Antiarthritic activity of *Cynodon dactylon* (L.) Pers

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*Cynodon dactylon* (L.) (Poaceae) is traditionally used herb to treat fevers, skin diseases and rheumatic affections. The ethanolic extract of *C. dactylon* was found to be safe at all the dose levels (100, 200 and 400 mg/kg, orally) and there was no mortality up to the dose of 5000 mg/kg of extract when administered orally. *C. dactylon* showed significant antiarthritic activity against Freund’s complete adjuvant induced arthritis in rats. Treatment with *C. dactylon* significantly reduced the mean percentage change in injected and non injected paw, ankle diameter, clinical severity and significantly increased body weight. Results were confirmed using biochemical parameters; there was a significant improvement in the levels of Hb and RBC in *C. dactylon* treated rats. The increased levels of WBC, ESR, C-reactive protein (CRP) and TNFα were significantly suppressed in *C. dactylon* treated rats. *C. dactylon* showed protective effect in arthritic joints but it has been supported by an improvement in bone lesions rather than in cartilage lesions. It can be concluded that ethanolic extract of *C. dactylon* at a dose of 400 mg/kg is effective in improving haematological level, CRP and reducing TNFα level. Phytochemical screening showed the presence of alkaloids, flavonoids and glycosides in ethanolic extract. All the above results support the traditional uses of the plant in the treatment of rheumatoid arthritis.

Keywords: Arthritis, *Cynodon dactylon*, FCA, TNFα

Rheumatoid Arthritis (RA) is a chronic, autoimmune, progressive, systemic inflammatory disorder characterized by infiltration of synovial membrane in association with destruction of joints which is responsible for the deformity and disability. Literary data of the arthritis in female: male is 3:1 affecting about 1% population in developed countries. Now a days many steroidal, non-steroidal, immune-suppressive and disease-modifying antirheumatic drugs (DMARDs) are used to control the sign and symptoms of inflammation but many of the drugs cause unwanted side effect. With mentioned difficulties, arthritis research organization has progressed exponentially. *Cynodon dactylon* (L.) Pers. (Poaceae) is one of the most commonly occurring weeds in India having a great religious value. In Hindi it is popularly known as dhub, doob or harialil; other common names include durba (Bengali), garikoihallu (Kanarese), durua (Marathi), durua or haritali (Sanskrit), arugampullu (Tamil), garikagoddi (Telugu), dharo (Gujarati) and dhubkhabbal (Panjabi). *C. dactylon* grows throughout the year and is consumed by the domestic animals as food. It is also commonly used as a one of material in religious rituals in worshiping of Lord Ganapati in all parts of India. Traditionally farmers apply crushed leaves externally to fresh cuts and wounds. The whole plant is applied on forehead in headache. The roots in the form of paste with water are taken internally to treat fevers. The plant is folk remedy for anasarca, calculus, cancer, carbuncles, cough, hypertension, snakebites, stones, gout, fever, skin diseases and rheumatic affections. It exert antiinflammatory, diuretic and anti-emetic property. It has been reported to posses antidiabetic, antiulcer, diuretic, antimicrobial, hepatoprotective, cardioprotective and immunomodulatory activities. Due to lack of scientific data regarding the antiarthritic activity of *C. dactylon*, the present study has been carried out to provide pharmacological evidence for the folklore medicinal consideration of *C. dactylon*.

Material and Methods

Collection of plant material—Fresh leaves of *C. dactylon* were collected from local area of Jalgoan district, Maharashtra, India during July-September. This plant was identified and authenticated by Dr. J. Jayanthi, Scientist, Botanical Survey of India, Pune. Voucher specimens no.
(BSI/WC/Tech./2011/34(B)) have been kept in Botanical Survey of India, Pune, Maharashtra, India.

Animals—Adult male Wistar rats, weighing 180-220 g and albino mice of either sex weighing 25-30 g were used and acclimatized to laboratory condition for one week. All animals were housed in well ventilated polypropylene cages at 12:12 h light/dark schedule at 25±2 °C and 55-65% RH. The rats were fed with commercial pelleted rats chow and water ad libitum as a standard diet. Institutional Animal Ethics Committee approved the experimental protocol in accordance with the committee for the purpose of control and supervision of experiments on animals (CPCSEA).

Preparation of leaf extract—The leaves were collected and dried in shade and ground. Coarsely powdered plant material (1000 g) was subjected to successive extraction with ethanol (60–80 °C) in a soxhlet extractor at 45-50 °C to 40 cycles per batch for 2 batches. The extraction was continued until the solvent in the thimble becomes clear indicating the completion of the extraction. After each extraction the solvent was distilled off under vacuum below 50 °C using rotary evaporator. The yield was 4.3% (w/w).

Preliminary phytochemical studies—Preliminary qualitative phytochemical screening were done for the presence of different group of chemicals i.e. alkaloids, flavonoids, saponins, tannins, sterols, carbohydrates, and glycosides.

Acute oral toxicity of the extract—The mice were divided into 5 groups of 10 animals each. The mice were fasted for 6 h and had access to only water ad libitum before experimental study. Gr. I received only vehicle (distilled water). Groups II, III, IV and V received different doses of ethanolic extract of *C. dactylon* (EtCD) i.e. 1000, 2000, 3000 and 4000 mg/kg respectively. All the doses and vehicle were administered orally. The animals were observed for 72 h for mortality.

Induction of rheumatoid arthritis—The rats were divided into following 5 groups of 6 animals each: Gr. I: vehicle control; Gr. II: Dexamethasone (5 mg/kg, po); Gr. III: EtCD (100 mg/kg, po); Gr. IV: EtCD (200 mg/kg, po); and Gr. V: EtCD (400 mg/kg, po). RA was induced by a single intradermal injection using 15 gauge needle of 0.1 mL of freund’s complete adjuvant (FCA, Sigma) containing 1.0 mg dry heat-killed *Mycobacterium tuberculosis* suspended/mL sterile paraffin oil into sub-planter region of the left hind paw of rats. Before injection, animals were anaesthetized with ip injection of pentobarbital sodium at a dose 40 mg/kg of body weight of animals. FCA produced a pronounced local oedema after a few hours with a progressive increase reaching its maximum on 13th day of FCA injection. Treatment with EtCD and dexamethasone were started from day 13 to day 21. At the end of study, blood was withdrawn from ratino bulbar venous plexus under light anesthesia (pentobarbital sodium at a dose 40 mg/kg of body weight of animals, ip) with the help of a glass capillary. Immediately, after blood withdrawal from animals, animals were sacrificed by decapitation for knee joint collection for histological assessment. The serum separated from the blood was collected for further biochemical assays.

Assessment of arthritis

Body weight—Body weight was recorded for each animals daily from day 0 (after immunization) to day 21 at a specific time between 10:00-11:00 hrs.

Arthritic score—Animals were scored regularly from day 0 (after immunization) until day 21 by two investigators who were blind to the treatment. Each paw was graded according to the severity, extent of erythema, swelling of periarticular soft tissues, and the enlargement and distortion of the joints. Clinical score ranged from 0 (no sign) to 4 (severe lesions), yielding a maximum score of 16 per animal.

Hindpaw oedema—Volumes of both injected and non injected paw were recorded on the day 0 (after immunization) by plethysmometer (7140-UGO Basile, Italy). The primary lesion was determined by measuring the paw volume on day 0, 3, 6, 9, 12, 15, 18 and 21. The percentage inhibition of paw volumes of both the injected left paw and non injected right paw over vehicle control group were calculated.

Ankle diameter—The diameter of the inflamed paw joint was measured by vernier caliper for recording the distance moved by the screw gauge and the joint was compressed by pressing the gauge till pain was elicited as indicated by squeaking or leg withdrawal.

Biochemical assays—Haemoglobin content was estimated by the method of Drabkin and Austin. Red blood cell and white blood cell counts were made according to the method of Cheshbrough and Mc Arthur in an improved Neubauer chamber. Estimation of erythrocyte sedimentation rate was followed by the method of Westergren. C-reactive protein level was estimated using the ELISA kit obtained from Alpha Diagnostics Intl., USA.
Plasma tumour necrosis factor-α (TNF-α) assay—Plasma TNF-α concentration was determined with an ELISA commercial kit (Rat TNF-α ELISA kit, Sigma Aldrich, St. Louis, USA). At the end of the experiment, samples of blood (0.5 mL) were drawn from a tail vessel. The blood was collected in polyethylene tubes having 25 µL of heparin solution (4000 IU). The plasma samples obtained after centrifugation for 10 min at 3000 g and 4 °C were frozen at −80 °C until assay. In brief, 100 µL of standard, sample and control were added to each well of the coated microplate. After 3 h of incubation at 24 °C the microplate was decanted and the liquid discarded. Then, 100 µL of biotinylated anti-TNF-α antibody was added to each well. After 45 min of incubation at 24 °C and a further elimination of the liquid from the wells, 100 µL of Streptavidin–horseradish peroxidase conjugate was added. After incubation for a further 45 min and a washing of the wells, 100 µL of chromogen was added. The absorbance of each well was read spectrophotometrically at 450 nm. TNF-α values were expressed as pg/mL.

Histological assessment—Knee joints of FCA injected paw of each animals were collected at the end of study and fixed immediately for 24 h in 4% formaldehyde, then decalcified in rapid bone decalcifier for 6 h. An experienced pathologist, unaware of treatments, scored the condition of tibiotarsal joints. Histopathological changes were scored using the following parameters: (i) infiltration of cells was graded on the scale from 0 to 3, depending on the presence of inflammatory cells in the synovial tissues; (ii) inflammatory cells in the joint cavity were graded on a scale from 0 to 3 and expressed as exudates; (iii) a characteristic feature in FCA induced arthritis in rats is the progressive loss of articular cartilage and this destruction of articular cartilage was separately graded on a scale from 0 to 3, ranging from the appearance of dead chondrocytes to complete loss of the articular cartilage; (iv) bone erosion was scored on a scale ranging from 0 to 3, ranging from no abnormalities to complete loss of cortical and trabecular bone of the femoral head; (v) cartilage and bone destruction by pannus formation was scored ranging from 0 to 3, and (vi) vascularity (0, almost no blood vessels; 1, a few blood vessels; 2, some blood vessels; 3, many blood vessels). Histopathological changes in the knee joints were scored in the femur region on 5 semiserial sections of the joint, spaced 70 µm apart. Scoring was performed on decoded slides by two observers, as described earlier.

Statistical analysis—The values were expressed as mean ± SE. Statistical evaluation of the data was done by one-way ANOVA (between control and drug treatments) followed by Dunnett’s t-test for multiple comparisons and two-way ANOVA followed by Bonferroni’s multiple comparison test, with the level of significance chosen at P <0.001 using Graph-Pad Prism 5, San Diego, CA software.

Results

Phytochemical screening—Phytochemical screening of the ethanolic extract of C. dactylon showed the presence of alkaloids, flavonoids and glycosides. Saponins, tannins, sterols and carbohydrates were absent.

Acute oral toxicity of the extract—The EtCD was found to be safe at all the doses used and there was no mortality up to 5000 mg/kg dose of EtCD when administered orally. Therefore, in the present study 500 mg/kg dose was taken as the therapeutic dose and made variations by taking 100 mg/kg as lower dose and 400 mg/kg as higher dose.

Body weight—Progressive decrease in body weight in FCA induced arthritic rats was observed. The progressive decrease in body weight was significantly (P<0.001) reduced from day 15-21 as compared to vehicle treated animals while receiving with dexamethasone (5 mg/kg) and EtCD (100, 200, 400 mg/kg) (Fig. 1 a). These data demonstrated that EtCD prevented body weight loss in FCA induced arthritis in animals in dose dependent manner.

Arthritic score—The mean arthritis severity score in FCA induced arthritis plus vehicle group was progressive from day 12 and achieved values of about 9 in the last three days, which indicated that without drug treatment animals were observed two paws with arthritis. Clinical severity was significantly (P<0.001) reduced from day 18, when treated with dexamethasone (5 mg/kg, po) and EtCD (100, 200, 400 mg/kg, po) (Fig. 1 b). Hindpaw oedema (injected paw)—Treatment with EtCD significantly (P<0.001) reduced the mean percentage change in injected paw swelling at 18th day evaluation and the percentage protection was 50.88, 59.98 and 61.04% in dose dependent manner at 100, 200 and 400 mg/kg respectively and at 22nd day evaluation, the percentage protection was 53.3, 62.2
and 66.3% in dose dependent manner at 100, 200 and 400 mg/kg respectively. However, the standard drug i.e. dexamethasone (5 mg/kg) exhibited significant (P<0.001) percentage protection as 61.62 and 71.5% at 18th and 22nd day respectively as compared with vehicle treated animals (Fig. 1 c).

**Hindpaw oedema (non injected paw)**—EtCD significantly (P<0.001) reduced the mean percentage change in non injected paw swelling at 18th and 22nd day evaluation in dose dependent manner (Fig. 1 d).

**Ankle diameter**—Inoculation of FCA produced gradual increase in swelling of injected paw (ankle diameter) which was significantly (P<0.001) decreased on day 22 while receiving EtCD (200 and 400 mg/kg) and dexamethasone (5 mg/kg) (Fig. 1 e)

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**Biochemical assays**

Biochemical parameters indicate that arthritic animals without any treatment showed a gradual decreased in levels of RBC and haemoglobin and there was a significant improvement with the treatment of dexamethasone (5 mg/kg) and EtCD (400 mg/kg). The increased levels of WBC, ESR and serum C reactive protein was significantly suppressed in dexamethasone (5 mg/kg) and *C. dactylon* (400 mg/kg) treated rats (Table 1).
Histopathological analysis

During histological assessment, pathological changes were observed in FCA induced arthritis in rats as well as EtCD and dexamethasone treated animals (Table 2). It was observed that few of neutrophils infiltrated into mildly edematous synovium and joint cavity. Destructive lesions in articular cartilage, formation of pannus into cartilage responsible for bone erosion and vascularity formation into joint space were also observed in FCA induced arthritis in rats (Fig. 3 a). Treatment with dexamethasone (5 mg/kg) (Fig. 3 b) and EtCD (400 mg/kg) (Fig. 3 e) significantly (P<0.001) suppressed inflammatory cells in synovial membrane and joint cavity. Recovery of destruction of articular cartilage, bone erosion and vascularity formation as compared to vehicle treated animals was observed in dose dependent manner. EtCD (100 mg/kg) showed insignificant effect against FCA induced arthritic animals (Fig. 3 c).

Discussion

Cynodon dactylon has shown the persuasive protective effect against the acute inflammation induced by carrageenan and autacoids induced oedema (carrageenan, serotonin, histamine and dextran). Keeping these information in view the present investigation was planned to study the protective effect of C. dactylon against chronic inflammation and rheumatoid arthritis induced by FCA. Recent studies have confirmed that adjuvant-induced arthritis is associated with both a decrease in bone formation and an increase in bone resorption due to an increased production of prostaglandins 21.

FCA induced arthritis is one of the excellent model which is extensively used to study the pathogenesis of RA for testing therapeutics. This experimental model shares several clinical and pathological features with RA. The acute stage of arthritis is characterized by signs of pause in body weight gain, clinical severity, hind paw and fore paw oedema, joint diameters increase 22. In the later, acute stages of disease (after 12th day of sensitization), rats with adjuvant arthritis were often relatively immobile due to the severity of paw swelling.

The present results showed that the ethanolic extract of C. dactylon significantly inhibited the development of chronic swelling induced by FCA. The decrease in body weight was significantly increased in arthritic animals while administering of dexamethasone (5 mg/kg, po) and EtCD (100, 200, 400 mg/kg, po). The loss of body weight in the
control (arthritic) animals could be due to reduced absorption of glucose and leucine in rat intestine in arthritic condition\textsuperscript{23}.

Paw swelling is one of the major factors in assessing the degree of inflammation and therapeutic efficacy of the drugs\textsuperscript{24}. Sub-planter injections of FCA into the rat paw induce inflammation as primary lesion with a maximum after 3 to 5 days. Secondary lesions occur after a delay of approximately 11 to 12 days which are characterized by inflammation of non-injected sites (hind leg, forepaws, ears and tail). Anti-inflammatory compounds do not inhibit secondary lesions, which are prevented or diminished by immune suppressive agents. Treatment with EtCD and dexamethasone significantly ($P<0.001$) reduced the mean percentage change in injected and non injected paw swelling at a dose 400 mg/kg. Paw swelling (ankle diameter) also suppressed with the treatment of EtCD (200 and 400 mg/kg) and dexamethasone (5 mg/kg).

The importance of T cells in the pathogenesis RA has been established and numerous studies performed to determine the cytokines and susceptibility factors involved in arthritis development\textsuperscript{25}.

In the present study, the arthritic rats exhibited a reduced RBC and Hb level and an increased ESR count. Anaemia is a common diagnostic feature with chronic
arthritis. The treatment with EtCD (400 mg/kg) improved the RBC and Hb level and the ESR count indicating the significant recovery from the anaemic condition.

White blood cells are a major component of the body's immune system. Increased WBC count is the indications for the infectious and inflammatory diseases. The migration of leukocytes is significantly suppressed in EtCD (400 mg/kg) treated rats as seen from the significant decrease in the WBC count. C-reactive protein is a member of the class of acute phase reactants and is used mainly as a marker of inflammation.

C-reactive protein values have been proved to determine disease progression or the effectiveness of treatments as its levels rise dramatically during inflammatory processes. The level of CRP was significantly reduced in C. dactylon (400 mg/kg) and dexamethasone treated animals.

Several areas of investigation have indirectly implicated TNFα an important contributor to cellular damage in FCA induced arthritic rats. The high levels of this cytokine can be interpreted as a progression of cartilage cell injury. The antiarthritic activity of EtCD lowered the TNF α concentration in plasma and consequently mitigated articular cell damage. Biochemical changes in patellar cartilage were also shown to be representative of FCA induced arthritic joints and found very sensitive to the inhibitory effect of proinflammatory cytokines. Thus, the present data were highly homogeneous because the decreased proteoglycan synthesis and aggrecan expression in cartilage resulted in a significant decreased cartilage staining in arthritic rats. The present results also showed significant protection to cartilage in knee joints while treated with C. dactylon and dexamethasone. C. dactylon prevented arthritis induced bone loss by an improvement in bone lesions rather than cartilage lesion. The contribution of synovitis to bone loss, ranging from focal erosions to periarticular or generalized osteopenia, has become a very active topic in RA.

Bone loss is the classical feature of adjuvant arthritis, in which an increase in the number of osteoclasts and the formation of osteoclast precursors from the monocyte/macrophage compartment in the synovium was reported within few days after induction of disease. Focal bone erosions and generalized bone loss are thought to be lower to an overload of pro-inflammatory cytokines. A decrease in bone resorption was also supported by the decreased urinary excretion of deoxypyridinoline in rats treated with C. dactylon. The bone-protective potency of C. dactylon is very provocative.

From the above results, it can be concluded that ethanolic extract of C. dactylon at a dose of 400 mg/kg is effective in improving haematological level, CRP and reducing TNFα level. Phytochemical screening of EtCD has shown the presence of alkaloids, flavonoids and glycosides. The potent activity may be attributed to the presence of these phytoconstituents. The ability of the extract to cause oedema inhibition produced by these inflammatory mediators suggests that it contains phytochemically active constituent(s) with antiarthritic properties. Amongst them, flavonoids may play a major role as they are proved as anti-inflammatory agents due to their inhibitory effects on enzymes involved in the production of the chemical mediators of inflammation. In view of the present work, extensive phytochemical studies are necessary to identify the active principle and which may enable us to elucidate exact mechanism of action.

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