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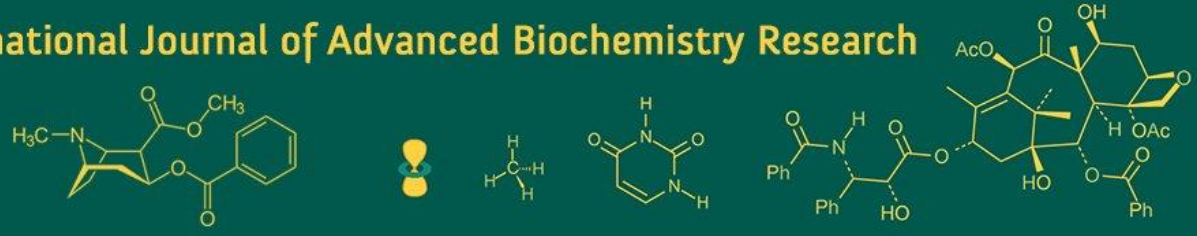

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Toxicity and efficacy of *Prosopis juliflora* seed pods extract on induced osteoarthritis in Wistar rats

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Abstract
The present study was planned to find out the LD_{50}, toxicity and efficacy of hydroalcoholic *Prosopis juliflora* seedpods extract (PJSPE) on experimentally induced osteoarthritis in Wistar rats. As per OECD test guideline 425, main test, oral gavage LD_{50} of PJSPE was greater than 2000 mg/kg body weight. Osteoarthritis was induced in 48 rats by intra-articular injection of monosodium iodoacetate (MIA, 1 mg/ joint). All the 48 rats were randomly divided into eight groups. Group I, II, III and IV served as a progressive phase while Group V, VI, VII and VIII served as reversal phase. Group I and V served as control and received only normal saline. Group II, III, IV of progressive phase and Group VI, VII, VIII of regressive phase received PJSPE dissolved in normal saline at doses of 250, 500 and 1000 mg/kg bodyweight respectively, by oral gavage daily for period of 28 days. The dosing was started from same day of induction of osteoarthritis in progressive phase while in reversal phase dosing was initiated after 28 days of osteoarthritis induction. All 48 rats of the study were survived up to terminal scarifies. Body weight, clinical pathology, organs weight, and organs of rats in the treatment groups of progressive and reversal phase did not showed any significant changes and were comparable to control of their respective phase. A 28 day oral dosing of 1000 mg/kg PJSPE did not show protective (in progressive phase) or ameliorative (in regressive phase) effect on MIA induced osteoarthritis. 1000 mg/kg was no observed adverse effect level.

Keywords: *Prosopis juliflora*, bodyweight, osteoarthritis, osteoarthritis, treatment groups

Introduction
Due to advancement in biological research, average life span of human being increases with decrease in death rates. However longer lifespan predisposed humans to various non-communicable diseases like heart attacks, cancer, asthma, diabetes as well as musculoskeletal disorders. When taking into both death and disability, all musculoskeletal disorders combined accounted for 6.7% of the total global disability-adjusted life years, which was the fourth greatest burden on the health of the world's population (third in the developed countries) (Woolf, 2015) [40]. Osteoarthritis (OA), rheumatoid arthritis, gout, low back pain, and neck pain are important musculoskeletal disorders responsible for aforesaid conditions.

Osteoarthritis (OA) is the most common joint disorder involving joint cartilage, underling bone, synovial membrane and characterized by progressive cartilage destruction,hypertrophy of bone at the margins, subchondral sclerosis, and range of biochemical and morphological alterations of the synovial membrane and joint capsule, pain, and loss of function (Pal *et al*., 2016) [21].In USA, approximately 10% in men and 13% in women are suffering from symptomatic knee OA and numbers are likely to increase due to the aging and obesity (Zhang & Jordan, 2010) [41]. According to one epidemiological study (Pal *et al*., 2016) [21], knee OA was found to be 28.7% in India. Gender (female are more prone to OA than male), obesity and sedentary works were associated factors for OA in India [2]. Till today, OA is considered be non-curable diseases with therapeutic strategies primarily aimed at analgesia and ameliorationjoint function. Intra-articular injections (hyaluronate, corticosteroid), arthroplasty or rarely autologous chondrocyte implantation into the damaged area are some therapeutic strategies of OA (Bennell *et al*., 2012; Hunter & Felson, 2006) [3, 8].

In one survey, almost 70,000 joint replacement surgeries were performed in India in the year 2011 (Pachore *et al*., 2013) [20]. Thereare many animal models that reproduce key aspects of human OAin terms of natural history, mechanisms, signs, and symptoms (Poole *et al*., 2010) [22]. Dunkin Hartley guinea pigs develop spontaneous OA at age of 18 months onwards, which is similar to those of human OA (Jimenez *et al*., 1997) [9], however much longer period is required. For rapid development of OA in laboratory animal surgery or chemical methods is used. Among surgical method, medial meniscal tear, partial medial meniscectomy, destabilization of the medial meniscus, and anterior cruciate ligament transection are frequently used method for induction of knee OA (Takahashi *et al*., 2018) [33]. Among chemical method, monosodium iodoacetate (MIA) rat model is well established, and the induced OA resembles human degenerative OA in terms of the histological and pain-related behaviours (Takahashi *et al*., 2018) [33].

From ancient era to till days, ayurveda is one of the leading systems of medicine used for treatment of many geriatricdiseases in India. In this traditional medicine system, most of the plants prevalent in India have been identified and their used in different diseases have been documented. However, some plants which were introduced in last one or two centuries in India are not well studied and their medicinal property yet to be documented. *Prosopis juliflora* is one of the most widespread hyper accumulating plants introduced in India nearly 130 years ago to prevent advancement of desert (Tewari *et al*., 2001) [35]. Because of *Prosopis juliflora*is not ancient Indian plant, their medicinal property are not documented in Indian ancient literature.

In some recent research, medicinal and other properties of *Prosopis juliflora*have been reported *viz*., as a part of layer diets (Khobondo *et al*., 2019) [11], hepato-protectant activity (Hassan *et al*., 2019) [7], antibacterial activity (Shah *et al*., 2018; Tajbakhsh *et al*., 2015; Thakur *et al*., 2014) [27, 32, 36] and, immunomodulation (Shah *et al*., 2018) [27]. In some unpublished research, *Prosopis juliflora*seed pods are effective in amelioration of clinical OA in humans (personal communication with Ayurvedacharya), however no preclinical studies have been carried out to support such hypothesis. Looking to the paucity of information present study was planned with following objectives. 1) To estimate LD_{50} of *Prosopis juliflora* seed pods extract by acute oral toxicity study (up-and-down procedure) in Wistar rats;2) To study the repeated dose 28-daysoral toxicity and efficacy of *Prosopis juliflora* seed pods extracton induced osteoarthritisin Wistar rats.

Experimental

Institutional Animal Ethics Committee (IAEC) approval

The protocols entitled "Acute oral toxicity study (up-and-down procedure) of *Prosopis juliflora* seed pods extract in Wistar rats" (VETCOLL/IAEC/2019/14/Protocol No. 4) and "Oral efficacy and toxicity study of *Prosopis juliflora* seed pods extract on induced osteoarthritis in rats" (VETCOLL/IAEC/2019/14/Protocol No. 5) were approved by the Institutional Animal Ethics Committee (IEAC) of the College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Gujarat, India.

Test compound Preparation: *Prosopis juliflora* seedpods extract (PJSPE) was used as a test compound. Fully matured seedpods of PF were collected around SDAU campus in the month of June 2019. The seedpods of PF were thoroughly washed with distilled water, and dry under shade in the laboratory. The dried PF seedpods were powdered by mixture grinder machine. Finally, powdered PF seedpods sieved through the kitchen strainer and fine powder was collected for extraction. Fifty gram of powdered PF seedpods was kept in 1000 mL conical flask and added 1000 mL distilled water. The closed conical flask kept in a shaker with shaking during initial six hours and no shaking for remaining eighteen hours. The mixture was filtered with filter paper. The water from the mixture was removed by heat in water bath temperature with 50 °C temperature. Finally, experiments were conducted using the residue collected from the previously mentioned process. For maintaining the constant volume of throughout the study, rats were dosed 1 ml/100g of body weight. The dose formulations were prepared by suspending the PJSPE in distilled water. For acute study, dose was prepared on daily basis, and for 28 days studies, dose formulations were prepared on weekly basis.

Animal Procurement

A total 48 female Wistar rats were procured from laboratory animal facility of Torrent Research Centre, GIDC Bhat, Ahmedabad, Gujarat 382424, India,for these two studies. All the procured female rats were kept under acclimatization for a period of 15 days enrolling in the studies.

Housing and environmental conditions

Animal management and treatment procedures complied with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. All the rats were housed in polypropylene cages at laboratory animal house facility in an environmentally controlled room with 22±3 ºC temperature and 30-70% humidity with continues monitoring. Light/dark cycles of 12/12 hours were provided throughout the acclimatization and study period. Corncob was used as bedding material. Rats had *ad libitum* access to standard pellet diet (VRK Nutritional Solution, Sangli, Maharashtra, India) and RO drinking water throughout the study period. The composition of pellet diet is presented in Table 3.2. Rats were identified by tail markings. All essential management procedures were adopted to keep the rats free from stress.

Acute oral toxicity study (up-and-down procedure) of PJSPE in Wistar rats

This experiment was design as per OECD Guidelines for the Testing of Chemicals, Section 4, Test No. 425 (OECD, 2008). A total five Wistar rat were dosed sequentiallyat 48 hours intervals. All five rats were fasted prior to dosing. Feed but not water was withheld from 5:00 p.m. on the day preceding dosing. Following the period of fasting, the rat was weighed, dose was calculated, and the test substance was administered in a single dose by gavage using a stomach tube. After dosing, feed was withheld for a further 3 hours. In first 30 minutes after dosing, rats were observed individually at least once, thereafter daily for a total of 14 days. The first animal was given a 175 mg/kg dose of the PJSPE. When the animal survived after 48 h, the next dose of the extract of 550 mg/kg was given to the second animal. When the second animal survived after 48 h, the next animal

was given a dose of 2000 mg/kg (upper bound dose). 2000 mg/kg PJSPE was given in two more animals in similar fashion. Due to three consecutive animals survive at the 2000 mg/kg, testing was terminated, and LD_{50} was derived.

Repeated dose 28-days oral toxicity and efficacy of PJSPE on induced osteoarthritis in Wistar rats

Induction of osteoarthritis (OA)

Osteoarthritis was induced by intra-articular injection of monosodium iodoacetate (MIA; cat. #I2512; Sigma-Aldrich, St. Louis, MO, USA). MIA dissolved in physiologic saline and administered in a volume of 50 µl using a 27-gauge, 0.5-inch needle (1 mg/joint). Anesthetized rats were given single intra-articular injection of MIA through the infrapatellar ligament of the femorotibial joint. Care was taken to ensure that the needle was not advanced too far into the cruciate ligament. OA was induced in all 48 rats on Day 1 of study.

Experimental design

The study was carried out on 48 MIA induced OA female Wistar rats. All the 48 rats were randomly divided into eight different groups of which Group I, II, III and IV served as a progressive phase while Group V, VI, VII and VIII served as reversal phase. Each group consisted of six rats. Group I and V served as control and received only normal saline as vehicle. Group II, III, IV of progressive phase and Group VI, VII, VIII of regressive phase received PJSPE dissolved in normal saline at doses of 250 mg/kg (low dose), 500 mg/kg (mid dose) and 1000 mg/kg bodyweight (high dose) respectively, by oral gavage daily for period of 28 days. The dosing was started from same day of induction of OA in progressive phase while in reversal phase dosing was initiated from day 29 of the study. The detail of experimental design is depicted in Table 1.

Table 1: Experimental design

Group	No of animals	Compound	Dose mg/kg b.wt/day
	Progressive phase (Dosing from same day of the induction of OA)		
I	101-106	Vehicle (Normal Saline)	(Control)
II	201-206	*Prosopis juliflora seed pods extract*	250 mg/kg b. wt
III	301-306	*Prosopis juliflora seed pods extract*	500 mg/kg b. wt
IV	401-406	*Prosopis juliflora seed pods extract*	1000 mg/kg b. wt
Regressive phase (Dosing from day 29th of the induction of OA)			
V	501-506	Vehicle (Normal Saline)	(Control)
VI	601-606	*Prosopis juliflora seed pods extract*	250 mg/kg b. wt
VII	701-706	*Prosopis juliflora seed pods extract*	500 mg/kg b.wt
VIII	801-806	*Prosopis juliflora seed pods extract*	1000 mg/kg b.wt

Observations for morbidity and mortality were made twice daily throughout the study. During acclimation period clinical observation were recorded once daily. Clinical observations were conducted at least twice on each day of dosing (Predose and post dose). The body weight of each rat was recorded one day of initiation of dosing and thereafter at weekly intervals throughout the study period. The body weight of progressive phase was taken on study day 1, 7, 14, 21 and 28 while in regressive phase body weight was taken on study day 29, 35, 42, 49 and 56. Relative organ weight was calculated on basis of terminal body weight recorded before necropsy.

The rats were fasted overnight prior to blood collection and necropsy. On study day 29 (Progressive phase) and 57 (Regressive phase), form all rats of particular phase, blood was collected from the retro-orbital plexus with the help of a heparinised capillary tube in clot activator for clinical chemistry and in EDTA for haematology. Haemoglobins (Hb), haematocrit (HCT), total erythrocytes count (TEC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobins concentration (MCHC), red cell distribution width (RDW%), total leucocytes count (TLC), neutrophils (Absolute and%), lymphocytes (Absolute and%), monocytes (Absolute and%), eosinophils (Absolute and%), total platelet count,, and mean platelet volume (MPV) were analysed on the day of collection using an Exigo Eos Vet, Boule Diagnostics AB, Sweden. Blood smears were prepared within 3 hours of collection and stained with Giemsa for evaluation of platelet, erythrocyte morphology and basophils count. Basophils percent were counted in blood smear and converted to absolute count. Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphates (ALP), gamma-glutamyl transferase (GGT), total protein, albumin, blood urea nitrogen (BUN), uric acid, creatinine, cholesterol, triglyceride, calcium, phosphorus, magnesium, and glucose were analysed on the day of collection using a Randox Monaco fully automatic clinical chemistry analyser, Randox Laboratories Ltd. UK.

All the rats of progressive and regressive phase were euthanized, on 29th and 57th day of the study respectively. The rats were fasted overnight prior to necropsy. Terminal body weights were collected prior to necropsy. All animals in the study were subjected to a full detailed necropsy. Both left and right femorotibial joint, peripheral nerve, skeletal muscle, brain, stomach, intestines, liver, kidneys, adrenals, spleen, heart, thymus, trachea, lungs, ovary, uterus, cervix, vagina, urinary bladder, and lymph nodes. The liver, kidneys, adrenals, thymus, spleen, brain, and heart of all animals were trimmed of any adherent tissue, and wet weight were be taken as soon as possible after dissection. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin.

Fixed tissues except bone with joints were trimmed, labelled, and washed under running tape water for 2 hours. Dehydration was done using ascending grade (30%, 70%, 90%, and 100%) of isopropyl alcohol. The dehydrated tissues were cleared by two changes of xylene and impregnated in melted paraffin wax. The entire tissue processing was performed in automatic tissue processor (Leica TP1020). The paraffin-impregnated tissues were embedded using Leica EG1160 paraffin embedding station and cooled on Leica EG1150 C Cold Plate. The 4 to 5 µ thick sections were cut using Leica RM2255 fully automated rotary microtome. The sections were taken on poly-L-lysine-coated (0.1% w/v in H2O) slides and stained with Harris Haematoxylin and Eosin (HE). The sections were deparaffinised by xylene, rehydrated through descending grade of isopropyl alcohol and water. The hydrated tissue sections were stained with haematoxylin,

differentiated in acid alcohol and blueing was carried out by ammonia water. Then tissue sections were stained with eosin, dehydrated with absolute isopropyl alcohol, followed by xylene wash, and mounted with DPX (Suvarna *et al*., 2013) [29]. The entire staining was performed in Gemini AS Automated Slide Stainer, Thermo Scientific.

After fixation in 10% neutral buffered formalin for 6 days, all the joints were decalcified with formic acid-sodium citrate method (Luna, 1968) [14] for a period of 12 days. After decalcification, as per Gerwin *et al*. (2010) (Gerwin *et al*., 2010) [5], frontal sectioning of jointswas performed by cutting into two approximately equal halves, an anterior, and a posterior one, along themedial collateral ligament in the frontalplane. Joints were processes routinely as describe earlier. Anterior and posterior half of the joint were embedded in a single paraffin block with the cut planes facingdown. Three 8 µsections were cut from each paraffin block atapproximately 200 mmsteps to obtain three sections from each half (anterior and posterior) of the joint. The first section was stained with HIM as describe earlier, while remaining two sections were stained with Toluidine blue to detect areas of proteoglycans loss in cartilage. Toluidine blue staining method was adopted from Gerwin *et al*. (Gerwin *et al*., 2010) [5] and Schmitz *et al*. (Schmitz *et al*., 2010) [26].

Microscopic scoring of OA was performed as recommendation of OARSI histopathology assessment system Pritzker *et al*. (Pritzker *et al*., 2006) [23] and modified Mankin score. The modified Mankin score was adopted fromTakahashi *et al*. (Takahashi *et al*., 2019) [33] with little modification as we used toluidine blue rather than Safranin O staining. On basis of cartilage structure, cellularity, toluidine blue staining intensity, and tidemark integrity, a total score 0-15 was given. In this system, higher values indicating more severe cartilage degeneration. We also used Pritzker *et al*. (Pritzker *et al*., 2006) [23] scoring system. In this system on basis of cartilage histopathology grade and stage assessment, the score ranges from 0 to 24, with higher values indicating more advanced cartilage degeneration was calculated.

The statistical analysis of data generated on various parameters was subjected to statistical analysis using 2-way analysis of variance (ANOVA). Pairwise comparisons with control, for each phase separately, was made using Dennett's test.

Results and Discussion

Acute oral toxicity study (up-and-down procedure) of PJSPE in Wistar rats

The five rats were dosed with 175, 550, 2000, 2000, 2000 mg/kg of PJSE respectively with oral gavage. All rats were survived up to 14 days period after dosing. There were not any variations in behavioural pattern or any clinical signs noted in rats during the 14-day observation period. On basis of acute oral toxicity of the PJSE utilizing the OECD 425 Main test, LD_{50} was calculated, and it was greater than 2000 mg/kg.

Plant products and herbal medicine are used to treat various pathological conditions of humans and animals since ancient era, due to its ameliorative effect or due to cultural belief. World Health Organisation (WHO) advocating the use of herbal medicine along with routine treatment and they also recommend performing scientific trials on herbal medicine to evaluate the efficacy and toxicity of various herbal medicine (WHO, 1993) [39]. Very few studies have been planned to estimate LD_{50} of various herbal medicine, as most of these products are considered as safe in humans as well as in animals in short term as well as long-term exposure. TraditionalLD_{50} study was conducted with different protocols however, most of new LD_{50} studies were conducted on basis of OECD 425 guideline with main test or limit test to derived LD_{50} of a different herbal compounds (Saleem *et al*., 2017; Shi *et al*., 2019) [24, 28]. Hassan *et al*. (Hassan *et al*., 2019) [7] conducted acute study to find out the LD_{50} of methanol extract of PJ leaves in rat. In their study, intraperitoneal PJ leaves extract administration gave LD_{50} value of 44.4 mg/ Kg. In this study LD_{50} is more than 2000 mg/kg. Probably the route of exposure (oral vs. intraperitoneal) as well as part of PJ (seedpods vs. leaves) have significant effects on LD_{50} in rats. In acute oral gavage toxicity study of *Prosopis farcta* extract in rat, LD_{50} was 540 mg/kg (Moayeri *et al*., 2018) [17]. In experiment conducted by Vasile *et al*. (Vasile *et al*., 2019) [38], LD_{50} of *Prosopis Alba* exudate gum was found to be >1000 mg/kg. This difference is probably due to different species *Prosopis* used in the studies. In one cattle study, PJ seedpods induced neuronal vacuolation, loss of neurons, degeneration of nerves and atrophy of masseter and other masticatory muscles after 200 days oral exposure (Tabosa *et al*., 2006) [30]. In goats, after 270 days oral exposure, neuronal vacuolation of trigeminal nuclei, Wallerian degeneration in mandibular and trigeminal nerves and atrophy of associatedmuscles were observed (Tabosa *et al*., 2000) [31]. Findings of these studies suggest that long term exposure (about 200 days or more) PJ have toxic effect in animals while short term exposure may be unable to produced appreciable gross or microscopic changes.

Repeated dose 28-days oral toxicity and efficacy of PJSPE on induced osteoarthritis in Wistar rats

All 48 rats of the study were survived up to terminal scarifies. Rats in the treatment group of progressive and reversal group did not show any in-life observations and were comparable to control of their respective phase. The body weight summary rats of all groups are presented in Table 2 and 3. Body weight of rats in the treatment group of progressive and reversal group did not show any significant changes and were comparable to control of their respective phase.

Table 2: Effect of *Prosopis juliflora* seedpods extract on weekly body weight (Mean ± SD, N=6, g) in female rats after daily oral administration for 28 days in progressive phase.

Group	I	II	III	IV
Days	**0 mg/kg**	**250 mg/kg**	**500 mg/kg**	**1000 mg/kg**
0 Day	311.40±27.594	306.78±31.892	313.82±27.950	312.75±27.950
7 Day	317.70±36.312	314.03±36.802	293.88±22.685	304.88±26.821
14 Day	325.35±30.332	317.13±39.281	313.58±25.760	292.633±27.211
21 Day	320.57±23.214	316.86±37.325	313.88±31.907	306.88±27.02
28 Day	330.43±27.569	324.21±38.185	316.05±23.277	308.033±28.473

Table 3: Effect of *Prosopis juliflora* seedpods extract on weekly body weight (Mean ± SD, n=6, g) in female rats after daily oral administration for 28 days in regressive phase.

Group	V	VI	VII	VIII
Days	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
29 Day	293.22±18.199	293.81±27.07	304.12±22.758	302.22±22.536
35 Day	306.27±16.452	304.06±24.327	293.88±22.685	312.917±18.383
42 Day	306.23±17.74	306.05±24.782	313.58±25.760	317.967±17.908
49 Day	304.12±22.75	315.75±22.109	313.88±31.907	317.46±18.835
56 Day	306.68±12.977	310.66±18.993	316.05±23.277	314.21±22.293

The haematology summary of rats of all groups is presented in Table 4 and 5. Haematology of rats in the treatment group of progressive and reversal group did not show any significant changes and were comparable to control of their respective phase. The clinical pathology summary of rats of all groups is presented in Table 6 and 7. Clinical pathology of rats in the treatment group of progressive and reversal group did not show any significant changes and were comparable to control of their respective phase.

Table 4: Effect of *Prosopis juliflora* seedpods extract on hematological parameters (Mean ± SD, n=6) in female rats after daily oral administration for 28 days in progressive phase.

Parameters	G I	G II	G III	GIV
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
RBC (10^6/ µL)	07.20±0.374	07.35±0.197	07.65±0.288	07.53±0.493
Haematocrit (%)	43.78±2.718	43.68±1.867	46.07±3.422	42.90±0.957
Haemoglobin (g/dL)	13.90±0.684	14.08±0.366	14.23±0.175	14.13±0.250
MCV (fL)	60.88±0.606	59.40±1.254	60.15±4.450	58.10±2.662
MCH (pg)	19.33±0.606	18.60±0.566	18.57±0.715	18.05±0.394
MCHC (g/DL)	30.62±2.190	19.15±0.373	18.68±0.445	18.50±0.746
RDW (%)	18.12±0.223	17.82±0.293	19.07±0.766	21.12±7.993
WBC (10^3/µL)	06.18±2.406	04.43±1.197	05.53±2.537	05.48±1.306
Neutrophils (%)	12.37±2.650	10.68±4.967	14.78±6.442	10.67±3.724
Lymphocytes (%)	82.48±2.843	84.27±5.201	80.85±7.133	84.06±3.616
Monocytes (%)	03.82±0.445	03.88±1.111	03.16±0.472	04.30±1.817
Eosinophils (%)	01.33±0.516	01.33±0.516	01.21±0.401	01.28±0.449
Basophils (%)	00.00±0.000	00.00±0.000	00.00±0.0 0	00.00±0.00
Neutrophils (10^3/µL)	00.75±0.427	00.47±0.325	00.67±0.326	00.52±0.255
Lymphocytes (10^3/µL)	05.18±2.093	03.60±1.623	04.58±2.217	04.62±1.170
Monocytes (10^3/µL)	00.25±0.122	00.20±0.128	00.22±0.075	00.28±0.113
Eosinophils (10^3/µL)	00.08±0.049	00.06±0.037	00.06±0.027	00.07±0.018
Basophils (10^3/µL)	00.00±0.000	00.00±0.00	00.00±0.000	00.00±0.00
Platelets (10^3/µL)	862.67±204.955	993.67±144.498	872.00±190.570	927.00±130.684

Table 5: Effect of *Prosopis juliflora* seedpods extract on hematological parameters (Mean ± SD, n=6) in female rats after daily oral administration for 28 days in regressive phase.

Parameters	G V	G VI	G VII	G VIII
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Neutrophils (%)	07.63±0.234	07.35±0.197	07.65±0.288	07.53±0.493
Lymphocytes (%)	45.33±3.501	46.40±3.258	43.78±3.561	40.10±12.603
Monocytes (%)	13.92±0.434	14.05±0.846	13.32±0.975	12.02±0.250
Eosinophils (%)	59.37±5.078	61.38±1.314	60.55±5.219	60.10±1.797
Basophils (%)	18.20±0.802	18.60±0.566	18.57±0.715	18.05±0.394
Neutrophils (10^3/µL)	30.62±2.190	30.32±2.523	31.07±1.633	30.07±0.472
Lymphocytes (10^3/µL)	14.00±0.551	14.03±0.468	13.90±0.544	13.80±0.282
Monocytes (10^3/µL)	3.33±1.218	03.62±0.886	04.53±1.127	03.88±1.609
Eosinophils (10^3/µL)	13.46±3.915	15.82±4.440	13.87±7.358	12.85±7.961
Basophils (10^3/µL)	79.96±4.786	78.62±4.639	79.85±8.999	82.57±9.481
Platelets (10^3/µL)	05.17±3.488	04.07±0.859	05.04±3.381	03.08±1.206
Neutrophils (%)	01.42±0.492	01.50±0.548	01.40±0.490	01.50±1.049
Lymphocytes (%)	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
Monocytes (%)	00.59±0.311	00.56±0.311	00.65±0.487	00.60±0.489
Eosinophils (%)	02.34±1.105	02.83±0.574	03.60±0.787	03.02±1.139
Basophils (%)	00.21±0.122	00.17±0.078	00.27±0.175	00.22±0.075
Neutrophils (10^3/µL)	00.07±0.031	00.90±1.154	00.75±1.594	00.07±0.060
Lymphocytes (10^3/µL)	00.00±0.000	00.00±0.000	00.00±0.000	00.00±0.000
Monocytes (10^3/µL)	948.67±298.382	1144.67±134.977	1149.50±225.494	1042.00±197.497

Table 6: Effect of *Prosopis juliflora* seedpods extract on biochemical parameters (Mean ± SD, n=6) in female rats after daily oral administration for 28 days in progressive phase.

Parameter	G I	G II	G III	G IV
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
ALT (U/L)	039.83±02.137	024.20±07.817	032.68±04.967	032.83±08.244
AST (U/L)	201.02±20.588	178.93±43.862	173.02±79.849	173.72±05.324
ALP (U/L)	053.33±17.682	052.83±08.954	053.50±10.597	056.17±0 6.243
GGT (U/L)	002.83±00.408	003.17±00.753	002.50±00.548	002.50±01.517
Total Protein (g/dL)	007.63±00.197	007.28±00.549	007.42±00.293	007.53±00.280
Albumin (g/dL)	004.38±00.332	004.20±00.473	004.12±00.366	004.30±00.179
Urea (mg/dL)	059.95±07.992	056.94±02.818	054.93±11.365	0061.48±6.173
Creatinine (mg/dL)	001.01±00.157	001.06±00.445	000.93±00.181	001.06±0.121
Uric Acid (mg/dL)	005.38±01.153	005.03±00.650	004.25±01.122	004.22±1.277
Glucose (mg/dL)	040.67±04.633	049.17±08.010	049.17±27.737	046.33±15.423
Triglyceride (mg/dL)	158.02±24.448	138.55±27.686	159.47±09.760	170.73±38.434
Cholesterol (mg/dL)	127.25±30.685	133.05±37.623	103.80±07.981	155.30±17.164
Calcium (mg/dL)	011.55±00.609	011.17±00.361	011.62±00.264	011.77±0.314
Phosphate (mg/dL)	006.90±00.919	007.87±01.533	007.28±00.519	006.78±0.549
Magnesium (mg/dL)	003.78±00.303	003.52±00.370	003.44±00.345	003.58±0.352

Table 7: Effect of *Prosopis juliflora* seedpods extract on biochemical parameters (Mean ± SD, N=6) in female rats after daily oral administration for 28 daysin regressive phase.

Parameter	G V	G VI	G VII	G VIII
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
ALT (U/L)	027.50±5.753	025.17±02.137	031.33±09.766	030.58±05.554
AST (U/L)	156.05±38.191	151.33±37.987	170.07±36.570	170.88±26.143
ALP (U/L)	027.50±05.753	056.83±17.680	62.17±22.658	051.00±11.6079
GGT (U/L)	000.83±00.753	001.00±00.632	001.00±00.632	000.83±00.408
Total Protein (g/dL)	007.35±00.389	007.87±00.459	007.80±00.310	007.50±00.415
Albumin (g/dL)	003.58±00.183	003.72±00.075	003.70±00.110	003.62±00.098
Urea (mg/dL)	099.86±14.146	90.91±14.080	094.61±28.277	093.80±15.215
Creatinine (mg/dL)	000.89±00.061	000.86±00.071	000.90±00.050	000.89±00.046
Uric Acid (mg/dL)	005.12±00.659	003.38±01.153	004.47±00.929	004.62±00.975
Glucose (mg/dL)	73.67±28.591	067.67±22.651	071.50±17.329	069.00±20.523
Triglyceride (mg/dL)	115.02±28.598	106.13±45.028	116.53±31.567	137.48±06.569
Cholesterol (mg/dL)	107.82±22.192	123.13±11.830	106.10±23.300	113.62±17.598
Calcium (mg/dL)	11.15±00.394	011.28±00.392	011.50±00.253	011.22±00.117
Phosphate (mg/dL)	006.35±00.822	006.72±00.768	006.05±00.536	006.25±00.999
Magnesium (mg/dL)	002.78±00.270	003.03±00.284	003.16±00.388	003.01±00.134

During full detail necropsy on study day 29th and 57th, rats from all treated group did not show any test article related changes in any organs and were comparable to controls of their respective phase. Absolute and relative organ weight of rats in the treatment group of progressive and reversal group did not show any significant changes and were comparable to control of their respective phase. The absolute and relative organ weigh summary are presented in Table 8 and 9 and, Table 10 and 11 respectively.

Table 8: Effect of *Prosopis juliflora* seedpods extract on absolute organ weight (Mean ± SD, N=6) in female rats after daily oral administration for 28 days in progressive phase.

Organ	G I	G II	G III	G IV
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Liver	10.69±1.659	10.79±1.971	09.43±0.891	10.53±1.594
Brain	01.99±0.163	01.92±0.229	01.945±0.102	01.97±0.160
Heart	01.12±0.070	16.50±07.960	01.00±0.069	01.125±0.203
Adrenals	00.37±0.379	00.12±0.026	00.10±0.012	00.13±0.027
Thymus	00.25±0.050	00.28±0.055	00.29±0.064	00.25±0.107
Spleen	00.75±0.223	00.69±0.136	00.56±0.142	00.51±0.200
Kidneys	01.99±0.218	02.07±0.172	02.26±0.248	02.01±0.199
Lungs	01.94±0.092	01.97±0.231	01.91±0.135	01.93±0.160

Table 9: Effect of *Prosopis juliflora* seedpods extract on absolute organ weight (Mean ± SD, N=6, g) in female rats after daily oral administration for 28 days in regressive phase.

Organ	G V	G VI	G VII	G VIII
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Liver	9.15±0.905	9.58±0.894	9.58±1.335	8.65±0.730
Brain	2.13±0.368	2.01±0.134	2.18±0.211	2.00±0.199
Heart	0.62±0.060	0.71±0.100	0.71±0.132	0.64±0.116
Adrenals	2.06±0.098	2.07±0.129	2.14±0.134	2.07±0.080
Thymus	1.08±0.112	0.96±0.027	1.13±0.202	1.00±0.137
Spleen	0.24±0.058	0.25±0.080	0.27±0.102	0.23±0.044
Kidneys	0.12±0.018	0.11±0.15	0.11±0.021	0.12±0.023
Lungs	1.83±0.138	2.01±0.170	1.91±0.174	1.80±0.094

Table 10: Effect of *Prosopis juliflora* seedpods extract on relarive organ weight (Mean ± SD, N=6, g) in female rats after daily oral administration for 28 days in progressive phase.

Organ	G I	G II	G III	G IV
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Liver	3.23±0.394	3.34±0.5.7	3.00±0.342	3.41±0.312
Brain	0.60±0.049	0.60±0.119	0.62±0.067	0.64±0.051
Heart	0.34±0.029	5.19±11.954	0.32±0.023	0.37±0.075
Adrenals	0.11±0.123	0.04±0.008	0.03±0.006	0.04±0.007
Thymus	0.07±0.018	0.09±0.017	0.09±0.017	0.08±0.038
Spleen	0.23±0.052	0.21±0.0031	0.18±0.043	0.17±0.070
Kidneys	0.60±0.038	0.64±0.087	0.72±0.109	0.65±0.034
Lungs	0.59±0.056	0.61±0.078	0.60±0.047	0.63±0.060

Table 11: Effect of *Prosopis juliflora* seedpods extract on relative organ weight (Mean ± SD, N=6, g) in female rats after daily oral administration for 28 days in regressive phase.

Organ	**G V**	**G VI**	**G VII-**	**G VIII**
	0 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Liver	2.98±0.296	3.09±0.329	2.90±0.476	2.77±0.335
Brain	0.69±0.113	0.65±0.049	0.66±0.098	0.64±0.076
Heart	0.20±0.021	0.23±0.023	0.21±0.015	0.21±0.045
Adrenals	0.67±0.039	0.67±0.033	0.65±0.059	0.66±0.32
Thymus	0.35±0.044	0.31±0.019	0.34±0.034	0.32±0.038
Spleen	0.08±0.022	0.08±0.023	0.08±0.024	0.07±0.014
Kidneys	0.04±0.007	0.04±0.005	0.03±0.006	0.04±0.007
Lungs	0.60±0.044	0.65±0.069	0.58±0.083	0.57±0.036

All organs (except joints) of Group IV and VIII did not show any significant changes and were comparable to Group I and V respectively. Histopathological observation of organs of Group I, IV, V and VIII are presented in Table 12.

Table 12: Effect of *Prosopis juliflora* seedpods extract on organ histopathology in female rats after daily oral administration for 28 days

Organ Name	**Observation**	**Severity**	**G1**	**G4**	**G5**	**G8**
Liver	Within normal limit	-	5	6	4	6
	Mononuclear cell infiltration	Minimal	1	0	1	0
	Microgranuloma	Minimal	0	0	2	0
	Bile duct hyperplasia	Minimal	0	0	1	0
Kidney	Within normal limit	-	4	5	3	4
	Tubular protein cast	Minimal	1	0	3	0
	Basophilic tubules	Minimal	1	1	0	1
	Lymphoid aggregoids	Minimal	0	0	0	1
Spleen	Within normal limit	-	5	6	6	6
	Pigmentation	Minimal	1	0	0	0
Brain	Within normal limit	-	6	6	6	6
Heart	Within normal limit	-	6	6	6	6
Lung	Within normal limit	-	6	6	6	6
Stomach	Within normal limit	-	6	6	6	6
Duodenum	Within normal limit	-	6	6	6	6
Jejunum	Within normal limit	-	6	6	6	6
Ileum	Within normal limit	-	6	6	6	6
Cecum	Within normal limit	-	6	6	6	6
Colon	Within normal limit	-	6	6	6	6
Rectum	Within normal limit	-	6	6	6	6
Adrenal	Within normal limit	-	6	6	6	6
Thymus	Within normal limit	-	6	6	6	6
Trachea	Within normal limit	-	6	6	6	6
Skeletal muscle	Within normal limit	-	6	6	6	6
Peripheral Nerve	Within normal limit	-	6	6	6	6
Uterus	Within normal limit	-	6	6	6	6
Ovary	Within normal limit	-	6	6	6	6
Vagina	Within normal limit	-	6	6	6	6
Urinary bladder	Within normal limit	-	6	6	6	6
Lymph node	Within normal limit	-	6	6	6	6

Abbreviation-WINL-Within Normal Limit

Table 13: Effect of *Prosopis juliflora* seedpods extract on femorotibial joint in female rats after daily oral administration for 28 days. Modified Mankins Scoring Method

Tibial plateu					
Animal Group	**Structure**	**Cellularity**	**TB Staining**	**Tidemark Integrity**	**Total score**
G1	2.33±1.506	1.67±0.816	1.83±0.408	0.50±0.548	6.33±2.338
G4	2.50±1.643	1.50±0.548	2.00±0.000	0.67±0.516	6.67±2.251
G5	4.00±0.000	2.00±0.000	3.17±0.753	0.83±0.408	10.00±0.894
G8	3.83±1.602	2.17±0.408	3.33±0.516	1.00±0.000	10.33±2.251
Femoral Condyle					
Animal Group	**Structure**	**Cellularity**	**TB Staining**	**Tidemark Integrity**	**Total score**
G1	1.67±1.211	1.33±0.516	2.00±0.000	0.00±0.000	5.00±1.095
G4	1.00±0.000	1.50±0.548	2.00±0.632	0.33±0.516	4.83±0.753
G5	2.50±1.643	1.50±0.837	2.83±0.408	1.00±0.000	7.83±2.483
G8	3.00±1.549	1.83±0.408	3.00±0.000	1.00±0.000	8.83±1.835

Table 14: Effect of *Prosopis juliflora* seedpods extract on femorotibial joint in female rats after daily oral administration for 28 days.Pritzker Scoring method (Score = grade × stage)

Tibial plateu				Femoral Condyle		
Animal Group	**Cartilage Grade**	**Cartilage Stage**	**Score**	**Cartilage Grade**	**Cartilage Stage**	**Score**
G1	2.33±1.211	2.17±0.408	5.17±2.994	1.50±0.837	1.17±0.408	2.00±2.000
G4	2.67±1.033	2.00±0.000	5.33±2.066	1.33±0.516	1.17±0.408	1.67±0.211
G5	3.17±0.408	2.67±0.186	8.67±3.882	2.33±0.816	1.50±0.548	3.83±2.401
G8	3.50±1.049	2.50±0.837	9.33±5.888	2.50±0.548	1.50±0.548	4.00±2.191

Intraarticular injection of monosodium iodoacetate successfully induced cartilage abnormalities in progressive and regressive phase. Three slides per joint/rat was evaluated microscopically in Group I, IV, V, VIII. In the study, rat suffered from OA showed lesions in articular cartilage of tibial plateau, femoral condyle, and meniscus with more severe lesions in medial tibial plateau. Depending upon the levels (of the section) and locations within the joints, articular cartilage showed, fibrillation, fissure, eburnation/denudation, fibrosis, thickening of subchondral bone plate, pannus, and osteophyte formation. Cartilage fibrillation was characterised by roughing of the surface of articular cartilage, while in fissures are characterised by vertical cracks without significant matrix loss. Eburnation/denudation severity was more in medial tibial plateau and characterised by focal extensive area of full thickness cartilage loss, with smooth shiny bone surface, empty osteocyte lacune, and increased bone density in subchondral area. Fibrosis (in OA context) characterised by either replacement of necrotic cartilage with fibrous tissue or replacement of bone marrow elements, and necrotic tissue with fibrous tissue. Within the fibrous tissue, variable numbers of multinucleated giant cells were evident. Osteophytes are characterised by formation of fibrocartilage like mass at the junction of synovium attached to cartilage or at the site of ligament or tendon insertions. In few cases, pannus formation was noted, characterized by formation of fibrovascular tissue that cover tibial plateau cartilage. In few rats, articular cartilage was markedly atrophied due to necrosis and sub-cartilage fibrosis was noted.

In progressive phase, on 29th day of the study, rat suffered from OA showed fibrillation, fissure, erosion, loss of proteoglycans content on toluidine blue staining and minimal fibrosis. Summary of modified Mankin score and Pritzkerscore of joints of are presented in Table 13 and 14 respectively. Lesions were more severe in tibial plateau than femoral condyle in Group I and IV. No significant changes were observed in Pritzker score of the Group I and IV.

In modified Mankin score, in control group, cartilage cellularity was slight decrease in four rats while moderately decrease in two rats. In Group IV, cartilage cellularity was slight decrease in four rats while moderately decrease in two rats. On toluidine blue staining, moderate loss of proteoglycans was evident in all six rats of Group I and IV (Figure 1). Superficial and transitional zone zones were more severely affected, and loss was staining was extended up to radial zone in few animals (Figure 2). Tidemark, a basophilic line between the cartilage and calcified cartilage was abnormal in three control rats and four rats of Group IV. The lesions in femoral condyle were less severe than tibial plateau and severity of modified Mankin score in Group IV was comparable to Group I. No significant changes were observed in modified Mankin score of the Group I and IV and findings of Group IV were comparable to Group I. Oral administration of PJSPE for 28 days did not show any effect on progression/advancement of histopathological lesions of OA in MIA induced OA at 1000 mg/kg dose.

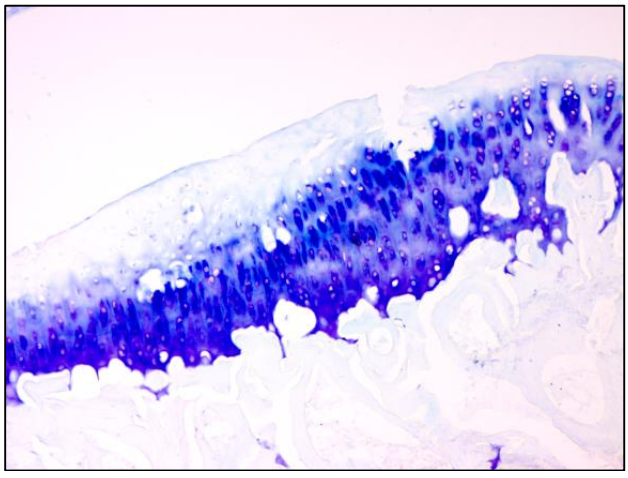

Fig 1: Group IV. Tibial plateau showin surface irregularity, cracks extended in radiag one and moderate loss of toluidine blue staining. OARSI score 6 (Cartilage grade 3 X stage 2). Modified Mankin score 9 (structure-4 point + cellularity-2 point + TBS-2 point + tidemark integrity-1 point). Toluidine blue, 100X

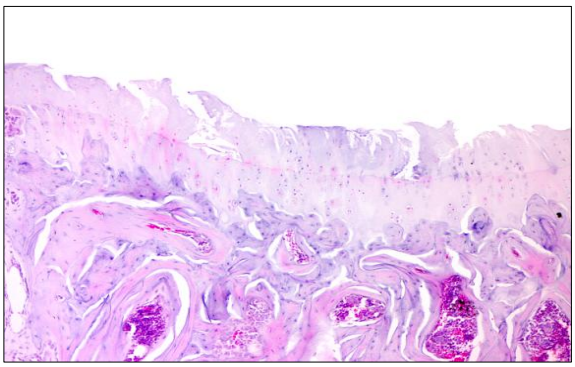

Fig 2: Group IV. Tibial plateau showing loss of cartilage matrix/erosion extended up to tidemark/calcified cartilage. OARSI score 8 (Cartilage grade 4 X stage 2). Subchondral area showing increased bone volume (subchondral sclerosis) with increased bone marrow distance from cartilage. H & E, 100X

In reversal phase, on 57th day of the study, rat suffered from OA showed all the lesions mentioned in 29th day of the study with more severity. Summary of modified Mankin score and Pritzkerscore of joints are presented in Table 13 and 14 respectively. On 57th day of the study, loss of cartilage (Figure 3), fibrosis (Figure 4) involved sub cartilage area and bone marrow space. Multinucleated giant cells numbers were increased in comparison to day 29th of the study.

No significant changes were observed in Pritzker score of the Group V and VIII.

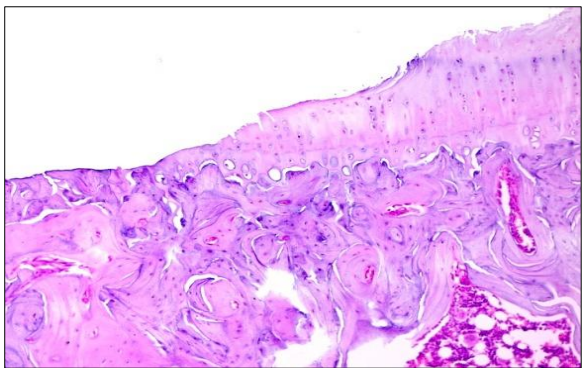

Fig 3: Group VIII. Tibial plateau showing focal extensive area of complete loss of cartilage (left side) and denudation and fibrillation. Subchondral sclerosis OARSI score 20 (Cartilage grade 5 X stage 4). Subchondral area showing increased bone volume (subchondral sclerosis). H & E, 100X

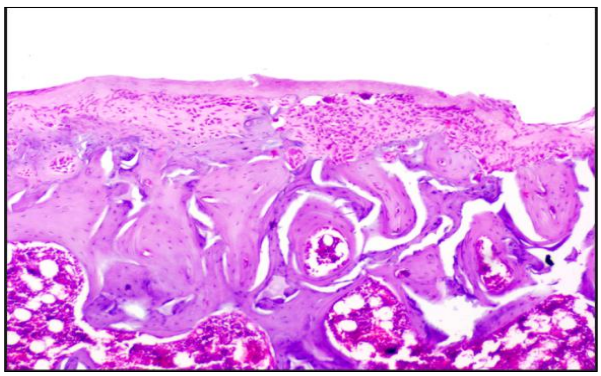

Fig 4: Group VIII. Tibial plateau showing marked necrosis and atrophy of the cartilage. Subchondral area showing fibrosis with few multinucleated giant cells and subchondral sclerosis. H & E, 200X

In modified Mankin score, in six rats of Group V and four rats of Group VIII, clefts/fissures were extended up to radial zone. Cartilage cellularity was moderately decrease in six rats of Group V and five rats of Group VIII. In one rat of Group VIII, cartilage cellularity was severely decrease. On toluidine blue staining, no staining was noted (severe loss of proteoglycans) in two rats of Group V and VIII, while three and four rats of Group V and VIII showed severe loss of toluidine blue staining. Tidemark loss, advancement, or duplication was noted in five rats of Group V and six rats of Group VIII. The lesions in femoral condyle were less severe than tibial plateau and severity of modified Mankin score in Group VIII was comparable to Group V. No significant changes were observed in modified Mankin score of the Group V and VIII. Oral administration of PJSPE for 28 days did not show any ameliorative effect on histopathological lesions of OA in MIA induced OA at 1000 mg/kg dose.

In the present study, oral administration of PJSPE at 1000 mg/kg for 28 days did not produce any pathological changes in organs. However, in past studies, dry ground pods of PF to cattle for 200 days or to goat for 270 days produced neuromuscular abnormality (Tabosa *et al*., 2000, 2006) [30, 31]. Study duration, species, and dose may be responsible for such discrepancy. In previous mentioned studies, cattle, or goat were fed ground pods of PF either 70% or 90% of dry matter basis for more than 200 days. Such high and long exposure of some unidentified toxicant present in PF may produce pathology in neutrons or muscles.

In the present study, monosodium iodoacetate at 1 mg/joint induced articular cartilage fibrillation, fissure, eburnation/ denudation, fibrosis, thickening of subchondral bone plate, pannus, and osteophyte formation. In the present study, lesions were milder in progressive phase (29th day of OA induction) in comparison to regressive phase (57th day of OA induction). Similar findings have been noted in previous studies (Guzman *et al*., 2003; Takahashi *et al*., 2018) [6, 33]. In present study, lesions were more severe in medial tibial plateau than lateral tibial plateau and femoral condyle, which is in accordance with previous studies (Guzman *et al*., 2003; Takahashi *et al*., 2018) [6, 33]. This may be attributed to biomechanics of the rat stifle joint, in which medial compartment naturally load higher than the lateral compartment of the femorotibial joint.

In 2010, OARSI published recommendations for histological assessments of OA in rat (Gerwin *et al*., 2010) [5]. Even though this is probably more accurate histological assessments of OA in rat, due to complexity and applicability to MMT model only, this system does not become popular. Even after publication of this recommendations, modified Mankin score, or OARSI score proposed by Pritzker *et al*. (2006) [23] are more commonly used to for histological scoring of OA in rat (Shi *et al*., 2019; Takahashi *et al*., 2019) [28, 34]. In present study, we used both the system to score OA. In our experience, OARSI score proposed by Pritzker *et al*. (Pritzker *et al*., 2006) [23] is more accurate and easier to apply to evaluate OA model in rat than modified Mankin score.

Forced ambulation, weight bearing test, tactile allodynia (Von Frey test), and radiographic examination are some tests used by research to access OA related pain, behaviour changes and cartilage abnormalities in past (Choi *et al*., 2019; Lee *et al*., 2018; Micheli *et al*., 2019; Morais *et al*., 2016) [4, 13, 16, 18]. Serum collagenases (MMP-3 and MMP-

13), C-telopeptides of type II collagen (CTX-II), insulin-like growth factor-1 (IGF-1) and Osteopontin (OPN) are some biomarkers used by various researchers to evaluate the effect of various compound in induced OA rat model (Bai *et al*., 2018; Katri *et al*., 2019; Lee *et al*., 2019; Madzuki *et al*., 2019) [2, 10, 12, 15]. In the present study, we have not evaluated any pain related behaviouralteration or OA-related serum, urinary or synovial biomarkers.

Researchers tested *Prosopis juliflora* extracts against some chronic disease's models of diabetes, Alzheimer and lethal conditions like cancer and found some ameliorative effect (Alsaadi & Al-Maliki, 2015; Sathiya & Muthuchelian, 2011; Utage *et al*., 2018) [1, 25, 37]. In some case, PF seed pods are effective in amelioration of clinical OA in humans (personal communication with Ayurvedacharya). In present study, PJSPE was nontoxic at 1000 mg/kg orally, however had no protective or ameliorative effect on MIA induced OA. This is probably the first in which LD_{50} of PJSPE was derived and *in vivo* toxicity and efficacy of *PJSPE* on experimentally induced osteoarthritis in Wistar rats was evaluated.

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