

## Potential of *Vigna radiata* (L.) sprouts in the management of inflammation and arthritis in rats: Possible biochemical alterations

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*Vigna radiata* (Fabaceae) is an important pulse crop widespread throughout the tropics and warm temperature regions. In this study, we evaluated the *in vitro* anti-inflammatory and *in vivo* antiarthritic activity of *Vigna radiata* sprouts in rats. The *in vitro* anti-inflammatory activity was determined by membrane stabilization and protein denaturation method. Whereas, the antiarthritic activity of the ethanolic extract of the sprouts was evaluated by complete Freund's adjuvant model with diclofenac sodium as the standard drug. Body weights, paw volume, biochemical parameters such as lipid peroxidation, total reduced glutathione, myeloperoxidase and lysosomal enzymes like cathepsin-D, N-acetyl  $\beta$ -D-glucosaminidase and  $\beta$ -D-glucuronidase were estimated. Treatment with ethanolic extract of *V. radiata* exhibited significant membrane stabilization activity and protein denaturation activity, and significantly attenuated the biochemical changes induced by administration of complete Freund's adjuvant. The findings of the present study suggest the possible role of *Vigna radiata* in the therapeutics of arthritis.

**Keywords:** Complete Freund's adjuvant, Green gram, Lysosomal enzymes, Membrane stabilization, Mungbean, Pulses, Rheumatoid arthritis.

Rheumatoid arthritis (RA), one of the most common autoimmune diseases, is a chronic progressive systemic inflammatory disorder characterized by symmetrical joint involvement affecting the synovial joints which is responsible for joint destruction ultimately leading to deformity and disability<sup>1</sup>. It has a worldwide distribution with an estimated prevalence of 1-2% and is having peak incidence in 30-40 years of age. In RA, the immune complexes in the synovial fluid activate the complement system which leads to the migration of polymorpho nuclear cells into the joint space triggering the release of various lysosomal enzymes and free radicals into joint space leading to damage<sup>2</sup>. The review by Das<sup>3</sup> discusses the role of complement system in the pathogenesis of RA and its therapeutic implications in depth.

Adjuvant model of RA has been widely used for preclinical testing of various agents which can be used in the therapeutics of the disease<sup>4-6</sup>. This method in rats was originally developed by Pearson and Wood<sup>7</sup>, wherein an intraplantar injection of complete Freund's adjuvant (FCA) in the rat hind paw induces inflammation. It has

been proposed that bacterial peptidoglycan and muramyl dipeptide are vital nonspecific immunogenic contributory components which are capable of synthesizing various inflammatory mediators. In turn, these synthetic products induce the production of a variety of enzymes which initiate cartilage and bone destruction<sup>8,9</sup>.

*Vinga radiata* (L.), Fam. Fabaceae, commonly known as Mungbean of Green gram, contain balanced nutrients including protein and dietary fibre, and significant amounts of bioactive phytochemicals. High levels of proteins, amino acids oligosaccharides, and polyphenols in mungbeans possibly contribute to paralysis, rheumatism, memory weakness, ulcers, leprosy and rickets, antioxidant, antimicrobial, anti-inflammatory, antitumor, antidiabetic, antiseptis, anti-hypertensive activities, and are involved in the regulation of lipid metabolism<sup>10-12</sup>. A recent study by Gnanaraj *et al.*<sup>13</sup> has demonstrated relationship between phospholipase C expression and response to abiotic stress such as drought and salt stress in green gram. Apart from these it serves as a protein rich source in the form of sprouts. Roots are also traditionally used as anti-inflammatory, aphrodisiac, refrigerant and emollient<sup>14</sup>.

Here, we explored *in vitro* anti-inflammatory and *in vivo* antiarthritic activity of *Vigna radiata* sprouts in experimental animals.

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## Materials and Methods

### Sprout formation and preparation of extract

*V. radiata* seeds were collected from local market, and a dried voucher specimen was authenticated by a taxonomist at Kakatiya University, Warangal, India and deposited in the herbarium of department of botany, Kakatiya University. Premium grade seeds of medium size were selected and washed with fresh water for 3-4 times, soaked in tap water at  $25 \pm 2^\circ\text{C}$  for 8 h, and were allowed to sprout for 2- 3 days till they attained a length of 1-2 cm by applying a fine water spray uniformly over the seeds at 4-5 h daily. These were then shade dried and ground to fine powder. The fine powder was extracted by maceration with sufficient quantity of ethanol for 7 days with intermittent shaking in a dark room. The filtrate is maintained in dark. To the marc, 100 mL fresh solvent was added and refluxed for 1h followed by filtration and finally both the filtrate were mixed together and concentrated to give ethanolic extract (VR). Percentage yield was noted and then stored in desiccators for further use<sup>15</sup>.

### Animals

Male Wistar rats (220-250 g) were obtained from Mahaveer Enterprises, Hyderabad, India. The rats were housed at  $25 \pm 1^\circ\text{C}$  and relative humidity of 45-55% under 12:12 light-dark cycle. The animals had free access to feed pellets and water *ad libitum*. The experimental protocol was approved (IAEC NO:1047/ac/07/CPCSEA) by the Institute Animal Ethics Committee (IAEC), and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, on animal experimentation.

### Acute toxicity studies

The acute toxicity study was carried out as per the procedure given in Organisation for Economic Co-operation and Development (OECD) Guideline No. 420<sup>16</sup>. After the sighting study (selection of the appropriate starting concentration for the main study), VR @ 2 g/kg body wt. was given to 5 animals, and were observed for 14 days for mortality and general behaviour.

### Membrane stabilization method

The effect of VR on haemolysis of Human Red Blood Cells (HRBC) induced by heat and distilled water was evaluated according to Shinde *et al.*<sup>15</sup>. Briefly, blood (2 mL) was collected from healthy volunteers and mixed with equal volume of sterilized

Alsever's solution containing dextrose, citrate and citric acid followed by centrifugation at 4000 rpm for 5 min. The packed cells were washed in normal saline at  $4^\circ\text{C}$  until use. The reaction mixture (4.5 mL) consisted of 2 mL hypotonic saline (0.25% w/v NaCl), 1 mL of phosphate buffer (pH7.4), 1 mL of test solution (100, 500, 1000  $\mu\text{g}/\text{mL}$ ) and 0.5 mL of 10% HRBC suspension. Positive control was run simultaneously with 1 mL of isotonic saline instead of test solution. Similarly, negative control was run with 1 mL isotonic solution instead of HRBC suspension Diclofenac sodium (DS) (100, 500, 1000  $\mu\text{g}/\text{mL}$ ) was used as a standard. The mixtures were incubated at  $37^\circ\text{C}$  for 30 min and centrifuged at 3000 rpm. The supernatant was separated and haemoglobin content was measured spectrophotometrically at 560 nm.

The percentage of membrane stability was calculated as follows:

$$\text{Percentage stabilization} = \frac{\text{OD of positive control} - \text{OD of test solution}}{\text{OD of positive control}} \times 100$$

### Protein denaturation method

Protein denaturation ability of VR was evaluated by the method of Biswakanth *et al.* (2012)<sup>17</sup>. Briefly, aqueous solution of bovine serum albumin and test or standard drug solutions (100, 500, 1000  $\mu\text{g}/\text{mL}$ ) were adjusted to pH 6.3 and were incubated at  $37^\circ\text{C}$  for 20 min followed by heating at  $57^\circ\text{C}$  for 3 min. The resulting solution was added with 2.5 mL of phosphate buffer (pH 6.3) and the absorbance was measured at 416 nm. Positive and negative controls were simultaneously run without test or standard drug and bovine serum albumin, respectively. The percentage inhibition of protein denaturation was calculated using the formula:

$$\text{Percentage stabilization} = \frac{\text{OD of positive control} - \text{OD of test solution}}{\text{OD of positive control}} \times 100$$

### Adjuvant induced arthritis

Male wistar rats (220-250 g) were randomly divided into 5 groups (n= 6); naïve, disease control, standard, VR 250 and VR 500. On the day 1, adjuvant arthritis was induced in all the animals except naïve by injecting 0.1 mL of complete Freund's adjuvant (FCA) into the sub planer region of the left hind paw<sup>18</sup> [complete fraction of *Mycobacterium butyricum* (10 mg/mL)] suspended in oil; Sigma Chemical Co., USA) Animals received the respective drugs dissolved in 0.1 % CMC daily in the morning at 10:00 h, from day 13 to day 21. After 21 days, rats were fasted

overnight and were sacrificed by cervical decapitation with prior anesthetization. The liver was rapidly removed and kept in ice cold saline and *in vivo* oxidant levels were measured. The blood from the animals was collected by retro-orbital plexus prior to sacrifice.

#### Body weight, paw volume and pain threshold

Body weight and paw volume of all the animals were measured on day 0, 5, 13, 18 and 21. The paw volume was recorded using plethysmometer (UGO basile, Italy) by double blind method. The mean change in FCA injected paw edema with respect to the initial paw volume was calculated on the respective days and percentage inhibition of paw edema with respect to control group was calculated using the following formula:

$$\text{Percentage inhibition} = 1 - \left[ \frac{\text{Mean change in paw volume of treated rat}}{\text{Mean change in paw volume of control}} \right] \times 100$$

Pain threshold for all the animals were estimated by means of randal selitto analgesymeter (UGO basile, Italy) on day 0, 5, 13, 18 and 21. Pain threshold was measured by the threshold required to show the paw withdrawal using a weight of 500 g applied to the hind paw<sup>19</sup>.

#### Estimation of serum cytokines

The quantitative analysis of the pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  and inflammatory cytokines IL-6 and IL-10 in serum of all the rats was done using commercial ELISA kits (R&D systems, USA) and ELISA reader according to the manufacturer's instructions.

#### Determination of liver lysosomal enzyme levels

An accurately weighed amount of the dissected liver tissue was washed and transferred to ice cold saline. About 10 % homogenates were prepared by homogenizing the tissues in 0.1 M Tris-HCl buffer (pH 7.4). The supernatant obtained after centrifugation of the homogenates was used for the estimation of N-acetyl- $\beta$ -D-glucosaminidase<sup>20</sup>,  $\beta$ -D-glucuronidase<sup>21</sup>, Acid phosphatase<sup>22</sup> and cathepsin D<sup>23</sup>.

#### Determination of Lipid peroxidation

A portion of the liver of known weight was minced into small pieces and then homogenized with 0.01 M Tris HCl buffer, pH 7.4. An aliquot of the tissue homogenate was treated with 3 mL of ice cold 10% trichloro acetic acid (TCA). The tubes were mixed well and 2 mL of thiobarbituric acid was added followed by boiling on water bath. After cooling, the tubes were centrifuged and the absorbance of

the supernatant was read at 532 nm. The malondialdehyde (MDA) content in the tissues was expressed as  $\mu\text{M/g}$  of tissue<sup>24</sup>.

#### Determination of reduced glutathione content

The tissue homogenate was deproteinized with an equal volume of 10 % TCA and was allowed to stand at 4°C for 2 h. The contents were centrifuged at 2000 rpm for 15 min. The supernatant was added to 2 mL of 0.4 M Tris buffer (pH 8.9) containing 0.02 M EDTA (pH 8.9) followed by the addition of 0.01 M 5, 5'-dithio-bis-2-nitro-benzoic acid. Finally, the mixture was diluted with 0.5 mL of distilled water, to make the total volume to 3 mL and the absorbance was recorded at 412 nm using UV spectrophotometer and the results were expressed as  $\mu\text{g GSH/g tissue}$ <sup>25</sup>.

#### Determination of Myeloperoxidase (MPO) activity

MPO activity was determined by a previously described method<sup>26</sup>. Briefly, 100 mg of liver tissue was homogenized in 0.5% DTAB solution in 50 mM potassium phosphate buffer (pH 6) and sonicated in ice bath for 10 s. The homogenates were freeze-thawed thrice and centrifuged for 15 min at 20000  $\times g$ . The level of MPO activity was measured spectrophotometrically: 0.1 mL of the test solution was mixed with 2.9 mL of 50 mM phosphate buffer, containing O-dianisidine dihydrochloride and 0.05% hydrogen peroxide. The change in absorbance at 460 nm was measured for 5 min using a Beckman spectrophotometer (Beckman DU 640B). The MPO activity was measured using the following formula:

$$\text{MPO activity} \left( \frac{\text{mU}}{\text{mg}} \right) = 1000 \times \frac{X}{\text{weight of tissue taken (mg)}}$$

Where

$$X = 10 \times \frac{\text{change in absorption per minute}}{\text{volume of supernatant taken in final reaction}}$$

#### Statistical analysis

All the data were statistically evaluated with ANOVA and the differences among groups were determined by Dunnett's multiple comparison tests using Graph pad prism 5.0. Values were considered to be significant when *P* value <0.05. All the results were presented as Mean  $\pm$  SD for six animals in each group.

#### Results

In the acute oral toxicity study, at a dose of 2 g/kg body wt. of VR, mortality was not observed. From the

results, test drug doses of 250 and 500 mg/kg body wt. were chosen for the efficacy studies.

#### Membrane stabilization and protein denaturation

In the *invitro* method of membrane stabilization, it was observed that VR group showed significant activity compared to positive control. Percentage stabilization was 77.99 and 94.25% at a concentration of 1000 µg/mL of VR and standard DS, respectively (Table 1). VR group exhibited maximum inhibition of protein denaturation, 82.84% at 250 µg/mL which was comparable with that of the standard DS with a maximum inhibition of 95.57% at the same concentration.

#### Effect of VR on body weight, paw volume and pain latency

The body weight of all the animals in naive group increased till day 21, whereas body weight of all the animals in control group were significantly decreased ( $P < 0.05$ ) till day 21 as compared to naive group. Further, the body weight of all the animals in VR or DS treated groups were increased significantly ( $P < 0.05$ ) as compared to control group (Fig. 1). Intraplantar administration of FCA in rats induced severe inflammation and redness over a period of 24 h. Oral administration of VR (250 or 500 mg/kg) ameliorated the alteration in paw volume on day 21 as compared to control (Table 2). Further, intraplantar administration of FCA significantly ( $P < 0.01$ ) decreased pain threshold as compared to normal rats. However, oral administration of DS (10 mg/kg) led to a significant increase in pain threshold from day 13 and day 18 when compared to 5<sup>th</sup> day of the respective group (Table 2). Further, VR (250 or 500 mg/kg) did not show significant change in the pain threshold compared to control group.

#### Effect of VR on serum cytokines

Induction of RA in animals led to a significant ( $P < 0.01$ ) elevation of pro-inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  when compared to naive group, whereas administration of DS (10 mg/kg) or VR (500 mg/kg) reduced the levels of these cytokines

compared to control group ( $P < 0.05$ ). However, lower dose of VR (250 mg/kg) was found to be non-significant. On the other hand, anti-inflammatory cytokines IL-10 and IL-6 were decreased significantly ( $P < 0.01$ ) in control group compared to naive animals. Treatment of arthritic animals with DS (10 mg/kg) or VR (500 mg/kg) elevated the levels of anti-inflammatory cytokines compared to control group ( $P < 0.05$ ) (Table 3).

#### Effect of VR on liver lysosomal enzyme levels

Induction of RA in animals led to a significant ( $P < 0.01$ ) decrease in the liver lysosomal enzymes when compared to healthy animals. However, daily administration of DS (10 mg/kg) or VR (250 or 500 mg/kg) led to the normalization of the liver lysosomal enzymes compared to control animals ( $P < 0.01$ ,  $< 0.05$ ,  $< 0.01$ , respectively) (Table 3).

#### Effect of VR on levels of ROS

The lipid peroxidation activity significantly increased ( $P < 0.001$ ) in tissue homogenate of RA induced group animals when compared to normal

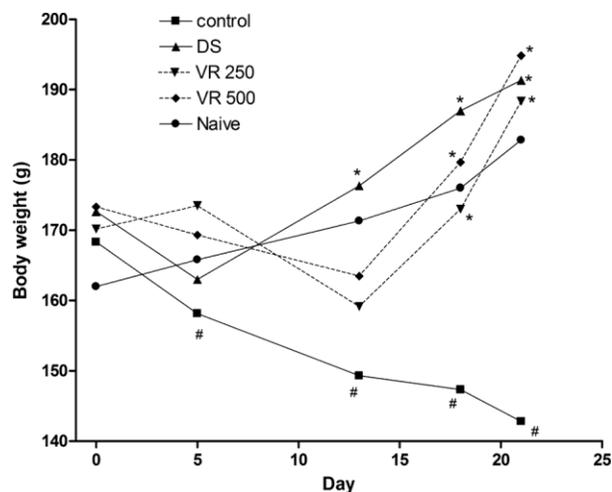


Fig. 1 — Effect of VR on body weight in FCA induced RA in rats. Data was analyzed by two way ANOVA followed by Bonferroni test. # $P < 0.05$  as compared to naive group, \* $P < 0.05$ , as compared to control group.

Table 1— Effect VR on HTBC membrane stabilization and protein denaturation

Concentration (µg/ml)	Membrane stabilization		Protein denaturation	
	VR (%)	DS (%)	VR (%)	DS (%)
100	59.34 ± 1.10	66.75 ± 1.28	64.25 ± 1.64	67.61 ± 1.29
500	71.29 ± 2.05	84.75 ± 2.09	77.22 ± 1.95	71.03 ± 1.56
1000	67.99 ± 1.28	94.25 ± 2.08	82.84 ± 2.39	95.57 ± 2.71

(n=3) Values are expressed as mean ± SEM

Table 2— Percentage inhibition of paw edema and Pain threshold by randal sellito test in adjuvant induced arthritis

	Percentage inhibition of paw edema			
	Day 5	Day13	Day 18	Day 21
Control	0	0	0	0
VR 250	10.22 ± 0.78	14.23 ± 0.95	24.88 ± 2.14	46.62 ± 0.84*
VR 500	10.26 ± 1.28	22.83 ± 1.02	32.97 ± 2.38*	64.38 ± 1.09**
DS	12.85 ± 0.64	24.96 ± 1.29**	38.54 ± 2.04**	70.29 ± 2.48**
	Pain threshold (g) by randal sellito test			
Naive	110.23 ± 5.76	114.89 ± 8.66	112.48 ± 9.49	109.23 ± 7.55
Control	74.98 ± 7.34 <sup>###</sup>	60.58 ± 6.46 <sup>###</sup>	58.96 ± 7.54 <sup>###</sup>	56.37 ± 5.88 <sup>###</sup>
VR 250	76.87 ± 4.99	75.43 ± 3.94	78.38 ± 8.63	81.67 ± 8.46
VR 500	75.84 ± 8.20	80.37 ± 4.96	85.64 ± 8.21	92.54 ± 9.56
DS	89.56 ± 9.34	95.98 ± 9.36**	98.56 ± 8.43**	102.22 ± 9.87**

(n=6) Data was analyzed by two way ANOVA followed by Bonferroni test. Values are expressed as mean ± SEM.

Table 3—Effect of VR on serum cytokines and liver lysosomal enzymes

	TNF $\alpha$ (pg/mL)	IL-1 $\beta$ (pg/mL)	IL-6 (pg/mL)	IL-10 (pg/mL)	N-acetyl- $\beta$ -D-glucosaminidase	Cathepsin-D	Glucuronidase
Naive	92.16 ± 0.06	110.45 ± 0.54	127 ± 0.73	119.08 ± 0.65	30.72 ± 1.40	41.22 ± 2.08	34.62 ± 0.80
Control	406.53 ± 2.64 <sup>###</sup>	631.24 ± 0.72 <sup>###</sup>	56.22 ± 0.85 <sup>###</sup>	57.16 ± 0.28 <sup>###</sup>	45.98 ± 1.10 <sup>###</sup>	62.96 ± 2.09 <sup>###</sup>	44.75 ± 0.45 <sup>###</sup>
VR 250	363.64 ± 0.84	426.76 ± 0.51	76.29 ± 0.78	86.45 ± 0.58	39.30 ± 1.00*	54.87 ± 1.65*	41.08 ± 0.43*
VR 500	248.55 ± 0.94**	426.76 ± 0.51*	91.69 ± 0.52*	101.75 ± 0.56*	35.94 ± 0.89**	50.56 ± 1.33**	36.94 ± 0.48**
DS	164.54 ± 1.43**	426.76 ± 0.51*	102.66 ± 1.05*	106.45 ± 0.54*	32.80 ± 1.11**	48.08 ± 1.83**	35.06 ± 0.32**

(n=6) Data was analyzed by one way ANOVA followed by Dunnet's test. Values are expressed as mean ± SEM. <sup>###</sup> $P < 0.01$  as compared to naive group, \* $P < 0.05$ , \*\* $P < 0.01$  as compared to control group.

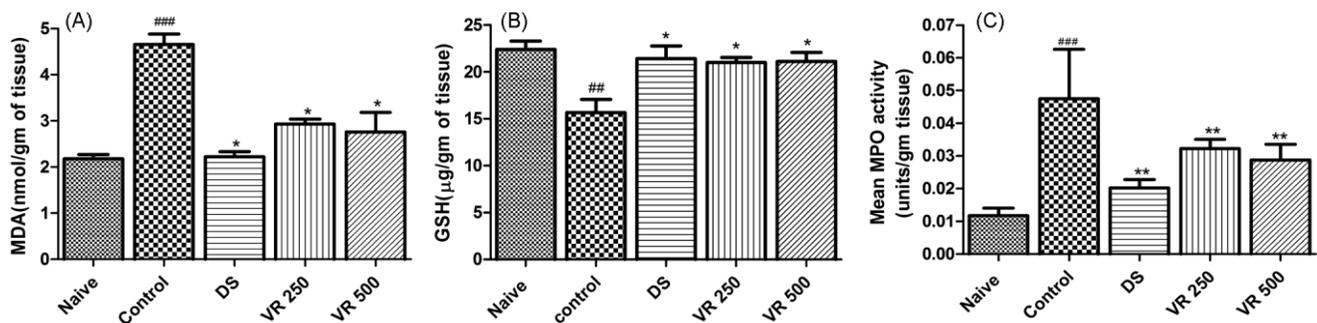


Fig. 2 — Effect of VR on alterations in (A) MDA; (B) GSH; and (C) MPO levels in FCA induced RA in rats. Data was analyzed by one Way ANOVA followed by Dunnet's test. <sup>###</sup> $P < 0.001$  as compared to naive group, \* $P < 0.05$ , \*\* $P < 0.01$  as compared to control group.

animals. However, VR or DS treated animals showed significant reduction in MDA levels ( $P < 0.05$ ). Likewise, a significant ( $P < 0.05$ ) increase in total reduced GSH content was observed in DS or VR (250 or 500 mg/kg) treated group compared to disease induced control animals (Fig. 2). Administration of DS (10 mg/kg) or VR at different doses for 21 days ameliorated the increase in MPO activity significantly ( $P < 0.01$ ) as compared to the control group.

## Discussion

HRBC membrane is analogous to liposomal membrane, which on stabilization of decreases the inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases which cause further tissue damage upon extra cellular release<sup>27</sup>. The present investigation demonstrates that VR enhances the stabilization of HRBC signifying its role in anti-inflammatory effect. The symptoms of

RA typically include abnormal weight loss, along with the inflammation of the joint. Increasing amount of evidence suggest that intraplantar administration of FCA in rat paw mimics these conditions in animals<sup>7,19</sup>. In consistent with the above reports, the present study demonstrated the decrease in body weight by administration of FCA along with the increase in the paw volume indicating the arthritic like inflammatory changes caused by CFA. However, administration of DS or VR showed significant protection against FCA induced changes as proved by better clinical condition of the animals.

Several reports suggest that intraplantar administration of FCA leads to the inflammatory changes due to migration of neutrophils and macrophages to the synovium causing inflammation of the paw<sup>28</sup>. In arthritic condition, the progression of disease is attenuated by a complex of enzymes which are produced by the microphages that have migrated to the synovium. These immune cells secrete TNF- $\alpha$  and IL-1 $\beta$  that stimulate the release of COX-2 and macrophage colony stimulating factors. In the present study, induction of RA led to the increase in these cytokines which were decreased on treatment with VR.

Lysosomal enzymes are membrane enclosed cytoplasmic granules within which acidic lytic enzymes of the cell are sequestered in a latent form. These enzymes such as N-acetyl- $\beta$ -D-glucosaminidase,  $\beta$ -D-glucuronidase, acid phosphatase and cathepsin D have been implicated in the loss of collagen in RA induced by FCA. In the present investigation, sustained elevation of these enzymes in liver was found in RA induced rats. Treatment with VR for 21 days led to the decline in these enzymes, thereby exhibiting a protective effect against the damage caused by the elevated enzymes. The increased levels of anti-inflammatory cytokines in VR treated rats demonstrate therapeutic effect of VR on the arthritic animals.

The migrated immune cells in the synovium release several components like proteases and nitrogen/oxygen metabolites leading to damage of surrounding tissues and ROS generation, respectively. The extent of lipid peroxidation was measured through MDA activity which is a pro-oxidant factor determining the oxidative damage. Similarly, MPO provides a quantitative measure of disease severity and measurement of MPO activity has been used as an indicator of neutrophil influx leading to release of free radicals<sup>29</sup>. In present investigation, MDA and MPO

content of liver were found to be significantly increased in RA induced rats compared to normal group indicating that administration of FCA led to the increased oxidative stress which was attenuated by administration of VR. This ameliorative effect can be attributed to the antioxidant like effect of quercetin. GSH is one of the vital compounds for maintaining the normal cell function against ROS induced oxidative damage. Depletion of GSH promotes the generation of ROS resulting in the loss of function and integrity of the cell<sup>30,31</sup>. Various studies have shown that colonic GSH concentrations were significantly lowered by FCA-induced RA<sup>32</sup>. Similarly, in the present study, there was a significant decrease in the GSH concentration following FCA administration which was ameliorated by the VR treatment confirming the antioxidant like effect.

RA is an inflammatory condition characterized by functional impairment and hyperalgesia of the joint<sup>33</sup>. The hyperalgesia in CFA-induced RA is mainly attributed to the production of prostaglandins along with the increase in substance P upregulation of neurokinin-1 receptor<sup>34</sup>. However, in the present study, administration of VR did not prevent the reduction of pain latency that is caused by administration of FCA indicating that nociception mediators such as substance P are not related to the beneficial effect of VR in RA.

In conclusion, VR exhibits a protective effect against FCA-induced arthritis which can be attributed to the combination of anti-inflammatory and antioxidant like effect capable of acting on various pharmacological targets with regard to arthritis. Thus, our results suggest that VR supplementation may be considered as an applicable approach in the therapy of rheumatoid arthritis.

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