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Anti-osteoarthritic activity of *Acacia nilotica* pods in Wistar rats

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Abstract

Osteoarthritis is one of the common joint degenerative disorders for which only symptomatic treatment is available. In the present study we have investigated anti arthritic activity of the Methanolic extracts of the pods of *Acacia nilotica* in papain induced osteoarthritis model which mimics the human arthritis. Osteoarthritis was induced in rats by intra-articular injection of 0.8mg of papain and 0.03M of cysteine on day 0, 4 and 7. Methanol extract of *A.nilotica* pods was administered to the animals in the doses of 100, 200, and 400 mg/kg for 15 days after the injection of papain and cysteine. Celecoxib (30 mg/kg) was used as the reference standard drug. The joint activity was assessed by wire hang test and measurement of the knee joint diameter. At the end of 15 days blood samples were collected for the analysis of TNFα, and IL-10 gene expression by Real Time PCR. The animals were sacrificed and the knee of the each animal was collected for the histological studies of the joint.Injection of papain with cysteine produced a significant increase in joint diameter and reduction in joint strength as depicted by decrease in wire hanging time. The Methanolic extract of *A.nilotica* pods produced time and dose dependent decrease in swelling of the knee joint along with significant increase in joint strength. There was a significant elevation of TNF-α as well as IL-10 gene expression in the rats treated with 400 mg/kg of test extract. Celecoxib or lower doses of *A. nilotica* produced slight increase in these levels. Histopathological studies revealed that the reversal of damages in the cartilage induced by papain and cysteine after administration of the extract.

Conclusion: Our data suggests potential anti-osteoarthritic effect of the pods of *A. nilotica* on papain and cysteine induced arthritic rats.

Key words: *Acacia nilotica* pods, Papain, Arthritis, TNFα & IL-10

Introduction

Osteoarthritis (OA) is primarily an inflammatory disease characterized by disturbance in the balance between synthesis and degradation of cartilage in the joint. A variety of inflammatory cytokines such as prostaglandins, leukotrienes, matrix metalloprotease, nitric oxide are involved in a cascade of the events leading to inflamed synovium and characteristic symptoms like joint pain, stiffness, and loss of mobility (Abramson *et al.,* 2001; Goldring *et al.* 2006). The prevalence of OA increases with age and other lifestyle disorders like obesity and hypertension. According to the World Health Organization (WHO), OA is one of the ten most disabling diseases in developing countries and of all forms of arthritis the prevalence of OA in Indian population is 22-37 per cent and it will continue to rise exponentially(Mahajan *et al.,*2005; Jarullah *et al.,*2009).

Despite efforts made in the development of a suitable drug for the management of OA, there is no successful medicine that can provide better management against benefit to risk ratio. Currently used drugs are just symptomatic and produce various adverse effects like gastric irritation and cardiovascular toxicity (Felson, 2009). Inhibition of

inflammatory markers like cytokines, interleukins, NO or MMP has been the strategies for a new drug discovery for the treatment of OA however, there has been a limited success (Livak and Schmittgen,2001; Chaubal*et al.,* 2003; Raghavendra*et al.,* 2006; Murat *et al.,* 2007; Felson 2009; Klein *et al.,* 2012; Verma*et al.,* 2012). WHO estimates that 80 per cent of the world's population recently used herbal medicine for some aspect of primary health care. It is likely to be more prevalent with increase in age and other lifestyle disorders like obesity and hypertension (Mahajan *et al.* 2005; Ramalingam 2009). Lifestyle changes, diet, exercise and nutrition supplementation play a key role in its prevention and management. Current therapy to manage this disorder is based on symptomatic relief from pain and joint stiffness through various anti-inflammatory as well as analgesic agents. In the severe form of this disease, joint replacement remains the only available option. Pharmaceutical industry also continues to examine their potential as sources of novel medicinal compounds for the betterment of those diseases for which synthetic therapies have not justified themselves(Katz, 2006). *Acacia nilotica*, (Lam.) wild, commonly known as *Babool,* is a widespread in the dry regions of Asia and Africa. The bark and leaves are traditionally used against cancer, congestion, diarrhea, fever, hemorrhoids, tuberculosis etc. The bark has been reported to exhibit chemoprevention of N-nitrosodiethylamine induced carcinogenesis probably attributed to polyphenols present in the bark (Singh *et al.,*2009). *A. nilotica* is reported to possess various activities such as anti-inflammatory, anti-microbial, anti-diarrheal etc. (Raghavendra *et al*., 2006; Saka, 2011; Verma *et al.,*2012). There are reports on isolation of many bioactive compounds from various parts of *A. nilotica* like gallic acid, catechin, kaemferol, rutin, apigenin, umbelliferone, niloticane, androstene and β–sitosterol(Sakthivel*et al.,* 2012). The pods are reported to have antifertility activity in male Wistar rats (Lampio*et al.,*2013)The methanol extract of the pods of *A. nilotica* have shown anti-inflammatory activity in various experimental animal models in our laboratory. Hence, we thought it is prudent to investigate the methanol extract of the pods in various models of OA. In the present study, we have tested effect of this extract on papain induced OA in rats. This model was selected as it resembles human OA when compared to cartilage degradation(Murat *et al.,*2007). The present study was designed to evaluate anti-osteoarthritic effect of *A. nilotica* using papain induced OA in rats. Further, we also investigated the effects of *A. nilotica* on peripheral inflammatory cytokines; TNF-α and IL-10.

Materials and methods

Preparation of the Methanolic extract

The ripe green pods were procured during winter season from in and around Pune region. The collected plant material was authenticated at Ramnarain Ruia College by Dr. Ganesh Iyer. The crude powder of the pods of *A. nilotica* was defatted using petroleum ether followed by Soxhlet extraction using methanol as solvent. This extract of *A. nilotica* was used for further phytochemical and pharmacological screening.

Phytochemical screening and HPTLC fingerprint of the methanol extract

HPTLC studies were conducted on the methanol extract in an effort to standardize the extract with respect to phytoconstituents present. A series of mobile phases were explored to arrive at an optimum mobile phase for maximum resolution of phytoconstituents. The HPTLC plates (precoated silica gel 60 F_{254} plates, Merck) were applied using a CAMAG LINOMAT IV Applicator and the developed plates were scanned at 254 nm and 366 nm using CAMAG SCANNER III and CATS 4 software.

Animals

Female Wistar rats weighing above 150 g were procured from HaffkineBiopharma, Mumbai. Animal use in this study was approved by the Institutional Animal Ethics committee (IAEC) (Approval No.: HITRT/IAEC/26/2012) of Haffkine Institute, Mumbai. They were housed in polycarbonate cages at room temperature (25±2°C) and humidity (75±5%) with 12:12 h light-dark cycle. Drinking water and feed were available to animals *ad libitum.* Acclimatization period of one week was given to the animals before starting the experiment.

Chemicals

OA inducing agent, papain was obtained as a gift sample from Enzyme India Ltd. Cysteine, an activator of papain was purchased from S.D. fine chemicals, Mumbai. RNA extraction kit from GE healthcare was used in the experiment for extraction of m-RNA. All other chemicals were of analytical grade and obtained from local suppliers.

Induction of OA & Treatment protocol

Animals were divided into six different groups of six animals each. The osteoarthritis was induced by intra-articular injection of 0.8 mg papain and 0.03Mcysteine given to four groups (Group 2-4) in right knee of each rat using 31 G needle as mentioned in literature (Murat *et al.,* 2007). Group 1 which served as control was treated with saline; Group 2 was positive control treated with standard drug Celecoxib (30 mg/kg) and other three groups (Group 3-5) were given the methanol extract of *A. nilotica* in the doses of 100, 200, and 400 mg/kg for 15 days after the injection of papain and cysteine. All the treatments were started after 8 days of papain injection to rats. At the end of 15 days, blood samples were collected for the analysis of TNFα, and IL-10 gene expression by Real Time PCR. The animals were sacrificed and the knee of the each animal was collected for the histology examination of the joint.

Evaluation of anti-osteoarthritic activity

Anti-osteoarthritic activity was assessed by evaluating knee joint diameter and joint strength of rats. For knee joint diameter, it was determined by using digital Vernier caliper (Aerospace, China). The knee joint strength was assessed by wire hang test. In this, a circular piece of wire mesh (1 cm × 1 cm spacing) was made. To perform the wire hanging test, the pail was inverted and an animal was placed inside the pail on the wire mesh. The mesh was then flipped around, thereby

inverting the animal over the open bottom such that it had to hold on to the cage wire in order to avoid falling (Klein *et al.,* 2012).

Effect of extract on change in expression levels of cytokine genes

Total RNA from blood was extracted from each sample using the Qiagen RNA isolation kit according to the manufacturer's instructions. Real Time PCR was performed using SYBR Green chemistry in a volume of 20 µl PCR reaction mixture (Takara, USA). The reaction was carried out on a real time PCR instrument (Applied Biosystems). Primers used in the study (Table. 1) were synthesized from Eurofins Genomics India Pvt. Ltd, Bangalore, India.Calculation of fold changes in gene expression was carried out using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Histopathology

On 24th day, rats were sacrificed using CO_2 asphyxia method after grossexamination of knee. After each sacrifice, right knee of each animal from all the groups were collected. The specimens were fixed in 10 per cent neutral buffered formaldehyde and stored at room temperature for 1 week. After decalcification, the tissue blocks were dehydrated in graded series of ethanol, cleared in xylene and were embedded in paraffin. Sections of 5-7 µm were prepared by using sliding microtome and stained with haematoxylin and eosin. The morphological differences observed in the sections were evaluated in each group and the mean scores were calculated. Histopathologicalanalysis were done by Dr. Chandrashekhar Mote at Innovet Diagnostic Laboratory, Pune.

Statistical analysis

The differences among experimental and control groups were determined using the Graph Pad 5.0 software. Comparisons among different groups were performed by analysis of variance using one way ANOVA test. Significant difference between means of different groups was assessed by Dunnett's post hoc-test. $P<0.05$ wasconsidered as statistically significant.

Results

Preliminary phytochemical screening and characterization of plant

The Methanolic extract was subjected to phytochemical analysis for evaluation of preliminary phytoconstituents. The crude drug parameters like ash values and extractive values were within the prescribed limits. (Table 2)

HPTLC fingerprints of the methanol extract on OA

HPTLC fingerprints of the methanol extract were generated after development of a suitable mobile phase - toluene: ethyl acetate: formic acid (6:3:1) for the separation of different phytoconstituents in the extract to be used as a tool for standardization of the methanol extract. Derivatization with suitable reagents (alcoholic ferric chloride solution (5%), Folin Ciocalteau reagent, conc. sulfuric acid, anisaldehyde sulfuric acid reagent, alcoholic potassium hydroxide solution indicated the presence of phenolic compounds in the extract (Table 3 and 4; Fig. 6,7 and 8).

Effect of Methanolic extract of *A.nilotica* on OA

After the administration of papain and cysteine, animals showed significant increase in knee diameter ($P<0.05$) on 8th day which sustained for 24 days. This increase was significantly ($p< 0.05$) prevented when the animals were treated with the standard drug celecoxib or extract. There was a time dependent and dose dependent decrease in the joint diameter when the rats were treated with *A. nilotica*. Celecoxib produced decrease in joint diameter compared to the control and it was maintained all throughout. On 24th day, the joint diameter of the rats treated with celecoxib or *A. nilotica* (400mg/kg) were not significantly different from the control indicating complete reversal of the inflammatory effect. (Fig. 1)

Effect of Methanolicextract of *A.nilotica* on Wire hang test

There was a significant reduction in the hanging time ($P<0.001$) in the animals induced with OA as compared to normal animals. There was partial increase in the hanging time by celecoxib as well as *A. nilotica* treated rats. The increase in the hanging time was time dependent and the maximum was observed at the end of 24 days with celecoxib and *A. nilotica* (400 mg/kg) (Fig. 2)

Effect of Methanolicextract of *A.nilotica* on expression of Cytokine genes

On 24th day, control animals and OA induced animals were compared for change in expression levels of cytokine genes such as TNF-α, IL-6 and IL-10 (Fig. 4). There was an increase in TNF-α and decrease in IL-10 and IL6 in the OA induced animals. These changes were found to be reversed when the animals were treated with extract. IL-10 was found to be increased in all groups after treatment. TNF-α was decreased in test group of 100 mg/kg and was increased in rest of the groups (Fig.3 & 4). The increase in IL10 and TNF-α by *A. nilotica* was found to be highest at 400 mg/kg.

Histopathology

The microscopic examination of induced control grouprevealed muscle degeneration and changes in the joint architecture, erosion of cartilage, chondrodystrophy and infiltration of mononuclear cells. Animals treated with test extract (100 mg/kg) showed very mild degenerationwhen compared to control group and mild damage when compared to induced group. At higher doses i.e. 200 and 400 mg/kg, there were minimal changes and the effects were almost similar to that of the 100 mg/kg test group (Fig. 5)

Discussion

OA can further lead to joint damage and permanent disability of limb thus causing serious problems in normal life of an individual. The present study demonstrates that the rats when injected with papain and cysteine intra-articularly, resulted in significant elevation of knee joint diameter ($P<0.05$) as well as significant reduction in fall time of animals ($P<0.001$). It is evident from wire hang test when compared with normal control group and exhibited the characteristic features of OA which were mainly gait disturbance, loss of free movement of limb, and withdrawal of paw. It was also seen that osteoarthritic rats showed increase in systemic TNF-α as well as decrease in IL-10. This was found to be in agreement with the previous reports (Ali A *et al.,*2012). The pods of *A nilotica* have been reported to contain gallocatechin, 5-O-gallate, methyl gallate, gallic acid, catechin, catechin 5-O-gallate, 1-O-galloyl-β -D-glucose, 1,6-di-O-galloyl-β-D-glucose and digallic acid(Plows *et. al.,* 1995).Phytoconstituents belonging to the galloylated sugars and tannins class have been reported to reduce the production of pro-inflammatory cytokines and mediators such as TNF-α, IL-16, NO (iNOS) and COX-2 on both protein and gene levels by blocking κβ nuclear translocation (Plows *et al.,* 1995). An exhaustive literature search indicates that the core chemical classes of anti-inflammatory agents from natural sources include polyphenols, flavonoids, terpenoidsand alkaloids. The phytoconstituents reported in *A. nilotica* corroborate our findings and the observed benefit in osteoarthritis.

In this study, animals treated with the test extract showed comparable reduction in knee joint diameter to that of standard treatment group. There was a time dependent and dose dependent decrease in the joint diameter when the rats were treated with *A. nilotica*. Celecoxib produced decrease in joint diameter to the control level and it was maintained all throughout the study. On 24th day, the joint diameter of the rats treated with Celecoxib or *A. nilotica* were not significantly different from the control indicating complete reversal of the inflammatory effect. This can be hypothesized that the effects of *A. nilotica* can be observed gradually and may not be just anti-inflammatory effects. OA is a chronic disease and the regression will also be the chronic process with *A. nilotica*.

Fall time of animals in wire hang test showed that when comparedto standard group, the test group of 100 mg/kg demonstrated more elevation in hanging time. There was partial increase in the hanging time by celecoxib as well as *A. nilotica* treated rats. The increase in the hanging time was found to be time dependent and the maximum was observed at the end of 24 days with celecoxib and *A. nilotica* (100 mg/kg).Though, the difference between control group and all other groups were significant, yet the animals were still not showing full recovery after the treatment. It appears that longer duration of treatment is required to get the effect with *A. nilotica*. However, more studies are required to be done.

This is the first experiment which was performed in order to see the effect of this extract on the systemic inflammatory cytokines. The present study revealed that after treatment with test compounds, pro-inflammatory cytokine, TNF-α was lowered in 100 mg/kg test group. Also IL-10, an anti-inflammatory cytokine was increased in all three groups. These results were in agreement with hypothesis of inflammation and recovery wherein it is believed that TNF-α rises in inflammation and IL-10 falls; whereas exact reverse happens in the recovery(Abramson *et al.,*2001; Ali *et al.,* 2012). Rise in TNF-α in the test groups of 200 and 400 mg/kg is assumed because of the possible hazard as well as toxicity of the extract. Elevation of TNF-α in standard group is not as significant as that of these two doses.

Treatment with the Methanolic extract reversed the damage caused in the cartilage which clearly indicated that the extract decreased the erosion of cartilage and other histopathological changes observed in the treatment group. It was also observed that the positive control group showed degraded cartilage, muscular degeneration around the joint.

Conclusions

In conclusion, our data suggest anti-osteoarthritic action of pods of *A. nilotica* on papain and cysteine inducedosteo-arthritic rats. This further validates its potential in the treatment of OA. Our studies also suggest involvement of TNF-α and IL-10 in the anti-osteoarthritic effects of *A. nilotica*. However, further studies are required to be carried out in order to find the mechanism of action for its anti-osteoarthritic effect.

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Table 1: Primer sequences used in Real Time PCR

Gene target	Primer sequence (5'-3')
TNF-α	F: CATCTGCTGGTACCACCAGTT R: TGAGCACAGAAAGCATGATC
IL-6	F: GAGAGCATTGGAAGTTGGGG
	R: CTTCCAGCCAGTTGCCTTCT
IL-10	F: AAACTCATTCATGGCCTTGTA
	R: TGCCTTCAGTCAAGTGAAGACT
GAPDH	F: GCCTTCTCCATGGTGGTGAA
	R: GGTCGGTGTGAACGGATTTG

Table 2: Physicochemical characterization of the pod powder of *A. Nilotica*

Parameters	Values obtain
Alcohol soluble extractives	42%
Water soluble extractives	45%
Total ash	4.98%
Water insoluble ash	2.25%
Acid insoluble ash	0.65%
LOD	8.96%

Table 3: HPTLC Data of Methanol Extract at 254 nm

Peak	Maximum Rf	Maximum height (mm)	Maximum area
1	0.30	140.3	5777.2
2	0.39	185.0	6336.5
3	0.50	618.3	25162.3
4	0.56	672.4	25322.1

Table 4: HPTLC data of Methanol Extract at 366 nm

Peak	Maximum Rf	Maximum height (mm)	Maximum area
1	0.16	42.0	1069.5
2	0.22	58.3	4010.8
3	0.35	81.0	3140.1
4	0.44	141.7	6698.8
5	0.53	81.4	1351.0
6	0.60	152.9	14931.6

Table 5: Change in the gene expression in induced animals compared to normal animals

Gene	Fold changes
TNF-α	1.12
IL-10	-1.02

Table 6: Change in the gene expression in treated animals compared to normal animals

Gene	Standard Control	*Acacia nilotica* (100mg/kg)	*Acacia nilotica* (200mg/kg)	*Acacia nilotica* (400mg/kg)
TNF-α	-1.08	-1.00	1.81	5.94
IL-10	1.75	1.25	1.34	2.97

Fig. 1: Effect of extract on knee joint diameter

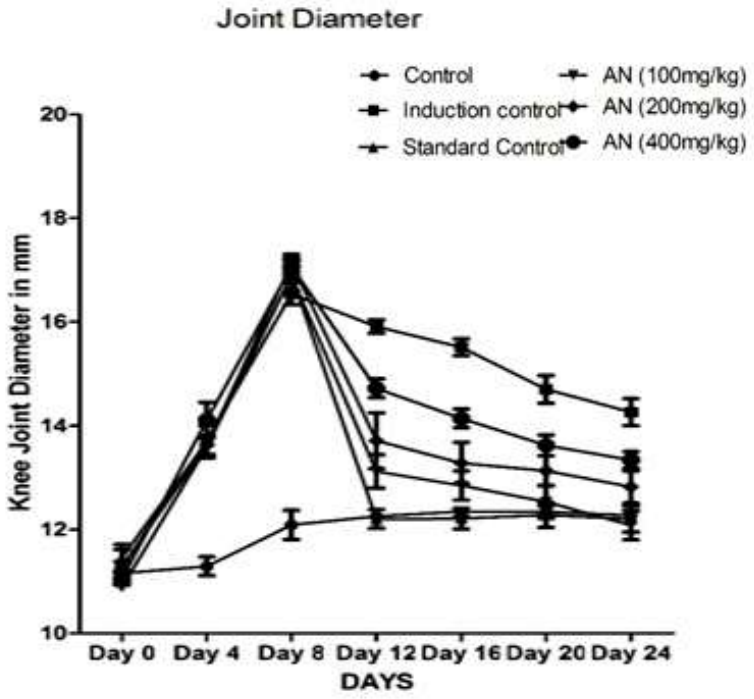


Fig. 2: Effect of extract on Wire Hang Test

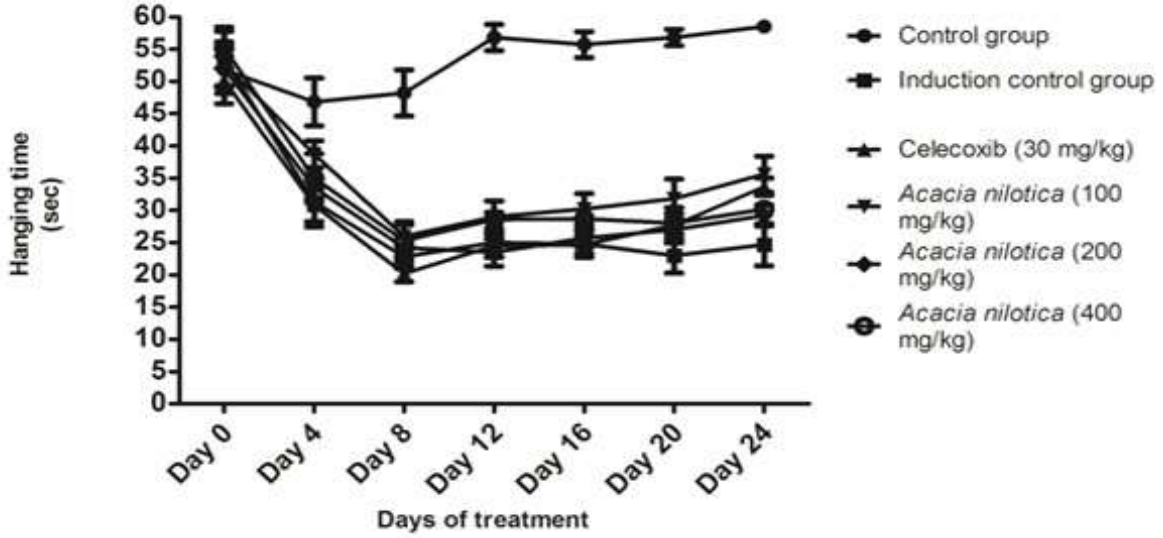


Fig. 3: Change in gene expression in positive control and normal control group

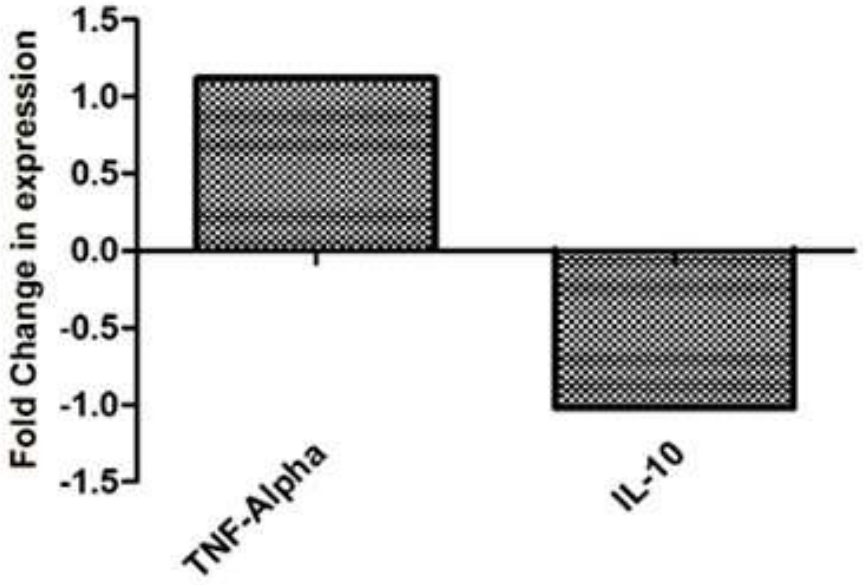


Fig. 4: Change in gene expression in test group compared to positive control group

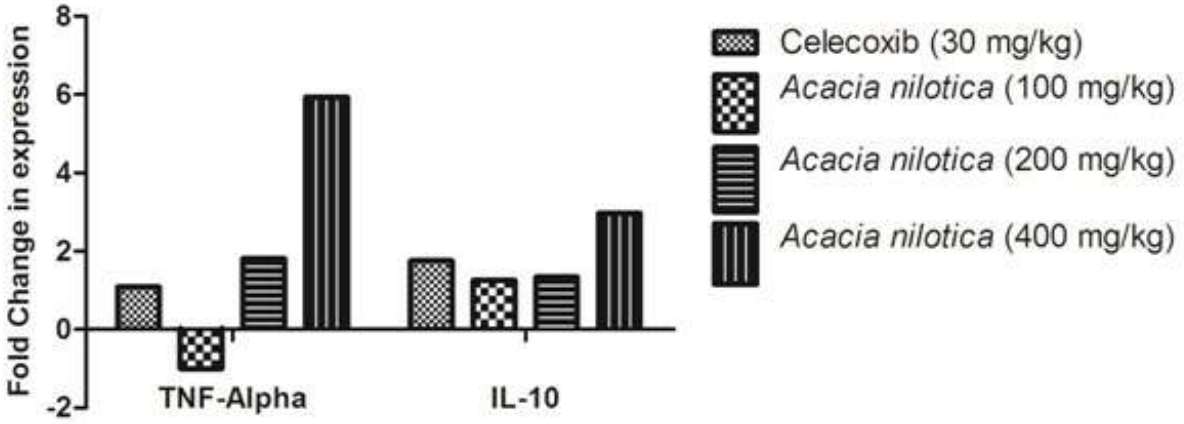
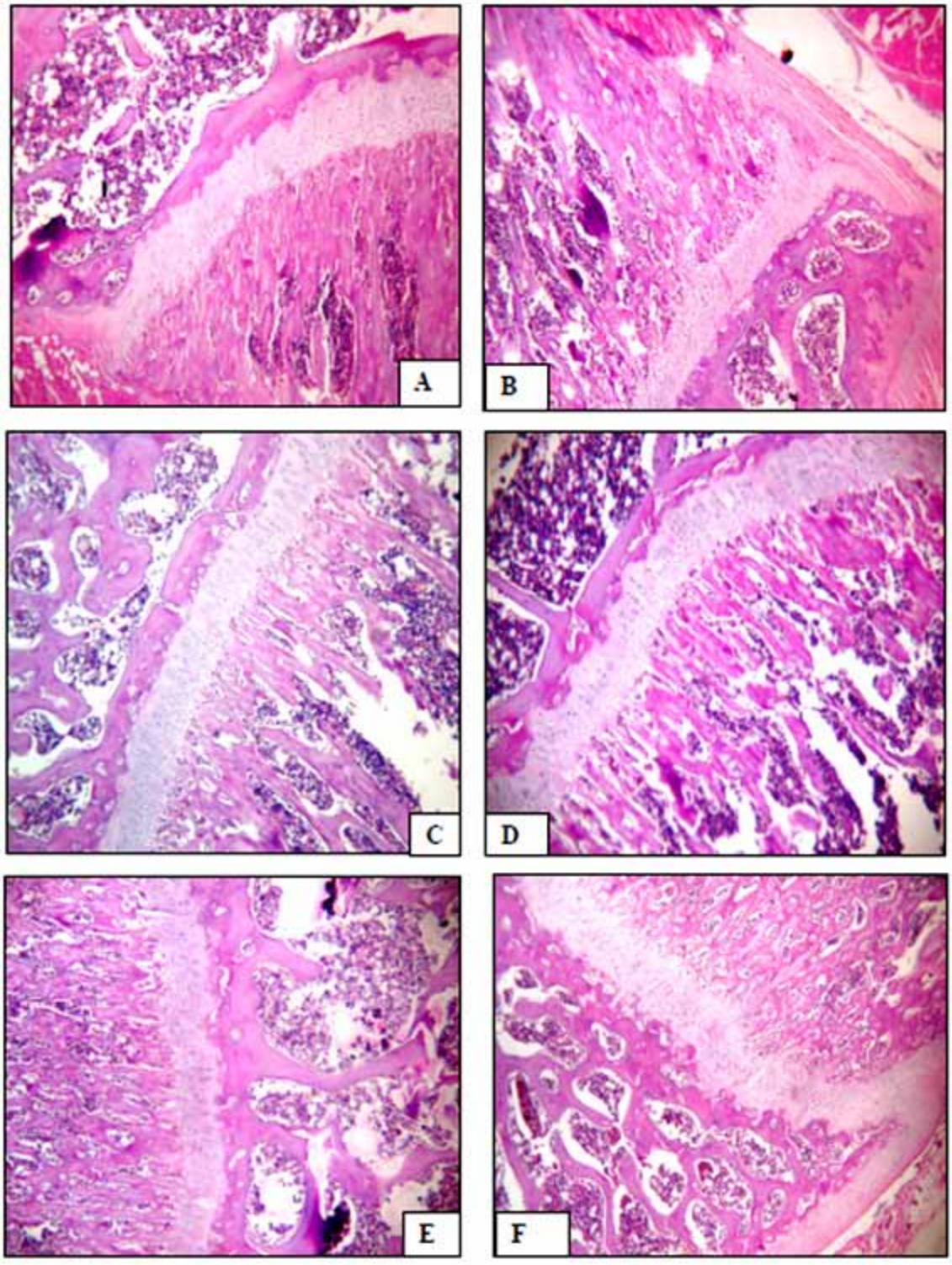


Fig. 5: Photomicrograph of joint tissues (10X)



(A) Standard control (B) Induction control (C) standard control (D) *Acacia nilotica* 100 mg/Kg (E) *Acacia nilotica* 200 mg/Kg (F) *Acacia nilotica* 400 mg/Kg

Fig. 6 : HPTLC fingerprints of methanol extract of *Acacia nilotica* at 254 nm

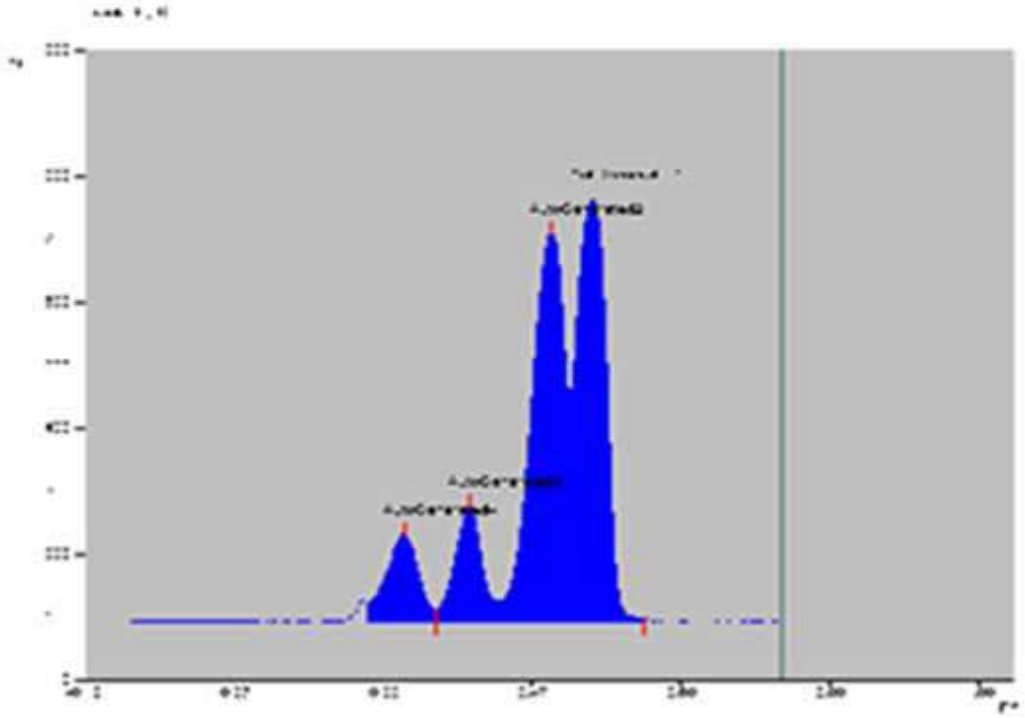


Fig. 7: HPTLC fingerprints of methanol extract of *Acacia nilotica* at 366 nm

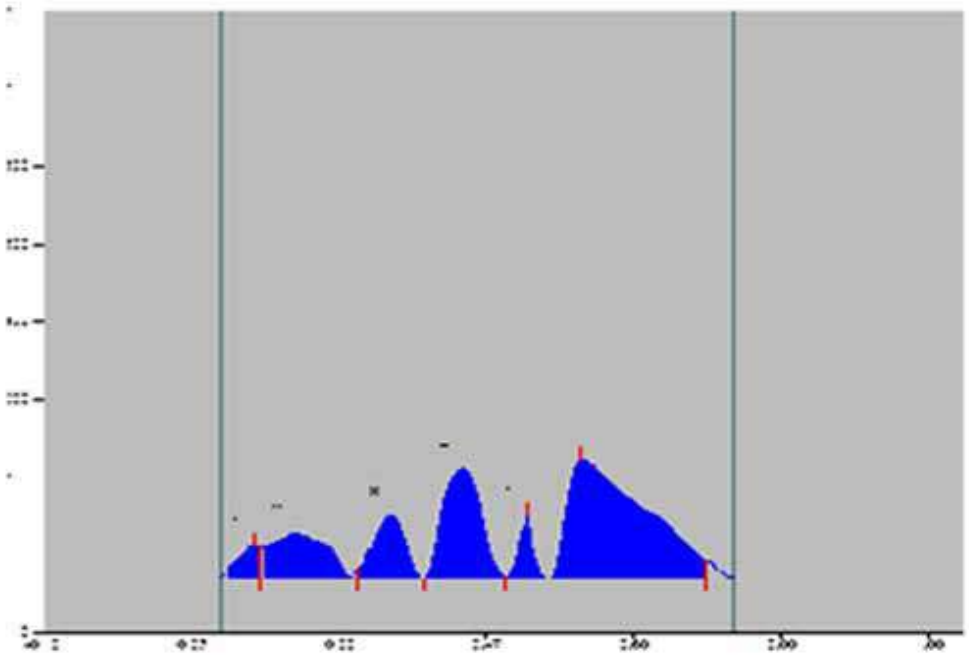


Fig. 8: HPTLC-UV spectral data of the components in the methanol extract of *Acacia nilotica*