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Original Article

Comparative effect of horse gram and black gram on inflammatory mediators and antioxidant status

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ABSTRACT

A balanced diet is important for the overall wellbeing of an individual. Pulses are an important part of a nutritive diet. Pulses have been consumed for at least 10,000 years and are among the most extensively used foods in the world. They are a rich source of protein and fiber, as well as a significant source of vitamins and minerals, such as iron, zinc, and magnesium. The purpose of this study was to compare the effect of two pulses, horse gram and black gram, on inflammatory mediators and the antioxidant enzymes. Two sets of experiments were conducted in rats which were fed with boiled and unboiled horse gram and black gram, at a dose of 100 mg/100 g body weight, for 21 days and 60 days. The results showed that horse gram supplementation for 21 days and 60 days significantly increased the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase and showed no significant changes in the activities of the inflammatory mediators such as cyclooxygenase, lipoxygenase, myeloperoxidase, nitric oxide synthase, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), etc. However, the black gram (with skin and without skin) supplementation significantly increased activities of the inflammatory mediators and showed a significant decrease in the antioxidant enzymes in both the 21-day and 60-day experiments. Thus, these preliminary results demonstrate the anti-inflammatory and antioxidant potential of horse gram and the proinflammatory effects of black gram in rats. This is in accordance with the dietary regime advised by Ayurveda practitioners, where horse gram is to be included and black gram is to be excluded from the diet for conditions such as rheumatoid arthritis. Further studies are to be conducted to validate the same.

1. Introduction

Consuming a well-balanced diet is essential for maintaining good health of the body. A nutritious diet helps to keep the immune system functioning properly and contributes to the overall wellbeing of the organism. Without good nutrition, the body becomes susceptible to disease, infection, fatigue, etc. Eating many different foods helps maintain a healthy and interesting diet which provides a range of different nutrients to the body. However, eating too much or too little of a particular food variety can cause issues. Some foods have

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antioxidant or anti-inflammatory potential and can be highly beneficial to the body, whereas some others may be detrimental by eliciting immune reactions in the body. Pulses are a very important food source particularly in Middle and South America, the Middle East, China, Africa, and Asia. India is one of the world's largest producers and consumers of pulses. Pulses have significant nutritional and health advantages for consumers. They are rich in proteins, carbohydrates, fiber, the minerals phosphate, calcium, and iron, and the vitamins of the B-complex. They are low in sodium and saturated fats. Pulses are an important source of protein for vegetarians and should be included in their diet. Soaking dried beans for several hours brings them back to life, activating enzymes, proteins, minerals, and vitamins [1,2]. Horse gram (*Macrotyloma uniflorum*) is one of the lesser known beans. Horse gram is a legume of the tropics and subtropics, grown mostly under dry-land agriculture. Studies have shown that unprocessed raw horse gram seeds not only possess antihyperglycemic properties, but also have qualities which reduce insulin resistance. Raw horse gram seed is rich in polyphenols, flavonoids and proteins, the major antioxidants present in fruits and other food materials [3,4]. Also, a solid state fermentation product, Kaulath, obtained from horse gram, showed an increased free radical scavenging property and can be included in the diet and in formulated foods [5]. Dehulling in combination with germinating the seeds has also been shown to improve the nutritive value of horse gram [6]. These studies in horse gram show the importance of including it in the diet. Black gram (*Vigna mungo*), also known as black lentil, white lentil and black matpe bean, is a bean grown in the Indian subcontinent. Ground into flour or paste, it is also extensively used in South Indian culinary preparations. *Vigna mungo* is used in traditional Indian (Ayurveda) medicine. Pharmacologically, extracts have demonstrated immunostimulatory activity in rats [7].

Inflammation is a localized protective reaction of cells/tissues of the body to any kind of noxious stimulus such as allergic or chemical irritation, injury, and/or infections. This initial response may further initiate a series of biochemical, immunological, and cellular events, ending with physical repair and restoration of function of the injured tissue. Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Without inflammation, wounds and infections would never heal [8]. The symptoms of inflammation are characterized by pain, heat, redness, swelling, and loss of function that result from dilation of the blood vessels leading to an increased blood supply and from increased intercellular spaces resulting in the movement of leukocytes, protein and fluids into the inflamed regions [9]. Prostaglandins, thromboxanes and leukotrienes are inflammatory mediators called eicosanoids and are synthesized by cyclooxygenases (COX) and lipoxygenases (LOX) in cell types that are associated with inflammatory disorders. COX-2 catalyzed synthesis of prostaglandin E2 plays a key role in inflammation and its associated diseases [10].

Free radicals are produced by chemical or biological aggression to the body caused by stress, physical damage, infection and cytotoxic or carcinogenic compounds. This may cause peroxidation of cell components such as proteins, lipids and can also break strands of DNA [11]. This oxidative damage caused by the free radicals is considered to play a causative role in ageing and several diseases including cataract, cognitive dysfunction, cancer, myocardial infarction, diabetes, arthritis and several heart diseases [12]. Reactive oxygen species are playing a dual role as deleterious and beneficial species, since they can be either harmful or beneficial to living systems [13]. Our bodies try to protect us from free radical damage by producing antioxidant enzymes that neutralize them. However, they are not capable of handling this function without nutrients and antioxidants provided by our diets. Antioxidants are protective molecules also referred to as free radical scavengers and hence prevent and repair damage done by these free radicals [14]. Antioxidants may also enhance immune defense and therefore lower the risk of cancer and infection. Fruits, pulses and vegetables, the main source of antioxidants in the diet, are associated with lower risk of degenerative disease [15].

The antioxidant enzymes help to maintain the oxidant/antioxidant status by tackling excess of free radicals in the system. The antioxidant enzymes should be studied in order to evaluate the extent of lipid peroxidation during inflammation. The enzymes involved in inflammatory processes are seen to be increased in many of the disease conditions. Hence, such inflammatory mediators should be studied in order to understand the progress of inflammation and also to figure out good therapeutic targets for inflammation. The present study is designed to evaluate the effects of two pulses, horse gram and black gram, on oxidative stress and inflammatory mediators in normal rats.

2. Materials and methods

2.1. Chemicals and solvents

Histopaque, linoleic acid, and arachidonic acid were purchased from Sigma-Aldrich Chemicals (St Louis, MO, USA) and Spectrochem Pvt. Ltd. (Mumbai, Maharashtra, India). All other chemicals and biochemicals used were of the highest grade available.

2.2. Collection of the pulses

Pulses, horse gram and black gram were purchased from the local market of Thiruvananthapuram district, India. Two types of black gram were purchased: with skin and without skin varieties.

2.3. Animal experiment

Female albino rats (Sprague–Dawley strain) of body weight 150–200 g which were breed and reared in the department animal house were used for this study. They were provided laboratory chow (Hindustan Lever Lab diet) and water *ad libitum* throughout the experimental period. The rats were housed in polypropylene cages in a room with temperature maintained at $26 \pm 1^{\circ}C$ and a 12 hour light and dark cycle. The animals received human care, in compliance with the

host institutional animal ethics guidelines. All experiments were conducted according to the guidelines of the animal ethics committee Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (registration number 218/CPCSEA) IAEC-KU-8/2012-13-BC-AH (22) according to the Government of India accepted principles for laboratory animal use and care.

The animals were fed with boiled and unboiled fractions of pulses along with the normal rat feed. For boiled fractions, the pulses (100 mg/g body weight) were cleaned, boiled, ground, and mixed with the rat feed. As for unboiled fraction, the pulses (100 mg/g body weight) were cleaned, powdered, and mixed with normal rat feed. The rats were divided into seven groups of six as follows: Group I: control rats, Group II: horse gram (100 mg/100 g body weight) boiled, Group III: horse gram (100 mg/100 g body weight) unboiled, Group IV: black gram (100 mg/100 g body weight) boiled (with skin), Group V: black gram (100 mg/100 g body weight) unboiled (with skin), Group VI: black gram (100 mg/100 g body weight) boiled (without skin), and Group VII: black gram (100 mg/100 g body weight) unboiled (without skin). Normal rats were fed with this diet for 21 days and 60 days as two separate experiments. At the end of the experimental period, animals were sacrificed after an overnight fast. The blood was collected in test tubes containing anticoagulant (heparin) to isolate the peripheral blood mononuclear cells and in tubes without anticoagulant for serum collection. Tissues such as liver and heart were removed, washed with ice cold saline, and kept in ice cold containers for the evaluation of various parameters.

2.4. Isolation of peripheral blood mononuclear cells

Isolation of monocytes was carried out as described by Radhika et al [16]. A 3-mL volume of Histopaque 1083 solution was placed in a 15-mL tube, and 3 mL blood was layered on top of this density gradient. After the centrifugation (400*g* for 30 minutes at room temperature) the blood cells were separated into two fractions: an upper white layer consisting of mononuclear cells plus the majority of platelets at the interface region, and a lower layer containing erythrocytes and granulocytes. The plasma layer on top was clear and contained no cells. First, the plasma layer was removed and discarded. From the buffy coat, the monocytes were carefully taken off by aspiration and washed with phosphate buffered saline. This was repeated twice. After that, the pellet was resuspended in phosphate buffered saline-Tween and subjected to freeze-thaw cycle three times. The resulting lysate was used as the enzyme source.

2.5. Assay of COX

The assay of COX was measured by the thiobarbituric acid (TBA) method of Shimizu et al [17]. The assay mixture contained Tris-HCl buffer, glutathione, hemoglobin, and enzyme. The reaction was started by the addition of arachidonic acid. Then, the reaction was stopped after 1 minute incubation at 25°C by addition of 0.2 mL of 10% trichloroacetic acid (TCA) in 1M HCl, mixed and 0.2 mL of TBA was added. The contents were heated in a boiling water bath for 20 minutes, cooled, and centrifuged at 112 × *g* for 3 minutes. The supernatant was measured at 532 nm for COX activity.

2.6. Assay of LOX

LOX was measured using a method by Axelrod et al [18]. A 70-mg sample of linoleic acid and equal weight of Tween 20 were dissolved in 4 mL oxygen free water and mixed back and forth with a pipette, avoiding air bubbles. A sufficient amount of 0.5N NaOH was added to yield a clear solution (0.55 mL) and then made up to 25 mL using oxygen-free water. This was divided into 0.5-mL portions and flushed with nitrogen gas before closing and kept frozen until needed. The assay mixture for 5-LOX contained 2.75 mL of borate buffer pH 9.0, 0.2 mL of sodium linoleate, and 50 mL of enzyme. The increase in optical density (OD) was measured at 234 nm. The assay mixture for 15-LOX contained 2.75 mL of phosphate buffer pH 6.5, 0.2 mL of sodium linoleate, and 50 mL of enzyme. The increase in OD was measured at 280 nm.

2.7. Biochemical estimations

Determination of myeloperoxidase (MPO) activity was done by a method by Bradley et al [19]. The sample was mixed with 50mM phosphate buffer (pH 6) containing 1.67 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide; 5N HCL was added to stop the reaction. The change in absorbance at 400 nm was measured. Nitric oxide synthase (NOS) was determined by the Salter and Knowles method [20]. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) were estimated as reported earlier [21]. The protein concentration of the samples was determined by a method by Lowry et al [22]. Tumor necrosis factor-alpha (TNF-α), interleukin-1-beta (IL-1β), IL-10, and monocyte chemoattractant protein-1 (MCP-1) concentrations were measured using enzyme-linked immunosorbent assay (ELISA) [23].

2.8. Analysis of mRNA expression of IL-1β and IL-10 by Reverse transcription- polymerase chain reaction (RT-PCR)

Total RNA was isolated from paw tissue using a Sigma RNA isolation kit, following the instructions provided by the manufacturer. RNA samples were spectrophotometrically quantified and then used as templates to generate cDNA. Specific PCR products were generated using the following primers: IL-1β: forward primer 5′-AACCTGCTGGTGTGTGAC GTTC-3′: reverse primer 5′-CAGCACGAGGCTTTTTTGTTGT-3′, TNF-α: forward primer 5′-TGCTCAGAAACACACGAGACGC-3′: reverse primer 5′-TTCAGCAGCCTTGTGAGCCAGA-3′, IL-10: forward primer 5′-AGAGCCCCAGATCCGATTTT-3′: reverse primer 5′-CATCAAGGCGCATGTGAACT-3′.

GAPDH: forward primer 5′-ATCCCATCACCATCTTCAAG-3′: reverse primer 5′-CCTGCTTCACCTTCTTG-3′.

All reaction products were analyzed after 30–35 amplification cycles, each of which involved consecutive 1 minute steps at 94, 65–70 and 72°C. IL-1β, TNF-α and IL-10 levels were compared to GAPDH RNA in RT-PCR studies. The PCR reactions were carried out in a Mastercycler Personel (Eppendorf, Germany) using thermal cycle conditions following suggestions of the manufacturer and according to primer

design. Agarose gel (1.5% w/v) were stained with ethidium bromide and relative gene expression levels were calculated by quantifying the bands using Gel/Chemi Doc (Biorad Laboratories, Berkeley, California).

2.9. Statistical analysis

The results were analyzed using SPSS/PC+, version 11.0 (SPSS Inc., Chicago, IL, USA). The data were tested for Gaussian distribution, prior to performing parametric analysis. One-way ANOVA was employed for a comparison test of significant differences among the groups. Pair fed comparisons between the groups were made by Duncan's multiple range test; $p < 0.05$ was considered significant.

3. Results

3.1. Effect of horse gram and black gram on the activities of inflammatory mediators

Activities of inflammatory mediators such as COX, LOX, MPO, and NOS were evaluated in 21-day and 60-day experiments, as shown in Figures 1 and 2. The data show significant ($p < 0.05$) increase in the proinflammatory mediators COX, 5- LOX, 15-LOX, MPO and NOS in both the boiled and unboiled groups of black gram. The activities of these enzymes showed no significant changes in the rats fed with the boiled and unboiled horse gram.

3.2. Effect of horse gram and black gram on proinflammatory and antiinflammatory cytokines and chemokines

Proinflammatory cytokines IL-1β and TNF-α, and antiinflammatory cytokine IL-10 were evaluated in the spleen cells by ELISA during the 60-day experiment, as shown in Figure 3. The horse gram fed group showed no significant increase in the levels of IL-1β and TNF-α, whereas black gram supplementation significantly ($p < 0.05$) increased the levels of these cytokines. By contrast, the level of antiinflammatory cytokine IL-10 was significantly ($p < 0.05$) decreased in the black gram fed group, while it was increased in the horse gram fed group. The PCR experiments showed the same results as above, that is, the mRNA expression of IL-1β and TNF-α were found to be upregulated and that of IL-10 downregulated in the black gram fed group. The chemokine, MCP-1, activity was also evaluated and black gram supplementation significantly increased MCP-1, whereas the horse gram fed groups did not show any significant increase in the activity of the chemokine.

3.3. Effect of horse gram and black gram on antioxidant enzymes and lipid peroxidation

The antioxidant status in liver and heart tissues of the rats was evaluated by determining the activities of antioxidant enzymes catalase, SOD, and GPx, and the lipid peroxidation by-product, MDA, in both the 21-day and 60-day experiments,

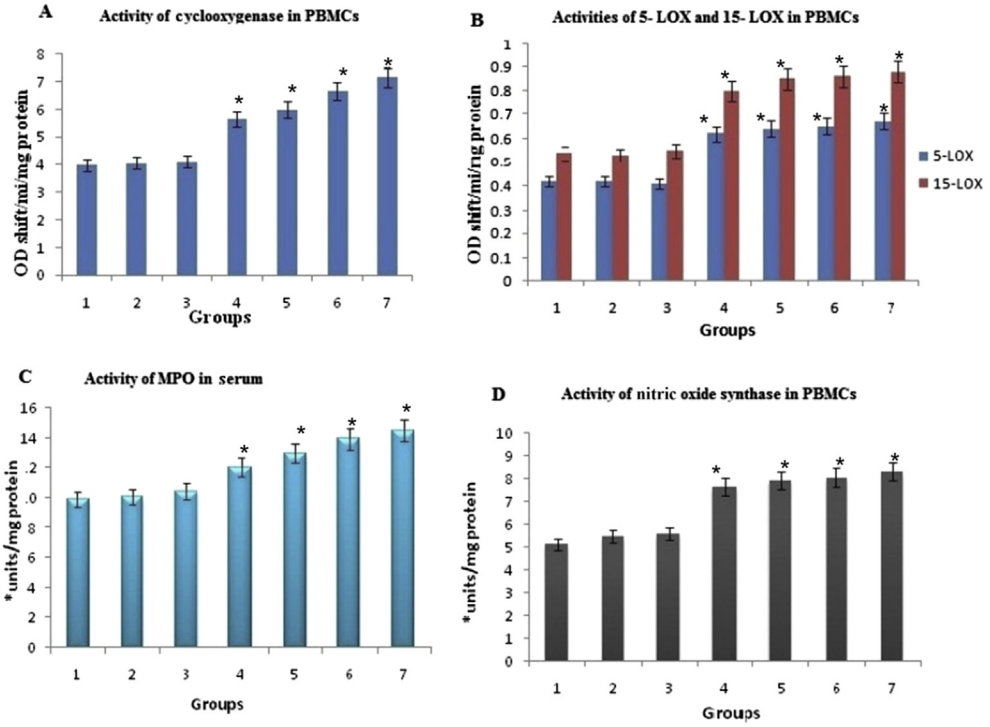

Figure 1 – Activities of inflammatory mediators in the 21-day experiment. Group I: control, Group II: horse gram boiled, Group III: horse gram unboiled, Group IV: black gram boiled (with skin), Group V: black gram unboiled (with skin), Group VI: black gram boiled (without skin), Group VII: black gram unboiled (without skin). Values expressed as average of six values ± SEM in each group. * Statistical difference compared with Group 1 at ($p < 0.05$). MPO units—degrading 1μM of peroxide/min at 25°C. NOS units—1nmol of NO produced/min at 37°C. LOX = lipoxygenase; MPO = myeloperoxidase; OD = optical density; PBMC = peripheral blood mononuclear cells; SEM = standard error of the mean.

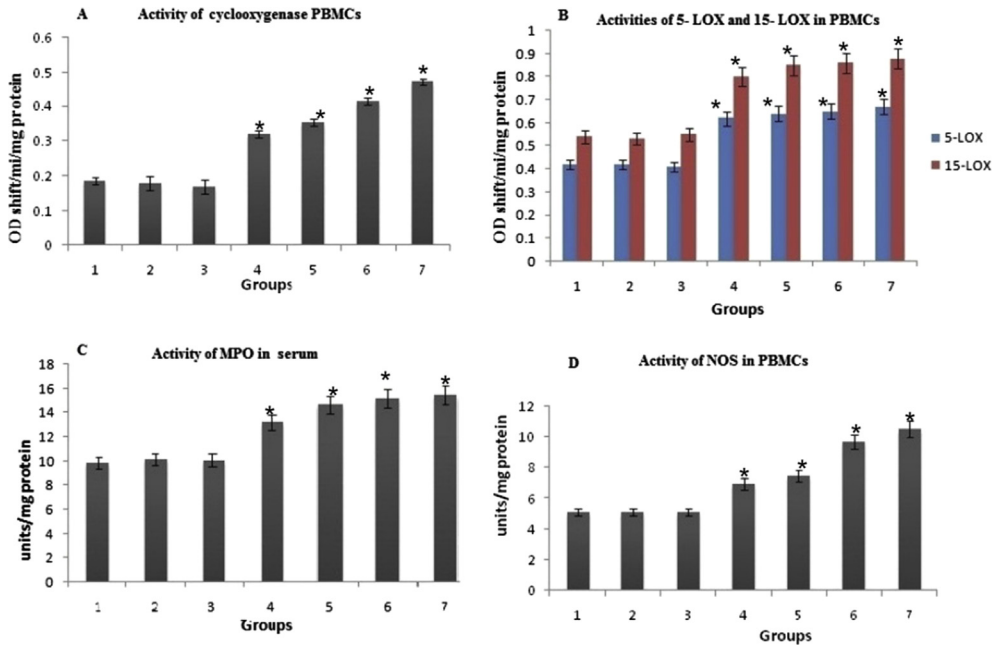

Figure 2 – Activities of inflammatory mediators in the 60-day experiment. Group I: control, Group II: horse gram boiled, Group III: horse gram unboiled, Group IV: black gram boiled (with skin), Group V: black gram unboiled (with skin), Group VI: black gram boiled (without skin), Group VII: black gram unboiled (without skin). Values expressed as average of six values ± SEM in each group. * Statistical difference compared with Group 1 at ($p < 0.05$). MPO units—degrading 1μM of peroxide/min at 25°C. NOS units—1nmol of NO produced/min at 37°C. LOX = lipoxygenase; MPO = myeloperoxidase; NOS = nitric oxide synthase; OD = Optical density; PBMC = Peripheral blood mononuclear cells; SEM = standard error of the mean.

which is given in Tables 1 and 2. The data from the tables shows that the activities of catalase, SOD, and GPx in liver and heart were significantly ($p < 0.05$) increased by horse gram supplementation, but the activities of these enzymes decreased significantly ($p < 0.05$) in both the forms of black gram administered to rats after the 21-day and 60-day experiments. The lipid peroxidation product MDA was found to be significantly ($p < 0.05$) increased in the black gram supplemented groups whereas horse gram showed significantly ($p < 0.05$) lower levels of MDA in Group II and Group III. Supplementation of these pulses for 60 days also showed the same effect in boiled and unboiled groups. Increased proinflammatory effect and the decreased antioxidant effect was observed in black gram, which was more prominent in black gram without skin; boiling and not boiling the pulses did not show much difference in their effects.

4. Discussion

Eating many different foods helps to maintain a healthy and interesting diet which provides a range of different nutrients to the body. However, eating too much or too little of a particular food variety can cause issues. Some foods have antioxidant or antiinflammatory potential and can be highly beneficial to the body, whereas some others may be detrimental by eliciting immune reactions in the body. In the present study, horse gram fed rats showed no significant increase in the COX enzyme, but including black gram in the daily diet for 21 days and 60 days significantly increased the activity of COX. Supplementation of black gram for 21 days and 60 days significantly increased 5-LOX and 15-LOX enzymes, whereas horse gram fed rats did not show any significant increase in the level of these enzymes in both experiments. COX is an enzyme that is responsible for the formation of prostanoid-like prostaglandins, prostacyclins, and thromboxanes, whereas LOX enzyme catalyzes the production of leukotrienes from arachidonic acid, which are each involved in the inflammatory response [24]. Extensive studies have been carried out to understand the role of COX and LOX in the progression of inflammation [25,26]. Studies with methanolic extract of *Dolichos biflorus* [27] and β-amyrin isolated from *Costus igneus* [28] show that inhibition of COX and LOX enzymes provides protection during inflammatory conditions. Our results show that horse gram has a protective effect as it did not increase the activity of COX enzyme. In comparison, black gram supplementation increased the activity of the enzyme indicating a proinflammatory stimulation, leading to prostaglandin synthesis.

After checking the effects in COX and LOX pathways, the effects of both pulses were evaluated in the NOS pathway, which produces NO. NO helps in the progression of vascular diseases [29], rheumatoid arthritis [30], etc. Horse gram supplementation showed no significant change in the level of NOS enzyme, but black gram significantly increased the NOS activity when compared to the control rats, in both 21-day and 60-day experiments. This suggests an increased synthesis of NO in the black gram fed group than in the horse gram fed group. Studies show that proinflammatory cytokines, such as TNF-α, can induce NO production and inducible nitric oxide

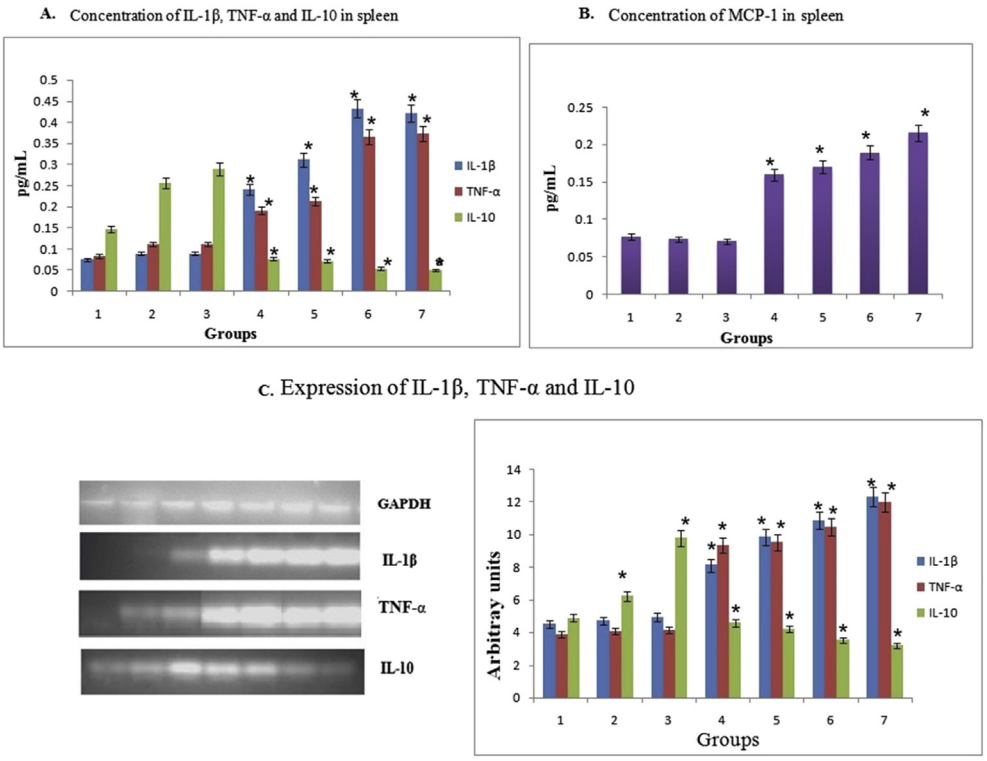

Figure 3 – Effect of horse gram and black gram on proinflammatory and anti-inflammatory cytokines and chemokines. Group I: control, Group II: horse gram boiled, Group III: horse gram unboiled, Group IV: black gram boiled (with skin), Group V: black gram unboiled (with skin), Group VI: black gram boiled (without skin), Group VII: black gram unboiled (without skin). Values expressed as average of six values ± SEM in each group. * Statistical difference compared with Group 1 at ($p < 0.05$). IL = interleukin, MCP-1 = monocyte chemoattractant protein-1; SEM = standard error of the mean; TNF-α = tumor necrosis factor-alpha.

synthase (iNOS) activity in colonic epithelial cells [31]. The increased activity of NOS in black gram fed groups suggests its proinflammatory effect in comparison to horse gram.

MPO plays an important role in the initiation, progression, and complications of many chronic inflammatory conditions like arthritis, atherosclerosis, etc. [32]. Several studies show that increased activity of the enzyme and its oxidant products are an important part of the inflammatory cascade produced due to endothelial dysfunction [33]. Black gram supplementation was found to significantly increase the activity of the enzyme, which again points to its proinflammatory effect, whereas horse gram showed no such significant increase in both experiments, suggesting its protective effect.

Cytokines play a major role in inflammation; while the proinflammatory cytokines help in the progression and maintenance of inflammation, the antiinflammatory cytokines try to reverse their effects. In the 60-day experiment, the levels of proinflammatory cytokines, IL-1β and TNF-α, in the spleen of black gram fed groups increased significantly, whereas the horse gram supplementation showed no significant increase. Macrophages are major producers of proinflammatory cytokines including TNF-α, IL-1β etc. and these are found to be increased in many disease conditions such as arthritis and diabetes [34]. IL-10 is an antiinflammatory cytokine that helps to maintain the balance by trying to bring down the inflammation. IL-10 levels were significantly decreased in the black gram fed group. This shows the imbalance between the proinflammatory and antiinflammatory cytokines during the supplementation of black gram. By contrast, horse gram supplementation maintained the balance in cytokine production, proving its beneficial effects. PCR results also show the same pattern in horse gram and black gram fed groups.

Similar results were obtained for the chemokine MCP-1. Chemokines are important proinflammatory molecules responsible for selectively recruiting monocytes, neutrophils, and lymphocytes and inducing chemotaxis of macrophages to the inflammatory site. The black gram fed group showed significantly increased activity of MCP-1, whereas the horse gram fed group showed no significant increase. Black gram, by increasing the synthesis of MCP-1, regulates the migration and infiltration of monocytes/macrophages. This has been demonstrated to be involved in various diseases such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, and insulin-resistant diabetes [35]. Thus, black gram supplementation showed increase in the proinflammatory enzymes, cytokines, chemokines etc. and decreased the antiinflammatory cytokine, proving the proinflammatory effects of black gram. This increase in inflammatory mediators can lead to overproduction of reactive oxygen free radicals in the system.

Reactive free radicals damage cells by initiation of lipid peroxidation that causes profound alteration in the structural

Table 1 – The activities of antioxidant enzymes and concentration of TBARS in the 21-day experiment.

	Parameters in liver				Parameters in heart			
	Catalase (units[a]/mg protein)	Superoxide dismutase (units[b]/mg protein)	Glutathione peroxidase (units[c]/mg protein)	Malondialdehyde (mM/100 g wet tissue)	Catalase (units[a]/mg protein)	Superoxide dismutase (units[b]/mg protein)	Glutathione peroxidase (units[c]/mg protein	Malondialdehyde (mM/100 g wet tissue)
I	68.59 ± 0.53	9.03 ± 1.87	0.2 ± 0.69	0.76 ± 1.42	9.94 ± 0.88	13.46 ± 2.19	0.81 ± 0.43	0.42 ± 1.69
II	93.07 ± 0.62*	14.19 ± 2.54*	0.79 ± 0.81*	0.53 ± 1.31*	13.51 ± 0.97*	16.9 ± 2.87*	1.59 ± 1.09*	0.26 ± 1.43*
III	94.59 ± 0.61*	14.39 ± 2.63*	0.85 ± 0.83*	0.49 ± 1.65*	14.65 ± 0.95*	17.29 ± 2.95*	1.71 ± 1.12*	0.23 ± 1.02*
IV	58.45 ± 0.45*	7.83 ± 1.42*	0.16 ± 0.55*	1.44 ± 3.21*	7.21 ± 0.79*	10.62 ± 1.62*	0.58 ± 0.61*	0.91 ± 2.89*
V	54.57 ± 0.43*	7.19 ± 1.33*	0.12 ± 0.48*	1.72 ± 3.44*	6.99 ± 0.52*	10.43 ± 1.58*	0.35 ± 0.42*	1.14 ± 3.25*
VI	52.41 ± 0.42*	5.79 ± 1.25*	0.07 ± 0.41*	2.78 ± 3.41*	6.51 ± 0.46*	8.87 ± 1.33*	0.13 ± 0.43*	2.26 ± 3.40*
VII	50.38 ± 0.45*	5.13 ± 1.06*	0.07 ± 0.36*	2.83 ± 3.56*	6.19 ± 0.42*	8.02 ± 1.05*	0.05 ± 0.37*	2.36 ± 3.51*

Group I: control, Group II: horse gram boiled, Group III: horse gram unboiled, Group IV: black gram boiled (with skin), Group V: black gram unboiled (with skin), Group VI: black gram boiled (without skin), Group VII: black gram unboiled (without skin), $NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidised), NADPH: nicotinamide adenine dinucleotide phosphate (reduced).
Values expressed as average of six values ± SEM in each group.
* Statistical difference compared with group 1 at ($p < 0.05$).
SEM = standard error of the mean.
[a] The amount of enzyme degrading 1.0μmol of H_2O_2/min 25°C.
[b] Enzyme concentration required to inhibit chromogen production by 50% in 1 minute.
[c] The amount of enzyme required to form 1.0mmol of $NADP^+$ from NADPH/min at pH 8.0 at 25°C.

Table 2 – The activities of antioxidant enzymes and concentration of TBARS in the 60-day experiment.

Group	Parameters in Liver				Parameters in Heart			
	Catalase (units[a]/mg protein)	Superoxide dismutase (units[b]/mg protein)	Glutathione peroxidase (units[c]/mg protein)	Malondialdehyde (mM/100 g wet tissue)	Catalase (units[a]/mg protein)	Superoxide dismutase (units[b]/mg protein)	Glutathione peroxidase (units[c]/mg protein)	Malondialdehyde (mM/100 g wet tissue)
I	68.55 ± 0.69	9.06 ± 2.37	0.23 ± 0.65	0.78 ± 0.26	9.95 ± 0.88	13.45 ± 2.12	0.81 ± 0.42	0.46 ± 0.34
II	105.58 ± 0.81*	15.38 ± 3.45*	0.51 ± 0.84*	0.57 ± 0.19*	16.5 ± 0.97*	18.36 ± 2.45*	1.92 ± 1.21*	0.17 ± 0.33*
III	112.54 ± 0.83*	16.57 ± 3.33*	0.68 ± 0.81*	0.48 ± 0.16*	16.9 ± 0.91*	19.5 ± 2.13*	2.09 ± 1.03*	0.14 ± 0.29*
IV	52.25 ± 0.55*	5.94 ± 2.42 *	0.27 ± 0.55*	1.42 ± 0.35*	6.20 ± 0.69*	7.22 ± 1.62*	0.48 ± 0.71*	0.74 ± 0.37*
V	48.15 ± 0.48*	5.68 ± 2.52*	0.25 ± 0.45*	1.64 ± 0.37*	6.09 ± 0.52*	6.92 ± 1.48*	0.35 ± 0.53*	0.79 ± 0.37*
VI	45.17 ± 0.41 *	4.22 ± 2.25*	0.08 ± 0.42*	2.47 ± 0.45*	5.68 ± 0.36*	6.37 ± 1.23*	0.22 ± 0.35*	0.91 ± 0.46*
VII	42.13 ± 0.36*	4.01 ± 1.5*	0.07 ± 0.37*	3.01 ± 0.52*	5.05 ± 0.32*	5.52 ± 1.03*	0.10 ± 0.32*	1.04 ± 0.52*

Group I: control, Group II: horse gram boiled, Group III: horse gram unboiled, Group IV: black gram boiled (with skin), Group V: black gram unboiled (with skin), Group VI: black gram boiled (without skin), Group VII: black gram unboiled (without skin), $NADP^+$: nicotinamide adenine dinucleotide phosphate (oxidised), NADPH: nicotinamide adenine dinucleotide phosphate (reduced).
Values expressed as average of six values ± SEM in each group.
* Statistical difference compared with Group 1 at ($p < 0.05$).
SEM = standard error of the mean.
[a] The amount of enzyme degrading 1.0μmol of H_2O_2/min 25°C.
[b] Enzyme concentration required to inhibit chromogen production by 50% in 1 minute.
[c] The amount of enzyme required to form 1.0mmol of $NADP^+$ from NADPH/min at pH 8.0 at 25°C.

integrity and functions of cell membranes. Horse gram supplementation showed significantly reduced MDA levels, suggesting decreased lipid peroxidation levels in heart and liver. In comparison, black gram supplementation showed significantly increased levels of MDA in both liver and heart. MDA is the major reactive aldehyde, resulting from the peroxidation of biological membranes. Studies show that the increased level of MDA is implicated in many inflammatory disorders such as rheumatoid arthritis [36], diabetes, and atherosclerosis [37]. In this study, horse gram maintained the antioxidant status compared to black gram which showed increased MDA levels, indicating the imbalance in reactive oxygen species (ROS) generation and the antioxidant status.

Many cellular defense mechanisms are involved in regulating the toxic effects of ROS. One major enzymatic antioxidant present in the body is SOD [38] and the supplementation of horse gram significantly increased the activity of SOD in liver and heart in the 21-day and 60-day experiments. However, black gram supplementation significantly decreased the activity of SOD in both experiments. SOD is widely distributed in the cells actively involved in cellular metabolism and offers protection from the harmful effects of super oxide anion. MnSOD has been implicated in a number of oxidative stress-related diseases [39]. SOD acts as an antioxidative enzyme in ROS-mediated lung inflammation and vascular disease such as ischemia and atherosclerosis [40]. The decreased activity of SOD in the black gram treated group further suggests its proinflammatory effect and the increased activity of SOD points to the beneficial effects of horse gram.

The activity of another important antioxidant enzyme, catalase, was found to be significantly increased in horse gram fed group when, in comparison, the black gram fed rats showed significantly reduced activity of catalase in liver and heart tissues. Catalase enzyme plays a major role in free radical scavenging and maintains the antioxidant status of the body [41]. This again proves the detrimental effect of black gram, as it reduces the activity of catalase which in turn increases the oxidative stress. However, horse gram maintained the oxidative balance and proved to have a protective effect on the rats. In the 21-day and 60-day experiments, horse gram fed rats showed significant an increase in the levels of the GPx, indicating a strong resistance against release of ROS, whereas black gram (with skin and without skin) supplementation showed significantly decreased activities of the antioxidant enzyme compared with the control rats.

From the results, it is clear that black gram disrupts the oxidant/antioxidant balance and such an imbalance in the antioxidant enzymes and release of free radicals possesses a risk of increasing the lipid peroxidation, which was proved by the increased MDA levels in black gram fed groups. However, horse gram supplementation maintained the antioxidant status by sustaining the oxidant/antioxidant balance, thus proving its protective effect.

These two pulses, horse gram and black gram, are widely consumed as part of the normal diet in many countries. The ancient treatment regime of Ayurveda suggests including horse gram in the daily diet of arthritic patients, while black gram has to be excluded from their diet. The results from this work suggest that horse gram, either boiled or unboiled, acts as an antiinflammatory and an antioxidant, whereas black gram, both with skin and without skin, supplementation possesses a proinflammatory and prooxidant effect, which provides a scientific validation for the strict regulation of black gram in arthritic diet. This is a novel study designed to check the effects of these two pulses on inflammatory mediators and oxidant stress in normal rats, hence, further studies are required to evaluate the effects of these two pulses in arthritic experimental models.

Conflicts of interest

All contributing authors declare no conflicts of interest.

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