

# Protective Effect of *Boerhavia diffusa* in Attenuating Pro-inflammatory Cytokines and Inhibition of Activated NF- $\kappa$ B-TNF- $\alpha$ -Nrf2 in Freund's Adjuvant-induced Rheumatoid Arthritis

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## ABSTRACT

**Background:** *Boerhavia diffusa* is widely used in Asian and African countries in the treatment of inflammatory disorders in adjunct with other herbal formulations. The current research work was devised with an aim to examine the protective effect of hydroalcoholic root extract of *Boerhavia diffusa* in rheumatoid arthritis. **Materials and Methods:** Hydroalcoholic extract of plant at varying doses and reference drug (indomethacin 3mg/kg), were administered daily for 21 days. The parameters such as joint diameter, level of antioxidant (GSH) and pro-oxidant (MDA) markers and histopathological evaluation were performed. Immunohistochemical analysis was carried out to evaluate the effect of *Boerhavia diffusa* on pro-inflammatory (IL-1), anti-inflammatory (IL-10), pro-inflammatory TNF- $\alpha$  and its receptor, TNF-R1, angiogenesis (VEGF), Nrf-2 and NF- $\kappa$ B. **Results:** Findings of study discovered that there is dose dependent significant ( $p < 0.05$ ) reduction in inflammation by varying doses of *Boerhavia diffusa* along with the reduction ( $p < 0.01$ ) in levels of oxidant stress markers. Notable decline in inflammation and joint dysfunction was found in *Boerhavia diffusa* 200 mg/kg dose with significance of  $p < 0.001$ . **Conclusion:** We concluded that the tuberous roots of *Boerhavia diffusa* show dose dependently attenuate paw edema, inflammation and reversed the bone damage through inhibition of activated pro-inflammatory mediators, Nrf-2 and specifically NF- $\kappa$ B-mediated production of cytokines.

**Key words:** *Boerhavia diffusa*, Rheumatoid Arthritis, NF- $\kappa$ B pathway, Angiogenesis, Inflammation.

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## INTRODUCTION

Inflammatory disorders such as Rheumatoid arthritis (RA) affect a wide range of population. The prevalence rate of RA in India in the year 2015 was found to be approximately 0.75% of total adult population.<sup>1,2</sup> The disease is characterized as persistent autoimmune-mediated inflammatory disorder with infiltration of cellular components and subsequent proliferation of synovial cells. It can also result in formation of pannus, destruction of cartilage and erosion of joints. The

destruction of joints progresses due to the involvement of infiltrating cells, other cytokines, proteolytic enzymes and presence of prostanoids.<sup>3</sup> The disease pathogenesis begins due to production of free radical species and presence of pro-inflammatory cytokines at the site of inflammation.<sup>4</sup> A variety of mechanistic pathways are involved in the pathogenesis of RA, some involved with the formation of free radical scavengers, activation of nuclear factor E2 related factor-2 and some other



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interplay between the deregulated expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IFN- $\gamma$ ) and anti-inflammatory cytokines (IL-10). Accumulation of macrophages, neutrophils and production of free radical producing enzymes in synovial fluid are believed to be the initial stage in RA. High level of ROS in the synovial cavity stimulates NF- $\kappa$ B, which further activate expression of pro-inflammatory cytokine.<sup>5</sup> Till date various reports have shown to inhibit activation of NF- $\kappa$ B and thus attenuate the joint destruction.<sup>6,7</sup> Consequently, there are numerous plant-based phytoconstituents that possess significant therapeutic value (such as inhibition of NF- $\kappa$ B) and have potential to be emerging as lead candidates for rheumatoid arthritis.<sup>8</sup> Therefore, identification of natural substances that protect the tissues from chronic inflammatory disorders would open new avenues to enhance RA therapy.

*Boerhavia diffusa* (BD) is used as natural medicine to relief pain and numerous other infirmities. Ayurvedic literature suggests that various parts of BD is used in various different ailments such as inflammation, asthma, rheumatism, hepatitis, leucorrhoea, blood pressure, urinary disorders, internal inflammation disorders.<sup>9</sup> Several preclinical studies demonstrated the protective role of BD as emetics,<sup>10</sup> pain and inflammation.<sup>11</sup> In addition to this, BD is also endowed with immunosuppressive activity,<sup>12</sup> immunostimulatory activity<sup>13</sup> anti-inflammatory activity,<sup>14-16</sup> anticonvulsant,<sup>17</sup> nephroprotective<sup>18</sup> and antibacterial activity.<sup>19</sup> The pilot studies on BD as anti-arthritic agent examined and presented in an international conference proceeding.<sup>20</sup> It offers cure for variety of ailments and to the best of our information and knowledge, it is the primary study to claim the tuberous roots of BD in attenuation of inflammation and bone damage effect till date. Therefore, the current study was envisioned to examine the potential effect of standardized root extract of BD on inflammation and bone damage in experimental model of RA. Second, if so, could the inhibition of activated NF- $\kappa$ B and Nrf-2 be the probable mechanistic pathway to attenuate rheumatoid arthritis in experimental animals.

## MATERIALS AND METHODS

### Investigational Study Animals

The research works involving use of animals, albino Wistar rats of five-seven weeks old, weighing 170-220 g. Animal study protocol was duly approved and reviewed by the institutional animal ethics committee (IAEC) of AIIMS, New Delhi, India (Protocol approval number

772/IAEC/13). The experimental animals were placed in standard laboratories and housed as per the protocol.

### Chemical and Reagents

Complete Freund's adjuvant (CFA) for inducing arthritis was procured from Difco Laboratories Inc., USA. Reference drug, Indomethacin (Purity > 98.5%) was procured from Sigma Chemical Co., (St. Louis, USA). TNF- $\alpha$  ELISA kit was purchased from Diclone SAS, France. Primary antibody of NF- $\kappa$ B, IL-1, IL-6, IL-10, Nrf-2, TNF- $\alpha$ , TNF-R1 was purchased from Santa Cruz, CA, USA.

### Investigational drug

The roots of BD were procured from the market in the month of March, 2013 and it is then dried in sunlight. The dried tuberous roots were authenticated by Prof. S.H. Ansari, Jamia Hamdard, New Delhi.

### Extraction procedure

Hydro alcoholic extract of BD was obtained in soxhlet apparatus using solvent water: ethanol (50:50). The semisolid extract were prepared by evaporating solvent to a temperature of 70°C at reduced pressure. The yield of crude extract obtained was found to be 4.33% w/w.<sup>18</sup>

### Determination of Boerhavinone B and Boerhavinone O in BD by High Performance Liquid Chromatography (HPLC) profiling

The hydroalcoholic extract of roots of *BD* was quantified for the presence of phytoconstituents (Boerhavinone O and Boerhavinone B) with the help of HPLC instrument equipped with C<sub>18</sub> column (Shimadzu, LC 2010A). The mobile phase consisted of gradient mixture of acetonitrile and water. The sample solution was then filtered via 0.45 $\mu$  membrane filter. The flow rate was accustomed to 1.0 mL/min. Volume of injection was adjusted to 20  $\mu$ L. The detecting wavelength for the estimation of Boerhavinone B and Boerhavinone O was set at 270 nm.<sup>18</sup>

### Experimental protocol

The animals were segregated into six groups ( $n=6$ ). The number of animals per group was selected on the basis of G power software analysis. Grouping was done in accordance to the below mentioned sequence.

Group 1 designated as normal control group in which normal saline at 1mL/kg/day was administered orally to animals for twenty one days.

Group 2 designated as CFA control group in which normal saline at 1mL/kg/day was administered orally to animals for twenty one days and subsequently a single subplantar injection of 0.1 mL CFA was also given on day 0 to the animals in left hind paw.<sup>20</sup>

Group 3 designated as indomethacin group in which the reference drug, indomethacin was administered to animals for twenty one days and subsequently a single sub plantar injection of 0.1 mL CFA was also given on day 0 to the animals in left hind paw.

Group 4-6 designated as investigational drug groups i.e BD at varying doses, low dose (50 mg/kg/day), middle (100 mg/kg/day) and higher (200 mg/kg/day) dose was administered to animals for study period of twenty one days and subsequently a single sub plantar injection of 0.1 mL CFA was also given on day 0 to the animals in left hind paw.

Therapeutic dose selection criterion for BD is translation of human dose to animal equivalent dose. Parameters assessed during the study period is measurement of joint diameter and after completion of study, i.e on 21 day, terminal blood collection (0.5 mL) was performed by retro-orbital plexus and it is further used for analysis. Animals were sacrificed after blood collection and the ankle joints were excised for further estimations.

#### Measurement of increase in joint diameter

The measurement of joint diameter of left hind paw was done on day 0, 3, 7, 14 and 21 using micrometer screw gauge. Screw gauge was to be placed transversally onto the ankle joint and the respective readings were studied from the scale at micrometer. The value of joint diameter on day 0 was considered as baseline and it is subtracting from measurements taken on 3, 7, 14 and 21 days. The values of increase in joint diameter were expressed in mm.<sup>21</sup> Arthritic index and stiffness scores were calculated in accordance to the assessment scale reported by Kaur *et al.* (2012) and Butler *et al.* (1992) respectively.<sup>22,23</sup>

#### Assessment of serum TNF- $\alpha$ level

The serum from the collected blood sample was separated by centrifuging the blood sample at 3000 *g*. Level of TNF- $\alpha$  in all serum samples were determined using ELISA kit (Diacclone SAS, France) using multimode ELISA plate reader (Biotek Instruments, USA) and the absorbance was recorded at 450 nm. The reference filter was used of wavelength 630 nm.

#### Quantification of oxidative stress markers in ankle joint tissue

The tissues present around the ankle joints of animals were excised and weighed. Measurement of oxidative markers (MDA, GSH and SOD) were carried out by preparing 10% homogenate of ankle tissue in 0.1M ice cold phosphate buffer at pH 7.4. The resultant homogenate was divided into three parts for assessment of MDA, GSH and SOD. First part of homogenate

used for the assessment of TBARS (thiobarbituric acid) and quantitatively expressed in terms of MDA content. The color adduct formed on interaction of MDA with thiobarbituric acid was read at 532 nm.<sup>24</sup> The second part of homogenate used to quantify GSH. For measurement of GSH, 10% tricarboxylic acid was added to homogenate (1:1) in equal parts and then centrifuged it for 10 min at 5000 *g* to obtain supernatant. The obtained supernatant produces yellow color on addition of DTNB (5,5-dithiobis 2-nitrobenzoic acid), as the thiol group of glutathione reacts with DTNB (pH 8.0) at 412 nm.<sup>25</sup> Third part of homogenate was used for the estimation of SOD. The homogenate was centrifuged at 5000 *g* for 10 min and inhibition of pyrogallol autoxidation (pH 8.4) was measured to calculate the enzyme activity of SOD.

#### Radiographic investigation

The radiographical investigation of ankle joints of hind paws of rats was performed on 21<sup>st</sup> day. It helps to examine the swelling of soft tissue, bony erosions and destruction of cartilage. All the animals were given intravenous anesthetics and placed over radiographic X-ray machine. The distance between the machine (Philips X12, Germany) and source was kept at 90 cm. Radiographic scoring of control and treatment groups (Figure 7A-7E) was performed on a scale of 0-3. Scale 0 defines no damage; scale 1, 2, 3 defines as mild, moderate and severe damage respectively (Figure 7F).<sup>26</sup>

#### Immunohistochemical analysis

The expression of various pro-inflammatory (TNF-R1, IL-1 $\beta$ ), anti-inflammatory (IL-10), VEGF and NF- $\kappa$ B were examined with the help of immunohistochemical (IHC) examination. The decalcified ankle joints were sectioned using cryotome. The thin frozen sections (6  $\mu$ m) were made using cryotome and the corresponding sections were processed to form slides. The slides were treated with 30% H<sub>2</sub>O<sub>2</sub> in methanol and block was prepared with bovine serum albumin for. Later on incubation with primary monoclonal antibodies against TNF-R1 (1:200), IL-1 $\beta$  (1:200), IL-10 (1:200), VEGF (1:200), NF- $\kappa$ B(1:200) it was conjugated with Horse Radish Peroxidase (HRP) as secondary antibody (1:2000). Finally, Treated with 3,3'-diaminobenzidine (DAB) to produced colorogenic immune reaction. Slides were then mounted with DPX to visualize TNF-R1, IL-1 $\beta$ , IL-10, VEGF and NF- $\kappa$ B activity under light microscope (Nikon ECLIPSE E600, Japan).

#### Western blot analysis

The protein level of Nrf-2, TNF- $\alpha$  and  $\beta$ -actin were determined in ankle tissue homogenate by immunoblot analysis. The ankle tissue were homogenized with pestle

and lysed in ice cold RIPA lysis buffer supplemented with protease inhibitor cocktail. The protein concentration in cell lysates was determined using BCA protein assay kit (Sigma-Aldrich, Inc.).

Grouping of ankle joint tissue homogenate was done in the following manner:

Group 1: Normal control ankle joint tissue homogenate

Group 2: CFA-control ankle joint tissue homogenate

Group 3: Indomethacin ankle joint tissue homogenate

Group 4: BD (200 mg/kg) ankle joint tissue homogenate

Protein (60 µg) was resolved on 10–15% SDS–PAGE and electroblotted and transfer onto PVDF membrane (MDI). The membrane was then incubated in 4% BSA followed by primary antibody incubation TNF-α (1:3000), Cell Signaling Technology, Inc., Nrf-2 (1:4000), BD Biosciences, USA. Membrane was washed with 0.8% tween-20 in PBS (phosphate buffer saline) followed by incubation with the HRP conjugated secondary antibody. Blots were washed after incubation and developed using Luminata Forte Western HRP Substrate, Millipore Corporation. The signal was detected by ImageQuant LAS 500 (GE Healthcare UK Limited) and band intensity was determined by densitometry, background staining were corrected and signal was normalized with the respective values of each lane to the signal for β-actin.

### Statistical analysis

All obtained data were recorded in triplicates as mean±SEM. The comparison between groups was calculated with the help of ANOVA (One-way analysis of variance). Post hoc test used to calculate significance was Dunnett's multiple comparison tests. All the representation was done using Graph pad Prism version 5.03, San Diego, CA, USA, where  $p < 0.05$  was considered as statistically significant.

## RESULTS

### Quantification of Boerhavinone B and Boerhavinone O in *B. diffusa* root hydroalcoholic extract

HPLC chromatogram of standard Boeravinone O (Figure 1a) HPLC chromatogram of standard Boeravinone B (Figure 1b) HPLC analysis of BD revealed that the percentage of Boeravinone O and Boeravinone B in the extract was 0.003% and 0.008% respectively (Figure 1c).

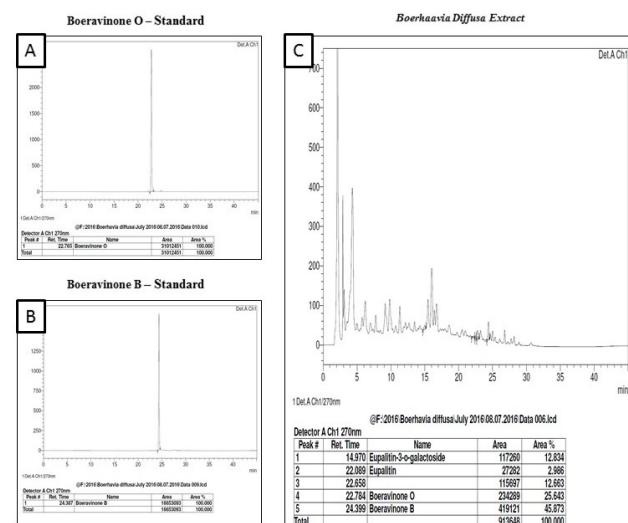
### Effect of BD on joint diameter

It was observed that after immunization with CFA, there was momentous augmentation in the joint diameter of injected animals. The maximal effect of CFA

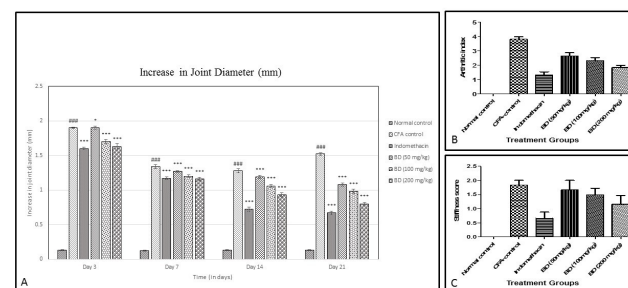
immunization was observed on 3<sup>rd</sup> day and afterwards the inflammation tends to decrease gradually in reference and treatment groups except CFA-control group. We also noticed a diminutive augment in joint thickness in CFA-control group on day 14. Significant inhibition ( $p < 0.001$ ) of joint inflammation was observed by standardized BD root extract (200 mg/kg) treated group and the alteration in ankle thickness of other treatment groups were not as marked as 200mg/kg group (Figure 2A).

### Effect of BD on arthritic score

Obtained data indicates that there was increment in arthritis score in CFA-control group as compared to normal control animals. However, the rats treated with BD (200 mg/kg) revealed significant decrement



**Figure 1:** HPLC chromatogram of *Boerhavia diffusa* root extract. Figure 1a: HPLC chromatogram of standard Boeravinone O; Figure 1b: HPLC chromatogram of standard Boeravinone B; Figure 1c: HPLC chromatogram of *Boerhavia diffusa* representing the presence of Boeravinone B and O.



**Figure 2:** Effect of *Boerhavia diffusa* in measurement of joint diameter in CFA-induced arthritis model. Figure 2A: Joint diameter measurement in CFA induced arthritis model. Figure 2B: Arthritic index, Figure 2C: Stiffness score. All values are Mean±SEM. Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison. # $P < 0.05$ , ## $P < 0.01$  vs normal control; \*  $P < 0.05$  \*\* $P < 0.01$  vs CFA-control.

in arthritis score as compared to CFA-control group (Figure 2B).

### Effect of BD on joint stiffness

The joint stiffness value was observed 1.83 in CFA-control with full restriction in movement of ankle. However, treatment with indomethacin (1.01) and BD at a dose of 200 mg/kg (1.14) almost completely reversed the joint stiffness as compared to CFA-control group (Figure 2C).

### Effect of BD on serum TNF- $\alpha$ levels

CFA injection increases the level of circulatory serum TNF- $\alpha$  in all the groups and momentous ( $p < 0.01$ ) increase was found in CFA-control group (Figure 3A). The quantification of serum TNF- $\alpha$  level was done on day 21 and the treatment with BD (200 mg/kg) significantly ( $p < 0.05$ ) reduces TNF- $\alpha$  level in serum.

### Effect of BD on oxidative stress

The level of reactive oxygen species was found increased in the CFA-control group and thus increases oxidative stress. The pro-oxidant (MDA) and antioxidant (GSH, SOD) level was measured to analyze the oxidative stress parameters. CFA immunization depletes the antioxidant level and consequently increases pro-oxidant level. We found increased level of MDA and decreased GSH, SOD in CFA group, whereas treatment groups ameliorate the depleted antioxidant (GSH, SOD) level and decreases the augmented MDA level (Figure 3B-3D). BD group

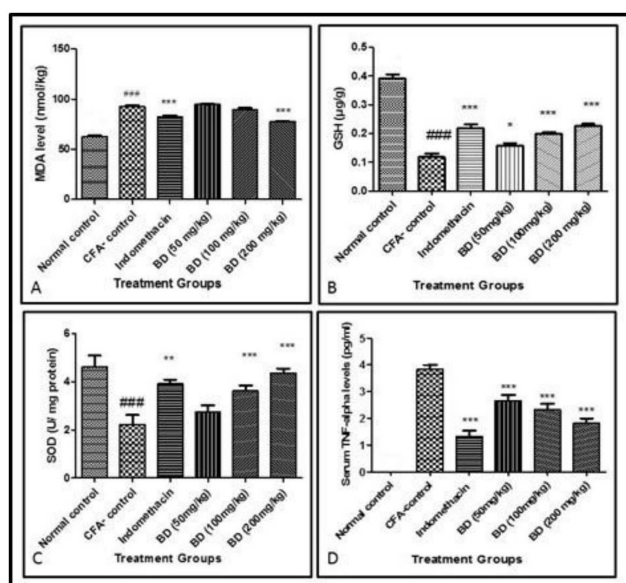
showed dose dependent augmentation in endogenous anti-oxidant markers and decreased pro-oxidant markers in contrast to CFA-control. BD (200 mg/kg) decreases significantly ( $p < 0.05$ ) pro-oxidant level as compared to CFA-control.

### Effect of BD on CFA-induced radiographic changes

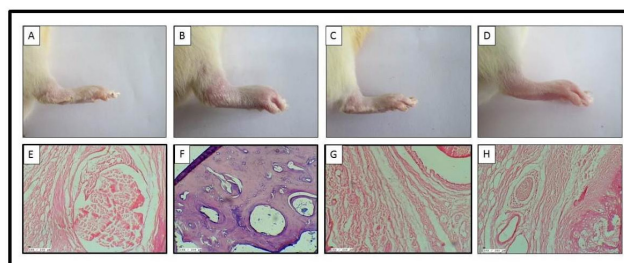
CFA-control group showed changes in osteophyte formation, bone matrix resorption and narrowing of joint space. The narrowing of joint space is accompanied with bony erosions and swelling of soft tissue (Figure 4B). However, the normal control animals did not showed any bone related changes (Figure 4A). BD (100 mg/kg) group exhibited less inflammation (Figure 4C), on the contrary reference group, indomethacin showed minimal destruction of cartilage, reduced swelling and less degree of diffusion of joint space (Figure 4D). BD (200 mg/kg) group (Figure 4E) showed minimal evidence of joint swelling. Scoring of radiographs of left hind paws of control and treatment groups were done and represented in Figure 4F.

### Effect of BD on synovial expression of pro-inflammatory cytokines (Immunohistochemistry of synovial joints)

Immunohistochemical staining of pro-inflammatory cytokines/cytokines receptor IL-1, IL-6, IL-10, TNF-R1, VEGF and NF- $\kappa$ B was performed in synovial tissue of CFA rats. Immunohistochemical analysis of CFA- control group demonstrated the massive protein expression of corresponding cytokines and transcription regulator (IL-1, IL-6, TNF-R1, VEGF and NF- $\kappa$ B) and decreased expression of IL-10, an anti-inflammatory marker in synovium. IL-1, IL-6, TNF-R1 and NF- $\kappa$ B protein expression were found to be lower, while higher expression of IL-10 in BD administered group in contrast to control. Synovial expression of these markers in indomethacin-treated groups was



**Figure 3: Effect of *Boerhavia diffusa* on oxidative stress and serum TNF- $\alpha$  level in CFA-induced arthritic model. All values are Mean $\pm$ SEM. Statistical analysis by One-way ANOVA followed by Dunnett's Multiple Comparison. # $P < 0.05$ , ## $P < 0.01$  vs normal control; \* $P < 0.05$  \*\* $P < 0.01$  vs CFA- control.**



**Figure 4: Radiographic analysis of CFA-induced rat joint. (A) Normal control; (B) CFA-control; (C) Indomethacin treated group; (D) *Boerhavia diffusa* (100mg/kg) treated group (E) *Boerhavia diffusa* (200mg/kg) treated animals; (F) Plot of the radiographic scores.**

intermediary to control and BD (200 mg/kg) treated animals (Figure 5).

**Effect of BD on Western blot analysis**

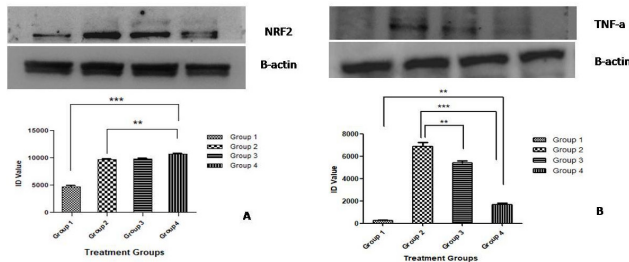
CFA-induced arthritis comprises variant interlinking pathways, so the expression of markers linked to these processes was studied with the help of western blot (Figure 6). Interestingly, the expression of TNF- $\alpha$  was also statistically ( $p < 0.001$ ) down regulated in BD (200 mg/kg); Group 4. However, indomethacin group also down regulate ( $p < 0.01$ ) the increased expression of

TNF- $\alpha$ , as compared to CFA-control group (Group 2) Figure 6B. Nrf-2, nuclear factor erythroid 2-related factor 2 regulates basal and induced expression of array of antioxidant response element dependent genes. Notably, the expression of Nrf-2 was upregulated in CFA-control, whilst BD (200 mg/kg) group showed tremendous increase in expression of Nrf-2 suggesting activation of antioxidant defense system Figure 6A.

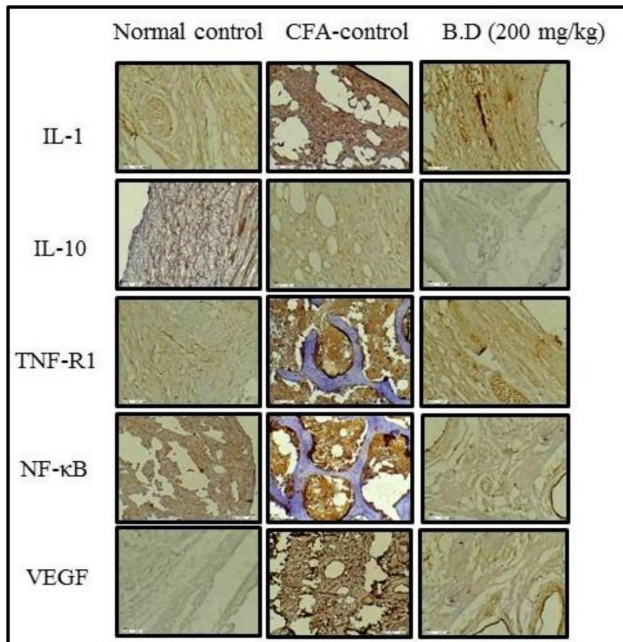
**DISCUSSION**

In the present study, it is examined whether oral administration of BD possesses anti-arthritic or anti-inflammatory activity in CFA-adjuvant model. Furthermore, the effects of BD on immunological parameters are also examined. To assess the immunological influences, the expression of pro-inflammatory (TNF-R1, IL-1) and anti-inflammatory (IL-10) cytokines were assessed. The prominent changes in histology of joints of all experimental animals were also explored. In addition, expression of NF- $\kappa$ B, Nrf-2 and VEGF was also investigated with the help of immunohistochemical examination.

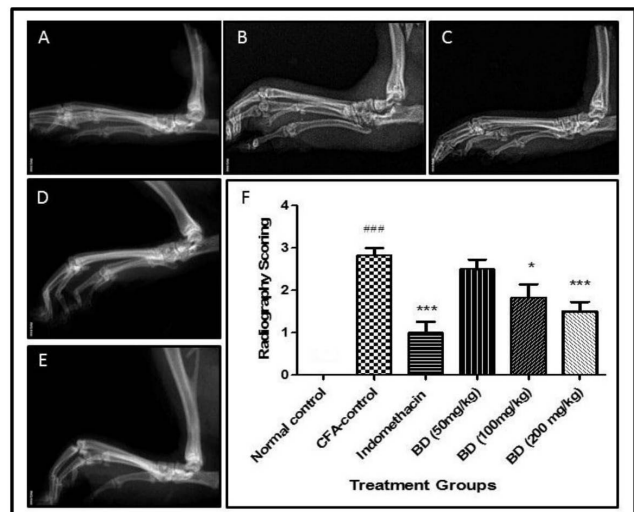
As previously discussed, RA is a provocative inflammatory disease that involves swelling of joint, inflammation of synovial membrane, erosion of bone and destruction of cartilage.<sup>22</sup> Numerous mediators are involved in the progression and onset of disease such as Tlymphocytes, cytokines, neutrophils, B-cells, monocytes. Various spheres of inflammation and immunity interplay in disease onset. A large number of studies have confirmed the central role of pro-



**Figure 6: Effect of *Boerhavia diffusa* on A: Nrf-2; B: TNF- $\alpha$ ; expression in rat ankle tissue homogenate, assessed by Western blot analysis. Group 1: Normal-control; Group 2: CFA-control; Group 3: Indomethacin; Group 4: BD (200 mg/kg). All values were expressed in Mean  $\pm$  SEM (N=6). \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .**



**Figure 5: Effect of *Boerhavia diffusa* on synovial pro-inflammatory cytokine/receptor TNF-R1, IL-1 $\beta$ , IL-6, IL-10, VEGF and NF- $\kappa$ B expression in synovial joint of rats in CFA-induced arthritic model. Sections are 6  $\mu$ m thick and photomicrographs are taken at 10X. (A) Normal control; (B) CFA-control; (C) *Boerhavia diffusa* (200mg/kg) treated animals. Bold arrows = DAB staining (yellow-brown) of the synovial membrane depicting presence of cytokines.**



**Figure 7: Radiographic analysis of CFA-induced rat joint. (A) Normal control; (B) CFA-control; (C) Indomethacin treated group; (D) *Boerhavia diffusa* (100mg/kg) treated group (E) *Boerhavia diffusa* (200mg/kg) treated animals; (F) Plot of the radiographic scores.**

inflammatory cytokines, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 in provoking inflammation and thus, contributes to the development of RA.<sup>27</sup> IL-1 and TNF- $\alpha$  trigger self-proliferation of synoviocytes and increases production of tissue enzymes (matrix-metalloproteinases) and resulted in destruction of cartilage in joints.<sup>28</sup> TNF- $\alpha$  initiate the process and it begin its arthritic genesis potency by inducing IL-1 release and simultaneously also triggers release of cytokines, endothelial adhesion molecules and collagen synthesis and induce osteoclast differentiation. It is reported in several reports that the imperative prevailing players that induces inflammation, cartilage destruction and bone erosion are IL-1, IL-6 and TNF- $\alpha$ .<sup>29</sup> Angiogenesis also plays an important role in formation of pannus in synovium of RA. Inhibition of increased expression of IL-1 helps in declining IL-6 and IL-8 (proangiogenic chemokine) with considerable inhibition of angiogenesis (VEGF) in synovium of RA. The present study, therefore, was designed to examine the role of IL-1, IL-10, TNF- $\alpha$ , VEGF, Nrf-2 and NF- $\kappa$ B in attenuation of bone damage by BD in CFA-induced model. Thus, results of the study demonstrated that the serum level of TNF- $\alpha$  was significantly increased in CFA-control rats in contrast to normal control group. Treatment with BD (200 mg/kg) manifestly reduced the serum level of TNF- $\alpha$  in comparison to CFA-control group. The decline in augmented production of proinflammatory cytokines might contributes to one of the mechanism for anti-inflammatory and anti-arthritic effect of BD. As we know that the key transcription factor which recruits the expression of pro-inflammatory cytokines is NF- $\kappa$ B.<sup>30</sup> Consequently, we also examined the effect of BD on inhibition of activated NF- $\kappa$ B. The immunohistochemical observations revealed downregulation of NF- $\kappa$ B and VEGF by root extract of BD (200 mg/kg). In this study, we also testified that BD treatment reduced oxidative stress in synovium of adjuvant-induced arthritis rats. Numerous reports also indicate that oxidative stress and ROS formed in CFA injected rats.<sup>31</sup> The present study revealed that immunization with adjuvant (BD) leads reduction of anti-oxidant markers (SOD and GSH) and over expression of pro-oxidants (MDA) in CFA-control rats compared to normal rats. Intake of BD (200 mg/kg) reinstates the discrepancy between pro-oxidant and antioxidant markers and therefore, exhibits potential role in reversing the damage caused by free radical species. Despite of protective role of BD on oxidative stress in treatment of RA, the severity of arthritis in CFA-induced model was also assessed by measuring arthritis score and

change in joint diameter. We found that BD at a dose of 200 mg/kg significantly reduced the increase in joint diameter ( $p < 0.001$ ) in CFA-induced rats. The effect of BD root extract was studied on immunohistochemical analysis of TNF-R1 to investigate the effect on pro-inflammatory cytokine/ receptor. The dysfunction in ankle joints was assessed with the help of radiographic examination and the damage to subchondral bone was investigated using scoring.<sup>26</sup> The radiographic analysis, primarily tarsals, metatarsals, phalanges of BD at higher dose exhibited protection against CFA-related changes. The radiographical score markedly increased in CFA-control group, whereas BD significantly lowered the radiographical score in the synovium of CFA-induced rats. These Figures established that BD alleviate the cartilage destruction, swelling, inflammation, oxidative stress in Freund's adjuvant induced animals. Lastly, the present study revealed that BD treatment is attenuating pro-inflammatory cytokines, angiogenesis and oxidative markers which are in confirmation that *B. diffusa* has the anti-arthritic activity.

## CONCLUSION

From the above findings, we conclude that BD is a potential therapeutic target for RA, heightening its role in the pathogenesis of inflammation and bone damage. This study shed new lights on the functional role of BD by supplementing it as a valuable complementary approach to alleviate the symptoms of RA. Furthermore, some clinical study are also needed to validate the potential therapeutic role of BD in RA.

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## CONFLICT OF INTEREST

The authors declare no Conflict of interest.

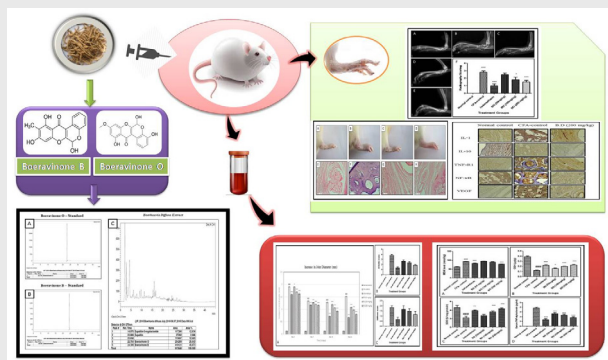
## ABBREVIATIONS

**RA:** Rheumatoid arthritis; **ROS:** Reactive oxygen species; **BD:** *Boerhavia diffusa*; **CFA:** Complete Freund's adjuvant; **TBARS:** ; **DTNB:** 5,5-dithiobis 2-nitrobenzoic acid; **HPLC:** High Performance Liquid Chromatography; **HRP:** Horse Radish Peroxidase; **DAB:** 3,3'-diaminobenzidine; **ANOVA:** One-way analysis of variance.

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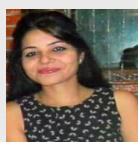
## PICTORIAL ABSTRACT



## SUMMARY

Tuberous roots of *Boerhavia diffusa* dose dependently attenuate paw edema, inflammation and reversed the bone damage through inhibition of activated pro-inflammatory mediators and specifically NF-κB-mediated production of cytokines.

## About Authors



**Dr. Ritu Karwasra** is actively involved in research for 10 years. She has received her doctorate from Department of Pharmacology, All India Institute of Medical Sciences, New Delhi, India and M.Pharm in pharmacology from Institute of Pharmaceutical Sciences, Kurukshetra University, Kurukshetra. She worked as ICMR-Centenary Postdoc Fellow in National Institute of Pathology, Safadrjhung Hospital Campus, New Delhi. She also worked as Research Associate in INMAS, DRDO, New Delhi. She passed all the examinations (10th, 12th, B.Pharm) with more than 75% and also cleared National Entrance Examinations, GPAT and PhD-AIIMS. She is life member of pharmacological society and Indian pharmaceutical association. She is actively engaged in research related activities on medicinal plants, especially in context to inflammatory disorders using cytokines biomarker and profiling studies. She has been associated in research on validation of pharmacoepial traditional medicine as SRF in AIIMS projects funded by Department of AYUSH and ICMR. She is actively involved in publishing research articles in various SCI-indexed journals with cumulative impact factor of 35. She has made significant contribution in the field of pharmacotherapy of inflammatory disorders.

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