Indian J.Sci.Res. 20(1): 12-19, 2018

ISSN: 0976-2876 (Print)
ISSN: 2250-0138(Online)

AMELIORATIVE EFFICACY OF DIETARY AMARANTH SPECIES ON JAK/STAT SIGNALING IN COLLAGEN INDUCED ARTHRITIS

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ABSTRACT

Rheumatoid arthritis is an autoimmune degenerative disease of the joints marked with articular cartilage degradation and is characterized by a series of molecular events leading to irreversible damage. Among the various signaling pathways activated, Janus Kinase/ Signal Transducer and Activator of Transcription (JAK/ STAT) pathway, upon stimulation by certain pro-inflammatory cytokines especially IL-6 leads to synovial destruction. Because of the side-effects of drugs used for RA, people depend on dietary modification. Green amaranths are used in daily diet of Indians as part of variety of culinary preparations. Among the different species of amaranths, *Amaranthus cruentus* (Ac), *Amaranthus viridis* (Av) and *Amaranthus hybridus* (Ah) were the easily available species commonly added with diet. The present study evaluates the dietary effect of three amaranths on JAK-STAT signaling pathway associated with inflammatory arthritis. Type II collagen (4mg/ml) was induced in rats (CIA), and after immunization period cooked Ac, Av and Ah at a dose of 500mg/100g body weight of each were supplemented along with normal feed. After 60 days of experimental period, blood and spleen were collected and analysed. The results on protein level activation of JAK1, JAK2, STAT1 and STAT3 showed a significant ($p < 0.05$) reduction in CIA rats supplemented with the three amaranth species. These results were well correlated with the decreased mRNA expression of IL-6 and MCP-1 expression. Myeloperoxidase and CRP were also found to be significantly ($p < 0.05$) decreased. Histopathological results of joints also confirmed the above results by showing a significant ($p < 0.05$) reduction of synovial hyperplasia and inflammatory cells in the three amaranth supplemented groups. In conclusion, dietary supplementation of these amaranths exhibit inhibitory effect on inflammatory JAK-STAT signaling pathway in collagen induced arthritis.

KEYWORDS: Rheumatoid Arthritis, Amaranthus, JAK/STAT, IL-6.

Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovial tissues in multiple joints that leads to joint destruction. Cumulative effects of several factors contribute to tissue destruction and indeed, the pathogenesis of RA is characterized by a series of molecular signaling events leading to irreversible damage. One of the most often utilized signal-transduction pathways is the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway. It is an intracellular signaling pathway activated by multiple immune cytokines and plays a key role in mediating inflammatory responses (Malemud and Pearlman., 2009). It has been implicated in the pathogenesis of various inflammatory and autoimmune diseases (Malemud 2010).

JAK is a novel family of cytoplasmic tyrosine kinases pre-associated with cytokine receptors and catalyzing STAT activation by phosphorylation. STATs are latent monomeric proteins that upon activation serve as transcription factors and are present in the cytoplasm of numerous cell types (Wiland et al., 2008). Dysfunctional intracellular signaling involving deregulated activation of JAK/STAT signaling play a prominent role in RA (Malemud 2011). Several cytokines that regulate immune responses in RA, such as IFN-γ, GM-CSF, IL-4, IL-6, IL-7, IL-10, IL-12, IL-15 and IL-21 use JAKs and STATs to transduce intracellular signals and evidences revealed that inhibition of JAK-STAT signaling contribute to reduced progression of disease pathology (Brennan et al., 2008). Also reflects an ongoing imbalance between pro-inflammatory and anti-inflammatory cytokines. These imbalances of cytokines in joints also triggers increase in synoviocyte proliferation and production of cascade of secondary mediators involved in the recruitment of inflammatory cells in the process of joint destruction (Camussi and Lupia, 1998).

The most common drugs used for RA as medication possess serious and adverse side-effects and hence people with RA are constantly trying to reduce the symptoms through dietary interventions. Vegetables provide a significant part of human nutrition, as they are important sources of nutrients, dietary fiber, and phytochemicals. Vegetable amaranths are the most popular vegetable crops in tropics especially in the tropical humid climate of Africa and Asia (Das et al., 2016). Green amaranths are rich source of lysine-rich protein, β-carotene, various vitamins, minerals and dietary fibres and also used in daily diet of Indians as part of variety of culinary preparations. Among the different species of amaranths, *Amaranthus cruentus* (Ac), *Amaranthus viridis* (Av) and *Amaranthus hybridus* (Ah) are the easily

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available edible species commonly added with diet. Amaranth is used to fight against gastroenteritis, stomach flu and also reduce tissue swelling from sprains. Moreover amaranth oil has been shown to prevent and treat those affected with hypertension and cardiovascular disease. Regular consumption of amaranth can reduce cholesterol levels and lower blood pressure (Das et al., 2016; Nana et al., 2012; Salvamani et al., 2016; Kraujalis et al., 2013). Hence the purpose of the present study is to evaluate the dietary effect of three amaranths on JAK-STAT signaling pathway associated with inflammatory arthritis in collagen induced rats.

MATERIALS AND METHODS

Chemicals and Solvents

Bovine type II collagen and antibodies were purchased from Sigma-Aldrich, India and cell Signaling technology, India. cDNA synthesis, tri reagent and all other chemicals and biochemicals used were from Thermo scientific, Mumbai and SRL chemicals, Mumbai, India.

Plant Collection and Preparation

Fresh three species of Amaranths namely *Amaranthus cruentus* (Ac), *Amaranthus viridis* (Av) and *Amaranthus hybridus* (Ah) were collected from the home garden and was authenticated by botanist Dr. G.Valsaladevi, Department of Botany, University of Kerala, India. Each species of amaranths were washed well and leaves were blanched separately in boiled water for 10 minutes (Virigina et al., 2012). These were separately supplemented into standard commercial rat feed at 500mg/100g body weight concentration.

Animals

Female Wistar rats of body weight 150-250g which were breed and reared in the department animal house were used for the experiment. 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±1℃ and a 12h light and dark cycle. This study was performed in accordance with the supervision of veterinary surgeon of the host institution. The animal use protocol was reviewed and approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (registration number 218/CPCSEA) IAEC-KU-6/2014-15-BC-AH (27) according to the Government of India accepted principles for laboratory animal use and care.

Experimental Grouping and Induction of Collagen Induced Arthritis (CIA)

Experimental animals were divided into five groups, each group consist of six rats namely, Group I - Normal rats

Group II - Collagen induced arthritic rats (CIA)

Group III - CIA rats supplemented with cooked Ac (500mg/kg bwt)

Group IV- CIA rats supplemented with cooked Av (500mg/kg bwt)

Group V - CIA rats supplemented with cooked Ah (500mg/kg bwt)

Normal and CIA groups received normal laboratory rat chow. Collagen induced arthritis was induced in Group II to V. For CIA immunization, bovine type II collagen was dissolved in 0.1M acetic acid at a concentration of 4mg/ml at 4˚C and emulsified in an equal volume of ice-cold incomplete Fruend's adjuvant. Each rat was given intradermal injections of 100µl at two sites on the back on day 0, and then received booster injection at two sites (100µl in divided doses) on day 7. Paw edema peaked on day 14. Rats that showed severe arthritis were excluded from the study. Normal non-immune rats were used as negative controls (Brand et al., 2007). After day 14 of CIA immunization, cooked Ac, Av and Ah (500mg/100g) were supplemented separately along with rat feed and fed to rats for 45 days for groups Ac, Av and Ah respectively, concurrently CIA control was given normal feed. The complete duration of experiment was 60 days. After the experimental period rats were sacrificed, blood and other tissues were collected for further analysis.

Biochemical Estimations

Serum MPO activity was determined by the method Bradley et al (1982). 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. CRP in serum was determined by using an immuno- turbidimetric kit (Agappe diagnostics).

Enzyme Linked Immuno Sorbent Assay (ELISA)

Spleen of respective experimental groups were collected and homogenized in lysis buffer was used for analysis. Protein level expression of MCP-1, IL-6, JAK1, JAK2, STAT1, STAT3 were determined by specific antibodies using ELISA. The expression was determined

with the corresponding protein concentration of the tissue sample. Protein concentration was determined by the method by Lowry et al (Lowry et al., 1951).

RNA Preparation and RT-PCR

Total cellular RNA was extracted from spleen of experimental animals using tri reagent (SRL chemicals) by the method by Chomczynski and Mackey et al (Chomczynski and Mackey, 1995). cDNA were synthesized from the corresponding RNA using kits, following manufacturer's instructions and these cDNA were amplified in PCR thermocycler (Eppendorf, Germany) to determine the gene expression using primers (Sigma Aldrich) specific for IL-6, MCP-1 and GAPDH following manufacturer's cycling parameters. 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. RT-PCR products were then electrophoresed on 1.5% agarose gel, and ethidium bromide stained bands were visualized and quantified by densitometry using gel documentation system (Bio Rad). GAPDH was used as an internal standard for the amplification process. The signals were expressed relative to the intensity of GAPDH in each sample. Oligonucleotide primers for PCR product amplification were as follows:

IL-6 forward 5′-CCACTGCCTTCCCTACTTCA-3′

reverse 5′-TGGTCCTTAGCCACTCCTTC-3′

MCP-1 forward 5′-GTGCTGACCC-CAATAAGGAA-3′

reverse 5′-TGAGGTGGTTGTGGAAAAGA-3′

Histopathological Analysis

After experimental duration rats were sacrificed and knee joints were collected for histopathological examination. First tissues were removed and cleaned, knee joint were fixed in 10% buffered formalin, decalcified in a solution of 10% EDTA in 0.1M phosphate buffer (pH 7-8) for approximately 8-10 weeks and then embedded in paraffin and visualized using hematoxylin-eosin staining.

Statistical Analysis

Results were expressed as mean with standard error of mean using the statistical program SPSS/PC+, version 17.0 (SPSS, Inc). Statistical evaluation was performed using one-way ANOVA, and significance in results was analyzed using Duncan's test of $P<0.05$.

RESULTS

Dietary Effect of Amaranth Species on Tissue Inflammatory Markers

CRP which is a preliminary marker of inflammation was found to be significantly ($p < 0.05$) elevated in the CIA control compared to normal. Whereas the three dietary supplemented groups Ac, Av and Ah showed a significant ($p < 0.05$) reduction in the CRP level, done using immuno-turbidimetric method. MPO is considered as a strong pro-inflammatory enzyme used to determine neutrophil activity was found to significantly ($p < 0.05$) increased in CIA control compared to normal. On the other hand, three dietary supplemented groups exhibited a significant ($p < 0.05$) decrease of MPO activity compared to CIA control (Table: 1).

Table 1: Dietary effect of Ac, Av and Ah on CRP and MPO activity.

Groups	Normal	CIA control	Ac	Av	Ah
C-Reactive Protein (mg/L)	**1.95±.0.17**	**10.50±0.38[a]**	**2.78±0.10[ab]**	**3.56±0.32[ab]**	**3.95±0.35[ab]**
Myeloperoxidase (*Units/mg protein)	**0.25±.009**	**0.59±.021[a]**	**0.34±0.12[ab]**	**0.45±.016[ab]**	**0.46±0.041[ab]**

Normal- rats supplemented with normal diet; CIA control- rats induced by collagen; Ac, Av and Ah- groups supplemented with cooked (500mg/100g) Ac, Av and Ah respectively after collagen immunization period. Values expressed as average of six values ± SEM in each group. [a]Statistical difference compared with normal at ($p < 0.05$), [b]Statistical difference compared with CIA control at ($p < 0.05$). *One unit of MPO activity defined as that degrading 1µm of peroxide per minute at 25˚C.

Dietary Effect of Amaranth Species on Activation of JAK and STAT

JAKs are cytoplasmic tyrosine kinases and STAT are transcription factor in cellular signaling pathways involved in regulating the immune and inflammatory process. Protein level expression of JAK1, JAK2, STAT1 and STAT3 revealed a pronounced expression ($p < 0.05$) in CIA control compared to normal. Whereas dietary supplementation of three

amaranths showed a downregulated expression ($p < 0.05$) compared to CIA control (Figure: 1).

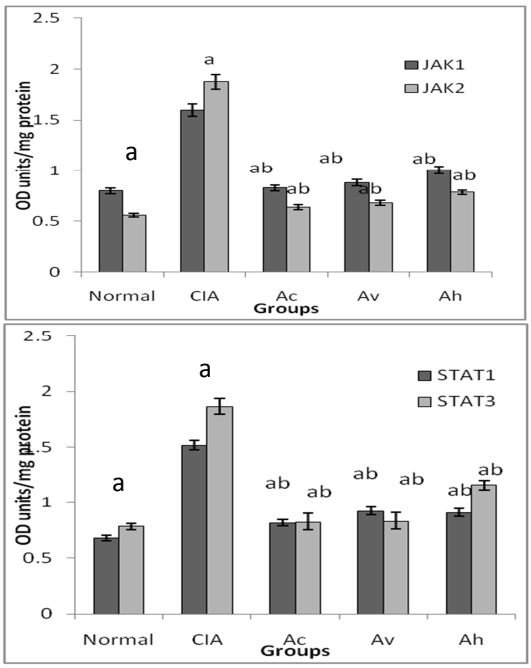


Figure 1: Dietary effect of Ac, Av and Ah on activation of JAK and STAT.

Normal- rats supplemented with normal diet; CIA control- rats induced by collagen; Ac, Av and Ah-groups supplemented with cooked (500mg/100g) Ac, Av and Ah respectively after collagen immunization period. Values expressed as average of six values ± SEM in each group. [a]Statistical difference compared with normal at ($p < 0.05$), [b] Statistical difference compared with CIA control at ($p < 0.05$).

Dietary Effect of Amaranth Species on IL-6 and MCP-1

IL-6 is a potent pro-inflammatory cytokine perpetuated through JAK/STAT signaling and also MCP-1 (Macrophage chemoattractant protein-1) is activated upon STAT activation. CIA control group showed a significant ($p < 0.05$) up-regulation of both Il-6 and MCP-1. Upon dietary supplementation of amaranths significantly ($p < 0.05$) down-regulated the protein as well as mRNA expression of IL-6 and MCP-1 (Figure: 2).

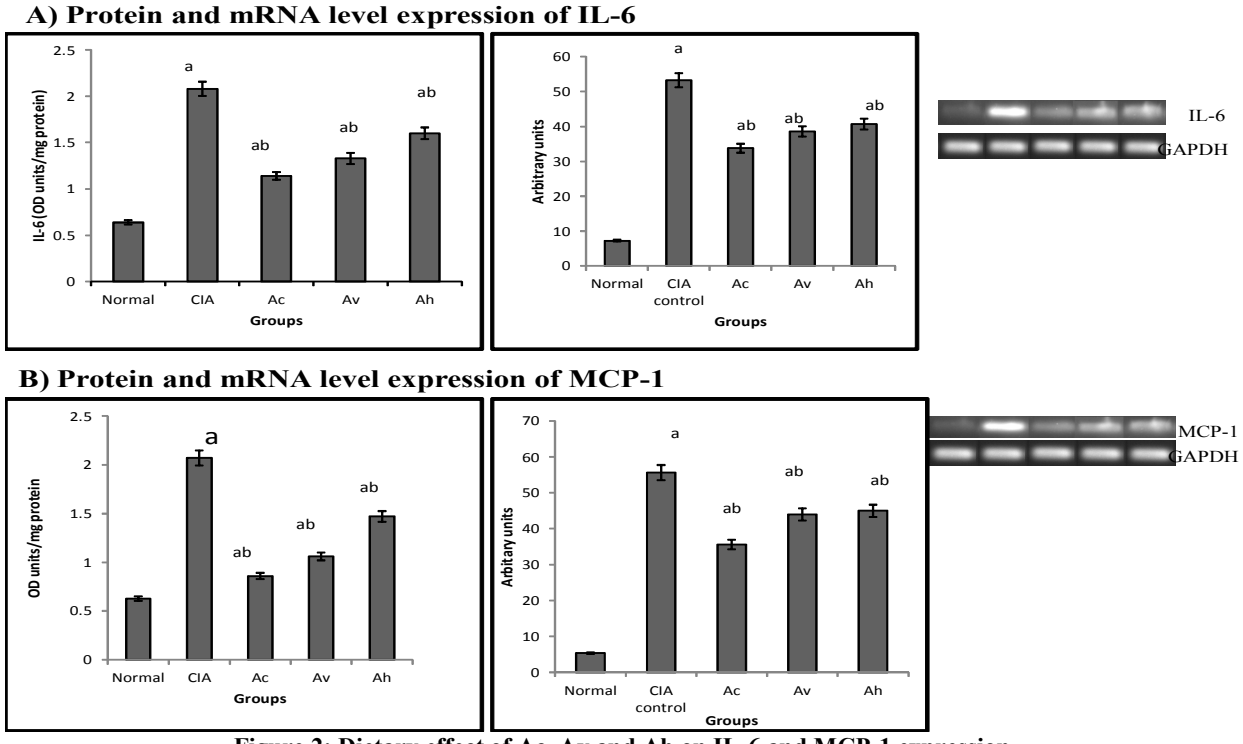
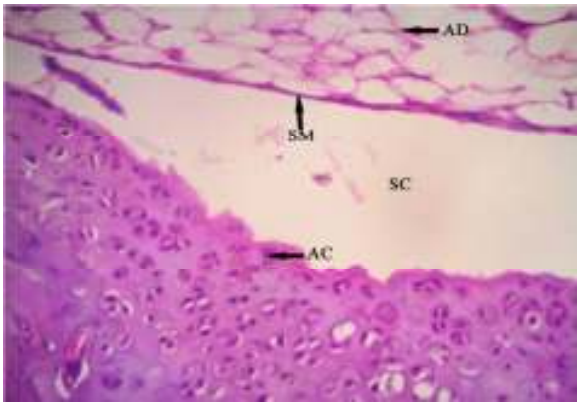


Figure 2: Dietary effect of Ac, Av and Ah on IL-6 and MCP-1 expression.

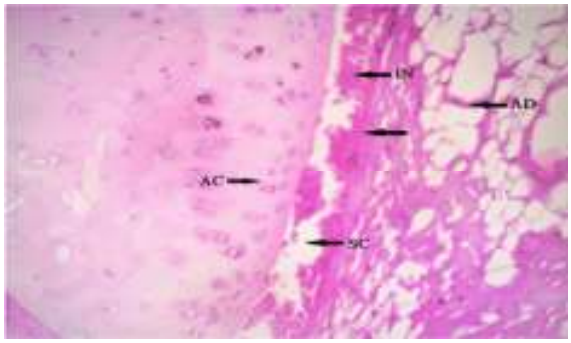
A) protein and mRNA level expression of IL-6. B) protein and mRNA level expression of MCP-1. Normal- rats supplemented with normal diet; CIA control- rats induced by collagen; Ac, Av and Ah- groups supplemented with cooked (500mg/100g) Ac, Av and Ah respectively after collagen immunization period. Values expressed as average of six values ± SEM in each group. [a]Statistical difference compared with normal at ($p < 0.05$), [b] Statistical difference compared with CIA control at ($p < 0.05$).

Dietary Effect of Amaranth Species on Histopathology of Joint Cartilage

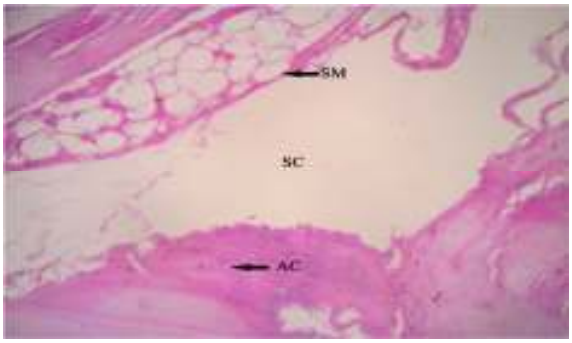
Knee joint cartilage collected after experimental period were decalcified and examined for cartilage destruction. CIA control group showed thickening of synovial and sub-intima membrane with synovial hyperplasia with infiltration of inflammatory cells. While the dietary supplemented groups Ac and Av exhibit a much significant reduction in synovial hyperplasia and infiltration of inflammatory cells but Ah showed mild hyperplasia (Figure: 3).



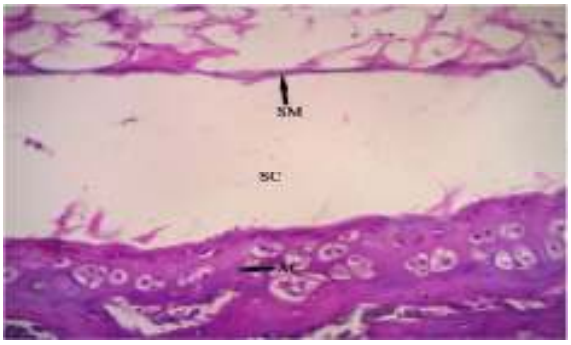
Normal



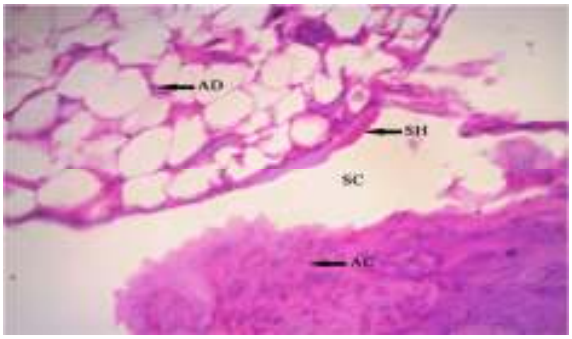
CIA control



Ac



Av



Ah

Figure 3: Dietary effect of Ac, Av and Ah on histopathology of joint cartilage.

Normal- rats supplemented with normal diet; CIA control- rats induced by collagen; Ac, Av and Ah- groups supplemented with cooked (500mg/100g) Ac, Av and Ah respectively after collagen immunization period. SM- synovial membrane, SC- synovial cavity, SH- synovial hyperplasia, AC- articular cartilage, IN- infiltration, AD- subintima membrane with supporting adipose tissue.

DISCUSSION

It is widely accepted that vegetables play a critical role in preventing the development of cardiovascular diseases, ageing-related diseases, obesity and cancers and improving human memory (Das, 2016). Therefore it is clear that there exist an association between

the consumption of vegetables and the risk of disease (Boeing et al., 2012). Several literatures support the positive impact of dietary intervention in decreasing disease activity in RA (Khanna et al., 2017). Inappropriate activation of a number of inflammatory pathways occurs in RA and there is growing evidence that modulation of the JAK/STAT pathway may represent a viable alternative therapeutic target in the treatment of RA (Walker and Smith, 2005). Hence the present study focused on whether dietary consumption of three amaranths Ac, Av and Ah can modulate the disease severity through the suppression of JAK-STAT signaling pathway in collagen induced arthritic rats.

During inflammatory condition there increase certain acute phase proteins. C- reactive proteins (CRP) is considered as an important marker in the earlier detection of inflammation. CIA group showed a marked increase in the CRP level which was significantly decreased on dietary supplementation of amaranths. Among the three, Ac shows a much significant reduction in CRP in collagen induced condition, suggests its effect in attenuating progression of inflammation. Myeloperoxidase (MPO) plays a central role in oxidant production by neutrophils in inflammatory sites and induces oxidative stress. It uses superoxide and hydrogen peroxide to catalyse the generation of anti-bacterial hypochlorous acid and free radicals (Kettle and Winterbourn, 1997). In the present study the result showed a diminished activity of MPO on dietary supplementation of Ac, Av and Ah in arthritic rat compared to CIA group. Thus dietary supplementation of amaranths inhibits the infiltration of neutrophils into the inflamed tissue thereby imparting an anti-inflammatory effect.

Activation of JAK/STAT by pro-inflammatory cytokines is now considered to be of major importance in driving chronic inflammation in RA (Aittomäki et al., 2014). IL-6 is one among those cytokines where its pathological role is perpetuated through the intracellular signaling pathway JAK/STAT. The present study exhibits an up-regulated protein expression of JAK1 and JAK2 in CIA control. JAK1 and JAK2 are receptor-associated tyrosine kinases involved in signal transduction and are being considered as targets for suppression in RA (Cohen et al., 2012). The three dietary amaranths Ac, Av and Ah exerted significant blocking effect on activation of both JAK1 and JAK2. Studies done in our lab revealed the inhibitory action of tricin, a flavonoid isolated from Njavara rice bran on JAK-STAT activation in LPS induced hPBMCs (Shalini, 2015). As STATs are downstream molecules in JAK/STAT pathway, the protein level expression of STATs were measured. Results showed a pronounced expression of STAT1 and STAT3 in CIA control group. Evidences from the previous studies showed Up-regulated levels of STAT3 mRNA in mononuclear cells from peripheral blood and synovial fluid, and elevated STAT1 expression in synovial fluid in active RA (Isomaki et al., 2015). STAT3 has been found to be responsible for joint degradation in RA (Mori et al., 2011). Dietary amaranths inhibited the role of STAT1 and STAT3 as transcription factors in collagen induced arthritis thereby attenuating joint degradation. Multiple mechanisms exist for down-modulating cytokine signaling by the JAK/STAT pathway. Inhibitory action of dietary amaranths may be attributed to the synergistic action of phytochemicals present in them which may either suppress dephosphorylation of JAKs or STAT thereby inhibiting activation of inflammatory genes.

To validate the functional importance of the suppressive activity of dietary amaranths on JAK-STAT inflammatory cascade in CIA model, further investigation was carried out to determine the pro-inflammatory effect of IL-6 and MCP-1 that include a STAT binding site in their promoter region. Several lines of studies have demonstrated that overproduction of IL-6 contributes to the pathogenesis of various autoimmune and inflammatory diseases (Ishihara et al., 2002). The CIA control group illustrates an enhanced expression of IL-6 and MCP-1. Accumulating evidence from collagen induced disease model revealed that IL-6 gene deficient mice showed a delayed onset and reduced severity of RA, detailing the pathological role of IL-6 (Alonzi et al., 1998; Sasai et al., 1999). However, dietary supplementation of these amaranths in CIA down-regulated the expression of both IL-6 and MCP-1 in spleen. There are evidences which proved the presence of flavonoids having antioxidant and anti-inflammatory potentials in three amaranths (Nana et al., 2012; Salvamani et al., 2016; Kraujalis et al., 2013), which may be reason for suppression of inflammatory cascade. The diminished production of IL-6 and MCP-1 in dietary amaranth supplemented groups may be partly due to the suppression of JAK-STAT activation involved in inflammatory signaling.

To confirm the results inhibitory activity of three amaranths in CIA rats, we checked the extent of recovery on histopathological of knee joint cartilage in dietary supplemented groups Ac, Av and Ah in CIA rats. The results on histopathology of knee joint cartilage of CIA control clearly depict the severity, from increased synovial

hyperplasia and increased infiltration of inflammatory cells. Studies done on supplementation of resveratrol, a polyphenol found in several food species which are components of the human diet, such as berries, peanuts, grapes and red wine decreased synovial hyperplasia and also infiltration in arthritic model (Riveiro et al., 2016). In the present study three dietary supplemented groups recovered the increased hyperplasia and also reduced the infiltration of inflammatory cells suggesting the protective response in ameliorating disease severity in RA.

As a whole, the present study demonstrated a protective mechanism of anti-inflammatory effect of three dietary amaranths in retarding the progression of joint destruction in RA by relieving inflammation through the inhibition of CRP as well as MPO. Furthermore, dietary amaranths showed a prominent effect in suppression of inflammatory cascade JAK/STAT, thereby inhibiting the overproduction of IL-6 and MCP-1 involved in pathogenesis of RA.

CONCLUSION

To conclude, Dietary intervention by adding cooked amaranths such as *Amaranthus cruentus, Amaranthus viridis* and *Amaranthus hybridus* along with our diet may boost our immune system from preventing the progression of chronic disease such as RA by exerting its protective effect through the suppression of inflammatory signaling pathway JAK/STAT associated with RA.

ACKNOWLEDGMENT

This study was financially supported by Kerala State Council for Science Technology and Environment (KSCSTE), Thiruvananthapuram, Kerala, India (No. 001/FSHP/12/CSTE). 27th Swadeshi Science Congress is also acknowledged for providing opportunity for presentation and publication of journal.

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