibition of use; and 3= deformed paw or joint with ankyloses, joint rigidity, and loss of function [18]

Effect of Co-AQF on vascular permeability test in FCA induced AA

On 28thday, 4 rats from each group anaesthetized and given evans blue (50mg/kg) via jugular vein and sacrificed after 4hr by anesthetic ether. From each knee joint fat pad, anterior and posterior synovial capsules were dissected, tissue reaped and weighed. Fat pad and Capsules are broken in to smaller pieces, mixed with acetone in 1%NaSO4 (7:3) and shaken continuously for 24h at room temperature. After centrifugation (10min at 2000rpm) 2ml of supernatant separated for measurement of absorbance at 620nm using UV- spectrophotometer. Amount of dye which is directly proportional to absorbance was calculated by extrapolating with standard curve prepared by using different concentrations of Evans blue solution (Figure-1). Percentage inhibition of joint infiltration was calculated by the equation, Percentage (%) inhibition = (Vc-Vt / Vc) x 100.

Where, Vt = Mean evans blue joint infiltration of treated groups, Vc = Mean evans blue joint infiltration of control group [19]

Effect of Co-AQF on release of histamine from blood in FCA induced AA

Remaining 4rats from each group are used. 5.0ml blood from each animal taken by cardiac puncture mixed with 6.0mg of ammonium oxalate, 4.5ml of distilled water, 0.5ml of 10-12N perchloric acid (HClO4) and kept at room temperature for 10minutes. Add 9volumes of 0.4N HClO4, homogenated, allowed to stand for 10minutes and then centrifuged. 4.0ml supernatant was mixed with 0.5ml 5N NaOH, 1.5gm solid NaCl, 10ml n-butanol and shaken for 5minutes to extract the histamine into butanol. Aqueous phase is removed and organic phase is trembled for 1minute with 5.0ml of Salt-saturated 0.1N NaOH for removal of histidine. After centrifugation, 8.0ml of butanol phase (organic phase) is mixed with 4.5ml 0.1NHCl, 15.0ml *n*-heptane and shake for 1min. Again centrifuged and aqueous phase is collected for measuring histamine fluorimetrically [20]. To 2ml aliquot of aqueous phase, add 0.1ml *O*-phthaldehyde (OPT), 0.2ml 3N HCl. Fluorescence intensity of resultant acidified solution (stable for 90min) was proportional to histamine concentration over the range of 0.005 to 0.5µg/ml. Standard curve obtained from 2ml aliquots of different concentrations prepared with standard histamine. Histamine concentration of treated groups was determined by extrapolating with the standard graph. (Figure 2) [21].

Effect of Co-AQF on ESR in FCA induced AA

Take citrated blood (1 part 3.8% Sodium citrate: 4 parts blood) in westrengren's pipette up to "0" mark and fix it on stand. Note time and take reading for ESR at the end of 1hr [22].

Effect of Co-AQF on Radiographic analysis in FCA induced AA

On 28thday, anesthetized (Ketamine hydrochloride-45mg/kg) rats individually placed on radiographic plate at a distance of 75cm from X-ray source, operated at 46kV peak, 4mA, exposure time 0.8sec. X-ray images obtained were assessed for radiographic changes [23].

Effect of Co-AQF on histopathological assessment in FCA induced AA

On 28thday, knee joints were dissected and fixed in 10%formalin [24]. Thereafter, joints decalcified, embedded in wax, sectioned and stained with haematoxylin and eosin. Histological analysis was carried out by a single observer, focusing on polymorph nuclear cell infiltration, tissue proliferation and cartilage erosions. Severity of lesions was given scores: 0= no change, 1= mild change, 2= moderate change and 4= marked change

Statistical Analysis

Results were expressed as Mean± SEM. Statistical comparison was made between treated groups and control group. Statistical difference between two means was determined by one-way ANOVA followed by Dunnett's multiple comparison test using Graph pad prism 6.0 software. Only those mean values showing statistical difference $p<0.001$, $p<0.01$, $p<0.05$ were considered as statistical significant and $p>0.05$ was considered as non-significant.

RESULTS

Acute toxicity study and Effect of Co-AQF on Carrageenan induced acute inflammation

Titled compound did not show any significant overall behavioral and toxicological effects at doses up to 550 mg/kg (p.o.) in the mice and the surviving mice appears normal and remained alive throughout the 14-days observation. The doses of 5 mg/kg and 10 mg/kg were selected for further studies. Co-AQF showed significant dose dependent inhibitory activity against carrageenan induced paw oedema inflammation (Table 1).

Effect of Co-AQF on FCA induced paw edema, paw width and paw height

Control group animals showed increased paw oedema gradually up to 28th day. Co-AQF showed significant reduced right paw oedema (Figure 3), right paw width (Figure 3), right paw height (Figure 3) from 14th day onwards and up to 28th day as compared to control group and dose depend protection were observed.

Effect of Co-AQF on DOA against FCA induced AA

Control group exhibited severe erythema and swelling, joint with ankylosis, joint rigidity and loss of function which reached maximum up to 28th day. The standard drug DS and Co-AQF treated animals exhibited a significant reduction in redness, swelling, and erythema at ankle joints as compared to control group.

Effect of Co-AQF on vascular permeability in FCA induced AA

Control group has showed significant increased infiltration of lymphocytes, indicated by increased evans blue extravasation which is due to increased

endothelial gap of vascular components at knee joint of rats. Treated groups showed significant reduced infiltration of lymphocytes as compared to control group. In treated groups, the percentage inhibition of joint infiltration is 28.59% and 44.53% in Co-AQF of 5mg and 10mg respectively. The significant decrease in extravasation of dye in and Co-AQF is due to reduced endothelial gap of vascular component at knee joints of rats (Table 2).

Effect of Co-AQF on histamine release from blood and on ESR in FCA induced AA

In control group animals showed significant elevated histamine content in blood In contrast, the standard DS and test compound Co-AQF showed significant (p<0.001) reduced release of histamine into the blood (Table 2). However ESR is significantly reduced (p<0.001) in all the treated groups, DS and Co-AQF as compared to control group (Table 2)

Effect of Co-AQF on Radiographic analysis in FCA induced AA

Radiographs taken on 28th day after FCA injection (a) control; shows severe swelling, deformity and increased joint spaces, (b) DS 5 mg/kg treated animals showing significant reduction in soft tissue swelling and bone erosive changes, (c) Co-AQF 5 mg/kg (d) Co-AQF 10 mg/kg shows significant reduction in swelling deformities and decrease in joint spaces (Figure 4).

Effect of Co-AQF on histopathological assessment in FCA induced AA

The extent of rat paw pathological conditions was graded on a semi quantitative scale. Light microscopy 10x. Paw joint tissue was fixed in 10% formaldehyde and 5-µm paraffin sections were stained with hematoxylin and eosin. Control group has shown destruction of cartilage and sub-chondral bone, disorganization of the joint space and replacement with mononuclear cells and fiber thickening, increased lymphocytes and plasma cells; DS 5 mg/kg, Co-AQF 5 mg/kg and Co-AQF 10mg/kg has shown mild Proliferation and infiltration of mononuclear cells, mild sub-chondral bone erosion and superficial cartilage damage (Figure 5)

DISCUSSION

Oedema develops after carrageenan inflammation, is a biphasic event. The initial phase is attributed to the release of histamine and serotonin. The oedema maintained between the first and the second phase is due to bradykinin [25]. Acute inflammation is a short-term process which is characterized by the typical signs of inflammation, such as swelling, pain, and loss of function due to the infiltration of the tissues by plasma and leukocytes. Among them, oedema is one of the fundamental actions of acute inflammation and it is an essential parameter to be considered when evaluating compounds with a potential anti-inflammatory activity [26]. The control group had shown significant increase in the paw-oedema volume after carrageenan induction in the plantar region of the right hind paw, this is due to the release of cascade of inflammatory mediators like histamine, serotonin, kinins, PGs and others. The quinazolinone metal complex Co-AQF showed significant inhibitory activity against carrageenan induced paw oedema inflammation. This observed activity may be due to the inhibition of cyclo-oxygenase enzyme and ultimately this leads to the inhibition of PG synthesis. The chronic inflammation involves the release of various inflammatory mediators like cytokines (IL-1a and TNF-a), granulocyte monocytes colony stimulating factor (GM-CSF), platelet derived growth factor (PGDF) and others. These mediators are responsible for the pain, destruction of cartilage and leads to severe disability. Paw swelling is one of the major factors in assessing the degree of inflammation and efficacy of the drugs. Adjuvant induced arthritis is non-specific immune response within the joint can also result in inflammatory and erosive disease [27]. Paw swelling is an index of measuring the anti-arthritic activity of various drugs and it is employed here to determine the activity of CO-AQF quinazolinone metal complex. Reference standard DS, CO-AQF administered groups showed marked reduction in paw volume when compared with the arthritic control group by inhibiting the release of inflammatory mediators.

The DOA scoring in control groups exhibited that severe redness, swelling, erythema at the ankle joints injected with FCA, this is due to the release of inflammatory mediators at the localized area. In addition to this, deformed paw and joint with ankylosis might be due to severe pannus formation and proliferation of synovium leading to cartilage damage [28]. In standard DS and CO-AQF administered groups there is a marked decrease in DOA scoring as compared to control group. The swelling of joint knees due to FCA induced arthritis, causes blood vascular permeability within 28 days of administration. Evans blue extravasation method is used to assess for plasma protein extravasation in the rat knee joint, because Evans blue has high binding affinity to plasma proteins. Normally, large plasma proteins and bound Evans blue dye cannot pass through the endothelial gaps and therefore gets restricted in the vascular component. Endothelial

cells undergo activation, expressing adhesion molecules and presenting chemokines, leading to enlargement of endothelial gaps as a result plasma protein and evans blue dye complex can escape to the interstitial tissues. The measurement of the amount of evans blue in the synovial capsule can provide us an index of the relative vascular permeability. The decreased extravasation reported for Co-AQF and DS was due to decreased endothelial gaps which is caused by decreased expression of adhesion molecules. The extraction of histamine into *n*-butanol from alkalinized perchloric acid tissue extracts, return of the histamine to an aqueous solution and condensation with OPT to yield a product with strong and stable fluorescence which is measured in a spectrofluorimeter. Preformed histamine is present in mast cell granules and gets released by mast cell degranulation in the inflammatory conditions. Thus released histamine gets involve in the immune reactions, which involves binding of antibodies to mast cells and releases neuropeptides and cytokines like IL-1, IL-6, IL-18 that plays an important role in the RA [29]. Co-AQF had shown the decreased level of histamine, release in blood and also observed decreased oedema, redness and swelling.

Increase in ESR is attributed to the accelerated formation of endogenous protein such as fibrinogen and a/ß globulin, and such a rise in the ESR indicates an active but obscure disease process [30]. In the present study Co-AQF treatment restored the altered hematological profile by decreasing the ESR provide support for its anti-arthritic effect.

Radiographic changes in RA conditions are useful diagnostic measures which indicate the severity of the disease. The radiographic (x-ray) analysis of the joints in the arthritis Control group showed the soft tissue swelling, bone deformities, bone erosive changes and bone resorption. This indicates the confirmation of arthritis. The significant reduction of above pathological changes were observed in Co-AQF and standard treated group as compared to control group. Histopathological analyses revealed that, in relation to the development of inflammatory and arthritic lesions in the paws, most of the FCA rats experienced progressive cartilage destruction and bone erosion, finally leading to complete ankylosis and malformation of the joints. In contrast, treatments with Co-AQF not only showed significant reduction of cellular infiltration, joint space narrowing, synovial hyperplasia, and pannus formation, but also markedly protected the affected joints against cartilage destruction and bone erosion.

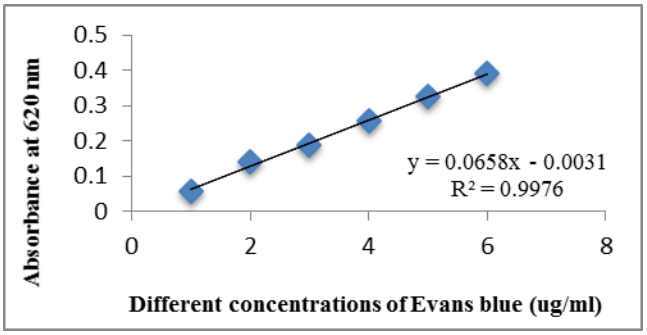

Figure 1: Standard curve of Evans blue for joint infiltration

CONCLUSION

Chemistry of quinazolonone compounds has been the subject of considerable interest though there had been scattered reports of investigation of the medicinal properties of such compounds. Quinazolinone was proved to be useful in cardiovascular, anti-folate and hypnotic drug discovery. Present investigation clearly demonstrated that Co-AQF possess anti-inflammatory, anti-arthritic activity. Further, studies are required to elucidate the detail mechanism of action of this agent at molecular level to explore the therapeutic benefits.

CONFLICT OF INTEREST

Authors report no conflict of interest. Authors alone are responsible for the content and writing the article.

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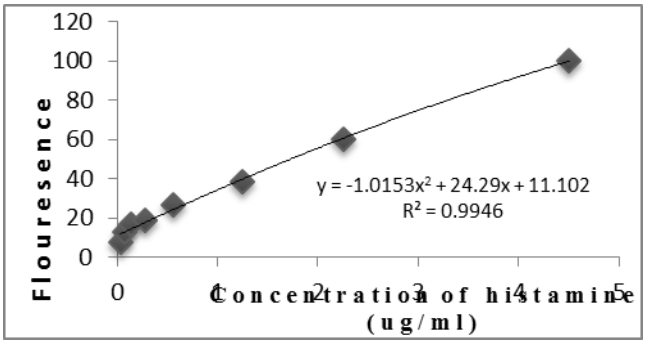

Figure 2: Standard curve for the estimation of histamine by flourimeter.

Table 1: Effect of Co-AQF on carrageenan induced acute inflammation.

Groups	Paw oedema Volume in ml (% of oedema inhibition)				
	½ hr	**1st hr**	**2nd hr**	**3rd hr**	**5th hr**
Control (10% tween80)	0.293±0.078	0.418±0.092	0.810±0.093	1.210±0.257	0.796±0.089
DS (5mg/kg)	0.350±0.044 ns (0.00%)	0.323±0.028 ns (21.95%)	0.270±0.049*** (66.66%)	0.215±0.035*** (82.23%)	0.256±0.036*** (67.59%)
CO-AQF (5 mg/kg)	0.145±0.028 ns (51.70%)	0.198±0.036* (53.65%)	0.458±0.024** (44.44%)	0.418±0.022*** (66.11%)	0.258±0.042*** (68.35%)
CO-AQF (10 mg/kg)	0.213±0.037 ns (27.58%)	0.270±0.035 ns (34.14%)	0.480±0.087* (40.74%)	0.446±0.057*** (63.63%)	0.325±0.014*** (59.49%)

*All values are expressed as mean ± SEM, and tested by One way ANOVA followed by Dunnett's multiple comparision test, n=6, **p<0.01, ***p<0.001 as compared to control group.*

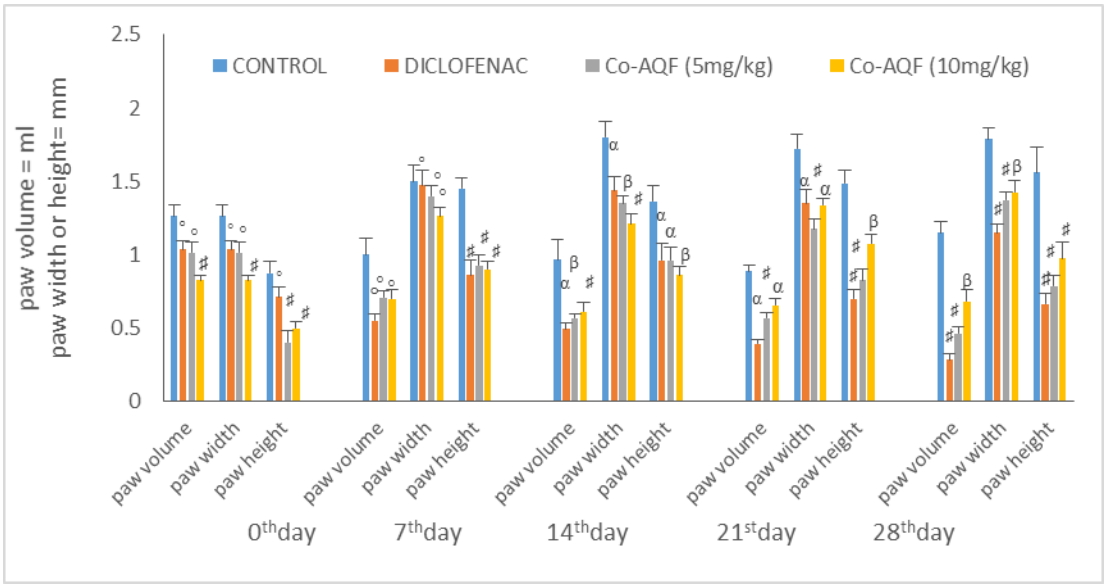

Figure 3: Effect of Co-AQF on FCA induced paw oedema, paw width and paw height. All values are expressed as mean ± SEM and tested by one way ANOVA followed by multiple comparisons Dunnet's test, n=8,0= non-significant, α= $p<0.05$, β=$p<0.01$, ♯= $p<0.001$ as compared to control group.

Table 2: Effect of Co-AQF on vascular permeability, histamine release from blood, ESR in FCA induced AA.

Groups	Concentration of Evans blue (µg/ml)	Percentage of Inhibition of Joint Infiltration	Concentration of Histamine (ug/ml)	ESR (mm/hr)
Control (10%v/v Tween80)	5.333±0.070	------	0.2740±0.0236	10.00±0.44
DS (5 mg/kg)	2.183±0.126***	59.06 %	0.1980±0.0080***	2.33±0.2108***
CO-AQF (5 mg/kg)	3.808±0.400**	28.59%	0.181±0.009***	1.667±0.2108***
CO-AQF (10 mg/kg)	2.958±0.400***	44.53%	0.040±0.003***	1.500±0.2236***

*All values are expressed as mean ± SEM and tested by one way ANOVA followed by multiple comparisons Dunnet's test, n=8,*p<0.05, **p<0.01, ***p<0.001 as compared to control group.*

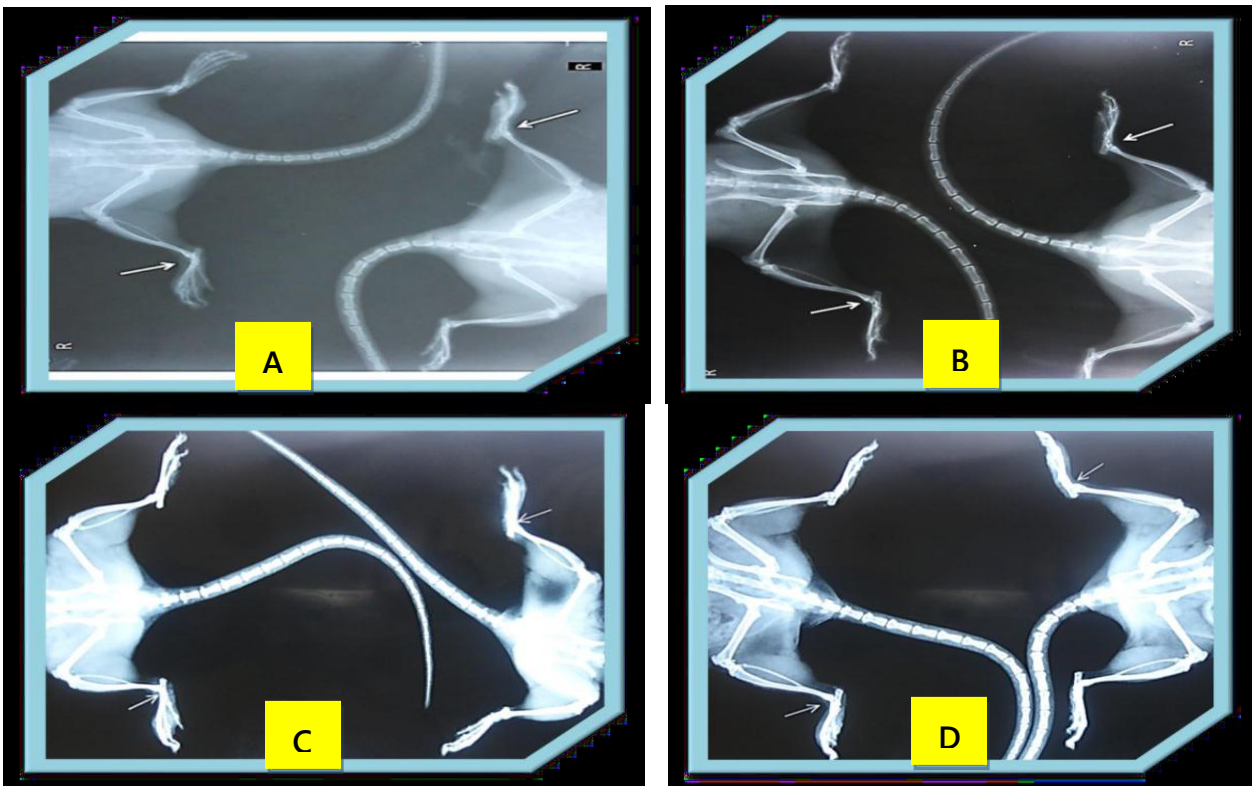

Figure 4: Effect of Co-AQF on Radiographic analysis in FCA induced AA. Radiographs taken on 28th day after FCA injection (a) control group shows severe swelling, deformity and increased joint spaces, (b) DS 5 mg/kg treated animals showing significant reduction in soft tissue swelling and bone erosive changes, Co-AQF 5 mg/kg (f) Co-AQF 10 mg/kg shows significant reduction in swelling deformities and decrease in joint spaces.

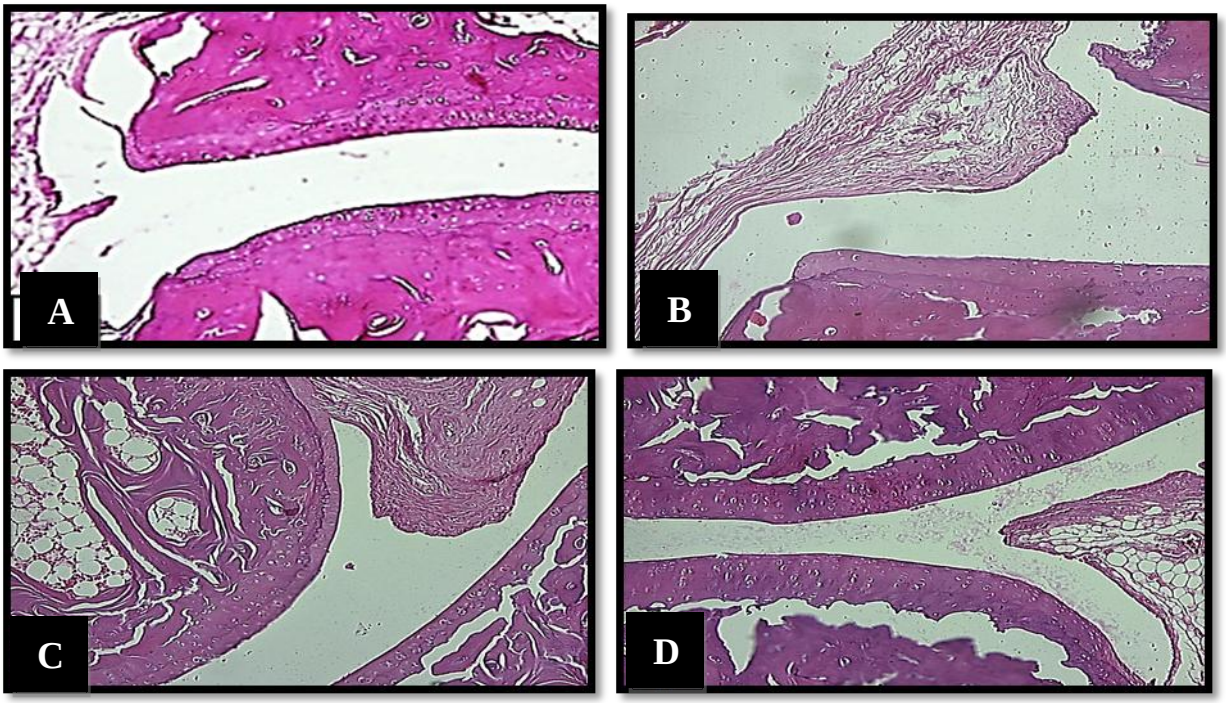

Figure 5: Effect of Co-AQF on histopathological assessment in FCA induced AA. (A) Control group shows destruction of cartilage and sub-chondral bone, disorganization of the joint space and replacement with mononuclear cells and fiber thickening, increased lymphocytes and plasma cells; (B) DS, (C) Co-AQF 5 mg/kg (D) Co-AQF 10mg/kg shows mild Proliferation and infiltration of mononuclear cells, mild sub-chondral bone erosion and superficial cartilage damage.

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