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Pharmacological screening of ethanolic extract of *Pithecellobium dulce* for antiarthritic activity in Rats

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Arthritis is an inflammatory joint disorder in which cartilage of the joint is gradually lost and categorized by swelling of joints, pain, and loss of function. The present study was conducted to explore the antiarthritic activity of ethanolic extract of *Pithecellobium dulce* against formaldehyde induced at sub plantar region of the left hind paw during days 1 and 3 of the study period. The changes observed in paw diameter during the study period, various biochemical, and haematological parameters were monitored. The Radiographic analysis and histopathology significantly improved after treatment with test extract *P. dulce* (250 mg/kg, b.w., *p.o.*) as compared to the standard treatment with indomethacin (10 mg/kg, b.w., *p.o.*).The results of the current investigation concluded that ethanolic extract of *P. dulce* possesses significant anti-arthritic activity against formaldehyde induced arthritis model, justifying its therapeutic role in arthritic conditions. The observed antiarthritic activity may be due to the presence of phytoconstituents such as alkaloids and flavonoids. *P. dulce* significantly suppressed the paw oedema in formaldehyde models (P <0.001). The Histopathological and radiographic studies of joints also showed a protective effect of *P. dulce*.

Keywords: Ethanolic extract, Formaldehyde, Joint disorder, Phytoconstituents, Radiographic studies.

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Introduction

Arthritis is an inflammatory joint disorder in which cartilage of the joint is gradually lost or an auto-immune disease by which the immune system of the human body assaults its own tissues. It is categorized by swelling of joints, pain, and loss of function. Osteoarthritis (OA) and rheumatoid arthritis (RA) are the most common arthritis. In the United States, they affect more than forty-five million individuals and are the topmost reason for physical inability among younger age. OA is a progressive non-inflammatory disorder that causes pain and limited joint movement due to loss of joint ligament. It is due to an association of old age, obesity, joints irritation, weakness of muscle and wearing down by friction. They are also called 'wear and tear' arthritis. It is a synovial joints disorder in which articular cartilage which is known for weight-bearing weakens and new bones form at the border of the joint and in the sub-chondral region. Unlike RA, OA disturbs articular ligament, while the membrane of the synovial joint often becomes swollen late in the disease. Two main differences between OA and RA are that OA disturbs the bigger joints like knees, hips because of wear and tear, while RA first attacks minor joints and later damages the cartilage. OA is the most common reason for hip knee replacement surgery[1-5].

RA is a chronic progressive inflammatory auto-immune disorder by which the defence mechanism of the human body attacks its own cartilage and joints lining. It not only affects synovial joints but also many other sites including the heart blood vessels and skins. In the female, RA is more common than male. The exact cause of RA is not yet clearly known but the progression of autoimmunity might be initiated by infection due to microbes, probably by viruses in hereditarily susceptible individuals. The main symptoms of RA are synovial membrane inflammation. If it is not treated, then the synovial membrane become thickens and synovial fluid accumulation occurs. It causes tenderness and pain and formed irregular granulation

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tissues known as Panus which adhere on the superficial layer of articular ligament and many times destroy the cartilage fully. Once the cartilage is damaged fibrous tissues becomes hardens and fuses to the joints, therefore it converts non-movable the final crippling result of RA development of the granulation tissues affects the alteration of fingers which characterized hands of RA subjects. The other common types of arthritis include Juvenile rheumatoid arthritis, fibromyalgia, psoriatic arthropathy, gout, systemic lupus erythematosus (SLE) and spondyloarthropathies[6-8].

Arthritis can be diagnosed by analyzing the symptoms, patient history, examining the joints and several imaging studies like X-ray, magnetic resonance imaging (MRI) and computed tomography (CT), scan of joints as well as by performing some diagnostic parameters like Blood, urine and synovial fluid[9].

There is no cure for arthritis and the objective of several therapies is to reduce the symptoms, especially pain and inflammation. The common medications used in arthritis are analgesics (acetaminophen, oxycodone, hydrocodone, tramadol), Non-steroidal anti-inflammatory drugs (NSAIDS: Ibuprofen, Naproxen sodium), Menthol or capsaicin creams, and immunosuppressant (cortisone, prednisone)[8].

P. dulce Benth belonging to the family Leguminosae is an evergreen spiny tree minor to average-sized, up to 18 m height, cultivated throughout the plains of India and in the Andamans. It is native to tropical America. In India, it is found on highways, nobody knows its cookery usage[10,11].

The plant contains Tannin, 25.36% fixed oil and 18.2% olein. Seeds contain the highest amount of total protein content (50.3 to 67.1%) in comparison to other parts. It also contains phospholipids, steroids, saponins, lipids, glycosides, glycolipids, and polysaccharides. Fatty acid analysis of Seeds was performed and yielded 9 saturated and 17 unsaturated fatty acids. Leaves contain quercetin, kaempferol, dulcitol, and afezilin. In Bark, 37% tannins of the catechol type are present. It also contains Vitamin E, B1, B2, B3, Calcium, phosphorus, and iron[10].

The leaves are used as an anticonvulsant, abortifacient, antidiabetic and antiulcer properties or could be applied as dressings for venereal sore and pain[12]. Leaves decoctions are generally used in intestinal disorder, indigestion and leprosy[13]. The pulp from the fruits is used to be astringent and homeostatic. The seeds are mainly used to treat ulcer[14]. This is used as antipyretic and eye inflammation[15] The present study was conducted to explore the antiarthritic activity of ethanolic extract of *P. dulce* against formaldehyde induced arthritis.

Materials and Methods

Plant material and extraction

The leaves part of *P. dulce* was collected in the month of November 2018 from Lucknow. The herbarium was prepared and submitted to the CSRI- National Botanical Research Institute (NBRI), Lucknow, India for authentication. Plants were authenticated and recorded with the reference number NBRI/CIF/2018/231.

The shade dried leaves of *P. dulce* were reduced to a coarse powder using a mechanical grinder. The amount of coarse powder was about 250 g. The powder was exposed to hot constant progressive extraction in Soxhlet assembly with increasing order of polarity solvents like petroleum ether, 90% aqueous alcohol (50 to 60 °C) controlled temperature. The extract was further concentrated and dried at 40 °C under reduced pressure. The dried extracts were kept in the desiccator for further assessment the yield of the extract was found 22.5% w/w

Phytochemical screening

The extracted material (leaf extract of *P. dulce*) was subjected to preliminary phytochemical analysis for the presence of phytoconstituents[16-21].

Animals

Healthy Albino Wistar rats (female) of approximately 10 to 12 Weeks age, weighing 125 to 150 g were purchased from the Laboratory Animal Services Division of Council of Scientific and Industrial Research- Indian Institute of Toxicology Research (CSIR-IITR) Lucknow. Animals were housed in polypropylene (72x36x44 cm) cages in the group of 5 per cage, with husk as bedding material. Cages were kept under measured temperature (23±2 ºC), humidity (55±5%), and twelve-hour light and twelve-hour dark cycles. They were fed with marketable pellets with water *ad libitum*. Animals were divided into four groups, Group 1 Normal control, Group 2 Negative control (normal saline), Group 3 Standard group (Indomethacin 10 mg/kg *p.o*), and Group 4 Test group (*P.dulce* ethanolic extract 250 mg/kg *p.o.*). Food was not allowed 1 hour before and till the completion of the behavioural study. All the experimental procedures were approved by the Institutional Animal Ethics Committee (IAEC) approval number AUUP/AIP/ M.Pharm/014/ 2018, (26/10/2018) of the Amity Institute of Pharmacy, Amity University Uttar Pradesh, Lucknow.

Experimental protocols

Formaldehyde induced arthritis

A volume of 0.1 mL formaldehyde was injected into rats in sub plantar surface of the left hind paw, on the first and third day. The standard drug was administered from day 1 to 10 and the test drug was administered orally from days 1 to -14. Formaldehyde (0.1%) was administered on 15th and 17th day and paw oedema was determined. On day 18th, paw oedema was evaluated in each group and compared with the control group by digital micrometre and Vernier callipers (Table 1)[22,23].

Biochemical estimation

Haematological screening

On the last day of the experiment, the blood sample of each animal was collected in heparinized test tubes by cardiac puncture and allowed to stand for 30 minutes to separate the serum. The serum was then centrifuged at 4000 rpm, after which total RBC, WBC was determined by hemocytometer, percentage of haemoglobin was measured by Sahlis method[24-28].

Total protein estimation

Various dilutions of Bovine serum albumin (BSA) solutions were formed by using stock BSA (1 mg/mL) with water in a test tube. The final volume was 5 mL in every tube. In these prepared dilutions, a 0.2 mL protein sample and two mL alkaline copper sulphate reagent was added and mixed. It was allowed to stand at normal temperature for ten minutes. Exactly 0.2 mL FCR (Folin Ciocalteau Reagent) was added to every tube and mixed thoroughly. It was then allowed to stand for 30 minutes. Readings were taken at 660 nm absorbance and a standard calibration curve was prepared to quantify the protein present in a given substance[29-30].

Table 1 — Qualitative phytochemical screening of *P. dulce* leaves

Organic compound	Test name	Result
Alkaloids	Hager's	+
	Wager's	+
	Mayer's	+
	Dragendorff's test	+
Tannins	Nitric acid test	+
Flavonoids	Alkaline reagent test	+
	Lead acetate test	+
Saponins	Foam test	+
Glycosides	Bontrager's test	+
	Legal's test	+
Terpenoids	Salkowski test	+
Carbohydrates	Molisch's test	+
	Benedict's test	+

Estimation of serum glutamic oxaloacetic transaminase activities (SGOT)

The Wistar rat was anaesthetized and by using the cardiac puncture method, the blood was collected (1 to 2 mL) in a microcentrifuge tube and centrifugated at 4000X rpm for 60 minutes. After centrifugation serum was collected and an SGOT kit was used in which R_1 (800 µL) and R_2 (200 µL) were added to the serum. The sample was run with auto analyzer[31-32].

Estimation of serum glutamic pyruvic transaminase activities (SGPT)

The Wistar rat was anaesthetized and by using the cardiac puncture method the blood was collected (1 to 2 mL) in a microcentrifuge tube and centrifugated at 4000X rpm for 60 minutes. After centrifugation, serum was collected and an SGPT kit was used in which R_1 (800 µL) and R_2 (200 µL) was added to the serum. The sample was run with auto analyzer[32-33].

Radiographic analysis

It was done by X-rays, or radiation like ionizing and non-ionizing to interpret the internal system of an object[34,35]. The different groups of Wistar rats were carried in a cage for X-ray investigation at Prabhat pet clinic and X-ray images were taken for examining the effects of rat's paw, or to check the bone erosions, swelling, and thinning the joint space.

Histopathology

Formalin (10%) was formulated by using 10 mL formaldehyde Solution in a beaker and total volume was made up to 100 mL with Distilled Water. After that, Rat knee tissue was dissected out and allowed to be fixed in 10% Formalin[36-37].

Results & Discussion

Phytochemical analysis of *P. dulce*

Phytochemical investigation of ethanolic extract of *P. dulce* showed the existence of saponins, cardiac glycosides, protein, saponins alkaloids, sugar, sterols anthraquinones, flavonoids, steroids, tannins, terpenoids, and amino acids Table 1.

Effect of *P. dulce* on paw diameter

Formaldehyde induces an increase in the paw thickness of arthritic animals as matched to the normal control (vehicle) representing joint inflammation and swelling due to the inflammatory mediators. The treatment with indomethacin (10 mg/kg *p.o*) and test *P. dulce* ethanolic extract (250 mg/kg *p.o*) presented a significant (P <0.001) decrease in the paw thickness as associated with the negative control by reducing

the issue of inflammatory mediators with promising anti-inflammatory activity Table 2.

Effect of *P. dulce* on biochemical parameters

Formaldehyde induction elevates the serum biochemical parameters like SGPT, SGOT, and Total protein when compared to the negative control causing injury to the hepatocytes releasing enzymes representing arthritis. Total protein in formaldehyde administration shows denaturation of proteins. The treatment with indomethacin (10 mg/kg *p.o.*) and test *P. dulce* ethanolic extract (250 mg/kg *p.o.)* significantly (P <0.001) decreased the SGPT and SGOT levels when compared to control groups whereas the treatment with indomethacin (10 mg/kg *p.o.*) and test *P. dulce* ethanolic extract (250 mg/kg *p.o)* also significantly (P <0.001) decreased the total protein levels when compared to control groups (Table 3).

Effect of *P. dulce* on haematological parameters

Formaldehyde induction may lead to changes in haematological parameters there was a significant decrease in RBC count and haemoglobin, count while WBC count was increased in arthritic rats when compared with the normal control rats. The drug treatment had significantly brought back the altered haematological changes in both developing and developed phases of adjuvant-induced arthritis. The treatment with indomethacin (10 mg/kg *p.o.*) and test *P. dulce* ethanolic extract (250 mg/kg *p.o.*) significantly (P <0.001) increased the RBC count and haemoglobin levels when compared to control groups whereas treatment with indomethacin (10 mg/kg *p.o.*) and test *P. dulce* ethanolic extract (250 mg/kg *p.o.*) significantly (P <0.001) decreased the WBC count (Table 4).

Radiographic analysis

The radiographic analysis of ethanolic extract of *P. dulce* in formaldehyde induced arthritic rats revealed changes in the knee viewpoint when compared to negative control arthritic groups. Fig. 1a osteoarthritis was not seen at stifle jointin X-ray of Right or left lateral view of Normal control group. The stifle joint was normal. Fig. 1b osteoarthritis was seen at stifle joint in X-ray of Right or left lateral view of Negative control group. Fig.1c prevented bony destruction and no swelling was shown in X-ray of Right or left lateral view of Standard group, (Indomethacin 10 mg/kg). Fig.1d no swelling and no bony erosion (osteoarthritis) was found in the Test group, (*P. dulce* extract 250 mg/kg).

Effect of *P. dulce* on joints histopathology

Histopathology of joints was performed in ethanolic extract of *P. dulce* in formaldehyde induced arthritic rats. Fig. 2a Normal control group shows normal synovial membrane (arrow) in Joint and joint space, Fig. 2b Negative control group shows Arthritis characterized by the presence of inflammatory cells neutrophils and lymphocytes joint space. Fig. 2c Standard group (Indomethacin 10 mg/kg) shows marked recovery of the synovial membrane in joint but mild inflammation reaction (bold arrow) in joint space. Fig. 2d Test group, (*P. dulce* extract 250 mg/kg) shows marked degeneration of bone (arrow) in Joint and synovial membrane.

Discussion

Osteoarthritis is measured by long-lasting inflammation and damage of synovial joints, causing disability and distortion. It is due to the presence of

Table 2 — Effect of *P. dulce* on paw diameter in formaldehyde induced arthritic

Groups	Day 0 (Mean±SEM)	Day 3 (Mean±SEM)
Normal Control	0.197±0.001	0.198±0.0013
Negative Control	0.354±0.011	0.368±0.012
Test	0.241±0.015***	0.241±0.007***
Standard	0.222±0.007***	0.213±0.003***

Values were expressed as Mean±SEM (n=5) ***(P <0.001) using one-way ANOVA followed by Tukeys post hoc test when compared to control groups.

Table 3 — Effect of *P. dulce* on SGPT, SGOT and Total protein

Groups	SGPT (IU/L)	SGOT (IU/L)	Total protein (g/dL)
Normal Control	52.9±0.27	55.09±0.030	0.465±0.0002
Negative Control	161.6±0.15	279.0±0.166	0.98±0.0005
Test	129.2±0.20***	167.82±0.312***	0.77±0.0003***
Standard	121.2±0.17***	129.2±0.131***	0.69±0.0004***

Values were expressed as Mean±SEM (n=5) ***(P <0.001) using one-way ANOVA followed by Tukeys post hoc test when compared to control groups.

Table 4 — Effect of *P. dulce* on WBC, RBC, and Hb

Groups	WBC (10^3/µL)	RBC (10^6/µL)	Hemoglobin (gm/dL)
Normal Control	9.348.0±224.31	2.8±011	14.4±0.46
Negative Control	10.456.0±12.87	7.3±0.19	12.0±0.14
Test	4.178.0±480.25	9.0±0.36	14.2±14.2
Standard	3.678.0±441.60	12.4±0.42	13.4±0.30

Values were expressed as Mean±SEM (n=5) ***(P <0.001) using one-way ANOVA followed by Tukeys post hoc test when compared to control groups.

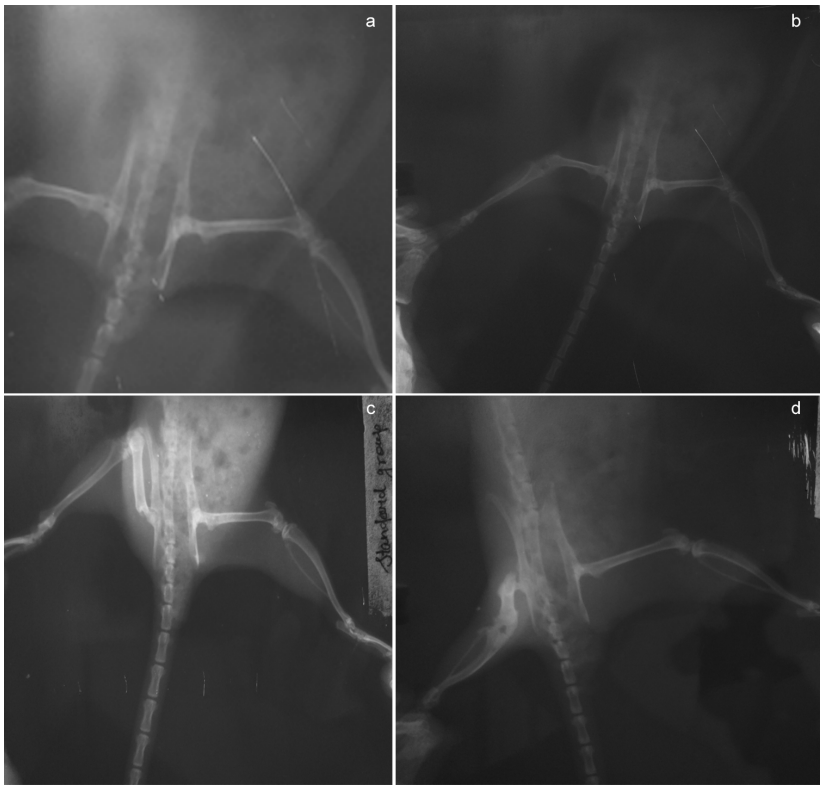

Fig. 1 — Radiography of Formaldehyde induced arthritis. a) Normal control, b) Negative control, c) Standard and, d) Test.

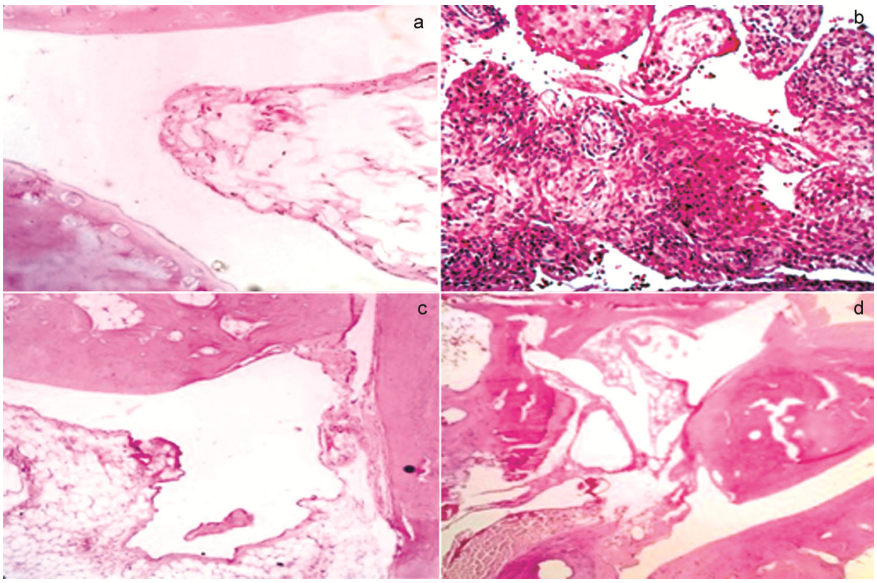

Fig. 2 — Histopathological investigations of *P. dulce* leaves extracts. a) Normal control, b) Negative control, c) Standard and, d) Test.

pro-inflammatory markers cytokines and leukotrienes. The main inflammatory markers are IL-1, TNF-α, IL-6, IL-15, IL-16, IL-17, IL-18, IFN-γ, and granulocyte-macrophage colony-stimulating factor, chemokines like IL-8, macrophage inflammatory protein-1 or monocyte chemoattractant protein-1[38].

Numerous studies have shown that several phytochemicals like saponins, flavonoids, and tannins which are present as secondary metabolites in the plant are useful in the treatment of arthritis. The phytochemical investigation of *P. dulce* revealed the existence of tannins, flavonoids, and saponins.

Therefore, *P. dulce* might serve as a potential source in the treatment of arthritis.

Inhibition of formaldehyde-induced oedema is one of the most suitable methods to evaluate the anti-proliferative activity and screen anti-arthritic agents. Formaldehyde develops localized inflammation in two phases. In the early phase (neurogenic phase), substance P is discharged, while in the late phase (inflammatory phase), histamine, serotonin, bradykinin, and prostaglandins are liberated, which ensues in pronounced vasodilation and permeability[39]. Thus, the destruction of oedema by *P. dulce* in the formaldehyde method revealed its anti-inflammatory and immune-modulatory effect.

The decrease in the body weight and paw thickness during arthritis is due to deficient absorption of nutrients through the intestine and treatment with anti-inflammatory drugs normalizes the process of absorption. The reduction in haemoglobin level and red blood cell count indicates the anaemic illness in the arthritic animal. This is because of irregular packing of iron in the synovial tissue and reticuloendothelial system and bone marrow failure respond to anemia[40]. The significant growth of white blood cell count in an arthritic animal might be because of stimulation of body defence mechanism against antigens and particularly reduced in *P. dulce* treated sets displayed its immune-modulation outcome. Haemoglobin, another significant factor that increases joint pain is because of the enhanced production of endogenous proteins, for example, fibrinogen and globulin. *P. dulce* in both doses and standard medication indomethacin repaired the haemoglobin back to the typical level so modifying it is an important part in arthritic conditions. Moreover, *P. dulce* in both repaired the levels of antioxidant in the liver which was disturbed by formaldehyde induced arthritis, this might be because of instantaneous anti-arthritic and anti-inflammatory potential of *P. dulce*. An elevated level of serum SGPT and SGOT in formaldehyde induced arthritic animals suggest the anti-inflammatory effect may be due to the rectification of biochemical parameter[41]. Standard and test extract significantly reduced the increased levels of SGOT and SGPTwhen matched to the arthritic control. The treatment with formaldehyde induced arthritis denatures a protein at the site of administration, which produces immunological reaction against the degraded product results in decrease in serum total protein levels[42]. The treatment with the test extract (250 mg/kg, b.w.) and the standard has shown significantly enhanced protein levels when compared to the arthritic control.

Radiographic changes in osteoarthritis conditions are valuable analytic estimates which show the improvement in arthritis. Swelling in soft tissue are the initial sign of radiography, while noticeable radiography alterations such as joint spaces reduction and bony erosions are viewed mainly in the last phases of joint pain. In formaldehyde induced ligament rodents (group II), swelling in soft tissue alongside joint spaces narrowing were seen which indicates the arthritic condition by bony damage. The standard medication indomethacin treated groups have prohibited this bony damage and there was no swelling of the joint. The extract treated groups also showed significant recovery from bony erosion representative of the protective role as anti-inflammatory and antiarthritis action.

Conclusion

Phytochemicals from plants, animals and minerals are consumed for the management of diseases that affect Humans. *P. dulce* showed a significant antiarthritic effect imparting anti-inflammatory effect might be due to the presence of phytochemicals contributed in the recovery of bone destruction. The results provide a systematic sign for antiarthritic use of *P. dulce* as an encouraging entity for a novel therapeutic agent of osteoarthritis. However, further research is needed to categorize and separate the possible phytoconstituents involved in the anti-arthritic activity and regulate the mechanism of action, which would facilitate the use of *P. dulce* in arthritic and inflammatory disease.

Conflict of interest

The authors declare no conflict of interest.

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