

**ANTI-ARTHRITIC AND SYNERGETIC ACTIVITY OF *WEDELIA*  
*CALENDULACEA* L. WITH METHOTREXATE IN ADJUVANT INDUCED  
ARTHRITIS WITH CARDIOPROTECTIVE ACTIVITY IN RAT**

**Aashish H. Panchal<sup>a\*</sup>, Rakesh K. Patel<sup>b</sup>, Anil Bhandari<sup>c</sup>**

<sup>a</sup> Asst. Professor, dept. of Pharmacology, L. J. Institute of Pharmacy, Ahmedabad, GUJARAT, INDIA, aashish\_sai13@yahoo.com

<sup>b</sup> Professor and Head, Dept. of Pharmacognosy, S. K. Patel College of Pharmaceutical edu. & Res., GANPAT UNIVERSITY, Kherva, Mehsana, GUJARAT, INDIA

<sup>c</sup> Dean, Faculty of Pharmacy, Jodhpur National University, Jodhpur, Rajasthan, INDIA

**Correspondence Author E-mail i.d:** aashish\_sai13@yahoo.com; **Ph:** 09898715582

### Summary

Untreated rheumatoid arthritis may lead to various complication in arthritic subject also most of the drug available in the market which produce various side effects including gastrointestinal disorders, cardiovascular risks, immunodeficiency, hepatotoxicity, renal toxicity and humoral disturbances. The present study was aimed at investigating the effect of *Wedelia calendulacea* alone or in combination with sub therapeutic dose of Methotrexate (1 mg/kg) is salvaging, oxidative stress, anti-arthritic action and cardioprotective actin with Methotrexate. S.D. rats were induced arthritis by sub plantar injection of 0.1 mL Complete Freund's Adjuvant (CFA) and pronounced arthritis was seen after 12 days of injection. Groups of animals were treated with std. and test drug from the day 13 up to days 28 by oral route. Various hematological (RBC, WBC, Hb, ESR and RA Factor), biochemical parameters (Homocecytein, TNF- $\alpha$ , IL-2 and CRP) and tissue parameters (SOD, Catalase and Lipid peroxidation of aorta) were measured before initiation and after completion of treatment in all groups. Methanolic fraction of methanolic extract (Me-OH/Me-OH) of *Wedelia calendulacea* shows significant anti-arthritic activity in CFA induced arthritic animals. Enhanced oxidative stress in terms of measured S.O.D., catalase and lipid peroxides were observed in arthritic control and methotrexate treated group. After the completion of treatment all rats were sacrificed under mild anesthesia and thoracic aorta was isolated. Relaxation response of ONOO<sup>-</sup> ( $10^{-9}$  M to  $10^{-3}$ M) and acetylcholine ( $10^{-9}$  M to  $10^{-5}$ M) on precontracted with Phenylephrine ( $10^{-5}$ ) in control, arthritic and treated rat thoracic aorta was measured. Relaxation response of ONOO<sup>-</sup> and Ach were increased in Me-OH-Me-OH extract of *Wedelia calendulacea* treated animals compare with the arthritic animals. While treatment with methotrexate showed the increase the ONOO<sup>-</sup> mediated relaxation but there is no any significance effect in Ach mediated relaxation. The present study indicates that the oral administration of the extract of *Wedelia calendulacea* L. showed anti-arthritic and cardioprotective activity. Concomitant administration of *Wedelia calendulacea* with Methotrexate was also found synergetic effect. So the extract of *Wedelia calendulacea* has the potential to be developed as a new combination therapy with the std. available drug for the management of arthritis and associated cardiovascular disorder.

**Key word:** Anti-arthritis, *Wedelia calendulacea*, vascular protective and methotrexate

## Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease, which is characterized by extensive destruction of the articular cartilage and bone. Prolong untreated arthritis may lead to increase oxidative stress, various chronic complication and cardiovascular complication. Cardiovascular complications associated with the arthritis are major cause for the increased morbidity and mortality in arthritic patients<sup>1-2</sup>. Steroids and NSAIDs are the most popular therapy; however biological response modifiers like tumor necrosis factor alpha and interleukin-1beta antagonists are newer strategies in the management of rheumatoid arthritis<sup>3</sup>. However, these drugs are known to produce various side effects including gastrointestinal disorders<sup>4</sup>, cardiovascular risks<sup>5</sup>, immunodeficiency, hepatotoxicity, renal toxicity and humoral disturbances. They generate ROS (reactive oxygen species) which cause the release of collagenases and elastases that contribute to cartilage destruction<sup>6</sup>. Particularly methotrexate act as a folate inhibitor and thereby increase serum homocysteine levels and which produced Vascular endothelial dysfunction in adjuvant induced arthritis<sup>7</sup>. Recently, there has been an increasing interest in free radical scavenging substances derived from herbs and the role of potential herbs in anti-arthritic therapy has been evaluated<sup>8</sup>.

The plant *Wedelia calendulacea* L. (Asteraceae) popularly known as “Bhangra” in English, and “Bringaraja” in Sanskrit, with yellow flower is a common remedy for various ailments. It has been cultivated as a common weed throughout India, particularly in cool and moist places. The plant contains coumestans i.e. wedelolactone (I) and demethylwedelolactone (II), polypeptides, polyacetylenes, thiophene-derivatives, steroids, triterpenes, flavonoids and nicotine<sup>9-12</sup>. Pharmacologically plant was used as a Immunostimulant<sup>13</sup>, Antibacterial<sup>14</sup>, wound healing<sup>15</sup> and Anticancer<sup>16</sup>. Particularly wedelolactone Inhibiting the IKK Complex<sup>17</sup>.

In this study, we have explored the influence of *Wedelia calendulacea* on treatment of arthritis and associated cardiovascular disorder. Therefore its ability to circumvent endothelial injury induced by methotrexate in Freund's Complete Adjuvant induced arthritis is being investigated.

## Material Methods

### Chemicals

Complete Freund's adjuvant (CFA) purchase from Sigma Aldrich and Methotrexate from the market preparation. All the solvents used for the extraction were of AR grade.

### Plant Material

The fresh leaves of plant *Wedelia calendulacea* L. (Asteraceae) were collected from the area of Sanand village, Nr. Ahmedabad, GUJARAT, INDIA, in November-December. The herbarium of this plant was identified and authenticated [Herbarium No. 1342/144] by Dr. H. B. Singh, Scientist F & Head, Raw Materials Herbarium & Museum, NISCAIR, New Delhi, INDIA.

### Preparation of Plant Extract

The coarse powder of the leaves (250 g) was extracted in soxhlet extraction with the sufficient quantity of Methanol for 24 hours. Solvent was removed under the room temperature to yield a viscous mass which was further mixed with the 80/120 silica gel for the column chromatography and prepare a free flowing powder. The powder mass was successively extracted with Petroleum ether (P.E), ethyl acetate (EtoAc) and methanol (MeOH) and each of the fraction was dried after evaporation of the solvent at reduced pressure

till solid to semisolid mass. Extracts were stored in an airtight container in refrigerator, which was further suspended in 1% w/v tween - 80 for the oral administration.

### Selection of Animal

Sprague Dawley rats of either sex of weighing 250-300g were procured from central animal facility of L. J. Institute of Pharmacy, Ahmedabad. They were fed with the standard food pellets (Pranav agro, Baroda) and water (*ad libitum*). They were housed in polypropylene cages maintained under standard conditions.

### Ethics

The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethics Committee, and was cleared by same before beginning the experiment (No. LJIP/IAEC/01/2011–2012).

### Acute Toxicity

Acute toxicity tests were performed on mice of either sex weighing between 20-30 g following OECD guidelines.

### Complete Freund's Adjuvant (CFA) Induced Arthritis<sup>18</sup>

One day before the initiation of study, the selected animals were randomly grouped into various groups such as normal control, arthritic animals and treated animal. On day 1, all the animals except normal control were injected into the sub plantar region of the left hind paw with 0.1 ml of complete Freund's adjuvant (This consists of 6 mg mycobacterium butyricum being suspended in heavy paraffin oil by thoroughly grinding with mortar and pestle to give a concentration of 6 mg/ml). After the injection of CFA the arthritic animals were kept as such without any drug treatment for 12 days. At the 12<sup>th</sup> day, animals were kept on fasting for 16 to 20 hours for blood collection. After the collection of blood it was utilized for evaluation of various serum biochemical parameters. According to the morphological and biochemical parameter they were further randomized in to different treatment groups according to study. The treatment were initiated from day 13 and continued up to day 28.

### Group of Animal

Group – I	:	Normal Control animals treated with 1% w/v tween-80
Group – II	:	Disease Control animals treated with 1% w/v tween-80
Group – III	:	Me-OH/Me-OH extract of <i>Wedelia calendulacea</i> 250mg/kg
Group – IV	:	Me-OH/Me-OH extract of <i>Wedelia calendulacea</i> 500mg/kg or (Me-OH/Me-OH -WC)
Group – V	:	Me-OH/Me-OH extracts of <i>Wedelia calendulacea</i> 750mg/kg
Group – VI	:	Positive control methotrexate 1 mg/kg orally
Group – VII	:	Methotrexate 1 mg/kg orally + Me-OH/Me-OH -WC

Each group having 6 animals

After 28<sup>th</sup> day the severity if the secondary lesions were evaluated by the various physiological, serum biochemical parameters and histological study for all the group;

### Arthritis assessments<sup>19</sup>

The arthritic severity in each paw was graded from 0 to 4: grade 0, paws with no swelling and focal redness; grade 1, paws with swelling of finger joints; grade 2, paws with mild swelling of ankle or wrist joints; grade 3, paws with severe inflammation of the entire paw; and grade 4, paws with deformity or ankylosis. Each paw was graded and the four scores were totaled so that the possible maximum score per rat was 16.

**Measurements of paw volumes<sup>20</sup>**

The paw volumes of the treated and untreated animals were measured using a plethysmometer at initial day (0day) and Final day (28<sup>th</sup> day). In all the models, the degree of edema formation was determined as increase in paw thickness. Increase in paw thickness and percent inhibition was calculated as under.

$$\text{Increase in Paw Volume} = V_D - V_0$$

Where,

$$\begin{aligned} V_D &= \text{Paw Volume at different day,} \\ V_0 &= \text{Paw Volume at 0 day} \end{aligned}$$

$$\% \text{ Inhibition in paw volume} = \frac{V_{d_0} - V_{t_{D28}}}{V_0} \times 100$$

Where,

$$\begin{aligned} V_{d_0} &= \text{Increase in Paw Volume at 0 day (diseased control)} \\ V_{t_{D28}} &= \text{Increase in Paw Volume at 28<sup>th</sup> day (treated animal)} \end{aligned}$$

**Blood analysis**

The blood samples were collected from rat tail vein under light anesthesia in heparinized centrifuge tubes. The plasma was separated by centrifugation (5000 rpm, 5 min at 4 °C) and Various hematological parameters like hemoglobin (Hb), RBC, WBC, platelets ESR and RA factor<sup>21</sup> and biochemical parameter like TNF- $\alpha$ , Homocysteine, IL-2 and CRP were determined by usual standardized laboratory method with the specific kits.

**In vivo antioxidants study****Assay for SOD activity<sup>22</sup>**

Isolated thoracic aorta was cleaned of surrounding fat and homogenized in 50 mM PBS buffer pH 7.0 using homogenizer. Homogenate was then centrifuged at 4°C; 15,000 rpm for 10 min. Supernatant was used for the estimation of SOD activity by auto-oxidation method as described by Misra et al., 1972.

**Assay for catalase activity<sup>23</sup>**

Catalase activity was measured according to Grover et al., 2009. Thoracic aorta was homogenized (20 mg of tissue/ml of PBS, pH 7.0) and centrifuged at 4°C (15,000 rpm for 10 min). The supernatant obtained was used for the assay. The degradation pattern of exogenously added H<sub>2</sub>O<sub>2</sub> by catalase enzyme present in 200  $\mu$ l of tissue supernatant was monitored at 240 nm in spectrophotometer at 15s interval for 5 min and its activity calculated. Catalase activity is expressed as U/mg of protein. Protein was estimated by Lowry's method.

**Lipid peroxidation assay<sup>24</sup>**

The concentration of MDA [thiobarbituric acid reactive substance (TBARS)] was assayed using the method described by Beltowski et al., 2000. 1ml of tissue supernatant of thoracic aorta was mixed with 1ml of 10% trichloroacetic acid and allowed to stand for 30 min at 37 °C. Then 1ml of 0.67% (w/v) thiobarbituric acid and 20 $\mu$ l of 20% butylated hydroxytoluene (BHT) and the sample were heated at 95°C for 30 min in boiling water bath. After cooling to room temperature, 2ml of n-butanol was added and vortex immediately and centrifuged for 5

min at 5000 rpm. The organic layer was removed and its absorbance was measured at 532 nm. The concentration of MDA is expressed as nM of MDA/mg of tissue.

#### **Preparation of rat aortic spiral preparation**

On 28st day from the CFA injection, rats were sacrificed by cervical dislocation under mild anesthesia and thoracic aorta was isolated from the diaphragm by dissection and placed in oxygenated Krebs Henseleit solution (KHS) at 4°C. The aorta was then carefully dissected out, cleaned of connective tissues with the help of sharp iris scissor and care was taken not to damage the vessel. Then the aorta is spirally cut into 2.5 cm segments. Each spiral aortic preparation was mounted in a 10 ml organ bath containing a modified Krebs Henseleit solution (The composition of Krebs solution was in mM; KHS; NaCl-118, KCl-4.7, KH<sub>2</sub>PO<sub>4</sub>-1.2, MgSO<sub>4</sub>-1.2, CaCl<sub>2</sub>-2.5, NaHCO<sub>3</sub>-25 and glucose-5.5, sucrose-10) of pH 7.4 (at every hour pH was checked and adjusted if required) and osmolarity (280-308 mmol/Kg). The solution was continuously aerated with carbogen (95 % O<sub>2</sub> + 5% CO<sub>2</sub>) at 37°C. A resting tension of 2 g was applied, which had determined to be the optimal resting tensions. Changes in the isometric contraction were recorded on student's Physiograph using isometric force transducer (INCO Pvt. Ltd., ambala). During the equilibration period and throughout the experiment the KHS in the organ bath was changed at every 15-20 minutes to minimize the variability of the responses.

#### **Experimental protocol for vascular reactivity study**

After 2 hours of equilibration, the rings were challenged with 80 mM KCl until a plateau was achieved by two-equipotent response. This was done to check the viability of the tissue. The 15 min of gaps should be kept in between each response so that it can come to its maximum resting state. Concentration response curve for relaxation by ONOO<sup>-</sup> and acetylcholine on aorta precontracted with Phenylephrine PE (10<sup>-5</sup>M) were constructed in normal control, arthritic and treated (std. & test compound) rat thoracic aortas.

#### **Analysis of dose response curves**

The measured relaxation in mm were expressed in mean ± S.E.M for calculation of % Relaxation and Rmax in control, arthritic and treated. The % Relaxation were calculated with the maximal relaxation to ONOO<sup>-</sup> & Acetylcholine as 100% in control, arthritic and treated. % Relaxation for each experiment was plotted against log [M] conc. of the ONOO<sup>-</sup> & Acetylcholine. This will useful for the calculation for pD<sub>2</sub> value. % Inhibition of relaxations was calculated for control, arthritic and treatments group.

#### **Statistical analysis**

The result was expressed as mean ± S.E.M., n=6 where n represents the number of rats. Statistical difference between two means were calculated by unpaired Student's t-test & One-way ANOVA followed by Tukey's multiple comparison test by using statistical computer software Graph pad Prism 5.0. Only those mean values showing statistical difference p<0.05 was considered as statistically significant.

## **Results**

#### **Toxicity study**

The maximum non-lethal dose of Me-OH fraction of Methanolic extract of *Wedelia calendulacea* were found to be 5000 mg/kg; hence 1/10<sup>th</sup> of the dose was taken as effective dose (500 mg/kg) and for the anti- arthritic activity three dose were taken for evaluating the dose dependent activity (250 mg/kg, 500 mg/kg & 750 mg/kg).

**Physiological parameter****Arthritis index**

Fig. 1 depicted that the arthritis index is high on 12<sup>th</sup> day for all group and it was significantly decreased on the 18<sup>th</sup> day and 28<sup>th</sup> day in treatments group.

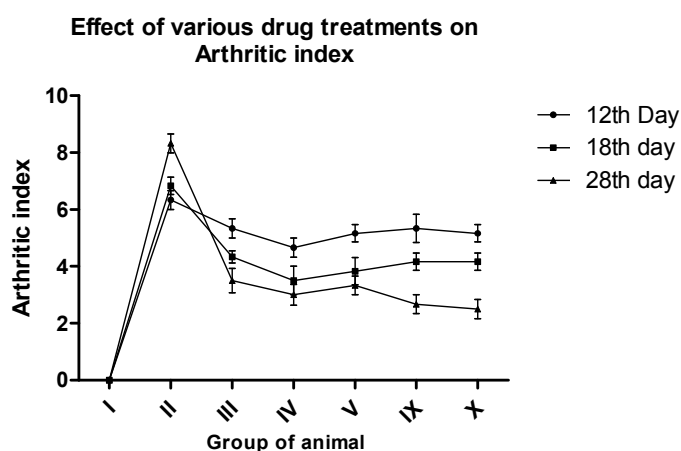


Fig.1 Effect of various treatment on arthritis assessed with arthritic index (mean±S.E. n = 6) in rats.

**Effect of the various treatments on paw volume in arthritis induced rats**

Table 1 depicts the time course of arthritis after the administration of Complete Freund's Adjuvant and inhibition of inflammation after various treatments. On the 12<sup>th</sup> day, hind paw swelling increased significantly and appeared persistent in the Complete Freund's Adjuvant treated group up to the end of the study. In CFA model, the Me-OH/Me-OH extract of *Wedelia calendulacea* inhibited induced paw oedema by 78.81 % at a dose of 250mg/kg, 84.74% at a dose of 500mg/kg and 70.33% at a dose of 750mg/kg. Paw volume was significantly decreased after administration of methotrexate compared to the Freund's Complete Adjuvant treated group, inhibition in paw oedema by 92.79% at the dose of 1mg/kg P.O. The effects methotrexate and *Wedelia calendulacea* (500mg/kg) in combination inhibition in paw edema by 93.58% Arthritis was significantly reduced with *Wedelia calendulacea* (500mg/kg) used in combination with methotrexate compared with CFA induced group.

Table 1 Effect of various treatments of extract of *Wedelia calendulacea* on paw volume

Group/ Hematological parameter	Day	I	II	III	IV	V	VI	VII
Paw volume	Initial	1.03 ± 0.009	1.04 ± 0.013	1.04 ± 0.006	1.04 ± 0.012	1.04 ± 0.009	1.04 ± 0.012	1.04 ± 0.006
	Final	1.03 ± 0.009	1.82 ± 0.022 <sup>###</sup>	1.21 ± 0.008 <sup>***</sup>	1.16 ± 0.006 <sup>***</sup>	1.28 ± 0.104 <sup>***</sup>	1.10 ± 0.021 <sup>***</sup>	1.09 ± 0.020 <sup>***</sup>
% Inhibition				78.81%	84.74%	70.33%	92.79 %	93.58%

Values are expressed as mean ± S.D. n=6. \*\*\*p<0.001 compared with arthritic control. ###p<0.001 compared with normal control.

**Biochemical parameter****Effect of the various treatments on Hematological parameter**

Table 2 represents the hematological changes associated with arthritic condition. Levels of Hb and RBC were decreased significantly in arthritic rats with concomitant increases in WBC, RA Factor, ESR, Homocysteine, CRP, TNF- $\alpha$  and IL-1. These changes were reverted to near normal levels in *Wedelia calendulacea* and combination of *Wedelia calendulacea* and methotrexate treated animals. Combination showed a profound effect than the single drug treatment. No significant changes were observed in control and drug control animals.

Table 2 Effect of various treatments of extract of *Wedelia calendulacea* on various hematological and biochemical parameter

Group/ Hematological parameter	Day	I	II	III	IV	V	VI	VII
Hb (gm/dl)	Initial	14.17 ± 0.78	10.04 ± 0.59 <sup>###</sup>	09.23 ± 1.04	11.38 ± 0.72	10.96 ± 0.75	08.62 ± 0.98	11.58 ± 0.89
	Final	14.17 ± 0.78	09.87 ± 0.61 <sup>###</sup>	11.73 ± 0.86	13.88 ± 0.58 <sup>**</sup>	13.46 ± 0.67	11.12 ± 0.71	14.08 ± 0.84 <sup>**</sup>
RBC (million/cmm)	Initial	6.08 ± 0.58	3.42 ± 0.46 <sup>###</sup>	3.31 ± 0.65	3.63 ± 0.36	3.57 ± 0.44	3.52 ± 0.49	3.98 ± 0.41
	Final	6.39 ± 0.21	3.51 ± 0.14 <sup>###</sup>	4.04 ± 0.34	5.93 ± 0.60 <sup>**</sup>	5.87 ± 0.30 <sup>***</sup>	4.39 ± 0.47	6.21 ± 0.59 <sup>**</sup>
WBC (thousand/cmm)	Initial	07.25 ± 0.79	12.06 ± 0.66 <sup>###</sup>	11.42 ± 0.35	11.45 ± 0.69	11.61 ± 0.48	12.01 ± 0.80	11.85 ± 0.68
	Final	07.37 ± 0.46	12.62 ± 0.50 <sup>###</sup>	09.25 ± 0.71	08.42 ± 0.70	08.62 ± 0.65	08.34 ± 0.9 <sup>**</sup>	08.30 ± 0.69 <sup>**</sup>
ESR (60min.)	Initial	4.12 ± 0.43	9.45 ± 0.47 <sup>###</sup>	9.32 ± 0.48	8.81 ± 0.23	9.59 ± 0.41	9.35 ± 0.40	9.12 ± 0.39
	Final	4.66 ± 0.19	10.10 ± 0.52 <sup>###</sup>	7.90 ± 0.86	5.86 ± 0.56 <sup>**</sup>	6.34 ± 0.44	5.33 ± 0.67 <sup>***</sup>	5.63 ± 0.58 <sup>***</sup>
RA factor (U/mL)	Initial	2.71 ± 0.21	15.95 ± 1.39 <sup>###</sup>	16.58 ± 1.58	15.84 ± 1.13	15.05 ± 1.80	17.70 ± 0.80	15.51 ± 1.75
	Final	2.81 ± 0.29	17.23 ± 0.98 <sup>###</sup>	13.79 ± 2.18	6.34 ± 1.20 <sup>**</sup>	7.68 ± 1.56 <sup>**</sup>	4.56 ± 1.11 <sup>**</sup>	4.18 ± 0.93 <sup>**</sup>
Homocecytein (micromole/L)	Initial	7.73 ± 0.28	13.98 ± 0.41 <sup>###</sup>	14.23 ± 0.87	16.38 ± 0.72	15.96 ± 0.85	13.62 ± 0.64	16.58 ± 1.00
	Final	7.89 ± 0.21	16.53 ± 1.55 <sup>###</sup>	11.79 ± 1.32 <sup>*</sup>	9.14 ± 1.25 <sup>**</sup>	9.75 ± 1.01 <sup>**</sup>	22.65 ± 1.55 <sup>**</sup>	8.12 ± 1.25 <sup>**</sup>
CRP (ng/mL)	Initial	354.88 ± 7.75	484.58 ± 9.69 <sup>###</sup>	489.25 ± 7.66	484.75 ± 8.12	470.25 ± 10.21	480.58 ± 6.72	482.75 ± 8.60
	Final	356.14 ± 9.10	6.14 <sup>###</sup>	483.42 ± 13.57	10.20 <sup>***</sup>	11.24 <sup>***</sup>	8.25 <sup>***</sup>	14.80 <sup>***</sup>
TNF-α (pg/mL)	Initial	0.50 ± 0.044	5.53 ± 0.81 <sup>###</sup>	5.54 ± 0.79	5.59 ± 0.37	5.43 ± 0.79	5.88 ± 0.79	5.40 ± 0.66
	Final	0.46 ± 0.018	5.16 ± 0.32 <sup>###</sup>	4.85 ± 0.26	2.55 ± 0.21 <sup>**</sup>	2.37 ± 0.25 <sup>***</sup>	1.51 ± 0.83 <sup>***</sup>	1.43 ± 0.17 <sup>***</sup>
IL-1 (pg/mL)	Initial	0.31 ± 0.034	2.76 ± 0.093 <sup>###</sup>	2.69 ± 0.080	2.61 ± 0.094	2.70 ± 0.080	2.72 ± 0.080	2.60 ± 0.093
	Final	0.33 ± 0.027	2.87 ± 0.10 <sup>###</sup>	2.35 ± 0.14 <sup>**</sup>	0.77 ± 0.089 <sup>**</sup>	0.92 ± 0.12 <sup>**</sup>	1.03 ± 0.10 <sup>**</sup>	0.73 ± 0.16 <sup>**</sup>

Values are expressed as mean ± S.D. n=6. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001 compared with arthritic control. ### p<0.001 compared with normal control.

### Effect of various treatments on various antioxidant parameters

Arthritis induced animals (group-II) showed decreased SOD and catalase however increased in MDA level. Treatment with methotrexate was not significantly improved the SOD, Catalase and MDA. Treatment with *Wedelia calendulacea* in varying dosage showed significantly increases the level of antioxidant enzymes like SOD and Catalase and reduces the level of lipid peroxidation in terms of MDA content. In combination showed more significantly increased in SOD, Catalase and decreased the level of lipid peroxidation in term of MDA.

Table 3 Effect of various treatments of extract of *Wedelia calendulacea* on various anti-oxidant parameter

Group/ Hematological parameter	I	II	III	IV	V	VI	VII
SOD (Unit/mg of protein)	6.79 ± 0.41	4.46 ± 0.36 <sup>###</sup>	4.86 ± 0.16	5.57 ± 0.16 <sup>*</sup>	5.62 ± 0.28	4.53 ± 0.28	5.66 ± 0.12 <sup>*</sup>
Catalase (U/mg of protein)	6.30 ± 0.42	4.06 ± 0.36 <sup>###</sup>	4.50 ± 0.16 <sup>*</sup>	5.13 ± 0.15	5.20 ± 0.28	4.11 ± 0.26	5.21 ± 0.13 <sup>*</sup>
MDA (nM of MDA/mg)	2.82 ± 0.64	5.49 ± 0.41 <sup>###</sup>	4.49 ± 0.46	3.86 ± 0.30 <sup>*</sup>	3.97 ± 0.28	5.39 ± 0.22	3.93 ± 0.25 <sup>**</sup>

Values are expressed as mean ± S.D. n=6. \* p<0.05, \*\* p<0.01, compared with arthritic control. ### p<0.01, \*\*\*\*P<0.001 compared with normal control and c p<0.05 compare with std. treated animals

### Results of vascular reactivity study

#### Comparison of peroxyinitrite and acetylcholine induced relaxation in arthritic and control rat thoracic aorta

Arthritis induced animals (group-II) showed impaired the relaxation of ONOO<sup>-</sup> and Ach as shown in fig.2.

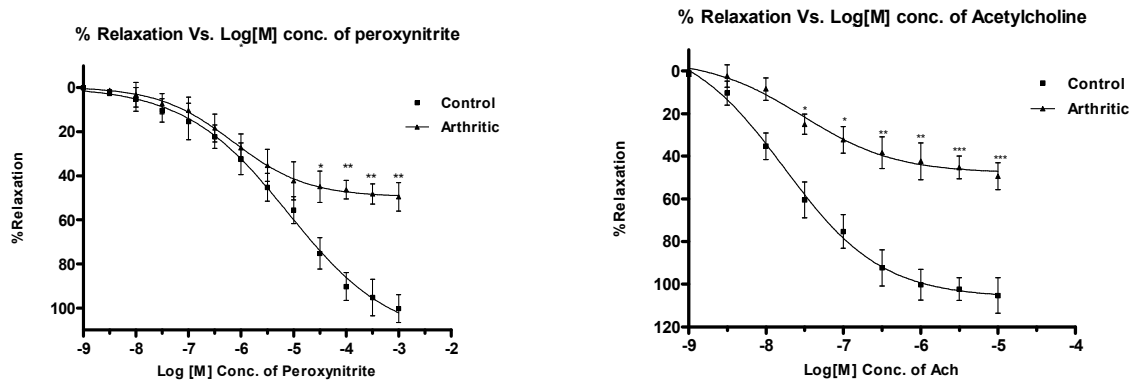


Fig. 2 Concentration response curve of ONOO<sup>-</sup> ( $10^{-9}$  M to  $10^{-3}$  M) and acetylcholine ( $10^{-9}$  M to  $10^{-5}$  M) induced relaxation in control and arthritic aortic strips, precontracted with PE (10-5 M). Values are expressed in means  $\pm$  S.E.M., n = 6. \*\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 Vs. normal control control

### Effect of varying dose *Wedelia calendulacea* treatment (two weeks) on peroxyntirite and acetylcholine induced relaxation in arthritic rat thoracic aorta

Treatment with *Wedelia calendulacea* in varying dosage showed significantly increases the relaxation of ONOO<sup>-</sup> and ach by dose dependent manner as shown in fig.3.

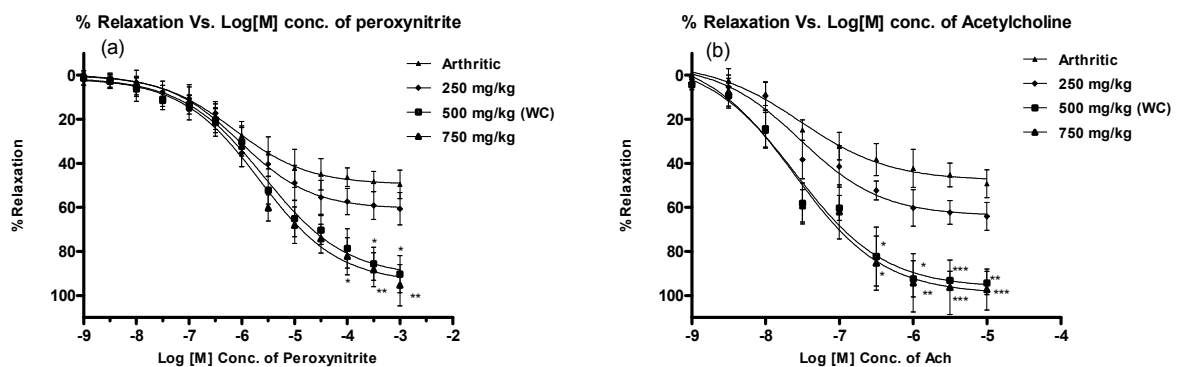


Fig. 3 Cumulative concentration response curves (CRCs) of ONOO<sup>-</sup> (a) and Ach (b) on endothelium intact aortic spiral preparations obtained from arthritic rats and 2 weeks *Wedelia calendulacea* (250, 500, & 750 mg/kg) treated arthritic rats. Each point is represented as Mean  $\pm$  S.E.M. n = 6. \*\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 Vs arthritic group.

### Effect of methotrexate and methotrexate *Wedelia calendulacea* (500mg/kg) treatment (two weeks) peroxyntirite and acetylcholine induced relaxation in arthritic rat thoracic aorta

Treatment with methotrexate showed the increase the ONOO<sup>-</sup> mediated relaxation but there is no any significance effect in Ach mediated relaxation. In combination with the *Wedelia calendulacea* (500mg/kg) showed more significantly increased in relaxation of ONOO<sup>-</sup> and ach as shown in fig.4.



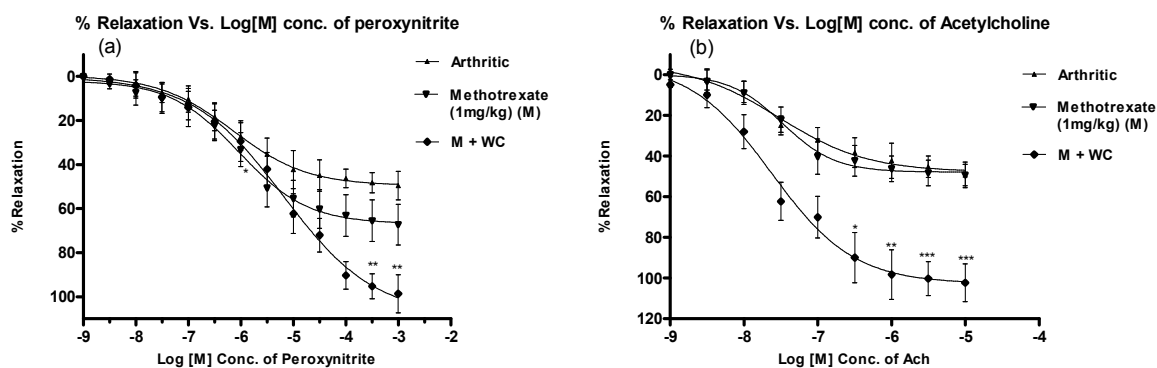


Fig. 4 Cumulative concentration response curves (CRCs) of  $\text{ONOO}^-$  (a) and Ach (b) on endothelium intact aortic spiral preparations obtained from arthritic rats and 2 weeks methotrexate (1mg/kg) and Methotrexate + *Wedelia calendulacea* (500mg/kg) treated arthritic rats. Each point is represented as Mean  $\pm$  S.E.M. n = 6. \*\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 Vs arthritic group.

Table 4 pD<sub>2</sub> values and % Rmax of  $\text{ONOO}^-$  and Ach induced relaxation in different treatment groups

Group	Relaxation	I	II	III	IV	V	VI	VII
Rmax	$\text{ONOO}^-$	100.34 $\pm$ 6.32	49.65 $\pm$ 6.45***	60.65 $\pm$ 7.36	90.34 $\pm$ 8.45*	95.32 $\pm$ 9.35**	67.32 $\pm$ 9.25	98.65 $\pm$ 8.65**
	Ach	105.32 $\pm$ 8.32	49.32 $\pm$ 6.35***	64.12 $\pm$ 6.52	94.66 $\pm$ 5.25**	97.56 $\pm$ 9.35***	49.32 $\pm$ 9.75	102.32 $\pm$ 9.86***
pD <sub>2</sub>	$\text{ONOO}^-$	5.29	6.12	6.02	5.61	5.73	5.97	5.41
	Ach	7.62	7.41	7.46	7.52	7.52	7.46	7.59

Values are expressed as mean  $\pm$  S.D. n=6. \*\*p<0.05, \*\*p<0.01, \*\*\*p<0.001 compared with arthritic control.

## Discussion

The present study illustrates the beneficial outcome of the plant drug *Wedelia calendulacea* in adjuvant induced arthritic rat model with respect to the hematological and cellular constituents. Also we have evaluated the ability of *Wedelia calendulacea* to synergize with methotrexate in ameliorating arthritis. Also investigated the impact of *Wedelia calendulacea* in cardiovascular protect action in alone or in combination with methotrexate in Adjuvant induced arthritis in animals.

In the present study, *Wedelia calendulacea* treatment in three different doses showed a significant protection against CFA induced arthritic changes in different physical parameters like paw volume and arthritic index.

As observed from the present study a similar decrease in Hb and RBC however increase in the WBC count and ESR levels in CFA rats<sup>25</sup>. The decrease in Hb and RBC levels in arthritic rats reflects the presence of anemia in these rats occur due to the decrease the plasma iron level. The decrease in plasma iron inturn was induced by IL1 in association with the acute phase response<sup>26-27</sup>. The increase in total WBC count in CFA rats<sup>28-29</sup>. The increase in WBC counts might be due to the stimulation of immune system against the invading pathogenic microorganism<sup>30</sup>. This is evident by the infiltration of inflammatory mononuclear cells in the joints of CFA rats<sup>6</sup>. ESR is an indirect measurement of acute phase response for determining the disease activity in RA<sup>31</sup>. Although CRP is a better marker for inflammation and though ESR is influenced by several factors such as the plasma concentration of fibrinogen, immunoglobulin's, RF and Hb, the increased level of ESR in arthritic rats adds information reflecting the chronicity and severity of the disease better than CRP<sup>32</sup>. Hence, a combination of the tests might be worthwhile. The abovementioned changes were brought back to near

normal levels upon different dose of *Wedelia calendulacea* extract treatments, which emphasizes the beneficial effect of the drugs on CFA.

The increase in level of NF- $\kappa$ B and TNF- $\alpha$  in paw tissues of adjuvant-induced arthritic rats has been reported to correlate with severity of arthritis during a period of 15–21 days after adjuvant inoculation, and not at day 4 and at day 10<sup>33</sup>. *Wedelia calendulacea* containing coumarins exerts its anti-inflammatory action may be suppressing synthesis of pro-inflammatory prostaglandins and leukotrienes<sup>34</sup>. *Wedelia calendulacea* containing coumarine wedelolactone suppress the activation of NF- $\kappa$ B inhibiting the IKK Complex a critical for the activation of NF- $\kappa$ B by mediating phosphorylation and degradation of I $\kappa$ B $\alpha$ <sup>17</sup>. Wedelolactone also down regulates the expression of tumor necrosis factor and interleukins which play a pivotal role in arthritis.

Oxidative stress implies an imbalance between the production of reactive oxygen species and the antioxidant defense system. Oxidative damages are a common feature of rheumatoid arthritis<sup>35</sup>. FCA induced arthritic rat model also mimic arthritis related oxidative damages caused due to ROS production. Catalase could cause anti-inflammatory effects by degrading hydrogen peroxide and preventing formation of other cytotoxic oxygen radical. Catalase activity in arthritic group of animals is decreased<sup>36</sup>. Lipid peroxidation leading to tissue damage and hence it is associated with aggravation of arthritis<sup>37</sup>. These products may be important in the pathogenesis of vascular complication in rheumatoid arthritis. The extent of lipid peroxidation is measured through malondialdehyde activity (MDA), a pro-oxidant factor, which determines the oxidative damage. In the present study, SOD, catalase and MDA content of the arthritis-induced animals (group II) were found to be significantly altered compared with the normal group. This indicates that the tissues are subjected to increased oxidative stress while a statistically significant reduction of lipid peroxide activity was observed in animal treated with extract of *Wedelia calendulacea*. So *Wedelia calendulacea* alone or in a combination showed potential antioxidant activity which reduces development of free radicals inside the vasculature and it may important for prevention of cardiovascular complications.

Several lines of evidence indicate that NO may be important in the pathogenesis of RA<sup>38</sup>. Evidence reviewed that NO can react with superoxide anion to produce peroxynitrite. ONOO<sup>-</sup> resulted in a vasorelaxant effect on PE (10<sup>-5</sup>M) precontracted Rat aortic strip. The cumulative addition of (10<sup>-9</sup>M to 10<sup>-3</sup>M) ONOO<sup>-</sup> led to a concentration-independent relaxation, reaching a maximum of 100%. These results are consistent with previous reports obtained in another types of arterial strips from different animals<sup>39-41</sup>. It is known that endothelium can release NO and PGI<sub>2</sub>, and that both can lead to smooth muscle relaxation<sup>42</sup>. Acetylcholine dilates vascular beds by stimulation of muscarinic receptors, primarily of the M<sub>3</sub> subtype, despite the lack of apparent cholinergic innervations of most arteries. The muscarinic receptors responsible for relaxation are located on the endothelial cells of the vasculature.

Present experiment shows that, in arthritic aortic strips, ONOO<sup>-</sup> and Ach induced relaxation was impaired as compared to control. This may indicate the development of vascular and endothelial dysfunction s in arthritic rats respectively. Treatment with the varying dose of *Wedelia calendulacea* showed that the significantly increase the ONOO<sup>-</sup> and Ach induced relaxation as compared to arthritic animals.

Methotrexate acts as a folate inhibitor and thereby increase serum homocysteine levels and which produced vascular endothelial dysfunction in adjuvant induced arthritis<sup>7</sup>. So present study showed that methotrexate increase the ONOO<sup>-</sup> mediated relaxation but there is no any significance effect in Ach mediated relaxation.

From the above findings we can reveal that *Wedelia calendulacea* has the potential to be developed as a new product for anti-arthritic and cardioprotective alone or combination with other market std. drug.

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